UNIVERSITA' DEGLI STUDI DI CAMERINO

School of Advanced Studies

DOCTORAL COURSE IN

Chemical and Pharmaceutical Sciences and Biotechnology

CURRICULUM

Pharmaceutical, Nutraceutical and Food Sciences

XXXV cycle

Impact of individual variability in rat models of alcohol use disorder

Ph.D. Candidate:

Sara De Carlo

Supervisor:

Prof. Roberto Ciccocioppo

Co-supervisor:

Prof. Nazzareno Cannella

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ABSTRACT

Alcohol use disorder (AUD) has been defined as a chronically relapsing disorder associated with compulsion in alcohol seeking and taking, loss of control over alcohol intake, and the emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability) when alcohol is no longer available. Just a small proportion of people consuming alcohol develops AUD. AUD aetiology lies in the complex interaction between genetic and environmental factors which makes some individuals more vulnerable to escalate alcohol use and develop AUD than others who show a resilient phenotype. Moreover, AUD patients also differ in their pattern of alcohol consumption and symptoms, and AUD treatments and medications are not effective in all of them. Several phenotyping and genotyping criteria have been proposed to group alcoholic patients into more homogeneous subpopulations. Similarly, different rat models appear to differ in their phenotypes and genetic background. Therefore, as a heterogeneous and multifactorial disorder, AUD investigation benefits from the use of animal models since each rat model may well resemble a specific subpopulation of AUD patients.

Focusing on three main objectives, the present thesis aims to investigate the impact of individual variability in different rat models of AUD to clarify how specific rat models can be suitable for the investigation of specific AUD populations and hence can contribute to the development of target therapies.

After providing an overview of the research background in **Chapter 1**, the first study exposed in **Chapter 2** aimed to develop a multisymptomatic animal model of AUD that would well mimic the high heterogeneity in alcohol phenotypes and treatment responses observed in patients. For this purpose, we selected as rat model the outbred NIH heterogeneous stock rats, a line that well resembles the genetically and phenotypically variability existing in the human population. Data collected from the screening of alcohol-related behaviors, allows me to group the HS population into three different clusters having different profiles of AUD-like behaviors. Responder and non-responder animals were individuated to naltrexone treatment, while memantine failed as alcohol-specific treatment, results that are both consistent with

clinical outcomes. Moreover, I also detected a relationship between the anxiety trait and naltrexone efficacy.

In **Chapter 3**, I focused on the role of the environment in inducing alcohol consumption. Specifically, I explored the short and long-term effects of adolescent sleep restriction on alcohol consumption and related behaviors in two different rat lines: the heterogeneous Wistar line and the genetically selected Marchigian Sardinian alcohol-preferring (msP) line. MsP rats mimic a specific AUD population in which ethanol abuse is associated with high comorbid anxiety and depression and their response to sleep restriction was homogenous and seemed to be driven by stress. In the outbred Wistar rats, instead, we observed interindividual variability in response to sleep restriction with a subgroup of animals showing a life-long lasting increase in alcohol consumption.

Finally, since recent evidence from our laboratory has highlighted the existence of interindividual variability also in the highly homogenous msP rat line, in **Chapter 4**, I investigated this aspect in relation to the effect of the neuropeptide S, recently proposed as a potential target to develop new treatments for AUD. To contribute to filling the gap in gender-related studies in the AUD field, female msP rats were used for the present study. Thanks to their phenotypical characteristics, msP rats may represent a good animal model to study the relationship between anxiety and alcohol consumption in females. From this study, two clusters of animals were identified showing different responses to the treatment with NPS and pointing out the possibility of differences in the NPS/NPSR system in female msP rats.

In conclusion, these studies together demonstrate how different rat lines may catch some aspects of the human disorder, making it possible to deeply explore the heterogeneity characterizing AUD and maximize the translational power of preclinical research in AUD.

PUBBLICATIONS

I. Borruto A.M., Fotio Y., Stopponi S., Petrella M., **De Carlo S.**, Domi A., Ubaldi M., Weiss F., Ciccocioppo R. (2021). NOP receptor antagonism attenuates reinstatement of alcohol-seeking through modulation of the mesolimbic circuitry in male and female alcohol-preferring rats. *Neuropsychopharmacology*, 46(12):2121-2131. doi: 10.1038/s41386-021-01096-1.

II. Cecarini V., Gogoi O., Bonfili L., Veneruso I., Pacinelli G., **De Carlo S.**, Benvenuti F., D'Argenio V., Angeletti M., Cannella N., Eleuteri A.M. (2022). Modulation of gut microbiota and neuroprotective effect of a yeast-enriched beer. *Nutrients*, 14(12):2380. doi: 10.3390/nu14122380.

III. Cannella N., Borruto A.M., Petrella M., Micioni Di Bonaventura M.V., Soverchia L., Cifani C., **De Carlo S.**, Domi E., Ubaldi M. (2022). A role for Neuropeptide S in alcohol and cocaine seeking. *Pharmaceuticals*, 15(7):800. doi: 10.3390/ph15070800.

IV. Benvenuti F., **De Carlo S.**, Rullo L., Caffino L., Losapio L.M., Ubaldi M., Soverchia L., Cannella N., Candeletti S., Fattore L., Romualdi P., Fumagalli F., Trezza V., Roberto M., Ciccocioppo R. (2023). Early social isolation differentially affects the glucocorticoid receptor system and alcohol-seeking behavior in male and female rats. *Neuropsychopharmacology (submitted).*

V. **De Carlo S**, Ciccocioppo R. (2023). Genetically selected alcohol-preferring rats as a model of alcohol use disorder. (i*n preparation*).

VI. Della Valle A., **De Carlo S.**, Ciccocioppo R., Ubaldi M. (2032). Deep learning approach for the recognition of behaviors in Forced Swim Test. (*in preparation*).

VII. Faniyan O., Simayi R., **De Carlo S.**, Ciccocioppo R., Bellesi M., De vivo L. (2023). Adolescent chronic sleep restriction promotes alcohol binge drinking and escalates alcohol consumption in msP rats. (*in preparation*).

VIII. Borruto A.M., Petrella M., **De Carlo S.**, Cannella N., Weiss F., Ciccocioppo R. (2023). Genetic deletion of nocipetin/orphanin FQ receptors does not alter associative memory in rats. (i*n preparation*).

Chapter 1

Introduction

1.1. ALCOHOL USE AND MISUSE: ETHANOL METABOLISM AND EFFECTS

Alcohol is a small, water and lipid soluble molecule which diffuses and distributes easily through cell membranes to be quickly absorbed in blood, and then it reaches all body tissues including the central nervous system (CNS) (Pleuvry, 2005). Once ingested, only a small amount (2-10% *v*/*v*) of alcohol is expelled through the urine, whereas most of it is metabolized by two main enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Edenberg, 2007).

Ethanol (EtOH) is how alcohol in standard beverages is chemically defined.

A standard drink (approximately 10-12 grams of ethanol) can increase the blood alcohol concentration (BAC) to about 20 mg/dL. In general, the liver can metabolize one drink per hour decreasing the BAC at a rate of 15-20 mg/dL, but there are individual variations in alcohol metabolism which depend on several factors including genetic factors (variations in the enzymes), age, weight, liver health and function, and gender (Birley, et al., 2008; Hahn & Burch, 1983; Kwo, et al., 1998; Thomasson, 2002).

Alcohol is a depressor of the CNS and it interacts with several enzymes, receptors, and neurotransmitters producing short- and long-term physical and physiological consequences including the development of alcohol use disorder (AUD) (Fadda & Rossetti, 1998; Harper & Matsumoto, 2005; Ross & Peselow, 2009). The early short-term effects of alcohol consumption include a sensation of well-being, lowered inhibition, talkativeness, enhanced sociability, self-confidence, and loss of coordination (American Psychiatric Association, 2013; Dubowski, 2006). Increases in BAC (200-300 mg/dL) worsen the individual's state of health: the subject starts to experience sedation, nausea, vomiting, mental confusion, disorientation, disturbances of vision, decreased response to stimuli, impaired consciousness, sleep, or stupor. Excessive BAC (300-400 mg/dL) can even cause impairment in circulation and respiration, complete unconsciousness (coma or anaesthesia), and even death in nontolerant individuals (American Psychiatric Association, 2013; Dubowski, 2006). Repeated episodes of heavy drinking bring a series of long-term effects, such as diminished grey and white matter in some brain regions, eventually causing brain

atrophy, alcoholic hepatitis, liver fibrosis, high blood pressure, stroke, irregular heartbeat, and cancer (Harper & Matsumoto, 2005; National Institute on Alcohol Abuse and Alcoholism, 2004; Pfefferbaum, et al., 1995; Piano, 2017). Finally, chronic use of alcohol can lead to developing AUD.

1.2. ALCOHOL USE DISORDER

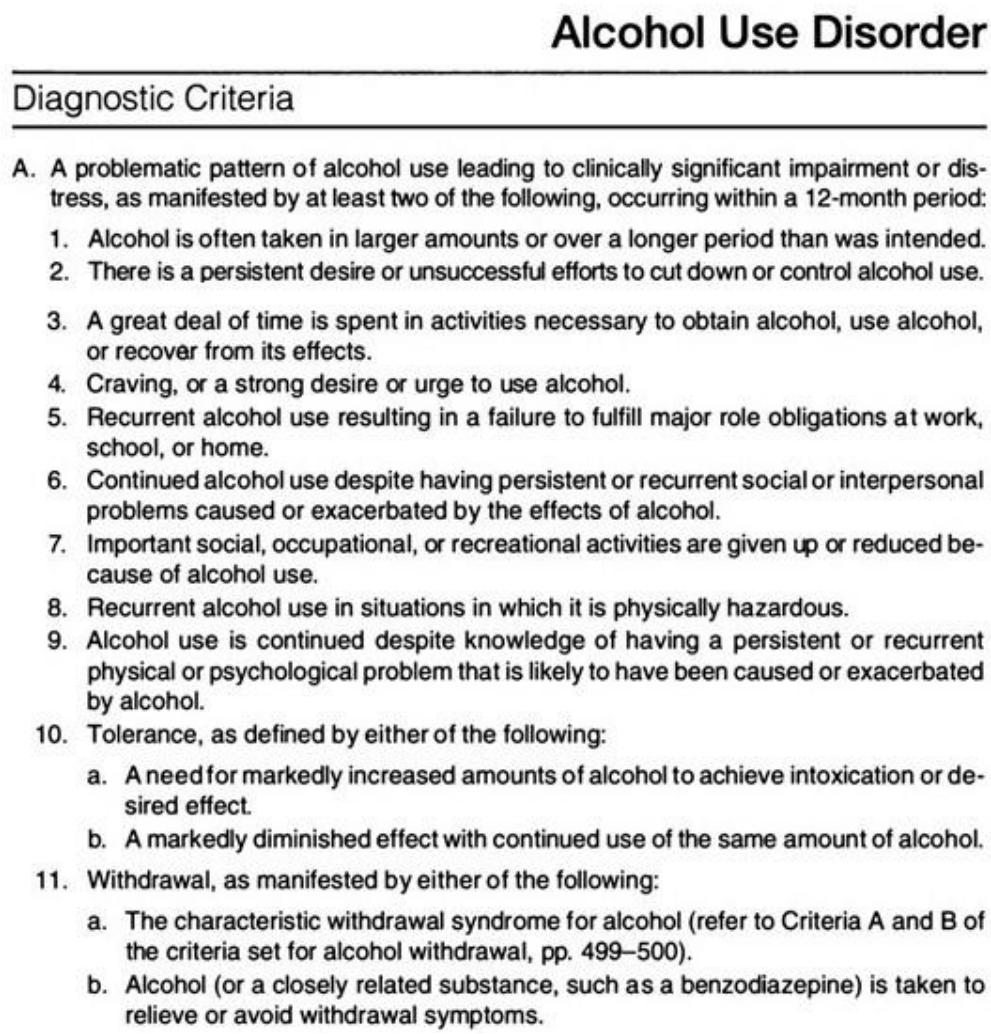
AUD is a heterogeneous and progressive brain disorder causing approximately 3.3. million deaths (5.3% of all deaths globally) and more than 200 diseases and injury conditions per year (World Health Organization, 2018). About 2.1 billion people worldwide (43% of adults) drink alcohol and 76.3 million people globally reported to suffer from AUD (World Health Organization, 2018). The first contact with alcohol usually occurs during adolescence and indeed in 2019, 7.0 million young people aged between 12 and 20 reported to have at least tried alcohol once as well as 414,000 adolescents (12-17 years old) referred to have AUD (Substance Abuse and Mental Health Services Administration, 2019). In 2019 the World Health Organisation (WHO) identified the European Region, the Region of the Americas, and the Western Pacific Region as the regions with the highest alcohol consumption among men and women (World Health Organization, 2021). Moreover, since the emergence of COVID-19 pandemic started, people reported using more drugs of abuse and alcohol than before (World Health Organization, 2021) and the number of alcohol-related deaths increased by about 25% (White, et al., 2022).

In light of these numbers becomes clear how AUD is a very relevant issue needed to be considered a serious public health problem.

1.2.1. AUD DIAGNOSIS AND THERAPEUTIC APPROACHES

According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (American Psychiatric Association, 2013) AUD (along with other substance use disorders (SUDs)) is characterized by cognitive - impairments in memory, decision-making, cognitive control, self-efficacy -, behavioral, and

physiological symptoms - withdrawal, tolerance, and craving - that altogether contribute to making the diagnosis. The AUD diagnosis is based on a total of 11 criteria grouped into four overall criteria: impaired control, social impairment, risky use, and pharmacological criteria. AUD is a spectrum disorder and its severity depends on the number of diagnostic criteria attested: it can be diagnosed as mild (if the patient meets 2-3 out of 11 criteria), moderate (presence of 4-5 criteria), or severe (6 or more criteria) with symptoms occurring within a 12-month period (American Psychiatric Association, 2013) (***Figure 1***).



Alcohol Use Disorder

Diagnostic Criteria

A. A problematic pattern of alcohol use leading to clinically significant impairment or distress, as manifested by at least two of the following, occurring within a 12-month period:

1. Alcohol is often taken in larger amounts or over a longer period than was intended.
2. There is a persistent desire or unsuccessful efforts to cut down or control alcohol use.
3. A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects.
4. Craving, or a strong desire or urge to use alcohol.
5. Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home.
6. Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol.
7. Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
8. Recurrent alcohol use in situations in which it is physically hazardous.
9. Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
10. Tolerance, as defined by either of the following:
    a. A need for markedly increased amounts of alcohol to achieve intoxication or desired effect.
    b. A markedly diminished effect with continued use of the same amount of alcohol.
11. Withdrawal, as manifested by either of the following:
    a. The characteristic withdrawal syndrome for alcohol (refer to Criteria A and B of the criteria set for alcohol withdrawal, pp. 499–500).
    b. Alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms.

Figure 1. DSM-5 AUD criteria (American Psychiatric Association, 2013).

Together with DSM-5 criteria, questionnaires are used to assess a patient's profile in clinical practice and research. Several of those have been developed and they can be filled by the physician or by the patient itself, such as the Alcohol Use Disorder Identification Test (AUDIT), Brief Drinker Profile (BDP), Severity of Alcohol Dependence Questionnaire (SADQ), and many others.

Behavioural treatments, mutual-support groups, and pharmacological medications are the three main classes of approaches currently used, usually in combination, to reduce or stop drinking and prevent relapse. Behavioural treatments are provided by licensed therapists, and they have the aim to change drinking behaviour by building motivation, teaching coping strategies, and improving skills. Mutual-support groups are based on peer support and they are at low or no cost. Four non-addictive pharmacological medications have been approved by the U.S. Food and Drug Administration (FDA) and the European Medications Agency (EMA): naltrexone, acamprosate, disulfiram, and, in Europe, also nalmefene.

Since opioid peptides participate to alcohol aetiology, it has been observed that the nonselective opioid receptor antagonists naltrexone and nalmefene can reduce alcohol intake activating the dopaminergic reward system in patients with some clinical characteristics (such as strong craving and family history) (Bogenschultz, et al., 2009; Gianoulakis, 1996; Gual, et al., 2014; Paille & Martini, 2014; Soyka, 2014). Acamprosate is a glutamate (Glu) receptor NMDA antagonist that re-established the excitatory/inhibitory (Glu/GABA) balance altered by chronic alcohol misuse, but its efficacy is inconsistent (Anton, et al., 2006; Lesch, et al., 2001). Lastly, disulfiram is always effective but requires a strong motivation by the patient: it inhibits the activity of ALDH interfering with alcohol metabolism and causing unpleasant effects like sweating, headache, nausea and vomiting (Mutschler, et al., 2016).

Treatments and medications for AUD are only discreetly effective, and quite often a treatment working for one person may not work for another one (Jonas, et al., 2014).

1.2.2. AUD AS A THREE-STAGES CYCLE

Over time AUD has been extensively investigated for its resistance to treatments as well as for being a heterogeneous and multifactorial disorder. Koob and Le Moal (1997) defined AUD as a chronically relapsing disorder associated with compulsion in alcohol seeking and taking, loss of control over alcohol intake, and the emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability, all contributing to what has been defined as a motivational withdrawal syndrome) when alcohol is no longer available (Koob & Le Moal, 1997). These key elements well match with AUD symptoms and criteria described in DSM-5 and previously exposed.

While on the one hand, the production of pleasant effects (i.e., positive reinforcement) and the relief from negative feelings and emotional discomfort (i.e., negative reinforcement) can motivate some people to consume alcohol, on the other hand, alcohol itself tends to enhance these negative states (including anxiety, irritability, and dysphoria) between bouts of consumption during abstinence. This phenomenon can motivate further drinking to alleviate an unpleasant emotional state and it can stuck the individual in a cycle of alcohol consumption (Armeli, et al., 2015; Koob & Schulkin, 2019). Indeed AUD, and more in general SUDs, has been framed as a three-stage cycle in which each stage is linked to and feeds on the others and an individual may go through this cycle for weeks, months, or even the same day (Koob, et al., 2020; Koob & Volkow, 2016). The three stages involve three domains mediated by three key brain regions: incentive salience – the basal ganglia; negative emotional states – the extended amygdala (AMG); executive function – prefrontal cortex (PFC) (Koob, et al., 2014; Koob & Le Moal, 1997) (***Figure 2***).

The first stage of the cycle is the **binge/intoxication stage**, during which a person experiences the rewarding effect of alcohol with a repeated activation of basal ganglia that reinforces drinking behavior increasing the likelihood to consume it again (positive reinforcement) and ultimately leading to habit formation and compulsive alcohol use. Basal ganglia involvement is also responsible for changes in the value of stimuli associated with alcohol drinking – people, places, paraphernalia, and every other stimulus presented at the moment of alcohol assumption - and in the way that

person responds to them (they become conditioned stimuli). In fact, neutral stimuli paired to the alcohol consumption become conditioned stimuli (conditioned reinforcement) and hence capable to trigger alcohol intense and urging desire (craving) becoming reinforcing themselves. The interruption of alcohol intake leads to withdrawal symptoms, namely negative emotional (dysphoria, irritability anxiety, emotional pain) and physical (sleep disturbances, pain, illness) states which generates a second motivational drive to alcohol. These symptoms act like negative reinforcement because the action performed to eliminate them increases the probability to perform again that response in the future. This is the second stage defined as **withdrawal/negative affect stage**. At this stage, the person no longer drinks alcohol for its pleasure and positive effects, but rather to avoid the negative states that abstinence produces. These negative states are a consequence of both a diminished activation in the basal ganglia's reward system (reward deficit) and an increased activation of the extended AMG responsible for the negative and unpleasant states (stress surfeit). Finally, protracted and repeated periods of abstinence as well as cue-induced and contextual craving contribute to the **preoccupation/anticipation stage**. At this stage, fundamental is the preoccupation about how to get alcohol since the craving has become imperative. The PFC is corrupted and the associated abilities of decision-making, contingencies, and outcomes representation, planning, and attention are now compromised (Koob, et al., 2014; Koob & Le Moal, 1997; Koob & Schulkin, 2019). Controlling behavioral responses is now particularly difficult for the individual, and impulsivity and compulsivity are the two relevant symptoms observable in AUD. Impulsivity has been defined by Moeller and colleagues as "a predisposition toward rapid, unplanned reactions to internal and external stimuli without regard for the negative consequences of these reactions to themselves or others" (Moeller, et al., 2001); hence, impulsivity is acting without forethought, without reflecting on the consequences of one's actions even choosing risky behaviors, and preferring immediate small rewards instead of waiting for more beneficial ones; it is a failure in motor inhibition. Instead, compulsivity is the persistence of behavioral responses despite negative and adverse

consequences or despite that response being inadequate and inappropriate to the situation. Compulsions are characterized by the inability to adapt behavior after negative feedback (Wolffgramm & Heyne, 1995). Habits are a type of compulsion, and they are responses, defined as conditioned, triggered by environmental stimuli (conditioned stimuli) regardless of the current desirability of the consequences of that response.

Considering the three-stage cycle described above, impulsivity often controls the early stages and compulsivity governs last stages. Shift from impulsivity to compulsivity is accompanied by a shift from positive reinforcement - increasing tension and arousal dominate before the impulsive behaviour and then pleasure, gratification or relief are felt as rewarding during the act - to negative reinforcement - removal of negative emotional state due to withdrawal trigger the compulsive act to experience relief from - into the drive of alcohol seeking and intake behaviour (Koob, 2014). However, it is important to highlight that impulsivity and compulsivity can also coexist in different stages of the cycle (Berlin & Hollander, 2014).

In conclusion, in AUD the initial recreational use of alcohol evolves into impulsive episodes of **binge drinking** in which alcohol is assumed in large quantities and in a short period of time with severe emotional and physical withdrawal symptoms. While many individuals remain in the first stage of binge/withdrawal without experiencing the other stages, others may begin to need alcohol compulsively to avoid the negative consequences of abstinence (**withdrawal/negative affect stage**). Strong preoccupation with alcohol obtaining and intense craving develops: stimuli associated with alcohol intake and the negative withdrawal acquire a strong power and alcohol is now a way to avoid stress, anxiety, dysphoria, and discomfort deriving from abstinence (**preoccupation/anticipation stage**).

Clarifying and understanding which changes in which brain areas are responsible for people's variability to step into the next stage of the cycle or not as well as to respond to some treatments and not to others, has been subjected of intense experimental research.

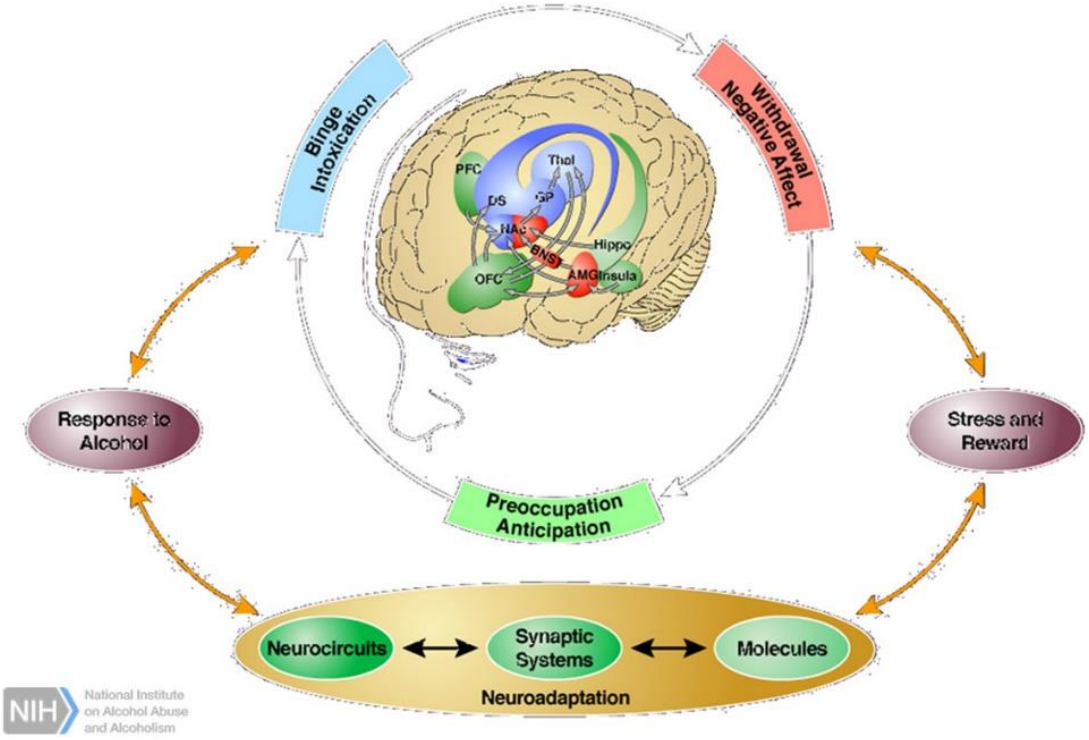


Figure 2. Schematic illustration of the neurocircuitry of addiction divided into the three-stages cycle responsible for the transition to the AUD: binge/intoxication, blue; withdrawal/negative effect, red; and preoccupation/anticipation, green. The same color-coded is maintained to individuate the neurocircuits involved. The Basal ganglia, including the nucleus accumbens (NAc), dorsal striatum (DS), globus pallidum (GP), and thalamus (Thal) as key elements of the binge/intoxication stage; the extended amygdala, including the central nucleus of the amygdala (AMG), bed nucleus of the stria terminals (BNST), and a transition area in the shell of the nucleus accumbens (NAc) as key elements of the withdrawal/negative affect stage; and the frontal cortex and allocortex, including the prefrontal cortex (PFC), orbitofrontal cortex (OFC), hippocampus (Hippo), and insula (Insula) as key elements of the preoccupation/anticipation stage. Molecular, synaptic, and neurocircuitry neuroadaptations combine to render the four key elements of the transition to addiction: increased incentive salience, decreased reward, increased stress, and decreased executive function *(Uhl, et al., 2019)*.

1.3. NEUROCIRCUITRY OF AUD

All stimuli (natural stimuli such as food, sex, social interaction, as well as substances of abuse) evaluated as rewarding and acting as positive reinforcement activate the same brain circuitry but the strength and intensity with which they do that is different

(NIDA, 2020). As claimed by the DSM-5 (American Psychiatric Association, 2013), all substances of abuse have in common a strong and intense activation of the reward brain circuitry, and all SUDs, including AUD, induce changes in brain circuits that persist even after detoxification and long periods of abstinence. The difference among substances of abuse relies on how they determine the release of neurotransmitters: briefly, alcohol does that by enhancing GABA release and reducing Glu release (***Figure 3***).

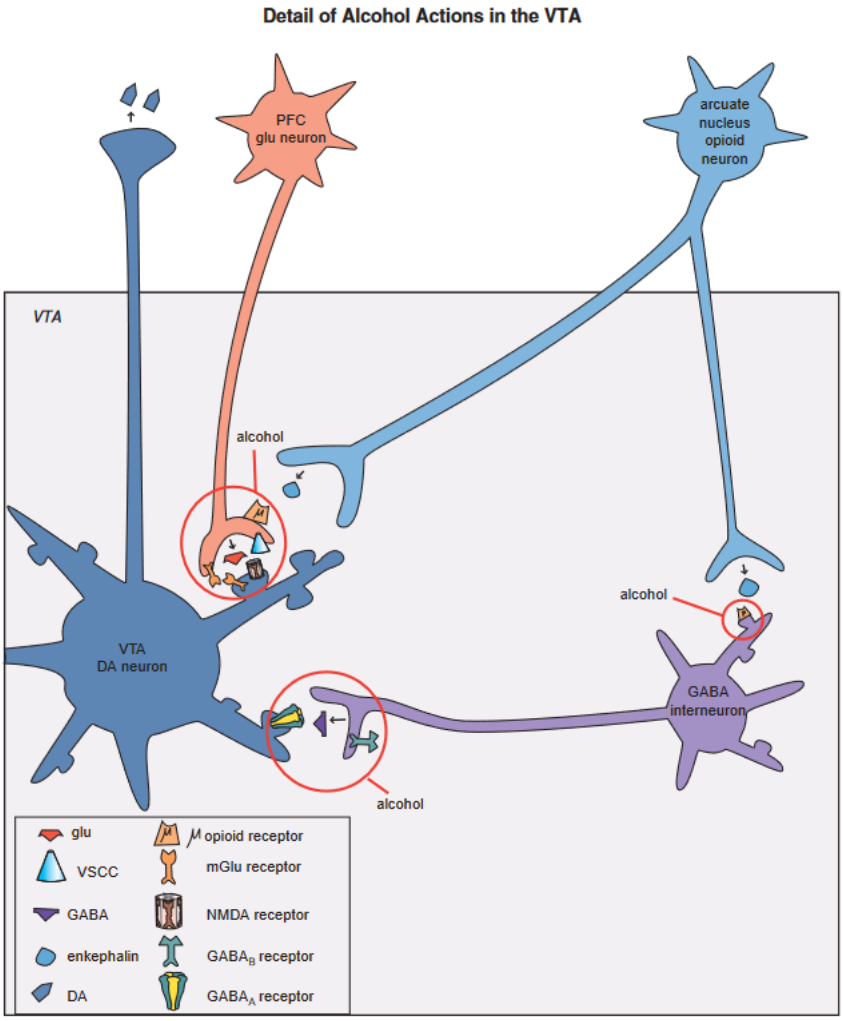


Figure 3. Representation of alcohol actions in the VTA. Opioid neurons synapse in the VTA with GABAergic interneurons and presynaptic nerve terminals of Glu neurons. Inhibitory actions of opioids at μ-opioid receptors cause disinhibition of DA release in the NAc. Alcohol either directly acts upon μ receptors or causes the release of endogenous opioids such as enkephalin. Alcohol also acts at

presynaptic metabotropic Glu receptors (mGluRs) and presynaptic voltage-sensitive calcium channels to inhibit Glu release. Finally, alcohol enhances GABA release by blocking presynaptic GABAB receptors and through direct or indirect actions at GABAA receptors *(Stahl, 2013)*.

The three stages of the addiction cycle involve neuroplastic changes in brain reward, stress, and executive function systems. Neuroadaptations observed in SUDs affect the so-called **reward system**, namely the **mesocorticolimbic system**: "meso" for the involvement of the ventral tegmental area (VTA) in the midbrain, "cortico" because cortices, especially the PFC, are implicated, and "limbic" for the strong contribution of structures belonging to the limbic system such as the nucleus accumbens (NAc), AMG, and hippocampus (Hippo) (***Figure 4***).

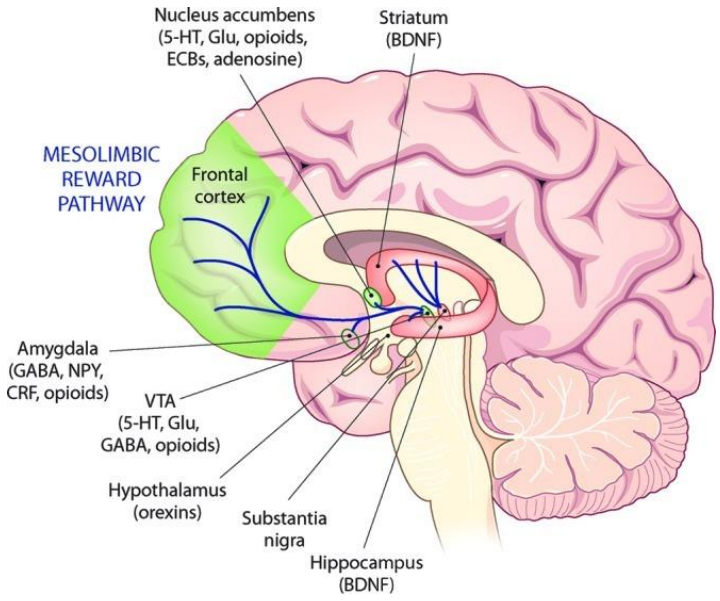


Figure 4. Schematic representation of the human mesocorticolimbic reward pathway and its neurotransmitters modulated by alcohol. 5-HT = serotonin, BDNF = brain-derived neurotrophic factor, CRF = corticotropin-releasing factor, ECBs = endocannabinoids, Glu = glutamate, NPY = neuropeptide Y, VTA = ventral tegmental area *(Gass & Olive, 2012)*.

The name of this circuitry has arisen from numerous evidence on its role in stimuli evaluation: it attributes valence, motivation, importance, and reinforcing value to stimuli, and energizes the goal-directed pursuit of stimuli with positive valance (acting as positive reinforcement) and then called rewards. To perform this task the first and important messenger used by the system is the neurotransmitter dopamine (DA) (Koob, et al., 1998; Lüscher, et al., 2020).

1.3.1. VTA, NAc AND PFC: NEURONAL CHARACTERISTICS AND CONNECTIVITY

The first brain area activated by alcohol is the VTA, which is involved in the neural response to a reward and on which the activity of the other areas depends. It is localized in the superior part of the midbrain and contains DAergic cell bodies that project to the limbic structures and cortical areas (Dobi, et al., 2010). Together with the nigrostriatal system, the DAergic projections from VTA represent the main DA system in the brain (Swanson, 1982). The VTA also contains populations of GABAergic and Gluergic neurons and all these three types of neurons internally communicate with each other regulating the DAergic and non-DAergic neurotransmission from the VTA to the connected structures (Carr & Sesack, 2000; Kalivas, 1993; Margolis, et al., 2006). Of all DAergic neurons in VTA, the majority of them (almost 85%) projects to the NAc, 50% of neurons projects to the AMG, and almost the 30% of them to the PFC (Swanson, 1982). The VTA receives its main excitatory Gluergic and cholinergic inputs from several brain regions including the PFC (Kalivas, 1993), as well as it also receives inhibitory GABAergic inputs from the NAc shell and the ventromedial ventral pallidum (Root, et al., 2015).

NAc is part of the ventral striatum (VS) and 90-95% of its neurons are GABAergic cells (Gerfen, 1992). Shell is the most external part of NAc: it receives DAergic inputs from the VTA and the substantia nigra (SN) and Gluergic inputs from the ventromedial PFC and insular cortex, infralimbic areas, basolateral AMG (BLA), ventral Hippo, and the paraventricular nucleus of the thalamus, and it projects GABAergic signals to ventromedial pallidum and VTA (Gabbott, et al., 2005;

Reynolds & Zahm, 2005; Zahm, 2000; Papp, et al., 2012; Britt, et al., 2012). The most internal portion of the NAc is called core: it receives DAergic inputs from the VTA and the SN as well as Gluergic inputs from the BLA, dorsomedial PFC, insular and OFC cortices, and prelimbic areas, whereas it sends GABAergic projections to the dorsolateral part of the ventral pallidum, ventrolateral SN, and VTA (Gabbott, et al., 2005; Reynolds & Zahm, 2005; Zahm, 2000). Core and shell seem to have different tasks: lesions of the NAc core impair the ability to flexibly adapt behavior based on changes in outcome value, whereas lesions of the NAc shell damage the ability of outcome-associated cues to bias action selection (Corbit, et al., 2001). The GABAergic efferences from the NAc originate from the medium spiny neurons (MSNs), cells that can be distinguished into two classes based on their projections' patterns: MSNs expressing DAergic 1 receptors (D1Rs) and projecting mainly to the VTA (direct striatal pathway) and SN, and MSNs expressing DAergic 2 receptors (D2Rs) and projecting mainly to the ventral pallidum (indirect striatal pathway) (Tripathi, et al., 2010; Zhou , et al., 2003), whereas just a small population of MSNs express both D1Rs and D2Rs and are mostly confined in the NAc shell (Bertran-Gonzalez, et al., 2008). Thanks to these extensive connections, the NAc has a key role in valence attribution to alcohol.

Moreover, the NAc plays a role in energizing the goal-directed behavior toward alcohol obtaining and consumption. The NAc is considered a sensory-motor interface allowing the limbic system to access the motor systems via the SN, determining the passage from a motivational evaluation of a reward to the implementation of an action to obtain it (Mogenson, et al., 1980).

The most frontal part of the frontal lobe takes the name of PFC and it can be divided into: a medial part that includes the anterior cingulate, precentral and dorsal prelimbic cortices dorsally, and the ventral prelimbic, infralimbic, dorsal peduncular and medial orbital cortices ventrally; a lateral zone composed of the OFC and the dorsal and ventral anterior insular cortices; a ventral PFC containing the ventral orbital and ventral lateral orbital cortices (Robbins , 2000). The majority of cells in the PFC are represented by Gluergic pyramidal neurons under the control of local

GABAergic interneurons (Wilson, et al., 1994), that with their ascending projections generate highly organized connections with several structures. These wide connections modulate other neurotransmitter systems and hence allow the PFC to exert an important role of control over several brain areas. Therefore, the PFC is fundamental for high-level cognitive and executive functions, such as decision-making, memory, attention, vigilance, arousal, emotion, stress, planning, social behavior, and many others. For instance, the PFC performs an inhibitor control over subcortical regions (top-down control) in order to block habit behaviors, selecting a new action and monitoring its execution (Ridderinkhof, et al., 2004; Moorman & Aston-Jones, 2015). The PFC, and especially the OFC, controls the flexibility of behavior by evaluating the specific situation, estimating possible outcomes, deciding about the best action to engage, and being able to change that action according to situation changes (Parkes , et al., 2018).

1.3.2. NEUROBIOLOGICAL MECHANISMS UNDERLYING THE THREE STAGES OF AUD

At the first **binge/intoxication stage** of the addiction cycle, the VTA processes alcohol and its properties, sending DA to the connected brain areas belonging to the mesocorticolimbic circuitry (Volkow, et al., 2007). DA release as the effect of alcohol intake is particularly fast and vast compared to the release as result of a natural reward. The main target of this huge DA release from VTA is the NAc shell in the limbic system (Koob, 1992). Here DA binds and activates both the low-affinity D1Rs (only activated by a sharp DA increase) and the high-affinity D2Rs (already stimulated by lower levels of DA) producing the strong rewarding effects of alcohol and triggering conditioned responses to it (Caine, et al., 2007; Steinberg, et al., 2014; Zweifel, et al., 2009).

With repeated exposure to alcohol, a shift from VTA to other brain regions occurs. Studies in animals have revealed that GABAergic neurons in NAc encode both the value of reward and cues contingent on it and the action necessary to achieve that reward (Nicola, et al., 2004; Taha, et al., 2007). Receiving DA from the VTA, the NAc

not only mediates alcohol-rewarding properties but also assigns motivational salience to what is contingent and associated with alcohol such that it acquires rewarding properties itself as well as the ability to predict alcohol arrival. D1Rs are those mainly involved in conditioned reinforcement (Ungless, et al., 2001) through the Gluergic projections from AMG (emotional reactivity), Hippo (memory), and PFC (salience attribution). In a recent optogenetic study, after mice have acquired optogenetic self-stimulation of DAergic neurons in the VTA, cue-induced relapse following a week of abstinence was accompanied by a potentiation of excitatory afferents onto MSNs expressing D1Rs in the NAc (Pascoli, et al., 2015). Repeated and constant alcohol consumption leads to sensitize D1Rs signalling. Progressively the release of DA in NAc in response to a conditioned stimulus increases so much that they induce alcohol craving and compulsive use even when alcohol pharmacological effects lessen. This mechanism is partly responsible for the individual to take again the substance (relapse) even after a long period of abstinence. Moreover, during abstinence increases in stress and anxiety-like responses also occur and those contribute greatly to the negative emotional state characterizing abstinence (Koob & Le Moal, 2005).

Repeated exposures to alcohol to lead within-system neuroadaptations in brain areas of the mesocorticolimbic circuitry. DAergic transmission in NAc strongly decreases during withdrawal (Volkow, et al., 2007) and it can be responsible for the loss of interest in normal rewards not related to alcohol. Emotional dysregulation is typical of the second stage of the cycle, the **withdrawal/negative affect stage**, and it is also caused by between-system neuroadaptation for chronic activation of the reward system as a result of heavy and constant alcohol consumption (Koob & Bloom, 1988). During withdrawal elevated adrenocorticotropic hormone, corticosterone, and corticotropin-releasing factor (CRF) are extensively released in the extended AMG as well as in the hypothalamic-pituitary-adrenal (HPA) axis. With repeated episodes of acute or prolonged withdrawal, these brain systems dysregulate, contributing to the negative emotional states typical of obstinance and responsible for relapse (Koob, et al., 2014; Piazza & Le Moal, 1996). The combination of decreased reward function and

increased stress function in VS and extended AMG is a powerful trigger of negative reinforcement that contributes to craving and compulsive alcohol-seeking and -consumption behavior.

Whereas at the beginning alcohol was voluntarily drunk for its reinforcing and rewarding properties, at this stage it is wanted, searched, and consumed to avoid or put an end to the aversive physical and physiological consequences that its absence causes. A loss of control characterized the **preoccupation/anticipation stage**. The transition from controlled to compulsive and impulsive drug seeking and consumption may be caused by a downregulation of D2Rs in NAc which is responsible for the activity imbalance of frontostriatal circuits underlying goal-directed behavior. Repeated exposure to alcohol reduces the number of D2Rs which act by inhibiting the indirect pathway and leads to hyperactivity of the NAc and reduction in the control capability of the PFC (Volkow, et al., 2006; Volkow, et al., 2009). The PFC becomes less and less active, whereas brain areas involved in habit formation and performing (the nigrostriatal pathway) start to be more engaged. A loss of prefrontal top-down control to mesolimbic regions is hence postulated. The dysregulation of the PFC acts in two directions: a cognitive impairment including poor working memory, inattention, loss of planning and decision-making, and a cue-induced craving and relapse. Indeed it has been demonstrated that even if the PFC is hypofunctional in normal conditions in people with AUD, it starts to strongly respond when those individuals are exposed to drug-conditioned cues compared to naïve controls (Goldstein & Volkow, 2011; Kilts, et al., 2001; Volkow, et al., 1996). Indeed, changes in DA firing in response to stress and conditioned stimuli further alter the strength of cortico-striatal Gluergic synapses altering signaling in D1R- and D2R-expressing MSNs in NAc (Paladini & Roeper, 2014).

In conclusion, chronic alcohol misuse leads to structural and functional alterations of several brain regions and their connectivity that persist even after detoxification and long periods of abstinence.

Identification of alcohol consumption-induced changes in the neurobiological circuits of the reward system provides key information about vulnerability, resilience, treatment, and recovery from AUD.

1.4. HETEROGENEITY OF AUD

1.4.1. CLINICAL CLASSIFICATIONS

As also recognized by DSM-5, not all individuals are equally vulnerable to developing AUD. There are people who after some initial assumptions won't re-intake alcohol, others that can remain at the initial phase of recreational use without becoming dependent, and others that, after a few intakes, quickly escalate in their consumption and develop AUD.

Alcohol consumption itself is not sufficient to develop AUD. A complex combination of genetic, psychopathological, and environmental factors makes some individuals more vulnerable to escalate alcohol use and develop AUD than others who show a resilient phenotype. Differences in pattern of alcohol consumptions and alcohol-related behaviors between patients with AUD have been also individuated.

In the past years, several clinical classifications of AUD have been proposed to facilitate the identification of the most appropriate treatment for different patients (Leggio & Addolorato, 2008; Roache, et al., 2008). Jellinek proposed the first classification in 1960 (Jellinek, 1960) dividing patients suffering from AUD into 5 groups based on their clinical characteristics, namely signs of physical dependence, craving, tolerance, and loss of control. Later, Cloninger and colleagues (Cloninger, et al., 1981) individuated type 1 and type 2 AUD patients and this classification is still the most used in AUD field. Type 1 AUD grouped both male and female patients in which the disorder raised in adulthood, after 25 years of age, mostly due to environmental events, while type 2 includes male patients with aggressive behavior that often also use other substances of abuse, and which initiated alcohol use early in life (before 25 years of age) having some relative affected by AUD (genetic predisposition as cause). The two types also differ in neurotransmitter deficits:

DAergic transmission deficit in type 1 and serotoninergic transmission deficit in type 2 (Mantere, et al., 2002; Storvik, et al., 2007). In the 1990s, based on a 5-year study and neurobiological, heredity, and psychopathological features, Lesch and colleagues suggested 5 categories named from type I to type IV (Lesch & Walter, 1996). Most recently, Moss and colleagues distinguished 5 subtypes using the National Epidemiologic Survey on Alcohol and Related Conditions and considering DSM criteria (Moss, et al., 2007).

Despite efforts to group AUD patients into more homogeneous subpopulations, AUD shows a vast heterogeneity in aetiology and phenotype which is very complicated to frame in a mere classification.

1.4.2. GENE X ENVIRONMENT INTERACTION IN AUD

Heterogeneity in AUD lies in both the aetiology and phenotype of the individual. Concerning the aetiology, AUD derives from a complex interaction between genetic factors and environment (GxE) (***Figure 5***).

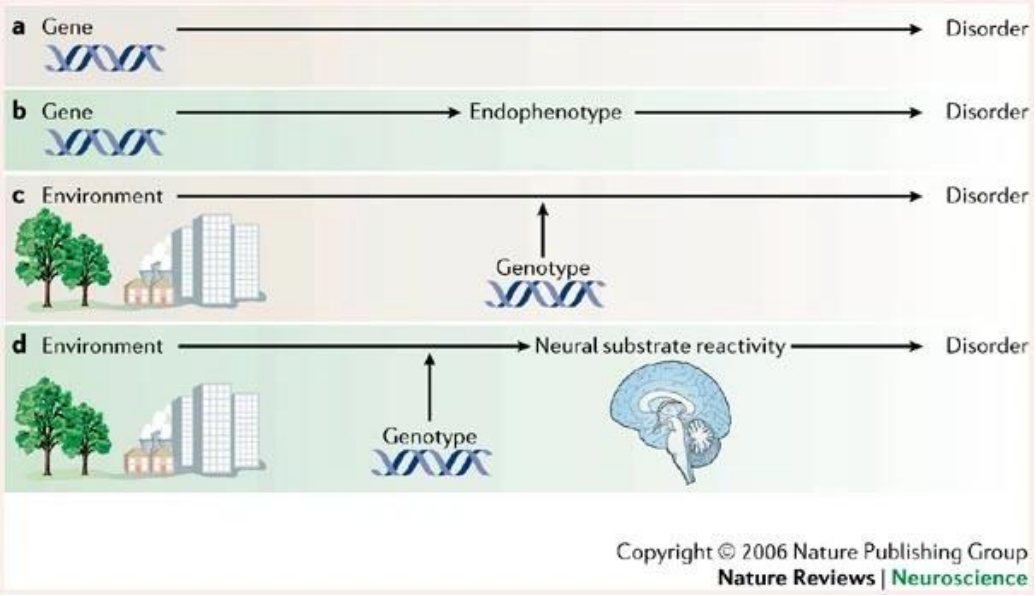


Figure 5. Different approaches used in psychiatric genetics research. **a.** A direct linear relations is assumed between genes and disorder. **b.** Intermediate endophenotypes are theorized between gene action and the disorder outcomes. **c.** the genotype is assumed to moderate the effect of environmental

pathogens on the disorder. **d.** Neuroscience complements the latter research by specifying the proximal role of nervous system reactivity in the gene-environment interaction *(Caspi & Moffitt, 2006)*.

Studies on twins who grew up in their biological family or who have been adopted have demonstrated a heritability for AUD between 50 to 60% (Heath, et al., 1997; Kohnke, 2008; Worst & Vrana, 2005) and a significantly higher rate of AUD was found in monozygotic twins compared to dizygotic twins (McGue, 1999). Several genes have been hypothesized to be involved in AUD aetiology revealing the polygenicity of this disorder: genes encoding for neurotransmitters, receptors, cell mechanisms, and metabolic functions (Goldman, et al., 2005; Rodd, et al., 2007). Furthermore, genetic factors seem to be involved not only in the initial alcohol use, but also in the individual quantity, modality, and frequency of alcohol consumption (Dick & Foroud, 2003; Hiroi & Agatsuma, 2005). More than 30 molecular targets have been identified as altering an individual's craving or consuming behavior (Litten, et al., 2012). Different types of people affected by AUD exist, and not everyone escalates alcohol use in the same way and goes through every single phase of the addiction cycle, rather different patterns can eventually lead to compulsive alcohol seeking and taking (George & Koob, 2010). Indeed, some relevant individual differences have been individuated in the sensitivity to the pharmacological effects of alcohol, frequency, and modality of alcohol consumption (e.g. binge drinking or not), resistance to abstinence symptoms, sensitivity to alcohol-associated stimuli and to stress in inducing relapse, and sensitivity to relapse (George & Koob, 2010). All these differences in AUD patients cannot be fully explained by genetics because AUD has not been linked to any specific gene.

The environment also plays a key role in AUD aetiology modulating genes' influence on the final individual phenotype (Caspi & Moffitt, 2006). For instance, even if genetic transmission of AUD has been demonstrated, it is also true that risky behaviors are transmitted from parents to children living in the household through direct and indirect teaching. Indeed, AUD tends to be more frequent in individuals who have grown up in a family environment tolerant to alcohol drinking (McGue, 1999;

Sloboda, et al., 2012) and higher rates of AUD have been observed in both males and females with high genetic risk developed in the aversive environment compared to those with low genetic risk and those with low environmental risk (Cloninger, et al., 1981; Cutrona, et al., 1994; Sigvardsson, et al., 1996). Peer influence is another social factor relevant to AUD risk (Kendler, et al., 2008), especially for adolescents: often the first contact with alcohol occurs in the peer group (Donovan, 2004; Mundt, 2011) and the positive experience associated with that event encourages and shapes adolescents' drinking behaviors (Chartier, et al., 2010). Among adolescents with high genetic AUD risk, the percentage developing AUD is higher among those having friends consuming alcohol compared to those whose peers do not consume alcohol (Dick, et al., 2007). Not only family and peers, but also other social factors - low socioeconomic status, resident instability, and low community supervision - contribute to increasing AUD risk, especially among the youngest (Winstanley, et al., 2008). Clinical studies reported that the heritability of AUD in adolescence was greater in urban than rural environments (Legrand, et al., 2008; Rose, et al., 2001).

The age of the first drink is an important factor in determining the probability to develop AUD and its characteristics (Pitkänen, et al., 2008; Robins & Przybeck, 1985). A recent survey by National Survey on Drug Use and Health (NSDUH) (Substance Abuse & Mental Health Data Archive (SAMHDA), 2018) showed that people who began drinking before age 15 were 5.6 times more likely to report suffering from AUD compared to those who started drinking at age 21 or later. Moreover, more variations in AUD symptoms seem to be associated with early drinking and attributable to a greater genetic influence than later drinking onset (Agrawal, et al., 2009).

Other prominent risk factors in AUD development are psychiatric disorders (that quite often have a base of heritability) and a history of trauma (physical maltreatment such as sexual abuse or violence). Depression, anxiety disorders, post-traumatic stress disorder (PTSD), and attention deficit hyperactivity disorder (ADHD) are comorbid with AUD (Blanco, et al., 2013; Conway, et al., 2006; Enoch, et al., 2008; Kushner, et al., 2011) and several neuropsychiatric disorders tend to emerge during the sensitive period of adolescence (Costello, et al., 2003; Kaplan, et al., 1998; Paus, et al., 2008).

Among these numerous risk factors, stress – especially if chronic and unpredictable - is a very potent one that not only can be responsible to relapse in AUD patients (Koob & Schulkin, 2019), but it can even facilitate alcohol consumption in adults without AUD because alcohol reduces negative feelings and tension caused by stressors thanks to its sedative effects (de Wit, et al., 2003). Moreover, increased sensitivity to psychosocial stress and increased responsiveness to anxiety-relief effects of alcohol have been reported in alcohol naïve people whose fathers suffer of AUD compared to people with no alcohol history in their family (Zimmermann, et al., 2004)

Finally, differences between men and women have been reported in several AUD-related aspects. Although historical data indicates a higher prevalence of AUD in men than in women (Wilsnack, et al., 2000), recent evidence has emerged about the narrowing of this gap (White, et al., 2015). In recent years, the rate of AUD in women has increased by 84%, relative to a 35% increase in men (Grant, et al., 2017) and during the COVID-19 pandemic the increase in alcohol consumption was higher in women than in men (Pollard, et al., 2020; Tucker, et al., 2022). Different reasons drive alcohol use and misuse in men and women (Peltier, et al., 2019; Schulte, et al., 2009). Whereas women usually initiate alcohol consumption as a coping strategy to ameliorate negative affective states (e.g., anxiety, depression, stress), in men drinking is often begun for recreational purposes, especially among young people (Buchmann, et al., 2010; Crutzen, et al., 2013; Oscar-Berman, et al., 2014; Peltier, et al., 2019). Numerous clinical studies have shown a greater association between a history of early life stress and childhood maltreatment and the likelihood of developing AUD in adulthood in women rather in men (Anda, et al., 2002; Dinwiddie, et al., 2000; Osofsky, et al., 2021). Moreover, sex differences in the AUD trajectory have been documented. Compared to men, women tend to shift from recreational alcohol use to compulsive drinking more rapidly (Becker, et al., 2017; Becker, et al., 2012), escalate their alcohol use quickly (Anglin, et al., 1987; Becker, et al., 2017; Bobzean, et al., 2014), and relapse in response to stressful events more likely (Greenfield, et al., 2007; Hudson & Stamp, 2011; Walitzer & Dearing, 2006). Finally, after a prolonged period of alcohol consumption, women have a higher risk of developing physical pathologies, such as

breast cancer, cardiovascular problems, and liver inflammation, than men (Ashley, et al., 1977; Smith-Warner, et al., 1998; Urbano-Márquez, et al., 1995).

All these data make evident how AUD is a complex multifactorial disorder in which the GxE interaction is fundamental in its aetiology, development, and characteristics. Since the range of potential environmental influences and the interaction between them and genetic background is vast, GXE research in animal models becomes extremely important to provide inferences about causation and predictors of treatment responses. Controlling genetic and environmental factors in animals would permit to clarify the neurobiology and genetics of AUD and how all different aspects involved in AUD interact with each other. The ultimate goal is to develop better and more effective medical interventions specific to the individual.

For this purpose, choosing the most suitable rodent model is fundamental.

Over the years, several animal models have been developed to better investigate the single or combined role of genetics and environment in the aetiology and progression of AUD.

1.5. ANIMAL MODELS OF AUD

1.5.1. METHODS TO INVESTIGATE AUD IN ANIMAL MODELS

Understanding the complex mechanisms by which alcohol acts on the brain inducing the AUD development over time has been possible through neuroimaging studies on humans but especially by using animal models.

As a complex and heterogeneous disorder, AUD investigation benefits from the use of animal models in preclinical research. Thanks to their behavioral, neuroanatomical, and neurochemical similarities (***Figure 6***) to humans, rodents have been extensively used to model and investigate human conditions and disease (Taylor & Alvarez, 2019).

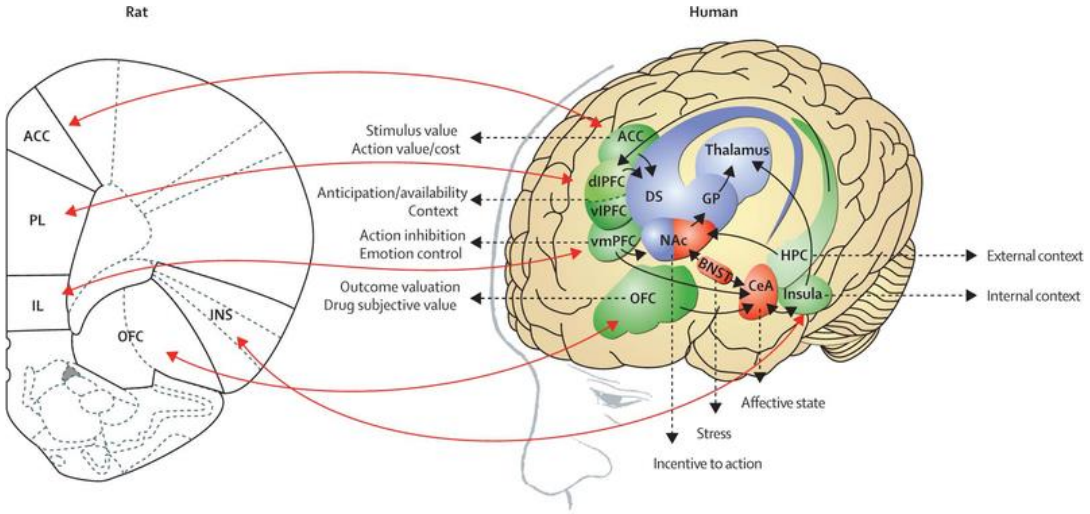


Figure 6. Representation of the correspondence between rat and human main brain regions of mesocorticolimbic circuitry involved in AUD. ACC = anterior cingulate cortex, PL = prelimbic cortex, IL = infralimbic cortex, OFC = orbitofrontal cortex, INS = insula, dlPFC = dorsolateral prefrontal cortex, vlPFC = ventrolateral prefrontal cortex, vmPFC = ventromedial prefrontal cortex, DS = dorsal striatum, GP = globus pallidus, NAc = nucleus accumbens, BNST = bed nucleus of the stria terminalis, CeA = central nucleus of the amygdala, HPC = hippocampus *(Koob & Volkow, 2016)*.

Of course, no animal model can capture all aspects of a complex psychiatric human disorder, however, it allows to separate single aspects and to investigate them in depth from both a behavioral/cognitive and biological perspective. Indeed, an animal model permits experimentally control factors such as genetics, environment, and drug exposure (alcohol or others) allowing to model the stages of the addiction cycle using different paradigms.

To ensure and easily reach high and specific levels of BAC, intragastric passive infusion (or gavage) and ethanol vapor exposure have been developed as principal methods. In both methods there is a passive administration of alcohol that has the disadvantage of not producing the same neurobiological and behavioral effects seen in voluntary consumption. Another procedure requiring a passive administration of alcohol is the conditioned place preference (CPP) or the conditioned place aversion (CPA). Through associative learning, these two methods measure the motivational

value of neutral or aversive stimuli and contexts that have been associated with alcohol effects (Morse, et al., 2000; Tzschentke, 2007).

Models in which animals consume alcohol voluntarily better replicate the behavioral phenotypes physically visible in human subjects. The main two experimental paradigms in this field are noncontingent and contingent alcohol administration. In noncontingent alcohol administration, namely the two-bottle choice (2BC) paradigm and the drinking-in-the-dark paradigm, the animal can choose to consume alcohol or tap water voluntarily allowing it to assess the rewarding properties of alcohol (Crabbe, 2014). Contingent alcohol administration consists of operant alcohol self-administration (SA), a procedure in which an animal has to perform a response, usually a lever pressing or a nose poke, to obtain a dose of alcohol. Alcohol acts as a reinforcer increasing the likelihood of the behavior that makes it available. Modifying the schedule of reinforcement - how many responses are required to obtain a dose of alcohol or which cues are contingent on alcohol delivery – is it possible to investigate the motivation for alcohol and its use despite aversive consequences.

Alcohol deprivation effect (ADE) (induced by forced abstinence or extinguishing the operant responding for alcohol) and reinstatement are experimental paradigms used to investigate alcohol craving and relapse. As happens in humans for relapse, reinstatement in animals can be induced by the presentation of a cue previously associate to alcohol delivery, priming of alcohol (small dose), or stress (physical or pharmacological).

Furthermore, a recent line of research has focused on using animal models that can better resemble human variability to alcohol response. Taking advantage of strain differences and individual variability in response to alcohol and environmental events, it is possible to investigate genetic and neurobiological processes underlying individual resilience or vulnerability to develop AUD. For instance, since animals do not usually drink enough alcohol to develop dependence, rodents from inbred strains with sensitivity to or propensity to drink alcohol were genetically selected. Several alcohol-preferring lines of rats have been created so far and they have proved to well model different and specific subpopulations of AUD patients (Ciccocioppo, 2013;

Crabbe, et al., 2010). Moreover, using animal models to explore the influence of developmental stage in response to alcohol is particularly relevant to clarify differences in vulnerability when alcohol consumption is initiated for example in adolescence, and understand the distinct drug use trajectories that are observed later in adulthood. Finally, the use of animal models is also relevant to explore sex differences observed in men and women.

Applying all these different animal models in preclinical research on AUD can help to individuate neurobehavioral, neurochemical, and neurophysiological correlates associated with alcohol vulnerability or resilience. The final goal of using animal models with phenotypic and genotypic heterogeneity in AUD investigation is to develop novel treatments personalized to the patient's specific characteristics, needs, and clinical and symptomatologic picture.

1.5.2. THE USEFULNESS OF DIFFERENT RAT MODELS

An animal model to be considered valid must meet some fundamental criteria resembling the human condition in several aspects: 1 – the model should mimic the disease in a number of fundamental behavioral aspects, which are specific to it, and it should not show features that are not seen clinically (**face validity**); 2 – there should be a similarity between the neurochemical, neurobiological, and psychobiological dysfunctions thought to elicit the disorder in the clinical population and in the animal model (**construct validity**); 3 – the model should accurately respond to the treatments employed for human patients and conversely, it should be insensitive to those treatments ineffective in humans (**predictive validity**).

The several rat models developed over the years can be distinguished according to their degree of heterogeneity in genetics and phenotypic traits.

Outbred rodents are bred specifically to maximize genetic diversity: because of an accurate rotational breeding scheme that intentionally prevents inbreeding, the result is a population of animals in which no two individuals are genetically identical. Outbred strains guarantee a good approximation to the genetic and phenotypic

variability observed in the human population. In the context of AUD, outbred strains are suitable for studies in which, holding constant environmental variables, the goal is to investigate individual differences on the bases of AUD vulnerability or resilience. In contrast, inbred strains are more in the clarification of the causal role of the environment in the development of the disorder. Inbred rodents are created to minimize genetic variability and generate a specific and reliable genetic background by inbreeding pairs of brothers and sisters over at least 20 generations. In this way, it is possible to obtain an inbred strain in which all rodents are genetically similar (~99% homozygous at all loci) in order to reduce experimental variability and increase reproducibility. Nonetheless, several preclinical studies have demonstrated that also among inbred strains marked differences in almost every animal characteristic may occur (Ho, et al., 2002; Lopez, et al., 2015; Näslund, et al., 2015; Nielsen, et al., 2000; Russo & Parsons, 2017). This phenomenon makes it possible to narrow down the range of genetic and environmental factors determining the variability still present in inbred lines.

Furthermore, at the two extremes of these animal models there are the highly heterogeneous outbred strains and the genetically selected lines.

Not a single animal model may mimic the human complex condition in its entirety and in a satisfactory way. Rather, any different animal model can catch just few aspects of the human AUD but allowing it to be thoroughly investigated.

To maximize the translational power of preclinical research in AUD, it is important to collect evidence form as many different animal models as possible. Combining the use of the different rat lines may be a crucial tool to achieve this objective.

1.5.3. THE NIH HETEROGENEOUS STOCK RATS

To preclinically study and clarify causal genes and phenotypes involved in several complex human traits, Heterogeneous Stock (HS) populations of rodents represent a valid and suitable animal model (Carrette, et al., 2021; Chitre, et al., 2020; Deal, et al., 2021; Heller, et al., 1998; Locurto, et al., 2003). The NIH Heterogenous Stock (NIH-HS) rats is an outbred strain of rats established at the National Institute of Health

(NIH) in 1984 (Hansen & Spuhler, 1984) through a rotational breeding strategy and 60 breeder pairs. As of early 2018, the colony had been through 81 generations of breeding.

The NIH-HS rats were derived from eight inbred progenitor strains: Black Agouti (ACI/N), Brown Norway (BN/SsN), Buffalo (BUF/N), Fischer 344 (F344/N), M520/N, Maudsley Reactive (MR/N), Wistar Kyoto (WKY/N), and Wistar Nettleship (WN/N) (***Figure 7***).

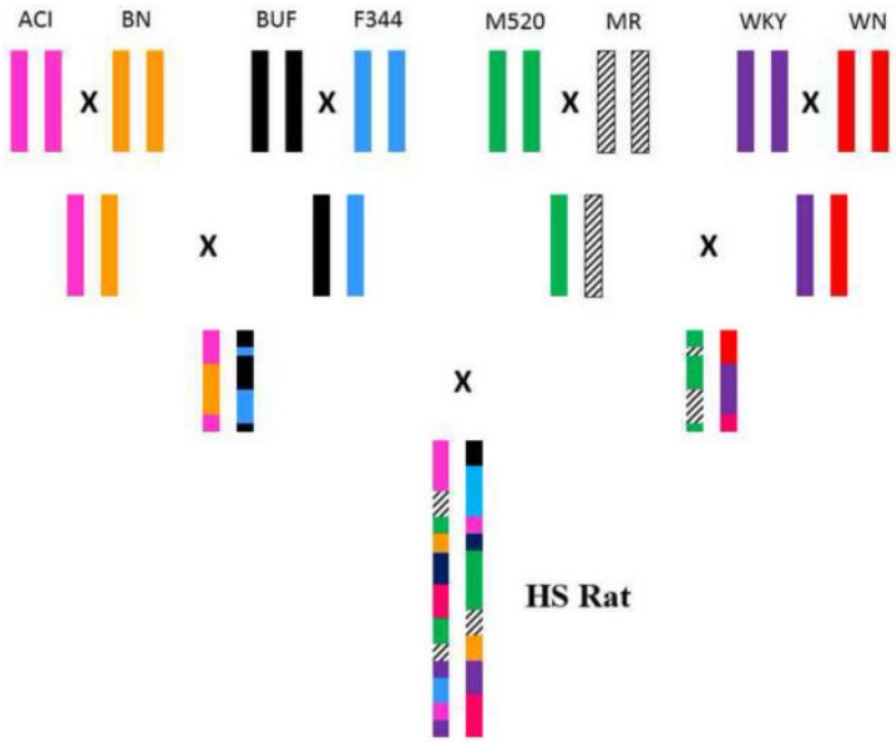


Figure 7. Schematic diagram of the development of HS rats from 8 inbred progenitor strains through more than 60 generations of outbreeding. Agouti (ACI/N), Brown Norway (BN/SsN), Buffalo (BUF/N), Fischer 344 (F344/N), M520/N, Maudsley Reactive (MR/N), Wistar-Kyoto (WKY/N) and Wistar-Nettleship (WN/N). The final result is that each HS rat has a unique, genetically random mosaic of founding animal chromosomes due to recombination that occurred over many generations *(Alam, et al., 2011)*.

As a result, each NIH-HS rat is genetically and phenotypically distinct and unique and therefore this rat line can well resemble the variation existing in the human population. The high degree of variability observed in NIH-HS rats made this outbred strain used to perform genome-wide association studies (GWAS) since they are an invaluable source of genetic diversity for selection studies. Indeed, the NIH-HS colony is a national resource funded through the National Institute on Drug Abuse (NIDA) Center of Excellence for GWAS in outbred rats to identify genetic loci underlying drug abuse behaviors.

NIH-HS rats have been previously used to screen alcohol drinking (Deal, et al., 2021; Li & Lumeng, 1984; Murphy, et al., 1987; Tabakoff & Culp, 1984; Zahr, et al., 2014), but no multiple alcohol use-related behaviors have been evaluated in them so far. One of the aims of my Ph.D. program was to screen NIH-HS rats for alcohol use-related behaviors and develop a multisymptomatic model of AUD able to spot vulnerable and resilient to AUD individual profiles and endorsed with high predictive validity to bridge the gap in pharmacological translational research (**Chapter 2**).

1.5.4. THE MARCHIGIAN SARDINIAN ALCOHOL-PREFERRING RATS

Rodent models of AUD, both inbred and outbred, show a substantial problem concerning the levels of alcohol intake. Rats do not voluntarily consume sufficient amounts of alcohol to reach BAC levels of intoxication (around 1g/L) and they metabolize alcohol three times faster than humans (Jeanblanc, et al., 2019). To overcome this issue and obtain a total volume consumed relevant enough to evaluate AUD in rodents, rat lines genetically selected for high ethanol preference or excessive alcohol drinking have been selected. These rat lines have demonstrated to be an invaluable source of information about AUD over the years.

The genetically selected Marchigian Sardinian (msP) line is one of them. It has been selected for its high ethanol preference starting from the 13th generation of Sardinian alcohol-preferring (sP) rats donated by Prof. Gessa (University of Cagliari, Italy) to the University of Camerino. After 20 generations of selective breeding, these rats have been re-named msP rats having husbandry conditions and genotypic and phenotypic

characteristics different from the original sP rats (Borruto, et al., 2021b; Ciccocioppo, 2013; Ciccocioppo, et al., 2006).

Over the years, msP rats have been highly investigated to characterize their behavior, neurobiology, genetics, and response to drug treatments.

As an animal model of genetic predisposition to high ethanol drinking, msP rats do not show aversion to alcohol but drink large amounts of it from the very first day of home cage presentation (Ciccocioppo, et al., 2006). Indeed, when msP rats are exposed to a continuous home-cage free 2BC paradigm between 10% ethanol solution (*v*/*v*) and tap water, they drink the pharmacologically relevant daily dose of approximately 7–8 g/kg of alcohol (Borruto, et al., 2021b; Ciccocioppo, et al., 2006). The vast majority of alcohol (80%) is consumed during the dark phase in drinking episodes organized into three bouts producing a BAC around 70-80 mg/dL that can even peak over 100 mg/dL with pharmacologically meaningful effects (Borruto, et al., 2021b; Ciccocioppo, et al., 2006).

In operant alcohol SA msP rats spontaneously and quicky acquires a robust ethanol lever-responding compared to the non-preferring Wistar control rats (Ayanwuyi, et al., 2013; Cannella, et al., 2016; Domi, et al., 2019). Confronted with Wistar rats, msP rats also show a stronger motivation for alcohol when tested in a progressive ratio (PR) schedule of reinforcement (Ciccocioppo, et al., 2006; Domi, et al., 2019). Moreover, following a withdrawal period during which lever pressing is extinguished, compared to Wistar controls, msP rats have a higher tendency to relapse in response to conditioned stimuli that predict alcohol availability or to stress in the absence of the primary reinforcer (Ayanwuyi, et al., 2013; Borruto, et al., 2021a; Cannella, et al., 2016; Ciccocioppo, 2013; Ciccocioppo, et al., 2004; Fotio, et al., 2021; Stopponi, et al., 2013).

Importantly, the high ethanol drinking showed by msP rats is associated with anxious and depressive-like traits as demonstrated by several behavioral tests, including the forced swimming test, elevated plus maze test, open field test, and marble burying test (Ayanwuyi, et al., 2013; Ciccocioppo, et al., 1999; Cippitelli, et al., 2015; Domi, et al., 2019; Natividad, et al., 2017; Stopponi, et al., 2018). Their high sensitivity to stress

may contribute to their excessive alcohol-drinking phenotype. In operant alcohol SA, when stress is induced by pharmacological treatment (Ayanwuyi, et al., 2013; Borruto, et al., 2021a) or by intermittent foot-shock (Hansson, et al., 2006) both msP and Wistar rats reinstate the alcohol-paired response but with a higher level in the msP line. Therefore, anxiety- and depressive-like symptoms showed by msP rats are attenuated by alcohol consumption and repeated intragastric alcohol administration (Ciccocioppo, et al., 2006; Ciccocioppo, et al., 1999).

Biochemical and electro-physiological data have demonstrated that the msP phenotype is due to two single-nucleotide polymorphisms at the CRF1 receptor locus responsible for CRF1 receptor overexpression and hyperactivity in certain brain areas, such as the central AMG (Natividad, et al., 2017; Stopponi, et al., 2018).

MsP rats have been also tested for the ADE, a model that resembles the AUD patients' relapse after a period of abstinence (Boening, et al., 2001; McBride, et al., 2002; Vengeliene, et al., 2005). After being exposed to chronic alcohol, msP rats display a robust ADE when they are re-exposed to alcohol after a forced abstinent period of 10 days (Perfumi, et al., 2005). This increase in drinking is transient and usually returns to baseline levels after a couple of days (Hölter & Spanagel, 1999; Vengeliene, et al., 2014).

Finally, using the "0/3crit model of addiction," based on the DSM-IV diagnostic criteria for addiction (American Psychiatric Association, 2000), msP and unselected Wistar rats were characterized for their alcohol-addiction phenotype (Domi, et al., 2019). The model consisted of a multidimensional experimental approach to identify subpopulations of rats that possess vulnerability (3crit) and resilience (0crit) to drug addiction-like behaviors by measuring three traits: (1) inability to refrain from drug seeking, (2) high motivation for the drug, and (3) maintenance of drug use despite negative consequences (Domi, et al., 2019). Significant variability among both msP and Wistar rats has been found and only a subset of rats (about 13%) were positive for all three AUD criteria. The number of msP animals classified as 3crit was three times higher than Wistar rats (9.5% vs 3.17%), whereas the 0crit group was mainly composed of Wistar rats. These results are consistent with human data: only a

proportion of individuals who chronically consume alcohol actually develop AUD and genetic factors have been demonstrated to account for 50-60% of this progression (Heath, et al., 1997; Wagner & Anthony, 2002; Worst & Vrana, 2005). Moreover, the amount of alcohol consumed positively correlated with anxiety-like behavior in msP rats but not in Wistar rats (Domi, et al., 2019).

All of this evidence together suggests that alcohol drinking in msP animals is motivated by negative reinforcement. Consequently, msP rats may represent a good rodent model to mimic a specific AUD subpopulation having genetic variations at the CRF1 receptor system (Ciccocioppo, et al., 2006) and in which alcohol is consumed to ameliorate negative affective symptoms associated with comorbid anxiety and depression (Koob & Le Moal, 2005).

1.5.4.1. SEX-RELATED BEHAVIORAL DIFFERENCES IN MSP RATS

Sex differences in AUD have been largely neglected for many decades and just recently this area of research has started to receive growing attention (Becker & Chartoff, 2019; Becker, et al., 2017). As discussed in the paragraph "**GENE X ENVIRONMENT INTERACTION IN AUD**", men and women differ in several aspects related to AUD and preclinical studies have confirmed these differences observed in humans. However, both clinical and preclinical research on AUD lack of a sufficient number of gender-related studies.

Most studies performed in rodents have been conducted only in males generating incomplete data to guide clinical trials (Landis, et al., 2012; Zucker & Beery, 2010). To emphasize the need to study both males and females at the preclinical level, the NIH issued a new series of guidelines a few years ago (Clayton & Collins, 2014; Collins & Tabak, 2014; Fattore & Melis, 2016).

In response to these recommendations, preclinical researchers in the field of SUDs have started to make substantial efforts to include both sexes in their investigations (Becker & Koob, 2016; Fattore & Melis, 2016). Our laboratory has recently begun a research program to investigate sex differences in alcohol consumption and alcohol-related behaviors in msP rats and heterogeneous Wistar controls.

The first series of experiments were performed in the 2BC paradigm (10% alcohol solution vs. tap water) to compare basal levels of voluntary alcohol intake in male and female msP and Wistar rats (Borruto, et al., 2021a). Since in Wistar rats the level of drinking was very low, it was impossible to detect differences between males and females in this strain. Differently, msP rats consumed a high volume of ethanol, and female msP rats took significantly higher amounts of alcohol compared with male msP rats.

Another relevant characteristic of msP rats is their high levels of anxiety- and depressive-like symptoms that are attenuated by alcohol drinking. Both naïve male and female msP rats exhibited higher anxiety- and depressive-like behavior compared to Wistar rats. However, alcohol drinking reduced anxiety-like behavior only in males when tested in the elevated plus maze test. On the other hand, the immobility time (a measure of depression) in the forced swimming test was significantly reduced in both male and female msP rats following alcohol drinking. Lastly, msP rats of both sexes showed higher freezing in response to foot-shock stress compared to unselected Wistar rats and alcohol consumption attenuated the freezing time both in male and female msP rats (Borruto, et al., 2021b). These differences are not due to a different pain/sensitivity threshold of the two rat lines (Cippitelli, et al., 2015; Hansson, et al., 2006). Rather we hypothesize that male and female msP rats are both characterized by traits that confer negative mood conditions that co-segregated with alcohol drinking during genetic selection. The relevant difference between male and female msP seems to be linked to their motivation for alcohol. From the results presented above it can be inferred that female msP rats tend to consume alcohol for its antidepressant properties more than males. This evidence is consistent with human data showing that AUD women are more likely than men to be also diagnosed with PTSD (Kessler, et al., 1995).

These preliminary findings are very promising and confirm how alcohol-related sex differences in AUD patients can be detected also in msP rats supporting the translational value of this type of investigation.

Of course, further studies are needed, especially in the field of individual variability that has been assessed only in male msP rats. For this purpose, part of my Ph.D. project was to investigate this aspect in female msP rats in response to a potential pharmacological treatment for AUD that acts on stress and arousal modulation (**Chapter 4**).

1.6. RESEARCH OBJECTIVES AND SIGNIFICANCE

AUD represents a major public health problem and the actions of alcohol on the nervous systems are complex and still not fully understood. Reasons for using alcohol may differ between individuals and situations. Different factors influence alcohol abuse vulnerability, including sex, age, genetics/family history, environment, copresence of other psychiatric disorders and stress. Moreover, the developmental trajectories of the disorder as well its characteristics and symptoms vary from patient to patient. The efficacy of pharmacotherapies for AUD is limited to drug-responsive sub-population, thus making the identification of patient subgroups that are most likely to respond to a treatment crucial already at the research and development stage of a drug's life. Mimicking with animal models the variability observed in the human population can help achieving this goal, facilitating the development of innovative personalized therapies. The main objective of the studies present in this thesis was to investigate individual resilience and vulnerability in AUD using different rat models and show how choosing the one best suited to mimic specific subpopulations of alcoholics can provide a good model for studying that subpopulation. The first study (**Chapter 2**) was conducted to demonstrate how the outbred NIH heterogeneous stock rats can be a good model to investigate the genetic aspect of AUD. In the following study (**Chapter 3**), we investigated the role of the environment in triggering innate genetic vulnerability to alcohol use. We observed that in the outbred Wistar line, a protocol of chronic sleep restriction during adolescence leads a subset of animals to lifelong increased alcohol consumption. Early alcohol use is a key environmental factor that increases the risk to developing AUD and distinguish AUD patients. Finally, in the successive work (**Chapter 4**), we explored the interindividual variability in the genetically selected msP female rats in response to the treatment with neuropeptide S in the reduction of alcohol self-administration. Here we observed heterogeneity in the responses of female rats unlike previously seen in males of the same line, thus highlighting how gender differences are a topic that requires further investigation in the field of AUD.

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Chapter 2

Development and pharmacological validation of a behavioral model of individual vulnerability to alcohol use disorder in genetically heterogenous NIH stock rats

2.1. ABSTRACT

Alcohol is the most easily accessible and consumed addictive substance worldwide, and its chronic and prolonged use can eventually lead to developing alcohol use disorder (AUD). AUD is a heterogeneous and progressive brain disorder, ranging from mild, time-limited, alcohol-related problems to severe, chronic, and relapsing presentation often termed addiction. An intricate interplay of polygenic, environmental, and neurobiological factors characterizes AUD. Due to the multisymptomatic nature of AUD, it is difficult to find new therapeutic drugs, and those already approved are not fully effective in the whole alcoholic population. Indeed, promising targets often fail clinical expectations, probably due to the genetic and phenotypic homogeneity of rat strains mostly used in preclinical research. Here we hypothesized a multisymptomatic preclinical model of individual vulnerability to AUD would offer better translational results than group-based approaches revealing responder and non-responder individuals to pharmacological treatments. For this purpose, the NIH Heterogeneous Stock (HS) rats were used and subjected to a multisymptomatic screening for AUD-like behaviors allowing us to identify three AUD-like clusters of HS rats showing differences in alcohol-related behaviors. Then, to validate our model in inverse translational pharmacological approach, we tested a drug already approved for AUD therapy, naltrexone (NTX), and another one that failed in clinical trials, memantine on alcohol self-administration. We hypothesized that NTX but not memantine would selectively reduce alcohol self-administration and that rats would show heterogeneous responses to treatment. NTX showed different efficacy in reducing alcohol SA in the three AUD-like Clusters while memantine was equally effective in all three. In addition, while the effect of NTX was alcohol specific, the efficacious dose of memantine also decreased the SA of the natural reward saccharin, confirming our hypothesis. Therefore, we next focused on NTX and divided the population into NTX responder and non-responder groups and found that non-responder rats can be predicted by innate anxiety levels assessed by the elevated plus maze. In conclusion, NIH-HS rats can be used to model individual vulnerability to AUD endorsed with high predictive validity.

2.2. INTRODUCTION

AUD is a chronically relapsing disorder associated with compulsive alcohol seeking and taking, loss of control over alcohol intake, and emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability) when alcohol is no longer available (Koob, 2014). Alcohol is one of the most highly used and abused drugs worldwide (Sudhinaraset, et al., 2016) but its consumption is not sufficient to develop AUD. Recent data demonstrated that just a subpopulation up to 5% of people who consume alcohol develops AUD (World Health Organization, 2021). The reasons behind this number lie in the large heterogeneity of both AUD aetiology and phenotype.

Approximately 50-60% of AUD vulnerability is due to genetic predisposition (Edenberg, 2002; Heath, et al., 1997; Köhnke, 2008) and several environmental factors are responsible for triggering the inner genetic susceptibility.

AUD patients are not all the same: differences in alcohol pattern consumption, related behaviors, and response to drug treatment have been identified in AUD patients making them a very heterogeneous population. The only three drug currently approved by the FDA have in fact limited effectiveness (Litten, et al., 2018) which varies between patient subgroups (Heilig, et al., 2011; Mann, et al., 2018) making increasingly necessary to move toward personalized treatments (Heilig, et al., 2016b; Heilig & Leggio, 2016a; Mann & Hermann, 2010).

For instance, naltrexone (NTX) (one of the three treatment approved by FDA) is a panopioid receptor antagonist that prevents the pleasurable effects of alcohol by blocking the μ-opioid receptor (Sudakin, 2016). Its effectiveness has been shown to depend on different factors. Comparing NTX effect in individuals with family history of alcoholism and individuals without alcoholic relatives, NTX demonstrated to perform better in the first group of people which was characterized by lower basal lever of β-endorphin and greater increase of β-endorphin as an effect of alcohol consumption (King, et al., 1997). Moreover, NTX is differently efficient based on ethnicity. A single nucleotide polymorphism of the OPRM1 gene (Asn40Asp) has been associated with an increased response to NTX (Koller, et al., 2012; Ray, et al.,

2010) and it has been found more expressed in Asian population (Hernandez-Avila, et al., 2007; Ray, et al., 2012).

Quite often promising drugs studied in preclinical experiments failed clinical expectations (Jonas, et al., 2014). This was the case of memantine (MEM), a non-competitive ionotropic glutamate NMDA receptor (NMDAR) antagonist approved for Alzheimer's disease treatment (Reisberg, et al., 2003; Rogawski & Wenk, 2003; Thomas & Grossberg, 2009). Alcohol inhibits NMDAR (Criswell, et al., 2003; Hoffman, et al., 1989; Lovinger, et al., 1990) as well as the synaptic responses mediated by NMDARs (Roberto, et al., 2004; Wang, et al., 2007; Zhao, et al., 2015), whereas chronic alcohol consumption increases both the function of NMDARs and NMDAR-mediated glutamatergic synaptic transmission (Chandler, et al., 1997; Gulya, et al., 1991; Smothers, et al., 1997) being involved in alcohol-seeking and craving. Given the involvement of glutamatergic transmission and NMDAR in alcohol effects, memantine was assessed in preclinical experiments demonstrating to efficiently reduce alcohol consumption, craving, and withdrawal symptoms in both rats and mice (Hölter, et al., 1996; Piasecki, et al., 1998). Based on this promising preclinical data, it was expected that memantine would give satisfactory results in clinical trials. Instead, data were discordant: using 20 and 30 mg doses, Krishnan-Sarin and colleagues were able to reduce alcohol craving (Krishnan-Sarin, et al., 2015), whereas with a similar dose range (15 and 30 mg) Bisaga and Evans did not obtain a significant reduction in alcohol craving (Bisaga & Evans, 2004). Regarding alcohol consumption, although one study showed that 20 mg reduced it (Muhonen, et al., 2008), similar experiments did not confirm this result (Montemitro, et al., 2021; Krishnan-Sarin, et al., 2015).

Difficulties in translatability between preclinical and clinical data may be due to limitations in rat lines usually used in preclinical research. Thanks to their genetic and phenotypic relative homogeneity, these rat lines guarantee the experimental reproducibility of the study, but, at the same time, they are less suited to study the role of inter-individual variability in the aetiology and development of addictive

behaviors as well as in the response to pharmacological treatments. (Parker, et al., 2014).

To overcome this issue and better evaluate the individual genetics and phenotypic differences found in human population, in the present study we used the outbred NIH heterogenous stock rats as preclinical animal model. This strain of rats is a genetically random mosaic of eight inbred founding strains. Through an accurate breeding system, each NIH-HS rat is genetically and phenotypically distinct and unique. Consequently, the NIH-HS rats closely resemble the variability existing in human population (Solberg Woods & Palmer, 2019).

Investigating and characterizing alcohol-related behaviors in the NIH-HS rats for the first time using a multisymptomatic approach, we aimed to develop an animal model of AUD that would well mimic the heterogeneity in alcohol phenotypes observed in patients. Additionally, to validate our animal model, we applied a reverse translational approach testing the efficacy of a drug approved for alcoholism therapy (NTX) and a drug failed in clinical trials (memantine) in rats showing different vulnerability to AUD-like behavior.

We hypothesized that the combination of a multisymptomatic behavioral approach and genetic variability in AUD would offer a model with high predictive validity. Specifically, we expected that NTX and memantine would show heterogeneous efficacy as it has been seen in humans, highlighting subgroups of animals with different treatment efficacy and revealing non-responder individuals.

2.3. MATERIAL AND METHODS

2.3.1. ANIMALS

NIH-HS rats (n=20/sex) were obtained from Wake Forest University (North Carolina, USA) and weighed 270 g (male) and 180 g (female) at the beginning of the experimental procedure. Rats were housed four per cage according to their sex and under a reversed 12:12h light/dark cycle (lights off at 7 AM) in a temperature (20-22° C) and humidity (45-50%) controlled room. Food (4RF18, Mucedola, Settimo

Milanese, Italy) and tap water were provided *ad libitum*. Rats arrived at the University of Camerino's animal facility aged six to seven weeks and were left undisturbed for one week to acclimate. Thereafter, animals were handled 5 min daily for 5 days by the same operator who performed the experiments. Experiments were conducted during the dark phase of the light/dark cycle.

All procedures were conducted in adherence with the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3.2. DRUGS

Alcohol solutions (5% and 10% *v/v*) used in the 3- and 2-bottle choice paradigm and the SA procedure were prepared by diluting 95% *v/v* alcohol (F.L. Carsetti s.n.c., Camerino, Italy) with tap water.

Naltrexone hydrochloride (Sigma, St. Louis, MO) was dissolved in 0.9% saline and administered subcutaneously (s.c.) at the doses of 0.0, 0.3, and 1.0 mg/kg in a volume of 1 mL/kg, 30 min before the alcohol SA sessions.

Memantine (Memantina Mylan 20 mg, coated tablets, Mylan Italia S.r.l.) was suspended in tap water and orally administered via gavage at the doses of 6 mg/kg, 12 mg/kg and 25 mg/kg in a volume of 4 ml/kg, 1 hour before SA sessions.

Drug doses were chosen based on published data (Bisaga & Evans, 2004; Jimenez-Gomez & Shahan, 2007; Krishnan-Sarin, et al., 2015; Muhonen, et al., 2008; Walker & Koob, 2008; Williams & Broadbridge, 2009).

Rats were habituated to the treatment administration procedures for three consecutive days before starting pharmacological tests by correspondent vehicle injections.

2.3.3. SELF-ADMINISTRATION APPARATUS

Operant training and testing were performed in SA chambers (Med Associates, St Albans, VT, USA) equipped with two retractable levers (one active and one inactive) located in the front panel of the chamber and a drinking reservoir (volume capacity:

0.30 mL) placed in between and connected with a syringe pump. The visual stimulus was presented via a house light located on the wall opposite to the levers. Each chamber was enclosed in ventilated sound-attenuating cubicles. Behavioral sessions were controlled and recorded by a windows compatible PC equipped with Med-PC-5 software (Med Associates).

2.3.4. OVERVIEW OF EXPERIMENTAL PROCEDURES

After leaving the HS rats undisturbed for a week to acclimatize them to the new environment, their drug naïve behavior was evaluated. To phenotypically characterize each rat, a battery of behavioral tests was performed before alcohol exposure over the course of 4 days. Rats were divided into groups of ten, and five male and five female rats were tested per day. On the same day, each rat performed all tests, from least invasive and anxiogenic to most invasive and anxiogenic. A break of one hour was given between each test to decrease the chance that behavioral responses were altered by the prior test.

The innate level of locomotor activity, anxiety-like behavior, and pain sensitivity were screened by the following behavioral tests in the following order:

- Open Field (OF) test;
- Elevated Plus-Maze (EPM) test;
- Tail immersion (TI) test.

Afterward, to accustom rats to the taste of alcohol, they were given continuous access to alcohol for two weeks. At the beginning of the dark cycle, rats received free access to three bottles (tap water, 5%, and 10% alcohol solution) whose position was alternated each day to avoid a potential side preference effect. Fluid intake was measured every 24 hours weighing the bottles.

Then, to facilitate the operant training, rats were subjected to a single overnight 15 hours session during which they only had access to the right lever. Under a fixed-ratio 1 (FR1) schedule of reinforcement, the right lever pressing produced the delivery

of 0.1 mL of water. Standard chow food pellets were available on the floor of the operant chamber.

Finally, alcohol SA sessions and tests were run according to the following timeline:

1. Alcohol SA under FR1 schedule of reinforcement;
2. Alcohol SA under PR schedule of reinforcement;
3. Extinction and cue-induced reinstatement;
4. Alcohol seeking behavior;
5. Effects of NTX pre-treatment on alcohol SA;
6. Foot-shock punishment on alcohol SA;
7. Effects of memantine pre-treatment on alcohol SA;
8. Continuous ethanol 2BC paradigm;
9. Quinine adulterated alcohol drinking in 2BC paradigm;
10. Saccharin SA training under FR1 schedule of reinforcement;
11. Effects of NTX pre-treatment on saccharin SA;
12. Effects of memantine pre-treatment on saccharin SA.

2.3.4.1. EXPERIMENT 1: Pre-alcohol exposure behavioral screening

Open field test

The open field (OF) test allows detecting the animal locomotor activity and exploratory behavior. Each OF arena consisted of a plexiglass arena (43.4 x 43.4 x 30.3 cm) that tracks location and locomotion using 16 evenly spaced infrared (I/R) beams (Med Associates, St Albans, VT, USA). Rats were placed in the arena and left free to explore for 1 hour in a sound-attenuated room illuminated by a dim red light (~ 30 lux). The distance travelled in centimeters was automatically recorded.

Elevated Plus Maze test

The elevated plus maze test (EPM) was performed to evaluate innate anxiety-like behavior in rodents since it is based on their natural aversion to spending time in open spaces. The black wooden apparatus was elevated 50 cm above the floor and consisted of two open arms crossed by two enclosed arms (50 cm long x 10 cm wide, 40 cm high walls), arranged such that the respective closed and open arms were opposite to each other. The EPM tests were conducted in a sound-attenuated room illuminated by dim red light. Each 5-min trial started when the animals were individually placed in the center of the maze, facing the open arm opposite where the experimenter was. An arm entry was defined by the presence of all four paws inside it and the time spent in each arm was video-tracked and scored by a trained experimenter.

Tail immersion test

To evaluate pain perception and response to an acute thermal stimulus, the tail immersion test was used. The tip of the rat's tail (2 cm) was immersed in a basin filled with warm water (50-52°) and the time required to withdraw the tail was recorded. To avoid burning the rat tail, a 10-s cut-off time was applied, i.e. if the rats did not show tail withdrawal reflex within 10 s, the test was interrupted, and the latency was reported as 10 s (de Guglielmo, et al., 2014; Langford & Mogil, 2008; Zhou, et al., 2014).

2.3.4.2. EXPERIMENT 2: Characterization of AUD-like behavior in operant SA

On the initial three bottle choice screening, we verified that rats did not show a preference for either 5% or 10% alcohol solution, therefore all following experiments were conducted with the 10% solution.

Alcohol SA training under FR1 schedule of reinforcement

Rats were trained to self-administer 10% alcohol (*v*/*v*) in 30-min daily sessions on FR1 schedule of reinforcement. Operant sessions started with levers insertion and ended with levers retraction. Each response to the right (active) lever resulted in the delivery of 0.1 mL of 10 % alcohol solution. The delivery of the solution was followed by a

contingent illumination of the house light and a 5-s time-out (TO) period during which the reinforced lever remained inactive. Left (inactive) lever presses had no scheduled consequences. The number of operant lever presses to both the active and inactive levers and the number of reinforcements received were recorded for each session.

EXPERIMENT 2.1: Alcohol SA under PR schedule of reinforcement

Once a stable alcohol SA baseline was reached, motivation for alcohol was measured in a PR schedule of reinforcement in which the number of lever presses or the ratio required to receive one dose of 10% ethanol was progressively increased. The PR is an operant schedule that allows measuring the maximum amount of work an animal is willing to carry out to obtain the reward, reflecting its motivation for it (Richardson & Roberts, 1996). The maximal number of responses a rat produces to obtain one reward is referred to as the break point (BP) and is considered a measure of motivation for the reinforcer. The following PR schedule of reinforcement was used: 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100 and 104 (Domi, et al., 2019; Economidou, et al., 2006). Each alcohol delivery was paired with a 5 s illumination of the house light. Session was terminated when 30 min had elapsed since the last reinforced response.

EXPERIMENT 2.2: Extinction and cue-induced reinstatement test

Rats were retrained to self-administer 10% alcohol solution in FR1 schedule of reinforcement and subsequently they were subjected to eleven daily-30-min extinction sessions to extinguish the responses to the alcohol-paired lever. During this phase, sessions began by extension of the two levers and responses to the active lever activated the delivery mechanism but did not result in the delivery of alcohol or the presentation of the contingent house light. The number of responses to both the active and the inactive levers was recorded for all extinction phases and test sessions.

The reinstatement test occurred after 11 extinction sessions and the day after the last extinction session. It lasted 30 min and it was conducted under the same conditions used during the conditioning phase, except that alcohol was not delivered, i.e., active

lever responses resulted in the house light presentation without 10% ethanol availability.

EXPERIMENT 2.3: Alcohol seeking behavior

After cue-induced reinstatement test, rats were trained to additional alcohol SA sessions to restore a stable baseline of operant training. Then, to evaluate animal's craving (Bienkowski, et al., 2004; Grimm, et al., 2011; Sayette, et al., 2000), a seeking test session was performed during which the active lever presses did not result in the delivery of alcohol but were reinforced by the illumination of the house-light with the same contingencies described for alcohol SA. Alcohol seeking score was calculated as the ratio between the number of active lever presses at the seeking test and the number of active lever presses at the last day of alcohol SA.

This test as well as all other test sessions described below were not conducted on Mondays to eliminate possible rebound of responding after weekends off.

EXPERIMENT 2.4: Foot-shock punished alcohol SA

After a period of retraining to self-administer 10% ethanol in FR1 schedule of reinforcement, alcohol taking despite adverse consequences was evaluated. SA session conditions were identical to SA training (i.e., 30-min session, delivery of 0.1 mL of 10% alcohol solution associated with 5-sec house light on) but contingently to alcohol delivery, a 0.5-second foot-shock (0.2 mA for the first three sessions and then 0.25 mA for the remaining fourteen sessions) was provided as punishment. The resistance score was calculated as the ratio between punished alcohol deliveries and the mean alcohol deliveries of the last three non-punished sessions (Domi, et al., 2021), obtained over 14 SA sessions under a 0.25-mA shock punishment.

2.3.4.3. EXPERIMENT 3: Characterization of AUD-like behavior in 2BC paradigm

EXPERIMENT 3.1: Continuous ethanol 2BC paradigm

At the end of all the alcohol SA procedures and tests, to determine individual rats' basal daily alcohol intake as well as their preference for alcohol compared to tap water (Koob, et al., 2003), a continuous ethanol 2BC paradigm was performed for two

weeks. Animals were housed individually with continuous access to two bottles, one containing alcohol 10% (*v*/*v*) and one containing tap water. Liquid consumption was measured by weighing the two bottles every 24 hours at the beginning of the dark phase. Bottles positions were switched daily to avoid potential side preference effects. Food intake was measured once a week and animals were weighed twice a week. Alcohol and water intake was calculated as absolute values at each interval time and are expressed as gram per kilogram (g/kg) to control for the influence of body weight differences (Finn, et al., 2007).

EXPERIMENT 3.2: Quinine adulterated alcohol drinking in 2BC

After two weeks of free 2BC, the 10% alcohol solution (v/v) solution was adulterated with quinine. Four increasing quinine concentration were used, each one maintained for three consecutive days: 10, 25, 50 and 100 mg/L. Water and alcohol + quinine bottles' position were switched daily to avoid side preference effect. Fluids consumption and preference were measured as previously described for the (Shaw, et al., 2020).

2.3.4.4. EXPERIMENT 4: Effects of NTX pre-treatment on alcohol SA

Animals were retrained to self-administer 10% ethanol in FR1 schedule of reinforcement and once a stable alcohol SA baseline was acquired, the effect of NTX treatment was tested. Each rat received each dose according to a Latin square within-subjects counterbalanced design. Thirty min before the beginning of the SA session, animals were s.c. injected with NTX (0.3 or 1.0 mg/kg; 1 mL/kg) or its vehicle. Drug treatment was performed every fourth day. Treatments were administered every fourth day, and the first day after each drug injection, rats remained undisturbed in their home cages, whereas on the 2nd and 3rd day after the drug injection baseline alcohol self-administration was re-established.

2.3.4.5. EXPERIMENT 5: Effects of memantine pre-treatment on alcohol SA

FR1 alcohol SA was again baselined and then the effect of memantine was evaluated. Following the same experimental procedures described for NTX, memantine (6.0, 12.0 and 25.0 mg/kg) was orally administered by gavage 1 hour before session according to a Latin square within-subjects counterbalanced design.

2.3.4.6. EXPERIMENT 6: Effects of the two pharmacological treatments on saccharin SA

Saccharin SA training under FR1 schedule of reinforcement

Finally, to verify the specificity of NTX effect on alcohol consumption, the effect of NTX on saccharin SA was tested. Rats were trained to self-administer 0.2% (*w*/*v*) saccharin in 30 min daily sessions under a FR1 schedule of reinforcement as previously described for alcohol SA.

EXPERIMENT 6.1: Effects of NTX **pre-treatment on saccharin SA**

Once rats acquired a stable saccharin SA baseline the effect of NTX (0.3 or 1.0 mg/kg; 1 mL/kg) on saccharin SA was tested according to the same procedures described above for alcohol.

EXPERIMENT 6.2: Effects of memantine pre-treatment on saccharin SA

Once rats acquired a stable saccharin SA baseline the effect of memantine (25.0 mg/kg) on saccharin SA was tested according to the same procedures described above for alcohol.

2.3.5. CLUSTERING OF RAT POPULATION BASED ON MULTISYMPTOMATIC ALCOHOL-RELATED BEHAVIORS

2.3.5.1. K-MEANS CLUSTERING BASED ON SIX AUD-LIKE BEHAVIORS

To the completion of the entire protocol, individual scores in 6 behavioral criteria were selected for the cluster analysis of AUD-like phenotypes:

I. Breakpoint reached in the PR session;
II. Cue-induced reinstatement expressed as the difference in active lever response between the cue-induced reinstatement test and the average of the last three extinction sessions;
III. Alcohol seeking behavior expressed as the ratio between the number of active lever pressing in the first extinction session and that of the last day of alcohol SA;
IV. Resistance to foot-shock punishment expressed as resistance score was calculated as the average of the last three resistance scores at the last three punished SA sessions. Each resistance score was obtained by dividing the number of rewards on the punished SA session by the average of the last three not-punished SA sessions;
V. Alcohol intake averaged over the last 3 days of 2BC;
VI. Resistance to quinine adulteration at 25 mg/L (the dose with higher variability in data distribution) expressed as the ratio between the average of the last 3 days of alcohol adulterated with quinine intake and the baseline (average last 3 days) alcohol intake.

The z-scored individual score in each of the 6 alcohol related behaviors were used for the k-means cluster analysis with 10 iterations and K=3.

2.3.5.2. COMPUTATION OF INDIVIDUAL EFFECT SIZE OF DRUG TREATMENT

To assess the contribution of each animal to each drug treatment efficacy (NTX or memantine), we computed each rat's individual effect size (IES) within its corresponding treatment and dose.

The active lever response expressed by each individual under each NTX dose and vehicle were z-scored. The individual z-scored active lever response of each dose was then subtracted from the vehicle z-score to calculate the IES. Comparing each difference, we could estimate the contribution of each individual to the distribution

of data in response to the treatment. IES computation and interpretation are illustrated in ***Figure 8***.

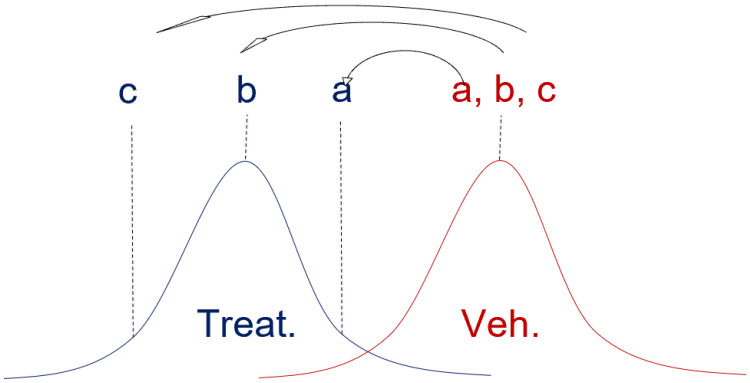

Figure 8. Illustration of the method to calculate individual effect size (IES) and its interpretation. The method can be generalized to both right and left shift induced by treatments. In here a left shift is assumed. Comparing subjects' position within the vehicle (Veh) and treatment (Treat) distribution there can be observed three kinds of contribution to treatment inducing shift of the distribution (group effect size): - subject b maintained the same relative position within treatment and vehicle distributions, i.e. it moved in line with group effect size; - subject c's position in treatment distribution shifted leftward compared to its position under the vehicle, i.e. the drug had a stronger effect than the average group effect size in subject c; - subject a's position in treatment distribution shifted rightward compared to its position under the vehicle, i.e. the drug had a weaker effect than the average group effect size in subject a. Therefore, subtracting treatment Z-score to vehicle Z-score of each subject it can be inferred that:

If $Z_{Veh} - Z_{Treat} < 0$ (subject a), then the subject shows a response to treatment weaker than the group's average.

If $Z_{Veh} - Z_{Treat} = 0$: (subject b), then the subject shows a response to treatment in line with the group's average.

If $Z_{Veh} - Z_{Treat} > 0$: (subject c), then the subject shows a response to treatment stronger than the group's average.

2.3.6. STATISTICAL ANALYSIS

Behaviors from the multisymptomatic screening (alcohol-naïve screening and alcohol-related behaviors) were analysed via one-way ANOVAs or Kruskal-Wallis tests (when data were not normally distributed) with cluster as between-subjects

factor. Data from the cue-induced reinstatement were analysed by two-way ANOVA with cluster as between-subjects factor and experimental phase as within-subjects factor. The effect of the two drug treatments on the whole HS population was assessed by one-way ANOVAs or Friedman test (non-normally distributed data) with dose as between-subjects factor. The memantine effect data were not normally distributed and hence Wilcoxon test was performed. To compare the effect of the two drug treatments on the three AUD-like Clusters as well as on responder and non-responder NTX-Clusters, two-way ANOVAs were run with cluster as between-subjects factor and dose as within-subjects factor. The two NTX-Clusters were compared on their anxiety-like behavior by running unpaired t-test. Correlation analyses were performed via Pearson's two-tailed test. ANOVAs were followed by Holm-Sidak or Sidak post-hoc analysis as appropriate, while non-parametric tests were followed by Dunn's post-hoc analysis as appropriate.

Statistical significance was set to conventional $p < 0.05$ for ANOVAs and non-parametric tests, and $p < 0.05$ (uncorrected) and then corrected by Bonferroni for Pearson correlations.

2.4. RESULTS

Three clusters (AUD-like Clusters) were identified through the k-means cluster analysis (***Figure 9***).

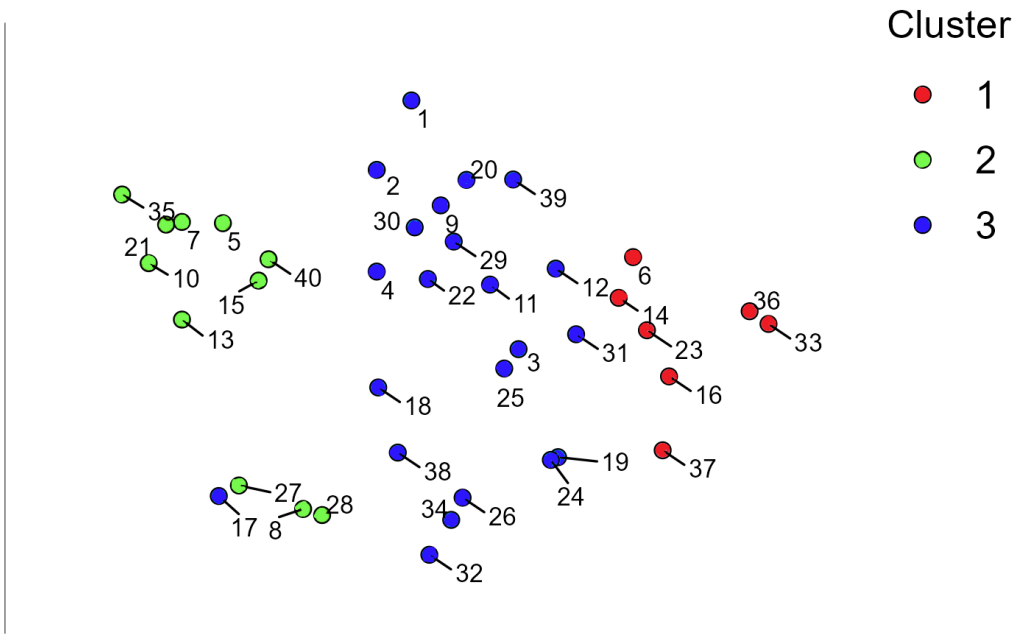

Figure 9. t-SNE plot of animals' allocation in 3 clusters defined by the k-means clustering, AUD-like Clusters. **Cluster 1**, n = 7; **Cluster 2**, n = 11; **Cluster 3**, n = 22.

K-means yielded three clusters (***Figure 9***): Cluster 1 accounted for 7/40 of rats (17.5% of the population) and it is mainly composed of males (5 males vs. 2 females); Cluster 2 accounted for 11/40 of animals (27.5% of the population) whose 5 males and 6 females; Cluster 3 is the larger group accounting for 22/40 of rats (55% of the population) with 10 males and 12 females (***Figure 10***).

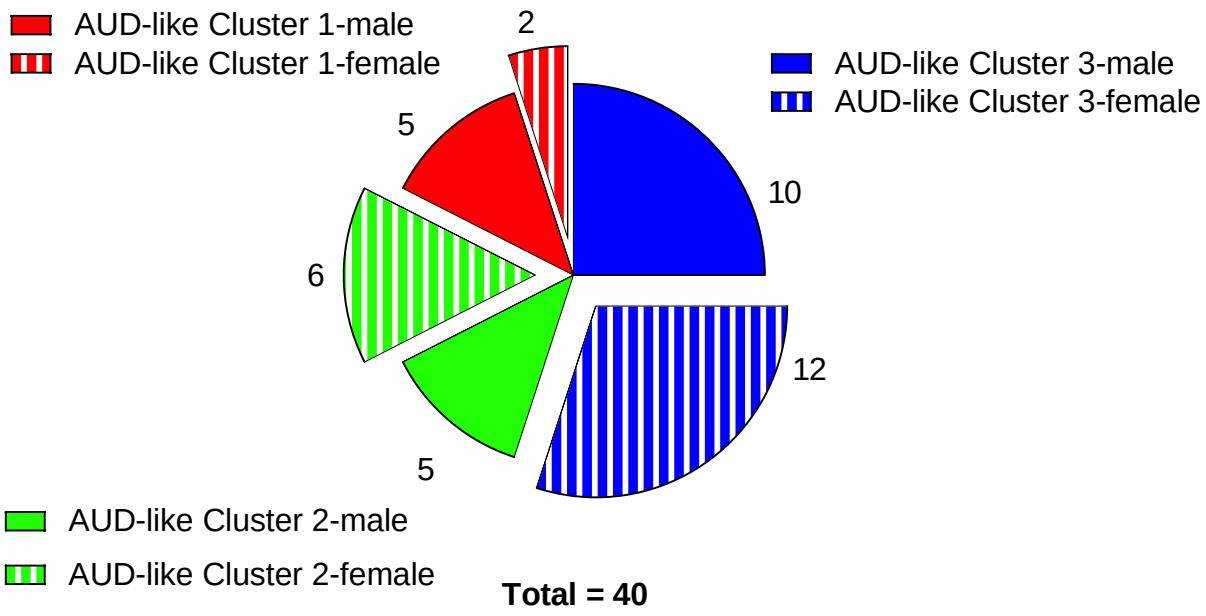

Figure 10. Sex prevalence with AUD-like Clusters. **Cluster 1** is the one composed of fewer subjects but also has proportionally more males. The others two clusters are better sex balanced. **Cluster 3** is the most numerous.

2.4.1. EXPERIMENT 1: Pre-alcohol exposure behavioral screening

Before alcohol SA, rats were screened for their innate levels of locomotor activity, anxiety, and pain sensitivity. Therefore, we retrospectively analysed these behavioral traits in the three AUD-like Clusters. One-way ANOVAs showed no difference between the three clusters in their locomotor activity [$F(2,37) = 0.48$, $p > 0.05$], anxiety-like behavior [$F(2,37) = 1.05$, $p > 0.05$], and pain sensitivity [$F(2,37) = 0.78$, $p > 0.05$] (***Figure 11***).

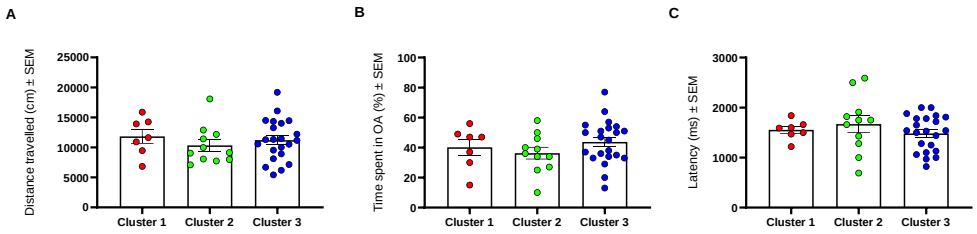

Figure 11. Screening of exploratory activity, anxiety, and pain sensitivity in drug-naïve HS rats. No difference was found between the three AUD-like Clusters (**Cluster 1**, **Cluster 2**, **Cluster 3**) in their locomotor activity expressed as distance travelled in the OF test (***A***), anxiety-like behavior expressed as the percentage of time spent in the open arms (OA) (***B***), nor in their pain sensitivity expressed as latency to pull the tail out of hot water (***C***). Data are presented as mean (±SEM).

2.4.2. EXPERIMENT 2: Comparison of operant alcohol-related behavior between AUD-like clusters

EXPERIMENT 2.1: Alcohol SA under PR schedule of reinforcement

To explore rats' motivation for ethanol, a PR was performed, each rat's BP was recorded, and the three AUD-like Clusters were then compared. Kruskal-Wallis test revealed a statistically significant difference between the three clusters ($X^2(3) = 10.24$, $p < 0.01$). Multiple comparisons showed a significantly higher BP in Cluster 1 compared to Cluster 2 [$p < 0.05$] and Cluster 3 [$p < 0.01$] (***Figure 12A***).

EXPERIMENT 2.2: Cue-induced reinstatement test

After a period of extinction during which active lever pressing was not reinforced by either alcohol delivery or presentation of the contingent house light cue, rats' cued relapse-like behavior was assessed. Two-way ANOVA revealed an overall effect of cluster [$F(2,37) = 7.51$, $p < 0.001$], experimental phase (extinction *vs.* cue-induced) [$F(1,37) = 90.25$, $p < 0.0001$], and the interaction between them [$F(2,37) = 15.07$, $p < 0.0001$]. Sidak post-hoc analysis revealed that alcohol use robustly reinstated alcohol-seeking behavior in Cluster 1 ($p < 0.0001$) and Cluster 3 ($p < 0.0001$), but not in Cluster 2 (***Figure 12B, upper panel***). Moreover, comparing the three clusters, Sidak post-hoc analysis found no difference between them in the extinction phase ($p > 0.05$), whereas

it revealed that Cluster 1 showed a statistically significantly higher reinstatement ratio than Cluster 2 ($p < 0.0001$) and Cluster 3 ($p < 0.0001$) (***Figure 12B, upper panel***). No differences were found between the extinction and reinstatement phases on the control inactive lever in all clusters [$F(1,37) = 0.011$, $p > 0.05$] (***Figure 12B, lower panel***).

EXPERIMENT 2.3: Alcohol seeking behavior

HS rats were evaluated for their alcohol-seeking behavior as a measure of craving comparing the number of active lever responses on the last day of alcohol SA with those that reached the first day of a cued extinction session (i.e., active lever responses were reinforced but alcohol paired cues but not alcohol). The seeking score was calculated as a ratio between these two values. Kruskal-Wallis test found no statistically significant differences between the three clusters ($X^2(3) = 4.18$, $p > 0.05$) (***Figure 12C***).

EXPERIMENT 2.4: Foot-shock punishment on alcohol SA

In the punished reward test, animals were presented with an aversive stimulus (foot-shock) associated with the subsequent administration of alcohol. The resistance score for each session, a mean of the resistance scores of the last three punished sessions was used to compare clusters of rats. No differences were detected from the Kruskal-Wallis test between the clusters ($X^2(3) = 5.76$, $p > 0.05$) (***Figure 12D***).

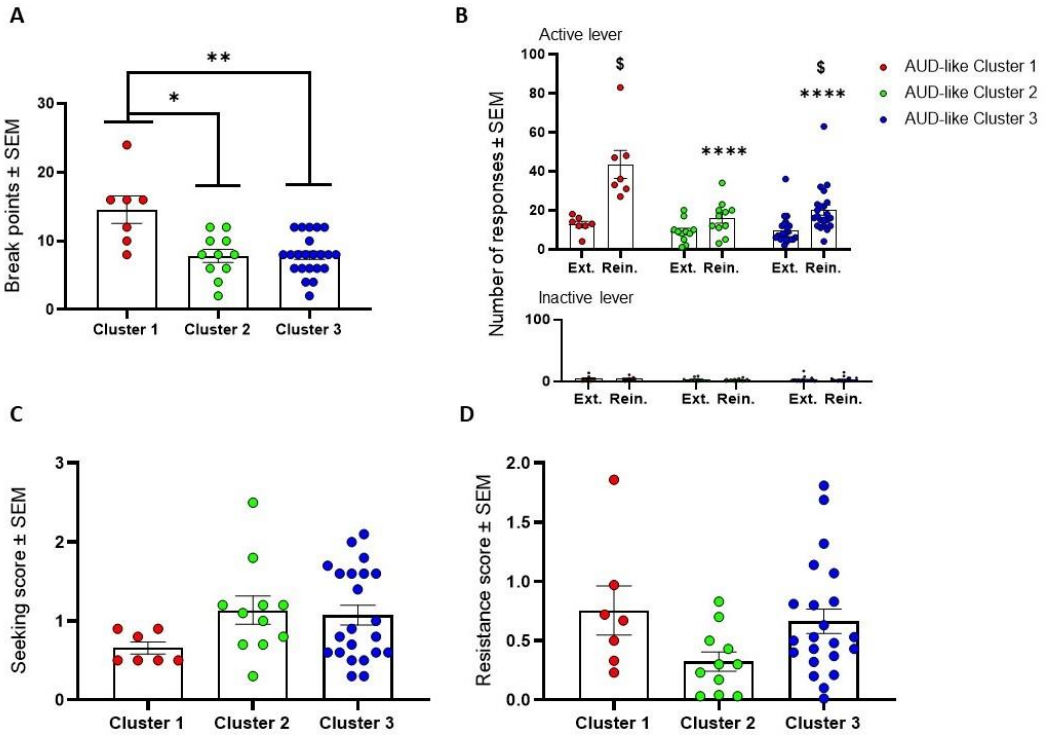

Figure 12. Four alcohol-related behaviors (***A*** - Progressive Ratio; ***B*** - Cued-induced reinstatement; ***C*** - Seeking behavior; ***D*** - Foot-shock punished SA) were compared between the three HS rats AUD-like Clusters (**Cluster 1**, **Cluster 2**, **Cluster 3**). ***A.*** Motivation for 10% ethanol measured by the BP during a PR schedule of reinforcement. The BP was higher in **Cluster 1** compared to the other two clusters. ***B.*** Number of responses to the active and the inactive lever during both extinction and reinstatement test. The cue-induced reinstatement (Rein.) compared to the extinction (Ext.) phase (average of the last three sessions increased alcohol seeking) in **Cluster 1** and **Cluster 3** as indicated by the higher number of active lever presses. Cue presentation also induced a higher relapse in **Cluster 1** compared to **Cluster 2** and **Cluster 3**. ***C.*** Alcohol seeking score 24 h after the last self-administration session. No differences were found between clusters. ***D.*** Resistance scores in foot-shock punished alcohol SA session. The mean of the resistance scores of the last three punished sessions was used to confront the three clusters. No statistically significant difference was found between the clusters. Data are presented as mean (±SEM). *p < 0.05, **p < 0.01, ****p < 0.0001 when Clusters are compared, $p < 0.0001 when the Rein. is compared to the Ext. in the same cluster.

2.4.3. EXPERIMENT 3: Characterization of AUD-like behavior in 2BC paradigms

EXPERIMENT 3.1: Continuous ethanol intake in 2BC paradigm

Basal levels of alcohol intake were detected through continuous access to 10% alcohol and tap water under the 2BC paradigm. Kruskal-Wallis test revealed a significant difference between the three clusters ($X^2(3) = 22.69$, $p < 0.0001$). Multiple comparisons showed a lower consumption of alcohol in Cluster 2 compared to Cluster 1 ($p < 0.001$) and Cluster 3 ($p < 0.0001$) (***Figure 13***).

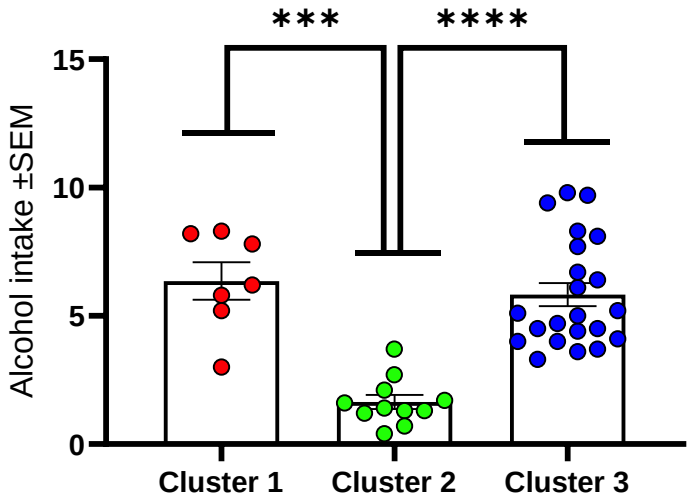

Figure 13. Alcohol intake in 2BC was compared in the three HS rats AUD-like Clusters (**Cluster 1**, **Cluster 2**, **Cluster 3**). ***A.*** Mean of the last three days of alcohol intake in the 2BC paradigm was used to compare the three clusters on their ethanol consumption. **Cluster 2** significantly consumes less alcohol when compared to **Cluster 1** and **Cluster 3**. No difference was found between **Cluster 1** and **Cluster 3**. Data are presented as mean (±SEM). ***p < 0.001, ****p < 0.0001.

EXPERIMENT 3.2: Quinine adulterated alcohol drinking in 2BC

Maintenance of alcohol intake despite quinine adulteration was measured. Four different doses of quinine (10, 25, 50, and 100 mg/L) were tested. Comparing the four doses of quinine, one-way ANOVA found a main effect of treatment [$F(3,117) = 190.3$,

p < 0.0001] and the subsequent post-hoc analysis revealed a statistical significant difference between each dose compared (each dose *vs.* each dose: p < 0.0001), except for the comparison between 50 and 100 mg/L (p > 0.05). Hence, quinine adulteration dose-dependently decreased alcohol intake (***Figure 14A***). To verify the specificity of quinine on alcohol consumption, water was adulterated using the doses of 2.5, 5, 10 and 25 mg/L. One-way ANOVA showed an overall effect of treatment [F(4,152) = 174, p < 0.0001]. Post-hoc test revealed that both doses significantly reduce alcohol intake compared to alcohol without quinine (each dose vs. vehicle: p < 0.0001) (***Figure 14B***). Already at the lower dose of 2.5 mg/L rats began to decrease their water consumption. Conversely, at 10 mg/L most of rats still drink water, and at 50 and 100 mg/L most of them stopped to consume alcohol, whereas. For this reason, the dose of 25 mg/L was selected to calculate the quinine adulteration score used as criteria to group rats into the AUD-like Clusters based on k-mean cluster analysis.

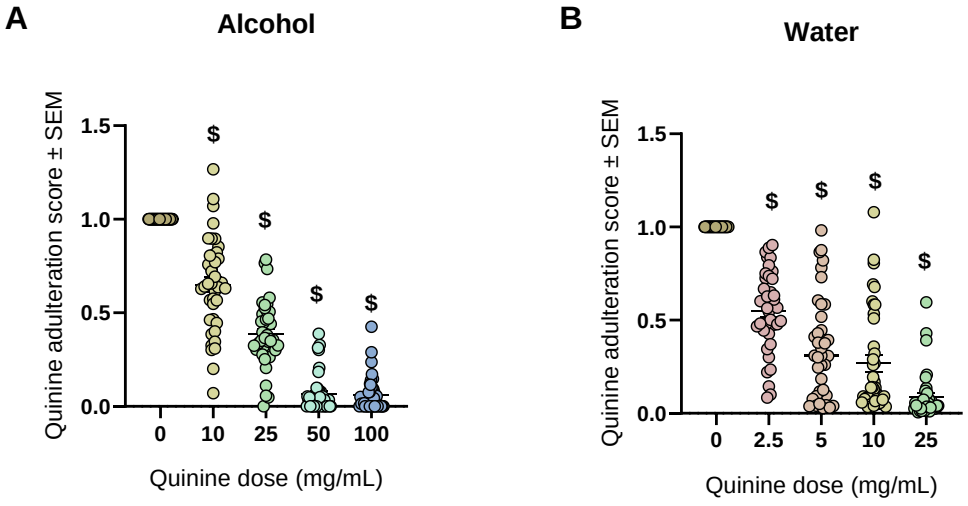

Figure 14. Quinine dose/quinine adulteration score curve in HS rats. ***A.*** Rats significantly reduced alcohol intake in response to quinine adulteration at all doses tested (10, 25, 50, and 100 mg/L). ***B.*** Rats significantly reduced water intake in response to quinine adulteration at both doses tested (10 and 25 mg/L). Data are presented as mean (±SEM). $p < 0.0001 *vs.* the corresponding vehicle treatment.

One-way ANOVA found a significant difference between the three clusters [$F(2,37) = 17.51$, $p < 0.0001$]. Holm-Sidak post-hoc analysis revealed differences between Cluster 1 and Cluster 3 ($p < 0.001$) and between Cluster 2 and Cluster 3 ($p < 0.0001$), whereas no difference was found between Cluster 1 and Cluster 2 ($p > 0.05$) (***Figure 15***).

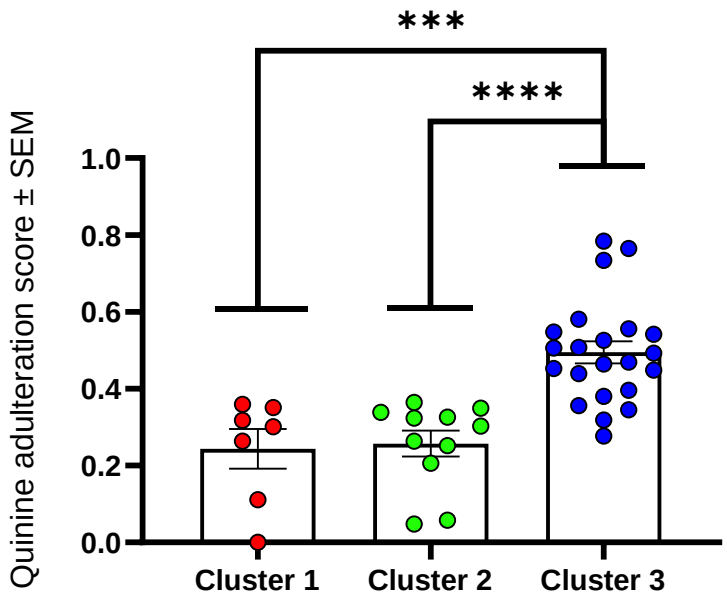

Figure 15. Resistance to quinine adulteration comparing the three HS rats AUD-like Clusters (**Cluster 1**, **Cluster 2**, **Cluster 3**). Quinine adulteration score was used to compare the three clusters. **Cluster 3** significantly consumed more alcohol compared to the other two despite quinine. Data are presented as mean (±SEM). ***$p < 0.001$, ****$p < 0.0001$.

2.4.4. EXPERIMENT 4 and 6.1: Specificity of NTX effect

EXPERIMENT 4: Effects of NTX pre-treatment on alcohol SA

The effect of NTX (0.3 and 1.0 mg/kg) or its vehicle was tested on alcohol SA in HS rats. Five rats that self-administered less than 10 rewards under vehicle condition were excluded from the analysis. One-way repeated measures ANOVA on the whole population found a significant difference between the doses used [$F(2,68) = 27.47$, $p < 0.0001$]. Holm-Sidak multiple comparisons test revealed an efficacy of both doses of

NTX (0.3 mg/kg: $p < 0.001$; 1.0 mg/kg: $p < 0.0001$) compared to the vehicle treatment (***Figure 16A***). Analysis of inactive lever responses found no significant effect [$F(2,68) = 2.13$, $p > 0.05$]. We next verified whether NTX differently affected alcohol SA in the three AUD-like Clusters. Two-way ANOVA showed an overall effect of dose [$F(2,64) = 21.46$, p 0.0001] and cluster [$F(2,32) = 3.98$, $p < 0.05$] but no interaction [$F(4,64) = 0.49$, $p > 0.05$]. Sidak post-hoc test revealed no difference between the three clusters in their alcohol SA under vehicle treatment ($p > 0.05$ for each pairwise comparison). Instead, the post-hoc showed that at 1.0 mg/kg NTX decreased alcohol rewards in all three clusters (Cluster 1: $p < 0.05$; Cluster 2: $p < 0.01$; Cluster 3: $p < 0.0001$), whereas at 0.3 mg/kg NTX significantly reduced alcohol SA in Cluster 1 ($p < 0.05$) and Cluster 3 ($p < 0.05$), with no effect in Cluster 2 ($p > 0.05$) (***Figure 16B***).

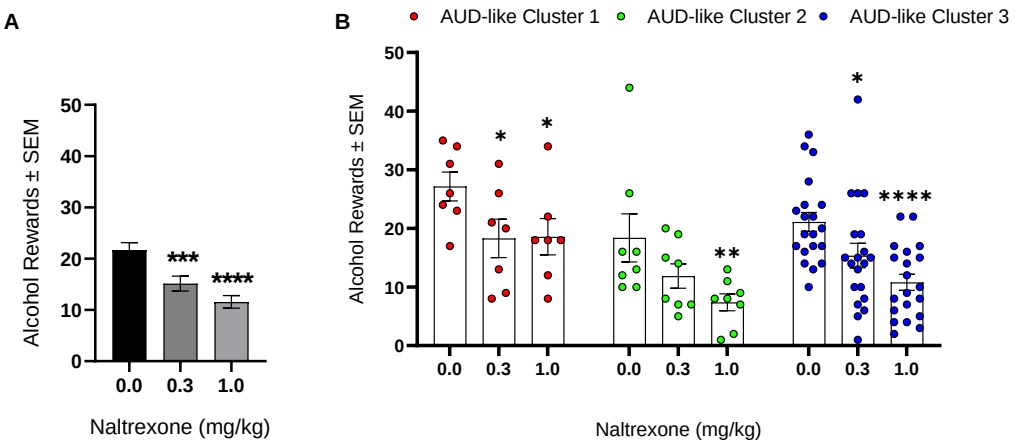

Figure 16. Male and female HS rats were treated with NTX (0.0, 0.3, and 1.0 mg/kg) i.p., 30 min before the alcohol SA session. ***A.*** At both doses, NTX significantly reduced the number of alcohol rewards compared to the vehicle treatment. ***B.*** At the dose of 1.0 mg/kg NTX successfully reduced alcohol SA in all clusters, whereas the lower dose of 0.3 mg/kg was only effective in **Cluster 1** and **Cluster 3**. Data are presented as mean (±SEM). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 *vs.* the corresponding vehicle treatment.

EXPERIMENT 6.1: Effects of NTX pre-treatment on saccharin SA

We next verified the specificity of the action of NTX by testing its effect on a natural reward, namely on saccharin SA. Four female rats were excluded for health issues. Friedman test demonstrated a significant difference between the NTX doses tested [$X^2(3) = 23.87$, $p < 0.0001$]. Post-hoc analysis revealed that only at 1.0 mg/kg NTX reduced saccharin rewards in all animals ($p < 0.0001$) (***Figure 17A***). Analysis of inactive lever responding found no significant effect [$F(2,60) = 2.19$, $p > 0.05$]. The effect of NTX on saccharin SA was further evaluated on the three AUD-like Clusters. Two-way ANOVA analysis found the only main effect of dose [$F(2,56) = 13.34$, $p < 0.0001$], neither effect of cluster [$F(2,28) = 0.71$, $p > 0.05$] nor interaction between the two factors [$F(4,56) = 0.94$, $p > 0.05$]. No difference between the three clusters in their saccharin SA under vehicle treatment ($p > 0.05$ for each pairwise comparison) was found by the post-hoc analysis. Sidak post-hoc test revealed that at 0.3 mg/kg NTX had no effect on any cluster, whereas its higher dose of 1 mg/kg NTX reduced the number of saccharin rewards in both Cluster 1 ($p < 0.01$) and Cluster 3 ($p < 0.001$), but not in Cluster 2 ($p > 0.05$) (***Figure 17B***).

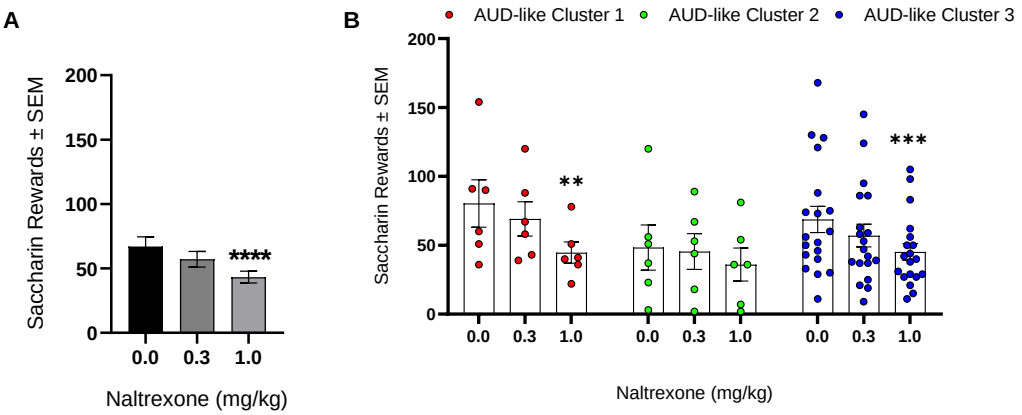

Figure 17. NTX (0.0, 0.3, and 1.0 mg/kg) was tested on saccharin SA in HS rats treated s.c., 30 min before the session. ***A.*** At the higher dose, NTX significantly reduced the number of alcohol rewards compared to the vehicle treatment. ***B.*** When NTX specificity was evaluated in the three AUD-like Clusters, the dose of 1.0 mg/kg reduced saccharin SA in **Cluster 1** and **Cluster 3**, with no significant effect in **Cluster 2.**

Data are presented as mean (±SEM). **p < 0.01, ***p < 0.001, ****p < 0.0001 *vs.* the corresponding vehicle treatment.

2.4.5. EXPERIMENT 5 and 6.2: Specificity of memantine effect

EXPERIMENT 5: Effects of memantine pre-treatment on alcohol SA

Memantine (6, 12, and 25 mg/kg), or its vehicle was tested in HS rats. Eight rats that self-administered less than 10 rewards under vehicle condition were excluded from this experiment. Friedman test found a statistically significant difference between the doses used ($X^2(4) = 44.94$, $p < 0.0001$). Dunn's post-hoc revealed that at 25 mg/kg memantine significantly reduced alcohol rewards compared to the vehicle treatment ($p < 0.0001$) (***Figure 18A***). Analysis of the inactive lever found no significant effect [$F(3,93) = 2.53$, $p > 0.05$]. Two-way ANOVA analysis applied to the three AUD-like Clusters found the only overall effect of dose [$F(3,87) = 18.34$, $p < 0.0001$], and neither the effect of cluster [$F(2,29) = 1.07$, $p > 0.05$] nor interaction between the two factors [$F(6,87) = 0.37$, $p > 0.05$] were statistically significant. Post-hoc analysis did not show a difference between the three clusters under vehicle treatment ($p > 0.05$ for each pairwise comparison). Sidak post-hoc test revealed that the higher dose of memantine significantly decreased alcohol rewards in Cluster 1 ($p < 0.05$) and Cluster 3 ($p < 0.0001$), whereas in Cluster 2 approached but did not reach statistical threshold ($p = 0.055$). The other two doses tested were ineffective on all clusters (***Figure 18B***).

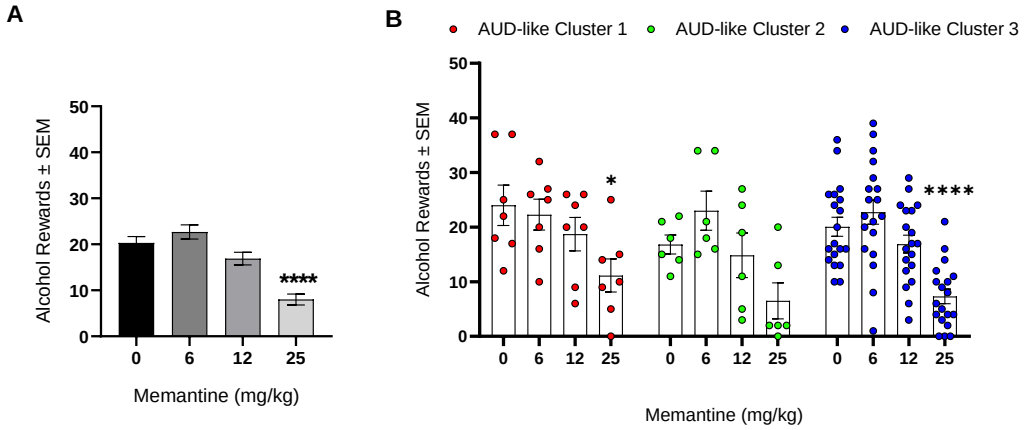

Figure 18. HS rats were treated with memantine (0, 6, 12, and 25 mg/kg) gavage, 1 h before the alcohol SA session. **A.** The higher dose of 25 mg/kg significantly reduced the number of alcohol rewards compared to the vehicle treatment. **B.** In the three AUD-like Clusters, at 25 mg/kg memantine successfully reduced alcohol SA in **Cluster 1** and **Cluster 3**, with a trend in **Cluster 2**. Data are presented as mean (±SEM). *$p < 0.05$, ****$p < 0.0001$ *vs.* the corresponding vehicle treatment.

EXPERIMENT 6.2: Effects of memantine pre-treatment on saccharin SA

Finally, we also tested the specificity of memantine effect on the natural reward saccharin SA. One female rat was sacrificed before performing the current experiment because of health issues. Since the only dose of 25 mg/kg memantine was effective on alcohol SA, only this dose was tested on saccharin SA. Wilcoxon test demonstrated that memantine also reduced saccharin SA in all HS animals [$W(32) = -518$, $p < 0.0001$] (***Figure 19A***). Analysis of inactive lever responding found no significant effect [$t(31) = 0.96$, $p > 0.05$]. Applying two-way ANOVA on AUD-like Cluster, the only overall effect of dose was found [$F(1,29) = 66.46$, $p < 0.0001$], and neither effect of cluster [$F(2,29) = 0.88$, $p > 0.05$] nor interaction between the two factors [$F(2,29) = 0.18$, $p > 0.05$]. Sidak post-hoc analysis showed no difference between the clusters under vehicle treatment ($p > 0.05$ for each pairwise comparison) but instead it revealed that at 25 mg/kg memantine significantly reduced saccharin SA in all clusters (Cluster 1: $p < 0.001$; Cluster 2: $p < 0.001$; Cluster 3: $p < 0.0001$) (***Figure 19B***).

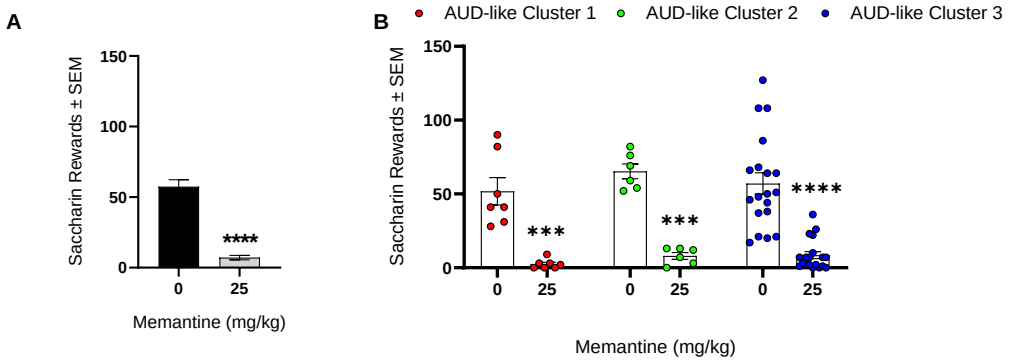

Figure 19. Memantine (0.0, and 25 mg/kg) specificity was evaluated on saccharin SA. ***A.*** At 25 mg/kg memantine reduced saccharin SA. ***B.*** Moreover, the higher dose selected significantly reduced saccharin SA in all AUD-like Clusters. Data are presented as mean (±SEM). ****p < 0.0001 *vs.* the corresponding vehicle treatment.

2.4.6. EXPERIMENT 7: Correlation between behaviors evaluated on the multisymptomatic screening and effects of NTX pre-treatment on alcohol SA

NTX and memantine experiments demonstrated that NTX but not memantine is efficacious and selective toward alcohol seeking and therefore all further analyses were conducted exclusively on NTX data.

We initially explored the relationship between the IESs induced by of the two doses of NTX tested and the rats' behaviors screened when they were drug naïve (anxiety-like behavior, locomotor activity, and pain sensitivity). Bonferroni corrected significant threshold was set at p = 0.015 (Curtin & Schulz, 1998; Zhao, et al., 2021). Pearson correlation coefficients (r) are reported in ***Figure 20A***. We found that the time spent in the open arms (OA) of the EPM test is negatively correlated with the IES value at 0.3 mg/kg NTX (r = -0.45, p < 0.01), meaning that the higher is the time spent in the OA of the EPM (less anxiety-like behavior), the lower the IES value at 0.3 mg/kg (i.e., the weaker response to the treatment) (***Figure 20B***).

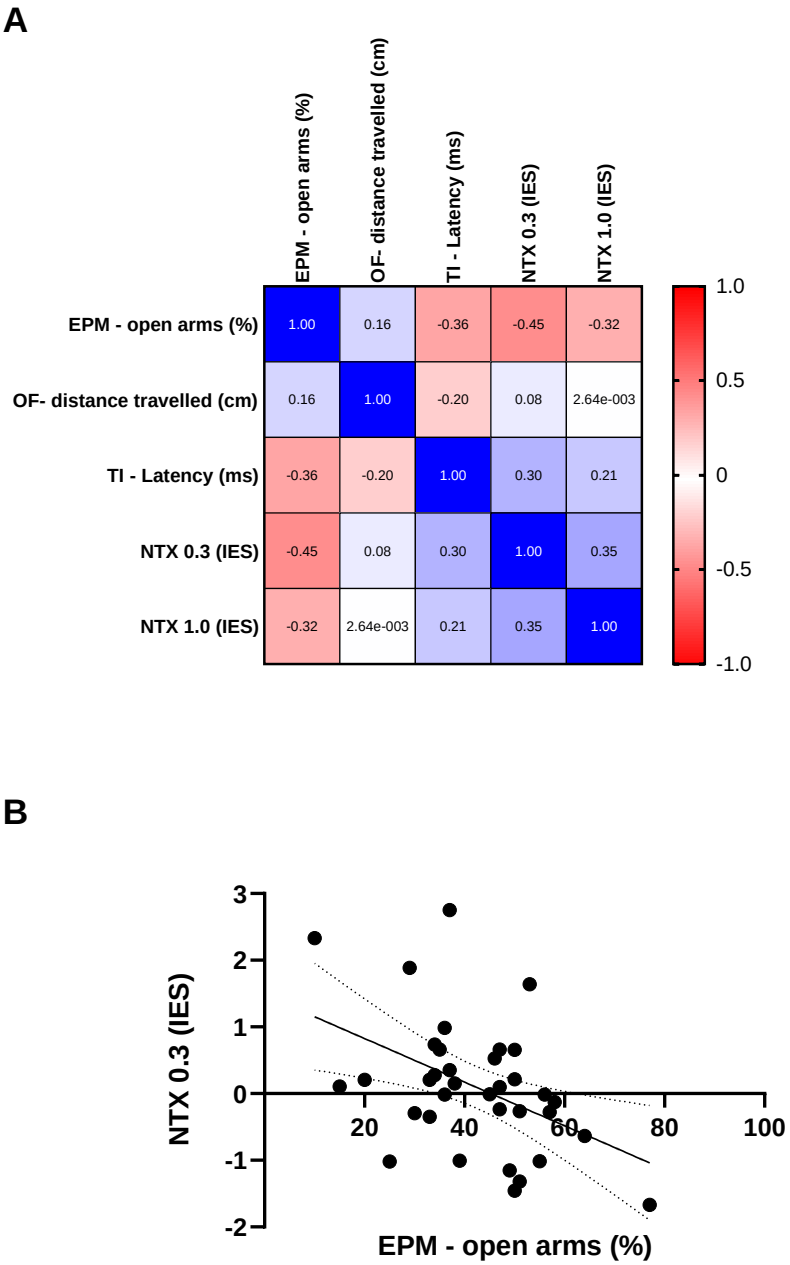

Figure 20. A. Heatmap of Pearson's correlation analysis between innate phenotypes (anxiety-like behavior in the EPM test expressed as the percentage of time spent in open arms, locomotor activity in the OF test expressed as the distance travelled in cm, pain sensitivity in the TI test expressed as the latency to withdraw the tail in ms) and NTX response on alcohol SA (expressed as IES values). Pearson correlation coefficients (r) are shown. ***B.*** Scatter plot of correlation between the percentage of time spent in the OA of the EPM test and the IESs at 0.3 mg/kg NTX. Continuous and dotted lines represent best fit linear regression and 95% interval, respectively.

Moreover, we also checked the relationship between the effect of the two doses of NTX (the IESs) tested and the rats' alcohol-related behaviors we used to group them in the three AUD-like Clusters (motivation, cue-induced reinstatement, seeking behavior, resistance to footshock punishment and quinine adulteration, and alcohol intake). Pearson correlation coefficients (r) are reported in ***Figure 21***. No significant correlation emerged between the response to the two doses of NTX and any alcohol-related behavior tested after Bonferroni's correction (Curtin & Schulz, 1998; Zhao, et al., 2021).

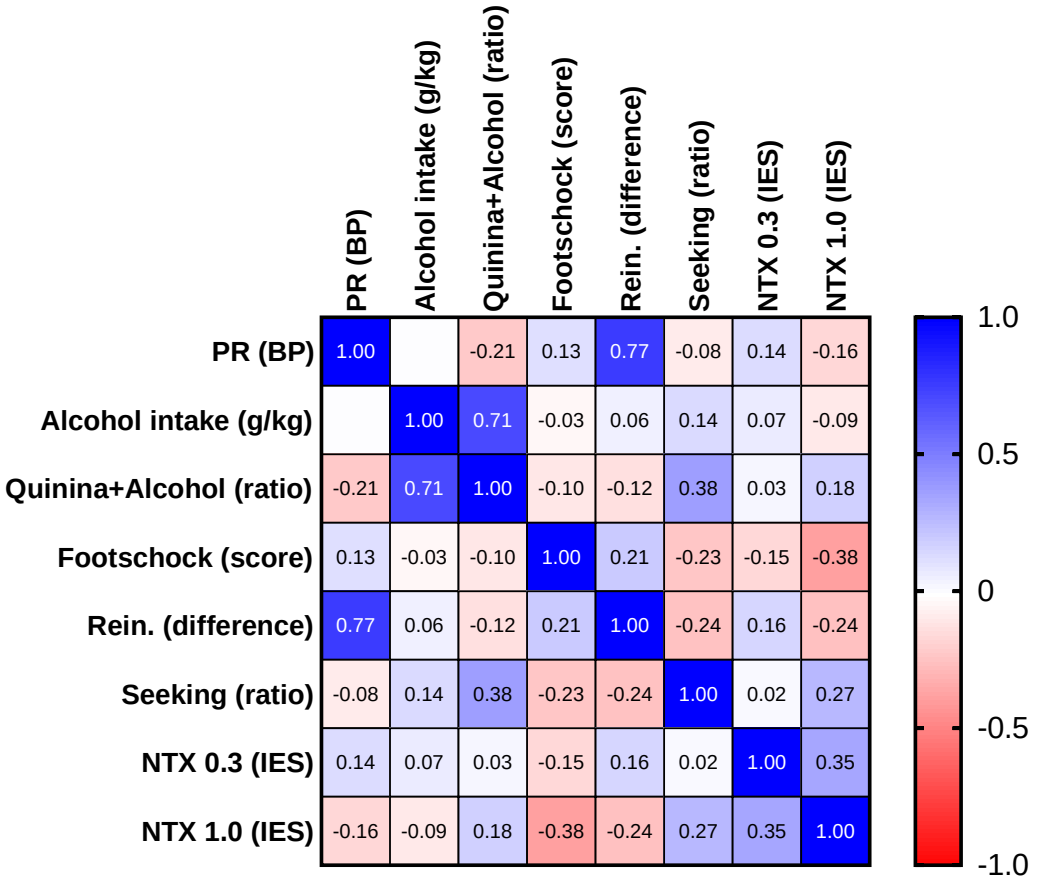

Figure 21. Heatmap of Pearson's correlation analysis between alcohol-related behavior (motivation in the PR session expressed BP, alcohol intake in the 2BC expressed as g/kg, quinine adulteration of alcohol intake as g/kg, resistance to foot-shock punishment expressed as resistance score, cue-induced reinstatement (Rein.) expressed as the difference in active lever response between the cue-induced reinstatement test and the average of the last 3 days of extinction, and seeking behavior expressed as the

ratio between the number of active lever pressing in the first extinction session and that of the last day of alcohol SA) and NTX response on alcohol SA (expressed as IES values). Pearson correlation coefficients (r) are shown.

To further investigate the effect of NTX and better understand this correlation we found, we decided to cluster rats based on their IESs. K-means cluster analysis (K=2, 10 iterations) was used to divide rats based on their IES to treatment (NTX) (***Figure 22***, NTX-Clusters).

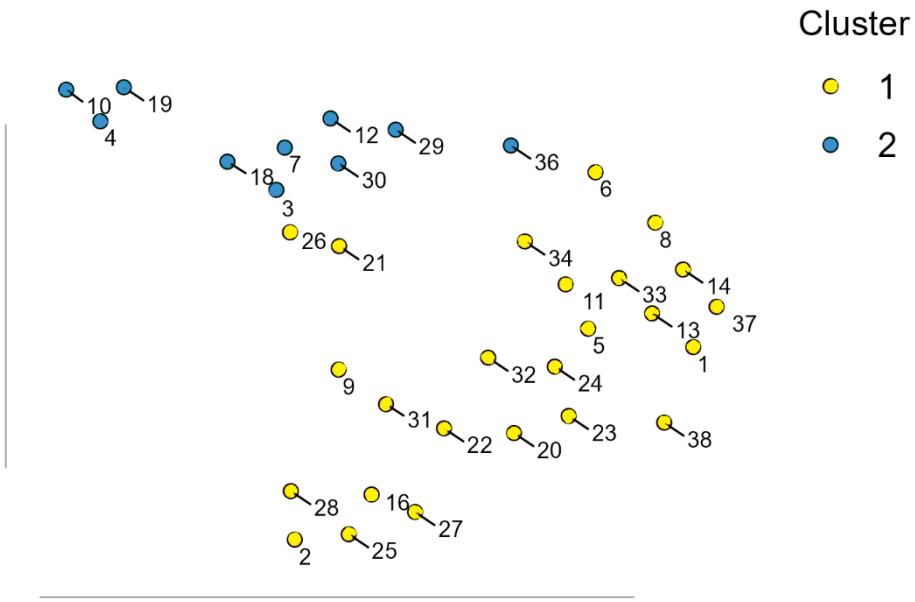

Figure 22. t--SNE plot of animals' allocation in 2 clusters defined by the k-means clustering. Each individual ISE for all the doses of NTX (0.3 and 1.0 mg/kg) was used to distribute rats in 2 clusters, NTX-Clusters. **Cluster 1**, n = 25; **Cluster 2**, n = 10.

Then, we checked the effect of NTX on alcohol SA expressed by the two NTX clusters. Two-way ANOVA revealed a main effect of dose [$F(2,66) = 40.9$, $p < 0001$] and treatment x cluster interaction [$F(2,66) = 11.11$, $p < 0.0001$], but no effect of cluster [$F(1,33) = 0.25$, $p > 0.05$]. Sidak post-hoc analysis showed that at 1.0 mg/kg NTX treatment decreased the number of reinforcements received by both clusters (NTX-

Cluster 1: $p < 0.001$; NTX-Cluster 2: $p < 0.0001$), whereas at 0.3 mg/kg NTX decreased alcohol rewards only in NTX-Cluster 2 ($p < 0.001$) (***Figure 23A***). When we compared the two NTX-Clusters on their response to NTX under saccharin SA to test the specificity of NTX treatment, two-way ANOVA found an overall effect of dose [$F(2,58) = 10.29$, $p < 0.001$], but neither effect of cluster [$F(1,29) = 0.018$, $p > 0.05$] nor treatment x cluster interaction [$F(2,58) = 0.19$, $p > 0.05$]. Sidak post-hoc revealed that at 1.0 mg/kg NTX was effective in NTX-Cluster 1 ($p < 0.001$), but not in NTX-Cluster 2 ($p > 0.05$), while at 0.3 mg/kg NTX did not affect saccharin SA in either cluster (***Figure 23B***). Therefore, based on these results we concluded that NTX-Cluster 1 is composed of non-responder rats and NTX-Cluster 2 consists of responder rats to the NTX treatment for alcohol SA. Based on the negative correlation we previously showed, we expected that the group of rats with the weaker response to NTX, namely NTX-Cluster 1, should also shows less anxiety-like behavior when tested in the EPM before alcohol exposure. Comparing the 2 NTX-Clusters for their time spent in the OA of the EPM, t-test analysis confirmed that Cluster 1 spent more time in the OA compared to Cluster 2 [$t(32) = 2.37$, $p < 0.05$] (***Figure 23C***).

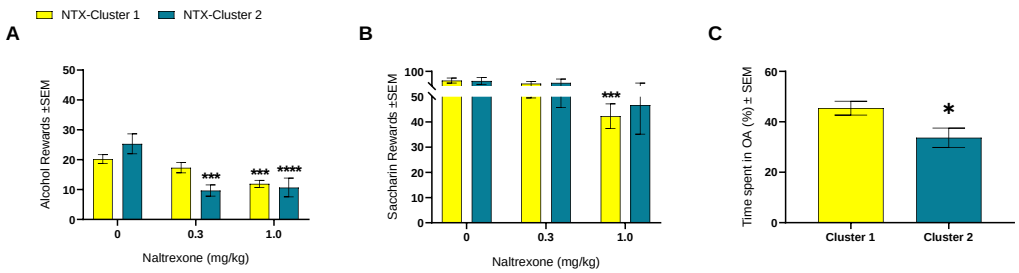

Figure 23. Comparison of the two NTX-Cluster defined by the k-means clustering. ***A.*** NTX-**Cluster 2** significantly reduced alcohol SA in response to both doses of NTX tests, whereas only at 1.0 mg/kg NTX was effective in NTX-**Cluster 1** in reducing the number of alcohol rewards. ***B.*** At 1.0 mg/kg NTX significantly reduced saccharin SA in NTX-**Cluster 1** revealing this cluster as the non-responder one. C. When the two NTX-Clusters were compared for their anxiety-like behavior before alcohol exposure, NTX-**Cluster 1** showed to be less anxious, spending significantly more time in the open arms of the EPM than NTX-**Cluster 2**. Data are presented as mean (±SEM). ***A-B***: ***$p < 0.001$, ****$p < 0.0001$ *vs.* the vehicle treatment in the same cluster for NTX treatment; ***C***: *$p < 0.05$ *vs.* NTX-**Cluster 1**.

Finally, we checked for the sex prevalence in the two NTX-Clusters (***Figure 24A***): NTX-Cluster 1 accounted for 5 males and 5 females (50% per sex), whereas NTX-Cluster 2 accounted for 14 males (56%) and 11 females (46%). The AUD-like Clusters prevalence in NTX-Clusters is shown in ***Figure 24B***: NTX-Cluster 1 accounted for just one rat of AUD-like Cluster 1 (10%), 2 rats of AUD-like Cluster 2 (20%), and 7 rats of AUD-like Cluster 3 (70%); NTX-Cluster 2 accounted for 6 animals of AUD-like Cluster 1 (24%), 6 rats of AUD-like Cluster 2 (24%), and 13 animals of AUD-like Cluster 3 (52%).

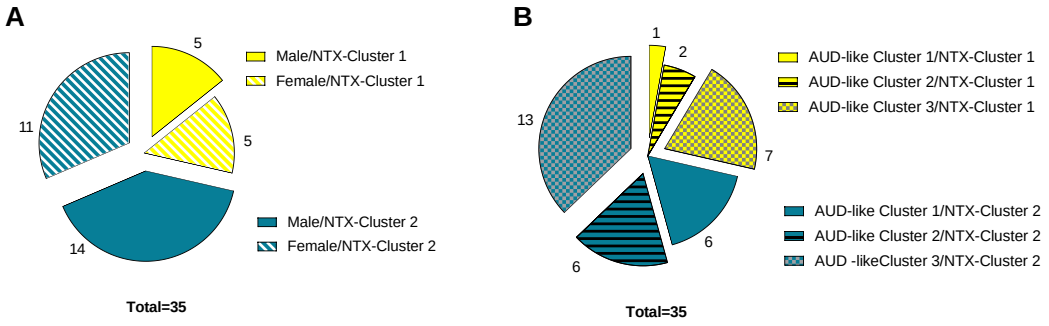

Figure 24. A. Sex prevalence within NTX-Clusters. NTX-**Cluster 1** is the one composed of fewer subjects but also has an equal distribution of males and females (5/sex), whereas NTX-**Cluster 2** is the most numerous and within it, the number of males and females is essentially equal because there are generally more males than females (44% female and 56% male). ***B.*** AUD-like Clusters prevalence within NTX-Clusters. AUD-like Cluster 1 is more prevalent in **Cluster 2** than **Cluster 1** as well as AUD-like Cluster 2, whereas AUD-like -Cluster 3 proportionally is more prevalent in **Cluster 1**.

2.5. DISCUSSION

NIH-HS rats were previously demonstrated to be a valid preclinical model of individual variability in vulnerability or resilience to developing opioid addiction-related behaviors (Allen, et al., 2021). This rat line has been previously used to screen alcohol drinking (Deal, et al., 2021; Li & Lumeng, 1984; Murphy, et al., 1987; Tabakoff & Culp, 1984; Zahr, et al., 2014), however this was the first attempt to screen multiple alcohol-related behaviors. We hypothesized that this model would be endorsed with a high predictive validity to screen pharmacotherapies for AUD. Limitations and difficulties in translatability between preclinical and clinical outcomes may be due to the use of rat lines with limited heterogeneity. While these lines guarantee the experimental reproducibility performing studies in which two homogenous groups of rats (belonging to the same strain) are compared regarding the functioning of a molecule, they also prevent the assessment of inter-individual variability. Since it has been documented that AUD is a multifactorial and heterogenous disorder (Maisto, et al., 2022; Litten, et al., 2015) in which medical treatments are only discretely effective (Ray, et al., 2019), using highly heterogenous rat lines would help to develop more specific and appropriate pharmacological treatment according to the patient's genetic and phenotypical characteristics.

To create a multisymptomatic preclinical model of individual variability of AUD, a fist cohort of NIH-HS rats (males and females matched) was initially screened for their innate behavior before being exposed to alcohol. Then, we characterized them for their alcohol-related behaviors to identify different individual susceptibility in the development of AUD.

AUD patients differ in their alcohol-related behaviors and show different symptoms. Indeed, according to the DMS-5, the AUD diagnosis is based on a total of 11 criteria, and to be diagnosed with AUD an individual does not need to meet all of them (American Psychiatric Association, 2013). Patient may distinguish from one another depending on which of the 11 different symptoms (criteria) they show, and the severity of the diagnosis depends on the number of diagnostic criteria attested (see **Chapter 1**, paragraph "**2.1. AUD DIAGNOSIS AND THERAPEUTIC**

APPROACHES"). To properly apply these criteria on our animal model, we divided them into 3 macro areas: macro area 1 includes behaviors associated with compulsivity and persistence on alcohol drinking despite negative consequences (criterion 1 and criteria 5-9); macro area 2 contains behaviors associated with motivation for alcohol drinking (criteria 2, 3, and 10); macro area 3 includes behaviors related with the risk to relapse (criteria 4 and 11). Therefore, similarly to what was previously proposed by Deroche-Gamonet and colleagues (Deroche-Gamonet, et al., 2004), we allocated the six alcohol related behaviors that we screened with the three DSM-5 macro areas.

Our characterization model included the analysis of:

1- Increased motivation towards alcohol, measured through the PR schedule of reinforcement (macro area 1);

2- Amount of alcohol consumed through the 2BC paradigm (macro area 1);

3- Continue alcohol seeking despite negative consequences, assessed by the foot-shock protocol (macro area 2);

4- Compulsive excessive drinking, determined through the quinine adulteration paradigm (macro area 2);

5- Relapse in alcohol consumption, measured by the cue-induced reinstatement protocol (macro area 3);

6- Alcohol seeking behavior and craving, evaluated considering the first day of extinction in which alcohol is not available (macro area 3).

We chose to test two different parameters for compulsive-like behavior (foot-shock punishment and quinine adulteration) to better characterize HS rats' behavior (Hopf & Lesscher, 2014). In the foot-shock procedure, the foot-shock punishment followed the seeking response of active lever pressing and preceded the alcohol delivery, and then it models the human behavior of responding perseveration despite adverse consequences (Domi, et al., 2019; Marchant, et al., 2013; McDonald, et al., 2021). On the other hand, quinine is a bitter substance that added to a fluid produces taste aversion. Then, adulteration of alcohol in the 2BC paradigm makes it possible to determine aversion-resistant drinking behavior (Hopf, et al., 2010; Turyabahika-

Thyen & Wolffgramm, 2006; Vengeliene, et al., 2009; Wolffgramm, et al., 2000). Using both procedures, we could highlight two different aspects of compulsive-like behavior: while the resistance to the foot-shock punishment underlines a compulsive behavior in which the rat continues to seek and consume alcohol despite adverse consequences (physical pain) (seeking punishment), quinine adulteration focuses on a form of compulsivity based on a taste aversion due to the bitter quinine added, so that an addicted animal continues to drink even if they did not like alcohol taste anymore (taking punishment).

According to the 6 alcohol-related behaviors assessed, we could group rats into clusters through a k-means cluster analysis (K = 3) based on the z-scores of each behavioral parameter.

As previously shown, the three clusters differ between them under some of the 6-alcohol-related behaviors. The three AUD-like Clusters individuated did not differ in their innate behavior (locomotion, anxiety-like behavior, and pain sensitivity) before being exposed to alcohol. Moreover, no statistically significant difference between them was found in their alcohol-seeking behavior and resistance to the foot-shock punishment. Instead, differences were revealed in the other criteria considered. AUD-like Cluster 1 (the smallest one, n = 7) was demonstrated to be composed of rats with the highest motivation for alcohol and reinstatement induced by a cue compared to the other two clusters. Although AUD-like Cluster 3 (the largest one, n = 22) consumed an amount of alcohol similar to AUD-like Cluster 1, it showed the higher maintenance of drinking despite quinine adulteration, and it also relapsed following cue presentation. AUD-like Cluster 2 (n = 11) did not relapse following cue presentation, and it showed the lowest alcohol intake, also under quinine adulteration. Cluster 2 and Cluster 3 are well balanced with regard to gender distribution, whereas Cluster 1 has a predominance of males (71.43%) than females, but it is important also to consider that it is also the smallest cluster.

Thereafter, to validate the predictive validity of our model, two doses of NTX (0.3 and 1.0 mg/kg) and three doses of memantine (6, 12, and 25 mg/kg) were assessed on the three clusters individuated.

NTX is one of three FDA-approved drugs for the treatment of AUD but there is a gap between the homogeneous efficacy demonstrated in preclinical studies and the heterogeneous efficacy observed in clinical practice. Several studies reported that the effects of NTX differ according to the individual phenotype shown by the patient (Hartwell, et al., 2020; Maisel, et al., 2013). Hence, we evaluated NTX efficacy in our NIH-HS rats showing different AUD-like behaviors. In the whole HS population, at 0.3 mg/kg NTX specifically reduced alcohol SA, whereas the higher dose of 1 mg/kg was also reduced saccharin SA. When we verified NTX effect on the three AUD-like Clusters, we observed that the lower dose of 0.3 mg/kg was specific for alcohol in Cluster 1 and Cluster 3, and the higher dose of 1.0 mg/kg selectively reduced alcohol SA only in Cluster 2. AUD-like Cluster 2 was characterized by less pronounced alcohol-related behaviors compared to the other two clusters, and then this result can be interpreted as the effect of the higher dose of NTX in suppressing the whole reward brain system in AUD-like Cluster 2. Indeed in several clinical trials has been a higher efficacy of NTX in heavy drinkers and patients with more severe AUD diagnosis (Davidson, et al., 1999; Kranzler, et al., 2009; O'Malley, et al., 1992; Pettinati, et al., 2006; Smith-Bernardin, et al., 2018; Volpicelli, et al., 1992).

Conversely, memantine is one of the drugs that failed clinical trials for AUD, although it showed promising results in the preclinical setting. We tested this compound as a negative control for the predictive validity of our AUD model. Therefore, we expected that memantine would fail in selectively reducing alcohol seeking. Our results showed that only the highest dose of memantine was effective in the three clusters but, as expected, its action was not specific for alcohol consumption as revealed when it was assessed on saccharin SA. Our evidence is consistent with clinical data (Montemitro, et al., 2021; Krishnan-Sarin, et al., 2015). However, there are discordant clinical evidence about the ability of memantine in reducing alcohol craving (Bisaga & Evans, 2004; Krishnan-Sarin, et al., 2015) and it could be interesting to evaluate these differences in NIH-HS rats in the future.

Finally, once the specific effect of NTX was identified as treatment for alcohol consumption, we decided to focus on this drug verifying whether any of the

behaviors tested could predict rats' response to NTX. Running Pearson correlations on both behaviors screened when rats were alcohol naïve and alcohol-related behaviors (the 6 alcohol-related behaviors), we found a negative correlation between anxiety-like behavior and the response at 0.3 mg/kg NTX on alcohol SA. This result underlines that the higher the time spent in the OA of the EPM (less anxiety-like behavior), the lower the IES value at 0.3 mg/kg (weak response to the treatment). To confirm this correlation, we decided to detect responder and non-responder rats to NTX treatment by k-means cluster analysis. Comparing these two NTX-Clusters on their response to NTX on both alcohol and saccharin SA, we identify the NTX-Cluster 1 as the non-responder cluster and the NTX-Cluster 2 as the responder cluster. In line with our hypothesis, we found the presence of 71.43% of non-responder subjects indicating that naltrexone has variable efficacy in our highly heterogeneous animal model. We compared the time spent in the OA of the EPM by the two clusters confirming the negative correlation: rats showing less anxiety-like behavior belonged to Cluster 1, the non-responder cluster.

The relationship between anxiety and alcohol use is complex and bidirectional. While alcohol consumption may be initially motivated by its ability to reduce anxiety and relief tension, it ultimately leads to increased anxiety during alcohol withdrawal (Koob, 2013). Several epidemiologic studies have demonstrated high comorbidity between alcohol use and anxiety disorders, but no causal connection has been demonstrated so far (Anker & Kushner, 2019; Grant, et al., 2004; Kessler, et al., 1996). The opioid system plays a crucial role in the neural modulation of anxiety. Clinical experiments with healthy volunteers suggest that anxiety is followed by the activation of the opioid system and the functional role of opioid endogenous release is to suppress anxiety (Duka, et al., 1982; Esquivel, et al., 2009; Liberzon, et al., 2002; Pickar, et al., 1982). Moreover, preclinical studies have shown that opioid agonists administration inhibits anxiety-like behaviors (Asakawa, et al., 1998; Zarrindast, et al., 2008; Zarrindast, et al., 2005), while the reduction in opioid activity increases anxiety-like behaviors (Burghardt & Wilson, 2006; Kõks, et al., 1999; Tsuda, et al., 1996). This evidence has raised concerns about the possibility that NTX can increase

anxiety blocking endogenous opioids. Nonetheless, clinical studies did not support this hypothesis and our result even suggests that innate anxiety traits can predict a better response to NTX as a pharmacological treatment for AUD. Indeed, it has been demonstrated that, contrary to expectation, patients with opioid or alcohol dependence treated with NTX reported gradual improvements in anxiety (Krupitsky, et al., 2006; Krupitsky, et al., 2016; Latif, et al., 2019; Volpicelli, et al., 1995). Volpicelli and colleagues have observed that NTX treatment attenuated the risk for excessive drinking relative to placebo in subjects with high baseline somatic distress (Volpicelli, et al., 1995). Conversely, a recent study failed to observe any significant predictor variables on response to naltrexone with respect to the presence of either depression or anxiety disorder antecedents (Rubio, et al., 2005). At this point, the effects of NTX on anxiety and the relationship between these two factors is difficult to ascertain, given inconsistencies across these clinical studies in baseline measurements, exclusion criteria regarding the stability of comorbidity, and the concomitant use of antidepressant and anxiolytic medications. For this reason, it would be interesting to unravel the issue using animal models and then testing NIH-HS rats on their anxiety-like behavior also during alcohol exposure and under NTX treatment to verify if NTX reduces anxiety in NTX-responder animals (here showing higher anxiety trait).

A limitation of the present study is the limited number of rats tested which is responsible for the three numerically unbalanced clusters we obtained from the k-mean cluster analysis. It is important to keep in mind that this is a first preliminary study and other cohorts of NIH-HS rats will be tested in the future using the same protocol here proposed. By definition, experiments with highly heterogenous animals require high numerosity to best capture all the varied characteristics observable in the human population. Indeed, our long-term aim is to run genome-wide association studies (GWAS) to connect different HS rat AUD-like genotypes with different underlying genetics, and GWAS can only be performed in large datasets (Visscher, et al., 2017).

Despite this caveat, our preliminary study results in some relevant promising evidence and paves the way for further studies using this rat line within the AUD field.

We detected three clusters of HS animals showing different profiles of AUD-like behavior. As predicted, NTX showed heterogeneous efficacy when tested in a model of individual variability in AUD-like phenotype, while memantine demonstrated not to be a valid treatment for alcohol misuse as already seen in clinical trials. Finally, a link between NTX efficacy and anxiety traits emerged, and further investigations are needed.

Altogether this evidence proves that the NIH-HS rats are a good animal model to investigate the genetic vulnerability and resilience in AUD. Advancement of understanding in the genetics and neurobiological basis of AUD will result to increasingly better identification of at-risk individuals or populations, and eventually significantly advance targeted prevention efforts as well as diminish the increasing prevalence of AUD. Medicine and pharmacology are progressively moving towards personalized treatments, and preclinical research can help in pursuing this goal through animal models increasingly suitable for this purpose.

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Chapter 3

Life-long effects of chronic adolescent sleep restriction on alcohol consumption behavior

3.1. ABSTRACT

Sleep loss and sleep difficulties can lead to neurodevelopmental maladaptations that can contribute to the emergence of mood disturbances and increased alcohol use. Early adolescent alcohol consumption is then associated with a higher risk of alcohol abuse disorder (AUD) and related problems in adulthood. The mechanisms involved in the effects of adolescent sleep restriction (ASR) on reward circuit maturation and subsequent risks of developing reward related disturbances are not fully understood. Moreover, not all teenagers suffering from sleep loss engage in alcohol use and misuse. In the present study, we examine the impact of ASR on alcohol consumption in adolescent and adult Wistar and Marchigian Sardinian alcohol-preferring (msP) rats. Groups of both rat lines were sleep restricted (SR) for 12 h/day for 20 days starting from mid-adolescence. Alcohol consumption was assessed every 2 days during the ASR using the 2-bottle choice (2BC) paradigm (choice between tap water *vs.* 10% ethanol solution). At the end of the entire procedure, locomotor activity and anxiety-like behaviors were assessed in adolescent rats. The 2BC paradigm was also used later in adulthood (at 3, 6, and 9 months of age) to evaluate the long-term effects of ASR and anxiety was again tested at 5 months of age. SR msP rats exhibited enhanced drinking immediately after alcohol re-exposure (binge drinking) compared to control animals not subjected to the ASR. This effect was transient and no longer visible later in adulthood. SR msP animals also showed an increased in locomotion during adolescence. In outbred Wistar rats we detected instead interindividual variability in response to the ASR. One subgroup of ASR rats significantly increased its alcohol consumption compared to the other SR animals and to the corresponding controls. This effect of ASR was life-long lasting. No effects on anxiety-behavior or locomotion were detected. In conclusion, our data showed enhanced but transient binge drinking in msP rats subjected to SR. Conversely, in Wistar rats sleep loss during the delicate developmental stage of adolescence seemed to enhance the propensity to drink maintained throughout adulthood, which, however, occurred in some individuals but not in others.

3.2. INTRODUCTION

Worldwide, alcohol is the most abused drug among adolescents (National Institute on Alcohol Abuse and Alcoholism, 2019): about 26% of teenagers report drinking by the age of 13-14 and about 42% of them report being drunk at least once by the age of 17-18 (Miech, et al., 2020). Starting drinking before the age of 15 increases by four times the probability of becoming alcohol dependent later in life (Grant & Dawson, 1998). Adolescence is a sensitive period of development defined by extremely high brain plasticity (Knudsen, 2004) and specific behaviors such as high social interactions, high levels of risk-taking, exploration, novelty, and sensation seeking (Spear, 2000; Spear, 2007). These features promote the acquisition of the adaptive and necessary skills for maturation and independence (Spear, 2000), but, at the same time, they can have negative implications. Impulsivity and risk taking behaviors may increase the likelihood of injury, death, sexual-related risky, depression, and anxiety. Moreover, they are strong predictors of drug and alcohol use among adolescents (Andrucci, et al., 1989; Kelley, et al., 2004; Wills, et al., 1994). These behavioral characteristics are linked to the unbalanced development of some brain areas involved in executive control and motivation (Giedd, et al., 1999) with the maturation of subcortical limbic regions occurring earlier than frontal cortical top-down control areas (Casey, et al., 2008; Casey & Jones, 2010). This unbalance is responsible for a heightened responsiveness to rewards (especially immediate rewards) and high emotional reactivity (Ernst, et al., 2005; Monk, et al., 2003; Montague & Berns, 2002). In addition, the immaturity of prefrontal regions would cause poor abilities in decision-making, reward evaluation, risk and consequences determination, as well as reduced impulse control (Bjork, et al., 2007; Eshel, et al., 2007; Nagy, et al., 2004). In adolescence the connections between limbic and cortical structures are still under construction and are highly vulnerable to environmental insults that may ultimately lead to reduced inhibitory top-down control from prefrontal regions towards limbic regions. Therefore, limbic areas can take over (bottom-up control) by determining enhanced propensity to experience behaviors associated with high emotional load,

high risk, and novelty seeking typical of adolescents (Casey, et al., 2008; Casey & Jones, 2010).

On the other hand, not all adolescents misuse alcohol and develop AUD.

Several environmental factors can induce adolescents to start and continue drinking alcohol, such as family history, traumatic experiences, stress, peer pressure, social environment and circadian factors (Bowen, et al., 2022; Chartier, et al., 2010).

Sleep is another key factor that recently emerged as a possible trigger of early alcohol use and misuse. Important changes in sleep pattern occur across life with total sleep time decreasing with age (Saksena, et al., 2020). At puberty, endogenous circadian rhythms and preferred sleep time start to shift later – a phenomenon called eveningness - (Randler, 2008; Roenneberg, et al., 2004). Hence, adolescents tend to prefer later sleep times (Crowley, et al., 2007; Randler, 2008) coming into conflict with social demands, especially with school schedules that require waking up early during weekdays (Hansen, et al., 2005). Then, over the weekend, they recover from weekdays by staying up later and sleeping until late morning or afternoon. Thereby, their circadian system responds to this weekend inconsistency by further delaying internal timing. It creates a weekday-weekend shift in sleep and a circadian misalignment referred to as "social jet lag" (Crowley & Carskadon, 2010). This situation causes circadian misalignment, increased daytime sleepiness, sleep disturbance, and sleep loss in adolescents (Carskadon, 2011; Crowley, et al., 2014; Touitou, 2013). Recent data have demonstrated that 71% of teenagers sleep less than 8 hours per night and 44% even less than 6 hours per night (Basch, et al., 2014; Keyes, et al., 2015).

Changes in sleep structure and behavior may impact the proper maturation of CNS structures including the mesocorticolimbic system (Anastasiades, et al., 2022; Goldstein & Walker, 2014). Late and variable bedtime, as well as poor sleep, are associated with depression, reduced reward responsiveness, sensation seeking, and impulsivity (Adan, et al., 2010; Caci, et al., 2004; Hasler, et al., 2010; Tonetti, et al., 2010), all characteristics depending on the reward brain system (Hasler, et al., 2013; Hasler, et al., 2012; Pasch, et al., 2010). Longitudinal studies have observed that the typical adolescent eveningness and circadian misalignment are associated with a

greater likelihood of alcohol use two years later (Hasler, et al., 2017; Pasch, et al., 2012; Tavernier, et al., 2015), worse AUD symptoms 3 to 5 years later (Hasler, et al., 2014; Tavernier, et al., 2015), and an earlier onset of AUD (Hasler, et al., 2016). A single night of sleep deprivation downregulates D2 receptors in the dorsal and ventral striatum (Volkow, et al., 2012a; Volkow, et al., 2008) which is associated with a greater propensity for risk-taking behavior (Linnet, et al., 2011) and an increased risk for compulsive drug consumption (Dalley, et al., 2007). Indeed, neuroimaging experiments have shown a reduction in D2/D3 receptor availability in cocaine abusers that has been associated with impaired prefrontal activity (Volkow, et al., 1993), relapse vulnerability (Martinez, et al., 2011), craving (Volkow, et al., 2012b), and poor treatment outcomes (Luo, et al., 2014). Cocaine abusers often suffer from sleep disturbances (Berro, et al., 2014), reduced sleep quality and duration (Johanson, et al., 1999; Valladares & Irwin, 2007). A recent study demonstrated that sleep duration in cocaine abusers predicted striatal D2/D3 receptor availability and mediated their reduction (Wiers, et al., 2016).

Despite this preliminary evidence collected in humans, little is known about how adolescent poor sleep can impact the brain development and induce alcohol misuse in individuals. As reported above, clinical evidence suggests that sleep loss can cause deficits in the top-down prefrontal control. It is therefore possible to assume that individual experiencing stronger bottom-up motivational drive, like adolescents, will have greater consequences due to poor sleep.

The present study aims to clarify whether SR during adolescence can enhance subsequent vulnerability to excessive drinking, using two different rat models of alcohol consumption. For the study outbred heterogeneous Wistar and genetically selected alcohol preferring msP rat lines were employed.

3.3. MATERIAL AND METHODS

3.3.1. ANIMALS

Male Wistar (n = 33) and msP (n = 24) rats were used for the present study. All subjects were bred in-house at the animal facility of the University of Camerino, Italy. Animals were weaned at postnatal day (PND) 21 and housed 2 per cage in environmentally controlled conditions (12:12h light/dark cycle; lights off at 7 AM; room temperature 20-22° C and humidity 45-50%). Food (4RF18, Mucedola, Settimo Milanese, Italy) and tap water were provided ad libitum. Rats weighed 103 g (Wistar) and 95 g (msP) at the beginning of the experimental procedure (PND 29). Animals were handled for 5 days, 5 min daily by the same operator who performed the experiments.

The SR protocol was conducted during the sleep light phase and the experiments during the dark active phase of the light/dark cycle.

All procedures were conducted in adherence with the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3.3.2. SLEEP DEPRIVATION BOXES

Adolescent sleep restriction (ASR) was performed using automated sleep deprivation (SD) boxes (AM Microsystem, Urbisaglia, MC, Italy). Similar systems have been demonstrated to effectively produce sleep deprivation in rats as validated by polysomnography (Hines, et al., 2013; Naylor, et al., 2012; Sims, et al., 2013; Wisor, et al., 2011; Wooden, et al., 2014). The apparatus consists of a Plexiglas cylinder (33 cm tall x 30 cm in diameter) with a metallic rotating bar at the base and a control panel to set parameters. In the SR protocol, bar rotation was set at 2 revolutions per minute (RPM) and its direction changed every 5 min to prevent adaptation to the bar rotation. The SD boxes were layered with con bedding and equipped with tap water and food provided *ad libitum*.

3.3.3. DRUGS

The alcohol drinking solution 10% (*v*/*v*) was prepared diluting 95% alcohol (F.L. Carsetti s.n.c., Camerino, Italy) with tap water. Saccharin (Sigma-Aldrich, Milan, Italy) was diluted to 0.1%, 0.05% and 0.03% (*w*/*v*) with tap water.

3.3.4. OPEN FIELD TEST

The OF test allows detecting the animal locomotory activity. At both PND 57 and PND 95 (3 months old) rats were placed in a Plexiglass arena (43.4 x 43.4 x 30.3 cm) able to track location and locomotion using 16 evenly spaced infrared (I/R) transmitters and receivers (Med Associates, St Albans, VT, USA). After 10 min of acclimatization, rats were left free to explore the arena for 40 min in a sound-attenuated room illuminated by a dim red light (~ 30 lux). The distance travelled in centimeters was automatically recorded.

3.3.5. ELEVATED PLUS MAZE TEST

To measure anxiety-like traits, rats were tested in the EPM test at PND 58. The black wooden EPM apparatus was elevated 50 cm above the floor and consisted of two open arms and two enclosed arms (50 cm long x 10 cm wide, 40 cm high walls) arranged such that the respective closed and open arms were opposite to each other. The EPM tests were conducted in a room illuminated by dim red light and sound-attenuated. Each 5-min trial started when the animals were individually placed in the center of the maze, facing the open arm opposite where the experimenter was. The number of entries in each arm - defined as the presence of all four paws in an arm – and the time spent in each arm were scored. The percentage of time spent in the open arms [(time in open arms/time in open arms + time in closed arms) X 100] was considered as an index of anxiety.

3.3.6. LIGHT/DARK BOX TEST

At 3 months of age, rats were tested again for their anxiety-like behavior using the light/dark test (L/D test). The apparatus consisted of two same-size chambers (30 x 30

x 40 cm) connected by a small opening (10 cm x 10 cm). One enclosed chamber with black wall was dark (2 lux), the other one was white, opened on the top and with a light (650 lux) placed 40 cm above the floor. The test started by placing the rat in the bright compartment facing the side opposite to the small opening giving access to the dark enclosed chamber. The time (s) spent in each compartment was measured for 5 min. Entry into a chamber is defined as stepping with all four paws in the chamber (Bourin & Hascoët, 2003). The percentage of time spent in the bright chamber [(time in bright chamber/time in bright chamber + time in dark chamber) X 100] was considered as an index of anxiety.

3.3.7. EXPERIMENTAL PROCEDURE

Adolescence is estimated between PND 28-42 (Spear, 2000) in rodents. During this interval sexual maturity occurs at approximately PND 32-34 in females and PND 45-48 in males (Sengupta, 2013). Adulthood starts approximately at PND 63 and at PND 70 rats are considered young adults (Sengupta, 2013). This period corresponds to 18-25 years in humans (Sengupta, 2013; Spear, 2015; Varlinskaya, et al., 2013).
Based on this evidence the 2BC paradigm began at PND 29 (early adolescence), whereas the SR protocol started at PND 35 (mid-adolescence); both ended at PND 55, before the rats became young adults.

Before starting the SR protocol, rats were habituated to the SD boxes for 2 days, 4 hours per day, by putting together the couple of rats normally pair-housed in the regular cage.

From PND 29 until PND 55 Wistar and msP rats were exposed to an intermittent 2BC procedure in their home cage according to the following schedule:

- 10% (*v*/*v*) alcohol + 0.1% (*w*/*v*) saccharin at PND 29, 31, 34, 37;
- 10% (*v*/*v*) alcohol + 0.05% (*w*/*v*) saccharin at PND 40;
- 10% (*v*/*v*) alcohol + 0.03% (*w*/*v*) saccharin at PND 43;
- 10% (*v*/*v*) alcohol at PND 46, 49, 52, 55;

Saccharin was initially added to the alcohol solution for two main reasons: first, to easily and quickly induce Wistar rats to drink during the rapidly occurring short adolescence (Koob & Weiss, 1990; Samson, 1986; Tolliver, et al., 1988); secondly, to mimic human adolescents' behavior characterized by initially alcohol drinking in the form of sweetened beverages (Roberts, et al., 2015; Rossheim & Thombs, 2013). The alcohol intermittent access was performed to increase alcohol consumption in rats over time (Hopf, et al., 2010; Kimbrough, et al., 2017; Pinel & Huang, 1976; Simms, et al., 2008; Spoelder, et al., 2015; Tomie, et al., 2006; Wise, 1973) and to mimic the prevalent weekend alcohol consumption displayed by teenagers (Gmel, et al., 2008; Kuntsche & Cooper, 2010; Kuntsche & Labhart, 2012; Parker & Williams, 2003).

The 10-h alcohol intake of the first 3 days of exposure was used as the baseline and as the criterion to equally distribute rats in the control (CNT) group (Wistar, n = 15; msP, n = 12) and the SR group (Wistar, n = 18; msP, n = 12).

The SR protocol started at PND 35 and continued until PND 55.

After 10 min of acclimatation to the SD boxes, rats in the CNT group were left undisturbed to sleep, whereas rats in the SR group were kept awake for 12 consecutive hours from 8 PM to 8 AM (Simasko & Mukherjee, 2009). Rats were subjected to SR or undisturbed sleep every day during the light phase. Every 2 days rats were exposed to alcohol in the 2BC paradigm during the dark phase.

After a day of break, at PND 57-58 all animals were tested for their locomotor activity and anxiety-like behavior, respectively in OF test and the EPM test.

Thereafter, rats were left undisturbed for about six weeks. At the age of 3 months, all rats were re-exposed to the 2BC paradigm as previously done to evaluated long-term effects of ASR.

At the age of 5 months, rats were re-tested for their anxiety-like behavior in the L/D test.

Wistar rats (now CNT group, n = 12; SR group, n = 11) were re-exposed to alcohol in the 2BC at 6 and 9 months old under the same conditions as before.

A schematic representation of the entire protocol is provided in ***Figure 25***.

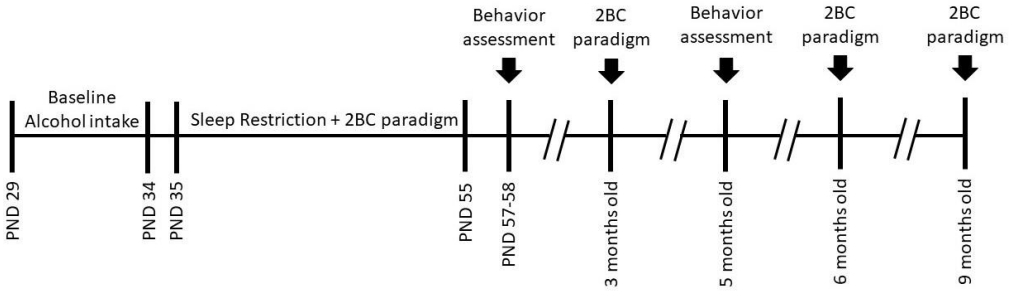

Figure 25. Schematic representation of the experimental protocol. From PND 29 to PND 34 (early adolescence) rats were given free access to 10% ethanol solution + 0.1% saccharin and tap water in the 2BC paradigm. At PND 35 (mid-adolescence) the SR protocol started. Rats were sleep restricted for 12 hours every day until PND 55. The 2BC was performed every 2 days gradually reducing saccharin until it is eliminated altogether. After a day of break, behavior assessment was performed at PND 57 and 58. Rats were left undisturbed until 3 months of age when the 2BC (10% alcohol solution vs. tap water) was resumed (for 4 days). At 5 months of age the behavior assessment was repeated. Finally at 6 and 9 months of age (middle-aged adulthood) rats' alcohol intake was evaluated using the 2BC procedure as before.

3.3.7.1. EXPERIMENT 1: Effect of ASR on contingent intermittent alcohol intake in the 2BC paradigm

Starting from PND 29 rats were exposed to 10% (*v*/*v*) alcohol solution in a 2BC paradigm for 10 hours from 9 AM to 7 PM to measure their alcohol drinking and preference (Koob, et al., 2003; Tabakoff & Hoffman, 2000). Initially, saccharin was added to the alcohol solution. Three exposures to alcohol sweetened with 0.1% (*w*/*v*) saccharin were taken as initial baseline (2BC at PND 29, 31, and 34).

The SR started at PND 35, and the 2BC was performed every 2 days gradually reducing saccharin concentration until rats were intermittently presented with 10% alcohol only from PND 46 to PND 55 (four alcohol exposures in total).

The 2BC was performed by single-housing rats and giving them free access to two bottles (tap water and 10% alcohol solution) whose position was alternated each day of alcohol exposure to avoid a side preference effect. Alcohol and water intake were measured after 30 min, 2 hours, and 10 hours by weighting the bottles.

Alcohol and water consumptions were calculated as absolute values at each time-interval and were expressed as grams per kilogram (g/kg) to control for the influence of body weight differences (Finn, et al., 2007; Rimondini, et al., 2003).

3.3.7.2. EXPERIMENT 2: Effect of ASR on behavior assessment in late adolescence

Twenty-four hours after the last alcohol exposure in 2BC and the last SR (PND 55), rats were behaviorally assessed to evaluate the possible effects of ASR. Their OF locomotory and EPM anxiety-like behavior were assessed at PND 57 and at PND 58, respectively.

3.3.7.3. EXPERIMENT 3: Effect of ASR on intermittent alcohol intake in the 2BC performed in adulthood (3 months old)

After the behavioral assessment at PND 57-58, rats were left undisturbed for six weeks. At 3 months of age, now adults, rats were re-exposed to the 2BC paradigm (10% ethanol solution vs. tap water): access to alcohol was given intermittently every 2 days, four a total of 4 alcohol exposures. As in Experiment 1, the 2BC tests started at 9 AM and lasted 10 hours. Fluid intake was monitored after 30 min, 2 hours, and 10 hours access. To avoid side preference development, bottles' position was alternated.

3.3.7.4. EXPERIMENT 4: Effect of ASR on anxiety-like behavior in adulthood

Later in adulthood, at 5 months of age, rats were tested again to investigate possible later effects of ASR on anxiety-like behavior in the L/D test.

3.3.7.5. EXPERIMENT 5: Effect of ASR on 2BC intermittent alcohol intake in adulthood (6 and 9 months old)

The effect of ASR on alcohol consumption later in life was evaluated at two other time points: at 6 and 9 months of age. Following the same protocol previously performed, rats were given free access to 10% ethanol solution and tap water for 10 hours, every

2 days. Fluid intake was measured after 30 min, 2 hours, and 10 hours and adjusted for rats' body weight (g/kg).

At both ages, rats were exposed to alcohol 4 times in total.

3.3.8. CLUSTERING CRITERIA BASED ON ALCOHOL INTAKE

Wistars and msP rats were independently clustered based on their 10-h alcohol intake of the last 3 exposures (normalized for the animal's body weight, g/kg). Rats in the top 34% of the distribution were labeled as "Drinkers". This criterion was arbitrarily chosen from seminal work by Deroche-Gamonet and colleagues (Deroche-Gamonet, et al., 2004). Considering that in the msP and in the CNT Wistar rats changes in the selection threshold from 25 to 40% had minimal effect on individual rat-group allocation, they were treated as a single population (Deroche-Gamonet & Piazza, 2014). This clustering let to the separation of Wistar rats into two groups: Sleep Restricted Drinkers (SRD; n = 6) and Sleep Restricted no-Drinkers (SRno-D; n = 12) (***Figure 26***).

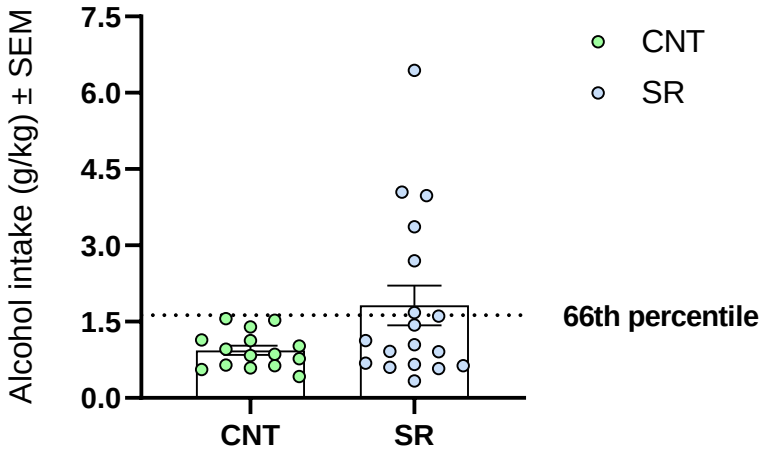

Figure 26. Mean values of 10-h alcohol intake of the last three exposures in CNT and SR Wistar rats. The dotted line indicates the 66th percentile of the distribution. As depicted in the figure, clusters were identified in the SR group only. Data are presented as mean (± SEM).

3.3.9. STATISTICAL ANALYSIS

Data from Wistar and msP rats were analysed independently. To analyse baseline alcohol drinking at 10-h and divide the rats into SR and CNT unpaired t-test was used. To verify no difference in the baseline alcohol consumption between SRD, SRno-D, and CNT in the Wistar line one-way ANOVA was used. Alcohol consumption during the ASR and later in adulthood was evaluated by three-way ANOVA with group as between-subjects factor and timepoint and exposure-day as within-subjects factors. Alcohol intake at each timepoint (30-min, 2-h, and 10-h) was further analysed by two-way ANOVA with group as between-subjects factor and exposure-day as within-subjects factor. OF test, EPM test, and L/D test data were assessed by one-way ANOVA for Wistar rats and unpaired t-test for msP rats. Data distribution for the L/D test in Wistar rats was not normally distributed and was therefore analysed by Kruskal-Wallis test.

The analyses were followed by the Newman-Keuls post-hoc test when appropriate, and statistical significance was conventionally set at $p < 0.05$.

3.4. RESULTS

3.4.1. EXPERIMENT 1: Effect of ASR on contingent intermittent alcohol intake in the 2BC paradigm

Wistar and msP rats were assigned to the SR or the CNT group based on their 10-h alcohol intake baseline. No difference between the SR and CNT group was detected either in Wistars ($t(31) = 0.97$, $p > 0.05$) or in msP rats [$t(22) = 0.97$, $p > 0.05$] (***Figure 27A,B***). Applying the cluster analysis after the SR emerged that the SR Wistars can be divided into two separate groups with different alcohol drinking levels. To evaluate whether these two groups differed in baseline drinking, a posteriori analysis was applied in Wistars to compare 10-h alcohol intake in the groups subsequently identified as CNT, SRno-D, and SR groups. One way ANOVA showed no differences [$F(2,24) = 0.21$, $p > 0.05$] (***Figure 27C***).

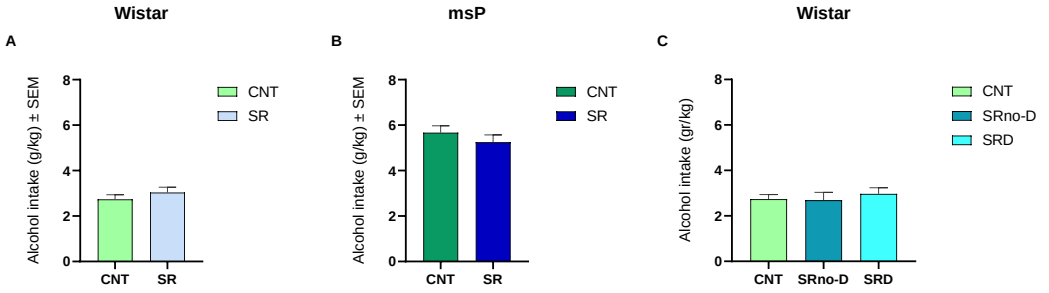

Figure 27. Average alcohol intake in 10-h of the first 3 alcohol exposures before starting SR protocol. Based on this value, rats were distributed into 2 groups that did not statistically differ in their alcohol consumption. Data are presented as mean (± SEM).

The effect of chronic SR was evaluated in adolescent Wistar and msP rats by exposing them to 10% alcohol solution in 2BC paradigm every two days. Since alcohol was initially sweetened with saccharin, the last 4 alcohol exposures to only 10% ethanol were analysed. In Wistar adolescent rats a three-way ANOVA showed a main effect of group [$F(2,30) = 28.45$, $p < 0.0001$], timepoint [$F(2,60) = 128.68$, $p < 0.0001$], group x timepoint [$F(4,60) = 30.61$, $p < 0.0001$] and group x exposure-day x timepoint [$F(12,180) = 1.9$, $p < 0.05$] interactions. Newman-Keuls post-hoc test showed that overall the SRD group is significantly different from the CNT ($p < 0.001$) and SRno-D ($p < 0.001$) groups. In particular, the SRD group differed from the CNT ($p < 0.001$) and SRno-D ($p < 0.001$) groups on the total alcohol consumed after 10 hours of 2BC exposure, whereas no difference between the three groups was detected at the two other timepoints. To further explore the effect of SR, data from each timepoint were analysed separately by single two-way ANOVAs. The analysis found a statistically significant difference between the three groups at all timepoints [30-min: $F(2,30) = 15.71$, $p < 0.0001$; 2-h: $F(2,30) = 21.32$; 10-h $F(2,30) = 31.2$, $p < 0.0001$]. The subsequent post-hoc test confirmed the SRD group as statistically different from the other two groups at each time point (30-min: SRD vs. CNT and vs. SRno-D, $p < 0.001$; 2-h: SRD vs. CNT and vs. SRno-D, $p < 0.001$;10-h: SRD vs. CNT and vs. SRno-D, $p < 0.001$) (***Figure 28A***). In msP rats, three-way ANOVA found an overall effect of group [$F(1,22) = 4.44$, $p < 0.05$], exposure-day [$F(3,66) = 11.06$, $p < 0.0001$], and timepoint [$F(2,44) =$

1023.51, $p < 0.0001$], exposure x timepoint interaction [$F(6,132) = 4.6$, $p < 0.001$], but no other significant interactions. To better understand the effect of SR on msP animals, data from each timepoint were analysed separately by single two-way ANOVAs. Results revealed an overall effect of group at 30-min [$F(1,22) = 6.06$, $p < 0.05$] and 2-h [$F(1,22) = 5.57$, $p < 0.05$], but not at 10-h [$F(1,22) = 2.65$, $p > 0.05$] (***Figure 28B***).

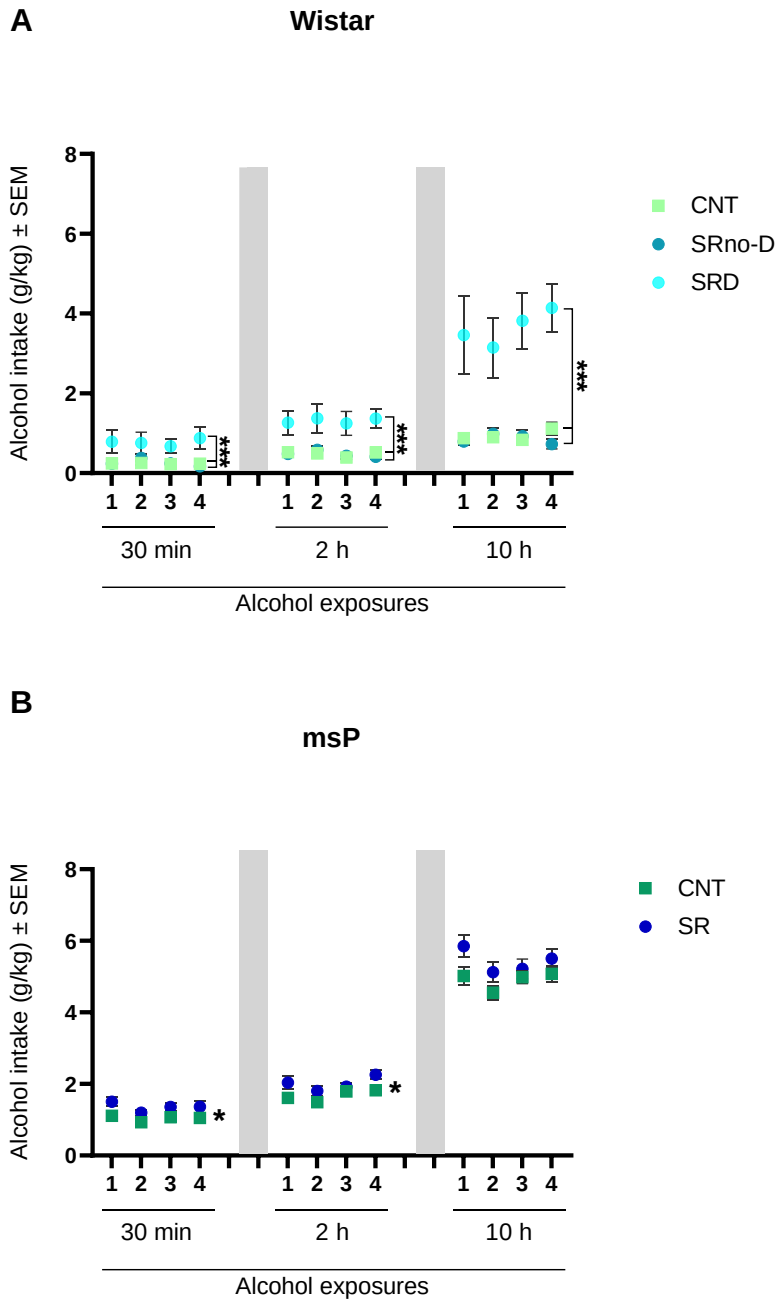

Figure 28. Wistar and msP rats' alcohol intake at 30-min, 2-h, and 10-h during the 4 ethanol exposures occurred every 2 days in adolescence in parallel with chronic SR. ***A.*** In Wistar rats, the SRD group significantly drink more alcohol than the CNT and SRno-D groups at all timepoints considered ***B.*** In msP animals, the SR group significantly drink more alcohol than the CNT group at 30-min and 2-h revealing binge drinking behavior. Indeed, the difference between the two groups is not evident at 10-h of alcohol exposure. Data are presented as mean (± SEM). *$p < 0.05$; in Wistar, difference between SRD and CNT, and between SRD and SRno-D: ***$p < 0.001$

3.4.2. EXPERIMENT 2: Effect of ASR on behavior assessment in late adolescence

At PND 55 rats were subjected to the last 12-h of SR and the last 10-h of alcohol exposure in the 2BC paradigm. After a day of rest, all animals were tested for their locomotory activity and anxiety-like behavior respectively in OF test and in the EPM test. No statistically significant difference was found between the three groups of Wistar rats by one-way ANOVA neither in the distance travelled in the OF test [$F(2,30) = 0.49$, $p > 0.05$] (***Figure 29A***) nor to the time spent in the open arms in the EPM test [$F(2,30) = 0.56$, $p > 0.05$] (***Figure 29B***). In msP animals, t-test analysis revealed an increase in locomotor activity in SR rats compared to controls in the OF test [$t(22) = 2.5$, $p < 0.05$] (***Figure 29C***), whereas no difference between the two groups was showed by t-test in the EPM test [$t(22) = 0.37$, $p > 0.05$] (***Figure 29D***).

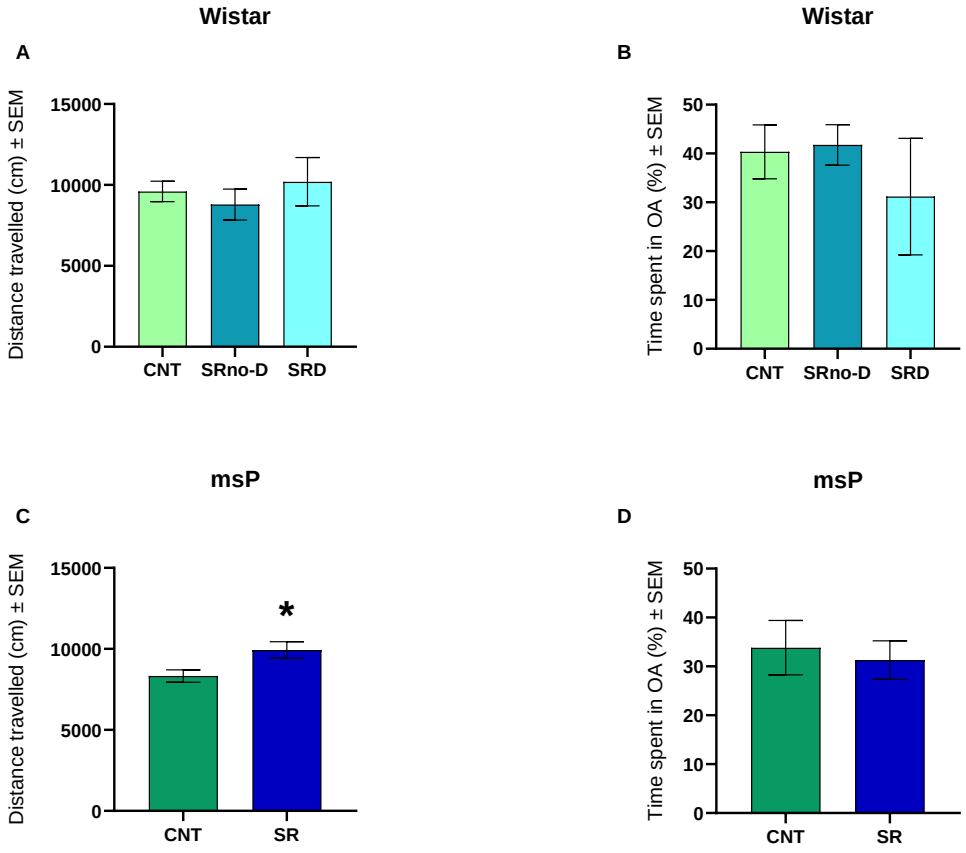

Figure 29. Behavior assessment after ASR. ***A-B.*** No difference was found between the three groups of Wistar rats in their locomotor activity indicated as the distance travelled in the arena (***A***) and anxiety-like behavior showed as the percentage of time spent in the open arms (OA) of the EPM apparatus (***B***).

C. msP SR rats displayed an increase in locomotion compared to the CNT group. ***D.*** The two msP groups did not differ in their anxiety-like behavior. Data are presented as mean (± SEM). *p < 0.05.

3.4.3. EXPERIMENT 3: Effect of ASR on intermittent alcohol intake in the 2BC performed in adulthood (3 months old)

Animals were left undisturbed for about one month and a half and then, at 3 months old, were re-exposed to the 2BC procedure (same condition as before: every 2 days, 3 timepoints) to evaluate the long-term effect of ASR on their alcohol consumption. Tree-way ANOVA found a main effect of group [$F(2,30) = 16.13$, $p < 0.0001$], timepoint [$F(2,60) = 142.25$, $p < 0.0001$], and group x timepoint interaction [$F(4,60) = 15.24$, $p < 0.0001$]. Post-hoc analysis revealed that SRD group differs from the CNT ($p < 0.001$) and SRno-D ($p < 0.001$) group, and its alcohol intake is higher at 10-h compared to the other two groups (vs. CNT: $p < 0.001$; vs. SRno-D: $p < 0.001$). To verify whether the ASR effects were still visible also at 30-min and 2-h, data from each timepoint were analysed separately by single two-way ANOVAs. Two-way ANOVA revealed an overall effect of group at 30 min [$F(2,30) = 17.04$, p 0.0001], 2-h [$F(2,30) = 19.72$, $p < 0.0001$], and 10-h [$F(2,30) = 16.94$, $p < 0.0001$]. The post-hoc analysis confirmed that the SRD group maintained its higher alcohol consumption even later in adulthood, compared to the CNT and the SR group at each timepoint (30-min: SRD vs. CNT and vs. SRno-D, $p < 0.001$; 2-h: SRD vs. CNT and vs. SRno-D, $p < 0.001$;10-h: SRD vs. CNT and vs. SRno-D, $p < 0.001$) (***Figure 30A***). Conversely, running three-way ANOVA on alcohol intake data from msP rats, we did not find any statistical effect of group [$F(1,22) = 0.01$, $p > 0.05$], and no interactions (***Figure 30B***).

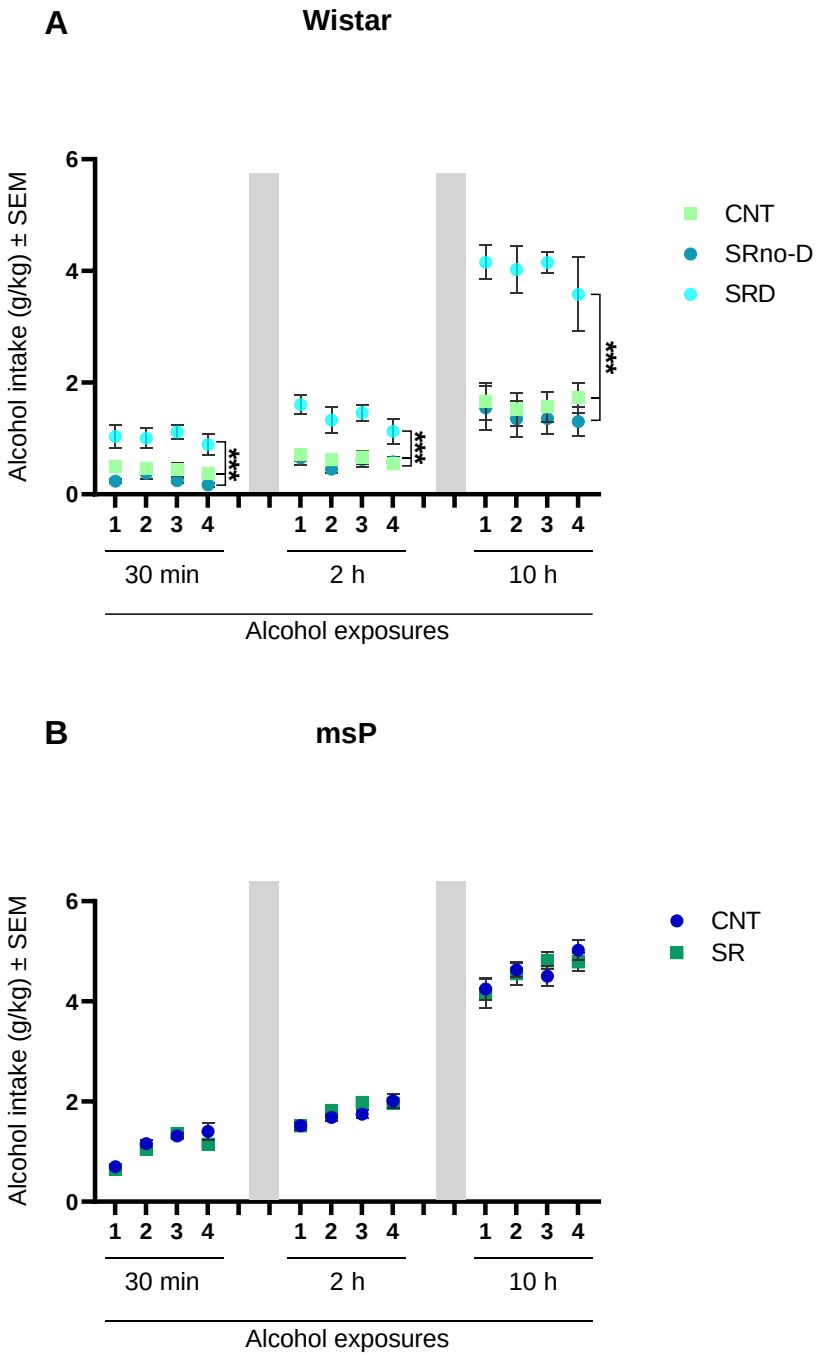

Figure 30. After a break period of one month and a half, animals were re-exposed to alcohol in the 2BC paradigm, following the same procedure used in adolescence. ***A.*** In Wistar animals, the SRno-D group showed to still drink significantly more alcohol than the other two groups as long-term effect of ASR. ***B.*** In msP rats, at 3 months of age, the two groups no longer differed in the amount of alcohol consumed at any of the timepoints considered. Data are presented as mean (± SEM). In Wistar, difference between SRD and CNT, and between SRD and SRno-D: ***$p < 0.001$.

3.4.4. EXPERIMENT 4: Effect of ASR on anxiety-like behavior in adulthood

Rats were re-tested for the possible later effect of ASR on their anxiety traits in the L/D test, 2 months after being exposed to alcohol (2BC paradigm) at 3 months of age. Kruskal-Wallis test did not reveal any statistically significant difference between the three groups of Wistar rats [$X^2(3) = 1.79$, $p > 0.05$] (***Figure 31A***). T-test analysis found no difference between CNT and SR groups in msP animals [$t(22) = 1.29$, $p > 0.05$] (***Figure 31B***).

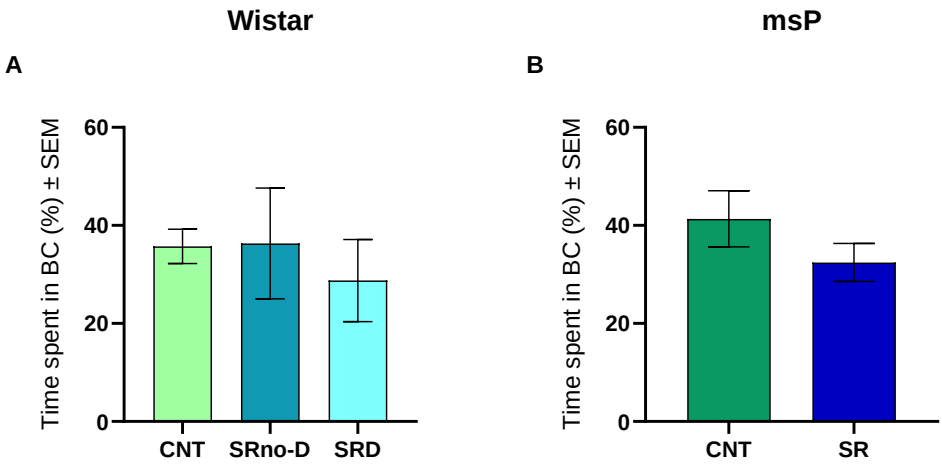

Figure 31. Behavior assessment in adulthood, 5 months of age. Anxiety-like behavior is indicated as the percentage of time spent in the bright chamber (BC) of the light/dark box. ***A.*** No difference was found between the three groups of Wistar rats. ***B.*** In msP animals, the two groups did not show any significant difference. Data are presented as mean (± SEM).

3.4.5. EXPERIMENT 5: Effect of ASR on 2BC intermittent alcohol intake in adulthood (6 and 9 months old)

Finally, considering the long-term effect of ASR at 3 months of age in Wistar rats, we assessed whether its effect on increased alcohol consumption may be prolonged even later in life, at 6 and 9 months of age. Since the binge drinking effect was lost at 3

months of age in msP rats belonging to the SR group, these animals were not further evaluated.

At 6 months of age, three-way ANOVA revealed a main effect of group [$F(2,20) = 11.57$, $p < 0.001$], timepoint [$F(2,40) = 119.48$, $p < 0.0001$], and group x timepoint interaction [$F(4,40) = 5.03$, $p < 0.01$], whereas no other interactions were detected. Newman-Keuls post-hoc test showed that the SRD animals still remained different in their alcohol intake compared to the CNT ($p < 0.001$) and SRno-D ($p < 0.001$) groups, and as previously seen, just at the 10-h timepoint (vs. CNT: $p < 0.001$; vs. SRno-D: $p < 0.001$). Single two-way ANOVAs performed on each timepoint showed a group effect at 30 min [$F(2,20) = 13.58$, $p < 0.001$], 2-h [$F(2,20) = 9.76$, $p = 0.001$], and 10-h [$F(2,20) = 8.71$, $p < 0.01$] and subsequent post-hoc tests revealed that the SRD group remained the highest drinker group compared to the other two groups (30 min: SRD vs. CNT and vs. SRno-D, $p < 0.001$; 2-h: SRD vs. CNT, $p < 0.01$, SRD vs. SRno-D, $p < 0.001$;10-h: SRD vs. CNT, $p < 0.01$ and vs. SRno-D, $p < 0.001$) (***Figure 32A***).

At 9 months of age, three-way ANOVA found an overall effect of group [$F(2,20) = 9.05$, $p < 0.01$], exposure [$F(3,60) = 4.74$, $p < 0.01$], timepoint [$F(2,40) = 219.77$, $p < 0.0001$], and group x timepoint [$F(4,40) = 8.81$, $p < 0.0001$]. Post-hoc test confirmed that even at 9 months of age SRD rats still consumed more alcohol than the CNT ($p < 0.01$) and SRno-D ($p < 0.001$) animals at timepoint 10-h (vs. CNT: $p < 0.01$; vs. SRno-D: $p < 0.001$). Then, we performed single two-way ANOVAs on each timepoint to verify whether SRD animals still differed from the other two groups also at 30 min and 2-h of alcohol. At 30 min a main effect of group has been found [$F(2,20) = 5.46$, $p < 0.05$], as well as at 2-h [$F(2,20) = 5.71$, $p = 0.01$] and at 10-h [$F(2,20) = 10.16$, $p < 0.001$]. Post-hoc analysis confirmed that SRD animals still consume significantly more alcohol than CNT (30 min: $p = 0.01$; 2-h: $p < 0.01$; 10-h: $p < 0.01$) and SRno-D (30 min: $p < 0.01$; 2-h: $p < 0.01$; 10-h: $p < 0.001$) rats even later in life as effect of ASR (***Figure 32B***).

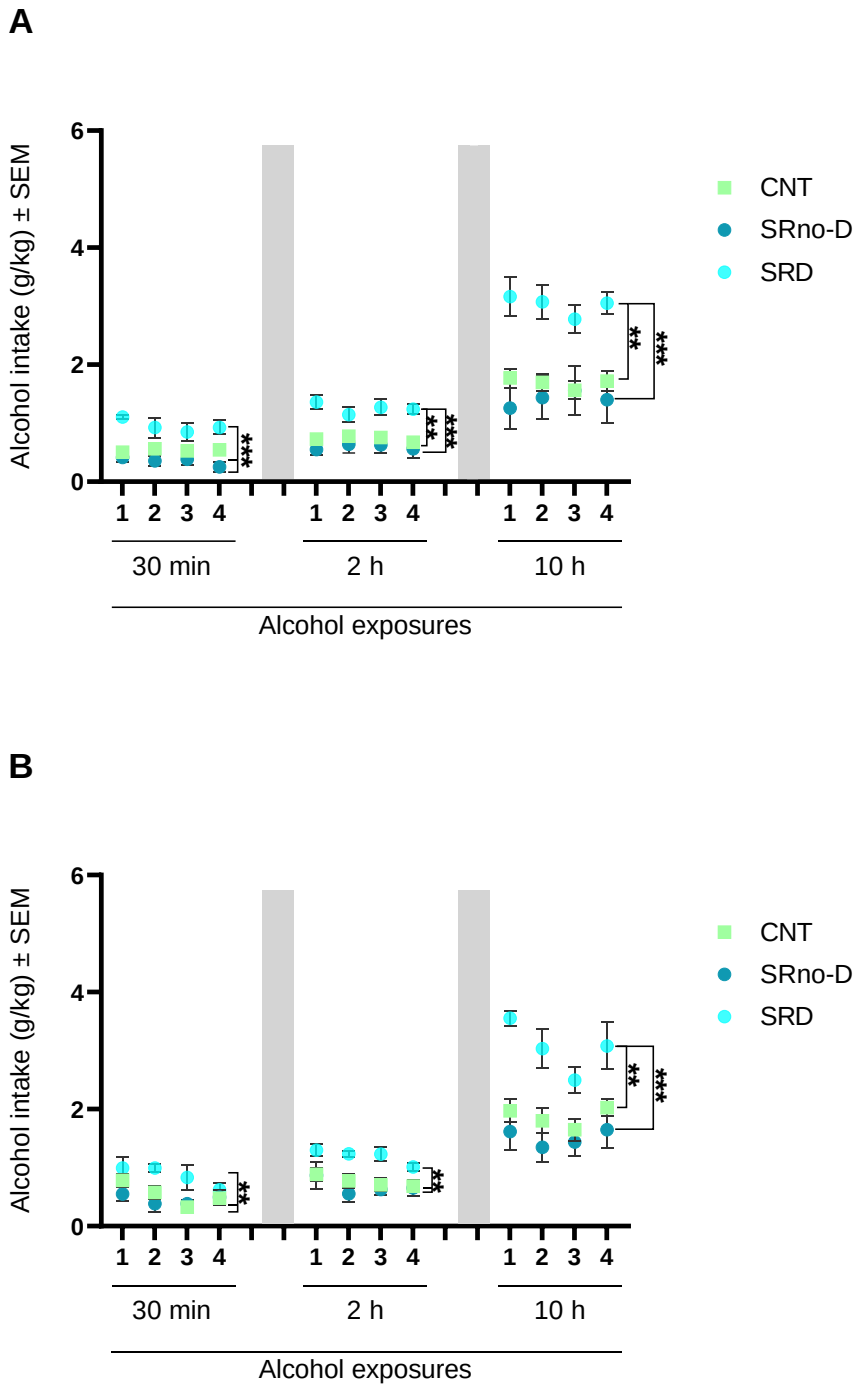

Figure 32. Wistar rats were re-exposed to alcohol in the 2BC paradigm (same procedure as before) to evaluate possible life-long effects of ASR. The SRD group remains the one that drinks significantly more than the other two groups even at 6 (***A***) and 9 months (***B***) of age. Data are presented as mean (± SEM). Difference between SRD and CNT, and between SRD and SRno-D: **$p < 0.01$, ***$p < 0.001$.

3.5. DISCUSSION

A bidirectional relationship between sleep disruptions and reward-related behavior has emerged in recent evidence. Circadian misalignment, sleep disturbance, and sleep loss during adolescence are associated with increased substance use and related problems (McKnight-Eily, et al., 2011; Paiva, et al., 2016; Pasch, et al., 2012; Roberts, et al., 2009; Sivertsen, et al., 2015). Adolescents, who physiologically tend to go to bed late, might be chronically subjected to a misalignment between the sleep/wake schedule and internal circadian timing. The delayed circadian phase-associated sleep loss may affect the neural circuitry of reward processing, leading to an increased alcohol use. In the present study, we evaluated this hypothesis using two different animal models.

Two different responses to ASR have been observed in the two rat lines here tested. Whereas SR msP rats displayed a momentary higher motivation for alcohol in the first two hours of the 2BC paradigm suggesting an increased urge to drink (i.e., resembling a binge drinking episode), in outbred Wistar rats a subgroup of animals exposed to ASR began to consume significantly more alcohol compared to the other SR rats and the CNT group; this effect was persistent and lasted into late adulthood.

The msP rat line has been selected for its high alcohol consumption and preference and these two phenotypic traits have been robustly segregated over the years through careful breeding processes (Borruto, et al., 2021; Ciccocioppo, et al., 2006). Consequently, msP rats are highly homogeneous in alcohol preference and intake. This aspect could explain the reason why we did not observe individual variability in their alcohol consumption in response to SR with high drinking levels occurring in all tested subjects. Instead, our results highlight an enhanced urge to drink following SR, which was evident after 30 min and 2 h of alcohol exposure that occurred during the adolescence but was not maintained later in adulthood. MsP animals are characterized by anxious phenotype and depressive-like symptoms and several data collected by our laboratory have suggested that their motivation for alcohol drinking may be linked to alcohol ability to attenuate anxiety (Borruto, et al., 2021; Ciccocioppo, 2013; Ciccocioppo, et al., 2006). We might then speculate that their

adolescent binge drinking behavior might result, at least in part, as an immediate stress-coping response to SR so much so that at 10 h the total amount of alcohol consumed is equivalent between the two groups. Moreover, in support of this hypothesis, we observed that when msP were no longer subjected to SR in adulthood, the alcohol consumed by the two groups did not differ at any timepoint in any alcohol exposure. Interestingly, msP SR rats showed increased locomotor activity in the OF test compared to the CNT animals of the same line. Repeated exposure to stressful stimuli can indeed elicit behavioral hyperactivity in rodents (Kalivas & Duffy, 1989; Kalivas & Stewart, 1991; Rougé-Pont, et al., 1995). The increased locomotion observed in SR msP rats might then corroborate our hypothesis of a stressful response to SR.

Differently, in Wistar rats we detected a steadily increased alcohol consumption in response to ARS, as expected from our hypothesis. Repeated exposure to alcohol caused a downregulation of D2Rs in NAc which leads to hyperactivity of the NAc, reduction in the control capability of the PFC, and then a reduction in the mechanism of top-down regulation (Volkow, et al., 2006; Volkow, et al., 2009). Decreased striatal expression of D2/D3 receptors has been also proposed as a potential mechanism by which sleep loss impairs prefrontal activities and enhances risk and reward seeking (Liu, et al., 2016; Volkow, et al., 2012a; Volkow, et al., 2008; Wiers, et al., 2016). Hence, despite non evaluated here, it is possible that the enhanced drinking observed in a subgroup of Wistar rats may be linked to sleep-loss induced downregulation of D2/D3 receptors. At morphological levels, numerous changes occur in mesocorticolimbic regions during adolescence in both humans and animals. The process of overproduction and consequent loss (pruning) of synapses have been observed in adolescence as an example of developmental plasticity whereby the brain is ontogenetically shaped on the bases of experience to effectively accommodate environmental needs (Rakic, et al., 1994). General DA receptors tend to peak in rodents' striatum during early/mid-adolescence and decrease thereafter to reach adult levels by PND 60 (Andersen, et al., 2000; Tarazi & Baldessarini, 2000). In particular, a functional predominance of D2Rs over D1Rs has been observed in rats from approximately PND 20-35 using MRI (Chorlian, et al., 2013). The period of

increase and subsequent receptors pruning, especially for D2Rs, corresponds to the period within which we performed SR in rodents (PND 35-55). Our chronic ASR may have altered the normal and physiological pruning process thus affecting D2Rs in adolescence, causing impairment of the developing prefrontal areas and their activity, thereby strengthening bottom-up motivational drive by limbic areas, and ultimately inducing alcohol seeking and consumption.

We also observed that not all adolescent Wistar rats subjected to ASR increased their alcohol intake. A subpopulation of individuals accounting for 6/18 animals (33.3% of the SR group) displayed significantly increased alcohol consumption compared to control rats and to the other animals exposed to SR. Remarkably, this enhanced alcohol intake was maintained throughout adulthood suggesting that permanent changes may occur after ASR. The ASR seems not to affect all animals, but rather appears to do so only in a subset of individuals probably by triggering in them an innate individual predisposition to alcohol use. Recent studies have implicated circadian genes in the neuroadaptive processes underlying drug reward and addiction (Falcón & McClung, 2009; Manev & Uz, 2006; McClung, 2007a; Perreau-Lenz & Spanagel, 2008; Rosenwasser, 2010; Spanagel, et al., 2005a) as well as DA-related genes in interindividual vulnerability to sleep loss and behavioral response to it (Bodenmann, et al., 2009; Goel, et al., 2011; Greer, et al., 2016). In humans, polymorphisms in both the *Clock* and *Period 2* (*Per2*) genes are considered risk factors for the development of SUDs and they are more prevalent in AUD patients (Kovanen, et al., 2010; McClung, 2007b; Spanagel, 2009). Polymorphism in the *Per2* gene in humans has been associated with reduced D2Rs expression in the striatum (Shumay, et al., 2012). The effects of these two genes on the activity of the reward system have been investigated in preclinical studies. Mice carrying a mutated *Clock* gene had a higher reward system response to cocaine administration than control animals suggesting the *Clock* gene as a direct regulator of the DA activity in the reward circuitry (Lamont, et al., 2007). Instead, rodents with a mutant *Per2* gene display enhanced cocaine consumption and alcohol drinking behavior (Abarca, et al., 2002; Perreau-Lenz, et al., 2009; Spanagel, et al., 2005b). On the other hand, a polymorphism

of the gene encoding for the human DA transporter (DAT) has been shown to influence neural responses to sleep loss: the DAT allele (9R carries) is associated with higher phasic DA activity and individuals carrying it revealed greater striatal responses to monetary reward after sleep deprivation (Greer, et al., 2016). Moreover, a polymorphism of the gene of catechol-O-methyltransferase (COMT), an enzyme responsible for the breakdown of catecholamines, has also been implicated in individual sleep physiology and homeostasis (Goel, et al., 2011) and altered DA signalling in the PFC associated with impaired performance on a working memory task after sleep loss (Bodenmann, et al., 2009).

Altogether this genetic evidence highlights the complex bidirectional relationship between circadian characteristics and reward-related behaviors.

Several genes could be involved in triggering the alcohol consumption vulnerability observed in our subgroup of SR Wistar rats and further studies need to be done to reveal the mechanisms responsible for our results.

Our evidence suggests that Wistar rats and the present protocol could be a good model to investigate the genetic and neurobiological factors that predispose some individuals to alcohol misuse in response to sleep difficulties in adolescence. Of considerable importance, although it has been widely demonstrated that sleep loss facilities drug-seeking in rodents that have been previously trained to drug self-administered (Aalto & Kiianmaa, 1984a; Aalto & Kiianmaa, 1984b; Aalto & Kiianmaa, 1986; Bjorness & Greene, 2018; Doyle, et al., 2015; Karimi-Haghighi & Haghparast, 2018; Puhl, et al., 2013; Puhl, et al., 2009; Reséndiz-Flores & Escobar, 2019), our study established a causal role between sleep loss and initiation of alcohol misuse. To our knowledge, only two other experiments explored the effect of SR in stimulating alcohol consumption but using different protocols. García-García and colleagues performed SR (through gently touching) and in parallel they monitored alcohol intake in the 2BC paradigm in adult male Wistar rats and for a more limited time (4 hours of SR for 7 days) (García-García, et al., 2021). Their results are consistent with ours and showed that SR promoted alcohol consumption compared to unrestricted-sleep rats. Instead, Atrooz and colleagues performed a SR and use an automated SD

apparatus more similar to ours. They sleep-restricted Sprague-Dawley male rats for 14 days (6-8 h/day), from PND 19 to PND 32 (much earlier than when we did) and they assessed their alcohol consumption at PND 39 for 5 consecutive days using the 2BC paradigm (tap water vs. 5% alcohol solution) (Atrooz, et al., 2022). The authors observed that SR rats consumed a larger volume of alcohol compared to control rats, as we also detected in our experiment. They also assessed anxiety- and depressive-like behaviors (EPM and sucrose splash test respectively) revealing an increase in both in the SR group compared to the control group. da Silva Rocha-Lopes et al. also observed increased anxiety but following chronic REM SR in adolescent rodents (from PND 21, for 21 consecutive days, 18 h/day) (da Silva Rocha-Lopes, et al., 2018). Unlike them, we did not observe an increase in anxiety-like behavior in SR rats, neither in SRD nor in SRnoD. Differences in the type of SR method used, total sleep or specific sleep stage disrupted, the developmental period in which the SR is performed, duration and timing of SR, as well as the strain of rats used, may be responsible for these different anxiety-related outcomes. Our data suggest that chronic SR from mid-adolescence may not lead to increased anxiety neither in adolescence nor later in adulthood. Even though a relationship between sleep loss and increased anxiety has been identified (Fuligni & Hardway, 2006; Lemola, et al., 2013; Orchard, et al., 2020; Palagini, et al., 2019; Van Dyk, et al., 2019), the direction of this relationship is still unclear. Clinical experiments usually evaluated the effects of acute sleep restriction (one or few days) on anxiety reporting poorer emotional regulation (Pires, et al., 2016). The effects of prolonged and chronic SR have not been studied in humans to date for ethical reasons and we have no preclinical data with which to compare our work.

In conclusion, with the present work, we established a preclinical model for studying the short- and long-term individual impact of adolescent sleep restriction on alcohol consumption. This first pilot experiment opens the way for several aspects to be evaluated, such as genetic and neurobiological bases of resilience and susceptibility to sleep loss and developing alcohol misuse, reward-related behaviors and affective behaviors that can be affected by ASR, and possible sex-based differential behavioral

responses to ASR. Since AUD has been recognized as a high public health priority (World Health Organization, 2018), identifying risk factors that contribute to the initial and escalation of alcohol use would be an important key to developing and providing better-targeted treatment.

Certainly, our experiment highlights how poor sleep during adolescence should not be underestimated but rather considered a possible relevant risk factor for alcohol misuse, not only in adolescence but even later in adulthood. Healthy sleep in adolescence could help to prevent or delay adolescent alcohol consumption and the potential subsequent development of AUD. Noteworthy, a clinical trial on 18 adolescent participants reported that a multicomponent sleep intervention reduced substance use and increased sleep duration was associated with reductions in substance-related problems (Britton, et al., 2010).

Moreover, social demands might be adjusted accordingly to the need for a full good sleep for adolescents. Several clinical studies have already evaluated the effects of later school start time revealing a relevant increase in teenagers' physical and mental health, safety, and academic achievement and performance (Boergers, et al., 2014; Dunster, et al., 2018; Li, et al., 2013; Lufi, et al., 2011; Marx, et al., 2017; Vedaa, et al., 2012; Meltzer, et al., 2021; Winnebeck, et al., 2020; Wolfson, et al., 2007). Therefore, a combination of later school start times and sleep interventions at the individual level could help to align adolescents' biological rhythms with the environment and perhaps mitigate future substance abuse problems.

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Chapter 4

Neuropeptide S differently modulates alcohol self-administration in female genetically selected Marchigian Sardinian alcohol-preferring rats

4.1. ABSTRACT

Neuropeptide S (NPS) possesses unique pharmacological properties being anxiolytic and promoting arousal at the same time. Its potential role in the neurobiology of alcohol use disorder (AUD) has been recently proposed due to relevant findings in both clinical and preclinical studies. Nevertheless, a huge sex gap exists in the literature on this subject. Previous preclinical studies were performed using male Wistar and Marchigian Sardinian alcohol-preferring (msP) rats and they have revealed that the effect of NPS is strongly influenced by the strain of the animal. In particular, it has been demonstrated that NPS reduces alcohol self-administration (SA) in male msP animals by reducing their innate anxiety. Indeed, msP rats are characterized by excessive alcohol consumption comorbid with heightened anxiety and depressive-like phenotypes. Recent experiments have shown that female msP animals, unlike males, may not drink solely to emolliate anxiety. Here we evaluated the effect of NPS on operant alcohol SA in female msP rats. Our data show a heterogeneous response to the NPS treatment in these animals so that two different clusters of rats have been individuated. In Cluster 1 NPS reduced alcohol SA, whereas in Cluster 2 it increased inactive lever pressing during alcohol SA sessions. These results suggest a complex action of NPS infusions in female individuals of this line of rats. This individual variability among animals belonging to a highly homogenous rat line has been recently observed in male msP for behaviors closely related to their main trait of high alcohol preference. Based on the results from the present work, we hypothesized that a genetic variability could exist for the NPS gene in female msP rats, which can be responsible for their different response to the NPS treatment.

4.2. INTRODUCTION

AUD is a complex and multifactorial psychiatric condition often associated with anxiety (Grant, et al., 2004; Ersche, et al., 2010). Up to 50% of individuals treated for alcohol use and misuse also met diagnostic criteria for one or more anxiety disorders (Chan, et al., 2008; Kushner, et al., 2005).

The neuropeptide S (NPS) is a 20 amino acid neurotransmitter expressed by small clusters of neurons located within three brainstem areas: the parabrachial area, the peri-locus coeruleus, and the sensory trigeminal nucleus (Liu, et al., 2011; Xu, et al., 2007; Xu, et al., 2004). Its receptor (NPSR) is expressed in limbic areas such as the hypothalamus, hippocampus, and amygdala, and it plays a role in motivated behavior (Liu, et al., 2011; Xu, et al., 2007). The NPS has a fascinating opposite effect on animal behaviors: central administration of this neuropeptide increases locomotor activity, decreases sleep, and enhances alertness as well as reduces anxiety in several stressful paradigms (Leonard, et al., 2008; Rizzi, et al., 2008; Xu, et al., 2004). This ability to regulate both arousal and anxiety indicates a potentially important role of the NPS in the neurobiology of AUD. Indeed, both variants of the NPSR gene have been associated with vulnerability to developing AUD in humans (Reinscheid, et al., 2005). A recent clinical study associated increased NPSR activity with more severe AUD and increased alcohol consumption (Laas, et al., 2015). In addition, several preclinical studies strongly indicate the involvement of the NPS system in alcohol-related behavior (Badia-Elder, et al., 2008; Cannella, et al., 2009; Cannella, et al., 2016; Enquist, et al., 2012; Ruggeri, et al., 2010; Ubaldi, et al., 2016).

Interestingly, preclinical experiments have highlighted that the effect of NPS is strongly influenced by the genetic background of the animal. The NPS pro-arousal effect on alcohol-related behaviors is evident in the only non-preferring rat lines. Cannella and colleagues demonstrated that when NPS was given concomitantly with the presentation of discriminative cues signalling drug availability, it promoted the reinstatement of alcohol seeking in Wistar rats, but not in msP rats (Cannella, et al., 2009; Cannella, et al., 2016). Conversely, the effect of NPS administration on alcohol consumption reduction has been specifically reported on rat lines genetically selected

for their high alcohol preference. NPS reduced alcohol intake in a 2BC paradigm in Indiana alcohol-preferring (P) rats and operant SA in msP, with no effect in non-preferring control lines (Badia-Elder, et al., 2008; Cannella, et al., 2009; Cannella, et al., 2016). The line-selective reduction in alcohol consumption was probably due to the anxiolytic properties of NPS (Cannella, et al., 2016; Dine, et al., 2015; Ionescu, et al., 2012; Leonard, et al., 2008; Lukas & Neumann, 2012; Rizzi, et al., 2008; Vitale, et al., 2008; Xu, et al., 2004). Both msP and P rats are characterized by an innate anxious phenotype, and their alcohol consumption is predominantly driven by the anxiolytic effect of the substance (Borruto, et al., 2021b; Ciccocioppo, et al., 2006; Domi, et al., 2019; Stewart, et al., 1993). Therefore, the reduction in anxiety determined by NPS consequently leads to a reduction in msP rats' motivation for alcohol.

To the best of our knowledge, only one preclinical study investigated the effects of NPS on reward-related behavior in female rodents (Badia-Elder, et al., 2008), despite increasing evidence in both humans and laboratory animals have highlighted sex differences in the SUD. Several studies suggest that men and women differ in risk trajectories for the development of AUD and in AUD-related behaviors (Sharrett-Field, et al., 2013). Women more than men consume alcohol as a coping strategy to attenuate negative affective states (e.g., anxiety, depression, and post-traumatic stress disorder) (Crutzen, et al., 2013; Guinle & Sinha, 2020; Peltier, et al., 2019). Similarly, preclinical studies have reported that female msP rats consume higher amounts of alcohol when compared to males (Borruto, et al., 2020; Borruto, et al., 2021b). Moreover, stress plays an important role in all phases of AUD, but its consequences are more pronounced in females than in males: women are more likely to relapse in response to stressful events than men (Greenfield, et al., 2007; Hudson & Stamp, 2011; Walitzer & Dearing, 2006). When a pharmacological stressor, such as yohimbine, is used to induce alcohol reinstatement in male and female msP rats, the latter relapse more highly than males (Borruto, et al., 2021a).

MsP rats, therefore, prove to be a good model to study the relationship between anxiety and alcohol consumption in female subjects (see also **Chapter 1**, paragraph 5, subgraph "**5.4.1. SEX-RELATED BEHAVIORAL DIFFERENCES IN MSP RATS**").

To help bridge the gap in studies of sex differences, the present work aimed to investigate the effect of NPS on alcohol consumption in female msP rats.

Recent data have highlighted the existence of a certain amount of individual variability even in the highly genetically homogenous msP line (see also **Chapter 1**, paragraph 5, subgraph "**5.4. THE MARCHIGIAN SARDINIAN ALCOHOL-PREFERRING RATS**"). Indeed, individual differences have been detected in msP rats in operant SA under fixed ratio condition as well as in relapse to alcohol seeking elicited by both conditioned stimulus (cues previously associated with alcohol SA) and the pharmacological stressor yohimbine (Egervari, et al., 2018). Although selected breeding and genetic pressure have successfully reduced individual vulnerability for the exact traits used to select animals (excessive alcohol drinking and alcohol preference), individual differences persist for other closely related behaviors. Consequently, in the present study, we also intended to examine possible individual differences in female msP rats in response to NPS treatment on alcohol SA.

4.3. MATERIAL AND METHODS

4.3.1. ANIMALS

Female msP rats (n = 29) bred at the School of Pharmacy, University of Camerino, weighing 200 g at the beginning of the study were used. Pairs of rats were housed in a room with an artificial 12 h/12 h light/dark cycle (lights off at 8:00 a.m.) at constant temperature (20–22°C) and humidity (45–55%). Animals were offered free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy). Rats were handled for 5 minutes a day by the same operators who performed the experiments to familiarize them with human contact. All training and experimental sessions were conducted once a day during the nocturnal phase of the light/ dark cycle.

All procedures were conducted in adherence with the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

4.3.2. INTRACRANIAL SURGERY

Animals were anesthetized via intramuscular injection of 100–150 μl of a solution containing tiletamine hydrochloride (58.17 mg/ml) and zolazepam hydrochloride (7.5 mg/ ml). A guide cannula (0.65-mm outside diameter) for drug injections was stereotaxically implanted and cemented to the skull according to coordinates in millimeters with reference to bregma (anteroposterior (AP), −1.0; lateral (L), −1.8; and ventral (V), -2.0) (Cannella, et al., 2009; Cannella, et al., 2016; Paxinos & Watson, 1988). To alleviate post-operative pain, rats were treated with 2.5 mg/kg of ketoprofen administered subcutaneously for two days. Surgery was followed by a 7-day recovery period, during which the rats were left undisturbed in their home cage. Before the beginning of the experiments, the animals received intracerebroventricular (ICV) injections of saline to habituate them to the drug administration procedure. Appropriate cannula placement was verified before the experiment by ICV injection of 100 ng of angiotensin II; only animals showing a clear dipsogenic response (consumption of at least 6 ml of water within 5 min) were used for further experimentation.

4.3.3. DRUG INJECTION

NPS, a generous gift of Dr. R. Guerrini, University of Ferrara, Italy, was dissolved in sterile isotonic saline and administered intracerebroventricularly in a volume of 1 μl/rat using a stainless-steel injector 2.5 mm longer than the guide cannula. The injector was connected to a 10-μl Hamilton syringe. Once the injector was introduced into the guide cannula, the NPS (or vehicle) solution was infused by gently pushing the syringe piston. After the infusion, the injector was left in place for 1 min before removal to avoid liquid backflow. The NPS or vehicle solution was injected 5 min before SA sessions.

4.3.4. OPERANT TRAINING OF ALCOHOL SA

Operant training and testing were performed in self-administration chambers (Med Associates, St Albans, VT, USA) equipped with a drinking reservoir (volume

capacity: 0.30 ml) placed between two retractable levers (one active and one inactive) located in the front panel of the chamber. Visual stimulus was presented via a house light located on the wall opposite the levers. Each chamber was enclosed in ventilated sound-attenuating cubicles. Behavioral sessions were controlled and recorded by a windows compatible PC equipped with Med-PC-5 software (Med Associates).

Rats were trained to self-administer 10% alcohol (*v*/*v*) in 30-min daily sessions on a FR1 schedule of reinforcement. Operant sessions started with levers insertion and ended with levers retraction. Each response to the right (active) lever resulted in the delivery of 0.1 mL of 10 % alcohol solution. The delivery of the solution was followed by a contingent illumination of the house light and a 5-s TO period during which the reinforced lever remained inactive. Left (inactive) lever presses had no scheduled consequences. The number of operant lever presses to both the active and inactive levers and the number of reinforcements received was recorded for each session.

4.3.5. EFFECT OF ICV NPS TREATMENT ON ALCOHOL SELF-ADMINISTRATION

Rats were trained to self-administer 10% (*v*/*v*) alcohol using the procedure described above. Once saccharin was faded out, each alcohol delivery was paired with the illumination of the chamber's house light, which signalled a 5-second TO period. Following the acquisition of a stable baseline of alcohol self-administration, NPS treatment began. Five minutes before the beginning of the self-administration session, rats received an ICV injection of NPS (0.1, 1.0, or 2.0 nmol/rat) or its vehicle in a counterbalanced order. Drug treatment was performed every fourth day. On the first day after each drug injection, the rats remained in their home cages, whereas on the 2nd and 3rd days after each drug injection, baseline alcohol self-administration was re-established.

4.3.6. COMPUTATION OF IES AND K-MEANS CLUSTERING

To the completion of the experimental protocol, to assess the contribution of each animal to the NPS drug treatment efficacy, we computed each rat's individual IES within its corresponding dose.

First, we z-scored each subject's value of number of rewards obtained at each treatment. Then, the individual z-score of each dose was subtracted from the vehicle z-score to calculate the IES. Comparing each difference, we could estimate the contribution of each individual to the distribution of data in response to the treatment (***Figure 33***).

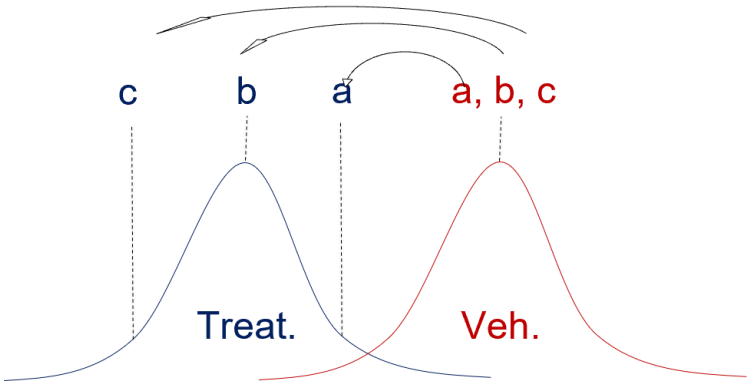

Figure 33. Illustration of the method to calculate individual effect size (IES) and its interpretation. The method can be generalized to both right and left shift induced by treatments. In here a left shift is assumed. Comparing subjects' position within the vehicle (Veh) and treatment (Treat) distribution there can be observed three kinds of contribution to treatment inducing shift of the distribution (group effect size): - subject b maintained the same relative position within treatment and vehicle distributions, i.e. it moved in line with group effect size; - subject c's position in treatment distribution shifted leftward compared to its position under the vehicle, i.e. the drug had a stronger effect than the average group effect size in subject c; - subject a's position in treatment distribution shifted rightward compared to its position under the vehicle, i.e. the drug had a weaker effect than the average group effect size in subject a. Therefore, subtracting treatment Z-score to vehicle Z-score of each subject it can be inferred that:

If $Z_{Veh} - Z_{Treat} < 0$ (subject a), then the subject shows a response to treatment weaker than the group's average.

If $Z_{Veh} - Z_{Treat} = 0$: (subject b), then the subject shows a response to treatment in line with the group's average.

If $Z_{Veh} - Z_{Treat} > 0$: (subject c), then the subject shows a response to treatment stronger than the group's average.

The IESs for all the doses treated were subjected to k-means cluster analysis with 10 iterations and the number of groups defined *a priori* as 2.

4.3.7. STATISTICAL ANALYSIS

Behavioral data were analysed via between- and within-factors ANOVAs as appropriate. The effect of NPS on all rats was analysed via one-way ANOVA with NPS dose as between-subjects factor. ANOVAs were followed by Dunnett's post-hoc analysis when appropriate. Significance was conventionally set at $p < 0.05$. The effect of NPS on the two clusters of msP rats was analysed via two-way ANOVA with cluster as a between-subjects factor and NPS dose as within-subjects factor. The analyses were followed by the Newman-Keuls post-hoc test or the Sidak's post-hoc test when appropriate, and statistical significance was set at $p < 0.05$.

4.4. RESULTS

EFFECT OF ICV NPS TREATMENT ON ALCOHOL SELF-ADMINISTRATION

The effect of NPS was assessed on alcohol SA in female msP rats. All rats showed positive results on the angiotensin II test; therefore, all rats were included in the statistical analysis. When the number of alcohol rewards was evaluated via one-way ANOVA, the statistical analysis found an overall effect of dose [$F(3,84) = 9.79$, $p < 0.0001$]. Dunnett's post-hoc analysis showed that NPS treatment decreased the number of reinforcements received by msP rats compared to the vehicle treatment (vehicle *vs*. 0.1 nmole/ICV: $p < 0.01$; *vs*. 1.0 nmole/ICV: $p < 0.01$; *vs*. 2.0 nmole/ICV: $p < 0.001$) (***Figure 34***).

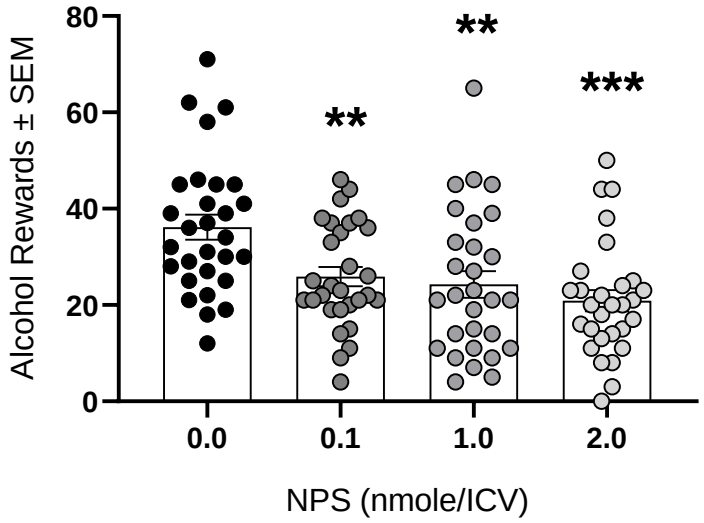

Figure 34. NPS treatment (0.0, 0.1, 1.0, and 2.0 nmole/ICV) was demonstrated to be effective at all doses tested compared to the vehicle treatment. Data are presented as mean (± SEM). **p < 0.01, ***p < 0.001.

To further investigate NPS efficacy on female msP rats and individuate responder and non-responder subjects, animals were clustered in two groups through the k-means cluster analysis (***Figure 35***).

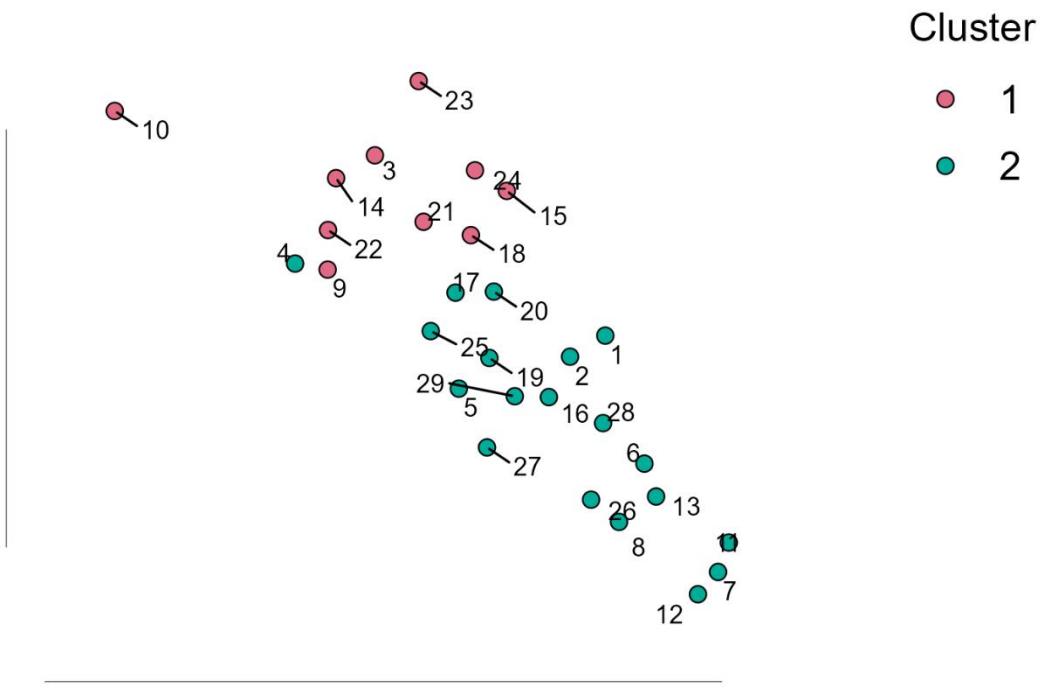

Figure 35. t-SNE plot of animals' allocation in 2 clusters defined by the k-means clustering. **Cluster 1**, n = 10; **Cluster 2**, n = 19.

Two-way ANOVA revealed an overall effect of dose [$F(3,81) = 18.42$, $p < 0.0001$] and dose x cluster interaction [$F(3,81) = 8.88$, $p < 0.0001$]. Newman-Keuls post-hoc test revealed that at 0.1 ($p < 0.01$), 1.0 ($p < 0.01$) and 2.0 ($p < 0.001$) NPS significantly reduced the number of alcohol rewards obtained by rats in Cluster 1, while it was not effective in Cluster 2 (***Figure 36***). Moreover, Sidak's post-hoc test found a significant difference between Cluster 1 and Cluster 2 at the vehicle treatment ($p < 0.01$) demonstrating that female rats in Cluster 1 significantly self-administer more alcohol than those in Cluster 2 and the NPS treatment reduced their alcohol SA bringing it to the average level of rats in Cluster 2 (***Figure 36***).

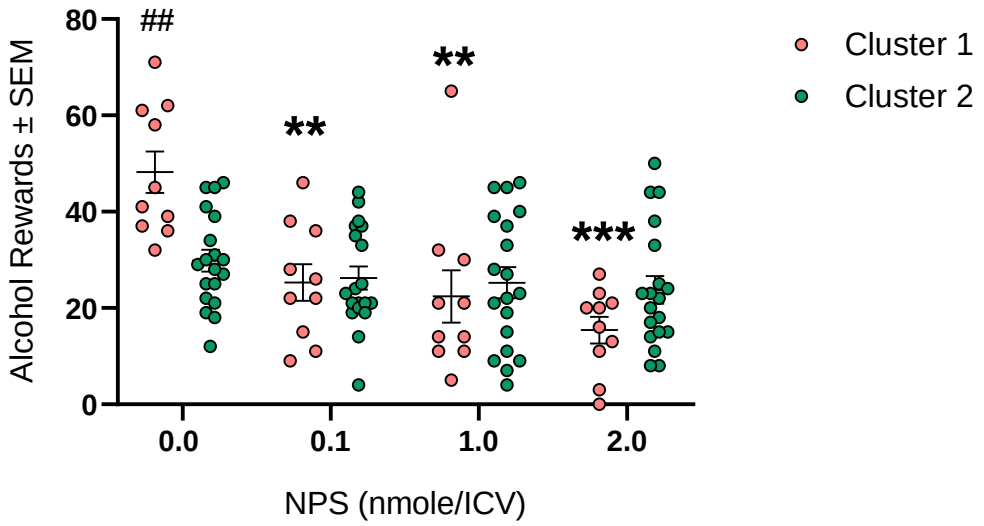

Figure 36. NPS (0.0, 0.1, 1.0, and 2.0 nmole/ICV) efficacy in the two Clusters individuated by the k-means cluster analysis. The two clusters are significantly different in their alcohol SA at the vehicle treatment, with **Cluster 1** self-administering more alcohol than **Cluster 2**. All doses of NPS reduced the number of alcohol rewards in **Cluster 1** when compared to the vehicle, whereas no effect of NPS treatment was found in **Cluster 2**. Data are presented as mean (± SEM). ##p < 0.01 *vs*. **Cluster 2** under the same conditions; **p < 0.01, ***p < 0.001 *vs*. vehicle treatment in the same cluster.

Then, the effect of NPS treatment was verified on the inactive lever-pressing behavior of all female msP rats. Interestingly, one-way ANOVA showed that NPS treatment increased the inactive lever-pressing in all female msP rats [$F(3,84) = 6.28$, $p < 0.01$] and Dunnett's post-hoc analysis found that all the doses tested were effective compared to the vehicle treatment (vehicle *vs*. 0.1 nmole/ICV: $p < 0.05$; *vs*. 1.0 nmole/ICV: $p < 0.01$; *vs*. 2.0 nmole/ICV: $p < 0.01$) (***Figure 37***).

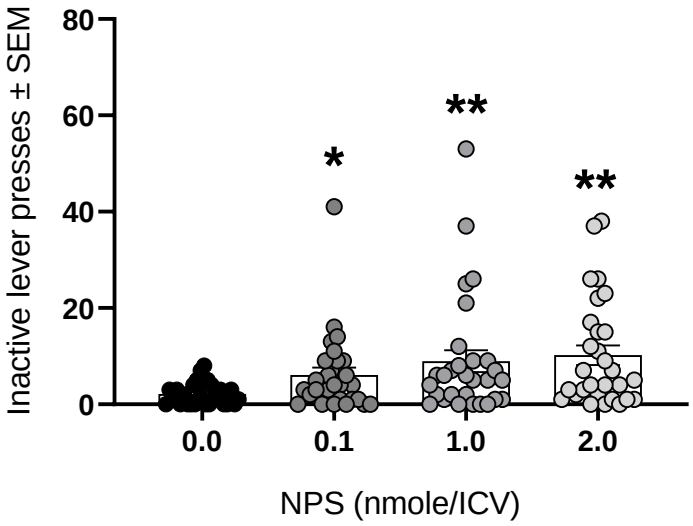

Figure 37. NSP treatment (0.0, 0.1, 1.0, and 2.0 nmole/ICV) overall increased the number of inactive lever presses in all female msP rats treated compared to the vehicle treatment. Data are presented as mean (± SEM). **p < 0.01, **p < 0.01.

To better understand this result, we analysed the effect of NPS on inactive lever-pressing behavior in the two clusters of female msP rats individuated. Two-way ANOVA found a main effect of dose [$F(3,81) = 4.8$, $p < 0.01$] and the subsequent post-hoc analysis revealed that at 0.1 ($p < 0.05$) and 2.0 ($p < 0.01$) nmole/ICV NPS significantly increased the inactive lever-pressing in Cluster 2, with no effect in Cluster 1 (***Figure 38***).

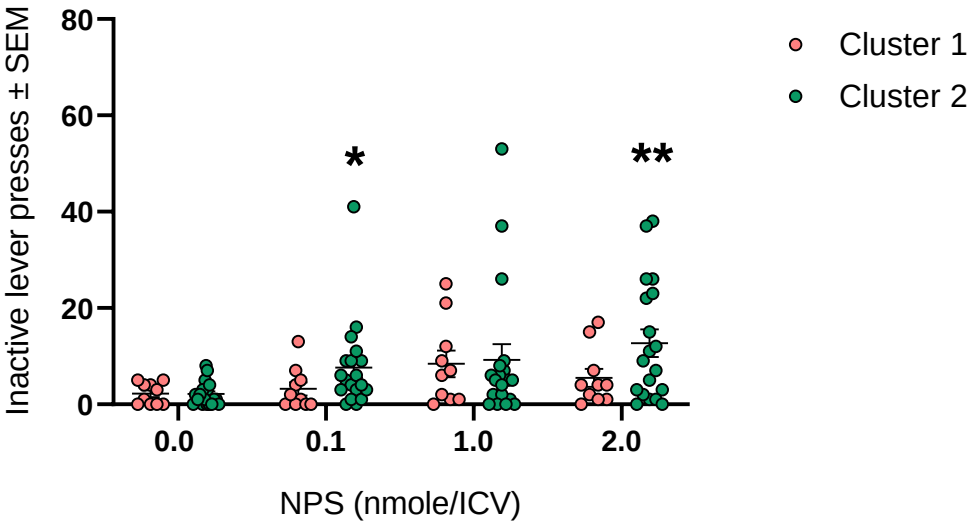

Figure 38. NPS (0.0, 0.1, 1.0, and 2.0 nmole/ICV) effect on the inactive lever-pressing behavior in the two Clusters individuated by the k-means cluster analysis. At 0.1 and 2.0 nmole/ICV, NPS significantly increases the number of inactive lever presses in **Cluster 2**. Data are presented as mean (± SEM). *$p < 0.05$, **$p < 0.01$ *vs*. vehicle treatment in the same cluster.

4.5. DISCUSSION

Although sex differences have been established on several aspects related to AUD in both humans and rodents, there is still a large gap in this field of research.

Recently the NPS/NPSR system has been proposed as a potential target to develop new treatments for AUD, but further investigations are needed, especially related to possible sex differences. Although interesting preclinical evidence has revealed differences between men and women on how the NPS/NPSR1 system may modulate SUD-relevant behaviors (Glotzbach-Schoon, et al., 2013; Laas, et al., 2015; Raczka, et al., 2010), only one preclinical experiment has evaluated the effect of NPS treatment on female rats before us (Badia-Elder, et al., 2008).

The present study aims to fill this gap by investigating the effect of NPS on alcohol drinking and related behavior in female msP rats. Our results revealed a heterogenous response at the NPS treatment based on which we individuated two different clusters of msP rats. Together with our previous findings on male msP rats (Cannella, et al., 2016), this evidence reveals the fascinating existence of sex differences in NPS response.

Comparing male msP and Wistar rats, we previously observed the anxiolytic effect of NSP on the genetically selected line, and the pro-arousal effect on the outbred strain, with no individual difference in any of them (Cannella, et al., 2009; Cannella, et al., 2016). Here, when we administered NPS to female msP animals, although overall NPS was effective in reducing alcohol SA at all doses tested on all rats, we recorded a very heterogeneous response. Therefore, we calculated the IES to better understand the contribution of each animal to the overall response to the NPS treatment. Using this parameter, we performed a k-means cluster analysis that allocated rats into two different clusters. When we compared the two clusters, operant alcohol SA was reduced in only Cluster 1, whereas inactive lever pressing was significantly increased in only Cluster 2. This effect of NPS on active lever pressing in Cluster 1 cannot be attributed to changes in locomotor performance because presses on the control inactive lever do not differ between vehicle and NSP treatment. The decrease in alcohol rewards obtained by Cluster 1 rats may be instead

due to the anxiolytic effect of NPS: since alcohol drinking in msP animals is associated with their anxiety traits and motivated by tension-relief purposes, NPS can reduce the reinforcing value of alcohol by reducing anxiety in these animals as already hypothesized for male msP rats. Conversely, increased inactive lever pressing in Cluster 2 rats may be attributed to an increase in locomotion, leading to the speculation that this effect could be due to the pro-arousal impact of NPS.

The detection of individual differences in response to treatment with NPS is not surprising. It has recently emerged that a certain amount of individual variability is also maintained in highly genetically homogenous rat lines (Ayanwuyi, et al., 2013; Cippitelli, et al., 2008; Domi, et al., 2019; Egervari, et al., 2018).

Although the selected breeding for the alcohol preference trait robustly segregated that trait, ensuring line stability and reducing individual variability related to that precise trait to zero, individual differences persist for other closely genetic traits (Domi, et al., 2019; Egervari, et al., 2018) and the NPS gene can be one of them. A single nucleotide polymorphism (SNP) was found in the human NPSR gene, which is located at triplet position 107 of the NPSR1 gene on chromosome 7p14.3 and changes from Ile to Asn in the active centre of the receptor binding, replacing the second base of the codon from T to A (Reinscheid, et al., 2005). Clinical studies revealed an association between the AA genotype and increased anxiety (Laas, et al., 2014a) as well as between the T allele and fear reaction and generalization in healthy humans (Raczka, et al., 2010; Okamura, et al., 2007). Moreover, AUD was associated with the A allele in women and the T allele in men. The T allele leads to an increased NPSR activity (Laas, et al., 2015) and individuals carrying this allele have poor impulse control (Laas, et al., 2014b), which is a risk factor for substances abuse (Fontenelle, et al., 2011). On the other hand, the A allele is linked to lower NPSR activity and women carrying the A allele are more vulnerable to affective disorders (such as anxiety and depression), which have been demonstrated to be other risk factors for AUD (Laas, et al., 2015; Laas, et al., 2014a). Considering how the human SNP is capable of changing the phenotypic traits of individuals, it cannot be excluded that similar variations in the rodent NPSR gene can be observed in female msP rats

belonging to the two different clusters identified. These possible differences in genes encoding for NPS/NPSR in female msP rats could therefore be responsible for their different response to NPS treatment.

Unlike msP males, the reasons behind alcohol consumption in msP females are not entirely clear. Alcohol drinking decreased depressive-like behavior in both sexes in the forced swim test, while it reduced anxiety-like behavior only in male msP animals but not in females in the elevated plus maze (EPM) test (Borruto, et al., 2021b). Both male and female msP rats are characterized by traits that confer negative mood conditions and co-segregated with alcohol drinking during genetic selection. However, the motivation for alcohol in males seems to be linked to its ability to attenuate both anxiety and depression, whereas in females alcohol drinking appears to be more linked to its antidepressant properties. Looking at our data, the two clusters of female msP rats did not drink the same quantity of alcohol before starting the NPS treatment. Rats in Cluster 1 drink significantly more alcohol than rats in Cluster 2, and those are the same animals in which NPS treatment successfully reduced alcohol self-administration. This observation tempts us to speculate that Cluster 1 consists of animals characterized by a more anxious phenotype than the rats in Cluster 2 and that these higher anxiety traits drive their higher alcohol consumption. For this reason, NPS might have acted anxiolytically in these subjects reducing their alcohol consumption.

Interestingly, the only other study that assessed the effect of NPS on genetically selected alcohol-preferring female rats, obtained undefined outcomes (Badia-Elder, et al., 2008). Whether on the one hand, NPS reduced ethanol intake in alcohol-preferring (P) rats, but not in alcohol-nonpreferring (NP) animals, on the other hand, less clear-cut are the results on anxiety and locomotion. Indeed, the authors observed neither any significant reduction in the anxiety-like behavior in the EPM test nor an increase in the locomotor activity in the open field (OF) test as the effect of the NPS treatment. The only indication of an anxiolytic effect of the NPS was an increase in the amount of time spent in the center of the OF arena following infusions of 0.6 nmole of NPS in P, but not in NP rats. These findings do not seem to clearly show

which is the effect of NPS on this line of female rats, similar to what seen in our present experiment. Of course, msP and P rats are not the same line and show similarities (selected for their alcohol preference, anxious profile, and dysregulation of the CRF system) as well as differences between them (Bell, et al., 2012; Ciccocioppo, 2013; Ciccocioppo, et al., 2006), but the evidence obtained by Badia-Elder and colleagues together with ours open the possibilities that the NPS effect on alcohol-preferring female animals can be more complex to what seen in males of the same line.

The present experiment needs to be replicated and expanded. To corroborate our speculations, once individuated the two clusters of female msP rats based on their response to NPS, they should be tested for their anxiety-like behavior (EPM) and locomotor activity (OF) under and without NPS treatment. We might expect that female msP rats here individuated in Cluster 1 will show higher levels of anxiety-like behavior attenuated by NPS infusions compared to rats in Cluster 2 which instead will increase their general locomotor activity in response to NPS.

In conclusion, our preliminary results suggest a complex picture of the role of NPS in genetically selected alcohol-preferring female rats. Substantial individual variability has been detected in female msP rats when monitored for their alcohol intake under operant self-administration condition. This initial difference between rats seems to imply a different response to NPS. Future studies will need to better detail the mechanism underlying the different action of NPS in female alcohol-preferring animals. The present study reaffirms the need to include females in preclinical studies and to analyse them as subjects endowed with specific characteristics that may differ from those of males. Moreover, these preliminary findings corroborate the observation that sex differences and heterogeneity in AUD-related behavior can be detected also in msP rats supporting the translational value of this type of investigation.

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Chapter 5

Conclusive remarks

In conclusion, the main findings of this thesis work can be summarized as follows:

- Outbred NIH-HS rats are a good animal model to investigate the genetic aspect of AUD in terms of individual vulnerability and resilience as well as the genetic predictors of treatment response.
- NIH-HS animals can be used as a multisymptomatic preclinical model of individual variability in AUD offering high predictive validity.
- The effect of sleep loss in adolescence can be better investigated using outbred strains, such as the outbred Wistar line. Indeed, through this rat model, we could better mimic the human population in which not all teenagers with sleep difficulties also consume large quantities of alcohol.
- Poor sleep during adolescence shouldn't be underestimate but considered a relevant risk factor for alcohol misuse, not only in adolescence but even later in adulthood.
- Social demands might be adjusted according to sleep physiological changes in adolescents.
- Clinicians should pay more attention to adolescent sleep problems and consider treating them to prevent alcohol misuse and possible subsequent development of AUD later in life.
- NPS/NPSR system has been proposed as a potential target to develop new treatments for AUD, but further investigations are needed, especially concerning gender differences.
- Individual variability can emerge even in a highly homogeneous rat line like the msP rats. Genetic pressure has stably segregated alcohol-abuse-related traits, but individual differences persist for other closely related behaviors and genes, and the NPS/NPSR system can be one of them.

- The fundamental need to include females in preclinical studies is affirmed. Female subjects must be analysed as having their unique characteristics that may differ from those of males.

- Altogether the three studies presented here emphasize the necessity to develop targeted and personalized therapies to treat AUD according to the individual patient's characteristics.

- Genetic background, neurobiological characteristics, age-of-onset, experienced events, drinking patterns, comorbidities, and gender are just a few factors involved in the aetiology and development of alcohol use and misuse and AUD and addressed and investigated in the present thesis.

- Additionally, the three studies exposed shed light on how combining different rat lines can maximize the translational power of preclinical research in AUD.

GLOSSARY

2BC:	Two-bottle choice
ACI/N:	Black Agouti
ADE:	Alcohol Deprivation Effect
ADH:	Alcohol Dehydrogenase
ADHD:	Attention Deficit Hyperactivity Disorder
ALDH:	Aldehyde Dehydrogenase
AMG:	Amygdala
ASR:	Adolescent Sleep Restriction
AUD:	Alcohol Use Disorder
AUDIT:	Alcohol Use Disorder Identification Test
BAC:	Blood Alcohol Concentration
BDP:	Brief Drinker Profile
BLA:	Basolateral Amygdala
BN/SsN:	Brown Norway
BUF/N:	Buffalo
CRF1R:	Corticotropin releasing factor type 1 receptor
CNS:	Central Nervous System
CNT:	Control
COMT:	Catechol-O-methyltransferase
CPA:	Conditioned Place Aversion
CPP:	Conditioned Place Preference
CRF:	Corticotropin-Releasing Factor
D1R:	DAergic 1 Receptor
D2R:	DAergic 2 Receptor
DA:	Dopamine
DSM-IV-TR:	Diagnostic and Statistical Manual of Mental Disorders, Fourth edition, Text Revision
DSM-5:	Diagnostic and Statistical Manual of Mental Disorders, Fifth edition
EMA:	European Medications Agency
EtOH:	Ethanol
F344/N:	Fischer 344
FDA:	Food and Drug Administration
FR:	Fixed Ratio
GxE:	Gene x Environment
GABA:	γ-Aminobutyric acid
GLU:	Glutamate
GWAS:	Genome-Wide Association Studies
HIPPO:	Hippocampus
HPA axis:	Hypothalamic-Pituitary-Adrenal axis
HS:	Heterogeneous Stock
i.p.:	Intraperitoneally

ICV:	Intracerebroventricular
ISE:	Individual Effect Size
L/D:	Light/Dark
MEM:	Memantine
MR/N:	Maudsley Reactive
MSN:	Medium Spiny Neuron
MSP:	Marchigian Sardinian alcohol-preferring
NAc:	Nucleus Accumbens
NIDA:	National Institute on Drug Abuse
NIH:	National Institute of Health
NIH-HS:	NIH Heterogenous Stock
NMDA:	N-methyl-d-aspartic acid or N-methyl-d-aspartate
NMDAR:	NMDA Receptor
NP:	alcohol-nonpreferring
NPS:	Neuropeptide S
NPSR:	Neuropeptide S Receptor
NSDUH:	National Survey on Drug Use and Health
NTX:	Naltrexone
OFC:	Orbitofrontal Cortex
PER:	Period
PFC:	Prefrontal Cortex
PND:	Postnatal Day
PR:	Progressive Ratio
PTSD:	Post-Traumatic Stress Disorder
s.c.:	Subcutaneously
SA:	Self-Administration
SADQ:	Severity of Alcohol Dependence Questionnaire
SAMHDA:	Substance Abuse & Mental Health Data Archive
SD:	Sleep Deprivation
SN:	Substantia Nigra
SNP:	Single Nucleotide Polymorphism
SP:	Sardinian alcohol-preferring
SR:	Sleep Restriction
SRD:	Sleep Restricted Drinkers
SRno-D:	Sleep Restricted no-Drinkers
SUD:	Substance Use Disorder
TO:	Time-Out
VT:	Ventral Striatum
VTA:	Ventral Tegmental Area
WHO:	World Health Organisation
WKY/N:	Wistar Kyoto
WN/N:	Wistar Nettleship

Appendix

Scientific contributions

pharmaceuticals

MDPI

Review

A Role for Neuropeptide S in Alcohol and Cocaine Seeking

Nazzareno Cannella, Anna Maria Borruto, Michele Petrella, Maria Vittoria Micioni Di Bonaventura, Laura Soverchia, Carlo Cifani, Sara De Carlo, Esi Domi and Massimo Ubaldi *

School of Pharmacy, University of Camerino, Via Madonna delle Carceri 9, 62032 Camerino, Italy; nazzareno.cannella@unicam.it (N.C.); annamaria.borruto@zi-mannheim.de (A.M.B.); michele.petrella@liu.se (M.P.); mariavittoria.micioni@unicam.it (M.V.M.D.B.); laura.soverchia@unicam.it (L.S.); carlo.cifani@unicam.it (C.C.); sara.decarlo@unicam.it (S.D.C.); esi.domi@unicam.it (E.D.)
* Correspondence: massimo.ubaldi@unicam.it; Tel.: +39-0737-403310

Abstract: The neuropeptide S (NPS) is the endogenous ligand of the NPS receptor (NPSR). The NPSR is widely expressed in brain regions that process emotional and affective behavior. NPS possesses a unique physio-pharmacological profile, being anxiolytic and promoting arousal at the same time. Intracerebroventricular NPS decreased alcohol consumption in alcohol-preferring rats with no effect in non-preferring control animals. This outcome is most probably linked to the anxiolytic properties of NPS, since alcohol preference is often associated with high levels of basal anxiety and intense stress-reactivity. In addition, NPSR mRNA was overexpressed during ethanol withdrawal and the anxiolytic-like effects of NPS were increased in rodents with a history of alcohol dependence. In line with these preclinical findings, a polymorphism of the NPSR gene was associated with anxiety traits contributing to alcohol use disorders in humans. NPS also potentiated the reinstatement of cocaine and ethanol seeking induced by drug-paired environmental stimuli and the blockade of NPSR reduced reinstatement of cocaine-seeking. Altogether, the work conducted so far indicates the NPS/NPSR system as a potential target to develop new treatments for alcohol and cocaine abuse. An NPSR agonist would be indicated to help individuals to quit alcohol consumption and to alleviate withdrawal syndrome, while NPSR antagonists would be indicated to prevent relapse to alcohol- and cocaine-seeking behavior.

Keywords: alcohol; cocaine; relapse; stress; anxiety; arousal

Citation: Cannella, N.; Borruto, A.M.; Petrella, M.; Micioni Di Bonaventura, M.V.; Soverchia, L.; Cifani, C.; De Carlo, S.; Domi, E.; Ubaldi, M. A Role for Neuropeptide S in Alcohol and Cocaine Seeking. *Pharmaceuticals* **2022**, *15*, 800. https://doi.org/10.3390/ph15070800

Academic Editor: Giuseppe Biagini

Received: 21 April 2022
Accepted: 23 June 2022
Published: 27 June 2022

1. Introduction

The neuropeptide S (NPS) is a 20 amino acid neurotransmitter expressed by small clusters of neurons located within the parabrachial area, the peri-locus coeruleus (LC) and the sensory trigeminal nucleus [1]. The NPS receptor (NPSR) is expressed in brain nuclei such as the hypothalamus, hippocampus, amygdala and other limbic areas playing a role in motivated behavior [1,2]. Preclinical studies showed that NPS evokes a robust anxiolytic activity when administered centrally. These effects of NPS were assessed in several behavioral tests, in which NPS increased the time spent in the light area in the light–dark test, increased the number of the entries in the central zone of open field (OF) test, although this effect could be secondary to the increased locomotion, and increased the time spent in the open arms in the elevated plus maze (EPM) test [3,4]. A functional polymorphism of the NPSR gene was also associated with anxiety in humans [5]. NPS reduced the conditioned fear response, social avoidance and promoted fear extinction in rodents with mechanisms involving GABAergic pathways in the lateral and basolateral amygdala (LA, BLA) [6,7]. Additional studies showed that NPS injection into the endopiriform nucleus (EPN) reduced freezing and risk assessment behavior, suggesting that an NPS-mediated circuit comprising the EPN and BLA is involved in the processing of contextual fear memories [8]. On the other hand, studies on humans have led to apparently contradictive results. It has been found that a genetic variant of the human NPSR gene

results in a functional boost of the NPSR, increasing the sensitivity to the agonist about tenfold [9]. This variant consists of a A > T polymorphism that leads to a change in one amino acid (Asn > Ile) of the NPSR protein. Noteworthy, individuals with the T allele showed a more conspicuous fear reaction to stimuli paired with painful electric stimulus then the individuals carrying the A allele. Collectively, these data indicate that the NPS system can be linked with a distorted interpretation of fear stimuli and its dysregulation might be associated with panic disorder [10,11]. The amygdala seems to be involved in this effect as demonstrated by fMRI studies that indicated a significant association of the T allele with amygdala responsiveness to fear-paired stimuli [12,13]. To reconciliate the apparently opposite results of the preclinical and clinical studies, it has been suggested that the levels of NPS critically affect the modulation of arousal and anxiety [14]. Previous studies have demonstrated that NPS activates the hypothalamic–pituitary–adrenal (HPA) axis. The microinjection of NPS into the paraventricular nucleus of the hypothalamus (PVN) increased adrenocorticotropic hormone (ACTH), corticosterone plasma levels and elicited a significant reduction of palatable food intake [15,16]. Moreover, NPS treatment increased the release of corticotropin-releasing factor (CRF) and arginine–vasopressin in hypothalamic explants [17]. Thus, the sustained NPS activity of the T genotype in humans could provoke an intense increase in arousal that might cause a correspondent stimulation of the HPA axis, triggering the insurgence of panic disorder. The NPS/NPSR system interacts with numerous others neurotransmitter systems implicated in stress, arousal, sleep–wake cycle and ingestive behavior. Double-labeling confocal microscopy of rat hypothalamus demonstrated that axons containing NPS are adjacent to Hcrt-1/Ox-A-positive neurons, that also express NPSR, suggesting a functional relationship between the two systems. Consistently, the hypocretin-1/orexin-A (Hcrt-1/Ox-A) selective receptor OX1 antagonist SB334867 blocked the exacerbation of drug seeking induced by NPS [18,19]. Moreover, centrally administered NPS evoked c-Fos expression in Hcrt-1/Ox-A neurons of the lateral hypothalamus (LH), the perifornical area (PeF) and in the dorsomedial hypothalamic nucleus (DMH) [20,21]. The corticotropin-releasing factor (CRF) system is also implicated in the effects of NPS on drug seeking and arousal. NPS failed to prime reinstatement of cocaine seeking and to stimulate locomotor activity in CRF receptor 1 (CRF1) knockout mice. Accordingly, the blockage of the CRF1 receptor by the antagonist antalarmin in wild-type mice blocked the NPS-induced reinstatement of cocaine seeking and increased locomotor activity. Interestingly, CRF knockout mice responded to the anxiolytic effect of NPS that were not blocked by antalarmin, indicating that the CRF system does not mediate the role of NPS in anxiety [22]. Evidence of a direct interaction between the NPS and CRF systems was also reported by a study showing that restraint stress increased c-Fos expression in NPS-expressing brain stem neurons co-expressing CRF1 receptor [23]. NPS also interacts with the glutamatergic neurotransmission, as a study demonstrated an activation of glutamatergic neurons in the EPN [8] and glutamatergic neurons in LC and trigeminal nucleus co-express NPS [2]. Recently, a circuit that is activated by stress and involves NPS/NPSR, OX1 receptors, NK1 receptors, mGlu5 receptors and CB1 receptors has been described [24]. The interaction with the CRF, orexin and glutamatergic systems can account for the pro-arousal functions of the NPS system and its involvement in stress modulation. On the other hand, the anxiolytic effects of NPS could be mediated by its action on amygdaloid GABAergic activity [8]. It was also demonstrated that a cluster of central amygdala (CeA) GABAergic neurons projects to the brainstem nuclei that express NPS, and this circuit is involved in the retrieval of fear memories [25]. Similarly, a GABAergic neuronal ensemble in the CeA, identified as protein kinase C δ (PKCδ + neurons) GABAergic positive neurons, was found to be crucial in driving compulsive drinking in a subset of rats and regulating the fear response through their brainstem projections [26,27]. It would be interesting to assess whether these inhibitory neurons also co-express NPS. Anxiety behavior induced by nerve injury was relieved by NPS through the increase of GABA release in the amygdala [28]. However, in another work, the anxiolytic-like effect of NPS was blocked by SHA68, a NPSR antagonist, but not by the GABA-A receptor antagonist

picrotoxin [29], suggesting that the interaction between the NPS and GABAergic system in mediating anxiety could occur by indirect pathways. A recent study links NPS to the activity of the ventrolateral preoptic nucleus (VLPO), an important brain area for non-rapid eye movement (NREM) sleep, through a GABAergic mechanism that could have an important role in the sleep/wake cycle [30]. The anxiolytic effect of NPS has also been associated with oxytocinergic (OXTergic) activity, which recently has shown to correlate positively with the magnitude of alcohol self-administration and anxiety-like behavior [31]. NPSR is considerably expressed in the OXT neurons of the PVN, where NPS activates these neurons. Notably, the blockade of the PVN OXTergic neurons prevented the anxiolytic-like effect of NPS [32]. The physio-pharmacological profile of NPS is somehow paradoxical, being characterized by apparently antipodal features. As described above, the activation of this system modulates the fear response and produces anxiolytic-like effects. However, it also displays a pro-arousal effect, reducing sleep, enhancing alertness, increasing locomotor activity and facilitating spatial memory [3,4,6,33,34]. Mice lacking the expression of the NPS precursor displayed reduced arousal and the impairment of long-term memory [35]. The precursor of the NPS gene is present and highly conserved in all vertebrates, with the only exception represented by fish [36]. This highly conserved nucleotide sequence of the NPS gene indicates that the peptide has been subjected to considerable evolutionary pressure, suggesting a critical functional role. One appealing possibility is that the NPS system has evolved its physiological characteristics to permit the organism to confront dangerous situations in which intense arousal and alertness, together with mitigated anxiety and fear, could be needed as an effective coping strategy. The ambivalent nature of NPS effects can also help to explain findings that associated the two variants (A/T) of NPSR polymorphism with alcohol use disorders (AUD) in clinical cohorts of men and women diagnosed with AUD. Interestingly, AUD was associated with the A allele in females and the T allele in males. As mentioned before, the T allele leads to a higher activity of NPSR [37]. Previous studies have shown that individuals carrying this allele have poor impulse control [38], which is a risk factor for drug abuse [39]. On the other hand, it is well known that affective disorders, such as anxiety and depression, are risk factors for alcohol abuse and females carrying the A allele were more vulnerable to anxiety disorders [37,40]. Therefore, a possible interpretation is that the higher alcohol consumption shown by A allele female carriers is linked to their lower NPSR activity with consequent higher levels of anxiety [37]. The pharmacological targeting of the NPS/NPSR system could lead to the development of novel drugs useful to treat various disorders including anxiety and drug abuse.

Stress plays a major role in drug abuse and, despite the well-characterized mechanism by which stress promotes drug abuse, there is no approved drug that targets the stress system. Thanks to its dual effect on stress, the NPS system is a potential target to develop drugs targeting the stress system to treat drug abuse. For this reason, this review will describe the preclinical data supporting the role for the NPS/NPSR system in addiction-related behaviors and the pharmacological approaches that could lead to future therapeutical treatments.

2. Neurobiology of NPS

In the rat, NPS precursor mRNA is localized in a few discrete brain stem nuclei, showing the highest level of expression within the peri-LC, the lateral parabrachial nucleus (lPBN), and the principal trigeminal sensory nucleus; sparse expression has also been identified in the DMH and the amygdala [2,3]. Similar findings were reported in the mouse, although with some significant differences. Indeed, NPS expression in the mouse brain is even more restricted, being found only in the peri-LC and the lPBN (Kölliker–Fuse (KF)) nucleus of the lateral parabrachial area [1,41]. Noteworthy, NPS is often expressed together with other neurotransmitters and neuropeptides, indicating that NPS could be released in conjunction with them on the neural targets of the NPS-synthetizing neurons. Most of the peri-LC NPS-positive neurons express glutamatergic, but not GABAergic markers, suggesting that they co-release glutamate as neurotransmitter. Intriguingly, peri-LC NPS-expressing cells do not colocalize with the catecholaminergic marker tyrosine hydroxylase,

indicating that these neurons represent a distinguishable non-noradrenergic (NAergic) cluster of cells in the peri-LC. Additionally, only few lateral peri-LC NPS-positive neurons express cholinergic markers, and they do not colocalize with CRF. Conversely, in the lPBN, many NPS-expressing neurons are also positive for CRF and galanin. Lastly, many NPS-positive neurons of the rat principal trigeminal sensory nucleus are believed to be of glutamatergic nature [1–3]. Recently, a study revealed the presence of NPS mRNA-expressing neurons in the human brainstem as well. Like in rodents, NPS is present in a cluster of neurons localized in the lPBN (spanning from the medial to the lateral subregions, including the KF nucleus); however, only few NPS-positive neurons were found in the human LC area, suggesting the presence of marked regional differences in NPS expression sites between the rodent and human brain [42]. In contrast to the focal localization of NPS, the pattern of expression of NPSR is much more dispersed within the rodent brain, being found in cortical regions, thalamic nuclei, the amygdala complex, hypothalamic regions and in the midbrain [2,41,43]. A schematic representation of NPS precursor and NPSR1 transcript distribution in the rat brain is provided in Figure 1. By acting on neurons and terminals expressing its receptor, NPS can alter the release of several neurotransmitters and thereby exert a direct or indirect modulation of the function of a very wide range of targets within the brain. For instance, NPS regulates amygdaloidal functions acting through different parallel pathways [7,8,44]. In the mouse, it enhances the glutamatergic tone to medial intercalated (mITC) GABAergic cells, presumably by acting on presynaptic NPSRs expressed in LA principal neurons. This phenomenon consequently increases the feedforward inhibition onto neurons in the CeA, which represents the main output nucleus of the amygdaloid complex [7]. In addition, NPS increases the feedforward inhibition toward BLA principal neurons, through a putative mechanism of action involving a direct excitation of principal neurons located in the endopiriform nucleus (EPN) [8]. A substantial difference has been found in the rat brain, as in control conditions, NPS failed to produce any significant effect in the monosynaptic glutamatergic release and feedforward GABAergic inhibition evoked into the CeA by the electrical stimulation of the BLA and entorhinal cortex, respectively [45]. Intriguingly, NPS was effective in an arthritis pain model, where it increased the mITC-mediated feedforward inhibition and decreased the release of glutamate into the CeA, indicating that specific conditions, such as the development of neuropathic pain, can produce plastic changes in the NPS–NPSR system [45]. Recent findings support the notion that NPS can also modulate neurotransmitter release in other brain regions. In the ventral hippocampus, NPS decreased basal glutamatergic neurotransmission and impaired long-term potentiation at the level of the CA3-CA1 synapses [46,47]. Furthermore, a recent study demonstrated that NPS indirectly inhibits the sleep-promoting galanin-expressing neurons in the VLPO by enhancing their GABAergic inputs, presumably through a direct depolarization of local galanin-negative GABAergic neurons [30]. The central administration of NPS increased cFos expression in the tuberomammillary nucleus wakefulness-promoting histaminergic neurons [21]. Direct and indirect evidence indicates that NPS interacts with monoaminergic signaling. NPSR is expressed in the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNC) [2,41,43,48], suggesting that they could play a role in regulating the activity of the mesocorticolimbic dopamine (DA) pathway. Accordingly, neurochemical studies demonstrated that intra-VTA NPS microinfusion stimulates DA release in the nucleus accumbens (NAc) [49]. Similarly, the central injection of NPS increased the accumulation of DA and its metabolite 3,4-Dihydroxyphenylacetic acid in the medial prefrontal cortex (mPFC) in vivo [50]. However, only small amounts of the evoked DA release were detected ex vivo in cortical synaptosomes [51], indicating that synaptic terminals are presumably not the site of action for the NPS-dependent regulation of cortical DA release. Other information on the relationship between NPS and DA function arose from studies demonstrating that NPS stimulates the activity of SNC neurons, as suggested by an increased cFos immunoreactivity following NPS treatment, and enhanced release of local DA in the SNC following central NPS administration [48,52]. Concordantly, NPS successfully re-

versed the Parkinsonian-like motor deficits produced by the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) in mice and rats, and counteracted the decreased local DA release in the SNC produced by 6-OHDA treatment [48,53]. Although there is substantial agreement on the stimulatory properties of NPS on DAergic function, conflicting results have been reported regarding its role in the modulation of reward-related phenomena. Indeed, while several investigations observed no effects of NPS in producing conditioned place preference (CPP) or aversion [54–56], other work found a bidirectional effect of the peptide, where a lower dose (0.1 nmol) of NPS produced aversion, while a higher dose (1 nmol) exhibited rewarding-like properties [57]. This latter study also reported that rats moderately self-administer NPS intracranially in a cue-assisted operant paradigm [57]. However, given the known pro-cognitive and pro-attentive properties of NPS [3,58], this set of data could be interpreted as facilitation sign tracking induced by NPS [56,57] (see below for a more comprehensive discussion). Few studies analyzed the effects of NPS on regulating serotonin (5-hydroxytryptamine, 5-HT) release. NPS perfusion inhibited the evoked release of 5-HT in cortical and amygdaloidal synaptosome preparations [51,59]. Conversely, Si and colleagues [50] found that the central injection of NPS did not change mPFC concentrations of 5-HT and its metabolite 5-HIAA detected by in vivo microdialysis in rats. These discrepancies could depend on marked methodological differences given the moderate expression of NPSR in the 5-HTergic Raphe nucleus [2], which are not preserved in synaptosome preparations. Additional studies are needed to further establish how NPS regulates the 5-HTergic system and the potential functional readouts of these modulations. A number of reports, analyzing the action of NPS in memory formation and consolidation, have identified a functional interplay between NPS and the NAergic system. Recently, Okamura and colleagues [60] demonstrated that pretreatment with the beta-adrenergic receptor antagonist propranolol was effective in blocking the NPS-dependent enhancement of inhibitory avoidance memory consolidation. Similarly, propranolol abolished the pro-mnemonic effects of NPS on the novel object recognition test both when administered intracerebroventricularly (i.c.v.) or into the BLA [61], suggesting that the NPS-dependent memory enhancement could partially depend on an increased amygdaloidal NAergic tone. However, it is important to emphasize that NPSR is not expressed in the LC, the main brain source of NA, indicating that NPS does not directly excite LC NAergic cells. Therefore, the NPS-dependent modulation of NAergic function likely depends on NPSRs located in NAergic synaptic terminals or in other NAergic neuronal sources. Alternatively, NPS could interact with the NAergic system indirectly by modulating the activity of NPSR-expressing brain regions that, in turn, project to the LC. Supporting the notion that the NPSR expressed in NAergic terminals could play a role in the effects of the peptide, NPS inhibited the evoked release of NA in ex vivo cortical synaptosomes [51]. Finally, central infusions of NPS significantly enhanced the plasma concentration of adrenaline [62], indicating that adrenergic receptors located outside the brain could contribute to some extent to the effects of the peptide. Noteworthy, the biological activity of NPS is believed to be partially mediated by its interaction with other neuropeptidergic systems. NPSRs are expressed in Hcrt-1/Ox-A neurons [18,20,21], and NPS-positive axons are localized in the proximity of these neurons [18]. Accordingly, central NPS administration enhanced cFos expression in Hcrt-1/Ox-A-positive cells in the LH, in the DMH and in the PeF [18,20,21,63]. Additional indirect information on the interplay between NPS and the Hcrt-1/Ox-A system arises from behavioral experiments demonstrating that the self-administration of NPS is abolished in the presence of the Hcrt-1/Ox-A receptor (Ox1) selective antagonist SB-334867 [57]. Similarly, SB-334867 counteracted the exacerbation of cue-induced restatement of alcohol and cocaine seeking in rats produced by intra-LH microinfusions of NPS [19,20], an effect mediated by Hcrt-1/Ox-A receptors localized in the PVN and the bed nucleus of the stria terminalis (BNST), but not by those located in the LC and VTA [18]. On the other hand, a dense network of Hcrt-1/Ox-A fibers has been found near to NPS-expressing neurons in the periLC and, to a lesser extent, in the KF nucleus in mice. Furthermore, these data are corroborated by the fact that NPS-positive neurons in the periLC express Hcrt-2/Ox-B

receptors [1]. Although additional work is needed to better establish, from a functional perspective, the existence of an orexinergic modulation of NPS release, altogether these findings suggest that the interaction between NPS and hypocretin/orexin systems could be of a bidirectional nature. NPS promotes the activation of the hypothalamic–pituitary–adrenal (HPA) axis, as indicated by the increased plasma concentrations of ACTH and corticosterone following intra-PVN NPS administration. This effect is believed to be indirect, since NPS did not promote ACTH release from anterior pituitary segments, but it did stimulate the release of CRF and vasopressin (but not neuropeptide Y) in hypothalamic explants [17]. Thus, it has been proposed that the activation of the HPA axis by NPS is mediated by the release of CRF and/or vasopressin from the PVN [17]. Intriguingly, it has been shown that the interaction between CRF and NPS systems is bidirectional. Indeed, CRF-positive fibers are localized in close proximity to periLC NPS-expressing neurons, and CRF perfusion directly depolarizes and increases the neuronal activity of periLC NPSergic cells in a CRF1 receptor-dependent manner [23]. Finally, a functional link between NPS and the OXT system within the PVN has also been reported. In fact, the NPSR is expressed in PVN neurons expressing OXT, and NPS perfusion activated OXT neurons in brain slices and induced the local release of OXT in vivo [32].

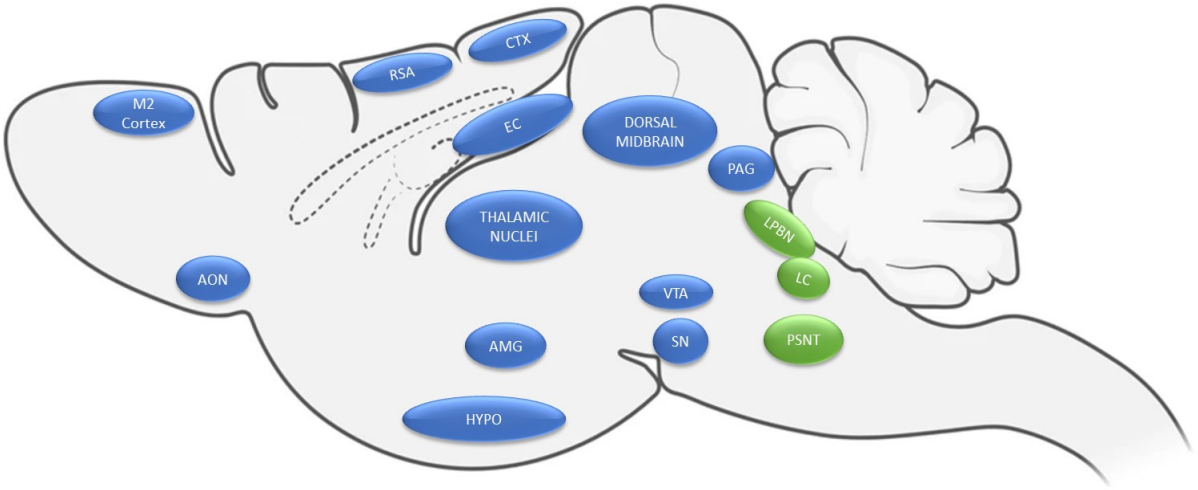

Figure 1. Schematic representation of NPS precursor and NPSR1 transcript distribution in the rat brain. Green circles (PSNT: principal sensory trigeminal nucleus, LPBN: lateral parabrachial nucleus and LC: peri-locus coeruleus) represent areas of NPS precursor mRNA expression, while blue circles (M2cortex: secondary motor cortex, AON: anterior olfactory nucleus, RSA: agranular retrosplenial cortex, CTX: cortex, EC: entorhinal cortex, thalamic nuclei, AMG: amygdala, HYPO: hypothalamus, dorsal midbrain; VTA: ventral tegmental area; SNC: substantia nigra, PAG: periaqueductal gray area) depict brain regions with NPSR1 transcript expression.

The neurobiological interactions of NPS are summarized in Table 1. A schematic representation of the relationship of NPS/NPSR with other neurotransmitter systems is reported in Figure 2.

Table 1. Neurobiological effects of NPS.

Brain System	Animal	Sex	Route of Administration	Experimental Procedure	Effect	Ref.
DA system	Wistar rats	Male	Intra-VTA injection	In vivo microdyalisis	↑ DA release in the Nac	[55]
	Sprague Dawley rats	Male	Central injection (i.c.v.)	In vivo microdyalisis	↑ DA release in the mPFC	[56]
	Swiss mice	N/A	Bath perfusion	Ex vivo synaptosomes	Little ↑ effects on evoked DA release in cortical synaptosomes	[57]
	Wistar rats/Swiss mice	Male	Central injection (i.c.v.)	cFOS immunodetection	↑ cFOS expression in SNC DA neurons	[54,58]
	Wistar rats	Male	Central injection (i.c.v.)	In vivo microdyalisis	↑ DA local release in the SNC of 6-OHDA treated rats	[54]
5-HT system	Swiss mice	N/A	Bath perfusion	Ex vivo synaptosomes	↓ evoked 5-HT release in cortical and amygdaloidal synaptosomes	[57]
	Sprague Dawley rats	Male	Central injection (i.c.v.)	In vivo microdyalisis	No effects on 5-HT in the mPFC	[56]
NA system	Swiss mice	N/A	Bath perfusion	Ex vivo synaptosomes	Little effects on evoked DA release in cortical synaptosomes	[57]
Limbic system	C57BL/6J mice	N/A	Bath perfusion	Ex vivo patch-clamp recordings	↑ glutamate release on mITC neurons and ↑ feedforward inhibition on CeA neurons	[15]
	GAD67-GFP mice	N/A	Bath perfusion	Ex vivo patch-clamp recordings	↑ feedforward inhibition on BLA principal neurons and ↑ excitation of EPN principal neurons	[16]
	Sprague Dawley rats	Male	Bath perfusion	Ex vivo patch-clamp recordings	No effects on evoked glutamate release and feedforward inhibition the CeA of control animals ↓ evoked glutamate release and ↑ feedforward inhibition in the CeA in a neuropathic pain model	[51]
	C57BL/6N mice	Male	Bath perfusion/intranasal	Ex vivo field potential recordings	↓ Paired pulse ratio and impaired LTP in CA3-CA1 synapses of the ventral hippocampus	[52,53]

Table 1. *Cont.*

Brain System	Animal	Sex	Route of Administration	Experimental Procedure	Effect	Ref.
Hypothalamus and HPA axis	Long Evans, Sprague Dawley, Wistar rats	Male	Central injection (i.c.v.)	cFOS immunodetection	↑ cFOS expression in LH, DMH and PeF Hcrt-1/Ox-A neurons	[25,27,28]
	Sprague Dawley rats	Male	Central injection (i.c.v.)	cFOS immunodetection	↑ cFOS expression in TMN histaminergic neurons	[28]
	C57BL/6J mice	Male	Bath perfusion	Ex vivo patch-clamp recordings	↑ neuronal activity of non-galanin VLPO neurons ↓ neuronal activity of galanin VLPO neurons by ↑ GABA release	[37]
	Wistar rats	Male	Bath perfusion	Ex vivo GCamp6 calcium signaling	↑ OXT neuronal activity	[39]
			Central injection (i.c.v.)	In vivo microdyalisis	↑ OXT local release	
	Wistar rats	Male	Central injection (i.c.v.)	Plasma concentration	↑ concentration ACTH and corticosterone	[24]
			Bath perfusion	Hypothalamic explants	↑ CRF and vasopressin release from anterior pituitary segments, no effects on ACTH and NPY	

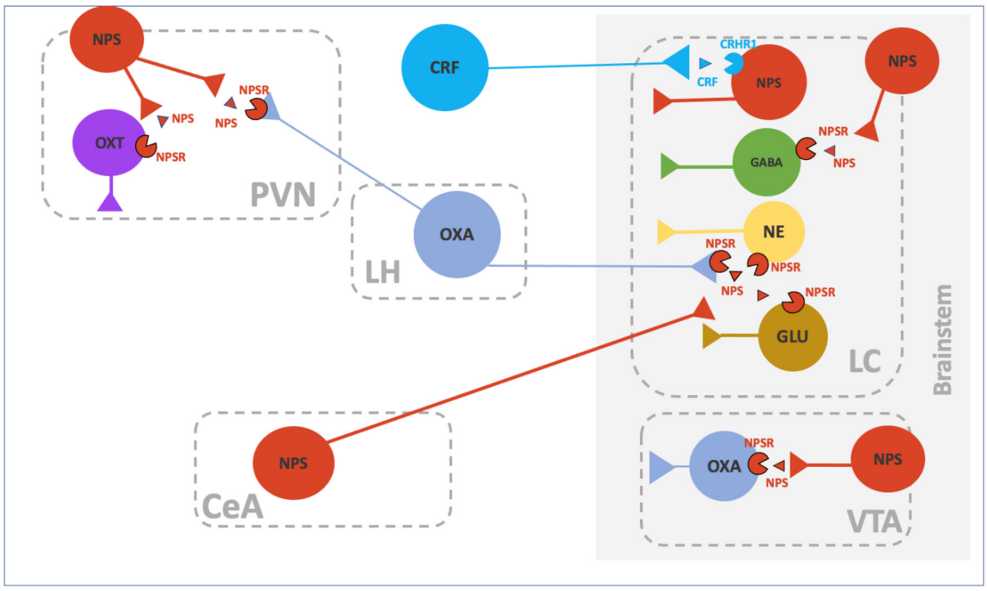

Figure 2. Schematic representation of the interaction of the NPS/NPSR system with other neurotransmitter systems. Ventral tegmental area (VTA); hypothalamic para ventricular nucleus (PVN): central amygdala (CeA); lateral hypothalamus (LH); locus coeruleus (LC).

3. Role of NPS in AUD

AUD is a chronic relapsing disorder often associated with anxiety and maladaptive impulsivity [64–66]. The pro-arousal/anxiolytic profile of NPS suggested a potential role of this neuropeptide in the neurobiology of AUD [3]. Indeed, both variants of the NPSR gene have been associated with vulnerability to develop AUD [9]. In addition, compelling evidence of the involvement of the NPS/NPSR system in alcohol-related behavior has been provided by preclinical studies.

3.1. NPS on Alcohol Drinking and Operant Self-Administration

Preclinical experiments in which the exogenous administration of NPS was tested on alcohol self-administration in the rat consistently reported a selective effect on rat lines genetically selected for their high alcohol preference. NPS reduced alcohol intake in a two-bottle choice (TBC) paradigm in Indiana alcohol-preferring (P) rats and operant self-administration on Marchigian-Sardinian alcohol-preferring (msP) rats, while it was not effective in non-preferring control lines [19,67,68]. A possible interpretation of this effect could be that the observed reduction in alcohol consumption was due to the NPS anorectic effects [15,69,70]. Indeed, alcohol represents an important source of calories for P and msP rats [71]. However, the reduction in alcohol self-administration was selective for alcohol-preferring lines [19,67,68] at doses that failed to reduce food intake [67]. In addition, the anorectic effect of NPS was observed in rat lines not selected for high alcohol preference [15,69,70]. Therefore, if this was the case, a reduction of self-administration should have been observed in non-preferring lines as well. A more compelling interpretation, supported by experimental evidence, is that the reduction in alcohol self-administration observed in alcohol-preferring rats [19,67,68] could be derived from the well-known anxiolytic properties of NPS [3,4,7,33,46,72–75]. Both msP and P rats express an innate anxious phenotype, and the anxiolytic effect of alcohol is a major driving force to consumption in these rats [71,76–78]. It is therefore likely that NPS decreased alcohol self-administration selectively in preferring lines by reducing the reinforcing value of the drug via its anxiolytic properties. In line with this interpretation, Enquist and colleagues demonstrated that NPS reduces alcohol consumption in a mouse TBC test, and that NPS anxiolytic and anti-depressive properties were enhanced in alcohol-exposed mice [55]. Psychological traits of withdrawal syndrome are key players in the development of AUD, as they are correlated with relapse risk and compulsive consumption [79,80]. Alcohol-intoxicated Wistar rats, expressing both physical and psychological withdrawal-like syndrome, showed increased NPSR gene expression in several nuclei of the amygdala and hypothalamic areas compared to non-intoxicated controls [81]. NPS alleviated withdrawal-induced anxiety in a defensive burying test [81]. This suggests that increased expression of NPS in stress-related areas could be an adaptive response to counteract anxiety symptoms associated with withdrawal. Interestingly, P and msP rats constitutively express psychological aspects typical of withdrawal syndrome, such as depression, anxiety, and heightened stress vulnerability [71,76,77]. Innate neurobiological and pharmacological response traits characteristic of a post-dependent state were reported in msP [82–85]. In line with post-dependent Wistars, the effect of NPS on alcohol consumption in P and msP rats was associated with the anxiolytic properties of this neuropeptide [19,67,68]. Furthermore, mice exposed to chronic alcohol drinking revealed increased anxiety and depression that was reverted by NPS pretreatment. The anxiolytic/antidepressant effects of NPS in these mice were mediated by the BLA, where NPS increased the amplitude of evoked GABA-mediated IPSCs [55]. It is also interesting to note that rats selectively bred for high anxiety (HAB) have a higher NPSR activity [86] akin to the human Ile107 risk variant [9,37] and that exogenous NPS treatment showed an anxiolytic effect in HAB rats, but not in their low-anxiety breeding counterpart [86]. Altogether, these data support the view that NPS reduced alcohol self-administration through its anxiolytic properties. In addition, data suggest the intriguing hypothesis that msP and P rats might have an increased NPSR expression and/or activity, making them more responsive to the anxiolytic effects of NPS, which would explain

why NPS reduced alcohol self-administration in preferring rats, but not in non-preferring rats [19,67,68]. Though highly speculative at the present time, it is possible that the gain of function associated with the Ile107 NPSR variant, within specific brain areas, could co-segregate with traits predisposing to high anxiety and alcoholism vulnerability and it might represent a trait protecting against these predispositions. Somehow in contrast with the lack of effect of NPS on alcohol self-administration in Wistar rats [19], the inhibition of NPSR transmission by the antagonist NCGC00185684 decreased alcohol self-administration in this line [87]. Upon NPS stimulation, NPSR exerts its action by ERK phosphorylation, intra-cellular Ca2+ mobilization and increased cAMP levels, with a three-to-four times higher potency on the ERK pathway [9,87]. NCGC00185684 blocked in vitro NPS-induced ERK phosphorylation studies and in vivo alcohol-induced ERK phosphorylation in the CeA and in the shell region of NAc, therefore this was proposed as the mechanism of action by which NCGC00185684 decreased alcohol self-administration [87]. This encourages a possible interpretation for the lack of an exogenous NPS effect on alcohol self-administration in Wistars. Indeed, the ceiling ERK phosphorylation induced by alcohol might have covered NPS effects, leaving self-administration level unaltered, whereas NCGC00185684, by preventing alcohol-induced ERK phosphorylation, decreased alcohol self-administration. As to why NPS agonism decreased self-administration selectively in preferring lines [19,67,68], whereas NPS antagonism decreased self-administration in heterogeneous Wistar rats [87], remains unclear. The CeA has been proposed as a site of action of NCGC00185684 in non-dependent Wistar rats [87], whereas neurobiological adaptations of the NPS/NPSR system observed in post-dependent Wistar rats included increased expression of NPSR in the BLA, PaV and LH, but not in the CeA [81]. In addition, the anxiolytic properties of NPS mediating the reduction of alcohol consumption in mice have been demonstrated to ground on the BLA [55]. Therefore, it would be worth testing how alcohol-preferring lines respond to NCGC00185684, and test whether NCGC00185684 in Wistar and NPS in msP and P rats act through different neurocircuitries.

3.2. NPS on Reinstatement of Alcohol Seeking

Exposure to the environmental stimuli associated with alcohol experience and their interaction with stressful events is recognized as a major factor augmenting relapse risks [88,89]. We demonstrated that in outbred Wistar rats, NPS can prime the reinstatement of alcohol seeking [68] and exert a facilitatory role on cued reinstatement through interrogation of the hypothalamic Hcrt-1/Ox-A system [18,19]. The downstream activation of Hcrt-1/Ox-A clearly indicates that the relapse facilitatory action of NPS is mediated by the stress/pro-arousal component of this neuropeptide's psychopharmacology [3]. Because Hcrt-1/Ox-A, the downstream modulator of NPS facilitatory role on relapse [18,19], was reported to mediate alcohol self-administration as well [90–92], the co-existence of NPS's facilitatory action on relapse with the lack of effect on self-administration in Wistar may appear controversial. However, while the site of action by which hypocretin/orexin modulates alcohol self-administration is the VTA [93], this area plays no role in the Hcrt-1/Ox-A-mediated facilitation of relapse induced by NPS [18]. In fact, in a series of histological and pharmacological studies, we explored the neurocircuitry by which NPS facilitates cued reinstatement. We demonstrated that site-specific pretreatment with the selective OX1 receptor antagonist SB334867 blocked NPS-induced facilitation of relapse-like behavior when SB334867 was delivered within the BNST and PVN, but not when the pretreatment was given in the VTA and LC [18]. Our findings were further corroborated by histological analyses demonstrating that: (i) NPS fibers run in close opposition to Hcrt-1/Ox-A neurons in the LH [18]; (ii) LH Hcrt-1/Ox-A neurons express NPSR [18]; (iii) NPS induces cFos activation in hypothalamic Hcrt-1/Ox-A neurons [20]; (iv) retro-tracing marker injected within the PVN and BNST (i.e., the sites where SB334867 blocked NPS) co-labels with LH Hcrt-1/Ox-A neurons [18]. Altogether, our findings demonstrate that NPS facilitates relapse-like behavior through LH Hcrt-1/Ox-A neurons, which, in turn, interrogate the extended amygdala via BNST and the HPA axis via PVN (see also [17]). In summary,

alcohol self-administration and relapse studies demonstrated that, consistently with its dual pro-arousal/anxiolytic profile, NPS exerts a double action on alcohol seeking behavior. The stress-related component of NPS psychopharmacology promotes relapse via the interrogation of LH Hcrt-1/Ox-A neurons and, in turn, by Hcrt-1/Ox-A-responsive BNST and PVN neurons. On the contrary, the action on alcohol self-administration seems to depend on the anxiolytic action of NPS that could be mediated by the BLA.

4. NPS and Reward

As discussed above, NPS did not affect drug self-administration in outbred rodent lines when self-administration was maintained by a positive reinforcement mechanism. Indeed, NPS decreased alcohol self-administration in P rats [67], msP rats [68], and mice seeking alcohol to alleviate their anxious state [55]. However, when tested on outbred lines in a non-dependent state, NPS failed to affect cocaine [20], alcohol [19,67] and nicotine (Cannella et al., unpublished observation) self-administration. It might still be interesting to assess whether NPS plays a role when excessive drinking and alcohol reinforcement is mediated by nicotine administration [94,95]. We and others reported that NPS induced neither place preference nor aversion, in a place conditioning paradigm [54–56]. Moreover, Li and colleagues reported that NPS blocked the acquisition of morphine CPP [54]. Altogether, these data indicate that NPS is devoid of rewarding properties per se. However, in favor of a possible rewarding effect of NPS, it was reported that NPS can increase DA release in the mPFC and NAc [49,50]. In addition, Cao and coworkers demonstrated that rats can actively self-administer NPS, and NPS self-administration was reduced by the selective D1-like receptor antagonist SCH 23390, and by the selective OX1 antagonist SB-334867 [57]. In the same work, and in contrast with others [54–56], it was reported that 1 nmol of NPS induced CPP and 0.1 nmol induced aversion [57]. Thus, the work from Cao et al. would indicate a rewarding effect of NPS in contrast with the rest of the literature. However, an alternative explanation could be proposed to reconcile this apparently contrasting result on NPS's reinforcing effects. In the work of Cao and colleagues, intraventricular self-infusions were paired with discrete cue light. Notably, this experiment included a control group that self-administered saline in the same condition as the NPS-treated group (self-administered about 20 infusions of saline/session), suggesting that rats actively pressed to visualize the discrete cue (unfortunately an inactive lever control was not included in the experimental design). Though significantly higher, the NPS-reinforced groups showed a level of self-administration only 0.5 times higher than the saline control group [57]. Therefore, an alternative interpretation could be that the observed NPS self-infusion behavior was secondary to NPS's ability to facilitate sign tracking. This interpretation would be consistent with the pro-cognitive and vigilance-enhancing properties of NPS [7,8].

5. Role of NPS in Cocaine Seeking

As for alcohol-related behaviors, the role of the NPS system in the regulation of cocaine properties is under extensive scrutiny. Since the publication of the first paper in 2009 [22], more publications exploring the effects of either activation or inhibition of NPS receptors on cocaine-related behaviors have been published. Here, we report an up-to-date overview of the current literature on the role of the NPS system in modulating cocaine intake and the reinstatement of cocaine seeking.

5.1. Role of the NPS System in Cocaine-Induced Reward

To date, few studies have explored the effects of exogenous NPS on cocaine self-administration. Our laboratory reported that i.c.v. infusion of NPS (1.0, and 2.0 nmol) failed to reduce cocaine self-administration [20]. Moreover, in the same study, we show that intraperitoneal injection (i.p.) of the selective NPS receptor antagonist SHA 68 (30.0, and 60 mg/kg) [96] did not modify cocaine intake under the same schedule of reinforcement [20]. In another set of experiments [97], we obtained similar results using another selective NPS receptor antagonist NPSR-QA1 [98]. This compound (15 and 30 mg/kg, i.p.) blunted

food self-administration in rats, without affecting cocaine intake [97]. However, it was recently shown that the NPSR antagonist RTI-118 [99] was able to reduce cocaine and food self-administration in rats [100]. Interestingly, RTI-118 was able to selectively reduce cocaine self-administration at the lower doses (10–20 mg/kg, i.p.), without affecting food self-administration, suggesting a selective effect for cocaine at this range of doses. The higher solubility of RTI-118 in water at physiological pH compared to SHA 68 could lead to better bioavailability, thereby explaining the discrepancies between the two studies [96]. More recently, another study found that RTI-118 (3.0, 10.0 and 32.0 mg/kg, i.p.) produced a dose-dependent blockade of the cocaine-induced facilitation of intracranial self-stimulation (ICSS) in rats at a range of doses that induced little or no effect on ICSS when administered alone [101]. Further studies are needed to better clarify the different effects of NPSR antagonists on cocaine self-administration and to elucidate the role of the NPS/NPSR system in cocaine reinforcement.

5.2. Role of the NPS System in the Reinstatement of Cocaine Seeking

More straightforward is the effect of NPS system in the regulation of cocaine seeking and relapse [20,22,96,97]. In 2009, Paneda and colleagues demonstrated that i.c.v. administration of NPS (0.45 nmol) was able to facilitate the reinstatement of cocaine-seeking behavior in mice [22]. This effect was dependent on the manipulation of the CRF system, as it was prevented by pretreatment with the CRF1 receptor antagonist antalarmin (30 mg/kg, i.p.) [22]. Accordingly, in CRF receptor knockout ((CRF1 (-/-)) mice, NPS (0.45 nmol) failed to reinstate extinguished lever pressing for cocaine and to stimulate locomotor activity [22]. These results indicate that the NPS-induced reinstatement of cocaine-seeking was mediated by stress-like effects. Over the following years, these results were replicated in rats [20]. I.c.v. and intra-VTA administration of NPS facilitated the reinstatement of cocaine-induced CPP in mice [101], corroborating the initial findings on cocaine-seeking behavior [20,22]. In addition, the NPSR antagonist SHA 68 (50 mg/kg, i.p.) blocked the stress-induced reinstatement of extinguished cocaine CPP [101]. In 2011, our laboratory demonstrated that i.c.v. or intra-LH infusions of NPS (1.0 and 2.0 nmol) promoted cocaine-seeking behavior in a discriminative cue-induced reinstatement model, whereas a smaller, but significant effect was detected when the peptide was delivered into the PeF, but not into the DMH or the CeA. Accordingly, the administration of SHA 68 (30.0 and 60.0 mg/kg) decreased lever pressing induced by environmental stimuli previously associated with cocaine availability [20]. Similarly, in another study, we reported that the other two NPS receptor antagonists, NPSR-QA1 (15.0 and 30.0 mg/kg, i.p.) and (D-Cys(tBut)5)NPS (10.0, 30.0 and 60.0 nmol) were able to reduce the cue-induced reinstatement of cocaine seeking, with a stronger effect for (D-Cys(tBut)5)NPS (10.0 and 30.0 nmol) when it was specifically microinjected in the PeF and the LH, but not in the CeA [97]. Likewise, Schmoutz and colleagues [96] reported that the NPS receptor antagonist RTI-118 (1.0, 5.0, 10.0 and 20.0 mg/kg, i.p.) decreased the primed-, yohimbine- and cued reinstatement of cocaine seeking. Overall, these studies indicate that NPS receptor antagonism may be a useful strategy to prevent relapse to cocaine, whereas the activation of NPS receptors through NPS infusion promotes cue-induced relapse to cocaine-seeking behavior. This latter effect can be explained by the general peptide's ability to increase goal-oriented behaviors [3]. However, in a paradigm of discriminative cue-induced reinstatement (cocaine paired with a tone vs. saline paired with house light), we demonstrated that the i.c.v. administration of NPS did not affect lever responding for cues previously associated with saline delivery [20], thus strengthening the idea that the exacerbation of reinstatement induced by NPS was not secondary to its action on locomotor activity or arousal. Together with the CRF system, the hypothalamic Hcrt-1/Ox-A system is implicated in the regulation of NPS's effect on cocaine relapse as well. Indeed, we reported that NPS increased cFos expression in the hypothalamic Hcrt1/Ox-A cells [19,20], and intra-LH injections of NPS (0.5 nmol) markedly increased the cue-induced reinstatement of cocaine seeking [20], an effect that was abolished by pretreatment with the selective

Ox-1 receptor antagonist SB-334867 (10mg/kg, i.p.). In agreement with these data, more recently, Chou and collaborators [102] demonstrated that i.c.v. infusions of NPS (1 nmol) augmented cFos-containing orexin neurons in the LH and the Ox-A level in the VTA [102]. This latter effect was prevented by the NPS receptor antagonist SHA 68 (50 mg/kg, i.p.), suggesting that NPS activates an orexinergic neurocircuitry involving the hypothalamus and the VTA. Noteworthy, the NPS-induced reinstatement of cocaine CPP was suppressed by systemic (10 mg/kg, i.p.) and intra-VTA (15 nmol) injection of SB-334867, suggesting a crucial role of the orexinergic signaling in the VTA in mediating such effect [102]. Overall, these results indicated that NPS is released under stressful conditions and activates LH orexinergic neurons to facilitate orexin release in the VTA, subsequently leading to the reinstatement of cocaine CPP through Hcrt1/Ox-A receptor signaling. However, considering that it was demonstrated that restraint stress can activate both the Hcrt1/Ox-A and the CRF systems [103], and CRF signaling is involved in modulating NPS's facilitation of cocaine-seeking [22], a primary contribution of the CRF system cannot be ruled out. The data described so far demonstrated that the NPS system modulates cocaine relapse through the activation of both the Hcrt1/Ox-A and the CRF systems [20,22,102,103], but the precise mechanisms of action are not clear yet. Several reports have shown that there may be direct interactions between CRF and Hcrt1/Ox-A systems, especially in the VTA [104]. Indeed, it was demonstrated that CRF-immunopositive cells are in direct contact with Hcrt1/Ox-A neurons in the LH, and that several Hcrt1/Ox-A cells expressed CRF receptors [105]. In addition, Sakamoto et al. (2004) reported that Hcrt-1/Ox-A activates approximately 96% and 45% of CRF-containing neurons in the PVN and the CeA, respectively [106]. This, in turn, increases CRF and vasopressin expression in the PVN and activates the HPA axis [107]. It is well established that both Hcrt1/Ox-A and CRF increase VTA DAergic neuron activity and potentiate NMDAR-mediated synaptic transmission in these cells [104,108–110]. When microinjected in the VTA, both neuropeptides promote DA release in the NAc and PFC [104,111–113], and they induce reinstatement to cocaine seeking in rats [111,114]. However, they may promote reinstatement to cocaine seeking by independent mechanisms. Indeed, it was shown that the effect of intra-VTA administration of Hcrt1/Ox-A can be abolished by Hcrt1/Ox-A antagonist SB-408124, but not by CRF receptor antagonism [114], and the Ox1 receptor antagonist SB-408124 did not block CRF-dependent foot-shock-induced reinstatement [114]. Moreover, the same work demonstrated that the reinstatement of cocaine seeking by intra-VTA infusion of CRF is completely glutamate-dependent, whereas reinstatement induced by intra-VTA Hcrt1/Ox-A infusion is not, suggesting a separate mechanism of action within this circuitry [114]. Taken together, these data indicate that these two peptidergic systems can work in parallel through distinct mechanisms and that NPS could modulate the two systems independently. A deeper understanding of these complex interactions would provide useful tools to find more effective therapies to treat cue- and stress-induced relapse in abstinent cocaine-dependent individuals.

A summary of the main preclinical findings reviewed above is provided in Table 2.

Altogether, the preclinical and genetic data indicate that NPS is likely to play a role in drug abuse. This makes the NPS a potential target to treat drug use disorders. Yet the panel of molecules developed to target NPSR is small, and to the best of our knowledge, none of them have entered clinical trials. However, it is interesting to observe that the orexin/hypocretin system is a major downstream target by which NPS exacerbates the reinstatement of drug seeking, and a certain number of trials are testing orexin antagonists in patients diagnosed AUD, opioid and cocaine use disorders (Table 3).

Table 2. Main preclinical findings on probing the NPS/NPSR system in alcohol and cocaine seeking.

Drug of Abuse	Strain	Drug	Route	Paradigm	Result	Ref.
Alcohol	P rats	NPS	i.c.v.	TBC	Reduced alcohol intake	[66]
Alcohol	Wistar rats	NPS	i.c.v. and LH	Cued reinstatement	Exacerbated seeking	[18]
Alcohol	Wistar rats	NPS	i.c.v.	Post-dependent alcohol withdrawal	Alleviated symptoms	[80]
Alcohol	Mice	NPS	i.c.v.	TBC	Reduced alcohol intake	[54]
Alcohol	Wistar rats	NPSR antagonist NCGC00185684	i.p.	Fixed ratio self-administration	Decreased self-administration	[86]
Alcohol	Wistar rats	NPS	LH	Cued reinstatement	Exacerbated seeking	[17]
Alcohol	Wistar and msP rats	NPS	i.c.v.	Reinstatement of seeking and self-administration	Reinstated seeking in Wistars and reduced self-administration in msP	[67]
Cocaine	Wild-type and CRF1 KO mice	NPS	i.c.v.	Reinstatement of seeking	Reinstated seeking in wild-type but not CRF1KO mice	[21]
Cocaine	Long Evans rats	NPS	i.c.v. and LH	Cued reinstatement	Exacerbated seeking	[19]
Cocaine	Wistar rats	NPSR antagonist RTI118	i.p.	Cued reinstatement of seeking and self-administration	Decreased both	[99]
Cocaine	Long Evans rats	NPSR antagonist NPSR-QA1	i.p.	Cued reinstatement of seeking	Reduced seeking	[96]

Table 3. Clinical trial on drug use disorders targeting the orexin system.

NCT Number	Disease	Treatment	Phase
NCT03897062	Alcohol Use Disorder	Suvorexant	2
NCT04229095	Alcohol Use Disorder	Suvorexant	2
NCT04287062	Opioid Use Disorder	Suvorexant	2
NCT04262193	Opioid Use Disorder	Suvorexant	2
NCT03789214	Opioid Use Disorder	Suvorexant	2
NCT05145764	Opioid Use Disorder	Suvorexant	2
NCT04818086	Opioid Use Disorder	Lemborexant	1 and 2
NCT02785406	Cocaine Use Disorder	Suvorexant	2
NCT03937986	Cocaine Use Disorder	Suvorexant	1

6. Conclusive Remarks

Here, we reviewed the evidence that NPS is a modulator of catecholamines, GABA and glutamate activities. NPS also interacts with key players of both the peripheral and central stress response system—specifically with Hcrt-1/Ox-A and CRF. This wide spectrum of interactions is associated with a unique physio-pharmacological profile, as this neurotransmitter promotes arousal and is anxiolytic at the same time. The pharmacological traits and neurobiological interactions of NPS indicated this neuropeptide as a new player in the stress response neurosystem. The dual pharmacology of NPS as player of the stress system is reflected by its effect on drug self-administration and the reinstatement of drug seeking. On the one hand, NPS reduced alcohol self-administration in rodents consuming alcohol to self-medicate their innate or withdrawal-induced anxiety state, and this effect was associated with the anxiolytic effect of NPS. On the other hand, NPS primed extinguished alcohol and cocaine seeking, and this latter effect was demonstrated to be mediated by CRF. In addition, NPS exacerbated the cued reinstatement of alcohol and cocaine seeking through Hcrt-1/Ox-A. The emergence of the NPS as a new player of the stress system involved in addiction is noteworthy as, although stress plays a major and well-consolidated role in addiction, no drugs targeting the stress system to treat addiction have hit the market so far. We predict that NPSR agonists would be indicated to help quitting alcohol consumption and to mitigate the psychological aspects of alcohol withdrawal syndrome; interestingly, the first NPSR agonist has been developed recently [115] and it would be interesting to test it on alcohol self-administration. Conversely, NPSR antagonists would be indicated to prevent relapse. The therapeutic potential of targeting the NPS system is not limited to this, though. Indeed, despite the exogenous administration of NPS having no effect on alcohol and cocaine self-administration in non-preferring rats, NPSR antagonists reduced the self-administration of both drugs. Future studies should be directed to characterize the neurocircuitries through which NPS reduces alcohol self-administration in preferring rat lines and to understand the neurobiological bases of the efficacy of NPSR antagonists where the exogenous administration of the peptide was ineffective.

Author Contributions: M.U., N.C., A.M.B., M.P. conceptualization. M.U., E.D., N.C. supervision. M.U., N.C., C.C., E.D. writing, review and editing. M.V.M.D.B., N.C., A.M.B., M.P., L.S., S.D.C. writing—original draft preparation. M.U., N.C. funding acquisition. M.U., E.D., M.P. visualization. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by grant FAR-2019 from the University of Camerino to M.U. and by grant 202213 from the Hetzler Foundation to N.C.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Modulation of Gut Microbiota and Neuroprotective Effect of a Yeast-Enriched Beer

Valentina Cecarini [1,†], Olee Gogoi [1,†], Laura Bonfili [1], Iolanda Veneruso [2,3], Giada Pacinelli [4], Sara De Carlo [4], Federica Benvenuti [4], Valeria D'Argenio [3,5], Mauro Angeletti [1], Nazzareno Cannella [4,*,‡] and Anna Maria Eleuteri [1,‡]

1 School of Biosciences and Veterinary Medicine, University of Camerino, Via Gentile III da Varano, 62032 Camerino, Italy; valentina.cecarini@unicam.it (V.C.); olee.gogoi@unicam.it (O.G.); laura.bonfili@unicam.it (L.B.); mauro.angeletti@unicam.it (M.A.); annamaria.eleuteri@unicam.it (A.M.E.)
2 Department of Molecular Medicine and Medical Biotechnologies, Federico II University, Via Sergio Pansini 5, 80131 Naples, Italy; venerusoi@ceinge.unina.it
3 CEINGE-Biotecnologie Avanzate, Via G. Salvatore 486, 80145 Naples, Italy; dargenio@ceinge.unina.it
4 Pharmacology Unit, School of Pharmacy, University of Camerino, Via Madonna delle Carceri 9, 62032 Camerino, Italy; giada.pacinelli@unicam.it (G.P.); sara.decarlo@unicam.it (S.D.C.); federica.benvenuti@unicam.it (F.B.)
5 Department of Human Sciences and Quality of Life Promotion, San Raffaele Open University, Via di Val Cannuta 247, 00166 Roma, Italy
* Correspondence: nazzareno.cannella@unicam.it; Tel.: +39-0737-403322
† These authors contributed equally to this work.
‡ These authors contributed equally to this work.

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Citation: Cecarini, V.; Gogoi, O.; Bonfili, L.; Veneruso, I.; Pacinelli, G.; De Carlo, S.; Benvenuti, F.; D'Argenio, V.; Angeletti, M.; Cannella, N.; et al. Modulation of Gut Microbiota and Neuroprotective Effect of a Yeast-Enriched Beer. *Nutrients* **2022**, *14*, 2380. https://doi.org/10.3390/nu14122380

Academic Editor: Serguei O. Fetissov

Received: 9 May 2022
Accepted: 7 June 2022
Published: 8 June 2022

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Abstract: Beer is the most consumed alcoholic beverage worldwide. It is rich in nutrients, and with its microbial component it could play a role in gut microbiota modulation. Conflicting data are currently available regarding the consequences of alcohol and alcohol-containing beverages on dementia and age-associated disorders including Alzheimer's disease (AD), a neurodegeneration characterized by protein aggregation, inflammatory processes and alterations of components of the gut–brain axis. The effects of an unfiltered and unpasteurized craft beer on AD molecular hallmarks, levels of gut hormones and composition of micro/mycobiota were dissected using 3xTg-AD mice. In addition, to better assess the role of yeasts, beer was enriched with the same *Saccharomyces cerevisiae* strain used for brewing. The treatment with the yeast-enriched beer ameliorated cognition and favored the reduction of Aβ(1-42) and pro-inflammatory molecules, also contributing to an increase in the concentration of anti-inflammatory cytokines. A significant improvement in the richness and presence of beneficial taxa in the gut bacterial population of the 3xTg-AD animals was observed. In addition, the fungal order, *Sordariomycetes*, associated with gut inflammatory conditions, noticeably decreased with beer treatments. These data demonstrate, for the first time, the beneficial effects of a yeast-enriched beer on AD signs, suggesting gut microbiota modulation as a mechanism of action.

Keywords: beer; Alzheimer's disease; amyloid; inflammation; microbiota

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease associated with memory impairment and cognitive decline and is the most common cause of dementia in the elderly. The brain regions mainly affected by the disorder are the hippocampus and cerebral cortex. These areas are interested by extensive deposition of protein aggregates, mainly extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles of the hyperphosphorylated form of the tau protein. The Aβ(1-40) and Aβ(1-42) peptides are principal components of plaques, and they are the product of the amyloidogenic processing of the amyloid precursor protein by the β- and γ-secretases [1]. Furthermore, dysfunctional proteolytic systems and high levels of both oxidative stress and inflammation

characterize the AD brain [2,3]. The inflammatory response initiates with the activation of microglia and the recruitment of astrocytes that release cytokines and other neurotoxic products that contribute to neuronal degeneration and cell death [4]. No definitive drugs are available for this condition and numerous efforts are directed toward the development of new therapeutic approaches able to prevent/ameliorate symptoms as well as to delay the onset of the disorder. Recently, an increasing number of studies are focusing attention on the effects of alcohol and alcoholic beverages on dementia and age-associated disorders including AD. However, conflicting data exist on this topic. In fact, several data reported that alcohol intake can be detrimental and can contribute to cognitive alterations thus increasing the risk of developing neurodegenerative disorders, mainly through induction of oxidative stress, glutamate-associated excitotoxicity and neuronal apoptosis [5]. On the contrary, other findings demonstrate that light to moderate alcohol consumption may have beneficial effects, reducing the risk of developing neurodegeneration [5]. Alcohol's neuroprotective effect depends upon several factors including the amount of intake and type of beverage consumed [5]. In this regard, alcoholic beverages that contain a reduced concentration of ethanol, such as beer, when taken in low or moderate amounts can help reduce the risk of developing AD [6,7], but the exact molecular mechanisms involved are still unclear. Beer is the most widely consumed alcoholic beverage and is extremely rich in nutrients and micronutrients. Beer's alcoholic content can range approximately from 0 to 15% *w*/*v*. Essential ingredients for brewing beer are barley, hops, water and yeasts, specifically *Saccharomyces cerevisiae*. Beer composition can vary from one type to another, and among the high number of nutrients, carbohydrates, protein/amino acids, minerals, vitamins and other compounds, such as polyphenols, are the most abundant [8]. Few data are currently available on the neuroprotective properties of beer. Previous findings on human postmortem samples demonstrated that moderate beer consumption, but not wine or spirits, reduced the prevalence of Aβ aggregation in the brain [9].

Furthermore, in addition to being an alcoholic beverage, similar to other fermented food and due to the fact of its microbial component, beer could have probiotic effects on gastrointestinal microbiota, a key component of the gut–brain axis, thus contributing to the maintenance of adequate cognitive and neurological functions. In fact, an increasing number of reports, including preclinical and clinical studies, are now suggesting that a proper modulation of gut microbiota by means of probiotics can ameliorate an AD condition, reducing the cognitive, physiological and neuroanatomical impairment and ameliorating the brain inflammatory and oxidative status [10–15].

The aim of this study was to evaluate if moderate consumption of unpasteurized beer could exert beneficial effects in 3xTg-AD mice, a reliable model of human AD, counteracting the cognitive decline and reducing the levels of major hallmarks of the disorder such as amyloid peptides and inflammatory cytokines. Possible effects on components of the gut-brain axis were also evaluated. Furthermore, in order to better highlight the role of yeasts in the modulation of gut microbiota/mycobiota, mice were also treated with an enriched formulation of the beer containing a higher concentration of the same *Saccharomyces cerevisiae* used for brewing beer.

2. Materials and Methods

2.1. Reagents and Chemicals

Unfiltered nonpasteurized beer with a 9% alcohol content was purchased from Kukà S.r.L. (Italy). In addition, 95% *v*/*v* alcohol was purchased from Carsetti S.r.L. (Italy) and diluted to 9%. SafAleTM T-58 yeast containing *Saccharomyces cerevisiae* and emulsifier E491 was purchased from Fermentis (Italy). Protease inhibitors tosyl phenylalanyl chloromethyl ketone (TPCK) and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF or Pefabloc) were purchased from Sigma-Aldrich S.r.L. (Milano, Italy). The amyloid beta 40 mouse enzyme-linked immunosorbent assay (ELISA) kit and amyloid beta 42 mouse ELISA kit for Aβ(1-40) and Aβ(1-42) peptide determination in brain homogenates were purchased from Invitrogen (Camarillo, CA, USA). The Rat/Mouse Ghrelin (active) ELISA

kit, Mouse Leptin ELISA, Rat/Mouse GIP (total) ELISA (Merk EZRMGIP-55K) and the multi-species GLP-1 Total ELISA (Merk EZGLP1T-36K) were bought from Merk group.

2.2. Animal Model

AD triple-transgenic mice, B6;129-Psen1tm1Mpm Tg (amyloid precursor protein (APP) Swe, tauP301L) 1Lfa/J (named 3xTg-AD), and the wild-type (wt) B6129SF2 mice (separate line) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). These transgenic mice contain 3 mutations associated with frontotemporal dementia or familial AD (APPSwe, tau MAPT P301L and presenilin-1 M146V). The animals displayed both a plaque and tangle pathology, with Aβ intracellular immunoreactivity detectable at 3 months of age and hyperphosphorylation of tau protein occurring by 12–15 months of age [16]. Experiments were conducted in accordance with the guidelines of the European Communities Council (86/609/ECC) for the care and use of laboratory animals and were approved by the Italian Ministry of Health (protocol: 1D580.28). Mice were housed in plastic cages (Makrolon, Covestro A.G., Filago, Italy) (4 animals per cage) in a temperature-controlled room (21 $\pm$ 5 °C) at 60% humidity on a 12 h light/dark reversed cycle (light was switched on at 8:00 p.m.). The mice were maintained on a laboratory diet (Mucedola, Italy) and tap water ad libitum.

2.3. Experimental Design

Eight-week-old 3xTg-AD and wt mice (n = 40/line, 50% female) were divided into 4 groups and treated for a period of four months as follows: one group received water (n = 10), one group received 9% alcohol (n = 10), one group received unpasteurized beer (n = 10) and one group received unpasteurized beer enriched with yeast (1.2×10^{11} CFU) (n = 10). This amount of yeast was added to the beer considering that total microbiota was estimated to be $\sim 10^{13}$–10^{14} microbial cells [17] and that fungi consisted of nearly 0.1% of the total microbes in the gut [18], thus approximately 10^{11}. Yeasts were daily dissolved in beer and given to the animals. Cages were equipped with two bottles, one containing the experimental beverage (i.e., alcohol, unpasteurized beer or yeast enriched beer) and the other containing water. Beverages were replaced every day, once a day, by the operator. The amount of ethanol, beer, yeast enriched beer and water consumed was measured daily by comparing the volumes in the bottles. Preliminary studies housing mice in single cages were performed to ensure that all animals drank the experimental beverages. Mice were monitored for the amount of water or beer consumed for a period of one week. Bottles were weighted twice a day in order to check the volume of the remaining solution. Both wt and 3xTg-AD mice drank approximately 6–7 mL of the experimental drink during the day. The liquid lost during handling by the experimenter or evaporation was estimated including the same sets of bottles on empty cages. During the treatment, body weight was monitored every week to ensure proper food intake. At sacrifice, blood, intestine with feces and brains were collected. Tissues and plasma, promptly treated with protease inhibitors (i.e., Pefabloc and TPCK), were stored at −80 °C.

2.4. Preparation of Brain Samples

Hippocampus (HIP) and prefrontal cortex (PFC) were homogenized (1:5 weight/volume of buffer) in 50 mM Tris buffer, 150 mM KCl and 2 mM EDTA, pH 7.5. Homogenates were immediately centrifuged at 13,000 rpm for 20 min at 4 °C, and an aliquot of the supernatant was used for Western blotting and other biochemical tests, whereas another aliquot was immediately supplemented with protease inhibitors (i.e., Pefabloc and TPCK) for ELISA determinations. The Bradford method was used to measure the protein concentration in homogenates using bovine serum albumin (BSA) as a standard [19].

2.5. Preparation of Plasma Samples

Blood samples were collected in tubes with 10% w/v (g/100 mL) of K_2-EDTA, centrifuged at 13,000 rpm for 20 min at 4 °C. Plasma was promptly added with proteases inhibitors.

2.6. Western Blotting

Brain homogenates (20 μg of proteins) were loaded on 12% SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Milano, Italy). Membranes were activated with methanol and blocked with 5% BSA in freshly prepared TTBS (Tween 20 plus Tris-HCl and NaCl, pH 7.5). Antibodies were diluted in 2% BSA in TTBS. Proteins were detected with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Milano, Italy) using a ChemiDoc MP system. Primary antibodies (1:500 dilution), used to detect pro- and anti-inflammatory cytokines, were from Abcam plc (Cambridge, UK), whereas secondary antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany, 1:500 dilution). Molecular weight markers (6.5–205 kDa) were included in each gel. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as a control to check equal protein loading (1:500 dilution). Membranes were stripped using a stripping buffer containing 200 mM glycine, 0.1% SDS and 1% Tween 20. Immunoblot images were quantified using ImageJ 1.52p software (NIH, Bethesda, MD, USA).

2.7. Behavioral Test

The novel-object recognition (NOR) test was used to evaluate mice memory integrity. Experimental procedures were performed during the dark phase of the light/dark cycle, from 8:00 a.m. to 3:00 p.m., by investigators blind to the experimental conditions as previously described [20]. Before the test, animals were handled for three days to accustom them to the experimenter. The NOR test was conducted over two days. The first day mice were allowed to explore the empty arena for 5 min to acclimate them to the experimental environment. The second day comprised two 10 min trials spaced 3 h apart. During the first trial (familiarization phase), mice were allowed to explore two identical (familiar) objects. During the second trial (test phase), mice were allowed to explore one familiar and one novel object. The time the rodent spent exploring each object during the test trial provided a measurement of memory integrity, as animals are expected to spend more time exploring the novel object. Objects were different in shape, color and texture and maintained throughout the study to obtain reproducible data. Preliminary experiments were conducted to verify that selected objects elicited the same amount of spontaneous investigation. The results are expressed as the NOR discrimination index (the ratio between the time spent exploring the novel object and the total time spent exploring both objects during the test trial).

2.8. ELISA for Aβ Levels Determination

HIP and PFC of the control and treated mice were assayed using ELISA to measure Aβ(1-40) and Aβ(1-42) levels. Based on preliminary tests, samples were diluted at 1:5 with diluent buffer provided with the ELISA kits. Plates were read at 450 nm on a visible plate reader (Biotrak, Amersham). Assays were performed according to the manufacturer's directions.

2.9. ELISA for Hormones Ghrelin, Leptin and GIP, and GLP-1

Plasma hormone concentrations were measured through sandwich ELISA using plasma treated with protease inhibitors (i.e., Pefabloc and TPCK). Plates were read at 450 nm on a visible plate reader, and the values were corrected from the absorbance at 590 nm after acidification of the formed products.

2.10. ELISA for Cytokines

The HIP, PFC, and plasma samples, from the wt and 3xTg-AD mice, added with protease inhibitors were also used to measure pro- and anti-inflammatory cytokines using the following ELISA kits: the IL-10 Mouse ELISA Kit, the IL-1β Mouse ELISA Kit, the TNF-α Mouse ELISA Kit, High Sensitivity and the IL-4 Mouse ELISA Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), following the manufacturer's instructions.

2.11. Microbiota and Mycobiota Analyses

As specified above, fecal samples from the wt and 3xTg-AD mice were collected at the time of sacrifice, immediately cooled on dry ice and stored at −80 °C until analysis. Genomic DNA was extracted using the 16 LEV Blood DNA kit and the Maxwell 16 instrument (both from Promega, Madison, WI, USA) as previously reported [21]. Two blank samples were also collected as the control of this analytical step to check for any environmental contamination occurring during the DNA extraction procedure. To deeply investigate the microbiome composition of all the collected samples, both bacterial and fungal communities were analyzed. For bacteria analysis, a 500 bp amplicon, covering the V4-V6 hyper-variable regions of the 16S rRNA gene, was obtained as previously described [22]. Then, a second-round PCR was performed to univocally tag different samples allowing for their multiplexing. In each PCR step, 2 negative controls were included to be further processed as contamination controls of the whole analytic procedure. The obtained multiple amplicon libraries were quality assessed (TapeStation, Agilent Technologies, Santa Clara, CA, USA) and quantified (Qubit, Thermo Fisher Scientific, Waltham, MA, USA) before being sequenced with the V3 300X2 PE MiSeq protocol (Illumina, San Diego, CA, USA), according to the specifications of the manufacturer. For fungi analysis, specific primers were used for ITS1 amplification [23]. After the first-round PCR to specifically amplify the target region, the amplicons were treated as specified above for the 16S rRNA amplicon. In addition, in this case, PCR controls were processed together with the samples to provide analytic controls for any environmental contaminant.

The FASTQ files were sent to the CRG bioinformatic facility (https://biocore.crg.eu/wiki/Main_Page, accessed on 28 October 2020) for primary data analysis. After an initial quality check with FastQC [24], sequences were processed using the mothur tool (version 1.44.1) [25], following the workflow described on the authors' website (https://mothur.org/wiki/miseq_sop/, accessed on 3 December 2020). Reference sequences for the bacterial 16S rRNA data analysis were obtained from the SILVA database, version 138 [26], and used for mapping the data and grouping the reads into operational taxonomic units (OTUs) in the mothur framework. Reference sequences for ITS data analysis were obtained from the UNITE database, version 4 February 2020 [27]. Secondary analysis of the metagenomic data was performed using the R packages "Phyloseq" v.1.30.0 [28] and "microbiome" v.1.8.0 [29] to include the estimation of alpha- and beta-diversity [30], and the identification of significantly enriched taxa in studied groups, using the R package "DESeq2" v.1.26.0 [31]. Moreover, the mothur output package was used for further analyses using the Microbiome Analyst tool [32]. Samples richness and/or evenness were evaluated, and the ANOVA test was performed to assess significant differences. Unweighted and weighted Unifrac distance measures were used to evaluate beta diversity coupled with the PERMANOVA test to verify the significance of the samples grouping. Differential abundance analysis was carried out using univariate statistical comparisons based on parametric tests (i.e., *t*-test/ANOVA); *p*-values were adjusted using the FDR method.

2.12. Statistical Analyses

Data presented in histograms are expressed as the mean values ± S.D. Statistical analysis was performed using Sigma-stat 3.1 software (SPSS, Chicago, IL, USA). Data were analyzed by one-way ANOVA, followed by the Bonferroni post hoc when appropriate. Wt and 3xTg-AD mice were analyzed separately. Statistical significance was set to the conventional $p < 0.05$.

3. Results

3.1. Effect of Beer Consumption on Cognitive Performance

The effect of the treatments was first evaluated on the consolidation process of memory and learning through the novel object recognition (NOR) test [33]. No significant difference was observed in the discrimination scores of wt mice (Figure 1). As for 3xTg-AD mice, ANOVA found no overall effect of treatments. However, since data observation suggested

that beer/yeast and beer treatments showed a better discrimination index than water, and being all independent groups, we also compared these two groups with water by *t*-test. Interestingly, we found that beer/yeast, but not beer alone, showed a discrimination index significantly higher than water-treated 3xTg-AD animals, indicating the beneficial effect of this treatment on hippocampus functions and recognition memory (Figure 1).

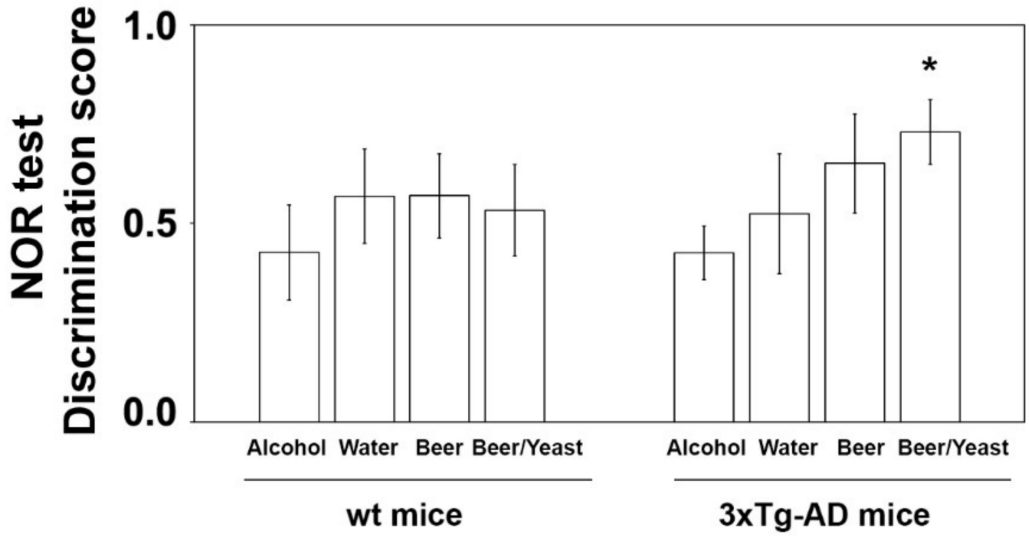


Figure 1. Effect of treatment on the NOR discrimination index in wt and 3xTg-AD mice. Treatment with alcohol, beer or beer/yeast did not affect discrimination index in wt mice, whereas yeast enriched beer (beer/yeast) significantly increased the NOR discrimination index in 3xTg-AD mice. Statistical significance: * $p < 0.05$ vs. water group.

3.2. Effect of Beer Consumption on Amyloid-β Levels

Accumulation of amyloid beta peptides into plaques is a major hallmark of AD. To evaluate the effect of the treatment on the amount of these proteins, we measured the levels of amyloid (1–40) and (1–42) in the hippocampus and frontal cortex of the control and treated animals. As shown in Figure 2, the treatments were not effective in reducing the levels of the Aβ(1–40) peptide, neither in the wt nor in the 3xTg-AD mice.

Conversely, regarding the Aβ(1-42) peptide, which is the most toxic and prone to aggregation, post hoc analyses revealed decreased levels in the HIP but not the PFC of the wt mice (Figure 3, panels A and B), whereas both the HIP and PFC of the 3xTg-AD mice showed significantly reduced amounts of this peptide (Figure 3, panels A and B). In detail, if compared to water, Aβ(1-42) in the HIP of the beer and beer/yeast 3xTg-AD groups showed, respectively, a 22 and 30% reduction (Figure 3, panel A) and in the PFC of the beer/yeast 3xTg-AD group a 20% reduction (Figure 3, panel B). These data globally suggest the ability of both beer treatments to act against one of the major hallmarks of AD pathology.

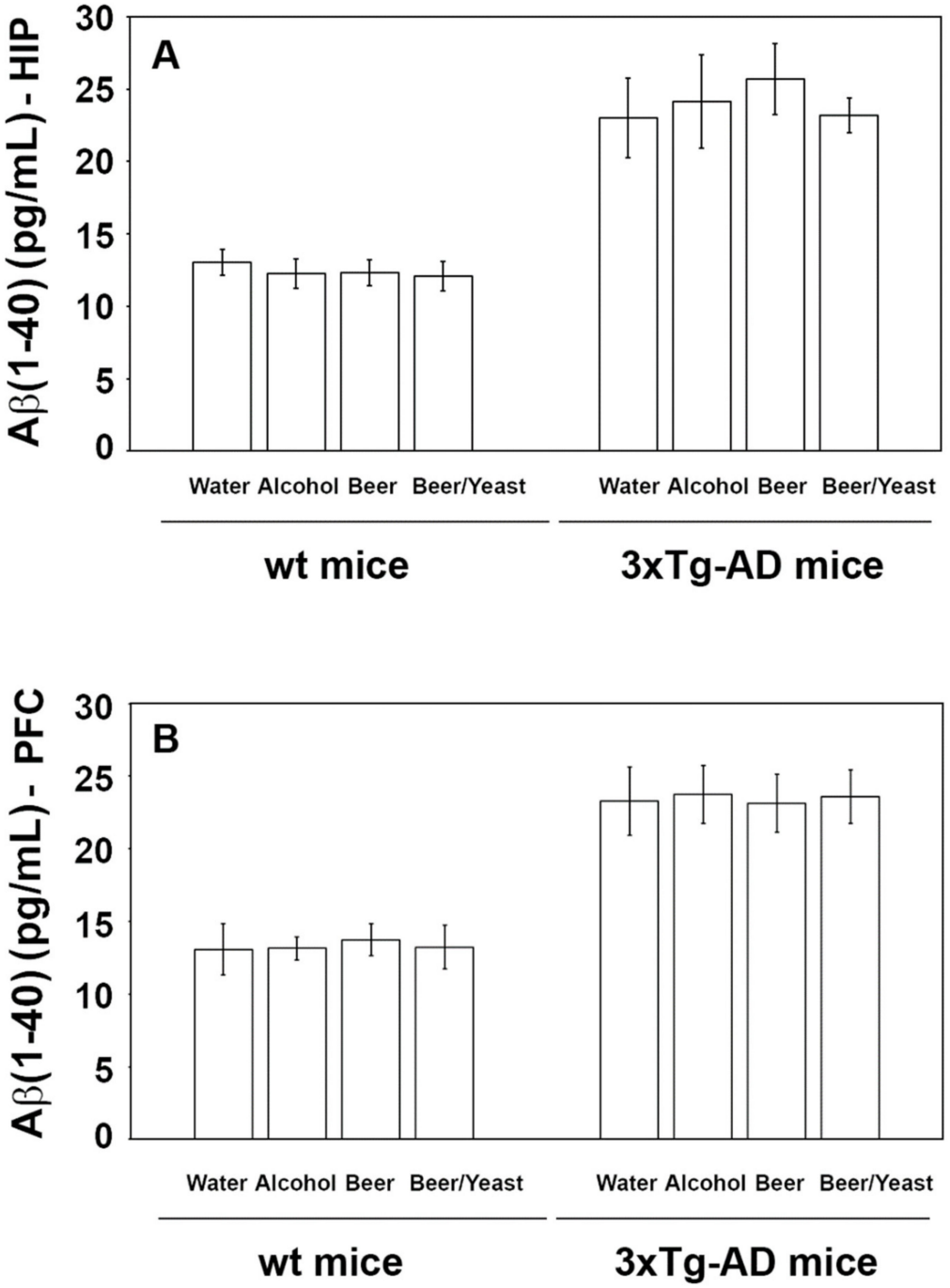


Figure 2. Levels of the Aβ(1-40) peptide measured by ELISA on brain homogenates of the wt and the 3xTg-AD mice treated with water, alcohol, beer and beer/yeast. Treatments did not affect the level of Aβ(1-40) in the HIP (panel **A**) and PFC (panel **B**) of the wt mice and 3xTg-AD mice. Data are expressed as pg/mL of Aβ(1-40).

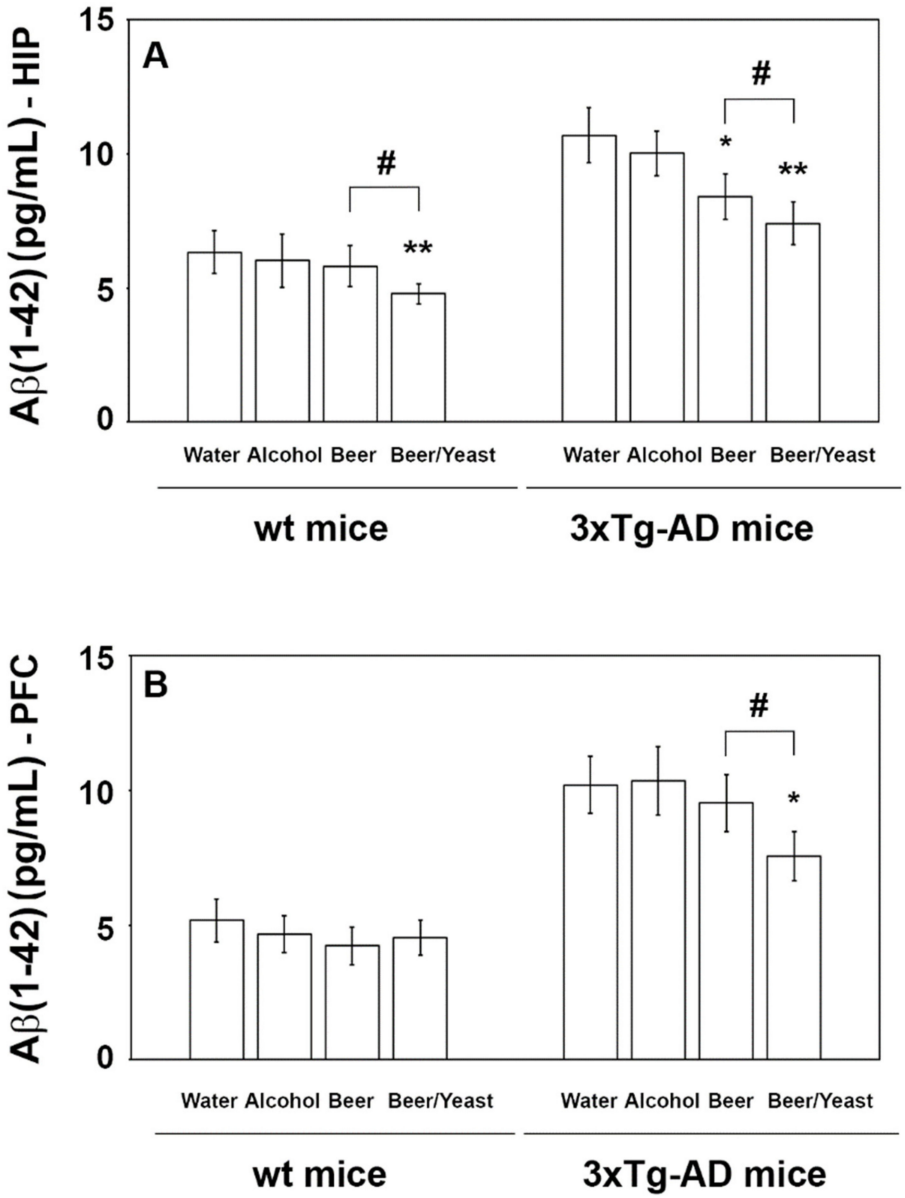


Figure 3. Levels of the Aβ(1-42) peptide measured by ELISA on brain homogenates of the wt and 3xTg-AD mice treated with water, alcohol, beer and beer/yeast. In wt mice, Aβ(1-42) was decreased in the HIP of mice treated with beer/yeast (**A**), but it did not change in the PFC (**B**). Beer and beer/yeast decreased the level of Aβ(1-42) in the HIP of 3xTg-AD mice (**A**). Beer/yeast decreased the level of Aβ(1-42) in the PFC of 3xTg-AD mice (**B**). Concentrations are expressed as pg/mL. (HIP: * $p < 0.05$, B vs. W and A; ** $p < 0.01$, B/Y vs. W and A; # $p < 0.05$, B/Y vs. B; PFC: * $p < 0.05$, B/Y vs. W and A; # $p < 0.05$, B/Y vs. B).

3.3. Effects of Beer Consumption on Cytokines Levels

Extensive inflammatory processes characterize the AD brain with increased amounts of pro-inflammatory molecules and decreased levels of anti-inflammatory cytokines [34]. To evaluate the possible effects of beer consumption on the inflammatory status of control and treated animals, both wt and transgenic mice, we measured the amounts of pro- (IL-1β and TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokines in both plasma and brain using ELISA kits and WB assays. Samples from the control and treated wt mice showed no difference in the levels of the cytokines TNF-α and IL-10 (measured in the plasma (Figure 4) and in the brain (Figure 5)) and IL-1β and IL-4 (measured in the brain (Figure 6)). Conversely, an evident modulation of the inflammatory condition was obtained in the 3xTg-

AD mice treated with the yeast-enriched beer formulation. In detail, comparing this group with water, the pro-inflammatory molecule TNF-α showed a 50% decrease in the plasma (Figure 4, panel A) and in both brain regions of the beer/yeast treated mice (Figure 5, panels B and D). IL-1β significantly decreased in the HIP (50% decrease) and PFC (60% decrease) of mice treated with beer/yeast (Figure 6, panels A and C). In the same samples, an evident increase was observed for the anti-inflammatory molecules IL-10 and IL-4. As for IL-10, the most evident increase was observed in the HIP of beer/yeast-treated mice (2.7-fold increase compared to the water group) (Figure 5, panel A). Finally, IL-4 showed a 1.73- and 2.36-fold increase, respectively, in the HIP and PFC of beer/yeast-treated mice compared to the water-treated animals (Figure 6, panels B and D).

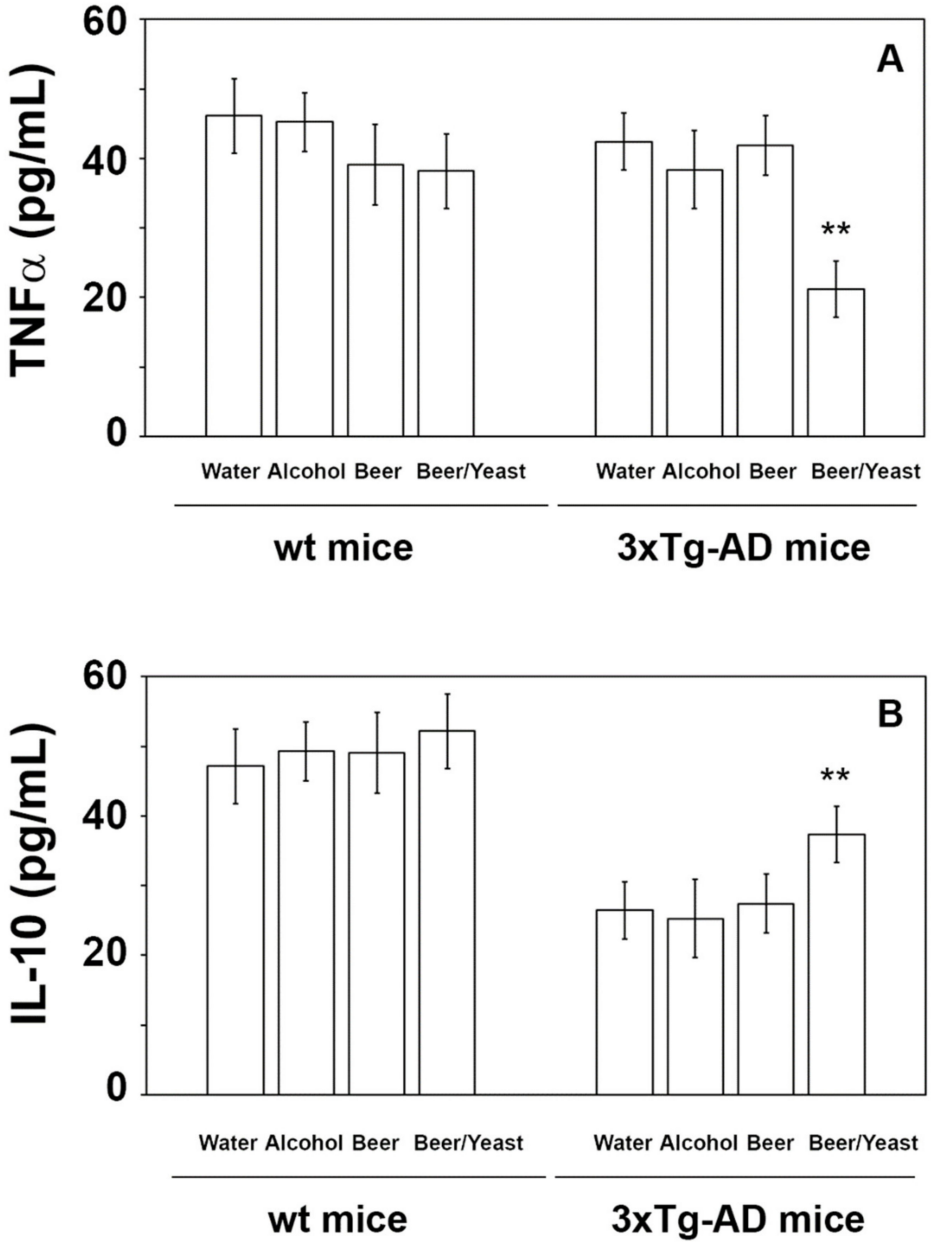


Figure 4. Levels of TNF-α (**A**) and IL-10 (**B**) measured by ELISA on plasma samples of the wt and 3xTg-AD mice treated with water, alcohol, beer and beer/yeast. No changes were detected in the wt animals, whereas TNF-α decreased and IL-10 increased in 3xTg-AD mice upon beer/yeast administration. Concentrations are expressed as pg/mL. HIP: hippocampus; PFC: prefrontal cortex. (** $p < 0.01$ B/Y vs. W, A and B).

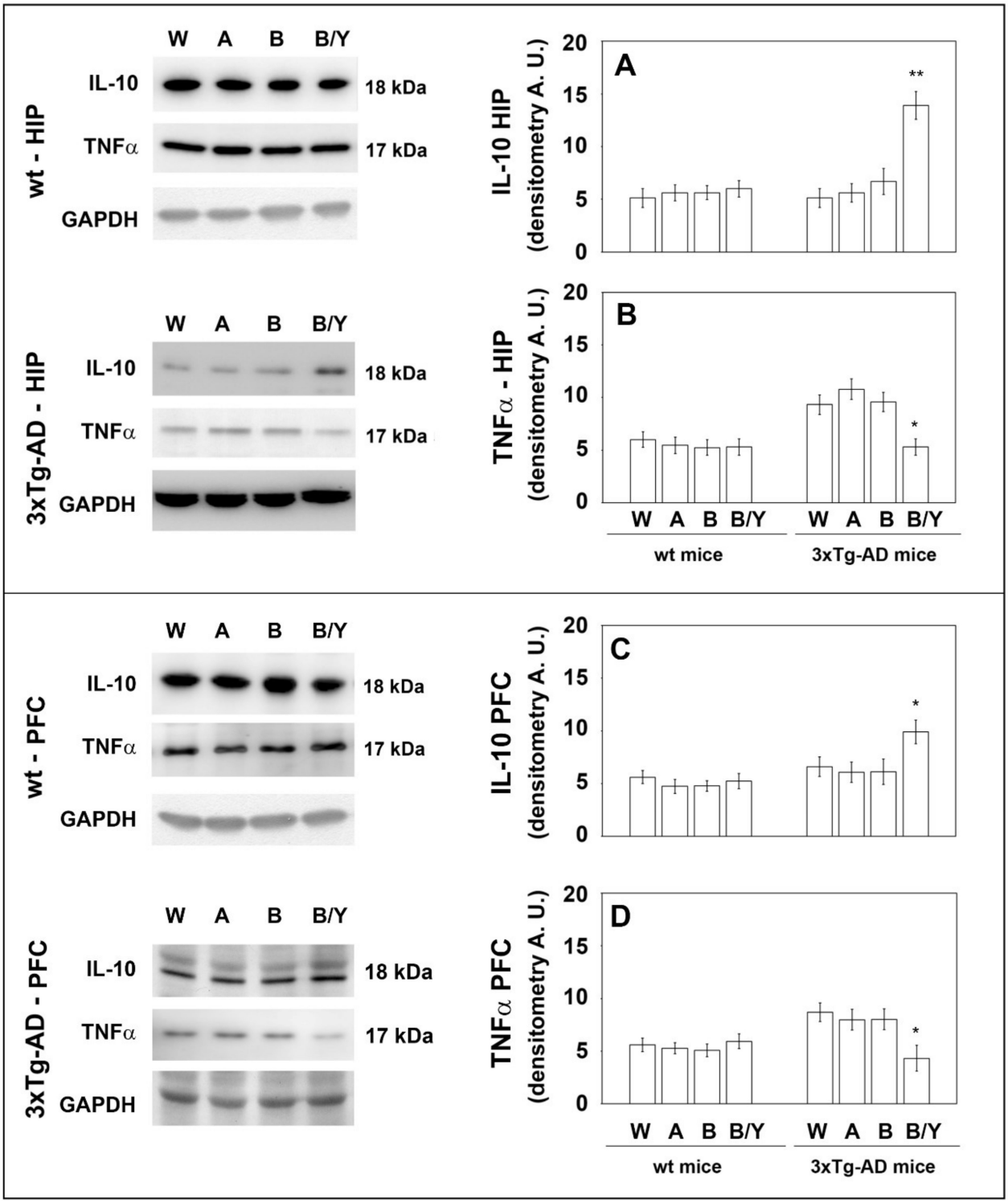


Figure 5. Levels of TNF-α and IL-10 measured by WB on brain samples of the wt and 3xTg-AD mice treated with water, alcohol, beer and beer/yeast. IL-10 expression increased in the HIP and PFC of 3xTg-AD mice (panels **A**–**C**), whereas TNF-α expression decreased in the tested brain regions (panels **B**–**D**). Representative immunoblots and densitometric analyses are shown (A.U.: arbitrary units). Equal protein loading was verified using an anti-GAPDH antibody. HIP: hippocampus; PFC: prefrontal cortex. Data points marked with an asterisk were statistically significant compared to the respective untreated cell line (* $p < 0.05$; ** $p < 0.01$, B/Y vs. W, A and B).

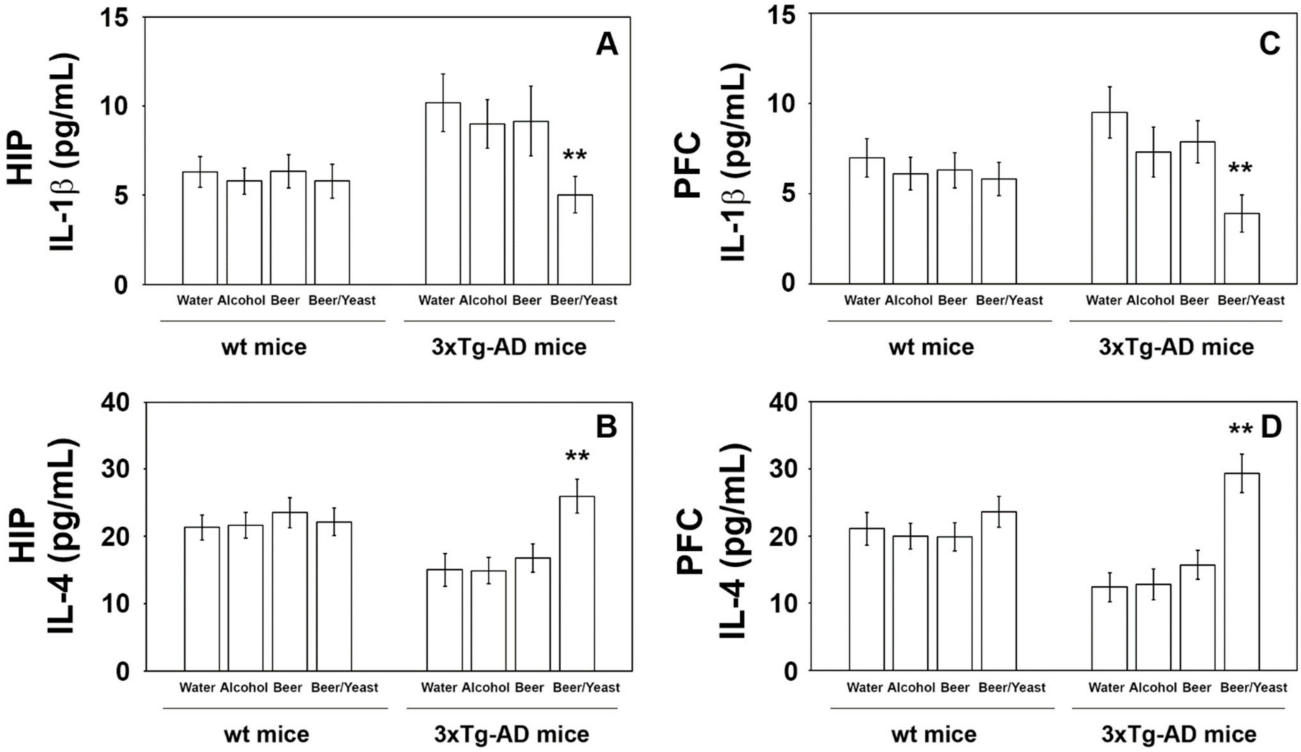


Figure 6. Levels of IL-1β (**A**–**C**) and IL-4 (**B**–**D**) measured by ELISA on brain homogenates of the wt and 3xTg-AD mice treated with water, alcohol, beer and beer/yeast. IL-1β and IL-4 showed, respectively, a decreased and an increased concentration in the HIP and PFC of B/Y-treated 3xTg-AD mice. Concentrations are expressed as pg/mL. HIP: hippocampus; PFC: prefrontal cortex. (** $p < 0.01$, B/Y vs. W, A and B).

3.4. Effect of Beer Consumption on Gut Hormones Levels

We then explored components of the gut–brain axis in order to assess its involvement in the obtained results. The effect of the treatment was evaluated on the concentration of gut hormones, such as leptin, ghrelin, GIP, and GLP-1, determined in plasma samples using ELISA kits. The results showed that treatment with beer and yeast-enriched beer did not significantly alter the levels of the four tested hormones compared to controls (Figure S1). In line with these findings, the body weight of treated mice showed no alterations during the treatment period with respect to the controls (Figure S2).

3.5. Bacterial Communities' Evaluation through 16S rRNA Analysis

An average of 29,030 reads/sample were obtained, allowing the identification of 126 different OTUs. The six negative controls (i.e., blanks), used to exclude any environmental contamination during DNA extraction and PCR amplifications, gave no reads and, thus, were removed from the data analyses. Considering that the gut microbiota of AD patients and animal models displays reduced diversity and a typical taxonomic composition compared to the microbiota of healthy controls [10,35], the presence of bacterial dysbiosis in the 3xTg-AD mice was verified, and the ability of treatments to promote the establishment of beneficial taxa was studied. In particular, since our data showed that in 3xTg-AD mice both beer and beer/yeast consumption were able to significantly modify Aβ(1-42) peptide and cytokines expression, we evaluated the effects of these two treatments on the AD microbiota composition.

Alpha diversity was measured to evaluate two key components: richness and evenness. Interestingly, the 3xTg-AD mice administered with water showed both a reduced richness (Figure 7, panels A and B) and evenness (Figure 8, panel C) with respect to the wt mice as assessed by the observed species, Chao 1 and Shannon indices. Moreover, within the

3xTg-AD mice groups, beer and beer/yeast treatments were able to affect both richness and evenness, which appeared to be restored at levels more similar to the wt mice (Figure 7, panels A, B and C). These results indicate that AD is associated to a reduced bacterial abundance and heterogeneity, and these features are improved at all taxonomic levels upon beer consumption.

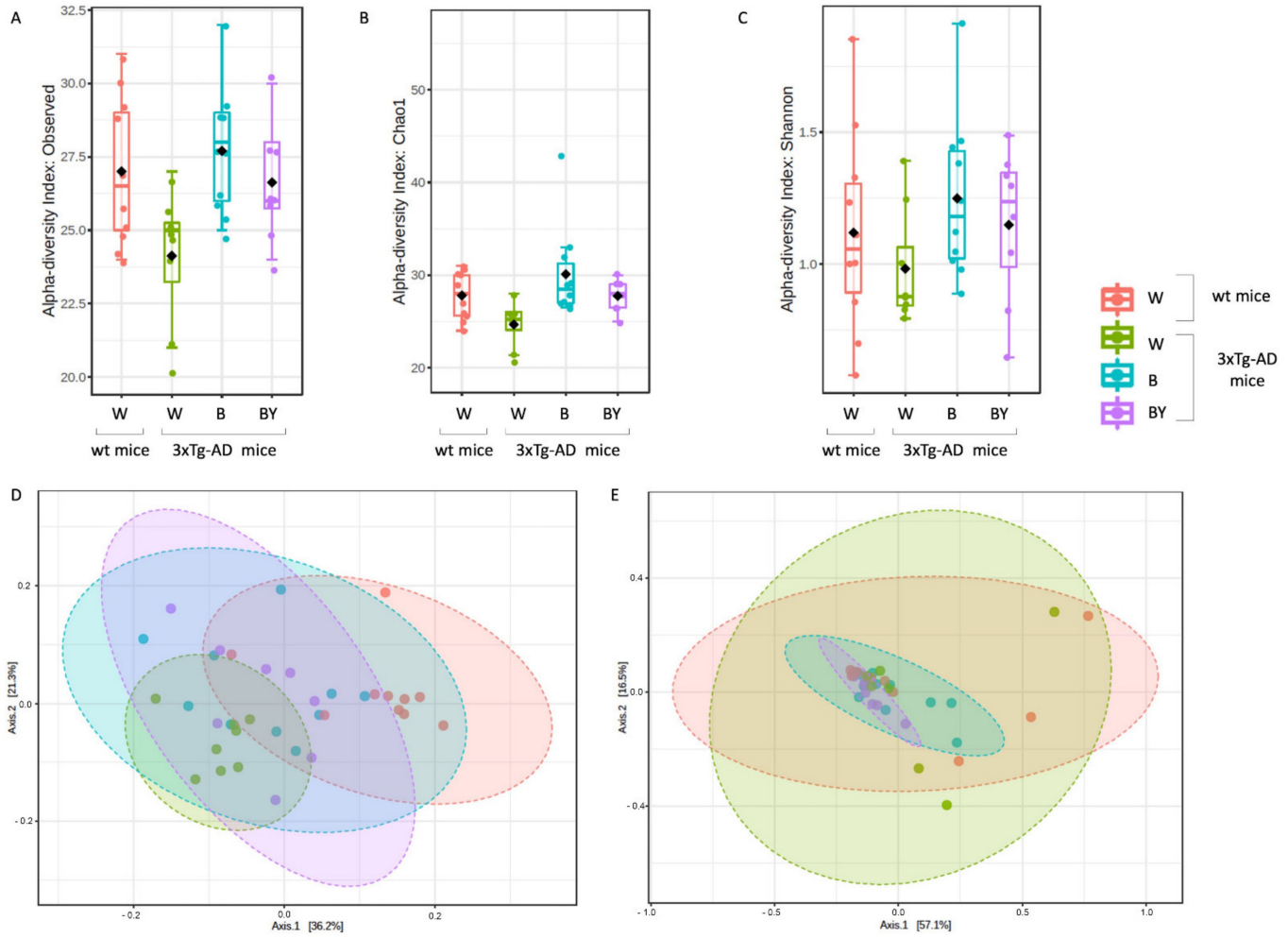


Figure 7. Alpha and beta diversity of the bacterial communities identified for each treatment in the wt (W) and 3xTg-AD mice (W, B and B/Y). Alpha diversity was measured using different metrics, observed species (p = 0.02, ANOVA, panel **A**), Chao 1 (p = 0.02, ANOVA, **B**) and Shannon index (p = 0.36, ANOVA, **C**), to evaluate the within-sample diversity and assess both the richness and evenness of each study group. Taken together, the plots show that the 3xTg-AD mice administered with water had a lower richness and evenness with respect to the wt mice, and that the treatments were able to positively affect the bacterial communities' heterogeneity. Beta diversity was also evaluated to check between-group diversity. Unweighted (panel **D**) and weighted (panel **E**) Unifrac distances were measured. Statistical significance was measured by PERMANOVA test ($p < 0.001$ and $p < 0.21$, respectively).

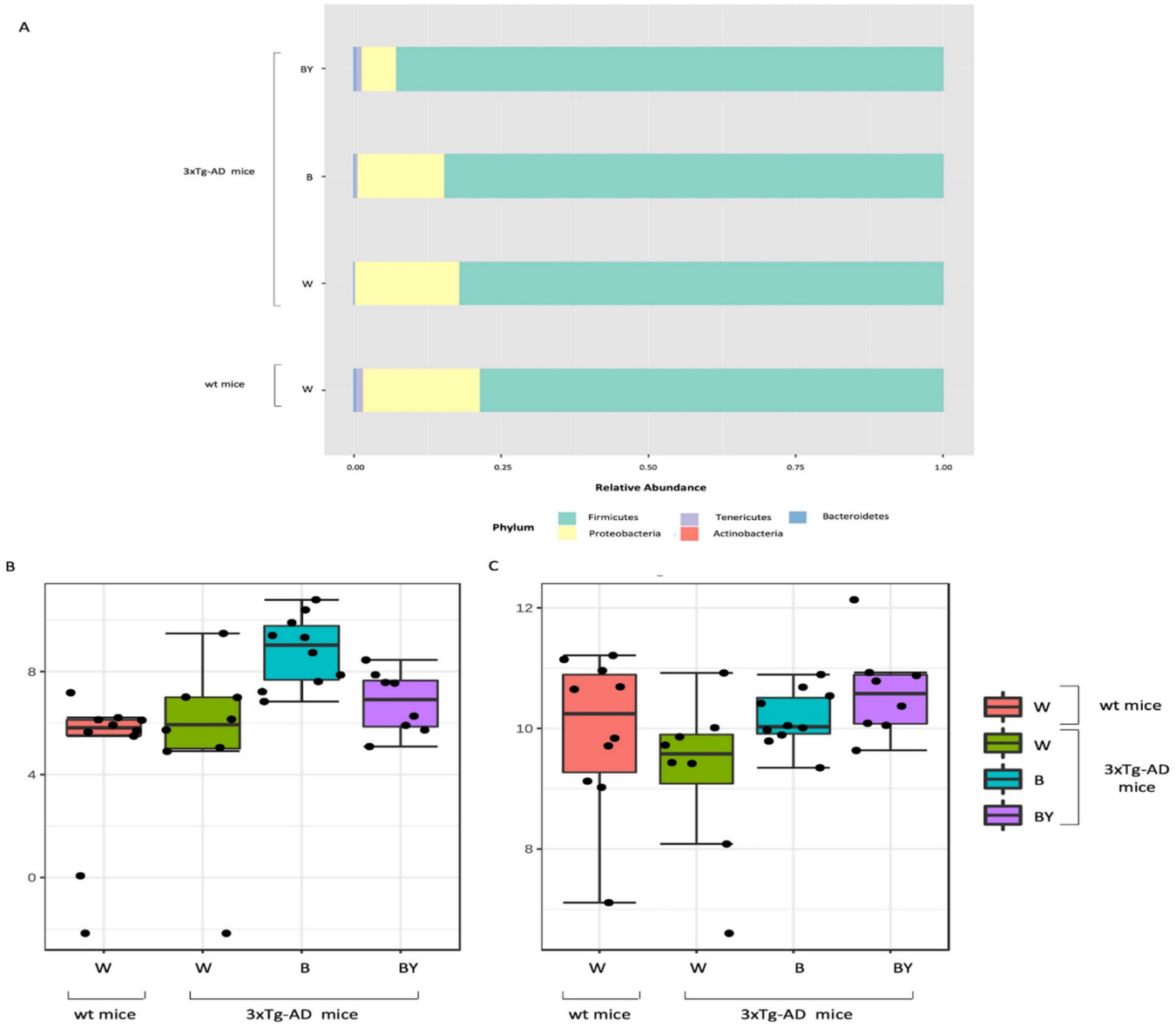


Figure 8. Taxonomic assignment of the gut bacterial communities. Phylum-level taxonomic assignment highlights a different microbial composition between the study groups. According to treatment, it is possible to observe an increase in Firmicutes and a reduction in Proteobacteria (**A**). Classical univariate analysis (i.e., *t*-test/ANOVA) was used to highlight significantly different taxa; at genus level, the genus *Bilophila* was increased in both the treated 3xTg-AD mice (**B**), while *Ruminococcaceae_unclassified* abundance seemed to be restored by the treatments, especially by beer/yeast (BY) administration (**C**).

To assess the presence of a different bacterial composition between the tested study groups, beta diversity analysis was also evaluated using both the unweighted (Figure 7, panel D, PERMANOVA, $p < 0.001$) and weighted (Figure 7, panel E, PERMANOVA, $p < 0.21$) Unifrac distance measures. Since the unweighted Unifrac is a quality-based parameter and the weighted Unifrac is a quantitative-based one, our data suggest that the differences between the compared groups are due more to the kind of taxa, rather than their relative abundances.

Taxonomy assignment showed different bacterial profiles in the individual samples at the phylum level. Merging samples/status, these differences were more evident: in total, five phyla were identified, with *Firmicutes* and *Proteobacteria* being the most abundant in all the studied groups (Figure 8, panel A). In particular, it was possible to observe a

reduction of *Proteobacteria* in the beer and beer/yeast treatments in respect to both the wt and 3xTg-AD mice administered with just water. In addition, the two treatments were featured by an increased abundance in both *Tenericutes* and *Actinobacteria* in respect to th eAD-W mice. Finally, the Bacteroidetes phylum appeared less abundant in all the 3xTg-AD mice, irrespective of treatment (Figure 8, panel A).

Thus, to highlight taxa significantly different between the tested conditions, classical univariate analysis (i.e., *t*-test/ANOVA) was performed. Interestingly, we found one phylum, two classes, two orders, two families and three genera significantly different (adjusted p-value < 0.05) among the four tested groups. All the significantly expressed taxa are listed in Table S1 (Supplementary Materials). These data confirm a significant reduction in the Bacteroidetes phylum in all the 3xTg-AD mice in respect to the wt; this difference was also present at the class (*Bacteroidia*), order (*Bacteroidales*), family (*Prevotellaceae*) and genus (*Prevotellaceae_unclassified*) levels. Moreover, we found a significant increase of the genus *Bilophila* (*Desulfovibrionaceae* family, *Desulfovibrionales* order, and *Deltaproteobacteria* class) in the B and B/Y groups of transgenic mice (Table S1, Supplementary Materials; Figure 8, panel B). Interestingly, the genus, *Ruminococcaceae_unclassified* (Firmicutes phylum), was reduced in the 3xTg-AD mice administered with water with respect to the wt, but their abundance was increased by both treatments, with a higher effect in the beer/yeast group (Figure 8, panel C).

Finally, to identify the taxa most likely to explain the differences between the study groups, linear discriminant analysis (LDA) effect size (LEfSe) was performed. As reported in Table S2 (Supplementary Materials), at the genus level, we found that the genera *Prevotellaceae_unclassified* and *Bilophila* were significantly more and less abundant, respectively, in the wt compared to the 3xTg-AD mice. Interestingly, the treatments seemed to be able to modify specific taxa resembling a relative abundance more similar to the wt mice in respect to the 3xTg-AD mice administered just with water.

3.6. Fungal Communities' Evaluation through ITS1 Analysis

Fungal-specific internal transcribed spacer (ITS) amplicon sequencing was performed to investigate associations between the fungal gut microbiota and AD and to evaluate a possible positive effect upon beer consumption. To this aim, we obtained a total of 469 OTUs with an average reads/sample equivalent to 95,184. Alpha and beta diversity were measured to assess the within and between groups variability of the identified fungal communities. In particular, observed species, Chao1 and Shannon diversity indices were evaluated to measure both richness and evenness within the tested groups. As shown in Figure 9, the 3xTg-AD mice had a significantly higher richness (panels A and B) and an increased, even if not significant, evenness (panel C). Interestingly, this feature seemed to be irrespective of treatment.

Then, beta diversity was measured as unweighted and weighted Unifrac distances (PERMANOVA test). The unweighted analysis showed a significant difference between the fungal communities of the compared conditions (Figure 9, panel D), not confirmed by the weighted test (Figure 9, panel E) as in the case of the bacterial communities.

Taxonomic assignment was then carried out. Despite a large fraction of unclassified OTUs, at the phylum level, the Ascomycota phylum was the most abundant in all of the study groups in respect to Basidiomycota (Figure 10, panels A and B). Interestingly, it is possible to observe in the untreated 3xTg-AD mice a reduction in both the Ascomycota and the Basidiomycota phyla with respect to the wt mice, partially restored by the treatments with a higher effect in the case of beer/yeast administration (Figure 10, panel A). However, the ratio between these two phyla did not seem to be affected (Figure 10, panel B). Thus, classical univariate statistical comparison (i.e., *t*-test/ANOVA) was performed highlighting the order *c_Sordariomycetes_unclassified* as the only differentially expressed taxa between the tested conditions. This order increased in the untreated 3xTg-AD mice, and its abundance was reduced by the beer and beer/yeast treatments (Figure 10, panel C); moreover, the

significant differences were present also at the family and genus levels. LEfSe analysis gave no significantly different results.

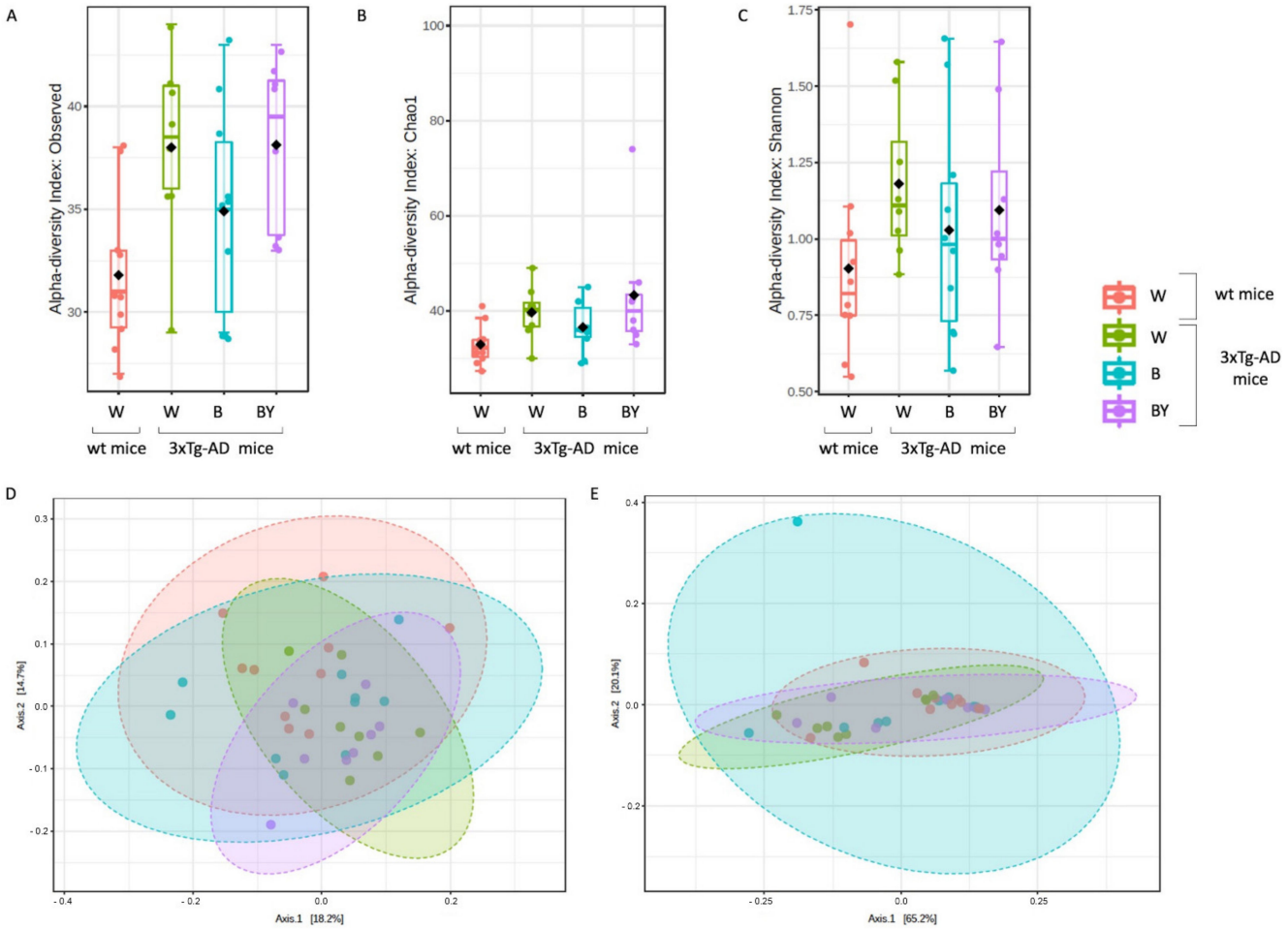
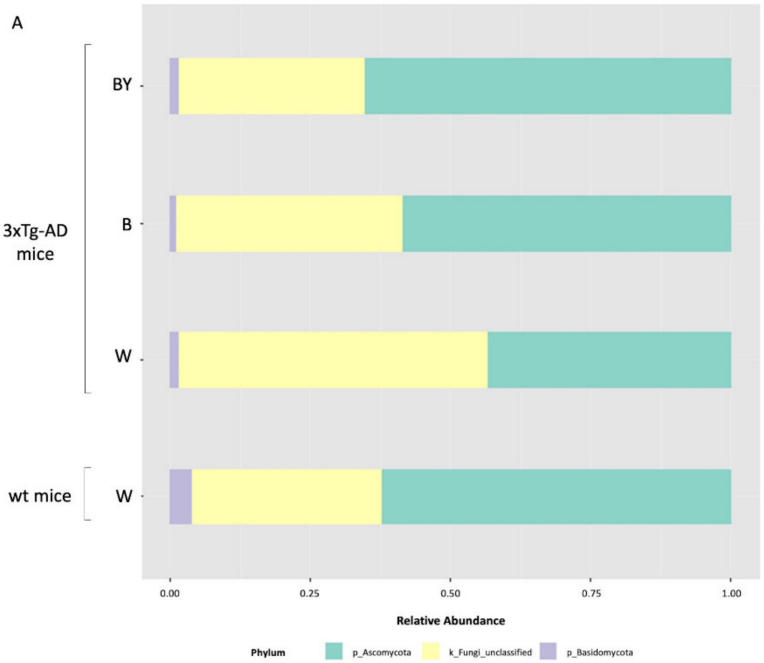


Figure 9. Alpha and beta diversity of the fungal communities identified in the wt and AD-treated and untreated (W, B and beer/yeast (B/Y)) mice. To evaluate the within-sample diversity and assess both the richness and evenness of each study group, the alpha diversity was measured using 3 different metrics, namely, observed species ($p = 0.01$, ANOVA, panel **A**), Chao 1 ($p = 0.04$, ANOVA, panel **B**) and Shannon index ($p = 0.33$, ANOVA panel **C**). 3xTg-AD mice had a higher richness and evenness with respect to the wt mice, and this seemed to not be affected by treatment. Beta diversity was also measured to evaluate the between-group diversity. Both unweighted (panel **D**) and weighted (panel **E**) Unifrac distances were measured using the PERMANOVA test to assess any statistical significance ($p = 0.028$ and $p = 0.22$, respectively).



B

	wt mice		3xTg-AD mice					
	W		W		B		BY	
	Abundance	Percentage	Abundance	Percentage	Abundance	Percentage	Abundance	Percentage
p__Ascomycota	681133	62.3	287855	43.4	568175	58.6	451225	65.3
p__Basidiomycota	40287	3.7	8217	1.2	7998	0.8	8794	1.3
B/A	0.06		0.03		0.01		0.02	

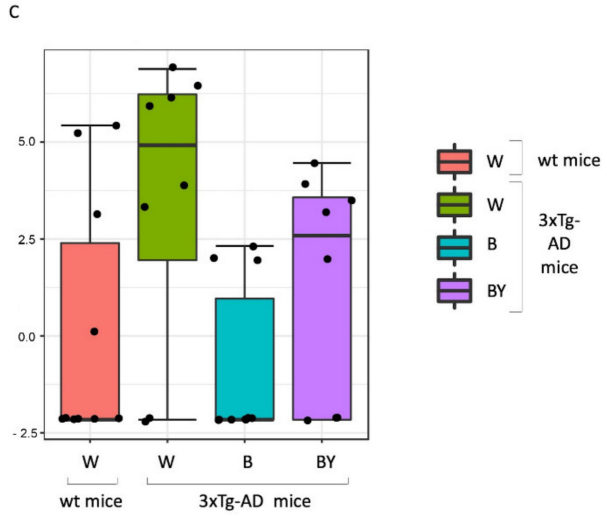


Figure 10. Fungal profiles at the phylum level obtained with the phylogeny-based taxonomy assignment approach. The identified phyla are reported for each study group (panel **A**). Basidiomycota and Ascomycota phyla reads abundance, percentage and ratio are also reported (panel **B**). The *c_Sordariomycetes_unclassified* order was the only significantly different taxa among the tested conditions (panel **C**).

4. Discussion

Beer is the most widely consumed fermented beverage in the world, produced from water, malt, hops and yeast, specifically *Saccharomyces cerevisiae* [36]. Emerging studies are now highlighting that moderate consumption of beer may be beneficial and favor healthy aging [37]. Aging results from the accumulation of molecular and cellular alterations, leading to a growing risk of developing disorders such as AD, which is characterized by massive deposition of Aβ peptides in senile plaques and other aggregates that lead to progressive cognitive dysfunctions [38]. Although no definitive treatment exists for AD, a proper modulation of gut microbiota composition is emerging as an effective strategy to ameliorate AD pathology [10,11,39]. For this reason, considering the presence of yeasts and of other microbes or probiotics in fermented beverages, it is reasonable to hypothesize that they could exert a protective effect through an action on gut microbiota. The present work investigated in wt and 3xTg-AD mice the potential beneficial effects of a four-month treatment with an unpasteurized beer, evaluating amyloid-β peptides amounts and inflammatory markers. In addition, shifts in gut microbes' population, both bacteria and fungi, were detected. The same beer used for the treatment was enriched with the yeast used for brewing beer to better elucidate the role of the microorganisms in the final effect.

Firstly, the mice's cognitive performances were analyzed with the NOR test. Discrimination indexes indicated that the treatment with the yeast-enriched beer positively affected the 3xTg-AD mice's cognitive functions. No effect on behavior was observed in the wt animals.

The observed beneficial effect on behavioral performance on short-term memory prompted us to focus on two important AD molecular hallmarks: the amount of amyloid peptides and the inflammatory condition. In agreement with our working hypothesis and in line with data from behavioral tests, biochemical results showed that the treatment with the yeast-enriched beer was more effective compared to beer alone, indicating the important contribution of the beer yeasts to the observed final effects. In detail, beer treatments successfully diminished the levels of the Aβ(1-42) peptide in the brain of treated AD animals and the addition of the yeast visibly strengthened the final effect, with an evident reduction in the peptide not only in the hippocampus but also in the cortex region of 3xTg-AD mice brain. Conversely, no changes in the Aβ(1-40) amyloid peptide amounts were detected in both the wt and 3xtg-AD mice. These results are in line with a post-mortem study performed by Kok et al. that investigated the association between the consumption of different alcoholic beverages and Aβ pathology, suggesting that beer intake may protect against Aβ aggregation in the brain [8].

AD is always accompanied by severe inflammation that slowly leads to neuronal death [34]. Moderate consumption of either wine or beer was previously associated with lower levels of systemic inflammatory markers in three different European areas [40]. Additionally, administration of iso-α-acids, bitter components of beer, suppresses neuroinflammation and improved cognitive function in a mouse model of AD [41]. In light of this evidence, we analyzed plasma and brain levels of pro-inflammatory (i.e., IL-1β and TNF-α) and anti-inflammatory (i.e., IL-4 and IL-10) cytokines, determining that the yeast-enriched beer stimulated a significant anti-inflammatory response in the 3xTg-AD mice. Conversely, treatment with beer did not significantly alter the plasma levels of the considered cytokines. Again, no effect was detected in the wt animals. These data, therefore, suggest that beer enrichment with the brewing yeast definitely improved beer's ability to decrease important toxic hallmarks of the pathology, such as the inflammatory status, further confirming previous findings on the beneficial effects of yeasts. In detail, these microorganisms, most of all *Saccharomyces cerevisiae*, were characterized for their probiotic effects and for their ability to favor the bioavailability of nutrients, thus improving the nutritional value of foods [42].

To better understand the mechanisms that promote the decrease in the investigated AD signs, we analyzed some of the components of the gut–brain axis, the intricate bidirectional communication system that integrates brain cognitive centers with intestinal functions through neuro-immuno-endocrine mediators [43]. In this regard, we first explored the levels of the gut hormones ghrelin, leptin, GIP and GLP-1 in mice plasma. However, no significant change was observed comparing the four experimental groups, in both the wt and 3xTg-AD mice. Then, we screened the microbiota composition for changes in the richness, that is, the number of species present in a sample, and in the evenness, the related differences in the abundance of species. Treatments with beer and beer/yeast significantly increased the richness in the gut bacterial population of the 3xTg-AD mice making the microbiota of these animals more similar to that of healthy subjects. Interestingly, the 3xTg-AD mice treated with the yeast-enriched beer showed an increase in *Firmicutes* and a simultaneous decrease in *Proteobacteria*. In light of previous studies demonstrating a reduction in the phylum *Firmicutes* and an enrichment of *Proteobacteria* in AD individuals compared to healthy subjects [44], these data demonstrate the positive impact of the treatment on bacterial population composition, suggesting that the modulation of gut microbiota may contribute to the final effect of the treatment. Interestingly, an increase was observed for the genus Bilophila, an anaerobic and sulfite-reducing bacterium and a member of the gut microbiota [45]. It is able to carry out organosulfonate respiration by using taurine and other sulfite donors for energy conservation and producing hydrogen sulfide. The latter bacterial metabolite has been reported as a risk factor for several diseases [46]. However, it was recently pointed out that hydrogen sulfide may have beneficial effects by acting as an antioxidant, signaling molecules and energy [47].

As for the fungal population, beside the very few data currently available on the entire set of fungal species residing in humans [48], it is now widely demonstrated

that these microorganisms can control important processes such as the regulation of the immune response and prevention and treatment of bacterial infections and intestinal complications [48,49]. Nevertheless, the mycobiota is still poorly investigated, the majority of metagenomic studies carried out so far being focused just on the bacterial counterpart. As a consequence, an accurate taxa identification is difficult due to the lack of comprehensive databases for fungal reads alignment and is reflected in the high number of unclassified reads. In our study, although minor changes were observed in this group of microorganisms upon treatments, a relevant and interesting shift was detected in the order *Sordariomycetes*, which increased in the untreated 3xTg-AD mice compared to the wt animals, whereas its abundance was reduced by beer and beer/yeast treatments. This is the first report of a relationship between AD and this fungal taxon that was instead previously associated with dysbiosis detected in a series of gut inflammatory diseases including Crohn's disease, colorectal cancer, myalgic encephalomyelitis and inflammatory bowel disease [50,51]. Interestingly, beer treatment successfully reduced the amount of these fungi in the gut of 3xTg-AD mice, eventually contributing to a reduction in the gut inflammatory condition.

This study provides supportive evidence for a beneficial role of fermented beverages in neurodegenerative disorders associated with aging. Collectively, our results indicate that a moderate intake of a yeast-enriched beer can successfully counteract AD major hallmarks and associated clinical manifestations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14122380/s1, Figure S1: Levels of gut hormones in control and treated mice; Figure S2: Mice body weight was monitored during the entire treatment period every week. The image shows body weight changes for control and treated wt and 3xTg-AD mice; Table S1: Significantly different bacterial taxa; Table S2: Linear discriminant analysis (LDA) effect size (LEfSe) between the differently treated AD and wt mice at the bacterial genus level.

Author Contributions: Conceptualization, A.M.E. and N.C.; methodology, V.C.; validation, V.C. and L.B.; formal analysis, V.C., L.B. and V.D.; investigation, O.G., I.V., G.P., S.D.C. and F.B.; data curation, V.C. and O.G.; writing—original draft preparation, V.C.; writing—review and editing, L.B., A.M.E., N.C., V.D. and M.A.; supervision and project administration, A.M.E. and N.C.; funding acquisition, N.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by ERAB: The European Foundation for Alcohol Research (grant number: EA1840).

Institutional Review Board Statement: The animal study protocol was approved by the Italian Ministry of Health (1D580.28, 625/2020-PR).

Data Availability Statement: Data is contained within the article or Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; in the decision to publish the results.

Abbreviations

AD: Alzheimer's disease; GIP, gastric inhibitory peptide; GLP, glucagon-like peptide-1; HIP, hippocampus; PFC, prefrontal cortex; NOR, novel object recognition; IL-1β, inteleukin-1β; TNF-α, tumor necrosis factor-α; Il-4, interleukin-4; IL-10, interleukin-10.

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ARTICLE

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NOP receptor antagonism attenuates reinstatement of alcohol-seeking through modulation of the mesolimbic circuitry in male and female alcohol-preferring rats

Anna Maria Borruto[1], Yannick Fotio[1], Serena Stopponi[1], Michele Petrella[1], Sara De Carlo[1], Ana Domi[1], Massimo Ubaldi[1], Friedbert Weiss[2] and Roberto Ciccocioppo[1]✉

In patients suffering from alcohol use disorder (AUD), stress and environmental stimuli associated with alcohol availability are important triggers of relapse. Activation of the nociceptin opioid peptide (NOP) receptor by its endogenous ligand Nociceptin/Orphanin FQ (N/OFQ) attenuates alcohol drinking and relapse in rodents, suggesting that NOP agonists may be efficacious in treating AUD. Intriguingly, recent data demonstrated that also blockade of NOP receptor reduced alcohol drinking in rodents. To explore further the potential of NOP antagonism, we investigated its effects on the reinstatement of alcohol-seeking elicited by administration of the α2 antagonist yohimbine (1.25 mg/kg, i.p.) or by environmental conditioning factors in male and female genetically selected alcohol-preferring Marchigian Sardinian (msP) rats. The selective NOP receptor antagonist LY2817412 (0.0, 3.0, 10.0, and 30.0 mg/kg) was first tested following oral (p.o.) administration. We then investigated the effects of LY2817412 (1.0, 3.0, 6.0 µg/µl/rat) microinjected into three candidate mesolimbic brain regions: the ventral tegmental area (VTA), the central nucleus of the amygdala (CeA), and the nucleus accumbens (NAc). We found that relapse to alcohol seeking was generally stronger in female than in male rats and oral administration of LY2817412 reduced yohimbine- and cue-induced reinstatement in both sexes. Following site-specific microinjections, LY2817412 reduced yohimbine-induced reinstatement of alcohol-seeking when administered into the VTA and the CeA, but not in the NAc. Cue-induced reinstatement was suppressed only when LY2817412 was microinjected into the VTA. Infusions of LY2817412 into the VTA and the CeA did not alter saccharin self-administration. These results demonstrate that NOP receptor blockade prevents the reinstatement of alcohol-seeking through modulation of mesolimbic system circuitry, providing further evidence of the therapeutic potential of NOP receptor antagonism in AUD.

Neuropsychopharmacology; https://doi.org/10.1038/s41386-021-01096-1

INTRODUCTION

Alcohol use disorder (AUD) is a chronic relapsing disease characterized by compulsive drinking and emergence of a negative emotional state when access to alcohol is prevented, heightening the risk of relapse to pathological drinking [1, 2]. In the last WHO report on the impact of alcohol on global health, it was reported that in 2016 alone more than 3 million deaths and 132.6 million disability-adjusted life years were caused by AUD (2018). This placed AUD as the fifth major risk factor for premature death and disability worldwide. Environmental factors such as drug-paired stimuli and stress are important elements that heighten vulnerability to relapse in abstinent detoxified alcoholics and present a major difficulty for the development of effective therapies to manage AUD [3–5]. Neurobiological mechanisms underlying relapse to alcohol-seeking are linked to profound counteradaptive changes in neuronal circuitries mediating motivation, emotions, and reward processing [6, 7]. Untangling these neuroadaptations is complex but essential to uncover the mechanisms of relapse to alcohol-seeking and to develop more efficacious therapies.

The nociceptin opioid peptide (NOP) receptor is the fourth member of the opioid subfamily of G-protein coupled receptors whose natural ligand is the 17 amino acid peptide Nociceptin/Orphanin FQ (N/OFQ) [8, 9]. Over the 25 years since receptor deorphanization, substantial progresses have been made to demonstrate that NOP can be a valuable therapeutic target for various pathological conditions. Clinical and preclinical studies have shown that NOP receptor agonists and antagonists attenuate pain and show promising effects in various psychiatric disorders such as major depression, anxiety, and addiction [10–12].

Preclinical data showing the efficacy of NOP agonism in preventing alcohol-seeking and relapse are particularly significant. In previous works we have demonstrated that activation of NOP by intracerebroventricular (i.c.v.) infusion of N/OFQ reduced stress-induced reinstatement of alcohol-seeking both in genetically selected Marchigian Sardinian alcohol-preferring (msP) rats and in

[1]School of Pharmacy, Pharmacology Unit, University of Camerino, Camerino, Italy. [2]Department of Molecular and Cellular Neuroscience, The Scripps Research Institute, La Jolla, CA, USA. ✉email: roberto.ciccocioppo@unicam.it

Received: 16 February 2021 Revised: 17 June 2021 Accepted: 28 June 2021
Published online: 20 July 2021

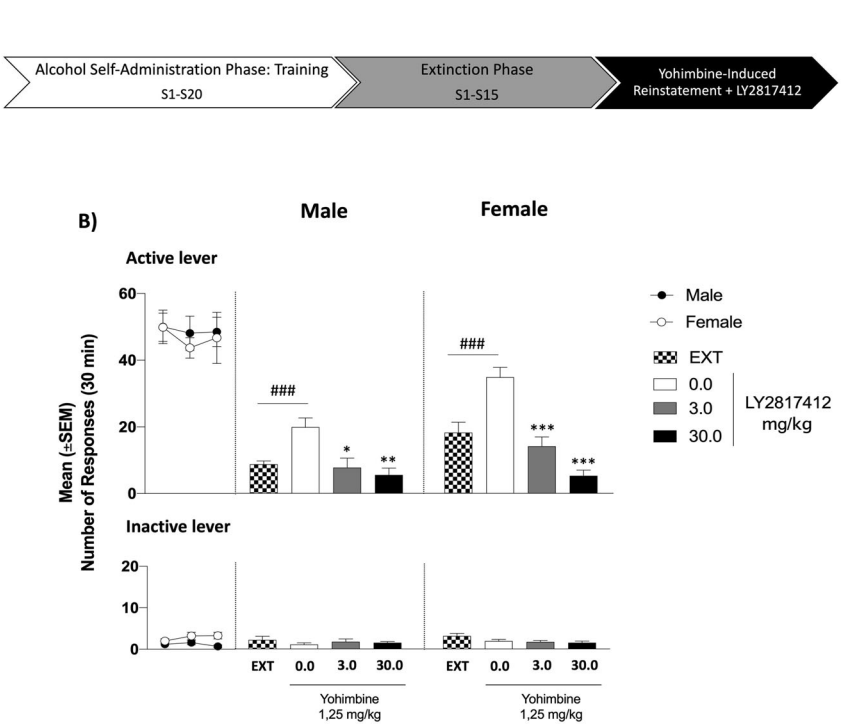


Fig. 1 Effect of Systemic Administration of LY2817412 on Yohimbine-Induced Reinstatement of Alcohol Seeking in Male and Female msP Rats. A Schematic representation of the experimental timeline. **B** Self-administration: black circles (male) and white circles (female) represent mean number of the responses during the last 3 days of alcohol self-administration sessions. No differences were denoted in the number of active or inactive lever presses during this phase. Extinction: mean number of lever presses during the last 3 days of extinction (EXT). Compared to extinction, male ($n = 10$) and female ($n = 10$) msP rats treated with yohimbine (1.25 mg/kg; i.p.) and LY2817412 vehicle (0.0) showed a significant reinstatement of responding. Administration of LY2817412 significantly reduced yohimbine-induced reinstatement both in males and females. Previously alcohol paired active and inactive lever presses are presented in the upper and lower panels, respectively. Values represent the mean (±SEM). $^{###}p < 0.001$, difference between EXT and rats treated with yohimbine plus LY2817412 vehicle (0.0); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, differences between rats treated with yohimbine and LY2817412 vehicle (0.0) and rats treated different doses of the antagonist.

post-dependent Wistar rats [13–15]. Moreover, we have showed that i.c.v. administration of N/OFQ significantly inhibited cue-induced reinstatement of extinguished alcohol-seeking in msP rats [13]. Recently, we found that blockade of NOP receptors by selective antagonists, also reduced alcohol drinking in rats and mice [16–18]. Furthermore, in an initial study we found that NOP blockade attenuated stress-induced reinstatement of alcohol-seeking in msP rats [16]. The mechanism through which both NOP receptor agonists and antagonists reduce alcohol drinking and reinstatement of alcohol-seeking is still unclear. Several hypotheses could be raised in the attempt to explain this paradoxical effect. For instance, NOP receptor agonists may depress N/OFQ signaling through receptor desensitization, leading to functional blockade of NOP receptors [16, 19]. Alternatively, it is possible that the effects of NOP receptor agonists and antagonists are mediated by different neurocircuitries or they may act at different levels within the same neural systems.

These findings prompted us to further investigate the pharmacological properties of NOP antagonists by exploring the efficacy of LY2817412, a potent and selective NOP blocker, on alcohol-seeking elicited by yohimbine or by environmental conditioning factors in an alcohol-preferring rat line. Moreover, to gather information on the action of NOP antagonists at the neurocircuitry level, we studied the effects of brain site-specific microinjection of LY2817412 on both yohimbine and cue-induced reinstatement. Guided by the role of the mesolimbic circuitry in mediating reinstatement and the distribution of NOP receptors in the brain [20, 21], we focused our attention on the ventral tegmental area (VTA), the central nucleus of the amygdala (CeA), and the nucleus accumbens (NAc). Finally, to determine the effect of sex in response to NOP antagonists, male and female msP rats were used.

MATERIALS AND METHODS

Animals

Male ($n = 101$) and female ($n = 102$) genetically selected alcohol-preferring msP rats were used. Experimental procedures were performed in accordance with the guidelines of the European Community Council Directive for Care and Use of Laboratory Animals and European legislation (2010/63/EU). Formal approval to conduct the experiments was obtained from the Italian Ministry of Health and the Organism Responsible for Animal Welfare of the University of Camerino (protocol no. 1D580.1). For details, see Supplementary Information.

Drugs

The following reagents and drugs were used: alcohol (10% v/v) prepared from alcohol 95% (FL Carsetti SNC, Camerino, Italy); saccharin (0.2% w/v; Sigma-Aldrich, Milan, Italy); yohimbine (Sigma-Aldrich, Milan, Italy); LY2817412 kindly provided by Eli Lilly (Indianapolis, IN, USA). For details, see Supplementary Information.

Intracranial surgery and infusion procedure

Bilateral guide cannulas (0.65 mm outside diameter) were aimed at the VTA, CeA and NAc with the following coordinates: [VTA: anterior/posterior (AP): −5.7 mm; medial/lateral (ML): ± 2.2 mm; dorsal/ventral (DV): −7.4 mm, 12° angle; CeA: AP: −2.3 mm, ML: ± 4.2 mm, DV: −6.5 mm; NAc: AP: + 1.5 mm; ML: ± 1.1 mm, DV: −5.5 mm] and female: [VTA: AP: −5.6 mm, ML: ± 2.0 mm, DV: −7.2 mm, 10° angle; CeA: AP: −1.8 mm, ML: ± 4.0 mm,

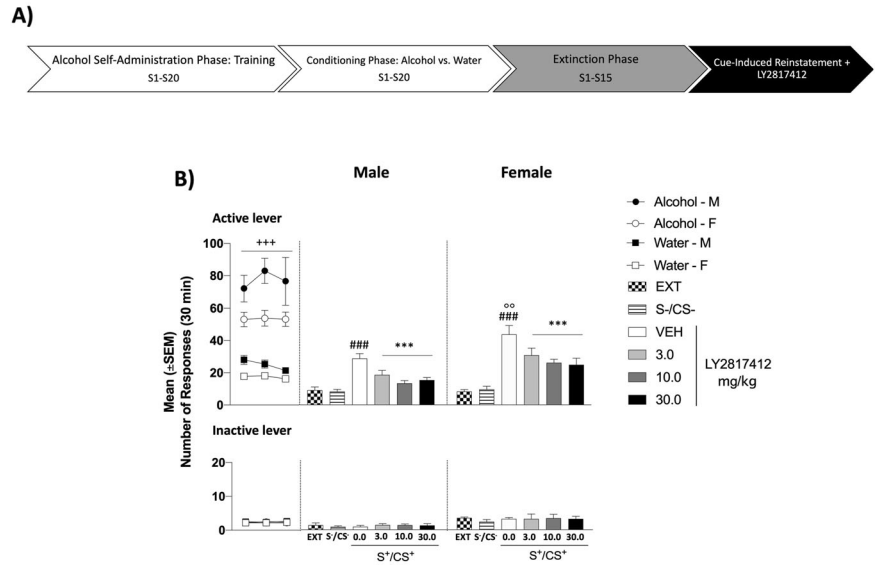


Fig. 2 Effect of Systemic Administration of LY2817412 on Cue-Induced Reinstatement of Alcohol-Seeking in Male and Female msP Rats. **A** Schematic representation of the experimental timeline. **B** Conditioning phase: black circles (male) and white circles (female) represent the responses during the last 3 days of alcohol self-administration sessions; black squares (male) and white squares (female) represents the responses during the last 3 days of water self-administration sessions during the discrimination phases. Analysis of this phase showed a significant time × drugs interaction for the active lever presses. No differences were denoted for the inactive lever. Extinction: mean number of lever presses during the last 3 days of extinction (EXT). Compared to extinction, male ($n = 8$) and female ($n = 10$) msP rats showed a significant reinstatement of lever pressing in response to alcohol cues (S^+/CS^+) but not to water (S^-/CS^-). Administration of LY2817412 significantly reduced cue (S^+/CS^+) induced reinstatement of alcohol seeking. Previously alcohol paired active and inactive lever presses are presented in the upper and lower panels, respectively. Values represent the mean (±SEM). $^{###}p < 0.001$, difference between EXT and rats exposed to alcohol paired cues (S^+/CS^+) treated with LY2817412 vehicle (0.0); $^{\circ\circ}p < 0.01$, difference in the reinstatement between male and female msP rats; $^{***}p < 0.001$, difference between rats presented with S^+/CS^+ and LY2817412 vehicle (0.0) and rats treated different doses of the antagonist.

DV: −6.4 mm; NAc: AP: + 1.40 mm, ML: ± 1.0 mm, DV: −5.2 mm]. For details see, Supplementary Information.

Alcohol and saccharin self-administration training

Operant training and testing were performed in standard self-administration operant chambers (Med Associate, Inc.). Male and female msP rats were trained to self-administer 10% (v/v) alcohol or 0.2% (w/v) saccharin for 5 days a week, in 30 min daily sessions under a fixed-ratio one schedule of reinforcement as previously described [22]. Alcohol and saccharin self-administration training was continued until animals reached a stable baseline of responding. After the acquisition phase for alcohol rats have been subjected to an extinction procedure followed by the relapse tests, whereas after saccharin training animals underwent directly to the testing phase. For details, see Supplementary Information.

EXPERIMENTAL PROCEDURES

Effect of systemic administration of LY2817412 on yohimbine-induced reinstatement of alcohol seeking in male and female msP rats

The experimental procedure consisted of three phases: operant training, extinction and reinstatement (for details, see the experimental timeline in Fig. 1A and Supplementary Information). Briefly, male ($n = 10$) and female ($n = 10$) msP rats was trained to self-administer 10% (v/v) alcohol in 30 min daily sessions. Training phase (total number of daily sessions: 20) continued until animals reached a stable baseline of responding. The mean of g/kg/30 min of alcohol consumed in the last 3 self-administration days was 1.30 for female rats and 0.86 for males. Rats were then subjected to 15 daily 30 min extinction sessions, followed by the reinstatement test. On the test days, animals were injected with either vehicle or LY2817412 (3.0 and 30.0 mg/kg; p.o.) 30 min prior to yohimbine (1.25 mg/kg; i.p.). Reinstatement sessions started 30 min after yohimbine administration. Experiments were carried out in a *Latin square* within-subjects counterbalanced design with a 3-day interval between drug tests during which animals were subjected to extinction sessions.

Effect of systemic administration of LY2817412 on cue-induced reinstatement of alcohol-seeking in male and female msP rats

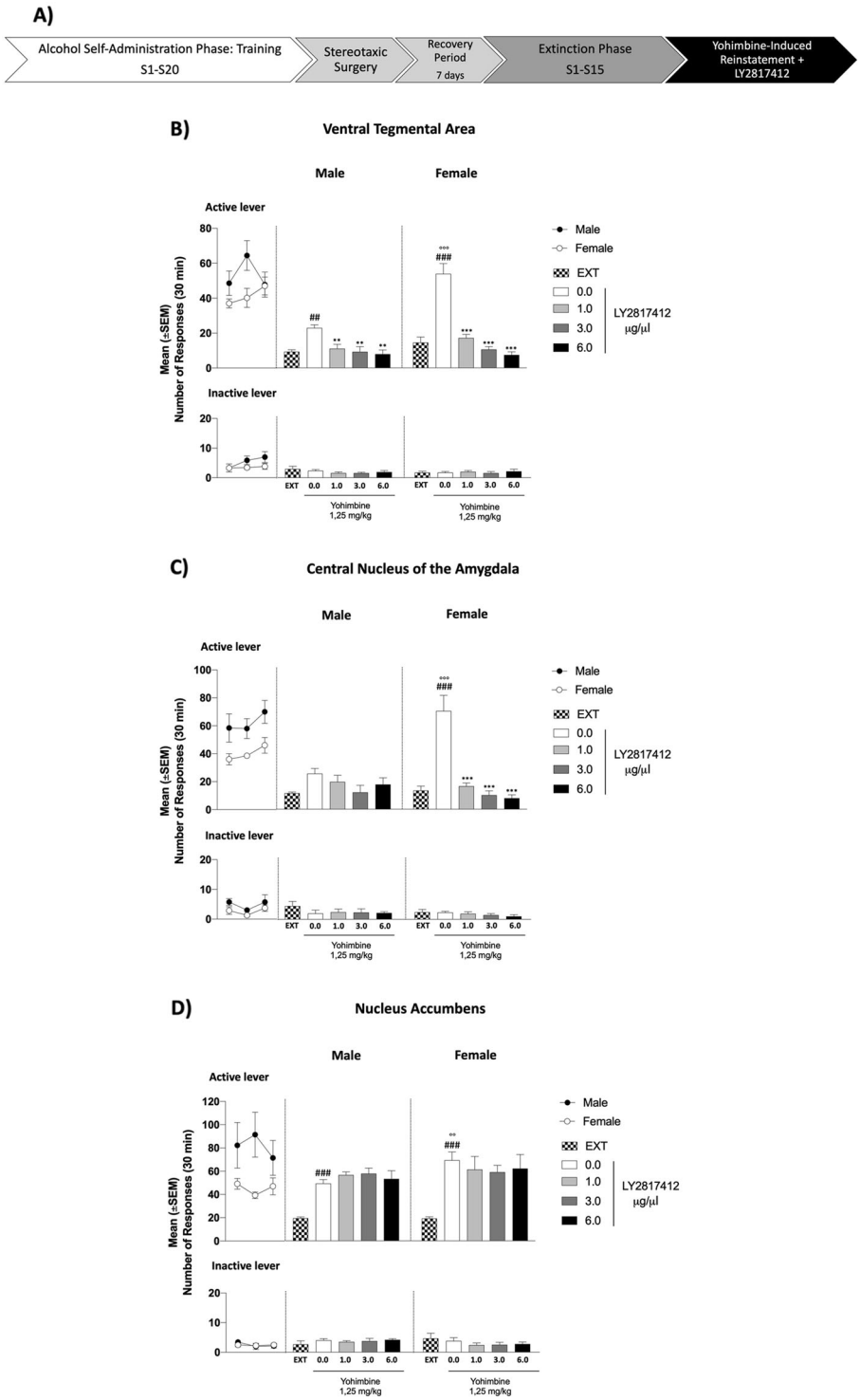
The experimental procedure consisted of four phases: operant training, conditioning, extinction, and reinstatement (for details, see the experimental timeline in Fig. 2B and Supplementary Information). Briefly, male ($n = 8$) and female ($n = 10$) msP rats were subjected to a self-administration/discrimination training procedure. During training the mean alcohol intake (g/kg/30 min) of the last 3 alcohol self-administration days was 1.44 for females and 1.01 for males. During the discrimination phase rats received a total of ten alcohol and ten water sessions. Discriminative stimuli (SD) predictive of alcohol (CS^+, odor of an orange extract) versus water availability (CS^-, odor of an anise extract) were presented during alcohol and water self-administration sessions, respectively. In addition, each lever press resulting in the delivery of alcohol was followed by a 5 s time-out period contingently paired with the illumination of the chamber's house light, while lever presses resulting in water delivery were accompanied by a 5 s time-out period paired with a 70 dB tone.

After completion of the conditioning phase, rats were subjected to a 15 daily 30 min extinction sessions during which lever pressing was no longer reinforced and cues were not present.

Followed the reinstatement tests in which the effect of LY2817412 (0.0, 3.0, 10.0, and 30.0 mg/kg; p.o.) was evaluated. The experiment was carried out in a *Latin square* within-subjects counterbalanced design with the drug given 1 h prior to the beginning of the sessions. Between reinstatement tests, animals remained confined in their home cages.

Effect of intracranial administration of LY2817412 on yohimbine- and cue-induced reinstatement of alcohol-seeking in male and female msP rats

For both yohimbine (1.25 mg/kg, i.p.) and cue-induced reinstatement, male and female msP rats were injected bilaterally with



LY2817412 (1.0, 3.0, 6.0 µg/0.5 µl/rat) or vehicle into the VTA (yohimbine: male/female $n = 12/9$; cue: male/female $n = 10/10$) the CeA (yohimbine: male/female $n = 9/9$; cues: male/female $n = 10/10$) or the NAc (yohimbine: male/female $n = 8/9$; cue: male/female $n = 9/10$). Experiments were carried out in a *Latin square* within-subjects counterbalanced design. During the last 3 self-administration training days the mean values of alcohol intake (g/kg/30 min) for yohimbine experiments were: male, 0.92 for VTA, 0.96 for CeA, 1.24 for NAc; female: 1.06 for VTA, 1.15 for CeA, 1.31 for NAc. For cue-experiments were: male, 0.92 for VTA, 0.93 for CeA, 0.95 for NAc; female: 1.58 for VTA, 1.53 for CeA, 1.44 for NAc. For details, see Supplementary Information (see also Supplementary Fig. 3 and Fig.4).

Effect of intracranial administration of LY2817412 on saccharin self-administration in male and female msP rats

To investigate the effect of NOP receptor blockade on 0.2% (w/v) saccharin self-administration, LY2817412 (1.0, 3.0, 6.0 µg/0.5 µl/rat) or its vehicle were bilaterally microinjected into the VTA (male/female $n = 13/12$) or the CeA (male/female $n = 12/13$).

Fig. 3 Effect of Intra-VTA, Intra-CeA and Intra-NAc Administration of LY2817412 on Yohimbine-Induced Reinstatement of Alcohol Seeking in Male and Female msP rats. A Schematic representation of the experimental timeline. **B**–**D** Self-administration: black circles (male) and white circles (female) represent the mean number of responses during the last 3 days of alcohol self-administration sessions. Self-administration: black (male) and white (female) circle represents the responses during the last 3 days of alcohol self-administration sessions. No differences were denoted in the number of active or inactive lever presses during this phase in all brain regions. Extinction: mean number of lever presses during the last 3 days of extinction (EXT). **B** Male ($n = 9$) and female ($n = 7$) msP rats were implanted with bilateral cannulas aimed at the VTA. Compared with EXT, yohimbine elicited a significant reinstatement of responding, both in male and in female rats. Intra-VTA administration of LY2817412 reduced the active lever responses elicited by yohimbine treatment in both sexes. **C** Male ($n = 7$) and female ($n = 7$) msP rats were implanted with bilateral cannulas aimed at the CeA. Compared with EXT, yohimbine elicited a significant reinstatement of responding in female but not in male subjects. Intra-CeA administration of LY2817412 reduced the active lever responses elicited by yohimbine treatment only in female rats. **D** Male ($n = 7$) and female ($n = 8$) msP rats were implanted with bilateral cannulas aimed at the NAc. Compared with EXT, yohimbine elicited a significant reinstatement of responding both in male and female msP rats. Values represent the mean (±SEM). $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, difference between EXT and rats treated with yohimbine plus LY2817412 vehicle (0.0); $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$, difference in the reinstatement between male and female msP rats; $^{**}p < 0.01$, $^{***}p < 0.001$, difference between rats treated with yohimbine and LY2817412 vehicle (0.0) and rats treated with different doses of the antagonist.

Experiments were carried out in a *Latin square* within-subjects counterbalanced design. A 3-day interval between drug tests was employed. Training data are reported as Supplementary Information (Supplementary Fig. 5).

Histological verification of correct cannula placement

Only data from rats with correct cannula placements were included in the statistical analysis [yohimbine: (male: $n = 9$ for VTA, $n = 7$ for CeA, $n = 7$ for NAc; female: $n = 7$ for VTA, $n = 7$ for CeA, $n = 8$ for NAc); cue: (male: $n = 7$ for VTA, $n = 7$ for CeA, $n = 8$ for NAc; female: $n = 7$ for VTA, $n = 8$ for CeA, $n = 8$ for NAc); saccharin: (male: $n = 10$ for VTA; $n = 10$ for CeA; female: $n = 9$ for VTA; $n = 9$ for CeA)]. For details, see Supplementary Information.

Statistical analysis

Appropriate ANOVAs were used with 'sex' as a between-subjects factor, treatment and 'time' as a within-subject factors. Where needed the Newman–Keuls *post-hoc* test was used. For details, see Supplementary Information.

RESULTS

Systemic administration of LY2817412 reduces yohimbine-induced reinstatement of alcohol seeking in male and female msP rats

During training the mean value of the last 3 days of alcohol self-administration was 48.87 ± 3.98 in males and 46.77 ± 3.98 in females. No significant differences were denoted in alcohol self-administration training both in the active lever [(sex: $F_{(1,\ 18)} = 0.14$, $p > 0.05$; time: $F_{(2,\ 36)} = 0.53$, $p > 0.05$; sex x time: $F_{(2,\ 36)} = 0.15$, $p > 0.05$)] and the inactive lever presses [(sex: $F_{(1,\ 18)} = 7.52$, $p < 0.05$; time: $F_{(2,\ 36)} = 1.06$, $p > 0.05$; sex x time: $F_{(2,\ 36)} = 1.35$, $p > 0.05$)]. During the extinction phase, the number of responding at the active lever progressively decreased to 8.73 ± 0.98 in male and to 18.23 ± 3.07 in female msP rats. As shown in Fig. 1B, overall ANOVA revealed a main effect of treatment, suggesting that administration of yohimbine reinstated the extinguished operant responding for alcohol (sex: $F_{(1,\ 18)} = 22.72$, $p < 0.001$; reinstatement: $F_{(1,\ 18)} = 28.52$, $p < 0.001$; sex × reinstatement: $F_{(1,\ 18)} = 1.08$, $p > 0.05$). A two-way ANOVA denoted that this effect was prevented by treatment with LY2817412 in both sexes (sex: $F_{(1,\ 18)} = 23.05$, $p < 0.001$; treatment: $F_{(2,\ 36)} = 31.68$, $p < 0.001$; sex × treatment: $F_{(2,\ 36)} = 3.48$, $p < 0.05$). Post-hoc analysis showed that both doses of LY2817412 tested, 3.0 and 30.0 mg/kg, were able to reduce yohimbine-induced reinstatement in male (3.0, $p < 0.05$, 30.0, $p < 0.01$) and in female ($p < 0.001$) msP rats. Responding at the inactive lever was negligible and unchanged either by yohimbine (sex: $F_{(1,18)} = 2.69$, $p > 0.05$; relapse: $F_{(1,\ 18)} = 3.05$, $p > 0.05$; sex × relapse: $F_{(1,\ 18)} = 0.02$, $p > 0.05$) or LY2817412 (sex: $F_{(1,\ 18)} = 0.57$, $p > 0.05$; treatment: $F_{(2,\ 36)} = 0.13$, $p > 0.05$; sex × treatment: $F_{(2,\ 36)} = 0.60$, $p > 0.05$) (Fig. 1B).

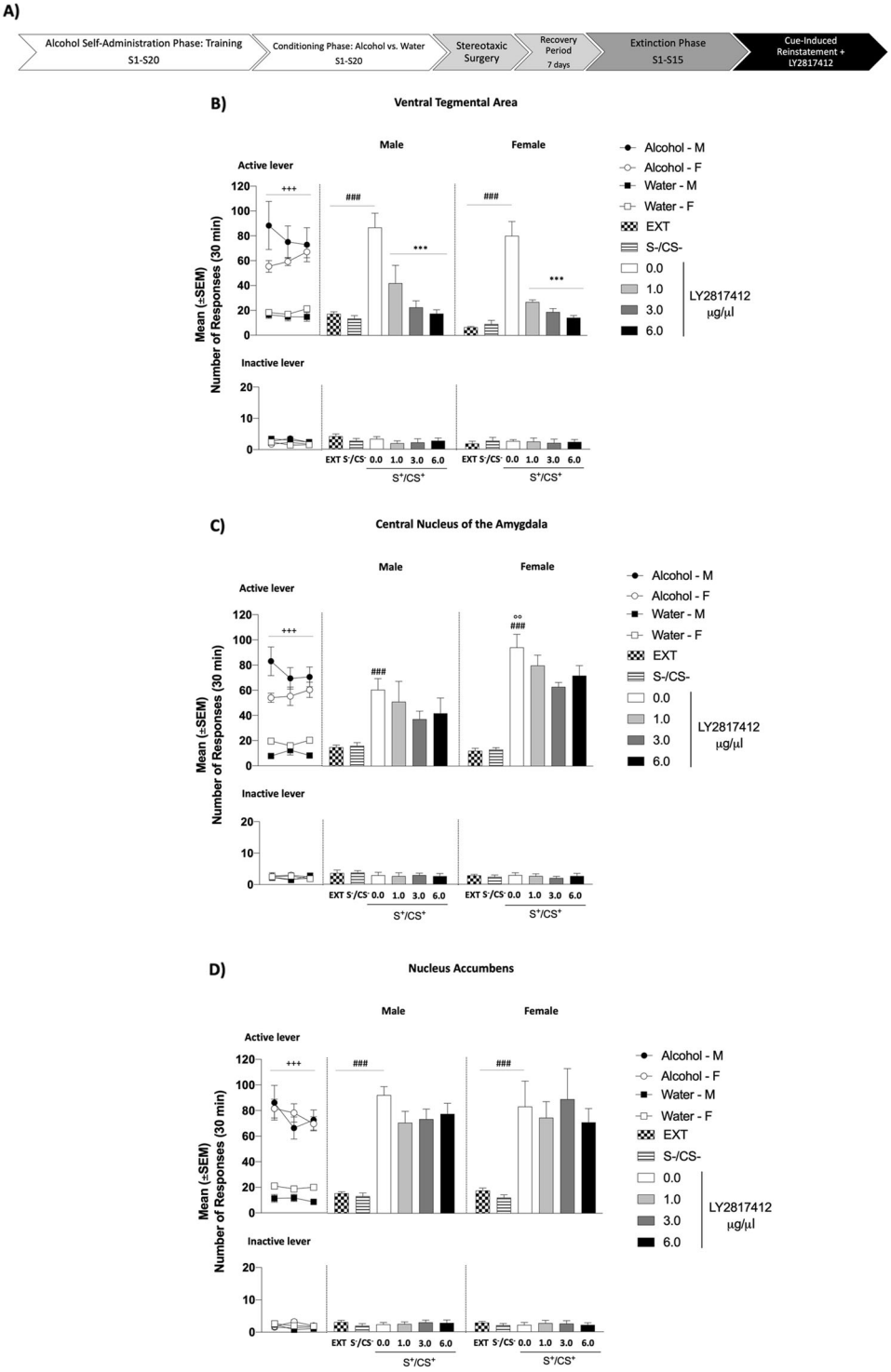
Systemic administration of LY2817412 reduces cue-induced reinstatement of alcohol seeking in male and female msP rats

During the conditioning phase, both male and female msP rats learned to discriminate between alcohol and water availability. At the end of this phase, the number of alcohol-reinforced responses was significantly higher compared to water-reinforced responses [(sex: $F_{(1,\ 16)} = 7.63$, $p < 0.05$; time: $F_{(2,\ 32)} = 68.31$, $p < 0.001$; time × sex: $F_{(2,\ 32)} = 4.13$, $p < 0.05$; drugs: $F_{(1,\ 16)} = 19.88$, $p < 0.001$; drugs × sex: $F_{(1,\ 16)} = 0.54$, $p > 0.05$; time × drugs: $F_{(2,\ 32)} = 32.54$, $p < 0.001$; time × drugs × sex $F_{(2,\ 32)} = 2.48$, $p > 0.05$)]. Alcohol-reinforced responses progressively diminished throughout the extinction phase from 77.25 ± 9.38 to 9.17 ± 1.98 in males, and from 55.37 ± 3.44 to 8.33 ± 1.10 in female msP rats. Two-way ANOVA revealed that presentation of cues predictive of alcohol availability significantly increased alcohol seeking [(sex: $F_{(1,\ 16)} = 3.74$, $p > 0.05$; reinstatement: $F_{(2,\ 32)} = 57.23$, $p < 0.001$; sex × reinstatement: $F_{(2,\ 32)} = 4.17$, $p < 0.05$)]. Post-hoc analysis revealed higher level of reinstatement in female msP rats compared to their male counterpart ($p < 0.01$, Fig. 2B). In addition, ANOVA revealed a main effect of treatment, with a decreased reinstatement elicited by alcohol-paired cues after systemic administration of LY2817412 [(sex: $F_{(1,\ 16)} = 10.06$, $p < 0.01$; treatment: $F_{(3,\ 48)} = 15.15$, $p < 0.001$; sex × treatment: $F_{(3,\ 48)} = 0.32$, $p > 0.05$)] (Fig. 2B).

Responses at the inactive lever were negligible and not significantly affected by the presentation of cues during the conditioning phase [(sex: $F_{(1,\ 16)} = 0.31$, $p > 0.05$; time: $F_{(2,\ 32)} = 0.00$, $p > 0.05$; time × sex: $F_{(2,\ 32)} = 0.02$, $p > 0.05$; drugs: $F_{(1,\ 16)} = 0.17$, $p > 0.05$; drugs × sex: $F_{(1,\ 16)} = 0.17$, $p > 0.05$; time × drugs: $F_{(2,\ 32)} = 0.05$, $p > 0.05$; time × drugs × sex $F_{(2,\ 32)} = 0.05$, $p > 0.05$)], reinstatement [(sex: $F_{(1,\ 16)} = 30.80$, $p < 0.001$; reinstatement: $F_{(2,\ 32)} = 1.32$, $p > 0.05$; sex × reinstatement: $F_{(2,\ 32)} = 0.36$, $p > 0.05$)] and LY2817412 treatment [(sex: $F_{(1,\ 16)} = 8.62$, $p < 0.01$; treatment: $F_{(3,\ 48)} = 0.11$, $p > 0.05$; sex × treatment: $F_{(3,\ 48)} = 0.05$, $p > 0.05$)] (Fig. 2B).

Intracranial administration of LY2817412 into the VTA and CeA but not into the NAc reduces yohimbine-induced reinstatement of alcohol seeking in male and female msP rats

To better investigate the neural substrates involved in the effect of the systemic LY2817412 treatment, we evaluated the effects of LY2817412 microinjections in the VTA, CeA, and NAc on yohimbine-induced reinstatement of alcohol seeking. During the training phase, all experimental groups acquired alcohol self-administration and reached a stable baseline of active lever responses, which was progressively decreased during the extinction phase [(VTA, male: from 53.59 ± 6.42 to 9.30 ± 1.05; female: from 42.0 ± 3.90 to 14.48 ± 3.22; CeA, male: from 62.14 ± 6.53 to 11.48 ± 0.79; female: from 40.19 ± 3.36 to 13.38 ± 3.17; NAc, male: from 81.71 ± 17.12 to 19.48 ± 1.05; female: from 45.17 ± 3.13 to 19.29 ± 1.92)] (Fig. 3B, D). No significant differences were denoted in alcohol self-administration training both in the active lever [VTA:



(sex: $F_{(1,\ 14)} = 2.28$, $p > 0.05$; time: $F_{(2,\ 28)} = 2.29$, $p > 0.05$; sex × time: $F_{(2,\ 28)} = 3.51$, $p > 0.05$); CeA: (sex: $F_{(1,\ 12)} = 8.92$, $p > 0.05$; time: $F_{(2,\ 24)} = 2.58$, $p > 0.05$; sex × time: $F_{(2,\ 24)} = 0.10$, $p > 0.05$); NAc: (sex: $F_{(1,\ 13)} = 5.04$, $p > 0.05$; time: $F_{(2,\ 26)} = 0.73$, $p > 0.05$; sex × time: $F_{(2,\ 26)} = 2.71$, $p > 0.05$)] and the inactive lever presses [VTA: (sex: $F_{(1,\ 14)} = 1.96$, $p > 0.05$; time: $F_{(2,\ 28)} = 1.67$, $p > 0.05$; sex × time: $F_{(2,\ 28)} = 0.96$, $p > 0.05$); CeA: (sex: $F_{(1,\ 12)} = 3.94$, $p > 0.05$; time: $F_{(2,\ 24)} = 2.19$, $p > 0.05$; sex × time: $F_{(2,\ 24)} = 0.10$, $p > 0.05$); NAc: (sex: $F_{(1,\ 13)} = 0.02$, $p > 0.05$; time: $F_{(2,\ 26)} = 1.24$, $p > 0.05$; sex × time: $F_{(2,\ 26)} = 0.82$, $p > 0.05$)]. As revealed by two-way ANOVA, administration of yohimbine significantly reinstated the operant response for alcohol in all the experimental groups [VTA: (sex: $F_{(1,\ 14)} = 23.62$, $p < 0.001$, reinstatement: $F_{(1,14)} = 118.60$, $p < 0.001$, sex × reinstatement: $F_{(1,\ 14)} = 28.11$, $p < 0.001$); CeA: (sex: $F_{(1,\ 12)} = 11.76$, $p < 0.01$; reinstatement: $F_{(1,\ 12)} = 44.24$, $p < 0.001$; sex × reinstatement: $F_{(1,\ 12)} = 16.26$, $p < 0.01$); NAc: (sex: $F_{(1,\ 13)} = 4.99$, $p < 0.05$, reinstatement: $F_{(1,\ 13)} = 101.51$, $p < 0.001$, sex × reinstatement: $F_{(1,\ 13)} = 6.46$, $p < 0.05$)] (Fig. 3). Post-hoc analysis revealed that female msP rats showed a more pronounced reinstatement than males following yohimbine treatment in all the

Fig. 4 Effect of Intra-VTA, Intra-CeA and Intra-NAc Administration of LY2817412 on Cue-Induced Reinstatement of Alcohol Seeking in Male and Female msP rats. A Schematic representation of the experimental timeline. **B–D** Conditioning phase: black circles (male) and white circles (female) represent the responses during the last 3 days of alcohol self-administration; black squares (male) and white squares (female) represent the responses during the last 3 days of water self-administration during the discrimination phases. Analysis of this phase showed a significant time × drugs interaction in all brain regions for the active lever presses. No differences were denoted for the inactive lever. Extinction: mean number of lever presses during the last 3 days of extinction (EXT). **B** Male ($n = 7$) and female ($n = 7$) rats were implanted with bilateral cannulas aimed at the VTA. Compared to EXT, rats exposed to alcohol-predictive of discriminative stimuli (S^+/CS^+) and treated with LY2817412 vehicle (0.0) reinstated active lever pressing. Intra-VTA administration of the drug attenuated the reinstatement elicited by the alcohol-predictive discriminative stimuli. **C** Male ($n = 7$) and female ($n = 8$) rats were implanted with bilateral cannulas aimed at the CeA. Compared to EXT, rats exposed to alcohol-predictive of discriminative stimuli (S^+/CS^+) and treated with LY2817412 vehicle (0.0) reinstated active lever pressing. Intra-CeA administration of the NOP antagonist did not prevent the effect of S^+/CS^+. **D** Male ($n = 8$) and female ($n = 8$) msP rats were implanted with bilateral cannulas aimed at the NAc. Compared to EXT, rats exposed to the alcohol-predictive stimuli (S^+/CS^+) elicited a significant reinstatement of responding. Intra-NAc administration of the NOP antagonist did not prevent the effect of S^+/CS^+. Presentation of water paired cues (S^-/CS^-) never affected operant responding that in all groups remained at extinction level. Values represent the mean (±SEM). $^{###}p < 0.001$, difference between EXT and rats exposed to alcohol paired cues (S^+/CS^+) treated with LY2817412 vehicle (0.0); °°$p < 0.001$, difference in the reinstatement between male and female msP rats; $^{***}p < 0.001$, difference between rats presented with S^+/CS^+ and LY2817412 vehicle (0.0) and rats treated different doses of the antagonist.

groups (VTA: $p < 0.001$; CeA: $p < 0.001$; NAc: $p < 0.01$). Yohimbine did not significantly increase active lever presses in male rats microinjected into the CeA ($p > 0.05$). When the effect of LY2817412 was evaluated, overall ANOVA showed a main effect of intra-VTA (Fig. 3B) and intra-CeA (Fig. 3C) treatments in preventing yohimbine-induced reinstatement of alcohol seeking behavior [VTA: (sex: $F_{(1,\ 14)} = 12.37$, $p < 0.01$, treatment: $F_{(3,\ 42)} = 67.46$, $p < 0.001$, sex × treatment: $F_{(3,\ 42)} = 17.86$, $p < 0.001$); CeA: (sex: $F_{(1,\ 12)} = 2.44$, $p > 0.05$, treatment: $F_{(3,\ 36)} = 25.25$, $p < 0.001$, sex × treatment: $F_{(3,\ 36)} = 13.48$, $p < 0.001$)]. Post-hoc analysis revealed that all doses of LY2817412 (1.0, 3.0, 6.0 µg/0.5 µl/rat) infused in the VTA attenuated yohimbine-induced reinstatement both in male ($p < 0.01$) and female ($p < 0.001$) rats. When injected into the CeA LY2817412 reduced yohimbine-induced alcohol-seeking only in female ($p < 0.001$). Microinjection of LY2817412 into the NAc (Fig. 3D) did not significantly affect yohimbine-induced reinstatement of alcohol seeking in both sexes [(NAc, sex: $F_{(1,\ 13} = 0.98$, $p > 0.05$, treatment: $F_{(3,\ 39)} = 0.03$, $p > 0.05$, sex × treatment: $F_{(3,\ 39)} = 1.08$, $p > 0.05$)].

The number of responses at the inactive control lever was very low throughout all the experiments and was not influenced by yohimbine [VTA: (sex: $F_{(1,\ 14)} = 2.15$, $p > 0.05$, reinstatement: $F_{(1,14)} = 0.23$, $p > 0.05$, sex × reinstatement: $F_{(1,\ 14)} = 0.23$, $p > 0.05$); CeA: (sex: $F_{(1,\ 12)} = 0.72$, $p > 0.05$; reinstatement: $F_{(1,\ 12)} = 1.44$, $p > 0,05$; sex × reinstatement: $F_{(1,\ 12)} = 1.14$, $p > 0.05$); NAc: (sex: $F_{(1,\ 13)} = 0.53$, $p > 0.05$, reinstatement: $F_{(1,\ 13)} = 0.04$, $p > 0.05$, sex × reinstatement: $F_{(1,\ 13)} = 0.74$, $p > 0.05$)] nor it was affected by LY2817412 [VTA: (sex: $F_{(1,\ 14)} = 0.00$, $p > 0.05$, treatment: $F_{(3,\ 42)} = 0.49$, $p > 0.05$, sex × treatment: $F_{(3,\ 42)} = 0.55$, $p > 0.05$); CeA: (sex: $F_{(1,\ 12)} = 0.41$ $p > 0.05$; treatment: $F_{(3,36)} = 0.48$, $p > 0,05$; sex × treatment: $F_{(3,\ 36)} = 0.57$, $p > 0.05$); NAc: (sex: $F_{(1,\ 13)} = 1.95$, $p > 0.05$, treatment: $F_{(3,\ 39)} = 0.84$, $p > 0.05$, sex × treatment: $F_{(3,\ 39)} = 0.29$, $p > 0.05$)] (Fig. 3).

Intracranial administration of LY2817412 into the VTA but not into the CeA and the NAc reduces cue-induced reinstatement of alcohol seeking in male and female msP rats

During the conditioning phase, all experimental groups learned to discriminate between alcohol and water availability (Fig. 4A–F) and showed a significant higher number of alcohol-reinforced responses when compared to water-reinforced responses [VTA: (sex: $F_{(1,\ 12)} = 0.95$, $p > 0.05$; time: $F_{(2,\ 24)} = 40.00$, $p < 0.001$; time × sex: $F_{(2,\ 24)} = 3.26$, $p < 0.05$; drugs: $F_{(1,\ 12)} = 23.26$, $p < 0.001$; drugs × sex: $F_{(1,\ 12)} = 1.66$, $p > 0.05$; time × drugs: $F_{(2,\ 24)} = 24.43$, $p < 0.001$; time × drugs × sex: $F_{(2,\ 24)} = 0.29$, $p > 0.05$); CeA: (sex: $F_{(1,\ 13)} = 1.14$, $p > 0.05$; time: $F_{(2,\ 26)} = 93.30$, $p < 0.001$; time × sex: $F_{(2,\ 26)} = 8.41$, $p < 0.01$; drugs: $F_{(1,\ 13)} = 41.62$, $p < 0.001$; drugs × sex: $F_{(1,\ 13)} = 7.43$, p; time × drugs: $F_{(2,\ 26)} = 24.24$, $p < 0.001$; time × drugs × sex: $F(2,\ 26) = 5.03$, $p > 0.05$); NAc: (sex: $F_{(1,\ 14)} = 0.82$, $p > 0.05$; time: $F_{(2,\ 28)} = 93.87$, $p < 0.001$; time × sex: $F_{(2,\ 28)} = 0.26$, $p > 0.05$; drugs: $F_{(1,\ 14)} = 52.17$, $p < 0.001$; drugs × sex: $F_{(1,\ 14)} = 3.19$, $p > 0.05$; time × drugs: $F_{(2,\ 28)} = 29.89$, $p < 0.001$; time × drugs × sex: $F_{(2,\ 28)} = 0.34$, $p > 0.05$)]. Alcohol-reinforced responses progressively decreased during the extinction phase [VTA, male: from 78.71 ± 13.90 to 17.33 ± 1.72; female: from 60.57 ± 2.87 to 6.52 ± 0.77; CeA, male: from 74.33 ± 5.68 to 14.90 ± 1.82; female: from 56.54 ± 4.19 to 12.17 ± 2.02; NAc, male: from 75.04 ± 9.11 to 15.67 ± 1.17; female: from 76.42 ± 3.98 to 18.21 ± 1.96]. ANOVA revealed that presentation of cues predictive of alcohol availability significantly increased alcohol seeking behavior in all the experimental groups [VTA: (sex: $F_{(1,\ 12)} = 1.91$, $p > 0.05$, reinstatement: $F_{(2,\ 24)} = 70.38$, $p < 0.001$; sex × reinstatement: $F_{(2,\ 24)} = 0.10$, $p > 0.05$); CeA: (sex: $F_{(1,\ 13)} = 3.95$, $p > 0.05$; reinstatement: $F_{(2,\ 26)} = 74.55$, $p < 0.001$; sex × reinstatement: $F_{(2,\ 26)} = 6.18$, $p < 0.01$); NAc: (sex: $F_{(1,\ 14)} = 0.12$, $p > 0.05$; reinstatement: $F_{(2,\ 28)} = 49.95$, $p < 0.001$; sex × reinstatement: $F_{(2,\ 28)} = 0.23$, $p > 0.05$)]. Post-hoc analysis showed that female rats with cannulas implanted in the CeA reinstated higher than male counterparts ($p < 0.01$, Fig. 4C). In addition, ANOVA revealed a main effect of treatment when LY2817412 was administered in the VTA [(sex: $F_{(1,\ 12)} = 1.13$, $p > 0.05$; treatment: $F_{(3,\ 36)} = 33.84$, $p < 0.001$; sex × treatment: $F_{(3,\ 36)} = 0.27$, $p > 0.05$)] (Fig. 4B), but not into the CeA [(sex: $F_{(1,\ 13)} = 12.32$, $p < 0.01$; treatment: $F_{(3,\ 39)} = 2.55$, $p > 0.05$; sex × treatment: $F_{(3,\ 39)} = 0.07$, $p > 0.05$)] (Fig. 4C) and the NAc [(sex: $F_{(1,\ 14)} = 0.00$, $p > 0.05$; treatment: $F_{(3,\ 42)} = 1.66$, $p > 0.05$; sex × treatment: $F_{(3,\ 42)} = 1.08$, $p > 0.05$)] (Fig. 4D).

Responses at the inactive lever were negligible and not significantly affected either by cues presentation during the conditioning phase (data not shown), reinstatement [VTA: (sex: $F_{(1,\ 12)} = 1.43$, $p > 0.05$; reinstatement: $F_{(2,\ 24)} = 0.10$, $p > 0.05$; sex × reinstatement: $F_{(2,\ 24)} = 2.10$, $p > 0.05$); CeA: (sex: $F_{(1,\ 13)} = 1.37$, $p > 0.0l5$; reinstatement: $F_{(2,\ 26)} = 0.11$, $p > 0.05$; sex × reinstatement: $F_{(2,\ 26)} = 0.45$, $p > 0.05$); NAc: (sex: $F_{(1,\ 14)} = 0.00$, $p > 0.05$; reinstatement: $F_{(2,\ 28)} = 1.94$, $p > 0.05$; sex × reinstatement: $F_{(2,\ 28)} = 0.04$, $p > 0.05$)] or by LY2817412 [VTA: (sex: $F_{(1,\ 12)} = 0.05$, $p > 0.05$; treatment: $F_{(3,\ 36)} = 0.43$, $p > 0.05$; sex × treatment: $F_{(3,\ 36)} = 0.21$, $p > 0.05$); CeA: (sex: $F_{(1,\ 13)} = 0.10$, $p > 0.05$; treatment: $F_{(3,\ 39)} = 0.11$, $p > 0.05$; sex × treatment: $F_{(3,\ 39)} = 0.16$, $p > 0.05$); NAc: (sex: $F_{(1,\ 14)} = 0.08$, $p > 0.05$; treatment: $F_{(3,\ 42)} = 0.21$, $p > 0.05$; sex × treatment: $F_{(3,\ 42)} = 0.17$, $p > 0.05$)] (Fig. 4).

Intracranial administration of LY2817412 into the VTA and the CeA does not reduce saccharin self-administration in male and female msP rats

During the training phase, all experimental groups acquired saccharin self-administration and reached a stable baseline of rewards [(VTA, male: 84.03 ± 8.55; female: 96.37 ± 6.09; CeA, male: 82.97 ± 7.32; female: 93.56 ± 3.04)] and active lever presses [(VTA,

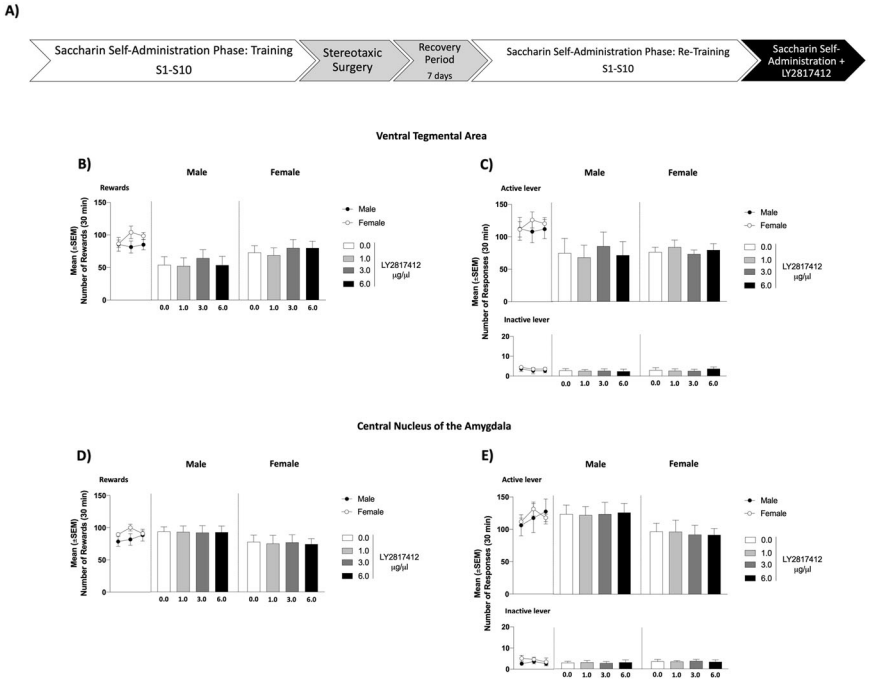


Fig. 5 Effect of Intra-VTA and Intra-CeA Administration of LY2817412 on Saccharin Self-Administration in Male and Female msP rats. A Schematic representation of the experimental timeline. **B–E** Black circles (male) and white circles (female) represent the mean number of rewards (**B, D**), active (**C**, **E** upper panel) and inactive (**C**, **E** lower panel) levers during the last 3 days of saccharin self-administration sessions**. B, C** Male ($n = 10$) and female ($n = 9$) msP rats microinjected into the VTA with LY2817412 did not show changes in saccharin reward or in the total number of lever pressing at both the active and inactive levers. **D, E** Male ($n = 10$) and female ($n = 9$) msP rats microinjected into the CeA with LY2817412 did not show changes in saccharin reward or in the total number of lever pressing at both the active and inactive levers. Values represent the mean (±SEM).

male: 110 ± 15.98; female: 119 ± 9.59; CeA, male: 117 ± 15.81; female: 120.44 ± 5.57)] (Fig. 5). No significant differences were denoted in saccharin self-administration training both in the number of rewards [VTA: (sex: $F_{(1,\ 17)} = 1.33$, $p > 0.05$; time: $F_{(2,\ 34)} = 1.24$, $p > 0.05$; sex × time: $F_{(2,\ 34)} = 2.83$, $p > 0.05$); CeA: (sex: $F_{(1,\ 17)} = 1.64$, $p > 0.05$; time: $F_{(2,\ 34)} = 1.22$, $p > 0.05$; sex × time: $F_{(2,\ 34)} = 1.48$, $p > 0.05$)] active lever [VTA: (sex: $F_{(1,\ 17)} = 0.20$, $p > 0.05$; time: $F_{(2,\ 34)} = 0.40$, $p > 0.05$; sex × time: $F_{(2,\ 34)} = 1.19$, $p > 0.05$); CeA: (sex: $F_{(1,\ 17)} = 0.03$, $p > 0.05$; time: $F_{(2,\ 24)} = 1.13$, $p > 0.05$; sex × time: $F_{(2,\ 34)} = 0.57$, $p > 0.05$)] and inactive lever presses [VTA: (sex: $F_{(1,\ 17)} = 1.02$, $p > 0.05$; time: $F_{(2,\ 34)} = 0.60$, $p > 0.05$; sex × time: $F_{(2,\ 34)} = 0.00$, $p > 0.05$); CeA: (sex: $F_{(1,\ 17)} = 1.44$, $p > 0.05$; time: $F_{(2,\ 34)} = 0.73$, $p > 0.05$; sex × time: $F_{(2,\ 34)} = 0.45$, $p > 0.05$)]. As revealed by two-way ANOVA, administration of LY2817412 did not modify the operant response for saccharin in all the experimental groups [rewards: VTA: (sex: $F_{(1,\ 17)} = 1.45$, $p > 0.05$, treatment: $F_{(3,\ 51)} = 1.62$, $p > 0.05$, sex × treatment: $F_{(3,\ 51)} = 0.40$, $p > 0.05$ (**Fig. B**); CeA: (sex: $F_{(1,\ 17)} = 1.97$, $p > 0.05$; treatment: $F_{(3,\ 51)} = 0.04$, $p > 0.05$; sex × treatment: $F_{(3,\ 51)} = 0.03$, $p > 0.05$ (**Fig. D**); active lever: VTA: (sex: $F_{(1,\ 17)} = 0.79$, $p > 0.05$, treatment: $F_{(3,\ 51)} = 0.23$, $p > 0.05$, sex × treatment: $F_{(3,\ 51)} = 2.68$, $p > 0.05$ (**Fig. C**); CeA: (sex: $F_{(1,\ 17)} = 3.30$, $p > 0.05$; treatment: $F_{(3,\ 51)} = 0.01$, $p > 0.05$; sex × treatment: $F_{(3,\ 51)} = 0.08$, $p > 0.05$ (**Fig. E**)]. Responding at the inactive lever was negligible and unchanged by LY2817412 [VTA: (sex: $F_{(1,\ 17)} = 0.29$, $p > 0.05$, treatment: $F_{(3,\ 51)} = 0.01$, $p > 0.05$, sex x treatment: $F_{(3,\ 51)} = 0.19$, $p > 0.05$); CeA: (sex: $F_{(1,\ 17)} = 0.58$, $p > 0.05$; treatment: $F_{(3,\ 51)} = 0.00$, $p > 0.05$; sex × treatment: $F_{(3,\ 51)} = 0.10$, $p > 0.05$] (Fig. 5).

DISCUSSION

The results demonstrated that systemic NOP receptor blockade by LY2817412 significantly attenuated yohimbine- and cue-induced reinstatement of alcohol-seeking in male and female msP rats. These data strengthen recent evidence indicating a role of NOP receptor antagonists in reducing alcohol drinking behavior [16–18]. Here LY2817412 produced a significant decrease of yohimbine-induced reinstatement, replicating the result of earlier work in which a similar effect was obtained with LY2940094, another selective NOP receptor antagonist [16, 23]. With the present work, we also discovered for the first time that yohimbine-induced reinstatement is reduced following blockade of NOP receptors in the VTA and in the CeA but not in the NAc. When we studied the effect of LY2817412 on cue-induced reinstatement, we again observed a significant reduction of lever pressing in msP rats. In this case, the effect was replicated following microinjection of the NOP antagonist into the VTA, but not into the CeA and the NAc. The effect of LY2817412 was specific for alcohol as intra-VTA and intra-CeA infusions of the compound did not modify lever pressing for saccharin. Consistent with this finding, in an earlier study, we found that LY2817412 reduced the intake of alcohol but not that of food and water in the rat [17]. In addition, when rats were treated with the NOP antagonist during the extinction phase (Supplementary Information), it did not affect lever pressing. This finding suggests that blockade of NOP receptors no longer produces its effects if operant behavior is decontextualized from alcohol drinking or is not triggered by yohimbine.

Administration of NOP receptor antagonist LY2817412 in the VTA reduces yohimbine- and cue-induced reinstatement

Noteworthy, previous studies showed that activation of NOP receptors prevents stress- and cue-induced reinstatement of alcohol seeking [14, 24–27]. For instance, i.c.v. administration of N/OFQ markedly inhibited footshock stress- and cue-induced reinstatement of alcohol-seeking in msP rats [13, 14], and subchronic treatment with MT-7716, a selective NOP receptor agonist, reduced yohimbine- and cue-induced reinstatement in msPs and in post-dependent Wistar rats [15]. Finally, SR-8993,

another selective NOP receptor agonist, prevented yohimbine-induced reinstatement in Wistar rats [27]. These effects observed following NOP receptor activation might depend on their ability to negatively modulate the activity of the mesolimbic dopamine (DA) system, which is known to play a crucial role in the regulation of stress- and cue-induced reinstatement [28, 29]. Notably, NOP receptors are widely expressed (~75%) on VTA tyrosine hydroxylase positive neurons [30] and N/OFQ inhibits DA neuronal activity in this region [31]. In agreement with these data, intra-VTA administration of N/OFQ attenuated basal DA release in the NAc [32]. Moreover, it was demonstrated that i.c.v. administration of the peptide dampened morphine- and cocaine-induced increases in extracellular DA levels in the NAc [33, 34]. Finally, retrodialysis of N/OFQ into the NAc attenuated the ability of cocaine to enhance local extracellular DA levels into the NAc [35]. Intriguingly, NOP receptor antagonism, produced similar effects as it resulted in a significant decrease of alcohol-induced enhancement of DA outflow in the NAc [16]. Stemming from this latter finding and considering the prominent role of the VTA in modulating alcohol reward and reinforcement, we sought to determine the effect of LY2817412 microinjection into this region [36–38]. Results revealed that intra-VTA infusions of this NOP antagonist reduced both yohimbine- and cue- induced reinstatement, making therefore plausible the hypothesis that receptor blockade could modulate alcohol-seeking via modulation of DA transmission in the VTA. An intriguing finding is that NOP receptor activation and blockade may produce overlapping neurochemical and behavioral effects and that the brain regions (i.e., the VTA and the CeA) mediating these actions are the same. The mechanisms through which this may occur are not yet clear, and to disentangle this complex phenomenon may not be easy, however, several hypotheses can be suggested. For example, it is possible that administration of non-physiological doses of NOP agonists may produce paradoxical antagonistic effects by depressing N/OFQ transmission through receptor desensitization. This possibility is supported by data showing that NOP receptors are subject to desensitization within minutes following administration of an agonist [39]. Consistent with the desensitization hypothesis there are also data showing that the efficacy of NOP agonists on alcohol drinking increases during repeated administration and is maintained for several days after treatment discontinuation [24]. An alternative hypothesis is that, despite having an effect within the same regions, NOP agonists and antagonists may act at different neurocircuitry levels. For instance, electrophysiological data from VTA slices suggested that NOP receptors are located both on DA cells and in presynaptic GABA and glutamate neurons. Hence, they can modulate DA function through both presynaptic and postsynaptic mechanisms, leading to sophisticated modulation of the activity of this catecholaminergic system [31, 40].

Administration of NOP receptor antagonist LY2817412 in the CeA, but not in the NAc, reduces yohimbine-induced reinstatement

A wealth of studies has shown that the α2-adrenergic receptor antagonist yohimbine induces alcohol craving in humans [41] and reinstates extinguished alcohol-seeking in rodents previously trained to self-administer alcohol [42–45]. The mechanism through which yohimbine evokes drug-seeking is complex and likely consists of a concomitant activation of the stress system and the invigoration of responding triggered by exposure to sensory cues [28, 46–49]. As a result of this intricate mechanism, corticotropin-releasing factor-1 receptor antagonism and blockade of DA transmission both reduced yohimbine-induced reinstatement of drug-seeking behavior [50–52]. The amygdala is known to play a pivotal role in mediating stress-related effects on alcohol [1, 53, 54]. Whereas, NOP receptor agonists exert marked anxiolytic and anti-stress effects [55, 56] through modulation of N/OFQ transmission in this region [57, 58]. The impact of NOP antagonists on such behaviors is under intense scrutiny and although in most of the studies receptor blockade has been found ineffective, in some reports, anxiolytic- and antistress-like activities have been reported [59, 60]. For instance, i.c.v. administration of the NOP antagonist UFP-101 reduced the latency of inhibitory avoidance [61]. In addition, the NOP antagonist LY2940094, attenuated fear-evoke immobility in mice, stress-induced hyperthermia in rats [60] and showed antidepressant activity in depressed alcoholics [62]. Furthermore, administration of JTC-801 and J-113397, other two NOP receptor antagonists, reversed anxiety-like behavior and modulated HPA axis activity following traumatic stress [63, 64]. More recently, in a model of inescapable electric foot-shock, administration of the NOP receptor antagonist SB-612111 reversed helpless-induced anxiety-like behaviors, whereas it increased anxiety levels in non-helpless mice, and was ineffective in non-stressed animals tested in the elevated plus-maze (EPM) test [65]. Together these data suggest that NOP receptor blockade may be particularly effective in exerting anxiolytic actions when this condition is associated with stress exposure. In msP rats, excessive alcohol drinking, anxiety-like predispositions, and hypersensitivity to stress have been co-segregated through genetic selection [39]. This multifaceted behavioral trait has been linked to an innate upregulation of the CRF1 and the NOP receptor systems in several stress-related regions, including the amygdala [66–68]. A tempting hypothesis is that intra-CeA administration of LY2817412 attenuated yohimbine-induced reinstatement of alcohol-seeking by alleviating the negative emotional load associated with the over-reactive stress system of msP rats [69, 70].

Electrophysiological studies revealed a significant role of NOP agonists in orchestrating both basal CeA synaptic functions and responsiveness to alcohol. For instance, activation of NOP receptors in this brain region reduced basal GABAergic synaptic transmission and counteracted the facilitatory effect of alcohol in both naïve and post-dependent rats [71, 72]. Activation of NOP receptors also diminished CeA basal glutamatergic transmission and at the same time reduced the inhibitory effects of alcohol on this neurotransmission [73]. Moreover, incubation with N/OFQ evoked a direct postsynaptic inhibition in all neurons recorded from the centromedial amygdala (CeM) [74] and in a subset of the total cells recorded from the CeA [71, 75]. NOP receptor blockade instead produced little effects in naïve control rats, but significantly increased both CeA GABAergic and glutamatergic neurotransmission in alcohol-dependent and chronic stress-exposed rats [57, 73]. Noteworthy, the antagonist prevented the effects of N/OFQ on both GABA and glutamate transmission but failed to prevent the effects of alcohol on glutamate [57, 71–73]. These data indicate that under basal condition CeA output activity is regulated in an opposite direction by NOP agonists and antagonists as expected. However, a history of alcohol exposure likely leads to adaptive changes of the system so that the effects of alcohol on glutamate remain sensitive to NOP agonists but is no longer influenced by receptor blockade. How these adaptive changes influence the effects of NOP agonists and antagonists on reinstatement behavior is unknown at present. But the fact that the CeA has been identified as an important neuroanatomical substrate mediating this action guarantee further investigations.

In conclusion, the present study demonstrates that the pharmacological blockade of NOP receptors by LY2817412 prevents relapse elicited by stress and environmental stimuli predictive of alcohol availability in msP rats. Furthermore, these effects were specifically mediated by the recruitment of the VTA and the CeA, but not the NAc. More studies are needed to better clarify the mechanisms through which NOP receptor blockade prevents reinstatement of alcohol-seeking behavior. The evidence established so far demonstrate that NOP receptor antagonists attenuate alcohol drinking and seeking opening to the possibility of developing these agents for AUD treatments.

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ACKNOWLEDGEMENTS

We wish thank Linda M. Rorick-Kehn for the scientific inputs and thoughtful comments on the work. We also thank Rina Righi, Agostino Marchi, Mariangela Fiorelli for animal care as well as Alfredo Fiorelli for his excellent technical support. Authors gratefully thank Federica Benvenuti, Marina Antonini, Martina Mondaini, and Maria Sole Centanni for their support in behaviorally testing the animals. This work was supported by the National Institutes of Health, grant RO1 AA014351 (to FW and RC) from the National Institute on Alcohol Abuse and Alcoholism.

AUTHOR CONTRIBUTIONS

AMB and RC were responsible for the study concept and design. AMB performed surgeries, behavioral testing, data analysis and wrote the paper. YF performed surgeries and behavioral tests. SS, AD, and SDC performed behavioral tests. MP, MU, FW, and RC provided critical revision of the paper for important intellectual content. RC and MP contributed to write the paper. All authors critically reviewed the content and approved the final version for publication.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41386-021-01096-1.

Correspondence and requests for materials should be addressed to R.C.

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Early social isolation differentially affects the glucocorticoid receptor system and alcohol-seeking behavior in male and female rats

F. Benvenuti,[1] S. De Carlo,[1] L. Rullo,[2] L. Caffino,[3] L.M. Losapio,[2] M. Ubaldi,[1] L. Soverchia,[1] N. Cannella,[1] S. Candeletti,[2] L. Fattore,[4] P. Romuladi,[2] F. Fumagalli,[3] V. Trezza,[5] M. Roberto,[6] R. Ciccocioppo[1]

[1]School of Pharmacy, Pharmacology Unit, University of Camerino, Camerino, Italy

[2]Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy

[3]Department of Pharmacological and Biomolecular Sciences, 'Rodolfo Paoletti', University of Milan, Milan, Italy

[4]CNR Institute of Neuroscience-Cagliari, National Research Council, Cagliari, Italy

[5]Department of Science, University "Roma Tre," Rome, Italy

[6]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA, USA

ABSTRACT

Adverse early life experiences during postnatal development can evoke long-lasting neurobiological changes in stress systems, thereby affecting subsequent behaviors including propensity to develop alcohol use disorder. Here, we exposed genetically selected male and female Marchigian Sardinian alcohol-preferring (msP) and Wistar rats to mild, repeated social deprivation from postnatal day 14 (PND14) to PND21 and investigated the effect of the early social isolation (ESI) on the glucocorticoid receptor (GR) system and on the propensity to drink and seek alcohol in adulthood. We found that ESI resulted in higher levels of GR gene and protein expression in the prefrontal cortex (PFC) in male but not female msP rats. In female Wistars, ESI resulted in significant downregulation of GR gene expression and lower GR protein levels. In male and female msP rats, plasma corticosterone levels on PND35 were similar and unaffected by ESI. Wistar females exhibited higher levels of corticosterone compared with males. Corticosterone levels were decreased by ESI in both sexes. In alcohol self-administration experiments we found that the pharmacological stressor yohimbine (0.0, 0.312, 0.625, and 1.25 mg/kg) increased alcohol self-administration in both rat lines, independently of ESI. After extinction, 0.625 mg/kg yohimbine significantly reinstated alcohol seeking in female rats only. ESI enhanced reinstatement in female msP rats. Overall, the present results indicate that repeated mild social deprivation during the third week of postnatal life affects GR expression in a strain- and sex-dependent manner, in which female msP rats are more sensitive to the effects of yohimbine-induced alcohol seeking in adulthood.

Keywords: Alcoholism; Reward; Glucocorticoid receptor; Alcohol drinking; Stress; Relapse

Introduction

Alcohol dependence is a multifactorial disorder whereby genetic and environmental factors interact to determine an individual's vulnerability or resilience to developing it [1-3]. Clinical and preclinical studies suggest that adverse social experiences during early stages of postnatal life are associated with alterations of synaptic and neuronal development that negatively affect proper development of the brain [4-6], leading to cognitive, emotional, and social impairment [7] and a greater susceptibility to psychiatric disorders [8], including alcohol use disorder and other substance use disorders [9-10]. Indeed, exposure to maltreatment and cumulative stressful life events before puberty, particularly in the first few years of life, is associated with an early onset of problem drinking in adolescence and alcohol use disorder and substance use disorders in adulthood [11-14]. Rodent studies showed that exposure to stress during early postnatal life modulates the rewarding effects of cocaine, amphetamine, and morphine in adulthood [15-18]. A higher propensity for alcohol drinking was also demonstrated [9, 19-25].

One key system that mediates the stress response is the hypothalamic-pituitary-adrenal (HPA) axis, and stress exposure in early life has been shown to significantly impact HPA axis function [26-28]. Despite some inconsistent reports, the general consensus is that stress exposure in early life results in HPA axis hyperactivity in adulthood, with increases in corticotropin-releasing factor signaling and impairments in glucocorticoid receptor (GR)-mediated negative feedback [28]. Preclinical models that attempt to mimic early traumatic events in rodents have primarily focused on the first 2 weeks of life. However, recent studies have shown that during the juvenile period in rodents (postnatal weeks 3-4), a large-scale reconfiguration of the neuronal epigenome and extensive synaptogenesis occur, similar to what occurs in humans during childhood [29]. In rodents, this developmental time-window is also characterized by the maturation of visual, motor, and social functions that are crucial for

interactions with the environment [30-32]. Notably, early social isolation (ESI) during the third postnatal week in mice increased cocaine-induced conditioning place preference compared with controls [33-34]. Although there has been a greater perception of the importance of social isolation during early adolescence for the subsequent development of psychiatric disorders, very little has been investigated with regard to problem drinking.

In the present study, we hypothesized that exposure to mild, repeated social isolation during the third postnatal week induces stable molecular changes at the level of the GR system that likely alter responses to alcohol reward and increases the susceptibility to alcohol seeking. To test this hypothesis, we explored the effects of ESI on GR function in the amygdala (Amy) and prefrontal cortex (PFC), two brain regions that are critically linked to stress and alcohol drinking in rodents, that were subsequently tested for their vulnerability to develop excessive drinking and seeking. To assess whether environmental stress interacts with heritable factors, we used unselected Wistar rats and genetically selected Marchigian Sardinian alcohol-preferring (msP) rats, a rodent line that is characterized by heightened alcohol consumption and stress sensitivity [35-36]. The majority of prior studies have focused on male rats only; very few reports have examined the consequences of early life stress in females [37]. Clinical evidence supports a higher association between a history of child maltreatment and a higher risk to develop drug use and psychiatric disorders in women than in men [38-40]. Women initiate alcohol consumption as a coping strategy more frequently than men to attenuate negative affective states, such as anxiety, depression, and posttraumatic stress disorder [41-43]. Women are also more likely to relapse in response to stressful events [44-46]. The vulnerability to several psychopathological conditions is also well known to display sex differences in experimental animals [47-48], with notable sex differences in HPA axis function [42, 49]. Therefore, we investigated the impact of ESI in both sexes.

Materials and Methods

Animals

Male (*n* = 28/line) and female (*n* = 25-27/line) msP and Wistar rats were used in the study. All rats were bred at the animal facility of the University of Camerino, Italy. MsP and Wistar dams were single-mated with an individual male rat of the same genotype until pregnancy could be verified. At this point, the male rat was removed from the cage, and pregnant females were single housed until delivery. The breeding colony was kept on a 12 h/12 h light/dark cycle (light on at 7 AM). All animals were disturbed as little as possible during the breeding process and had *ad libitum* access to food pellets (4RF18, Mucedola, Settimo Milanese, Italy) and tap water. The day of birth was considered postnatal day 0 (PND0). At birth, the litters were left undisturbed with their mothers until PND14, at which time half of the pups from each nest was subjected to the ESI protocol as described below. On PND21, all pups were weaned and housed in groups of the same sex and environmental condition in a new room on a 12 h/12 h light/dark cycle (light off at 8 AM). They were housed under same-sex/environmental condition groups of 3 or 4 per cage with *ad libitum* access to food and water. All subsequent experiments began on PND35, at which time they weighed approximately 150 g (males) and 130 g (females). Before starting the behavioral experiments, the rats were handled daily for 5 min for 3 days by the same researchers who performed the experiments. The experiments were conducted during the dark phase of the light/dark cycle.

All efforts were made to minimize animal suffering and reduce the number of animals used. All animal procedures were conducted in adherence to the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Early social isolation protocol

Early social isolation occurred between PND14 and PND21. Half of the pups from each nest were individually removed and placed in a cage with clean bedding for 30 min/day. After the 30 min separation time, they were returned to their home cages. Control pups were left undisturbed with their mothers in their home cages.

Tissue collection

On PND35, the animals were sacrificed under low stress conditions. Blood was collected from the trunk, and brain areas of interest were dissected for *in vitro* experiments and quickly frozen on dry ice. Tissues were stored at -80°C until the gene and protein expression analyses were performed.

RNA extraction and gene expression analysis by quantitative real-time polymerase chain reaction

Total RNA was extracted according to the method of Chomczynski and Sacchi (1987) [50]. Each sample was subjected to DNase treatment and converted to cDNA with the GeneAmp RNA PCR kit (Life Technologies Italia, Monza, Italy; catalog no. N8080143) as previously described [51]. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with a StepOne Real-Time PCR System (Life Technologies, Monza, Italy) using SYBR Green PCR MasterMix (Life Technologies, Monza, Italy; catalog no. 4309155). The relative expression of different gene transcripts was calculated using the ΔΔCt method and converted to relative expression ($2^{-\Delta\Delta Ct}$) for the statistical analysis [52]. All data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The specificity of each PCR product was determined by melting curve analysis, constructed in the range of 60°C to 95°C. The primer sequences that were used for PCR amplification were designed using Primer 3 and were the following: GAPDH (forward, 5'-

AGACAGCCGCATCTTCTTGT-3'; reverse, 5'-CTTGCCGTGGGTAGAGTCAT-3'), Nr3cl gene encoding for GR (forward, 5'-GAAAGCCATCGTCAAAAGGG-3'; reverse, 5'-TGGAAGCAGTAGGTAAGGAGA-3').

Preparation of protein extracts and Western blot analyses

PFC tissue was homogenized in a glass-glass potter using cold buffer that contained 0.32 M sucrose, 1 mM HEPES solution, 0.1 mM ethylene glycol tetraacetic acid (EGTA), and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4, in the presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. The homogenized tissues were centrifuged at 1000 × *g* for 10 min. The resulting pellet (P1), corresponding to the nuclear fraction, was resuspended in a buffer that contained 20 mM HEPES, 0.1 mM dithiothreitol, and 0.1 mM EGTA, with protease and phosphatase inhibitors. The supernatant (S1) was centrifuged at 9000 × *g* for 15 min to obtain the pellet that corresponded to the crude synaptosomal fraction, and the resulting supernatant S2 corresponded to the clarified fraction of cytosolic proteins. Total proteins were measured in the nuclear fraction and cytosolic fraction using the Bio-Rad Protein Assay, with bovine serum albumin as the calibration standard (Bio-Rad Laboratories, Segrate, Milan, Italy). Glucocorticoid receptor levels were evaluated in both the nuclear and cytosolic fractions. Ten micrograms of proteins for each sample were run on sodium dodecyl sulfate-10% polyacrylamide gel under reducing conditions and then electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories). Blots were blocked 1 h at room temperature with 10% bovine serum albumin in TBS buffer and then incubated with the anti-GR antibody (1:500, Thermo Scientific, USA). The results were standardized using β-actin (1:10,000, Sigma-Aldrich, Milan, Italy) as the control protein, which was detected by evaluating the band density at 43 kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories) and analyzed using Image Lab

software (Bio-Rad). Gels were run twice each. The results represent the average from two different Western blots and were averaged and normalized using a specific correction factor [53]. Examples of full-size original cropped immunoblots of protein expression levels that were measured in the nuclear and cytosolic fractions of the PFC are presented in Supplementary Fig. S1-2).

Analysis of plasma corticosterone levels

Blood samples from each rat were collected in tubes that contained ethylenediaminetetraacetic acid (EDTA; 250 μl × 2 ml of collected blood) as the anticoagulant agent. Plasma was separated by centrifugation at 6500 × *g* for 10 min. Corticosterone (CORT) levels were determined using an enzyme-linked immunosorbent assay (ELISA) with a commercial kit according to the manufacturer's instructions (Tecan, Italy).

Drugs

The alcohol drinking solution 10% (v/v) was prepared by diluting 95% alcohol (F.L. Carsetti, Camerino, Italy) with tap water. Yohimbine hydrochloride (17-hydroxyyohimban-16-carboxylic acid methyl ester hydrochloride) was purchased from Sigma-Aldrich, Italy, dissolved in sterile distilled water, and administered intraperitoneally (i.p.) at 0.0, 0.312, 0.625, and 1.25 mg/kg in a 1 ml/kg injection volume 30 min before the drug tests.

Self-administration apparatus

Operant alcohol self-administration training and drug testing were conducted in standard operant conditioning chambers (Med Associates, St. Albans, VT, USA) that were enclosed in ventilated sound-attenuating cubicles. Each chamber was equipped with two retractable levers in the front panel that were positioned laterally to the drinking reservoir and

connected to a syringe pump. A house light was located on the wall opposite the levers. A Windows-compatible computer with Med-PC-5 software (Med Associates) controlled the delivery of fluid, presentation of visual stimuli, and recording of behavioral data.

Experiments

EXPERIMENT 1: Effect of ESI on Nr3c1 gene expression, GR protein levels and plasma CORT levels. Twelve male (*n* = 6/environmental condition) and 12 female (*n* = 6/environmental condition) msP rats and 12 male (*n* = 6/environmental condition) and 12 female (*n* = 6/environmental condition) Wistar rats were sacrificed at the beginning of the dark cycle on PND35. Blood samples were collected to measure CORT levels. The PFC and the Amy were used for Nr3c1 gene expression and GR protein level analyses. This time point was chosen because late adolescence is an age at which rats are particularly sensitive to develop stress-related maladaptive molecular and behavioral changes.

EXPERIMENT 2: Effect of ESI on alcohol self-administration. To examine whether ESI-induced molecular changes at the level of the GR system affect the response to alcohol, alcohol-related behaviors were evaluated in a new cohort of rats. Behavioral training began on PND35. Sixteen male (*n* = 8/environmental condition) and 15 female (*n* = 8-7/environmental condition) msP rats and 16 male (*n* = 8/environmental condition) and 15 female (*n* = 6-7/environmental condition) Wistar rats were used in this experiment. On PND35, the rats were given intermittent access to 10% (v/v) alcohol in an additional water bottle in their home cage for 1 week. The purpose of this procedure was to avoid neophobic responses to alcohol in the operant chambers. After this acclimation period, operant training began. The rats were given 15 h access to a single lever (right lever) that produced 0.1 ml deliveries of water on a fixed-ratio 1 (FR1) schedule of reinforcement with *ad libitum* food available on the floor of the operant chamber.

Afterward, the animals were trained to respond for 10% (v/v) alcohol in 30 min daily sessions under a FR1 schedule of reinforcement. Operant sessions began with lever extension into the chamber and ended with lever retraction. Responses at the right (active) lever were reinforced with 0.1 ml of 10% (v/v) alcohol that was delivered in the drinking reservoir. Reinforcement delivery was followed by a 5 s timeout (TO) period, during which the house light was contingently illuminated. During the TO, active lever responses were recorded but not reinforced. Throughout the sessions, responses at the left (inactive) lever had no scheduled consequences. The number of operant responses at both the active and inactive levers and the number of reinforcers received were recorded. Alcohol self-administration training was performed 5 days weekly until the successful acquisition of a stable baseline of operant responding was achieved (18 sessions).

EXPERIMENT 3: Effect of ESI on alcohol self-administration on a progressive-ratio schedule of reinforcement. After the successful acquisition of operant responding under the FR1 schedule of reinforcement, the same cohort of animals in the previous experiment was switched to a progressive-ratio (PR) schedule of reinforcement to evaluate their motivation for alcohol [54-55]. The PR schedule is an operant schedule that measures the maximum amount of work an animal is willing to expend to obtain a reward, reflecting its motivation for it [56]. The breakpoint, defined as the last ratio completed by the animals to obtain one dose of 10% (v/v) alcohol, was used as a measure of motivation. Under the PR contingency, the response requirement that was necessary to receive one dose of 10% (v/v) alcohol was increased according to the following progression: 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104. Each alcohol-reinforced response resulted in the house light being turned on for 5 s, and sessions were terminated when more than 30 min elapsed since the last reinforced response.

EXPERIMENT 4: Effect of ESI on alcohol self-administration following yohimbine administration. Following the PR test, the animals were returned to an FR1 schedule of reinforcement to restore the alcohol self-administration baseline. Once stable self-administration responding was obtained (in 5 days) under this reinforcement schedule, the experiment started. The pharmacological stressor yohimbine was used at doses (0.312, 0.625, and 1.25 mg/kg) that were previously shown to increase alcohol-reinforced lever pressing in both Wistar and msP rats [55, 57]. To habituate animals to the injection procedure, physiological saline was injected i.p. three times before drug testing. Yohimbine (0.312, 0.625, 1.25 mg/kg) or its vehicle was administered i.p. 30 min before the self-administration session using a within-subjects counterbalanced Latin-square design. Drug tests were conducted every fourth day. Following each test day, the animals were allowed one day off, and a new baseline was then established over the next 2 days. The number of operant responses at both the active and inactive levers and number of reinforcers received were recorded.

EXPERIMENT 5: Effect of ESI on yohimbine-induced reinstatement of alcohol seeking. The experimental procedure consisted of three phases: operant training, extinction, and reinstatement. Briefly, the same cohort of animals that was used in the previous experiments was retrained for FR1 alcohol self-administration for 10 days to reestablish a stable baseline of operant responding. The rats were then subjected to 16 daily 30 min extinction sessions, during which lever presses were no longer associated with alcohol delivery, but the house light was still presented to allow for its concomitant extinction. At completion of the extinction phase, the rats were challenged with a single dose (0.625 mg/kg) of yohimbine, and reinstatement was evaluated. Because in the previous experiment, 0.625 mg/kg yohimbine was sufficient to increase alcohol self-administration in all groups, this dose was again used in the reinstatement

experiment. Additionally, previous studies demonstrated that this dose of yohimbine can reinstate alcohol seeking in both msP and Wistar rats [55]. Yohimbine (0.625 mg/kg) was administered i.p. 30 min before the 30 min reinstatement session that was conducted under identical conditions to extinction training. Total responses at the active lever were recorded and used to evaluate alcohol-seeking behavior. Inactive lever responses were also measured.

Statistical analysis

Data from msP and Wistar rats were analyzed independently for each experiment. Biochemical and molecular data have been initially evaluated by Shapiro-Wilk tests to confirm the normality of the distribution and by Grubb's test to identify outliers. After testing for the assumption of a normal distribution changes in mRNA and plasma CORT were analyzed using two-way analysis of variance (ANOVA), with ESI and sex as between-subjects factors. Changes in protein expression that were produced by ESI were analyzed using unpaired Student's *t*-test. Corticosterone data from two male (one from the ESI group and one from the control group) and two female (one from the ESI group and one from the control group) msP rats were excluded from the statistical analysis because their data deviated from the mean by 2 standard deviations, thereby violating the assumption of normality. Similarly, in the analysis of mRNA expression data from one female (from ESI group) msP rats and from one male (from control group) Wistar rats were excluded in the Amy. In the PFC, two male (one from the ESI group and one from the control group) and two female (one from the ESI group and one from the control group) msP rats and one female (from control group) Wistar rats were excluded. To evaluate the operant self-administration training data, the number of alcohol rewards was analyzed using three-way ANOVA, with ESI and sex as between-subjects factors and sessions as the repeated measure. The breakpoint for alcohol self-administration under the PR schedule of reinforcement was evaluated using two-way ANOVA, with sex and ESI as

between-subjects factors. The effect of yohimbine on alcohol self-administration was analyzed using three-way ANOVA, with sex and ESI as between-subjects factors and treatment as the repeated measure. Active and inactive lever responses were analyzed separately. The yohimbine-induced reinstatement results were analyzed using two-way ANOVA, with ESI as the between-subjects factor and reinstatement as the repeated measure. When appropriate, the Newman-Keuls test was used for *post hoc* comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

EXPERIMENT 1: Effect of ESI on Nr3c1 gene expression, GR protein levels and plasma CORT levels.

We tested the effect of ESI on *Nr3c1* gene expression in the Amy and PFC in male and female msP and Wistar rats on PND35. In the Amy in msP rats, the two-way ANOVA revealed no significant effect of sex ($F_{1,18} = 0.009$, $p > 0.05$) or ESI ($F_{1,18} = 1.6$, $p > 0.05$) and no sex × ESI interaction ($F_{1,18} = 0.04$, $p > 0.05$; **Figure 1A**). In Wistar rats, the overall ANOVA showed a main effect of ESI ($F_{1,19} = 5.9$, $p < 0.05$), but no significant effect of sex ($F_{1,19} = 2.8$, $p > 0.05$) or sex × ESI interaction ($F_{1,19} = 0.003$, $p > 0.05$; **Figure 1B**). The two-way ANOVA of *Nr3c1* gene expression in the PFC in msP rats revealed significant effects of sex ($F_{1,16} = 13.41$, $p < 0.01$) and ESI ($F_{1,16} = 8.5$, $p < 0.05$) and a sex × ESI interaction ($F_{1,16} = 12.5$, $p < 0.01$). The *post hoc* analysis showed that male msP rats that were subjected to ESI during the third week of postnatal life exhibited significantly higher *Nr3c1* mRNA levels in the PFC ($p < 0.001$). No significant difference was found for *Nr3c1* gene expression in female msP rats that were subjected to ESI compared with the respective control group ($p > 0.05$; **Figure 1C**). In the PFC in Wistar rats, the two-way ANOVA revealed a significant effect of sex ($F_{1,19} = 8.5$, $p < 0.01$), sex ($F_{1,19} = 54.8$, $p < 0.0001$) and a sex × ESI interaction ($F_{1,19} = 10.16$, $p < 0.01$). The *post hoc*

analysis showed that control females exhibited significantly higher *Nr3c1* mRNA levels in the PFC compared to males ($p < 0.0001$) and that females subjected to ESI displayed a significant downregulation of *Nr3c1* gene expression ($p < 0.001$). Conversely, in male Wistar rats, ESI did not result in any significant alteration of *Nr3c1* gene expression in the PFC ($p > 0.05$; **Figure 1D**).

Because no changes in *Nr3c1* gene expression were detected in the Amy in msP and Wistar rats, we next measured the ratio of GR protein levels in nuclear and cytosolic fractions as an index of receptor translocation from the cytosol to the nucleus only in the PFC. The protein expression analysis showed that in msP rats (**Figure 2A)**, ESI increased the nucleus/cytosol ratio of GR protein only in the PFC in male rats (male: +96%, $t_8 = 4.271$, $p < 0.01$; female: +17%, $t_{10} = 1.676$, $p > 0.05$). In Wistar rats (**Figure 2B)**, no changes were observed in male rats (+4%, $t_{10} = 0.278$, $p > 0.05$), whereas exposure to the ESI protocol reduced GR trafficking in female rats (-31%, $t_{10} = 2.431$, $p < 0.05$).

To verify whether the observed dysregulation of the GR system might be associated with altered levels of circulating glucocorticoids, we measured CORT levels in plasma in Wistar and msP rats on PND35. In msP rats, the two-way ANOVA revealed no significant effect of sex ($F_{1,15} = 0.5$, $p > 0.05$) or ESI ($F_{1,15} = 0.6$, $p > 0.05$) and no sex × ESI interaction ($F_{1,15} = 0.003$, $p > 0.05$; **Figure 2C**). In Wistar rats, the two-way ANOVA revealed main effects of sex ($F_{1,19} = 24.2$, $p < 0.0001$) and ESI ($F_{1,19} = 5.5$, $p < 0.05$) but no sex × ESI interaction ($F_{1,19} = 0.9$, $p > 0.05$; **Figure 2D**).

EXPERIMENT 2: Effect of ESI on FR1 alcohol self-administration

The effect of ESI on the acquisition alcohol self-administration was evaluated. In msP rats, the three-way ANOVA revealed overall effects of sessions ($F_{17,486} = 9.3$, $p < 0.0001$) and sex ($F_{1,486} = 78.5$, $p < 0.0001$) but no effect of ESI ($F_{1,486} = 3.5$, $p > 0.05$) and no interactions.

These results reflect a higher number of lever presses in male msP rats throughout training (**Figure 4A, left panel**) compared with females (**Figure 4A, right panel**). Similarly, the three-way ANOVA of self-administration data in male (**Figure 4B, left panel**) and female (**Figure 4B, right panel**) Wistar rats revealed significant effects of sessions ($F_{17,450} = 5.2$, $p < 0.0001$) and sex ($F_{1,450} = 28.9$, $p < 0.0001$) and a sessions × sex interaction ($F_{17,450} = 3.5$, $p < 0.0001$) but no effect of ESI ($F_{1,450} = 0.6$, $p > 0.05$) and no other interactions. Overall, these data suggest that the ESI procedure did not alter the acquisition of alcohol self-administration or responding for alcohol under the FR1 schedule of reinforcement in any of the groups tested, independent of sex and rat strain.

EXPERIMENT 3: Effect of ESI on alcohol self-administration on a PR schedule of reinforcement

After the acquisition of a stable baseline of alcohol self-administration under the FR1 contingency, the animals were tested in a PR schedule of reinforcement to evaluate their motivation for alcohol. In male and female msP rats, the two-way ANOVA showed a main effect of sex ($F_{1,27} = 4.9$, $p < 0.05$) but no effect of ESI ($F_{1,27} = 0.6$, $p > 0.05$) and no sex × ESI interaction ($F_{1,27} = 0.7$, $p > 0.05$; **Figure 3C**). These results suggest that male msP rats, independent of ESI, exhibited a higher motivation for alcohol. In male and female Wistar rats, the ANOVA revealed no significant effect of sex ($F_{1,25} = 0.3$, $p > 0.05$) or ESI ($F_{1,25} = 0.02$, $p > 0.05$) and no sex × ESI interaction ($F_{1,25} = 2.4$, $p > 0.05$; **Figure 3D**).

EXPERIMENT 4: Effect of ESI on alcohol self-administration following yohimbine administration

In msP rats, the three-way ANOVA revealed overall effects of treatment ($F_{3,108} = 11.02$, $p < 0.0001$) and sex ($F_{1,108} = 7.2$, $p < 0.01$) but no effect of ESI ($F_{1,108} = 0.9$, $p > 0.05$) and no

interactions, indicating that yohimbine enhanced alcohol-reinforced lever pressing in both male and females equally, and this effect was not influenced by ESI (**Figure 4A, upper panel**). The ANOVA of inactive lever responding showed a significant effect of sex ($F_{1,108} = 7.7$, $p < 0.01$) and a sex × ESI interaction ($F_{1,108} = 6.2$, $p < 0.05$) but no effect of treatment ($F_{3,108} = 2$, $p > 0.05$) or ESI ($F_{1,108} = 3.8$, $p > 0.05$) and no other interactions (**Figure 4A, lower panel**).

Similar to msP rats, in Wistar rats, the three-way ANOVA showed significant effects of treatment ($F_{3,100} = 6.9$, $p < 0.001$) ad sex ($F_{1,100} = 12.3$, $p < 0.001$) but no effect of ESI ($F_{1,100} = 0.002$, $p > 0.05$) and no interactions, indicating that yohimbine increased alcohol self-administration in all experimental groups (**Figure 4B, upper panel**). The ANOVA of inactive lever responding showed no significant effect of treatment ($F_{3,100} = 2.2$, $p > 0.05$), sex ($F_{1,100} = 0.01$, $p > 0.05$), or ESI ($F_{1,100} = 0.07$, $p < 0.05$) and no significant interactions (**Figure 4B, lower panel**).

EXPERIMENT 5: Effect of ESI on yohimbine-induced reinstatement of alcohol seeking

Rats that were trained under an FR1 schedule of operant alcohol self-administration were subjected to an extinction phase, during which lever pressing progressively decreased and then was tested for yohimbine-induced reinstatement. During training, the mean numbers of active lever presses relative to the last 3 days of alcohol self-administration in msP rats were the following: male controls (77.5 ± 6.6), male ESI (68.6 ± 7.8), female controls (45.8 ± 4), female ESI (57.4 ± 6.4). Responding in Wistar rats was the following: male controls (44.5 ± 4), male ESI (42.2 ± 5.4), female controls (43.4 ± 1.2), female ESI (31.4 ± 2.6). During the extinction phase, responding at the active lever progressively decreased. The mean numbers of lever presses during the last 3 days of extinction in msP rats were the following: male controls (12.5 ± 1.8), male ESI (9.5 ± 1.8), female controls (9.5 ± 1.9), female ESI (19.5 ± 6.6). Responding in Wistar rats was the following: male controls (8.9 ± 1.4), male ESI (11.4 ± 1.5),

female controls (19.2 ± 3.5), female ESI (11.5 ± 2.3). Following yohimbine administration, in msP rats, the three-way ANOVA showed overall effects of treatment ($F_{1,27}$ = 17.7, p < 0.001), sex ($F_{1,27}$ = 10.3, p < 0.01), and ESI ($F_{1,27}$ = 5.8, p < 0.05), a significant treatment × sex interaction ($F_{1,27}$ = 11.9, p < 0.01), a treatment × ESI interaction ($F_{1,27}$ = 4.4, p < 0.05), a sex × ESI interaction ($F_{1,27}$ = 9.6, p < 0.01), but no treatment × sex × ESI interaction ($F_{1,27}$ = 3.06, p > 0.05; **Figure 5A, upper panel**). Inactive lever responding was unaffected. The ANOVA revealed no effect of treatment ($F_{1,27}$ = 2.2, p > 0.05), sex ($F_{1,27}$ = 0.2, p > 0.05), or ESI ($F_{1,8}$ = 2.3, p > 0.05) and no interactions (**Figure 5A, lower panel**).

In Wistar rats, the ANOVA revealed overall effects of yohimbine treatment ($F_{1,25}$ = 29.5, p < 0.0001) and sex ($F_{1,25}$ = 12.3, p < 0.01) but no effect of ESI ($F_{1,25}$ = 0.9, p > 0.05). The overall ANOVA also revealed a significant treatment × sex interaction ($F_{1,27}$ = 6.9, p < 0.05) but no treatment × ESI interaction ($F_{1,25}$ = 0.2, p > 0.05), sex × ESI interaction ($F_{1,25}$ = 0.9, p > 0.05), or treatment × sex × ESI interaction ($F_{1,25}$ = 0.07, p > 0.05; **Figure 5B, upper panel**). Inactive lever presses were negligible and not significantly affected by yohimbine treatment ($F_{1,25}$ = 4.01, p > 0.05), sex ($F_{1,25}$ = 6.7, p < 0.05), or ESI ($F_{1,25}$ = 0.3, p > 0.05), with no treatment × sex interaction ($F_{1,25}$ = 14.3, p < 0.001), treatment × ESI interaction ($F_{1,25}$ = 0.003, p > 0.05), sex × ESI interaction ($F_{1,25}$ = 7.7, p < 0.01), or treatment × sex × ESI interaction ($F_{1,25}$ = 0.008, p > 0.05; **Figure 5B, lower panel**).

4. Discussion

The present results showed that repeated ESI experiences resulted in higher levels of GR gene and protein expression in the PFC in male msP rats on PND35. No changes in *Nr3c1* gene expression were observed in female msP rats or male Wistar rats. Contrary to male msP rats, female Wistar rats that were subjected to ESI exhibited a significant downregulation of *Nr3c1* expression and lower protein levels. ESI did not affect GR transcript or protein

expression in the Amy. Basal plasma CORT levels were unaffected by ESI in msP rats. In female Wistar rats, significantly higher CORT levels were detected compared with males, and ESI decreased it in both sexes. When rats were subsequently tested for basal alcohol self-administration, the results showed that the motivation for alcohol was unaffected by ESI in either msP or Wistar rats. The administration of yohimbine increased alcohol self-administration in both msP and Wistar rats. This effect occurred independently of ESI. In an extinction reinstatement paradigm, yohimbine administration significantly reinstated alcohol seeking in female msP and Wistar rats. Interestingly, ESI increased the yohimbine-induced reinstatement of alcohol seeking in female msP rats but not in Wistar rats.

In the present study, we applied ESI during the juvenile period (week 3), during which a large reconfiguration of the neuronal epigenome and extensive synaptogenesis occur [29]. Furthermore, this developmental time window is characterized by the maturation of functions that are crucial for interactions of rodents with their environment, such as visual, motor, and social abilities [30-32]. Although this developmental period is characterized by significant neuroplasticity, we did not observe major effects of ESI on drinking. Previous studies applied maternal separation during earlier maturation phases (weeks 1 and 2), showing significant changes in the function of multiple brain areas that are involved in stress/reward processing that is often associated with high alcohol drinking in adulthood [58-60]. Compared with the ESI protocol that was used herein, maternal separation during early life is likely associated with higher levels of physical stress in pups (e.g., hypothermia and alterations of lactation patterns), which may explain the different effects on drinking. Moreover, in these earlier studies, alcohol consumption was evaluated using home-cage two-bottle choice free drinking, whereas we used operant alcohol self-administration in the present study, which more directly captures the motivation for alcohol compared with its *ingesta* [9, 19-24]. Notably, consistent with our findings, Lesscher et al. (2015) reported that social isolation from PND21 to PND42

enhanced two-bottle choice home-cage drinking but did not influence operant responding for alcohol under FR or PR schedules of reinforcement [61].

Few earlier mouse studies that applied ESI during the third week of life demonstrated an increase in depressive-like behavior or enhanced cocaine-induced conditioned place preference in adulthood, suggesting greater motivation for this psychostimulant [33-34, 62]. Based on these data, we would have also expected greater motivation for alcohol, especially in msP rats because they present depressive- and anxiety-like traits that are attenuated by alcohol consumption [36, 63-65]. Contrary to this expectation, we did not observe any effect of ESI on the motivation for alcohol. The different drugs of abuse that were tested (i.e., cocaine *vs*. alcohol) and the fact that previous studies were conducted in mouse pups that were exposed to an additional stressor (i.e., the presence of a resident adult mouse) during the 30-min social isolation session may account for these discrepancies [33-34, 62].

To examine whether stressful stimuli later in life interact with ESI to influence alcohol intake, we also tested the effect of the pharmacological stressor yohimbine on alcohol self-administration and alcohol-seeking behavior. Yohimbine is an α_2 adrenergic receptor antagonist that increases norepinephrine cell firing [66] and enhances norepinephrine release in terminal areas [67-68]. Yohimbine induces anxiety-like responses in both humans [69-70] and laboratory animals [71] and craving in alcohol-dependent patients [72]. In the present study, yohimbine increased alcohol-reinforced lever pressing in all experimental groups, independently of rearing conditions, genotype, and sex, thus indicating that ESI does not alter the propensity to drink in response to this pharmacological stressor. A few earlier studies examined the effect of exposure to stressors other than yohimbine on alcohol intake in animals that were subjected to maternal separation during early life, revealing either no difference [73] or an increase in alcohol intake [22, 74-75].

To our knowledge, no prior studies have examined the consequences of early life stress

on later susceptibility to relapse in response to a yohimbine challenge. In the present study, based on the finding that 0.625 mg/kg yohimbine increased alcohol self-administration in both msP and Wistar rats, we tested this dose on the reinstatement of extinguished alcohol seeking. This dose is lower than the dose (1.25 m/kg) that was classically used in previous studies. We chose this dose to better capture potential interactions with ESI [55, 57, 76]. The results showed that this relatively low dose of yohimbine significantly reinstated alcohol seeking in female but not male rats. In previous studies, the significant reinstatement of alcohol seeking in male rodents was observed following 1.25 mg/kg yohimbine administration [55, 57, 76]. Therefore, our data suggest that females are more sensitive to yohimbine [77]. Consistent with this finding, previous studies reported higher yohimbine-induced reinstatement of cocaine seeking in female animals compared with male animals [78, 79]. This finding also aligns with clinical work that indicated that women who abuse cocaine or alcohol are more likely to relapse in response to stressful events [41, 80].

Notably, in msP rats, yohimbine elicited significant reinstatement of alcohol seeking only in females that were subjected to ESI. The reason for this difference between female msP and Wistar rats is difficult to explain based on the present molecular data. However, these two rat lines differ significantly in their HPA axis reactivity to ESI. In msP rats, CORT levels are similar in males and females and unaffected by ESI, whereas female Wistar rats have higher plasma CORT concentrations that are reduced following ESI. Additionally, ESI reduced GR expression in the PFC in female Wistar rats, but no effect was detected in female msP rats. These results confirm previous experiments showing that msP rats display evidence of dysregulated neuroendocrine function and higher sensitivity to stress compared to Wistar rats with differences between sexes [81].

In summary, the present results showed that repeated mild social deprivation experiences during the third week of postnatal life led to changes in GR expression in a strain-

and sex-dependent manner, and female rats were generally more sensitive to yohimbine-induced alcohol seeking. Moreover, based on the plasma CORT measurements, it is possible to highlight significant hypofunctionality of the HPA axis system in female msP rats compared with Wistar rats because glucocorticoid levels were as low as those in males and were insensitive to prior ESI.

FUNDING

This research was funded by NIH/NIAAA grant AA017447 (to MR) and PRIN 2017SXEXT5 (to RC) and by grants from MIUR Progetto Eccellenza

ACKNOWLEDGEMENTS

We are thankful to Giulia Bianchini for helping with the experiments, Rina Righi, Agostino Marchi, and Matteo Valzano for animal breeding and expert technical assistance, and Michael Arends for proofreading the manuscript.

AUTHOR CONTRIBUTIONS

Conceptualization, FF, LF, VT, PR, RC, MR, NC; Formal Analysis, FB, NC, MU; Investigation, LC, LR, LML, FB, MR, RC, MU, LS; Resources, FF, LF, VT, PR, MR, RC; Data Curation,SC, FB; LC, LR, Writing – Original Draft Preparation, NC, RC; Writing – Review & Editing, SC, FF, LF, VT, PR, FB, NC, MR, RC; Funding Acquisition, MR, RC, FF, LF, VT, PR. All authors approved the submitted version and agree to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.

COMPETING INTERESTS

The authors declare no competing interests.

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Figure Legends

Figure 1. Effect of early social isolation on *Nr3c1* gene expression in the Amy and PFC in msP and Wistar rats. (**A**, **B**) *Nr3c1* gene expression in the Amy in male and female msP and Wistar rats. (**C**, **D**) *Nr3c1* gene expression in the PFC in male and female msP and Wistar rats. The data represent $2^{-\Delta\Delta Ct}$ values calculated using the ΔΔCt method. Gene expression was normalized to GAPDH as the housekeeping gene. The data are expressed as the mean ± SEM. Male msP ESI *vs* female msP ESI: ###; male msP control *vs* male msP ESI: ***; male Wistar control *vs* female Wistar control: ####; female Wistar control *vs* female Wistar ESI: ***; male Wistar ESI *vs* female Wistar ESI: $$. Where not indicated, differences from controls were not statistically significant.

Figure 2. Effect of early social isolation on protein expression in the PFC and on plasma corticosterone levels in msP and Wistar rats. Ratio between nuclear and cytosolic GR protein levels in the PFC in msP (**A**) and Wistar (**B**) rats that were sacrificed on PND35. Representative Western blot bands are shown. Plasma glucocorticoid levels in male and female msP (**C**) and Wistar (**D**) rats are also depicted. The data are expressed as the mean ± SEM. Main effect of ESI: $*p < 0.05$; $**p < 0.01$; main effect of sex: $^{\#\#\#\#}p < 0.0001$. Where not indicated, differences from controls were not statistically significant.

Figure 3. Effect of early social isolation on alcohol self-administration in male and female msP and Wistar rats. Acquisition pattern of alcohol self-administration in male and female msP and Wistar rats. Early social isolation did not affect alcohol reinforcement under FR1 schedule in either male (**A, left panel**) or female (**A, right panel**) msP rats or male (**B, left panel**) or female (**B, right panel**) Wistar rats. The motivation for alcohol under the PR contingency in male or female msP rats (**C**) or male or female Wistar rats (**D**) was also not affected. The data are expressed as the mean ± SEM. Main effect of sex: $^{\#}p < 0.05$. Where not

indicated, differences from controls were not statistically significant.

Figure 4. Effect of early social isolation on alcohol self-administration following yohimbine administration in male and female msP and Wistar rats. Male and female msP and Wistar rats were treated with the pharmacological stressor yohimbine (0.0, 0.312, 0.625, and 1.25 mg/kg, i.p.) 30 min before the test sessions. Independent of ESI, yohimbine administration increased operant alcohol self-administration in male and female msP rats (**A**) and male and female Wistar rats (**B**). The data are expressed as the mean ± SEM number of (a) reinforced responses (rewards) at the active lever and (b) responses at the inactive lever. Main effect of yohimbine treatment: ***$p < 0.001$, ****$p < 0.0001$. Where not indicated, differences from controls were not statistically significant.

Figure 5. Effect of early social isolation on yohimbine-induced reinstatement of alcohol seeking in male and female msP and Wistar rats. Following alcohol self-administration training, male and female msP and Wistar rats were subjected to an extinction phase and then treated with yohimbine (0.625 mg/kg, i.p.). Thirty minutes later, the reinstatement of responding was evaluated. Extinction (EXT): mean number of lever presses during the last 3 days of extinction. Yohimbine administration elicited the significant reinstatement of responding in female msP rats but not in male msP rats. Early social isolation increased the level of reinstatement in female msP rats only (**A**). Similarly, yohimbine reinstated alcohol seeking in female but not male Wistar rats. Early social isolation did not potentiate the effect of yohimbine (**B**). The data are expressed as the mean ± SEM of (a) total responses at the active lever and (b) inactive lever. Sex × treatment interaction: *$p < 0.05$, **$p < 0.01$; treatment × ESI interaction: $^{\#}p < 0.001$; sex × ESI interaction: $^{\$\$}p < 0.01$. Where not indicated, differences from controls were not statistically significant.

Fig. 1

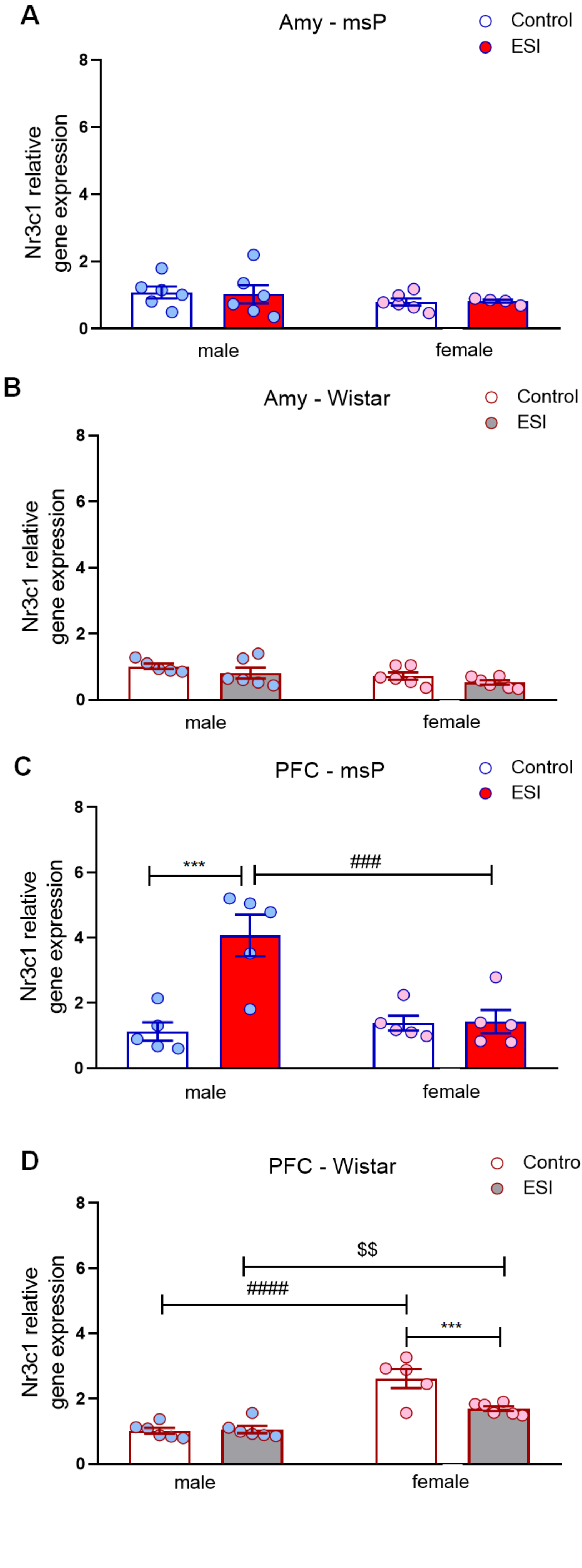
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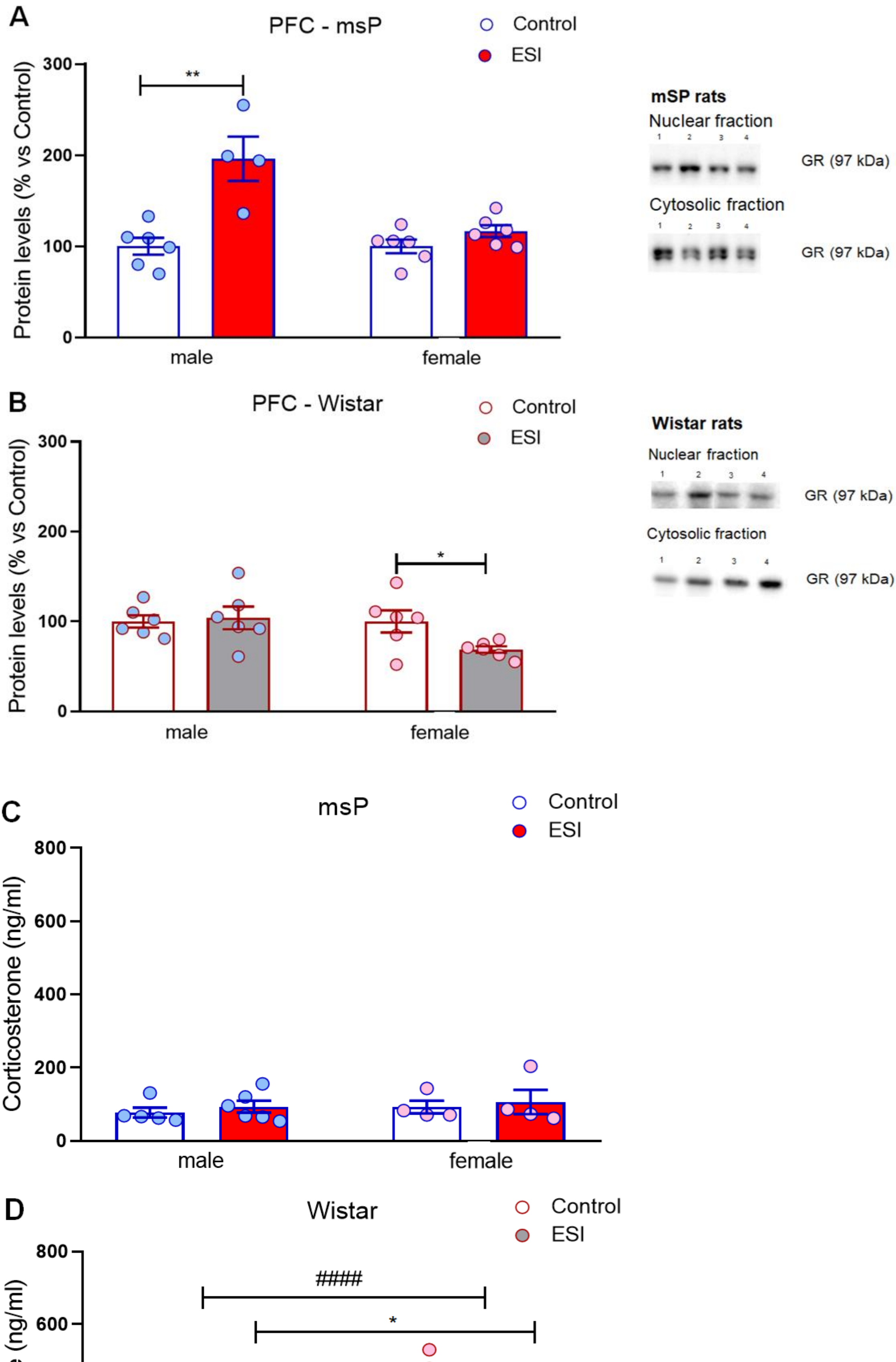
Fig. 2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female

Fig. 3

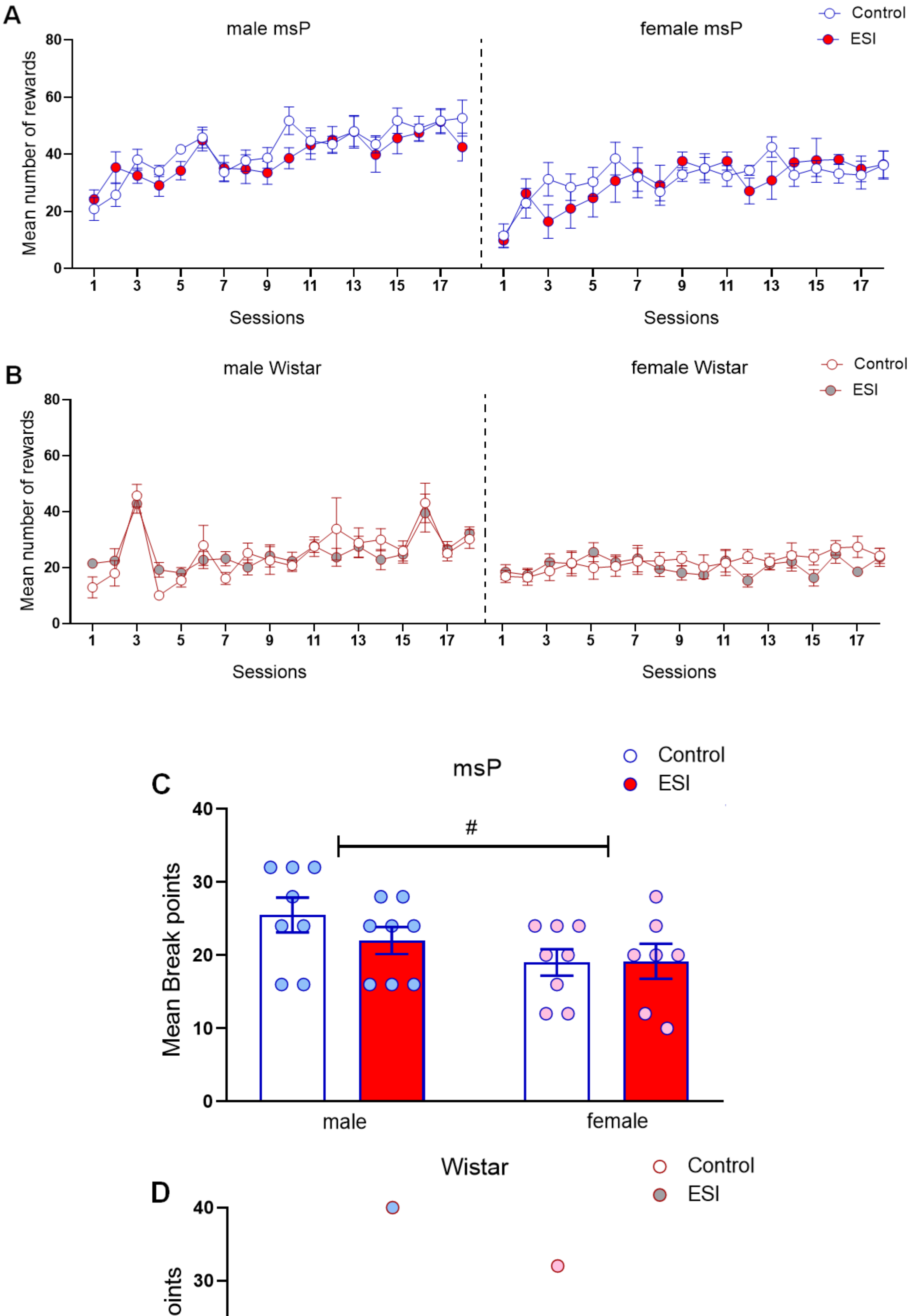

A
male msP
female msP
Control
ESI
Mean number of rewards
80
60
40
20
0
1 3 5 7 9 11 13 15 17
Sessions
B
male Wistar
female Wistar
Control
ESI
Mean number of rewards
Sessions
C
msP
Control
ESI
#
Mean Break points
40
30
20
10
0
male
female
D
Wistar
Control
ESI
Mean Break points
male
female

Fig. 4

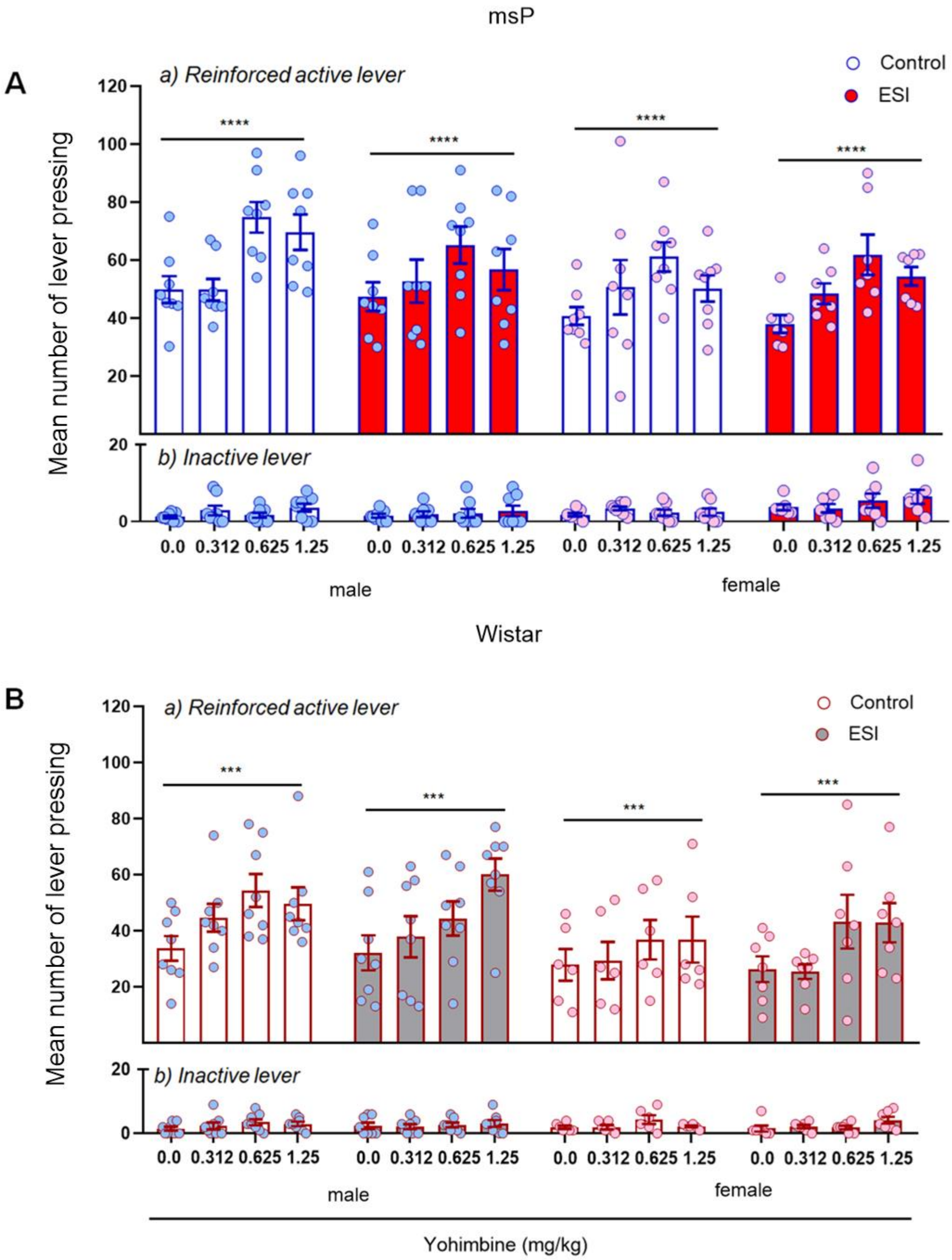
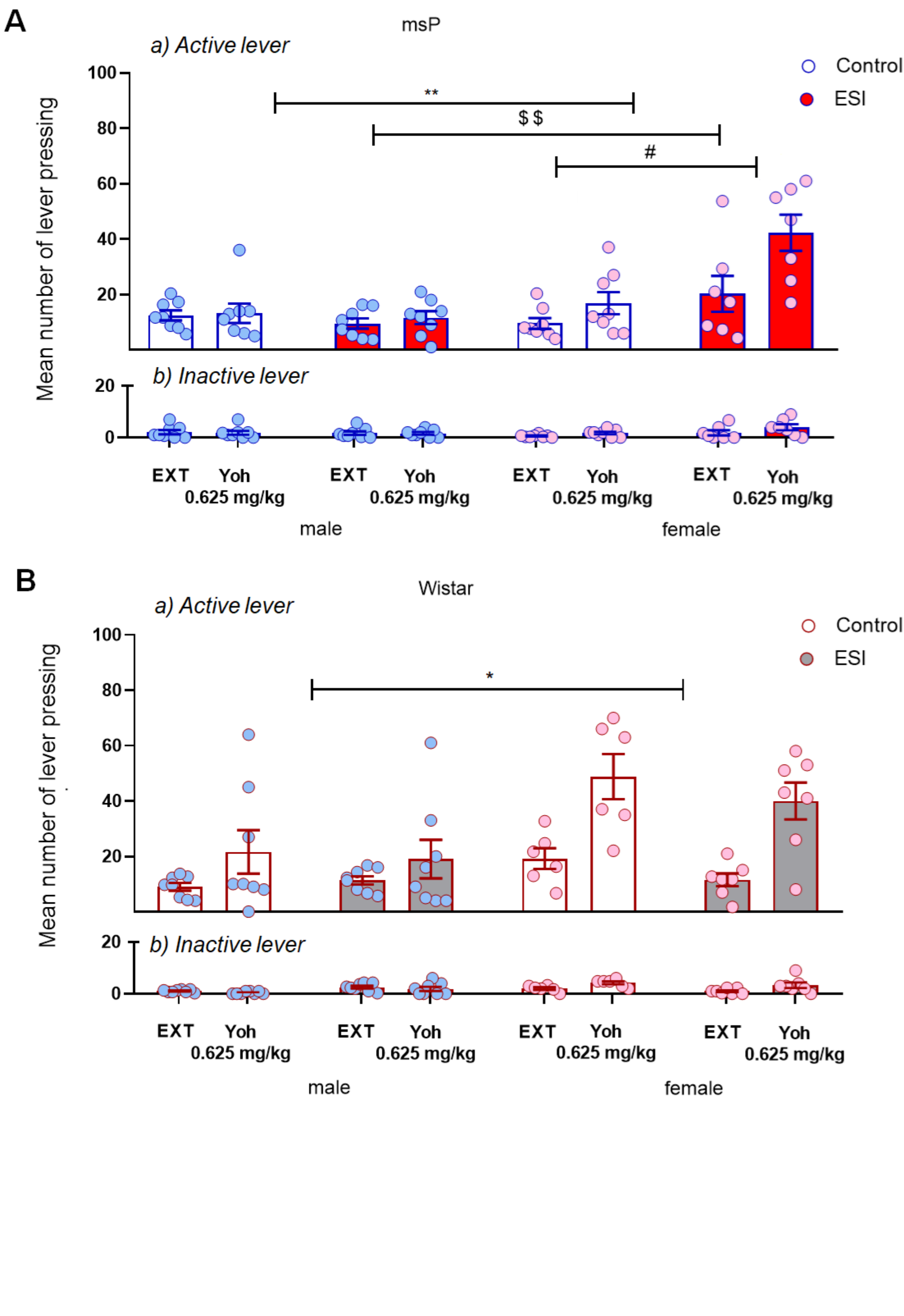

msP
A
a) Reinforced active lever
Control
ESI
Mean number of lever pressing
120
100
80
60
40
20
****
****
****
****
20
0
b) Inactive lever
0.0 0.312 0.625 1.25
0.0 0.312 0.625 1.25
0.0 0.312 0.625 1.25
0.0 0.312 0.625 1.25
male
female
Wistar
B
a) Reinforced active lever
Control
ESI
Mean number of lever pressing
120
100
80
60
40
20
***
***
***
***
20
0
b) Inactive lever
0.0 0.312 0.625 1.25
0.0 0.312 0.625 1.25
0.0 0.312 0.625 1.25
0.0 0.312 0.625 1.25
male
female
Yohimbine (mg/kg)

Fig. 5


A
msP
a) Active lever
Control
ESI
Mean number of lever pressing
100
80
60
40
20
**
$ $
#
b) Inactive lever
20
0
EXT
Yoh
0.625 mg/kg
male
female
B
Wistar
a) Active lever
*
b) Inactive lever