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ROLE OF THE NEUROPEPTIDE S SYSTEM ON ALCOHOL SELF ADMINISTRATION AND IMPULSIVITY IN LABORATORY RATS

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Abstract

Neuropeptide S (NPS) is a 20-amino acid neurotransmitter that exerts both pro-arousal and anxiolytic effects. The NPS system consists of the 20-amino acid neuropeptide S (NPS) and its receptor, the neuropeptide S receptor (NPSR). Previous research has associated the NPS system with alcohol use disorder (AUD) and impulsivity. AUD is a psychiatric condition caused by the interaction of excessive alcohol consumption with genetically predisposing factors. Impulsivity is a multifaceted behavior characterized by acts that are poorly conceived, prematurely expressed, risky, or inappropriate, often leading to undesirable consequences.

In this study, we verified the stimulatory, anxiolytic, and stress-coping roles of NPS in marchigian-sardinian alcohol-preferring (msP) rats using the open-field (OF), elevated plus maze (EPM), and fear conditioning (FC) tests. We investigated the effects of NPS on alcohol self-administration (ASA) in both male and female msP rats, verified whether the anxiolytic effect of NPS alone is sufficient to reduce alcohol seeking, and assessed whether NPS has the ability to suppress stress-induced relapse. We developed two Go/No-Go models of impulsivity, tested the effect of NPS on impulsivity in Wistar and msP rats, and examined the tetrapeptide RTI263—a truncated form of NPS that maintains the anxiolytic properties while being devoid of the stimulatory effect—in Wistar rats.

The results showed that NPS reduced ASA in both male and female msP rats through its anxiolytic and stress-coping effects, respectively. However, selective stimulation of the anxiolytic effect of NPS alone was sufficient to reduce alcohol seeking in females but not in males. The Go/No-Go models of impulsivity were validated with predictive validity, as verified by atomoxetine. MsP rats exhibited impulsive-like behavior compared to Wistar controls, which contributes to their heightened motivation for alcohol. NPS exerted an anti-impulsive effect on both msP and Wistar rats. The tetrapeptide RTI263 demonstrated a weaker effect than NPS in the impulsivity model.

In conclusion, this study provides novel insights into the role of the NPS system in AUD and impulsivity, highlighting the potential of the NPS system as a valuable target for future therapeutic interventions.

Key words

Neuropeptide S system, alcohol, stress, anxiety, fear conditioning, impulsivity

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CHAPTER 1

GENERAL INTRODUCTION

ALCOHOL

The consumption of alcohol has been a part of the human experience for thousands of years. The ease with which it can be obtained and the efficiency with which it is delivered have both contributed to its increasing prevalence across numerous nations and cultures, exerting a direct or indirect influence on various aspects of human life (Luo, 2021). This chapter aims to provide a concise overview of the existing knowledge and mechanisms related to alcohol-related symptoms, including alcohol use disorder (AUD), stress, anxiety, fear conditioning, impulsivity, and the neuropeptide S (NPS) system.

Pharmacology of Alcohol

Alcohol (ethanol) is a highly permeable organic compound with one hydroxyl group attached to an aliphatic carbon atom (Gold 2019; Patai 1971; Huberman 2022). It is both water- and fat-soluble (Patra et al. 2004) and can pass directly into cells, causing deleterious effects (Ilana Crome 2015; Berggren and Goldberg 1940; Huberman 2022), leading to significant physical and mental problems. Alcohol consumption has been shown to damage organ systems, particularly the brain, heart, liver, pancreas, and immune system. These effects can lead to mental illness, delirium tremens, irregular heartbeat, impaired neurocognitive function, and, in severe cases, death (American Psychiatric Association 2013; Romeo et al. 2007).

Upon consumption, alcohol is metabolized into acetaldehyde and acetate via a Nicotinamide Adenine Dinucleotide (oxidized) to Nicotinamide Adenine Dinucleotide (reduced) ratio (NAD-to-NADH ratio)-dependent pathway (Cederbaum 2012; Huberman 2022). Acetaldehyde is more toxic than alcohol, causing cell damage and death. Acetate is converted into empty energy and ATP, a process that is costly in terms of energy expenditure (Bose et al. 2019; Paton 2005; Huberman 2022). Alcohol consumption has a range of effects. Alcohol, acetaldehyde, and acetate can cross the blood-brain barrier and affect various brain regions indiscriminately (Peana et al. 2017; Huberman 2022). However, six notable effects require consideration: top-down control mechanism, serotonin, hypothalamic-pituitary-adrenal (HPA) axis, gut-brain axis, dopamine, and brain structure.

Top-down control mechanism

Alcohol affects neural pathways that mediate behaviors motivated by positive reinforcement, which are essential for survival (Tabakoff and Hoffman 2013). Alcohol disrupts the top-down control system (Renteria et al. 2018; Huberman 2022). The term "top-down control" refers to the regulatory process by which higher-order brain regions, such as the prefrontal cortex (PFC), influence lower-order brain regions, guiding behavior, attention, perception, emotions, and responses (Friedman and Robbins 2022). This system is critical for goal-directed actions, modulating automatic responses, and enabling deliberate decision-making in dynamic environments. In humans, it is involved in suppressing unwanted motor responses and promoting the selection of the most appropriate action (E. K. Miller and Cohen 2001; Friedman and Robbins 2022; Huberman 2022).

The impact of alcohol on the top-down control system is multifaceted. Alcohol diminishes the activity of neurons in the PFC that typically provide top-down inhibition (Tu et al. 2007; Huberman 2022), and regulates impulsive behavior, thinking, and planning. Suppression of PFC activity leads to reduced top-down control and an increase in habitual and impulsive behavior (S. Kim and Lee 2011; B. Li et al. 2020; Huberman 2022).

Alcohol also alters neural circuits that regulate habitual and impulsive behavior, reinforcing these tendencies (Renteria et al. 2018; C. R. Li et al. 2009; Huberman 2022). This occurs through synaptic plasticity, which facilitates information processing and behavioral learning. In AUD, alcohol modifies synaptic weights in circuits involving ventral tegmental area (VTA) dopamine neurons and their targets, as well as excitatory synapses on spiny projection neurons in the nucleus accumbens (NAc) that receive input from various brain regions (Luo 2021).

Alcohol binds to Gamma-Aminobutyric Acid Type A (GABAA) receptors, increasing Cl^- flow and enhancing inhibitory synaptic transmission by promoting the opening of Gamma-Aminobutyric Acid (GABA-) and glycine-gated channels (Mihic and Harris 1997). Additionally, alcohol may increase synapses in circuits controlling habitual behavior while reducing those regulating impulse control. This shift weakens the neural circuits responsible for self-regulation, leading to greater impulsivity (Huberman 2022).

Fortunately, this process is reversible. With sustained abstinence, neural circuits can be restored to their original state (Huberman 2022).

Serotonin and alcohol.

Alcohol ingestion significantly alters the activity of neurons that regulate serotonin release, a neurotransmitter functioning as a neuromodulator (Lovinger 1997; Huberman 2022). Also known as 5-hydroxytryptamine, serotonin is a monoamine

neurotransmitter that modulates neural circuits and transmits signals between nerve cells in the brain and body (Luo 2021). It plays a crucial role in regulating mood, well-being, cognition, reward, learning, memory, and physiological functions such as vomiting and vasoconstriction (Berger et al. 2009).

Alcohol and its metabolite, acetaldehyde, act as toxins at synapses—the connections between serotonergic neurons and other neurons—disrupting serotonin-related mood regulation (Tong et al. 2011; Huberman 2022).

Hypothalamic-pituitary-adrenal (HPA) axis.

Alcohol ingestion alters the HPA axis, which regulates physiological equilibrium in response to stress (Gianoulakis 1998; Huberman 2022). The hypothalamus signals the pituitary gland, which then secretes hormones into the bloodstream, prompting the adrenal glands to release adrenaline and cortisol—key components of the body's long-term stress response (W. L. Miller 2018; Leistner & Menke 2020). The HPA axis also plays a beneficial role in immune system regulation (W. L. Miller 2018). However, alcohol disrupts this system, leading to increased adrenaline and cortisol release even in the absence of alcohol consumption (Sheng et al. 2021).

Dopamine and alcohol.

Alcohol ingestion has been shown to influence dopamine levels (Di Chiara 1997; Huberman 2022). Chronic alcohol abuse leads to a long-lasting increase in excitatory input to VTA dopamine neurons (Luo 2021). Initially, alcohol consumption elevates dopamine transmission, enhancing mood and well-being. However, as alcohol is metabolized, dopamine and serotonin levels decline, leading to a subsequent drop in mood. This results in a cycle of alcohol consumption driven by the desire to regain the initial positive effects (Huang 2010; Yang et al. 2022; Huberman 2022).

Alcohol on Brain structure.

Alcohol consumption is negatively associated with global brain volume, brain macrostructure, brain microstructure, and both grey and white matter volume (Daviet et al. 2022). While heavy alcohol consumption is well known to contribute to neuronal loss, brain atrophy, and reduced white matter integrity, research by Daviet et al. suggests that even moderate to light alcohol intake is linked to declines in these brain structures (Daviet et al. 2022).

Grey and white matter are essential components of the central nervous system (CNS). Grey matter consists mainly of neuronal cell bodies, dendrites, unmyelinated axons, glial cells, and capillaries, playing a crucial role in processing and interpreting information (Mercadante and Tadi 2025). White matter, in contrast, is composed primarily of myelinated axons, which facilitate communication between different brain

regions and between the brain and spinal cord (Sampaio-Baptista and Johansen-Berg 2017).

Alcohol consumption has been shown to contribute to neocortical thinning and cerebral degeneration, particularly in the frontal lobes, vermis, and regions controlling the trunk and legs, especially in individuals with thiamine deficiency (Kandel et al. 2021).

Furthermore, alcohol alters neural circuitry and neurochemistry, as well as brain-to-body stress-related systems, which can reduce resilience, elevate baseline stress levels, and negatively impact mood (Koob and Volkow 2016; Koob 2003; Heilig et al. 2010; Huberman 2022).

Gut-brain axis.

Alcohol ingestion significantly impacts the gut-brain axis, a bidirectional communication network between the gastrointestinal tract and the CNS, including the brain (Appleton 2018; Huberman 2022; Sheng et al. 2021). This axis relies on neural, hormonal, and immune signaling pathways to regulate interactions between the brain and gut (Appleton 2018). Gut bacteria play a crucial role in immune function by transmitting electrical and chemical signals to the brain, promoting the release of neurotransmitters such as serotonin and dopamine, which positively influence mood (Y. Chen et al. 2021; Huberman 2022). However, alcohol disrupts the gut microbiota by destroying beneficial bacteria, impairing gut-brain communication, and negatively affecting overall health.

Neurobiological Bases of Alcohol Use Disorder

Genetically, alcohol has been shown to alter gene expression by modifying the binding of transcriptional regulatory proteins to themselves and to gene regulatory regions (Kandel et al. 2021). It has been hypothesized that some neurotic disorders and various forms of addiction may result from reversible defects in gene regulation (Kandel et al. 2021).

Multiple genes involved in serotonin and receptor synthesis, GABA receptors, top-down inhibition, the HPA axis, N-methyl-D-aspartic acid (NMDA) receptors, the NPS system, and dopamine, along with environmental, age-related, tolerance, and cultural influences, contribute to the development of AUD (Huberman 2022; Laas, Reif, et al. 2015). AUD is characterized by compulsive alcohol use despite long-term negative consequences, including loss of self-control and a tendency to relapse (Luo 2021). It is a serious physical and mental disorder caused by excessive alcohol consumption, leading to damage in vital organ systems, particularly the brain, heart, liver, pancreas, and immune system. This can result in severe health outcomes such as mental illness, delirium tremens, cardiac complications, impaired neurocognitive function, and, in extreme cases, death (American Psychiatric Association 2013; Romeo et al. 2007).

The World Health Organization (WHO) estimates that 400 million people (7% of the global population) have AUD, including 209 million (3.7% of the adult global population) who suffer from alcohol dependence (World Health Organization 2024). Geographically, AUD are least common in Africa and Asia, with higher rates in Europe and the Americas, as shown in Figure 1 (Landgeist 2022). Alcohol consumption is a major public health risk, contributing to 5.1% of the global burden of disease and injury (WHO 2016).

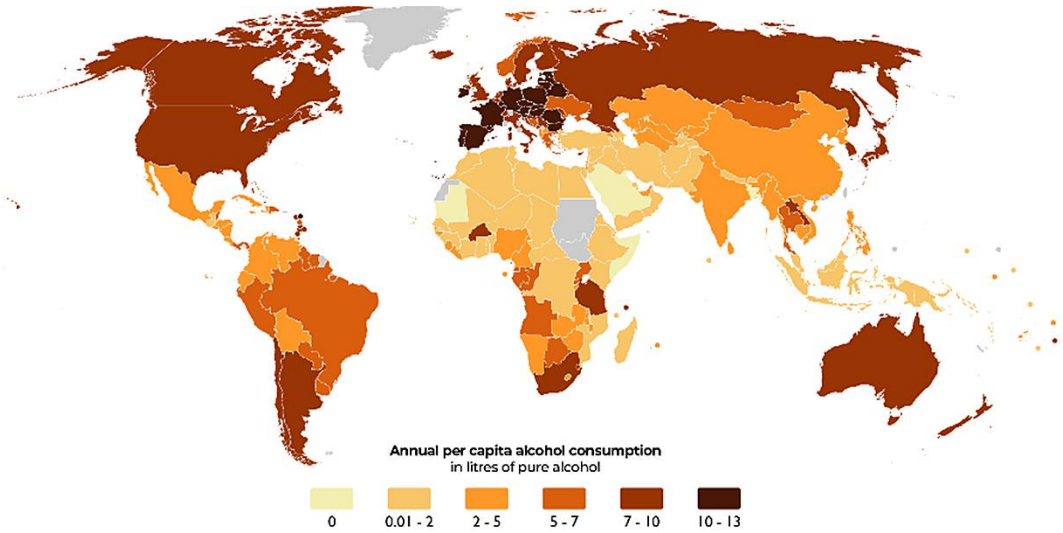

Figure1. Alcohol consumption worldwide in 2019 (adapted from Landgeist 2022) (Landgeist 2022)

Imaging studies in humans and animals have identified three major neurocircuits involved in AUD:

1. The **ventral tegmental area and basal ganglia**, which drive the binge/intoxication stage.
2. The **extended amygdala**, which drives the withdrawal/negative affect stage.
3. The **prefrontal cortex**, which drives the preoccupation/anticipation (craving) stage (Koob and Volkow 2016).

The complexity of alcohol's effects on brain neurotransmission and the multiple neurotransmitters involved in AUD neurocircuitry make it difficult to pinpoint the most critical contributing system (Huberman 2022). Notably, the **mesocorticostriatal dopamine system** plays a role in all three stages of AUD. Dopamine levels **increase** during binge/intoxication, **decrease** during withdrawal/negative affect, and **increase again** during craving (Koob and Volkow 2016). This fluctuation is linked to the rapid activation of low-affinity dopamine D1 receptors, which mediate drug reward and conditioned responses. However, stimulation of high-affinity dopamine D2 receptors appears insufficient for drug reward and may even limit it (Volkow et al. 2019).

Despite uncertainties regarding the precise molecular mechanisms underlying AUD, three key findings provide insight:

1. Alcohol stimulates the brain's reward system by increasing the activity of VTA dopamine neurons (Luo 2021). This elevates dopamine levels on output targets of VTA dopamine neurons, including the nucleus accumbens (NAc), which processes reward signals, and the PFC, which governs executive functions such as decision-making. VTA dopamine neurons receive excitatory input from many brain areas, including glutamatergic excitatory input from the PFC and GABAergic inhibitory input from local and NAc neurons (Luo 2021).
2. Alcohol modulates GABAergic activity, either by directly activating GABAA receptors or by inducing GABA release in the VTA, NAc, and amygdala (Koob and Volkow 2010).
3. Alcohol consumption triggers the release of opioid peptides in the VTA, central nucleus of the amygdala, and NAc, further reinforcing its rewarding effects (Koob and Volkow 2010).

Risk Factors Promoting Alcohol Use Disorder

The development of AUD is influenced by four primary risk factor categories: **biological, psychological, environmental-social, and behavioral patterns**. These factors often interact, increasing the likelihood of AUD (Chartier et al. 2017).

1. Biological Factors

- **Genetic Factors:** Numerous genes have been linked to AUD, particularly those involved in serotonin synthesis, receptor function, GABA receptors, top-down inhibition, the HPA axis, NMDA, the NPS system, and dopamine regulation (Huberman 2022; Laas, Reif, et al. 2015). The genetic underpinnings of AUD are complex and multifaceted.
- **Epigenetics:** Epigenetics refers to phenotypic changes that do not involve alterations in gene expression. Alcohol has been shown to induce epigenetic modifications, such as changes in DNA methylation and chromatin remodeling, which can increase AUD risk. Emotional stressors and social adversity may trigger epigenetic responses that alter reward signaling pathways, potentially predisposing individuals to alcohol use (Palmisano and Pandey 2017).
- **Transgenerational Epigenetic Inheritance:** Preclinical studies in rats suggest that addiction-induced epigenetic changes can be passed from parents to offspring, influencing behavioral phenotypes related to addiction risk, including AUD. These heritable changes include DNA methylation, histone modifications, and microRNA alterations. A family history of AUD increases susceptibility due to genetic predisposition. Further research is needed to determine the specific epigenetic alterations and behavioral consequences in human offspring (Heidari et al. 2023; Vassoler and Sadri-Vakili 2014).

- **Neurobiology:** Differences in brain chemistry, particularly within the dopamine and serotonin systems, play a role in alcohol cravings and AUD development (Ma and Zhu 2014; Clapp et al. 2008).
- **Gender:** Evidence suggests that while males are at higher risk for AUD, females may be more vulnerable to its effects, even at lower levels of alcohol consumption (White 2020).

2. Psychological Factors

Psychological factors, such as stress, anxiety, post-traumatic stress disorder (PTSD), and impulsivity, significantly influence AUD development, maintenance, and progression (Castillo-Carniglia et al. 2019; Morris et al. 2020).

- **Stress:** Chronic stress increases the likelihood of using alcohol as a coping mechanism. Poor stress management, trauma, or grief can contribute to alcohol dependence. Animal studies also indicate that stress can influence gene expression (Becker et al. 2011).
- **Anxiety:** Alcohol is often used to alleviate anxiety symptoms, but long-term use can worsen anxiety disorders (Kushner 1990).
- **PTSD and Trauma:** Individuals who have experienced trauma or adverse life events are at greater risk for AUD. Some with PTSD use alcohol to cope with intrusive memories, nightmares, and hyperarousal symptoms (Kessler 1995; Jacobsen et al. 2001).
- **Impulsivity:** High impulsivity is linked to AUD, as individuals with this trait may struggle with self-control and engage in risky behaviors, including excessive alcohol consumption (Shin et al. 2012; Dick et al. 2010).

3. Environmental-Social Factors

Environmental influences play a crucial role in AUD risk.

- **Family Influence:** Exposure to alcohol use or abuse within the family promote drinking behaviors.
- **Peer Influence:** Social circles that encourage heavy drinking increase AUD risk.
- **Cultural Norms:** Societal attitudes toward alcohol, including relaxed drinking norms, are associated with higher rates of AUD. Conversely, protective factors such as strong family and peer relationships, higher socioeconomic status, and education reduce the risk of AUD. Adverse conditions, including parental neglect or social exclusion, heighten the likelihood of developing AUD in later life (Buu et al. 2007; Hawkins et al. 1992; Borsari and Carey 2001; Room and Mäkelä 2000).
- **Alcohol Availability and Marketing:** Easy access to alcohol increases consumption risk. Aggressive marketing and media campaigns, particularly

targeting young people, significantly impact drinking behavior (Anderson et al. 2009; Smith and Foxcroft 2009).

4. Behavioral Patterns

- **Early Onset of Alcohol Use:** Beginning alcohol consumption at a young age is associated with a higher likelihood of developing AUD later in life (Hingson et al. 2006).
- **Binge Drinking:** Consuming large amounts of alcohol in a short period significantly increases the risk of alcohol dependence (Naimi et al. 2003).
- **Habitual Use:** Using alcohol regularly, particularly as a coping mechanism for emotions or stress, can lead to AUD (Zaorska et al. 2023).

Treatment for Alcohol Use Disorder

The complex nature of AUD, encompassing biological, psychological, environmental, social, cultural, and spiritual (biopsychosocial-spiritual) dimensions, necessitates multifaceted treatment approaches tailored to individual needs. These strategies include behavioral therapies, medications, lifestyle changes, long-term follow-up, and alcohol sale restrictions (Rezende-Pinto and Moreira-Almeida 2023; McHugh et al. 2010; Volkow et al. 2016; Anton et al. 2006).

1. Behavioral Therapies

Behavioral interventions play a crucial role in understanding and modifying alcohol consumption patterns. Common approaches include:

- **Cognitive Behavioral Therapy:** Focuses on identifying and modifying thoughts and behaviors related to AUD (Carroll and Kiluk 2017).
- **Motivational Enhancement Therapy:** Aims to increase motivation to stop drinking by addressing ambivalence (Kumar et al. 2021).
- **Contingency Management:** Provides tangible rewards for maintaining sobriety (Petry 2013).
- **12-Step Facilitation Therapy:** Prepares individuals to engage in community-based 12-step programs, such as Alcoholics Anonymous (Cooper-Sadlo and Chou 2019).

2. Medications

Several medications are approved for AUD therapy by Food and Drug Administration (FDA) (Akbar et al. 2018; Stokłosa et al. 2023):

- **Disulfiram:** Produces adverse reactions when alcohol is consumed, deterring drinking.

- **Naltrexone:** An opioid antagonist that reduces alcohol cravings and is effective for up to a year post-treatment.
- **Acamprosate:** Helps maintaining abstinence by restoring the brain's chemical balance and reducing cravings.
- **Benzodiazepines:** Helps in alcohol detoxification and withdrawal management.

The effectiveness of these medications depends on adherence to the treatment regimen. However, non-adherence—due to side effects or discontinuation—remains a common challenge in AUD treatment (Weiss 2004).

3. Lifestyle Changes

Developing healthy habits plays a crucial role in preventing and managing AUD:

- **Exercise:** Aerobic exercise, particularly endurance activities (e.g., marathon running), has been shown to reduce alcohol consumption, decrease relapse risk, and increase dopamine receptor D2 density (Lardier et al. 2021).
- **Avoiding Triggers:** Identifying and avoiding drinking triggers can help maintain sobriety.
- **Support Networks:** Strong social support from **friends, family, and peers** provides emotional and practical assistance in recovery.

4. Long-Term Follow-Up

Since AUD is a chronic condition, ongoing support is essential for sustained recovery. Regular check-ins with healthcare providers and participation in support groups can help prevent relapse (Moos and Moos 2007).

5. Alcohol Sale Restrictions

Regulating alcohol availability, particularly for adolescents, is an effective strategy in AUD prevention. Additionally, education and accessible treatment services play a key role in reducing alcohol-related harm (Gruenewald 2011).

A comprehensive review of existing literature highlights the complex interplay of biological, psychological, social, and cultural factors in AUD (MacKillop et al. 2022). While the molecular mechanisms underlying AUD remain incompletely understood, recent research has identified promising new treatment targets that aim to restore the brain stress and emotional regulation systems to homeostasis (Huberman 2022; Schank et al. 2012).

Given the strong link between stress, anxiety, PTSD, and AUD, reducing stress and anxiety levels may serve as an effective preventive strategy (Smith and Cottler 2018). This study examines the role of NPS in alcohol self-administration (ASA), stress,

anxiety, fear conditioning, relapse, and impulsivity, which will be explored in the next section.

NEUROPEPTIDE S SYSTEM AND ITS FUNCTIONS

Neuropeptide S

NPS is a 20-amino acid neurotransmitter first described in 2004 for its anxiolytic and arousal effects (Xu et al. 2004). As an excitatory neurotransmitter, it increases intracellular free Ca^{2+} and stimulates cyclic adenosine monophosphate (cAMP) synthesis at low nanomolar concentrations in a dose-dependent manner (Xu et al. 2004). The name "neuropeptide S" was proposed due to the presence of serine as the amino-terminal residue in the primary structure of the mature peptide across species (Botticelli et al. 2021). NPS shares no homology with other neuropeptides and is highly conserved among vertebrates (Shirsath et al. 2024).

Bioinformatic analysis of genome databases has shown that the NPS precursor gene is present in all tetrapods, with strong sequence conservation across mammals. The absence of this gene in fish, amphibians, and reptiles indicates that NPS is a relatively recent addition in vertebrate evolution (Rainer K. Reinscheid 2007). The remarkable conservation of the NPS gene suggests it plays a critical functional role, likely due to evolutionary pressures that enabled organisms to cope with dangerous situations requiring intense arousal, alertness, and reduced anxiety and fear (R. K. Reinscheid 2005).

NPS is typically co-released with other neurotransmitters and interacts with various brain systems and neurotransmitters, including orexin-A, corticotropin-releasing factor (CRF), oxytocin, and adenosine (Botticelli et al. 2021). It also modulates the activities of catecholamines, GABA, and glutamate (Cannella et al. 2022), and interacts with key components of the peripheral and central stress response systems, specifically Hypocretin-1/Orexin-A (Hcrt-1/Ox-A) and CRF (Cannella et al. 2022).

Once released, NPS can diffuse widely, affecting multiple targets and producing various effects. Over the past two decades, research has expanded our understanding of the pharmacology, neurobiology, physiology, genetics, and functions of the NPS system. It has been investigated for its involvement in numerous biological processes in both central and peripheral response systems, including arousal, anxiety, food intake, locomotion, cognition, mood, vigilance, wakefulness, fear, emotionality, stress, gastrointestinal functions, and addiction-like behaviors. The consequences of alcohol misuse are wide-ranging and can affect various physiological functions, including the immune system, sleep patterns, cognitive abilities (e.g., learning and memory), pain perception, energy levels, endocrine balance, obesity, social decision-making, panic disorders, schizophrenia, compulsive disorders, attention deficits, emotional regulation,

asthma, inflammation, nociception, inflammatory bowel disease, and endometriosis (Tobinski and Rappeneau 2021).

Neuropeptide S receptor (NPSR)

Neuropeptide S receptor (NPSR) is a member of the G protein-coupled receptor superfamily of integral membrane proteins (Xu et al. 2004). It couples with both Gαq and Gαs proteins to elevate intracellular Ca^{2+} and cAMP, thereby increasing cellular excitability (Park et al. 2021). Multiple human NPSR isoforms have been identified as products of alternative splicing of NPSR mRNA (S.K. Leonard and Ring 2011; Laitinen et al. 2004). This splicing produces up to eight variants of human NPSR (NPSR A, BLONG, BSHORT, C-G), which differ in protein sequence and membrane topology (Pulkkinen et al. 2006). Of these, only three isoforms—A, BLONG, and G—are likely to have the typical 7-transmembrane topology of G protein-coupled receptors. Current evidence indicates that only the hNPSR-A and hNPSR-BLONG isoforms produce functional receptors that are trafficked to the cell membrane (Vendelin et al. 2005). In contrast, rats and mice express a single NPSR isoform, which shares 89% homology with the human NPSR-A (Pulkkinen et al. 2006; S.K. Leonard and Ring 2011).

The **human NPSR gene**, located on chromosome 7, is characterized by multiple single nucleotide polymorphisms (SNPs) and splice variants (Botticelli et al. 2021). These variations result in functional differences in the NPSR. The rs324981 SNP has been extensively studied and linked with panic disorder, anxiety, fear evaluation, stress responsiveness, maladaptive personality traits, early-onset bipolar disorder, and other conditions, including sleep, allergic, and inflammatory diseases (Rainer K. Reinscheid and Ruzza 2021).

NPSR exists in two functional isoforms derived from an A>T SNP, resulting in an Asn-Ile amino acid exchange at position 107 (SNP591694 A > T; ref. SNP ID: rs324981) (Botticelli et al. 2021). This SNP is human-specific. The T allele (Asn107Ile mutation) leads to a gain of function in NPSR, with NPS/agonists activating the NPSR Ile107 isoform with approximately 10-fold higher potency than the NPSR Asn107 isoform (Lennertz et al. 2012). The NPSR107 Ile polymorphism is frequently linked to various disease manifestations, except in schizophrenia, where T allele carriers exhibited enhanced cognitive performance (Rainer K. Reinscheid and Ruzza 2021). The NPSR107 Ile polymorphism is also associated with alcohol use disorder, impulsivity, stress sensitivity, fear responses, anxiety, hyperactivity, neuroticism, panic disorder, and maladaptive personality traits, among others (Laas et al. 2014; Laas et al., 2015).

The A allele of the **NPSR1-Asn107 SNP** (rs324981) has been shown to encode lower NPSR function and is associated with allergic and inflammatory diseases. While this allele is postulated to have a protective effect, this has yet to be demonstrated independently (Rainer K. Reinscheid and Ruzza 2021). Furthermore, NPSR1-Asn107

has become the predominant allele in European and Asian populations (Rainer K. Reinscheid and Ruzza 2021).

A gain-of-function variant, **NPSR1-His206**, was identified in a single family, where it was associated with a dramatic reduction in total sleep time (Rainer K. Reinscheid and Ruzza 2021). This supports the hypothesis that the high-activity variant NPSR1-Ile107 is linked to delayed bedtime and highlights the role of NPS in arousal (Rainer K. Reinscheid and Ruzza 2021).

A **mutation in the NPS peptide**, Leu6-NPS (rather than Val6; rs4751440, G/C), has been shown to reduce agonist potency by a factor of 10-20, but no phenotypes associated with this variant have been reported (Robledo et al. 2012; Deng et al. 2013).

Neuropeptide S system

The **neuropeptide S system** consists of NPSR and its endogenous ligand, NPS (Botticelli et al. 2021; Y.-L. Xu et al. 2004). The NPSR system has been detected in various tissues and organs, including the placenta, bone marrow, peripheral blood, skin, skeletal muscle, uterus, ovaries, prostate, bladder, kidneys, adrenal glands, spleen, pancreas, liver, colon, small intestine, stomach, esophagus, mammary glands, thyroid, thymus, aorta, heart, lungs, salivary glands, pituitary, spinal cord, olfactory bulb, and brain regions such as the brainstem, midbrain, forebrain, cerebellum, cortex, hypothalamus, hippocampus, and others (Y.-L. Xu et al. 2004). The highest levels of expression are observed in the CNS and in peripheral tissues like the thyroid, salivary, and mammary glands (Y.-L. Xu et al. 2004). The distribution pattern of the NPSR system in the brain is only partially conserved between humans, rats, and mice, with some significant differences that vary among individuals.

In the human brain, NPS precursor mRNA has been detected in the pons, particularly in the medial and lateral parabrachial nuclei, the Kölliker-Fuse nucleus, and the adjacent lateral lemniscus and pontine central grey matter (Adori et al. 2015). NPSR mRNA is predominantly located in the rostral laterodorsal tegmental nucleus, the cuneiform nucleus, the microcellular tegmental nucleus, and the periaqueductal grey (Adori et al. 2015).

In the rat brain, NPS precursor gene expression is observed in the trigeminal principal sensory nucleus, the lateral parabrachial nucleus, the peri-locus coeruleus area, the pontine central gray matter, and scattered neurons in the amygdala and hypothalamic dorsomedial nucleus (Y.-L. Xu et al. 2004; Yan-Ling Xu et al. 2007; Adori et al. 2015; Tobinski and Rappeneau 2021). NPSR mRNA expression is widely distributed in the brain, with the strongest expression in the olfactory nuclei, amygdala, subiculum, certain cortical structures, and various thalamic and hypothalamic regions (Cannella et al. 2022). The presence of NPSR protein has been identified in the medial amygdala, substantia nigra pars compacta, subiculum, dorsal raphe, and several hypothalamic and

thalamic regions (S.K. Leonard and Ring 2011). Double-label in situ hybridization has shown that NPS precursor mRNA is predominantly expressed in glutamatergic neurons, corticotropin-releasing factor-positive neurons, and cholinergic neurons (Yan-Ling Xu et al. 2007). These NPS-expressing neurons project to regions such as the amygdala, hypothalamus, thalamus, and cortex, suggesting that NPS plays a role in modulating functions in these areas. The extensive projections of these neurons influence a range of physiological and behavioral processes, including arousal, stress, anxiety, impulsivity, and wakefulness (Yan-Ling Xu et al. 2007). However, the precise subcellular location of the receptor proteins remains to be clarified.

In mice, NPS precursor mRNA has been studied in two brainstem regions: the peri-locus coeruleus area and the Kölliker-Fuse nucleus in the lateral parabrachial nucleus area (S. D. Clark et al. 2011; X. Liu et al. 2011). In contrast, robust NPSR mRNA expression has been identified in the dorsal endopiriform nucleus, intra-midline thalamic and hypothalamic regions, basolateral amygdala, subiculum, and multiple cortical regions (S. D. Clark et al. 2011).

Role of NPS System in AUD

Substantial clinical and preclinical evidence suggests that the NPS system plays a role in AUD. In the context of human AUD, the NPS system is involved in five key aspects of alcohol's mechanism of action, as outlined in Chapter 1: top-down control mechanisms, serotonin, the HPA axis, the gut-brain axis, and dopamine.

1. **Top-down control mechanisms**: The NPS system modulates GABA neurotransmission. NPS enhances the activity of GABAergic neurons in specific brain regions, either directly or through indirect mechanisms involving other neurotransmitter systems (Jüngling et al. 2008). It also modulates the release of GABA from presynaptic terminals, either increasing or decreasing its availability depending on the context (Meis et al. 2011).
2. **Serotonin**: NPS modulates serotonergic neurons, particularly in the dorsal raphe nucleus, a major source of serotonin in the brain (Andrade and Haj-Dahmane 2013). By increasing the firing rate of serotonergic neurons, NPS increases serotonin release in target regions such as the medial prefrontal cortex and limbic system (Si et al. 2010). NPS binding to NPSR activates intracellular signaling cascades, such as increased cAMP and calcium signaling, which can affect neuronal excitability and synaptic plasticity (Y.-L. Xu et al. 2004; Park et al. 2021).
3. **HPA axis**: NPS activates the HPA axis, modulating CRF-positive neurons in the hypothalamus and stimulating CRF release from the paraventricular nucleus. This activation leads to the secretion of adrenocorticotropic hormone (ACTH) from the pituitary, which increases cortisol or corticosterone levels from the adrenal glands (K. L. Smith et al. 2006). NPS also fine-tunes the HPA axis

response to stress, enhancing arousal and vigilance, while exerting anxiolytic effects that buffer against excessive anxiety (Okamura and Reinscheid 2007).

4. **Gut-brain axis**: The NPS system plays a key role in lymphocyte proliferation and macrophage phagocytosis, processes crucial for compensating for immune deficiencies caused by imbalanced gut microbiota (Filaferro et al. 2013). NPS modulates parasympathetic and sympathetic outputs to the gut by acting on brain regions like the hypothalamus and brainstem. It also interacts with other neurotransmitter systems such as serotonin and GABA, which are important for regulating gut motility, secretion, and sensation (Y. Chen et al. 2021; Holzer and Farzi 2014).
5. **Dopamine**: NPS modulates dopaminergic regulation, particularly in the VTA, a critical region for dopamine production. NPS increases dopamine release in the VTA and key target areas, including the nucleus accumbens and prefrontal cortex (Mochizuki et al. 2010). NPS also regulates the mesocorticolimbic dopamine pathway, stimulating the release of extracellular dopamine and its metabolites (Mochizuki et al. 2010; Si et al. 2010). Additionally, NPS significantly increases dopamine activity in the mesolimbic pathway.

In a clinical context, Laas and colleagues reported that the NPSR gene variant and environmental factors contribute to AUD and alcohol consumption in humans (Laas, Reif, et al. 2015). They found that the A/T variants of the NPSR polymorphism are associated with AUD and exhibit differential sex effects. AUD and harmful alcohol use were more prevalent in female A-allele carriers during both adolescence and adulthood. In contrast, in males, AUD and harmful alcohol use were more common in T-allele carriers, particularly among those exposed to adverse environments at age 15. Alcohol use was also higher in male T-allele carriers at ages 15 and 18. However, for men at age 25, the A allele was associated with higher alcohol consumption (Laas, Reif, et al. 2015). Complex interactions between the rs324981 SNP, sex, and alcohol consumption were identified in Caucasian individuals. In men, T-allele carriers exhibited increased alcohol consumption in late adolescence and an elevated risk of AUD in young adulthood. In women, the A allele was identified as a vulnerability allele for both adolescence and adulthood (Laas, Reif, et al. 2015).

In rodents, NPS has been shown to exert a dual and somewhat paradoxical effect on alcohol consumption and seeking behaviors. These effects depend on the genetic background and emotional state of the animal. For example, in a study comparing Wistar rats and Marchigian Sardinian alcohol-preferring (msP) rats, intracerebroventricular NPS reduced alcohol reward in male msP rats, while having no effect or an opposite effect in Wistar rats (Cannella et al. 2016). Furthermore, central infusion of NPS significantly reduced the intake of 20% ethanol in a voluntary limited access paradigm and in the context of self-administration of ethanol in naive rodents (Enquist et al. 2012), as well as in msP rats (Cannella et al. 2016). While NPS did not alter alcohol self-administration in non preferring Wistar rats, infusion of NPS into the

LH increased this behavior (Cannella et al. 2009). Intriguingly, NPS reduced the intake of 15% ethanol in a two-bottle choice paradigm in female P (alcohol-preferring) rats, yet it had no effect in female NP (non-alcohol-preferring) rats (Badia-Elder et al. 2008).

Role of NPS System in Relapse

In the clinical context, compulsive alcohol seeking and relapse are recognized as the primary challenges associated with human AUD. The recurrence of alcohol use is primarily attributed to exposure to environmental stimuli previously associated with alcohol consumption (Cannella et al. 2019). Relapse models based on environmental conditioning rely on cues linked to the rewarding effects of alcohol (Janak 2013). When re-exposed to alcohol-related cues, individuals recall the pleasurable effects of alcohol, which can lead to the resumption of alcohol use (Chaudhri et al. 2008). It is clear that environmental conditioning is a long-term phenomenon and serves as a significant catalyst for relapse in AUD. This recurrence can occur even after several months of abstinence (Cannella et al. 2019; Monti et al. 2000; Sinha and Li 2007). Psychological factors, such as coping deficits (inability to cope with stress or emotions without alcohol) and cognitive distortions (e.g., the belief that one drink is harmless), often contribute to relapse.

Changes in the brain's reward system, particularly in areas like the mesolimbic dopamine pathway, PFC, and HPA axis, can increase sensitivity to alcohol-related cues and make relapse more likely (Blaine et al. 2016; Clapp et al. 2008).

Research has shown that NPS may reduce the likelihood of alcohol relapse. Relapse is often associated with a complex interplay of stress, anxiety, fear, cognitive control, emotional dysregulation, craving, and reward. NPS has been identified as a potential modulator of neural circuits regulating these processes. For example, NPS has been shown to reduce stress by affecting the HPA axis (K. L. Smith et al. 2006) and to regulate the brain's reward system, including the mesolimbic dopamine pathway, which plays a significant role in addiction and relapse (Si et al. 2010; Holanda et al. 2021).

Cannella and colleagues demonstrated that administration of NPS to the intra-lateral hypothalamus significantly increased alcohol relapse behavior in animals. This suggests that the lateral hypothalamus plays an important role in NPS's effect on alcohol relapse. Activation of the NPSR in the lateral hypothalamus increases the likelihood of alcohol seeking when triggered by environmental cues. The role of NPS in enhancing ethanol seeking was mediated by the Hcrt-1/Ox-A system, as administration of the orexin receptor type 1 receptor antagonist SB334867 blocked this effect (Cannella et al. 2009).

These findings highlight NPS as a promising target for the development of pharmacological interventions aimed at preventing relapse in individuals with AUD. However, further research is needed to validate these results in clinical settings.

Brief Description of Stress and its Effects

Stress is defined as the body's natural response to a challenge or demand (Chrousos 2009). It can be physical, mental, or emotional, and is triggered by both external and internal factors. When individuals perceive a situation as threatening, their bodies activate the 'fight or flight' response, releasing hormones such as adrenaline and cortisol to prepare for a response (Sharma and Pal 2021).

The sympathetic and parasympathetic nervous systems have distinctly different functions in response to stress. The parasympathetic nervous system conserves the body's resources and restores homeostasis. It is responsible for rest, digestion, and maintaining the body's basal heart rate, respiration, and metabolism under normal conditions (Kandel et al. 2021). In contrast, the sympathetic nervous system plays a critical role in the body's response to stress (Baak 2001). It regulates the 'fight or flight' response, which is triggered during emergencies to enable the body to react to sudden changes, whether emotional (e.g., stress) or physical (e.g., a fight, athletic competition, or blood loss). The sympathetic nervous system increases output to the heart, peripheral vasculature, sweat glands, piloerector muscles, and certain eye muscles (Kandel et al. 2021). Survival of an animal with an experimentally removed sympathetic nervous system depends on factors such as shelter, warmth, and protection from stress and emotional stimuli (Kandel et al. 2021).

Post-traumatic stress disorder (PTSD) manifests following exposure to a traumatic event, such as combat or physical abuse, which can be life-threatening. PTSD is characterized by recurrent anxiety, often triggered by memories of the original trauma. A notable feature of PTSD is the persistence of traumatic memories, which can last for decades and may be reactivated by various triggers and stressors. This phenomenon is attributed to the activation of the noradrenergic system by these reactivating stimuli, supporting the hypothesis that uncontrollable stress significantly amplifies noradrenergic function in the brain (Kandel et al. 2021).

Severe stress in animals results in intense presynaptic activity and sustained firing of postsynaptic adrenergic neurons, placing greater demands on neurotransmitter synthesis. In response, the tyrosine hydroxylase gene is induced to increase enzyme production. Elevated levels of tyrosine hydroxylase are observed in the cell body within hours and at nerve terminals days later (Kandel et al. 2021).

Several hormones and brain regions are involved in the stress response:

1. Hormones secreted by the anterior and posterior pituitary glands regulate various physiological functions related to stress (El Sayed et al. 2023).
2. Uncontrollable stress causes a significant increase in noradrenergic function in the brain (Kandel et al. 2021).

3. Corticotropin-releasing factor receptors are implicated in the stress response (Sukhareva 2021).
4. Social stressors in humans may reduce serotonin levels, with serotonin metabolism influenced by both environmental and genetic factors (Muscatell et al. 2021).
5. The hypothalamus plays a pivotal role in regulating stress by modulating hormone release and autonomic nervous system activity, affecting physical and immunological responses (Kandel et al. 2021).
6. The central amygdala regulates the output of the amygdala complex, which induces neuroendocrine responses, including stress hormone production, through projections to the hypothalamus and brainstem (Y. Xu et al. 1999).
7. The release of beta-endorphin and ACTH into the bloodstream in response to stress has been well documented. Beta-endorphin, a precursor of ACTH, is primarily expressed in the pituitary gland (Pilozzi et al. 2020).
8. The uptake of monoamines is a key regulatory step, with stress leading to an upregulation of amine transporter mRNA and a sustained increase in norepinephrine production (Kandel et al. 2021).
9. Certain peptides, such as γ-melanocyte-stimulating hormone, ACTH, and β-endorphin, are located in specific regions of the CNS that regulate complex stress responses (Kandel et al. 2021).
10. Stress has been shown to stimulate both opioid and non-opioid mechanisms of analgesia (Mogil et al. 1996) (Kandel et al. 2021).

A variety of factors, including development, hormones, stress, drug addiction, alcoholism, and learning, have been identified as capable of altering gene expression. These factors modify the binding of transcriptional regulatory proteins to regulatory regions of genes. It is hypothesized that neurotic illnesses and certain forms of drug addiction, including AUD, result from reversible defects in gene regulation (Kandel et al. 2021).

Role of NPS System in Stress Response

The NPS system plays a crucial role in modulating stress-related behaviors. 1) It has been shown to modulate the HPA axis, which regulates the stress response. NPS stimulates or regulates CRF neurons in the hypothalamus, fine-tuning the release of stress hormones such as cortisol (Kageyama et al. 2021). 2) NPS interacts with other neurotransmitters, including GABA, glutamate, dopamine, and serotonin, to modulate stress (Shirsath et al. 2024). 3) By acting on the locus coeruleus and other arousal-regulating areas of the brain, NPS enhances alertness in stressful situations, increasing arousal and improving the individual's ability to respond to stress (Okamura and Reinscheid 2007).

In rodents, the NPS system modulates the neuroendocrine stress response. NPS has been shown to significantly increase plasma ACTH and corticosterone (CORT) levels

(Zhu et al. 2010; K. L. Smith et al. 2006; Cohen et al. 2018). The timing of the CORT increase following NPS infusion suggests that NPS acts directly on the paraventricular nucleus to regulate HPA axis function (Tobinski and Rappeneau 2021). Importantly, applying NPS to hypothalamic explants increased corticotropin-releasing hormone and arginine vasopressin release, while NPS application to adrenal explants did not stimulate ACTH release, indicating direct effects of NPS on the hypothalamus (K. L. Smith et al. 2006). However, mice carrying the human-specific NPSR1-N107 variant showed no significant differences in plasma CORT levels compared to those with the ancestral NPSR1-I107 variant (Bengoetxea et al. 2021). Further research is needed to fully understand how the NPS system activates the stress system (Tobinski and Rappeneau 2021).

Regarding behavioral stress responses, central infusion of NPS was found to reduce stress-induced hyperthermia, a measure of stress impact on body temperature (Sarah K. Leonard et al. 2008; Rizzi et al. 2008). However, NPS did not significantly affect stress handling in the Forced Swim Test or Tail Suspension Test (Sarah K. Leonard et al. 2008; Wegener et al. 2012; Tillmann et al. 2019). Similarly, central infusion of NPS did not alter genetically mediated stress-coping strategies in an animal model of depressive-like behavior, the Flinders Sensitive Line rats (Wegener et al. 2012). Additionally, no significant effects of NPSR1 deficiency were observed on neuroendocrine or behavioral stress responses in mice. For example, NPSR -/- and +/+ mice showed no substantial differences in basal CORT levels (Germer et al. 2019; Kolodziejczyk and Fendt 2020), or stress-induced CORT responses, including responses to fear conditioning, forced swim testing, or methamphetamine challenge (Zhu et al. 2010; Kolodziejczyk and Fendt 2020), as well as stress-induced hyperthermia and stress-coping behavior in the Forced Swim Test and Tail Suspension Test (Ruzza et al. 2012; Zhu et al. 2010; Duangdao et al. 2009).

Brief Description of Anxiety and its Effects

Anxiety is a normal response to threatening situations or an emotional state of worry in the absence of immediate danger (Kandel et al. 2021). Perceived threats that cause anxiety can be direct or indirect, such as the absence of people or objects that provide security. Anxiety is adaptive; low to moderate anxiety signals potential danger and can aid in coping with difficult situations, contributing to personal growth. However, excessive anxiety is maladaptive, characterized by either its intensity or its provocation by events that are not actually dangerous. Anxiety becomes pathological when it is excessive, persistent, or no longer serves its function of signaling danger (Kandel et al. 2021).

Anxiety disorders are the most prevalent psychiatric disorders, affecting 10-30% of the general population. The classification of these disorders depends on clinical characteristics and responses to psychopharmacological agents. Major categories include panic disorder, PTSD, generalized anxiety disorder, social phobia, and

obsessive-compulsive disorder (Kandel et al. 2021). Generalized anxiety disorder is characterized by long-lasting worry (Kandel et al. 2021). Individuals with anxiety disorders experience persistent, unrealistic, or excessive worry about impending misfortune. This worry lasts for more than a few minutes, often extending for at least six months. Both subjective and objective manifestations accompany this anxiety (Kandel et al. 2021). Subjective manifestations include heightened awareness and a profound fear of impending disaster or death. Symptoms also include avoidance behavior and a desire to escape (Kandel et al. 2021). Indicative symptoms include:

- Motor tension (e.g., tremors, twitching, muscle aches, muscle tension, restlessness, sleep disturbance, fatigue)
- Autonomic hyperactivity (e.g., palpitations, increased heart rate, increased blood pressure, sweating, cold hands, dry mouth)
- Vigilance and scanning (e.g., nervousness, exaggerated startle response, difficulty concentrating) (Kandel et al. 2021)

Depression and anxiety frequently co-occur. The French neurosurgeon Henri Laborit showed that anxiety before surgery triggers the release of substantial histamine from mast cells. He theorized that histamines could contribute to the adverse effects of anesthesia, including sudden death (Kandel et al. 2021).

As with other mental illnesses, anxiety disorders are unlikely to be caused by a single transmitter system. Generalized anxiety disorder, for example, involves the GABAA receptor system or, more likely, an abnormal interaction between the GABAA receptor and the serotonergic system (Kandel et al. 2021). Many patients with anxiety disorders respond positively to selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (Kandel et al. 2021).

Modulating GABAergic inhibition has been shown to alleviate symptoms of anxiety disorders (Luo 2021). GABA (Gamma-Aminobutyric Acid) is the principal inhibitory neurotransmitter in the CNS, with GABAA receptors opening chloride channels. The influx of chloride hyperpolarizes and inhibits target cells (Kandel et al. 2021). The most commonly used anti-anxiety drugs bind to GABAA receptors, enhancing their function (Luo 2021). Both barbiturates and benzodiazepines exert anxiolytic effects by binding to GABAA receptors, increasing GABA transmission. However, it is important to note that barbiturates, benzodiazepines, and GABA have different binding sites on GABAA receptors. At elevated concentrations, barbiturates can act as allosteric agonists, independent of GABA, causing hyperpolarization of target neurons through chloride influx. In contrast, benzodiazepines increase the affinity of GABAA receptors for GABA and other GABA agonists, such as barbiturates or alcohol, without directly activating the receptors (Luo 2021). This results in increased Cl^- influx through chloride channels, prolonging the synaptic inhibition produced by GABA. Benzodiazepines act as allosteric agonists by enhancing the action of endogenous GABA, contributing to

their relative safety compared to barbiturates, as their maximum effect is limited by the amount of endogenous GABA (Luo 2021).

The GABAA receptor has distinct binding sites for GABA, barbiturates, and benzodiazepines. The receptor is allosteric, meaning that binding of one ligand (GABA, benzodiazepine, or barbiturate) affects the binding of the others, facilitating the action of GABA. Specifically, GABA binds more tightly if a benzodiazepine is also bound to its site on the receptor. These binding sites are distinct. Analysis of the GABAA receptor's primary structure suggests the presence of at least three subunits (α, β, γ), with benzodiazepine binding to the γ subunit. Research on the dissociation of the functions of GABAA receptor subunits α1 and α2 in promoting sedation and relieving anxiety is ongoing. For example, the sedative effects of benzodiazepines are attributed to their interaction with α1-containing GABAA receptors, while their anxiolytic properties are associated with α2-containing GABAA receptors (Luo 2021).

Serotonin is a neurotransmitter that affects feelings of anxiety by influencing various receptors in the body (Garcia-Garcia et al. 2014). SSRIs are non-addictive drugs that can reduce anxiety. Researchers are studying how serotonin works to alleviate anxiety (Luo 2021).

Following the seminal study by Xu and colleagues (Y.-L. Xu et al. 2004), which demonstrated the pro-arousal and anxiolytic properties of NPS in mice, substantial clinical and preclinical evidence has emerged supporting the role of the NPS system in modulating anxiety. This evidence suggests that the anxiolytic effects of NPS primarily result from its interaction with the NPSR. Binding of NPS to NPSR triggers signaling pathways that increase cAMP and calcium levels, enhancing neuronal excitability and synaptic plasticity in anxiety-related circuits (Park et al. 2021). Additionally, NPS has been shown to interact with other neurotransmitters, including GABA, glutamate, dopamine, and serotonin, indicating a multifaceted role in anxiety modulation.

Role of NPS System in Anxiety

Clinical studies have identified an association between the NPSR1 gene rs324981 A/T (Asn107Ile) polymorphism and anxiety. The AA genotype is linked to increased anxiety in healthy individuals, while individuals with the TT genotype show heightened anxiety sensitivity, particularly if they have experienced childhood trauma or a recent life-threatening event, compared to those with the AA genotype (Laas, Reif, Akkermann, et al. 2014).

In preclinical studies, NPS administration has been shown to reduce behavioral signs of anxiety in several anxiety tests, including the open field, elevated plus maze, light-dark box, and marble burying paradigms (Y.-L. Xu et al. 2004). Central administration of NPS produces anxiolytic-like effects, while also increasing locomotor activity at comparable doses (Y.-L. Xu et al. 2004). In tests like the open field, elevated plus maze,

and light-dark box, increased exploration is generally taken as an indication of reduced anxiety. However, this could be confounded by simply increased movement. Studies have separated the two components of these tests: one measures activity (e.g., total distance traveled, number of transitions), and the other measures anxiety (e.g., number of entries into unprotected areas, time spent in unprotected areas) (Rodgers and Johnson 1995). These two factors do not always correlate or show the same results (Y.-L. Xu et al. 2004; Ramos et al. 2008). The effects of NPS on movement appear to be mediated by corticotropin-releasing hormone receptor 1 in the brain, while its calming effects seem to be independent of this receptor (Pañeda et al. 2009; Tobinski and Rappeneau 2021).

The relationship between NPS and NPSR in anxiety modulation is also complicated, as evidenced by significant deficits in exploration and increased anxiety-related behavior observed in knockout models of the NPS precursor peptide (X. Liu et al. 2017). In contrast, knockout of NPSR1 had no major effect on locomotion or anxiety (Ruzza et al. 2012; Zhu et al. 2010; Pulga et al. 2012; Fendt et al. 2011). The outcomes of such studies depend on the specific behavioral test (e.g., anxiogenic conditions) and the mouse strain used (Tobinski and Rappeneau 2021).

Brief Description of Fear Conditioning and its Effects

Fear is a natural emotional response to a perceived threat or danger, representing a fundamental survival mechanism that triggers physical, emotional, and psychological reactions aimed at protecting the individual (Steimer 2002). These reactions are governed by the brain, particularly the amygdala, which processes emotional responses (Šimić et al. 2021). These reactions often manifest as recurrent episodes of fear, triggered by reminders of the initial trauma (Kandel et al. 2021).

Fear conditioning is a type of classical conditioning where an animal learns to associate a neutral stimulus with an aversive event, resulting in a learned fear response (Lissek et al. 2005). This process is frequently studied by psychologists and behaviorists to understand how fear responses are acquired, stored, and expressed. Fear conditioning generally includes cued fear conditioning (which includes auditory fear conditioning) and contextual fear conditioning.

In auditory fear conditioning, animals are trained using tones paired with fear-inducing stimuli, such as electric shocks. Following the training, the animals exhibit a freezing response when exposed to the auditory stimulus alone (Luo 2021). In cued fear conditioning, an electric shock follows the presentation of a cue (Luo 2021). In contextual fear conditioning, an animal is exposed to aversive stimuli, such as electric shocks, in a specific environment (the 'context'). When the animal is placed back in the same environment, it shows a fear response, such as freezing (Luo 2021).

A theory of emotion must explain the relationship between physiological states and cognition. Until the late nineteenth century, the conventional view was that the recognition of a significant event (e.g., the sight of one's dwelling engulfed in flames) initiates a conscious emotional experience (i.e., fear) in the cerebral cortex, which then signals peripheral structures like the heart, blood vessels, adrenal glands, and sweat glands. According to this traditional perspective, a conscious emotional event triggers reflexive autonomic responses in the body (Kandel et al. 2021).

However, extensive clinical and preclinical evidence now shows that the amygdala interacts with other brain regions, including the hypothalamus and brainstem, to manifest emotions physically, and with neocortical regions (such as the cingulate, parahippocampal, and prefrontal cortices) involved in conscious emotions, especially fear (Kandel et al. 2021). Research has demonstrated that both rodents and humans use analogous mechanisms in response to fear (Luo 2021).

The amygdala plays a pivotal role in fear conditioning (Luo 2021). A large body of research has shown that amygdala circuits are crucial in fear conditioning, both in rodent models and in humans. The amygdala complex includes divisions such as the lateral amygdala, basal amygdala, and central amygdala. Sensory input related to learned emotional states, especially fear and anxiety, enters the amygdala via the basolateral complex.

As shown in Figure 2, in auditory fear conditioning, information about the tone (CS) is transmitted to the lateral amygdala through a direct pathway from the auditory thalamic nuclei and an indirect pathway from the high-order auditory cortex. The foot shock (US) signal also reaches the amygdala through multiple pathways, including projections from the somatosensory thalamic nuclei to the lateral amygdala and from the pain pathway via the parabrachial nucleus to the central amygdala. The hippocampus provides contextual input to the amygdala through the basal amygdala. The amygdala processes this information, with flow from the lateral amygdala to the central amygdala, either directly or via the basal amygdala. The central amygdala then exports information to brainstem and hypothalamic targets, which regulate behavioral, endocrine, autonomic, and neuromodulatory systems (Luo 2021).

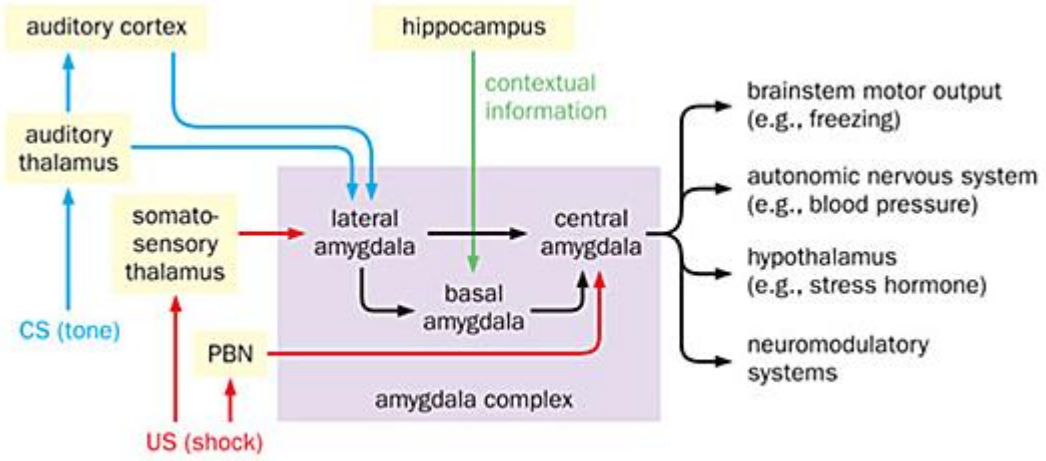

Figure 2. A simplified circuit diagram for fear conditioning (adapted from Luo 2021) (Luo 2021).

The prevailing academic consensus suggests that the amygdala is responsible for forming tone-shock and context-shock associations, while the hippocampus synthesizes the contextual information necessary for place memory formation. Therefore, both types of fear conditioning (auditory and contextual) involve the amygdala, but contextual fear conditioning also engages the hippocampus, whereas auditory fear conditioning does not (Luo 2021).

Furthermore, research has demonstrated that fear conditioning also involves neurons in the prefrontal cortex, which regulate conditioned fear behavior in a bidirectional manner (Luo 2021). Prefrontal cortical neurons are reciprocally connected to the basolateral amygdala and can send signals directly to the hypothalamus and periaqueductal gray. These regions regulate the body's motor, neuroendocrine, and autonomic outputs. The paraventricular nucleus of the thalamus, highly active during stress, sends signals to the central amygdala (essential for fear conditioning) and the nucleus accumbens, which processes information from the basolateral amygdala, prefrontal cortex, and midbrain dopamine neurons, helping regulate reward-seeking and punishment avoidance behaviors (Luo 2021). This network of brain regions involved in fear conditioning underscores the complexity of the interconnected circuits underlying behavior (Luo 2021).

The processes of fear conditioning and amygdala long-term potentiation share similarities with spatial learning and hippocampal long-term potentiation. Both processes involve molecular mechanisms such as calcium/calmodulin-dependent protein kinase II (CaMKII) autophosphorylation and postsynaptic NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking (Luo 2021).

Role of NPS System in fear conditioning

NPS plays a significant role in the process of fear conditioning. 1) NPS exerts its effects by binding to the NPSR, which is expressed in brain regions that regulate fear, such as the amygdala and prefrontal cortex. This binding modulates fear-related neural circuits and promotes adaptive responses to fearful stimuli (Chauveau et al. 2012; Justin P. Smith et al. 2014). 2) NPS also interacts with neurotransmitters like glutamate, serotonin, dopamine, and GABA, which help strengthen synaptic and neural plasticity in fear-related circuits.

From a clinical perspective, the NPSR gene rs324981 A/T (Asn107Ile) polymorphism has been associated with fear response (Laas, Eensoo, et al. 2015).

In preclinical investigations, NPS was found to reduce cued fear expression, facilitate its extinction, and decrease contextual fear reinstatement (Tobinski and Rappeneau 2021; Chauveau et al. 2012). Notably, mice carrying the human-specific hypo-functional variant of NPSR1 demonstrated enhanced extinction of conditioned fear (Bengoetxea et al. 2021). However, NPSR1 -/- mice showed no substantial changes in the expression of fear, whether cued, contextual, or social (Fendt et al. 2011; Germer et al. 2019). While NPSR1 knockout did not significantly impact cued fear extinction, it appeared to facilitate the extinction of social and contextual fear, with mixed results for the acoustic startle response (Kolodziejczyk et al. 2020).

Brief Description of Impulsivity and its Effects

Impulsivity is a multifaceted behavior, generally defined as a predisposition toward premature, poorly planned, unduly risky, or inappropriate actions in response to internal or external stimuli, without regard for the negative consequences to oneself or others. These actions often lead to adverse or maladaptive outcomes (Daruma and Barnes, 1993; McCown et al. 1993; Moeller et al. 2001; Bakhshani 2014; Hamilton et al. 2015). This behavior is characterized by acts that are poorly conceived, prematurely expressed, risky, or inappropriate, often with undesirable consequences, and lack appropriate forethought, thereby increasing the potential for negative outcomes (Evenden 1999).

A healthy level of impulsivity can be a positive aspect of our character, helping us seize opportunities and gain new experiences (Winstanley 2011). It can also aid in navigating certain social or environmental situations. For instance, 'functional' impulsivity can lead to positive results and is an important part of human behavior. Without it, people might not take acceptable risks or pursue new opportunities (Bevilacqua and Goldman 2013). However, elevated levels of impulsivity are considered undesirable and can be personally and socially costly (Winstanley 2011). Impaired impulse control affects many areas of life, leading to behaviors such as quick decision-making, difficulty waiting, interrupting others, sudden changes in topic, emotional outbursts, impulsive buying, excitability, and engaging in riskier behaviors like alcohol abuse. Maladaptive

impulsivity has been linked to several serious neuropsychiatric conditions, including obsessive-compulsive disorder, attention-deficit hyperactivity disorder, mania, antisocial behavior, schizophrenia, drug addiction, and AUD (Gomez and Corr 2014; Lombardo et al. 2012; Rogers et al. 2010; S. R. Chamberlain et al. 2018; Besson and Forget 2016).

From a clinical perspective, impulsivity can be categorized into various types, each exhibiting distinct behavioral patterns and neural mechanisms (Evenden 1999). Understanding these categories is essential for studying impulsivity in both normal behavior and clinical contexts. Table 1 and Table 2 present the primary categories of impulsivity, adapted from Evenden's review article on types of impulsivity (Evenden 1999).

Table 1. Varieties of impulsivity proposed by researchers who primarily conduct research on human subjects (Adapted from Evenden 1999) (Evenden 1999).

Type	Exposition
Dysfunctional impulsivity	Dysfunctional impulsivity is defined as the tendency to act with less forethought than most people of equal ability when this tendency is a source of difficulty (Dickman 1990).
Functional impulsivity	Functional impulsivity refers to the tendency to act with relatively little forethought when such suitable forethought is most effective (Dickman 1990).
Attentional impulsivity	Insufficient focusing of attention has been demonstrated to result in impulsivity (Dickman 1993). Difficulty maintaining attention and focus has been shown to lead to distractibility and difficulty delaying responses. Examples of this include struggling to complete tasks, rapidly shifting focus, or responding to irrelevant stimuli, and distractibility, as well as task-switching. Neurobiologically, it is associated with dysfunction in the dorsolateral PFC and parietal cortex, and dysregulated dopamine and norepinephrine systems. Prominent in ADHD and schizophrenia.
Reflection-impulsivity	Reflection-impulsivity is defined as the cognitive dimension employed to delineate the disparities in individuals' propensity to resolve response uncertainty. This can be measured by means of the matching familiar figures task (Kagan 1966) (Öğüt 2024).

Disinhibition	In the absence of withholding responses, omission of reward is frequently observed (Dickman 1993).
Inhibitory control	Inhibitory control is defined as the cognitive ability to suppress prepotent actions when such actions are unlikely to accomplish valuable results (Bari and Robbins 2013). For instance, the subject may experience difficulty in controlling their impulses, and find it challenging to wait (Buss and Plomin 1975).
Decision time	For instance, people often say the first thing that comes into their mind, or act on the spur of the moment (Buss and Plomin 1975).
Lack of persistence	For example, people often give up on things easily and quickly switch to other interests (Buss and Plomin 1975).
Sensation seeking impulsivity/risk-taking impulsiveness/boredom/ venturesomeness/ novelty seeking	Sensation-seeking impulsivity is defined as the tendency or willingness to seek out novel, exciting or risky experiences without considering the potential dangers involved and weighing the potential losses. Examples of such activities include skydiving, engaging in unprotected sexual activity, experimenting with drugs, driving recklessly, gambling large sums of money, making uncalculated business investments, Acts instantly on momentary whims (H. J. Eysenck 1993) (Cloninger 1987a). For instance, individuals who exhibit sensation-seeking behaviour are often characterised by a tendency to seek out new and exciting experiences and sensations, and to feel bored easily (Buss and Plomin 1975). Neurobiologically, it is associated with increased dopamine activity in the mesolimbic reward pathway, and reduced activity in the OFC and anterior cingulate cortex (ACC). Common in gambling disorder, bipolar disorder, substance use disorders, and adolescent behaviour.
Impulsiveness	Unconscious risk-taking with the tendency to make decisions without prior consideration or deliberation (H. J. Eysenck 1993). For instance, the subject frequently responds impulsively before the questions have been fully completed, and is prone to acting on the spur of the moment (Schalling et al. 1987).
Motor impulsivity	Acting or doing things without thinking (Patton et al. 1995). The propensity to prematurely act in a hasty manner, without fully processing the situation or

	contemplate the consequences, is exemplified by the following behaviours: blurting out answers, the abrupt articulation of responses, interrupting others, and engaging in sudden physical actions without forethought, acting without thinking. The neurobiology is linked to deficits in the PFC and basal ganglia, and dysregulated dopamine signalling may impair inhibitory control. Common in Attention-Deficit/Hyperactivity Disorder (ADHD) and Tourette's syndrome.
Cognitive impulsivity /decision-making impulsivity	The ability to make rapid cognitive decisions (Patton et al. 1995). Cognitive impulsivity, otherwise termed 'decision-making impulsivity', is defined as the tendency to make judgements or hasty decisions without suitable consideration, prioritising short-term rewards over long-term benefits. Instances of cognitive impulsivity include making hasty decisions, making impulsive purchases, choosing immediate gratification over delayed rewards, and gambling without assessing the risks. Neurobiology implicated in brain regions include the orbitofrontal cortex and ventral striatum, and increased sensitivity to reward (dopamine) and decreased activity in the PFC for evaluating outcomes. Common in substance use disorders, gambling addiction and bipolar disorder.
Non-planning	The present orientation of the subject is characterised by an absence of "futuring" (Patton et al. 1995)
Ideomotor	Acting without thinking (Barratt ES 1994)
Careful planning	Paying attention to details (Barratt ES 1994)
Coping stability	The orientation of the subject towards a future point in time (Barratt ES 1994).
Harm avoidance	Harm avoidance is a way of thinking about trait anxiety that is based on biology. This phenomenon is indicative of an inherent hereditary tendency to respond with considerable intensity to forms of punishment or frustration by the inhibition of ongoing behaviour (Markett et al. 2016). For instance, a carefree absence of inhibition, even in situations that demand focus (Cloninger 1987a).
Reward dependence/ impulsivity in delay discounting/temporal impulsivity	Preferring smaller, immediate rewards over larger, delayed rewards. For instance, the absence of persistent ambition with regard to deferred rewards (Schalling et al. 1987), choosing to receive $10 now rather than $20 a

	week later. Neurobiology involves dysfunction in the medial PFC, ventral striatum and ACC and greater activation of reward circuits for immediate outcomes. Implicated in addictive behaviours, obesity and financial irresponsibility.
Irritability/impulsive aggression	Impulsive aggression is the behaviour to react aggressively to something that makes you frustration or provocation without considering the consequences. For example, reacting violently in disagreement, verbal or physical outbursts triggered by minor annoyances, impatience, irritability, and verbal aggression (Schalling et al. 1987). The neurobiology involved is hyperactivity in the amygdala (emotional processing) and hypoactivity in the PFC (regulatory control), and low levels of serotonin are strongly implicated. Common in intermittent explosive disorder, borderline personality disorder and antisocial personality disorder.
Self-control	The term 'self-control' is typically employed to denote the process of deliberating between competing alternatives that arriving at disparate temporal junctures. For instance, weighting irritability, aggressivity and control of responses (Lecrubier et al. 1995).
Time needed for decision	In normals weighting time needed for decision and capacity for delay (Lecrubier et al. 1995).
Substance abuse disorders	For instance, the persistent desire or unsuccessful efforts to reduce or control substance abuse, and the allocation of a significant amount of time to activities necessary for the procurement of the substance (American Psychiatric Association 1994).
Inattention	For instance, the subject frequently experiences difficulties in maintaining concentration when engaging in tasks or recreational activities (American Psychiatric Association 1994).
Hyperactivity	For instance, an individual may vacate their seat in a classroom setting or in situations where remaining seated is customary (American Psychiatric Association 1994).
Criterion 7	Excessive involvement in pleasurable or recreational activities that carry a significant risk of adverse or painful consequences (American Psychiatric Association 1994).

Table 2. Varieties of impulsivity proposed by researchers who primarily conduct research on non-human subjects (Adapted from Evenden 1999) (Evenden 1999)

Type	Exposition
Response inhibition	Response inhibition, defined as the ability to suppress a response when confronted with fluctuating internal or external demands, underlies a series of behaviours deemed critical for adaptive functioning (Congdon et al. 2012). The involvement of serotonergic neurons is required for the manifestation of behavioural inhibition (Soubrié 1986).
Resistance to delay of reinforcement	Impulsives and individuals have been shown to lack the ability to tolerate delays in the receipt of anticipated rewards, and encounter difficulties in exercising patience and deferring gratification (Logue 1988).
Timing	Impulsive individuals demonstrate an inability to make accurate temporal estimations, frequently exhibiting temporal intervals that are too short.
Behavioural switching	Higher frequency of shifting between response alternatives (Ho et al. 1998).
Motor impulsivity	Failure to inhibit behaviour characterised by imprecise, hasty, and fast responses (D. Brunner and Hen 1997a).
Cognitive impulsivity	The distorted judgement of alternative outcomes, resulting in a loss of reward in the long term (D. Brunner and Hen 1997a).
Preparation	Not all the pertinent information has been considered prior to decision-making (Evenden 1998).
Execution	The behaviour chain is terminated prior to the achievement of the goal (Evenden 1998).
Outcome	The decision is made to opt for a more expeditious, albeit less significant, outcome, rather than in preference to a more protracted yet more valuable result (Evenden 1998).
Premature responding	Respond when the chance is given before discriminating information available (Evenden 1999).
Lack of persistence	The observed behaviour was quantitatively less than would be expected under normal circumstances (Evenden 1999).

Theorists posit that impulsivity encompasses a multifaceted array of behavioral processes, including urgency, lack of planning, lack of premeditation, risk-taking, sensation-seeking, disregard for future consequences, and insensitivity to punishment. The fourth edition of the Diagnostic and Statistical Manual identifies a range of psychiatric disorders collectively referred to as impulse control disorders, a classification also found in the World Health Organization's International Classification of Diseases, which includes a category for habit and impulse disorders (Winstanley 2011). Evidence suggests that impulsivity can be categorized into multiple forms, with the term 'impulsivity' used to describe behaviors ranging from motor disinhibition to maladaptive decision-making (D. Brunner and Hen 1997b; Moeller et al. 2001).

In the clinical context, numerous self-report questionnaires have been developed to assess various aspects of impulsivity, including the Eysenck Personality Questionnaire (Hans J. Eysenck and Eysenck 2016), the Barratt Impulsiveness Scale (Barratt, 1985), the UPPS scale (Whiteside and Lynam 2003), and the Dickman Impulsiveness Scales (Monterosso and Ainslie 1999). Questionnaires designed to assess multiple aspects of impulsivity are commonly used clinically, such as the 11-point Barratt Impulsiveness Scale, the Impulsivity Rating Scale, and the Karolinska Scale of Personality (Winstanley 2011). However, it is important to recognize the limitations of self-report measures, especially in individuals who exhibit impulsive behavior, as they may lack the capacity for introspection and may be unable to accurately perceive their own actions (Wilson and Dunn 2004; Jupp et al. 2013). Fortunately, computerized clinical psychometric tests that assess aspects of impulsive action or choice provide a more objective measure of impulsivity (Samuel R. Chamberlain and Sahakian 2007; Kertzman et al. 2006).

Among the various tests, delay discounting is the most commonly used metric for assessing impulsive choice. This test involves subjects making a series of choices between smaller, immediate rewards and larger rewards available after a delay. Individuals with impulsive tendencies typically prefer immediate rewards, even if they are smaller than those offered later (Richards et al. 1999). The Go/No-Go task and the stop-signal reaction time task are used to evaluate impulsive action behavior (Weafer et al. 2013). In the Go/No-Go task, subjects must respond to a specific cue, such as a light, and withhold a response when a different cue, such as a sound, is presented. Subjects with accelerated or erroneous responses or diminished behavioral inhibition are considered more impulsive (Riccio et al. 2002; Ficarella and Battelli 2019). In the Stop Signal Reaction Time Task, subjects perform a primary visual binary choice reaction time task, making a rapid decision based on a visual cue. In a subset of trials, subjects are instructed to inhibit their response when a stop signal appears, either auditory or visual, at any time before or after the initial stimulus. Impulsive individuals are more likely to fail to inhibit their responses after the stop signal (Logan et al. 1997; D. M. Eagle et al. 2008).

A crucial aspect of the study of impulsivity's biological basis is the observation that indices of impulsive behavior in humans are similar to those observed in animal models (Jupp et al. 2013; D. M. Eagle et al. 2008). Many studies have successfully translated delay discounting, Go/No-Go, and stop-signal tasks into operant-based models, which are commonly used to assess impulsivity in rodents (Winstanley 2011). Other operant-based methodologies assess motor impulsivity, such as the five-choice serial reaction time task (5CSRTT), which uses premature responses to a food-predictive brief light stimulus to evaluate impulsive action. This method has become popular because it simultaneously measures motor impulsivity and sustained or divided attention (T. Robbins 2002; Jupp et al. 2013; Winstanley et al. 2006; P. Kim et al. 2012).

Although simulating human behavior in rats remains a challenging goal, measuring impulsivity in a laboratory setting is complex due to the variety of behaviors it encompasses. Impulsivity involves multiple phenomena, each potentially governed by independent biological mechanisms (Evenden 1999; P. Kim et al. 2012). However, the core symptoms of impulsivity observed in rodents may be valuable for research, and several tests have been developed to measure it. These tests fall into three categories: punishment/extinction, reward-directed, and rapid-decision paradigms (P. Kim et al. 2012). In the punishment/extinction paradigm, impulsivity is demonstrated when subjects persist in responding despite punishing or unrewarded outcomes. In reward-driven paradigms, impulsivity is shown when subjects prefer a smaller, sooner reward over a larger, later one. In rapid decision-making paradigms, impulsivity is evaluated by observing subjects' tendency to make premature or uninhibited responses (Dougherty et al. 2005; P. Kim et al. 2012). Winstanley et al. provide a concise overview of these behavioral paradigms, categorizing them into two groups: those measuring impulsive action (motor disinhibition) and those measuring impulsive choice (impulsive decision-making) (Winstanley et al. 2006). In summary, impulsivity can be conceptualized as comprising two primary components: impulsive action and impulsive choice.

Impulsive choice is characterized by a diminished tolerance for delay or disordered, risk-based decision-making processes. This is exemplified by impulsive decision-making, where impulsive individuals tend to select smaller, immediate rewards more frequently than delayed, larger rewards (P. Kim et al. 2012). The assessment of impulsive choice primarily utilizes delay discounting paradigms (P. Kim et al. 2012).

Impulsive action is defined as the tendency to act prematurely, without prior deliberation, often referred to as "acting on impulse." This behavior is marked by an inability to suppress immediate responses, leading to potentially impulsive actions (Winstanley et al. 2006). Impulsive action is typically assessed using stop-signal reaction time tasks or Go/No-Go tasks (P. Kim et al. 2012).

The literature supports the hypothesis that impulsive action and impulsive choice are independent constructs (Broos et al. 2012). 1) SHR rats demonstrated impulsivity in a

delay-discounting task (Adriani et al. 2003; Aparicio et al. 2019) but not in a 5CSRTT (Van Den Bergh et al. 2006; Jupp et al. 2013). A study found no correlation between the performance of Wistar rats on 5CSRTT and delay discounting or a stop-signal reaction time task (Broos et al. 2012), a dissociation also observed in humans (Broos et al. 2012). 2) Katherine M. Nautiyal and colleagues present evidence that impulsive action and choice are distinct components of impulsivity, with different biological bases. They demonstrate that altering 5-Hydroxytryptamine (serotonin) receptor 1B levels 5-(HT1BR) modulates impulsive action but not impulsive choice. Factor analysis further shows that impulsive action and impulsive choice dissociate into independent components (Nautiyal et al. 2017). 3) Broos et al. showed that impulsive action and impulsive choice are differentially affected by pharmacological challenges with amphetamine and atomoxetine (Broos et al. 2012; Swalve et al. 2016). Nonetheless, impulsive action and impulsive choice may constitute discrete yet interconnected constructs, with shared underlying mechanisms, allowing the coexistence of both forms of impulsivity without a direct correlation between them (Jupp et al. 2013). For instance, RHA rats exhibit heightened impulsive behavior and increased impulsive choice behavior (Jupp et al. 2013; Moreno et al. 2010). Similarly, rats selected for high impulsivity on the 5CSRTT exhibit similar impulsive behavior in a delay discounting task (Robinson et al. 2009). These two forms of impulsivity, reflecting deficits in "failing to wait," may be associated with reward-value assessment mechanisms that are distinct from those involved in "waiting" per se (Jupp et al. 2013). Notably, the neural substrates involved in encoding reward value overlap with those implicated in impulsivity (Levy and Glimcher 2012). An individual may exhibit impulsivity in choice tasks but not in motor tasks (e.g., stop-signal), although the reverse is not typically true (Jupp et al. 2013).

Neurobiology of Impulsivity

Impulsivity is a multifaceted concept, influenced by independent neurobiological substrates, which may also interact and overlap. The neurobiological underpinnings of impulsivity are evident through complex interactions among brain regions, neurotransmitter systems, and genes. The contribution of various brain regions, circuits, neurotransmitter systems, and genes results in different manifestations of impulsive behavior. Below is a synopsis of the neurobiological mechanisms underlying impulsivity:

1. Brain Regions Involved in Impulsivity

- **Prefrontal Cortex**:
    1. Guzulaitis and colleagues demonstrated the neural basis of premature impulsive action in the PFC, showing that the lateral motor cortex plays a key role in impulsive behavior by influencing premature motor output in mice (Guzulaitis et al., 2022).

2. Numerous studies have identified alterations in the structure and function of specific PFC regions and their associated corticostriatal circuitry in individuals with impulsive behavior, supported by brain imaging in humans (Mitchell & Potenza, 2014).
3. Behavioral profiles in RHA rats indicate reduced medial PFC volume and function compared to RLA rats (Giorgi et al., 2019; Río-Álamos et al., 2017, 2019).
4. The medial PFC is critical for regulating reward-driven behavior and impulsivity. Dysfunctional signaling between the medial PFC and nucleus accumbens shell is believed to underlie the inability to suppress dominant responses (i.e., motor impulsivity). Chemogenetic activation of the ventromedial PFC to NAcSh pathway has been shown to reduce motor impulsivity (Anastasio et al., 2019).
5. Enhanced serotonin transporter function in the orbitofrontal cortex (OFC) has been linked to impulsive choice behavior (Darna et al., 2015).

- **Anterior Cingulate Cortex**:
  Veen and colleagues demonstrated that the ACC modulates premature responding, a form of motor impulsivity. Activation of Gi signaling in layer 5 pyramidal cells of the ACC reduced impulsivity, as confirmed by mGluR2 activation (Van Der Veen et al., 2021).
- **Basal Ganglia**:
  The basal ganglia play a vital role in regulating movement, balance, eye movements, and posture. They are interconnected with other motor areas and are involved in reward processing, reinforcement, addiction, and habit formation.
- **Amygdala**:
  Behavioral profiles in RHA rats are consistent with reduced amygdala volume and function compared to RLA rats (Giorgi et al., 2019; Río-Álamos et al., 2017, 2019).
- **Hippocampus**:
  Reduced hippocampal volume and function have been observed in RHA rats compared to RLA rats (Giorgi et al., 2019; Río-Álamos et al., 2017, 2019).
- **Thalamus**:
  The lateral thalamus plays an important role in decisional impulsivity. Dopamine receptors (D1 and D2) in the lateral thalamus have been shown to affect cognitive impulsivity (Wang et al., 2017).
- **Hypothalamus**:
  Freeman and Aston-Jones demonstrated that activation of medial hypothalamic orexin neurons was associated with enhanced accuracy in a Go/No-Go task, while no correlation was observed with lateral hypothalamic orexin neurons (Freeman & Aston-Jones, 2020).

2. Neurotransmitter Systems Involved in Impulsivity

- **Dopamine**:
  Dopamine is a key neurotransmitter involved in impulsivity regulation:
  1. Reduced dopamine signaling at the D1 receptor in the dorsal striatum reduces impulsivity, while signaling at the D2 receptor increases impulsivity (Eagle et al., 2011; Anderberg et al., 2016).
  2. Positron emission tomography studies have shown altered dopamine release and receptor availability in individuals with impulsive traits (Clark et al., 2012).
  3. Hynes et al. (2021) found that suppressing dopamine neurons in the VTA reduced motor impulsivity in male rats but increased risky decision-making in females.
  4. Dysregulated dopaminergic and noradrenergic function contributes to impulsivity, supported by the efficacy of ADHD medications, such as methylphenidate and atomoxetine (Del Campo et al., 2011; Jupp et al., 2013).
- **Serotonin**:
  Serotonin influences impulsivity:
  1. Decreased serotonin signaling increases impulsivity and choice behavior in rats. Activation of dorsal raphe serotonin neurons can attenuate impulsivity (Anderberg et al., 2016; Harrison et al., 1997).
  2. Serotonergic dysfunction, including reduced 5-Hydroxytryptamine (serotonin) receptor 2A (5HT2A) receptor binding and serotonin transporter function in the PFC, is linked to heightened impulsivity (Meyer et al., 2008; Kim et al., 2024).
  3. Enhanced serotonin transporter function in the OFC is associated with impulsive choice behavior (Darna et al., 2015).
  4. Low serotonin concentrations are observed in individuals with impulsive aggressive behavior (Krakowski, 2003; Mosienko et al., 2012).
  5. A mutation in the gene encoding monoamine oxidase A, which regulates serotonin metabolism, is linked to impulsive behavior and aggression, suggesting that serotonin's role in aggression is complex (Brunner et al., 1993).
- **Norepinephrine**:
  Norepinephrine plays a role in mood, attention, and arousal. Dysregulated levels impair concentration and increase distractibility.
- **Glutamate**:
  Overactivity of glutamate in PFC circuits disrupts cognitive control and increases impulsivity.
- **GABA**:
  Deficits in GABAergic signaling impair the brain's ability to inhibit impulsive behavior.

- **Corticosterone**:
  Chronic exposure to corticosterone during adolescence reduces impulsive behavior but increases impulsive choice in male Sprague-Dawley rats (Torregrossa et al., 2012).
- **Ghrelin**:
  Ghrelin, a neuropeptide released in response to low glucose levels, stimulates eating and increases impulsivity. It alters both motor and choice impulsivity (Anderberg et al., 2016).

3. Genes Involved in Impulsivity

Research into the hereditary basis of impulsivity has identified polymorphisms in genes involved in monoaminergic function, including those encoding the dopamine transporter (Congdon et al., 2008), serotonin receptors 5HT2A (Reist et al., 2004) and 5HT2B (Bevilacqua et al., 2010), and monoamine oxidase A enzyme (Liu et al., 2011). Additionally, the contribution of brain regions, circuits, neurotransmitter systems, and genes varies across different subtypes of impulsivity, giving rise to distinct manifestations of impulsive behavior.

Relationship Between Impulsivity and Alcohol Use Disorder

A substantial body of literature supports the association between impulsivity and AUD. Impulsivity appears to be linked with all stages of AUD (Lejuez et al., 2010).

1. The classification of alcoholics into type 1 or type 2 depends on personality variables and drinking patterns. For example, individuals with type 1 alcoholism typically exhibit low novelty seeking and high harm avoidance, with alcohol consumption typically beginning later in life. In contrast, those with type 2 alcoholism show high novelty seeking, low harm avoidance, and often start drinking during their teenage years (Cloninger, 1987b). The type 2 group has been found to have issues with impulse control and is highly heritable (Cloninger, 1981; Winstanley et al., 2010).
2. Extensive research supports the hypothesis that high levels of impulsive choice, as measured by delay discounting paradigms, correlate with increased alcohol consumption (Winstanley et al., 2010; Poulos et al., 1995).
3. Animals classified as highly impulsive based on task performance show a marked increase in alcohol consumption, particularly at higher concentrations, when given restricted-access self-administration procedures (Poulos et al., 1995).
4. Significant variability in the acute response to alcohol was observed among rats with "intermediate" levels of impulsive choice. Specifically, "high reactive" rats showed a dose-dependent increase in impulsive choice after alcohol administration, while "low reactive" rats showed minimal response (Poulos et al., 1998; Winstanley et al., 2010).

5. The data suggest a strong correlation between high impulsive choice and greater alcohol preference, indicating that impulsivity may be a vulnerability factor for higher alcohol consumption (Winstanley et al., 2010).
6. Impulsivity in rodents has been linked to an increased susceptibility to compulsive drug self-administration (Pattij et al., 2020; Trevor Robbins, 2012).

The relationship between impulsivity and AUD is debated, with some suggesting it is unidirectional, while others argue it is bidirectional. Impulsivity may predispose individuals to risky alcohol use, while alcohol use may impair self-control brain regions, creating a feedback loop that perpetuates alcohol use.

Role of NPS System in impulsivity

Laas and colleagues studied the NPSR1 gene functional polymorphism Asn107Ile (rs324981, A>T) in two age groups: younger and older individuals. They found that this NPSR1 A/T polymorphism is associated with impulsivity. The NPSR1 AA genotype had a positive effect on impulsivity, particularly in a positive family environment, and more so in males. In contrast, the NPSR1 TT genotype had a negative effect on impulsivity, especially in females (Laas, Reif, Kiive, et al., 2014; Laas, Eensoo, et al., 2015).

MARCHIGIAN SARDINIAN ALCOHOL-PREFERRING RATS (MSP RATS)

MsP rat is a distinct strain of laboratory rat selectively bred for its propensity to consume alcohol (Ciccocioppo et al., 2006). This model has become a key tool in research, serving as a valuable animal model for studying AUD and associated behaviors. It closely resembles the human alcoholic population, who often resort to alcohol consumption as a means of tension relief and self-medication due to their diminished capacity to engage in effective stress-coping strategies. Elevated stress and anxiety-like behaviors in msP rats have been assessed using tests such as the elevated plus maze and open field test. These behaviors mirror the co-occurrence of stress, anxiety disorders, and alcohol use in humans (Ciccocioppo et al., 2006).

Research has shown alterations in neurotransmitter systems in msP rats, including the GABAergic and dopaminergic systems (Borruto et al., 2021; Ciccocioppo et al., 2006). Additionally, changes in stress regulatory systems have been observed, such as dysregulation of the HPA axis, elevated corticotrophin-releasing factor receptor 1 system (Cippitelli et al., 2015; Hansson et al., 2007). A previous study demonstrated that NPS decreases alcohol intake in male msP rats (Cannella et al., 2016).

AIM OF THE THESIS

This thesis aims to investigate the effects of the NPS system on ASA and impulsivity in laboratory rats.

In Chapter 2 we tested the stimulatory, anxiolytic, and stress-coping roles of NPS in msP rats, and assess NPS's ability to reduce alcohol ASA in these rats. Since previous work has demonstrated that NPS do not affect alcohol self-administration in non-preferring Wistar rats, we evaluate whether the anxiolytic effect of NPS, in absence of its pro-arousal effect, is sufficient to reduce alcohol seeking in non-preferring lines. Finally, we determine if NPS can suppress stress-induced relapse.

In Chapter 3 we developed a rat operant model of impulsivity to compare impulsive behavior between msP and Wistar rats. And we explored the role of impulsivity in alcohol preference expressed by msP testing the effect of atomoxetine on ASA in msP rats.

In Chapter 4 we investigated the effect of NPS on impulsive bahavior and tested whether the anxiolytic effect of NPS, in absence of its pro-arousal effect, is sufficient to maintain the anti-impulsive effect of NPS.

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CHAPTER 2

ROLE OF THE NEUROPEPTIDE S SYSTEM ON ALCOHOL SEEKING BEHAVIOR

ABSTRACT

Background. Neuropeptide S (NPS) is a 20-amino acid neuromodulator with anxiolytic and pro-arousal effects. Alcohol use disorder (AUD) is influenced by excessive drinking and genetic predisposition. This study examines the role of NPS in anxiety, stress coping, and alcohol seeking in msP rats.

Methods. We assessed NPS's stimulatory effects using the open-field (OF) test, its anxiolytic role using the elevated plus maze (EPM), and its impact on stress coping using fear conditioning (FC). To determine whether NPS reduces alcohol self-administration (ASA), we conducted an operant ASA experiment. To examine whether its anxiolytic effects alone influence ASA, we tested RTI263 (a truncated form of NPS with anxiolytic but not stimulatory properties) in Wistar rats. Finally, we evaluated NPS's effect on yohimbine-induced reinstatement of alcohol seeking in msP rats.

Results. NPS increased locomotor activity in both sexes. Its effects on anxiety and stress coping differed by sex: in females, it facilitated fear extinction in the FC test but had no effect in the EPM test, whereas in males, it increased open-arm time in the EPM test but did not affect fear extinction. NPS reduced ASA in both sexes, while RTI263 reduced ASA only in female Wistar rats, suggesting that anxiolysis alone is insufficient to reduce alcohol seeking in males. NPS did not prevent yohimbine-induced relapse.

Conclusions. NPS reduces ASA in both sexes through different mechanisms, likely by facilitating stress coping in females and exerting anxiolytic effects in males. However, its anxiolytic effects alone do not reduce alcohol seeking in males. NPS is more effective in modulating innate anxiety and stress coping than in preventing relapse triggered by external stressors, suggesting a sex-dependent role in alcohol-seeking behavior.

INTRODUCTION

Alcohol use disorder (AUD) is a worldwide medical emergency, causing nearly three million deaths annually across the globe, accounting for 5% of all deaths. A substantial proportion of deaths attributable to cardiovascular diseases, malignant neoplasms, digestive diseases, and both unintentional and intentional injuries are linked to alcohol consumption (WHO 2024).

Stress is a major player in the trajectory to AUD. Stress-related psychiatric conditions, such as anxiety (Anker et al. 2019) and PTSD (Sonne et al. 2003) are often co-morbid with AUD. While both men and women consume alcohol to cope with affective negative states, stress seems to be a stronger driver of alcohol drinking in women than men (Peltier et al. 2019). For example, women are more likely than men to be diagnosed with co-morbid AUD-PTSD (Sonne et al. 2003), women with AUD exhibit higher levels of anxiety (King et al. 2003), and they report a higher frequency of heavy alcohol drinking to cope with negative emotional states (Abulseoud et al. 2013). Despite the critical role of stress in AUD, no drug targeting the stress system is currently approved for its pharmacotherapy (Cannella et al. 2019; Schank et al. 2012). Unfortunately, stress-targeting drugs that reached clinical trials failed to show efficacy in humans (Kwako et al. 2015; Schwandt et al. 2016).

The NPS is a stress-related brainstem neurotransmitter whose receptor (NPSR) is expressed throughout the brain (Xu et al. 2007). NPS is the only known endogenous compound that promotes arousal and activates the stress system, and yet it is anxiolytic at the same doses (Cannella et al. 2013; Smith et al. 2006; Xu et al. 2004). Interestingly, NPS has been shown to reduce anxiety (Rizzi et al. 2008; Wegener et al. 2012) and facilitate fear extinction in a fear conditioning test (Jungling et al. 2015; Jungling et al. 2008), a model of PTSD.

NPS also plays a dual role in alcohol-seeking behavior. Exogenous administration of NPS promoted relapse-like behavior in non-preferring Wistar rats (Cannella et al. 2009; Ubaldi et al. 2016), while it reduced alcohol self-administration in both Marchigian Sardinian (Cannella et al. 2016) and Indiana (Badia-Elder et al. 2008) male alcohol-preferring rats. Alcohol preferring lines, such as the Marchigian Sardinian alcohol preferring (msP) line, are characterized by innate negative affective states. The increased alcohol preference is considered a coping strategy in which alcohol is consumed to relieve these negative affective states (Borruto et al. 2021; Ciccocioppo et al. 2006).

Aim of This Chapter

In this study, we sought to verify the stimulatory, anxiolytic, and stress-coping role of NPS in male and female msP rats. We first tested whether NPS retains its stimulatory effect in an open-field test (OF), followed by assessment of its effects on generalized anxiety and consolidation of fear memories in an elevated plus maze (EPM) and fear conditioning (FC) test, respectively. Next, since anxiety and PTSD are conditions often co-morbid with AUD, we confirmed NPS's ability to reduce ASA in male msP rats and investigated whether this neurotransmitter retains the same effect in female msP rats as well. Then, to verify whether the anxiolytic effect of NPS alone is sufficient to reduce alcohol seeking, we tested the effect of RTI263—a truncated form of NPS that maintains its anxiolytic properties while being devoid of the stimulatory effects of NPS

(Clark et al. 2017; Huang et al. 2023) – on ASA. Finally, as stress is a trigger for relapse, we tested the effect of NPS on yohimbine-induced reinstatement of alcohol seeking (Curley et al. 2022) in msP rats.

MATERIALS AND METHODS

Animals

Male and female msP (University of Camerino breeding facility) and Wistar (Envigo) rats were aged 9 weeks at the beginning of experimental procedures. Rats were housed in group cages in a room with reversed 12 h/12 h light/dark cycle (lights off at 8:30 am), constant temperature (20-22°C), and humidity (45-55%). Food (4RF18, Mucedola, Italy) and tap water were availlable *ad libitum*. All procedures were conducted during the dark phase of the light/dark cycle and were in adherence with the *European Council Directive for the Care and Use of Laboratory Animals* and the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Surgeries

Rats were surgically implanted with an intracerebroventricular (ICV) cannula under isoflurane anaesthesia (1.5-2.5%). Guide cannulae were stereotaxically implanted and cemented to the skull. Coordinates referred to bregma were as follows: antero-posterior: -1.0 mm; medio-lateral: -1.8 mm; dorso-ventral: 2.0. To prevent postoperative pain, rats were treated subcutaneously with 0.1 ml of meloxicam (5 mg/ml). Surgeries were followed by a one-week recovery period, during which rats were left undisturbed in their home cages. The antibiotic Enrofloxacin (Baytril ®) was diluted in the drinking water (50 mg/100ml) for five days following surgery.

Cannula placement was verified before experiments by ICV injection of 100 ng of angiotensin II; only animals showing a clear dipsogenic response (consumption of at least 5 ml of water within 5 minutes) were used for further experimentation.

Drug Injection

NPS was a generous gift from Prof. R. Guerrini (University of Ferrara, Italy). RTI263 was a generous gift from Prof. Stewart Clark (University of Buffalo, USA). Drugs were dissolved in sterile isotonic saline and administered ICV in a volume of 1 µl/rat using a stainless-steel injector 2.5 mm longer than the guide cannula, connected to a 10-µL Hamilton syringe. Once the injector was inserted into the guide cannula, the drug solution (or vehicle) was infused by gently pressing the syringe's plunger. After infusion, the injector was left in place for 10 seconds before removal to prevent fluid backflow.

Yohimbine was dissolved in distilled water and administered intraperitoneally 30 min

before the session.

Operant Alcohol Self-Administration Training

Operant training and testing were conducted in self-administration chambers (Med Associates) equipped with a drinking reservoir (0.30 ml capacity) connected to an infusion pump, a house light, and two retractable levers, designated as the active and inactive lever, respectively. Rats were trained to self-administer 0.1 ml of 10% alcohol (v/v) solution in daily 30-minutes sessions according to a fixed ratio 1 (FR1) schedule of reinforcement on the active lever. No scheduled consequence was associated to inactive lever pressing. A 5-second time-out period, during which active lever presses were not reinforced, started contingently with pump activation. The house light was illuminated during the time-out. The number of rewards and active and inactive levers presses was recorded by Windows-compatible MedPC-5.

EXPERIMENTAL PROCEDURES

Experiment 1: Effect of NPS on Open Field, Elevated Plus Maze and Fear Conditioning Tasks in msP Rats.

Thirty-one female and twenty-four male alcohol-naïve msP rats were used in this experiment, that consisted of three consecutive tests: open filed (OF), elevated plus maze (EPM) and fear conditioning (FC). Tests were conducted in a sound-attenuated room illuminated by a dim red light. At least three days passed between the completion of one test and the commencement of the next. Before the beginning of the tests, rats were randomly assigned to a NPS dose (veh: 10♀+ 8♂; 0.5 nmole: 10♀+ 8♂; 1.0 nmole: 11♀+ 8♂).

The OF test was performed in eight OF arenas (43.18 × 43.18 cm) equipped with 16 evenly spaced infrared beams on each side to record rat location and movement. Data were acquired and analyzed by Windows-compatible Activity Monitor software (Med Associates). Rats received treatment immediately before being placed in the arena, and locomotor activity was recorded for 1h.

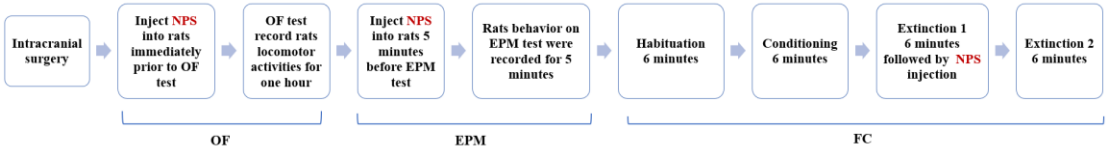
The EPM apparatus consisted of two closed and two open arms facing each other (100 cm long in total, 10 cm wide). The maze was elevated 100 cm above the floor. Rats received treatments 5 minutes before the test. They were individually placed in the center of the maze facing a closed arm and left exploring the maze for 5 min. Rat behavior was video recorded and analyzed by EthoVision XT 7. An entry was defined as the presence of the center of mass in an arm. The relative time spent in the open arms [time in open arms/total test time] was used as of index of anxiety.

The FC test was conducted on a 50 x 50 cm metallic grid embedded in a black wooden

box, equipped with a speaker. The test lasted three days and consisted of four trials, each lasting 6 minutes. On day 1 (habituation trial) rats were allowed to freely explore the apparatus. On day 2 in the morning, conditioning trial, a 1-second 2.0 mA foot-shock was delivered every minute starting 59 seconds after the beginning of the trial (i.e. six foot-shocks in total). Each foot-shock was anticipated by a 10 s cue tone that co-terminated with the shock. Six hours later, the test trial was identical to the conditioning trial, except that the foot-shock was not delivered. Rats were treated with NPS or vehicle immediately after the end of the test trial. 24 hours later, on day 3, rats entered the extinction trial, identical to the test trial.

Trials were videorecorded and the amount of time spent freezing was manually scored by a trained operator blinded to treatment conditions.

A flowchart of Experiment 1 experimental procedures is presented below.



Experiment 2: Effect of NPS on Alcohol Self-Administration (ASA) in MsP Rats.

Female and male msP rats (n = 8/sex) were trained to ASA as described above. After surgery, ASA was re-baselined before treatment started. Rats received ICV saline injections for two consecutive days to acclimate them to the treatment procedures. On test days, rats received ICV injection of NPS (0.1, 1.0 or 2.0 nmol/rat) or its vehicle 5 minutes before session. Treatment doses were delivered in a within-subjects counterbalanced order; test sessions were repeated every fourth day until each rat had received all doses. The first day after treatment rats remained in their home cages, the second and third day they underwent ASA baseline sessions.

A flowchart of Experiment 2 experimental procedures is presented below.

Experiment 3: Effect of RTI263 on Alcohol Self-Administration in Wistar Rats.

Fourteen female and fourteen male Wistar rats were trained to FR1 ASA. The test was identical to Experiment 2, except that the rats were treated with RTI263 (1.0 and 10.0 nmol/ICV), its reference drug NPS (2 nmol/ICV), and their vehicle. Next, the test was repeated, increasing the RTI263 dose to 100 nmol/ICV.

A flowchart of Experiment 3 experimental procedures is presented below.

Experiment 4: Effect of NPS on Yohimbine-Induced Reinstatement of Alcohol Seeking.

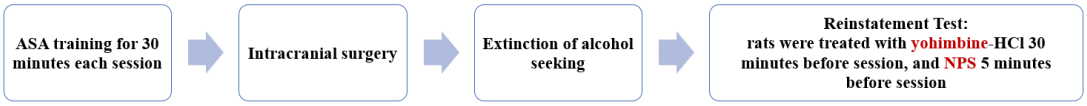
This experiment consisted of three phases: ASA training, extinction of alcohol seeking, and reinstatement test.

ASA Training: Female and male msP rats (n = 9/sex) underwent ASA as described above.

Extinction of Alcohol Seeking: After 20 ASA sessions, rats were subjected to 16 consecutive extinction sessions. The extinction sessions were identical to ASA sessions, except that active lever presses were reinforced by illumination of the house light and pump activation but did not result in alcohol delivery.

Reinstatement Test: The day after the last extinction session, rats were treated with 0.625 mg/kg of yohimbine-HCl. Thirty minutes later, they received an ICV injection of NPS (0.1, 1.0, or 2.0 nmol/rat) or its vehicle. The test session, which was identical to a standard extinction session, began five minutes later. NPS doses were delivered in a within-subjects counterbalanced order. Test sessions were repeated every fourth day until each rat had received all NPS doses; rats were subjected to extinction training on intervening days.

A flowchart of Experiment 4 experimental procedures is presented below.



STATISTICAL ANALYSES

Samples size was based on our previous works on NPS effects on ASA and anxiety (Cannella et al. 2016; Cannella et al, 2009).

Data were analyzed using appropriate between- and within-subjects ANOVAs. Approximation to normality of the distributions was verified using Q-Q plots of the residuals before conducting the tests. Statistical significance was set to conventional $p < 0.05$. ANOVA were followed by Dunnett's post-hoc for treatment vs. control comparisons or Tukey's post-hoc for pairwise comparisons when appropriate.

RESULTS

Experiment 1: Effect of NPS on Open Field, Elevated Plus Maze and Fear Conditioning Tasks in msP Rats.

One male vehicle rat lost the cannula implant and was excluded from analyses.

Two-way ANOVA (dose and sex as independent factors) on distance traveled in the open field arena, found an overall effect of NPS dose [$F(2, 48) = 8.213$, $p < 0.0001$, $\eta^2 = 0.242$], but no effect of sex [$F(1, 48) = 0.4584$, $p = 0.5$, $\eta^2 = 0.007$], and dose by sex interaction [$F(2, 48) = 1.48$, $p = 0.24$, $\eta^2 = 0.044$]. Dunnett's post-hoc test on the main treatment effect revealed that both 0.5 and 1 nmole NPS doses increased distance traveled in the open field arena (**Figure 1**). This result confirmed that NPS retains its stimulatory effect in the msP line when tested in a novel OF arena. The stimulatory effect of NPS on msPs was confirmed also in the absence of novelty (**Supplementary Figure S1**).

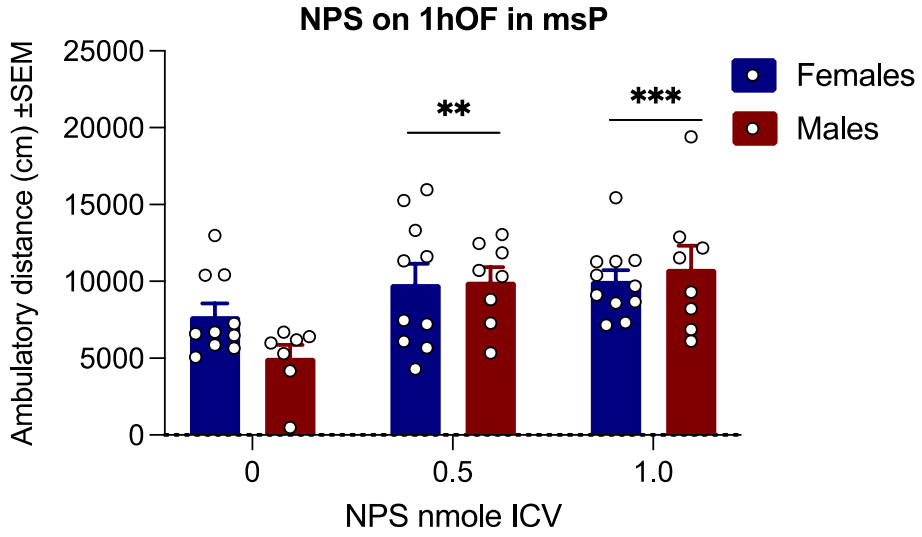


Figure 1. Effect of NPS on locomotor activity in msP rats. Both doses of NPS increased locomotor activity in both male and female rats. treatment on one hour OFT performance by female and male msP rats. Bars represent means ± SEM of total distance travelled in 1h in the OF arena. Statistical significance: **p<0.01 and ***p<0.001 vs 0 independently of sex.

During the EPM test, one female (0.5 nmole) and two male (0.0 and 1.0 nmole) rats fell from the maze and were excluded from analysis. A two-way ANOVA (dose and sex as independent factors) of relative time spent in the open arm (time in open arms/total test time) showed a significant effect of NPS dose [$F(2, 44) = 6.76$; $p = 0.003$, $\eta^2 = 0.213$], and no effect of sex [$F(1, 44) = 0.65$; $p = 0.42$, $\eta^2 = 0.010$]. Dose by sex interaction approached but failed to reach statistical significance threshold [$F(2, 44) = 2.67$; $p =$

0.08, $\eta^2 = 0.084$]. Observation of raw data suggested that the overall dose effect was probably driven by the male subjects. Therefore, we conducted a secondary one-way ANOVA for each sex separately. As expected, NPS failed to affect time in open arms in female [$F(2, 27) = 1.36$; $p = 0.27$, $\eta^2 = 0.092$] (**Figure 2A**), but did show an overall effect [$F(2, 17) = 3.92$; $p = 0.039$, $\eta^2 = 0.316$] in male msP rats. Dunnett's post-hoc analysis revealed that 1 nmole of NPS significantly increased the time spent in the open arm ($p < 0.01$; **Figure 2B**). The number of open arm entries was not affected by NPS (**Figure S2**), indicating that the effect observed on time in open arms was not secondary to locomotor effects.

Analysis of FC using a three-way ANOVA (dose and sex as independent factors, test trials as a repeated measure) found an overall effect of trial [$F(2, 92) = 26.02$; $p < 0.001$; $\eta^2 = 0.151$], no effect of sex [$F(1, 46) = 0.41$; $p = 0.52$; $\eta^2 = 0.004$] and treatment [$F(2, 46) = 1.9$; $p = 0.16$; $\eta^2 = 0.040$]; a significant trial by treatment interaction was observed [$F(4, 92) = 3.47$; $p = 0.011$; $\eta^2 = 0.040$] but trial by treatment by sex interaction was not significant [$F(4, 92) = 0.54$; $p = 0.706$; $\eta^2 = 0.006$]. Again, to further explore our results, we run secondary analyses in each sex separately. In female two-way ANOVA confirmed an overall effect of trial [$F(2, 56) = 22.13$; $p < 0.0001$, $\eta^2 = 0.2$], no overall effect of treatment [$F(2, 28) = 2.4$; $p = 0.11$, $\eta^2 = 0.068$] but there was a significant dose by trial interaction [$F(4, 56) = 4.44$; $p = 0.003$, $\eta^2 = 0.080$]. Tukey's post-hoc analysis revealed that all three groups of rats exhibited increased freezing time during the test trial compared to the conditioning trial. During the extinction trial, the vehicle group maintained a similar level of freezing as observed in the test trial, whereas the two groups treated with NPS showed significantly reduced freezing compared to the vehicle group. ($p<0.05$; **Figure 2C**). In male rats, two-way ANOVA confirmed an overall effect of trial [$F(2, 36) = 7.56$; $p = 0.002$, $\eta^2 = 0.114$], but no effect of treatment [$F(2, 18) = 0.36$; $p = 0.7$, $\eta^2 = 0.023$] or trial by treatment interaction [$F(4, 36) = 0.67$; $p = 0.62$, $\eta^2 = 0.020$]. Freezing time during the test trial was greater than during both the conditioning and extinction trials, regardless of NPS dose (**Figure 2D**).

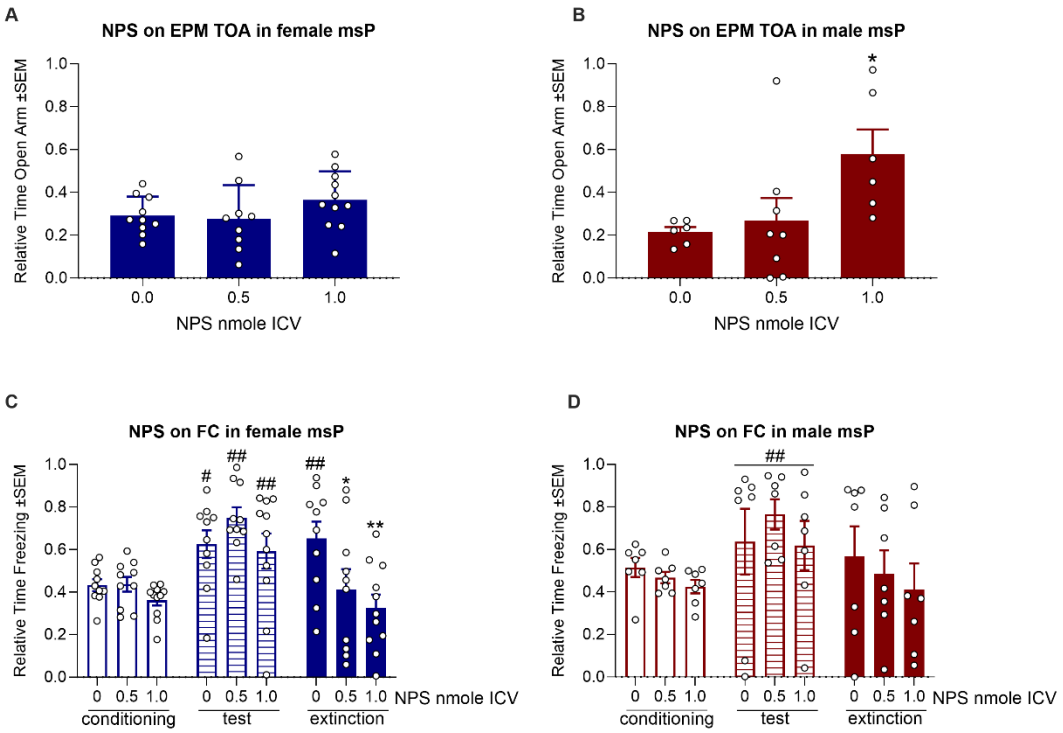


Figure 2. **A-B**) **Effect of NPS on EPM test in msP rats. A**) NPS did not affect the relative time spent in the open arm of a EPM apparatus in female rats. **B**) 1.0 nmole of NPS significantly increased the relative time spent in the open arm of a EPM apparatus in male rats, indicating an anxiolytic effect. **C-D**) **Effect of NPS on FC test in msP rats. NPS was administered immediately after test trial. C**) In female rats, there was no difference in relative time freezing between NPS dose groups during the trials that preceded treatment, i.e. conditioning and test trials, indicating a lack of group bias. In the test trial all groups showed increased freezing compared to conditioning trial, indicating acquisition of fear memory. In extinction trial vehicle treated rats maintained a level of freezing compared to test trial, while both doses of NPS facilitated the extinction of fear memory reducing the time spent freezing. **C**) In male rats, all groups showed increased freezing in test trial compared to conditioning trial, indicating acquisition of fear memory. No difference between NPS dose groups was observed in any trial. Bars and whiskers represent means ± SEM. Statistical significance: **B**) *p<0.05 vs 0; **C**) $^{\#}$p<0.05 and $^{\#\#}$p<0.01 vs same dose in conditioning trial, *p<0.05 and **p<0.01 vs 0 in the same trial; **D**) $^{\#\#}$-p<0.01 vs same dose in conditioning trial independent of dose.

Experiment 2: Effect of NPS on Alcohol Self-Administration in MsP Rats.

There was a solution backflow in one rat during the administration of 2 nmole of NPS; that data point was excluded from the analysis. Therefore, data were analyzed by mixed-effects two-way ANOVA, with NPS doses as repeated measure and sex as independent factor. Mixed effect ANOVA allows missing data points in repeated measure analyses. The analysis found an overall effect of NPS dose [F(3, 41) = 20.52;

$p < 0.0001$; $\eta^2 = 0.408$] and sex [$F(1, 14)=7.89$; $p = 0.014$; $\eta^2 = 0.091$], but no dose by sex interaction [$F(3, 41) = 1.5$; $p = 0.23$; $\eta^2 = 0.040$]. These results indicate an effect of NPS independent of sex. Dunnet's post-hoc test on the main treatment effect confirmed that all doses of NPS decreased the number of reinforcements received (**Figure 3A**). The number of inactive lever responses was unaffected by pharmacological treatment (**Figure 3B**): dose [$F(3, 41) = 1.37$; $p = 0.27$; $\eta^2 = 0.055$], sex [$F(1, 14)=0.03$; $p = 0.86$; $\eta^2 = 0.002$], dose by sex interaction [$F(3, 41) = 0.56$; $p = 0.69$; $\eta^2 = 0.034$].

Similar to as previously reported in male Wistar rats (Cannella et al. 2016). NPS was ineffective in female Wistar rats (**Figure S3**).

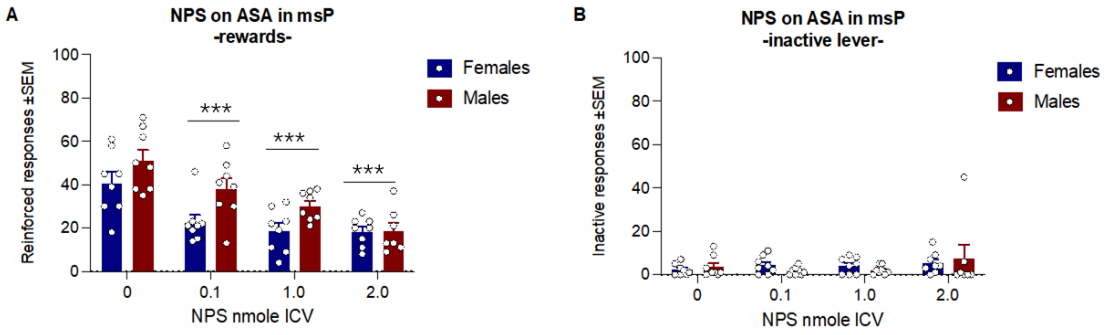


Figure 3. Effect of NPS on alcohol self-administration in msP rats. A) NPS reduced the number of 10% alcohol rewards in both male and female rats. **B**) NPS did not affect inactive lever responses. Bars and whiskers represent means ± SEM of reinforced active (**A**) and inactive (**B**) lever responses. Statistical significance: ***p<0.001 vs 0 independent of sex.

Experiment 3: Effect of RTI263 on Alcohol Self-Administration in Wistar Rats.

A female rat had its guide cannula blocked before the end of treatment and was therefore excluded from the analyses. A two-way ANOVA of rewards in the first test (i.e., RTI263 1–10 nmol dose range) found an overall effect of treatment [$F(3, 75) = 6.15$; $p = 0.0009$; $\eta^2 = 0.114$], no effect of sex [$F(1, 25) = 3.57$; $p = 0.07$; $\eta^2 = 0.043$], and a significant sex-by-treatment interaction [$F(3, 75) = 4.01$; $p = 0.01$; $\eta^2 = 0.075$]. Dunnett's post-hoc test indicated that RTI263 did not affect the number of rewards earned by Wistar rats; however, the reference drug NPS significantly decreased rewards in male rats ($p < 0.01$; **Figure 4A**). Analysis of inactive responses indicated an overall effect of treatment [$F(3, 75) = 5.43$; $p = 0.002$; $\eta^2 = 0.119$], driven by an increase in lever presses independent of sex induced by NPS (**Figure 4B**). No effect of sex [$F(1, 25) = 0.08$; $p = 0.77$; $\eta^2 = 0.001$] or treatment-by-sex interaction [$F(3, 75) = 0.17$; $p = 0.91$; $\eta^2 = 0.004$] was found.

To further characterize RTI263 dose/response curve, we subjected the rats to a second round of treatments increasing the dose of RTI263 to 100 nmol/ICV. Another female

rat lost guide cannula patency and was excluded from the analysis. Again, we observed an overall effect of treatment [$F(2, 48) = 7.04$; $p = 0.002$; $\eta^2 = 0.082$], no effect of sex [$F(1, 24) = 0.198$; $p = 0.66$; $\eta^2 = 0.005$], but a significant sex by treatment interaction [$F(2,48) = 6.2$; $p = 0.004$; $\eta^2 = 0.072$]. Dunnett's post-hoc analysis confirmed that the reference drug NPS decreased the number of rewards in males ($p < 0.001$) but did not affect it in female rats. However, in this case we also found that 100 nmole of RTI263 reduced ASA in female rats ($p < 0.01$; **Figure 4C**). Analyses of inactive lever presses confirmed an overall effect of treatment but no sex and sex by treatment interaction. Again this corresponded to an increase in lever presses induced by NPS independent of sex (**Figure 4D**).

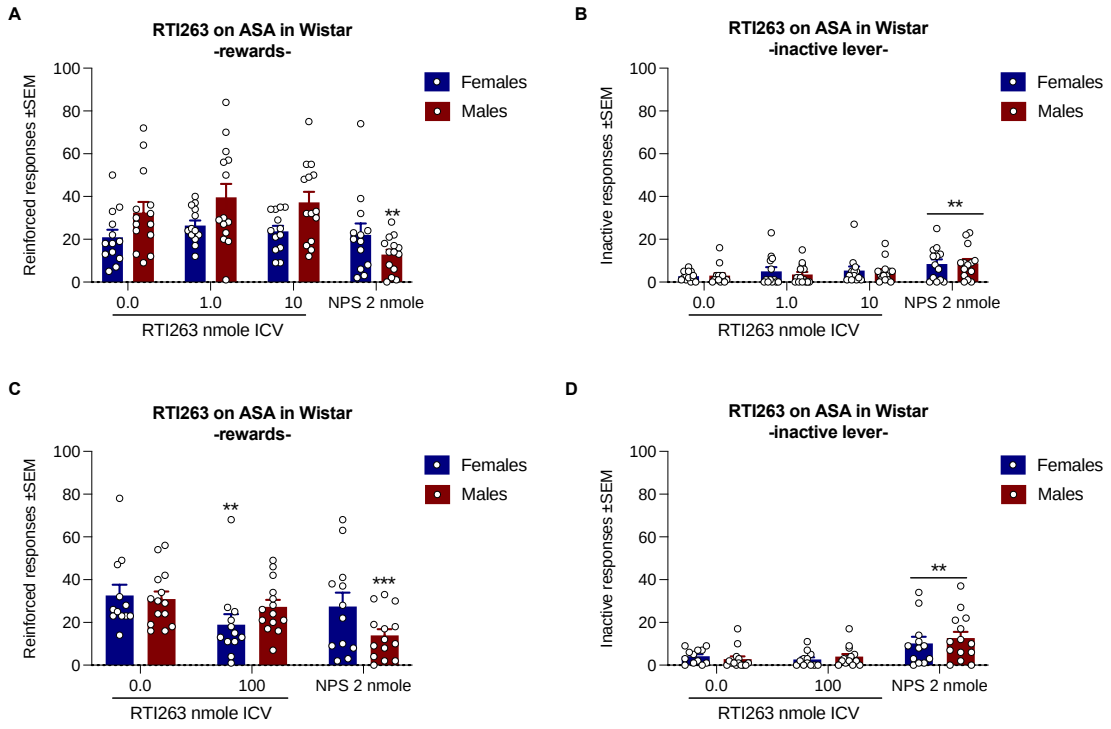


Figure 4. Effect of RTI263 and its reference compound NPS on alcohol self-administration in Wistar rats. A) RTI263 (1-10 nmol/ICV) failed to affect ASA in Wistar rats. NPS reduced 10% alcohol rewards in male rats. **B**) NPS, but not RTI263 (1-10 nmol/ICV) increased the number of inactive lever presses in both male and female Wistar rats. **C**) 100nmol/ICV of RTI263, but not NPS reduced ASA in female Wistar rats. NPS confirmed to reduce ASA in male rats. **D**) NPS, but not RTI263 (100 nmol/ICV) increased the number of inactive lever presses in both male and female Wistar rats. Bars and whiskers represent means ± SEM of reinforced active (**A, C**) and inactive (**B, D**) lever responses. Statistical significance: **A, C**) **p<0.01 and ***p<0.001 vs 0.0 dose same sex; **B, D**) ** p<0.01 vs 0 independent of sex.

Experiment 4: Effect of NPS on Yohimbine-Induced Reinstatement of Alcohol Seeking in msP rats.

During the last three days of ASA, male and female rats consumed an average of 42.85 ± 2.88 and 37 ± 2.64 alcohol rewards per session, respectively. Analysis of extinction training indicated an overall effect of time [$F(15, 240) = 4.82$; $p < 0.0001$; $\eta^2 = 0.096$], sex [$F(1, 16) = 7.21$; $p = 0.016$; $\eta^2 = 0.164$], and a significant time-by-sex interaction [$F(15, 240) = 2.85$; $p = 0.0004$; $\eta^2 = 0.057$]. Tukey's post-hoc test revealed that male rats extinguished alcohol-seeking behavior compared to extinction session 1 ($p < 0.0001$), while females maintained a comparable level of active lever responding throughout the extinction training (**Figure 5A**).

To analyze the effect of yohimbine on alcohol-seeking behavior, we compared the average lever presses of the last three days of extinction with responses in the yohimbine + NPS vehicle treatment condition. Analysis of active lever responding found an overall effect of condition (extinction vs. yohimbine) [$F(1, 16) = 14.34$; $p = 0.0016$; $\eta^2 = 0.118$] and sex [$F(1, 16) = 6.95$; $p = 0.018$; $\eta^2 = 0.227$], but no sex-by-condition interaction [$F(1, 16) = 0.11$; $p = 0.743$; $\eta^2 = 9.174 \times 10^{-4}$]. This is consistent with an overall higher response rate in female rats and with an increase in lever presses induced by yohimbine, independent of sex (**Figure 5B**). Inactive lever presses were low and not affected by condition [$F(1, 16) = 0.05$; $p = 0.827$; $\eta^2 = 0.004$], sex [$F(1, 16) = 0.73$; $p = 0.404$; $\eta^2 = 0.008$], or their interaction [$F(1, 16) = 0.05$; $p = 0.827$; $\eta^2 = 0.004$].

Analysis of the effect of NPS on yohimbine-induced alcohol seeking found an overall effect of NPS dose [$F(3, 48) = 3.63$; $p = 0.019$; $\eta^2 = 0.079$] and no effect of sex [$F(1, 16) = 1.35$; $p = 0.262$; $\eta^2 = 0.04$], while the dose-by-sex interaction approached but did not reach the significance threshold [$F(3, 48) = 2.64$; $p = 0.06$; $\eta^2 = 0.058$]. This prompted us to run secondary analyses within the two sexes separately. In males, no overall effect of NPS treatment was observed [$F(3, 24) = 1.21$; $p = 0.327$; $\eta^2 = 0.131$] (**Figure 5C**). Conversely, in females, there was an overall effect of treatment [$F(3, 24) = 5.15$; $p = 0.0068$; $\eta^2 = 0.392$], which, according to Tukey's post-hoc test, was driven by differences between the NPS 0.01 nmole/ICV dose and the other two doses of NPS, but not versus the vehicle (**Figure 5D**). Inactive lever presses were always low and not affected by treatment: males, [$F(3, 24) = 0.43$; $p = 0.735$; $\eta^2 = 0.051$]; females, [$F(3, 24) = 0.71$; $p = 0.557$; $\eta^2 = 0.027$]. Altogether, these results indicate that NPS did not affect yohimbine-induced alcohol seeking.

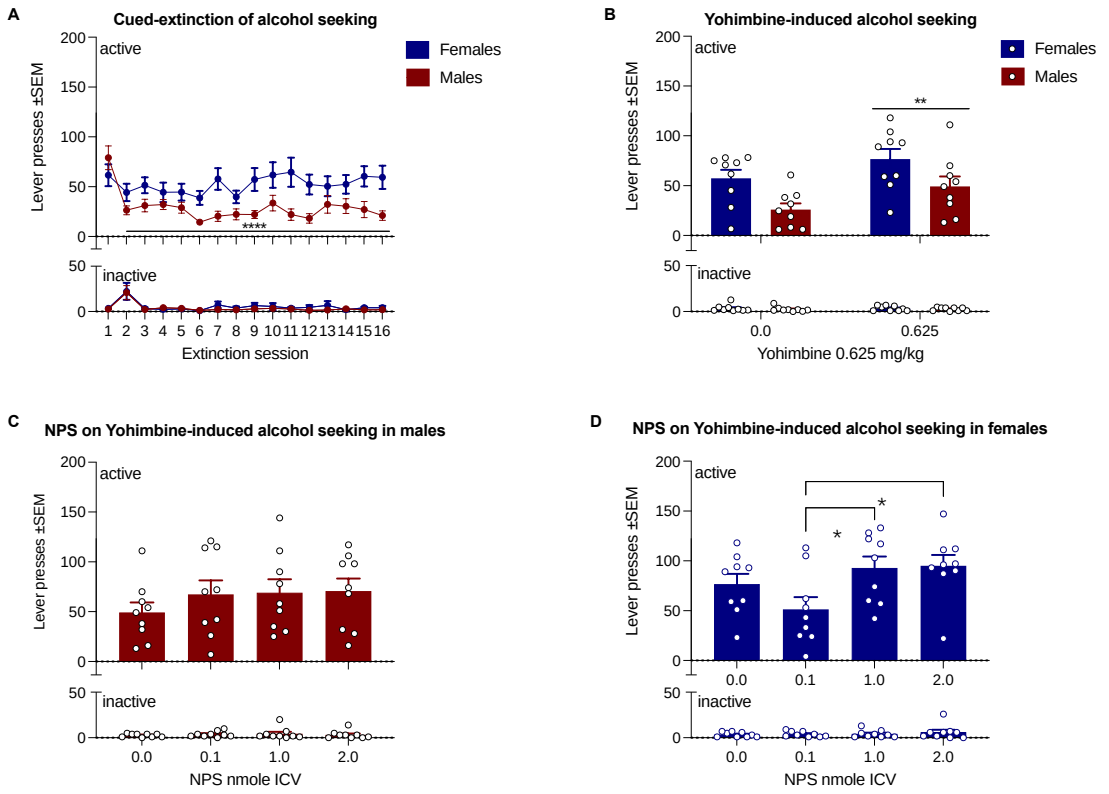


Figure 5. Effect of NPS on yohimbine-induced cued-alcohol seeking in msP rats. A) When subjected of 16 consecutive sessions of alcohol-paired cued lever pressing, male, but not female, msP rats extinguished alcohol seeking. **B**) Yohimbine increased cued-lever pressing independent of sex. **C, D**) NPS did not affect yohimbine-induced alcohol seeking neither in male (**C**) nor in female (**D**) msP rats. Bars and whiskers represent means ± SEM of lever responses. Statistical significance: **B**) ** $p<0.01$ vs 0 independent of sex. **D**) *$p<0.05$ between groups indicated by brackets.

DISCUSSION

In this study, we aimed to characterize the role of NPS in anxiety, stress response, and alcohol seeking in msP rats, a strain with a high preference for alcohol, which they consume to mitigate their innate negative affective state (Borruto et al., 2021; Ciccocioppo et al., 2006).

Given NPS's dual pharmacological nature as both stimulatory and anxiolytic, we first confirmed that it retains its stimulatory effects in msP rats. Both male and female msP rats exhibited increased locomotor activity following NPS treatment. Next, we examined its effects in the EPM and on fear memory consolidation. Our findings revealed that NPS had different effects depending on sex. In female msP rats, it facilitated fear extinction, but did not increase time spent in open arms in the EPM test

at a 1 nmole/icv dose. In contrast, in male msP rats, NPS increased open-arm time in the EPM test but had no effect on fear extinction in the FC test at the same dose.

A possible interpretation for the facilitation of fear extinction in female observed is that, as NPS promotes arousal and locomotion, this would reflect in less time freezing. However this is unlikely for two reasons: i) we observed the facilitation of fear extinction in female only, while the pro-arousal effect is common to both male and females; ii) we conducted extinction test 24h after NPS treatment, while the locomotor effect of NPS lasts no longer than an hour (Xu et al., 2004; Smith et al., 2006).

Our findings align with previous research showing that NPS exerts anxiolytic effects in the EPM in msP rats (Cannella et al., 2016) and mice (Rizzi et al., 2008), and facilitates fear extinction in the FC test in Wistar rats (Kawade et al., 2022). However, these studies were conducted only on male rats. A study by Wegener and colleagues investigated the anxiolytic and stress-coping effects of NPS on both male and female Flinders Sensitive Line (FSL), Flinders Resistant Line (FRL), and Sprague–Dawley (SD) rats. Their experiment showed that NPS reduced anxiety-like behavior in the elevated plus-maze in all strains, with no observed sex differences. NPS also did not modify stress-related behavior in the forced swim test in any strain, again with no sex differences (Wegener et al., 2012). Interestingly, our results mirror the effects of alcohol on the EPM and FC in msP rats, as reported by Borruto et al. (2021). They found that alcohol affects msP rats differently, with males drinking to reduce anxiety and females drinking to cope with stress. This evidence confirms the effects of alcohol in msP rats in the EPM and FC tests, suggesting that NPS is a promising target for alcoholism. Its effects are specific, as it can reduce alcohol self-administration in female msP rats by facilitating stress coping and in males through its anxiolytic effect.

Our findings on the effect of NPS parallel the anxiolytic and stress-coping effects of alcohol in msP rats, supporting the idea that NPS and alcohol share overlapping behavioral effects in anxiety and stress-related tasks. Since msP rats consume alcohol in response to anxiety (males) or stress (females), it is significant that NPS influences the same behavioral domains affected by alcohol. This overlap provided the rationale for testing whether NPS could impact ASA, as it may substitute for alcohol's anxiolytic and stress-relieving properties, thereby reducing the motivation to seek alcohol. Given that NPS reduces both anxiety (which drives male drinking) and stress coping (which drives female drinking), it could be an effective intervention for alcohol seeking in both sexes.

Thus, we tested the effect of NPS on ASA in msP rats and, as expected, found that NPS reduced ASA in both male and female msP rats. This suggests that NPS is a viable therapeutic target for alcohol use disorder.

It has been demonstrated that NPS has both anxiolytic and stimulatory effects. In an

experiment by Clark and colleagues, RTI263 showed anxiolytic and learning effects similar to NPS. However, RTI263 did not produce the same increase in locomotor activity as NPS (Clarck et al,. 2017). Given the established link between alcohol consumption and anxiety, the present study investigated the impact of RTI263 on ASA in Wistar rats. The results showed that RTI263 reduced ASA in female rats, but not in males. This suggests that the anxiolytic effect of NPS alone is sufficient to reduce alcohol seeking in female rats, but not in males.

The failure of NPS to reduce yohimbine-induced alcohol seeking in msP rats suggests that: NPS may be more effective in modulating innate anxiety and stress-related behaviors than in modulating external compound-induced stress-related drug-seeking behavior, which leads to more chaotic neuroplastic changes.

Although alcohol affects various aspects of human behavior, the stress and anxiety-induced potentiation of AUD appears crucial, as reducing stress and anxiety may decrease alcohol consumption (J. Anker, 2019; Joshua P. Smith & Randall, 2012). This highlights the importance of our study on the effects of NPS, a promising target for AUD, stress, and anxiety. Future research will need to explore the detailed mechanisms underlying NPS's multiple effects.

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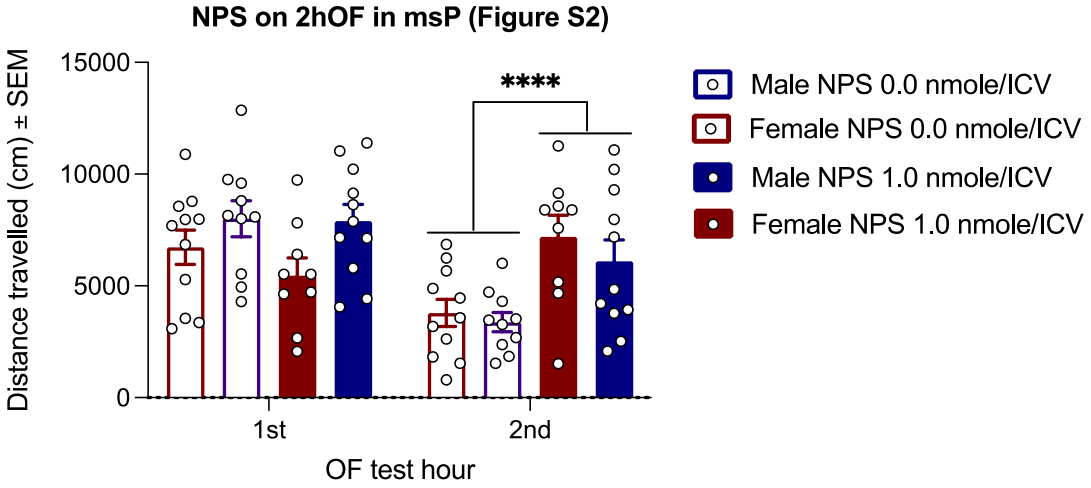

Figure S1. Two hours of locomotor activity were recorded in male and female msP rats. When the first hour had elapsed, acquisitions were paused, rats immediately treated ICV with either 1 nmole of NPS or its vehicle, acquisition restarted and recorded for the following hour. Three-way ANOVA found significant time by dose [$F(1, 37) = 35.27$; $p < 0.0001$； $\eta^2 = 0.106$] but not time by dose by sex [$F(1, 37) = 2.13$; $p = 0.15$； $\eta^2 = 0.006$] interaction. This result is consistent with a higher locomotor activity expressed by both male and female rats treated with 1 nmole of NPS during the second hour of acquisition. Bars represent means ± SEM of total distance travelled during 1h in the OF arena. Statistical significance: ***p<0.001: difference driving the overall dose by time interaction.

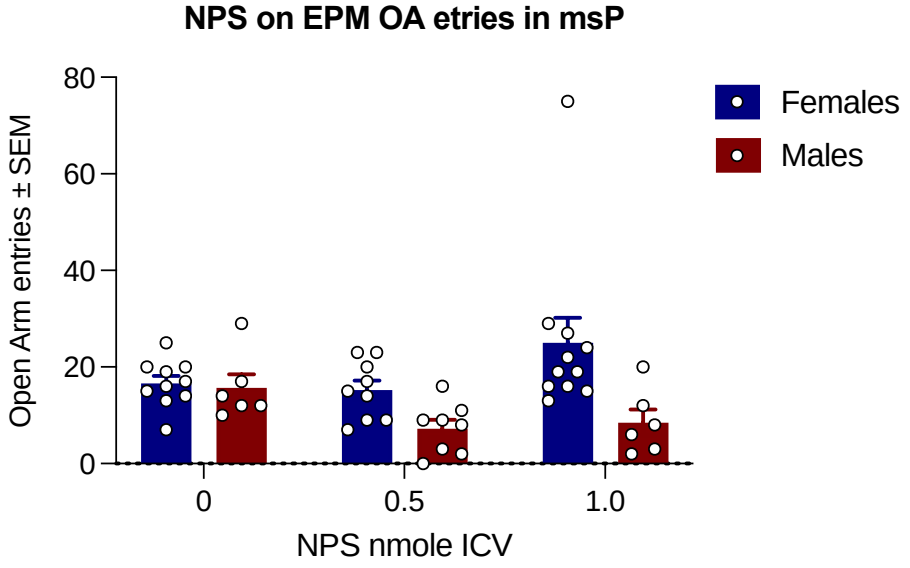

Figure S2. NPS failed to affect the number of open arm entries during EPM test. Dose [F (1, 44) = 1.57; p = 0.22; η^2 = 0.064]; sex [F(1, 44) = 8.94; p = 0.004; η^2 =0.163]; sex by dose [F(2, 44) = 2.45; p = 0.1; η^2 = 0.092]. Bars represent means ± SEM of total open arm entries.

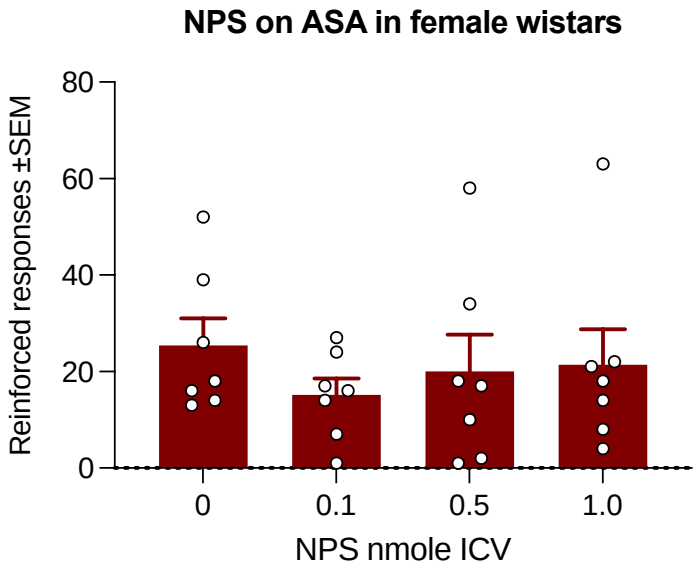

Figure S3. NPS did not affect alcohol self-administration in female Wistar rats. Experimental conditions were identical to experiment 2. Bars represent means ± SEM.

CHAPTER 3

IMPULSIVITY AS A RISK FACTOR FOR ALCOHOL USE DISORDERS: DEVELOPMENT AND VALIDATION OF A GO-NOGO MODEL OF IMPULSIVITY

ABSTRACT

Background: Impulsivity is a multifaceted behavior characterized by premature, risky, and inappropriate actions often leading to adverse consequences. It is increasingly recognized as a vulnerability factor for alcohol use disorders. In this chapter, we developed a rat operant self-administration model of impulsivity to investigate whether Marchigian Sardinian alcohol-preferring (msP) rats exhibit impulsive-like traits and whether this trait contributes to their alcohol-seeking behavior.

Methods: First, we developed and validated a between-trials Go/No-Go model and a within-trial Go/No-Go model of impulsivity in non-preferring Wistar rats, using atomoxetine for validation. Next, we selected the within-trial Go/No-Go model to compare impulsive behavior between Wistar and msP rats. Finally, we tested the effect of atomoxetine on alcohol self-administration (ASA).

Results: Atomoxetine validation confirmed that both Go/No-Go models exhibited predictive validity for impulsivity. Compared to Wistar rats, both male and female msP rats displayed higher impulsive traits. Additionally, atomoxetine reduced ASA in msP rats, though sex-dependent differences were observed.

Conclusions: Using a Go/No-Go model of impulsivity, we demonstrated that msP rats exhibit greater impulsivity than Wistar controls, contributing to their high alcohol preference.

INTRODUCTION

Impulsivity is a multifaceted behavior, typically defined as a tendency to engage in premature, poorly planned, or inappropriate actions in response to internal or external stimuli, without regard for the negative consequences to oneself or others. This often leads to adverse or maladaptive outcomes (Daruma & Barnes, 1993; McCown et al., 1993; Moeller et al., 2001; Bakhshani, 2014; Hamilton et al., 2015). The symptom is marked by risky or poorly conceived acts, frequently resulting in undesirable consequences due to a lack of forethought (Evenden, 1999).

Impulsivity is a key factor in several psychiatric disorders and is increasingly recognized as a vulnerability for AUD (Kozak et al., 2019). It also contributes to relapse

and treatment failure (Stevens et al., 2014; Lozano-Rojas et al., 2024; Winstanley, 2011). Chronic alcohol use can impair brain function and increase impulsive behavior (Huberman, 2022). Wilhelm et al. (2007) found that individuals with alcoholism or those consuming large quantities of alcohol tend to exhibit higher levels of impulsivity, as indicated by various measures, compared to those who either do not consume alcohol or consume it in smaller amounts. This may be due to factors predating alcohol exposure, such as genetics, or neuroadaptations resulting from alcohol exposure (Wilhelm et al., 2007). Pattij et al. reported that after self-administration of alcohol, impulsivity increased in rats (Pattij et al. 2020).

Over the past decade, trait impulsivity has received considerable attention as a risk factor for substance dependence in both clinical and preclinical studies. However, this relationship varies by impulsivity subtype and drug class (Mitchell & Potenza, 2014). Various forms of impulsivity have been identified as markers of vulnerability to AUD (Verdejo-García et al., 2008). For instance, impulsive choice predicts increased alcohol consumption, but the strength and extent of this relationship remain uncertain (Verdejo-García et al., 2008; Linsenbardt et al., 2017; Acheson et al., 2011; Lejuez et al., 2010). The interaction between impulsivity and AUD often creates a vicious cycle, as impulsivity facilitates alcohol use, and alcohol consumption, in turn, increases impulsivity—forming a positive feedback loop that perpetuates alcohol-seeking behavior (Littlefield et al., 2014).

Emerging evidence suggests that rodents selectively bred for excessive alcohol consumption, like humans with a family history of alcoholism, exhibit cognitive impairments, including impulsivity (Beckwith & Czachowski, 2016; Oberlin & Grahame, 2009; Walker et al., 2011). Additionally, alcohol-naive alcohol preferring rats show greater delay discounting compared to rodent populations without elevated genetic risk (Beckwith & Czachowski, 2014; Perkel et al., 2015). These findings suggest that impulsivity is not merely a consequence of excessive alcohol consumption but is instead regulated by shared genetic and neural mechanisms (Linsenbardt et al., 2017). Delay discounting, a behavioral measure of cognitive impulsivity, has been linked to AUD (Linsenbardt et al., 2017). Both alcoholism risk and delay discounting are heritable traits, with increasing evidence of a genetic relationship between them (Mitchell, 2011).

In summary, while the link between AUD and impulsivity is well-established in clinical and preclinical research, genetically predisposing factors can link impulsive traits to alcoholism. Both alcoholism and impulsivity are heterogeneous constructs influenced by factors such as sensation seeking, lack of planning, and positive or negative urgency (Littlefield et al., 2014). Therefore, early intervention and targeted treatments are crucial for preventing and managing these symptoms.

Aim of This Chapter

In this chapter we sought to determine whether marchigian sardinian alcohol preferring rats, in which high alcohol preference is associated with negative affective state such as high anxiety and stress response, also show increased impulsivity and if this trait also contribute to alcohol preference.

We set out developing two Go/No-Go models of impulsivity:

1. Between-trials Go/No-Go model, in which Go and NoGo inputs are presented in separate trials.
2. Within-trial Go/No-Go model, in which Go and NoGo inputs are presented within the same trial.

Then we chose the within-trial model to compare the impulsive behavior of non-preferring Wistar rats with msP. Finally, we tested whether the anti-impulsive drug atomoxetine is able to reduce alcohol self-administration in msP rats.

MATERIALS AND METHODS

Animals

Male and female Wistar rats (Envigo) and marchigian sardinian alcohol preferring (msP) bred at the University of Camerino were 9 weeks old at the beginning of experimental procedures. Rats were housed in groups of four in a room with reversed 12 h/12 h light/dark cycle (lights off at 8:30 am), constant temperature (20-22°C), and humidity (45-55%). Food (4RF18, Mucedola, Italy) and tap water were available *ad libitum*. All procedures were conducted during the dark phase of the light/dark cycle and adhered to the *European Council Directive for the Care and Use of Laboratory Animals* and the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Drugs

Atomoxetine-HCl (Sigma -Aldrich) was dissolved in sterile isotonic saline and administered intraperitoneally (IP) 30 minutes before test sessions.

Operant Self-Administration Apparatus

Operant training and testing were conducted in self-administration chambers (Med Associates) equipped with two retractable levers, a cue light above each lever, a house light located on the opposite wall and a beep-tone speaker. A drinking reservoir (0.30 ml capacity) located between the two levers was connected to an infusion pump. The apparatus was controlled by Windows-compatible MedPC-5.

EXPERIMENTAL PROCEDURES

Experiment 1: Development and Validation of a Between-Trials Go/NoGo Model of Impulsivity.

The between-trials Go/NoGo model of impulsivity was developed in 12 female and 9 male Wistar rats. Training consisted of four phases:

Phase 1; Self-administration training. The right and left levers were designated as active and inactive, respectively. Rats were trained to self-administer 0.1 mL of a 10% (w/v) sucrose solution in daily 30-minute sessions according to a fixed ratio 1 (FR1) schedule of reinforcement on the active lever. The cue lights above the active levers remained on throughout the session.

Phase 2; Go-Trial training. After self-administration SA training, they entered the Go Trial Training Phase. Session duration was reduced to 20 minutes and consisted of 20-second Go Trials spaced out by Inter-Trial-Intervals (ITI). Go Trials were identical to Phase 1: the levers were extracted, the active cue light - functioning as Go-signal - illuminated, and FR1 presses on the active lever were reinforced with 0.1 ml of 10% sucrose. A Go Trial ended, and the ITI began, if either a reward was delivered or 20 seconds elapsed before the rat pressed the active lever. In the first case, the trial was recorded as a "Correct-Go" trial, and in the second case, as a "Missed-Go" trial. During the ITI, the levers were retracted, and no cue was presented. The ITI lasted 5 seconds (short) if it followed a Correct-Go trial and 20 seconds (long) if it followed a Missed-Go trial; the longer ITI functioned as punishment for the missed response.

Phase 3; Introduction of a Pre-trial period. In this phase, a 3-second Pre-Trial period preceding the Go-Trial was introduced. During Pre-trials, levers were extracted, and the house light on the opposite wall was illuminated. If the rat did not press the active lever, the Pre-Trial was followed immediately by a Go Trial, as described in Phase 2. If the rat pressed the active lever during the Pre-Trial, the levers were retracted, and a long ITI began. Active lever presses during Pre-Trials were recorded as "Premature Responses."

Phase 4; Full between-trials Go/NoGo model. Finally, NoGo trials were introduced to complete the Go/NoGo model. Each trial started with a Pre-trial as described in Phase 3, that - unless a rat produced a Premature Response - was randomly (50:50) followed by either a Go trial as described in Phase 2 or a NoGo trial. During the 20-second NoGo trials, levers were extracted, and a continuous beep tone was on. Abstaining from pressing the active lever for the entire duration of the NoGo trial was reinforced with 0.1 ml of 10% sucrose, recorded as "Correct-NoGo" trial, and followed by a short ITI. Pressing the active lever during NoGo trials was not reinforced, recorded as "Missed NoGo", and punished with a long ITI.

This model yielded the following possible outcomes:

a- Pre-trial → active lever pressed → "Premature response" recorded → rat punished with long ITI
b- Pre-trial → active lever not pressed → random Go/NoGo trial begin

c- Go-trial→ active lever pressed → "Correct Go" recorded → sucrose reward followed by short ITI.
d- Go-trial→ active lever not pressed → "Missed Go" recorded → rat punished with long ITI.

e- NoGo-trial→ active lever not pressed → "Correct NoGo" recorded → sucrose reward followed by short ITI.
f- NoGo-trial→ active lever pressed → "Missed NoGo" recorded → rat punished with long ITI.

The between-trials Go/NoGo workflow is schematized in **Figure 1**

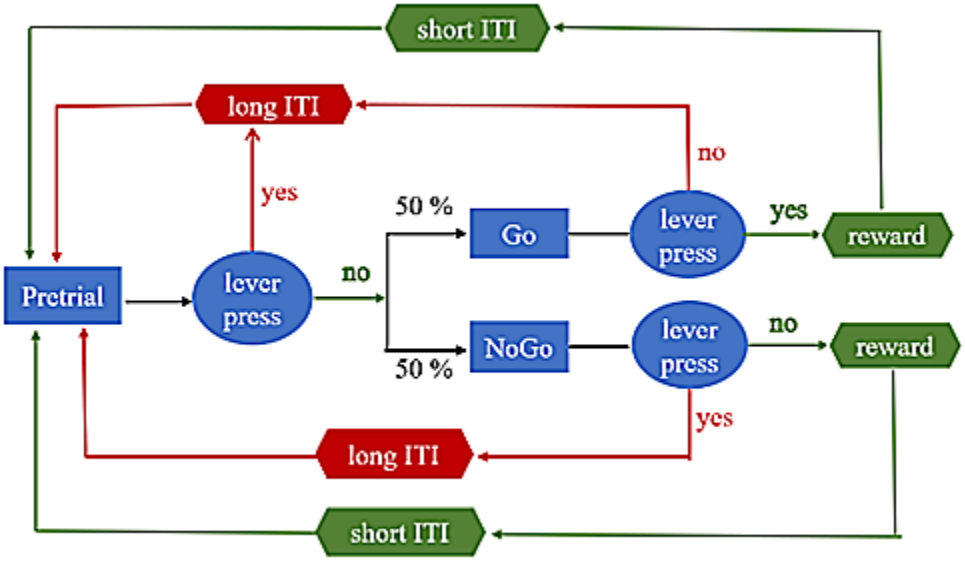

Figure 1. Between-trials Go/NoGo workflow.

Based on these outcomes, we extrapolated three behavioral variables:

1) number of premature responses (total outcome a)
2) missed NoGo ratio = total missed NoGo / total NoGo trials = (f) / (e+f)
3) missed Go ratio = total missed Go / total Go trials = (d) / (c+d)

Importantly, “missed ratio” measures are sufficient to fully describe animal behavior, as they correspond to “1- correct ratio” measures.

The inactive lever was presented and retracted contingently with the active lever. Inactive responding had no scheduled consequences and was recorded in every phase.

Model validation. To test the predictive validity of the between-trials Go/NoGo model, at the end of Phase 4 of training, rats were treated with atomoxetine HCl (1 and 5 mg/kg) or its vehicle. Treatment was repeated every fourth day until each rat had received all treatment doses in a within-subjects counterbalanced order**.** On the first intervening day, rats remained in their home cage, while on the second and third days, they were subjected to baseline between-trials Go/NoGo sessions. Animals were habituated to the treatment procedure for three days before treatment.

Experiment 2: Development and Validation of a Within-Trials Go/NoGo Model of Impulsivity.

The within-trial Go/NoGo model of impulsivity was developed in 8 male and 8 female Wistar rats. Training consisted of four phases:

Phases 1-3 were identical to those described above for the between-trials Go/NoGo model.

Phase 4; Full within-trial Go-NoGo model. Each trial started with a Pre-trial as described in Phase 3, which — unless a rat produced a Premature Response — was randomly (50:50) followed by either a Go-NoGo trial or a NoGo-Go trial. Specifically, each trial included both a 5-second Go phase and a 15-second NoGo phase. Go-NoGo trials started with a 5-second Go phase, during which the rat could press the active lever to obtain a reward. If the rat did not press the active lever, the trial would move into the 15-second NoGo phase, during which the rat had to abstain from pressing the active lever to obtain the reward. In NoGo-Go trials, the order of Go and NoGo phases was inverted. Pressing the active lever during a Go phase and abstaining from it during a NoGo phase were recorded as a “correct response”. Pressing the active lever during a NoGo phase and failing to press it during a Go phase were recorded as a “wrong response”.

Premature, Go, and NoGo cueing were identical to the between-trials GoNoGo model described above.

This model yielded the following possible outcomes:

a- Pre-trial → active lever pressed → “Premature response” recorded → rat punished with long ITI
b- Pre-trial → active lever not pressed → random Go-NoGo / NoGo-Go trial begin

c- Go-NoGo (Go phase)→ active lever pressed → "correct Go" recorded → sucrose reward followed by short ITI.
d- Go-NoGo (Go phase)→ active lever not pressed → "wrong Go" recorded → NoGo phase begin
e- Go-NoGo (NoGo phase)→ active lever not pressed → "correct NoGo" recorded → sucrose reward followed by short ITI.
f- Go-NoGo (NoGo phase)→ active lever pressed → "wrong NoGo" recorded → rat punished with long ITI

g- NoGo-Go (NoGo phase)→ active lever pressed → "wrong NoGo" recorded → rat punished with long ITI
h- NoGo-Go (NoGo phase)→ active lever not pressed → "correct NoGo" recorded → Go phase begin
i- NoGo-Go (Go phase)→ active lever not pressed → "wrong Go" recorded → rat punished with long ITI
j- NoGo-Go (Go phase)→ active lever pressed → "correct NoGo" recorded → sucrose reward followed by short ITI.

The within-trials Go/NoGo workflow is schematized in **Figure 2**

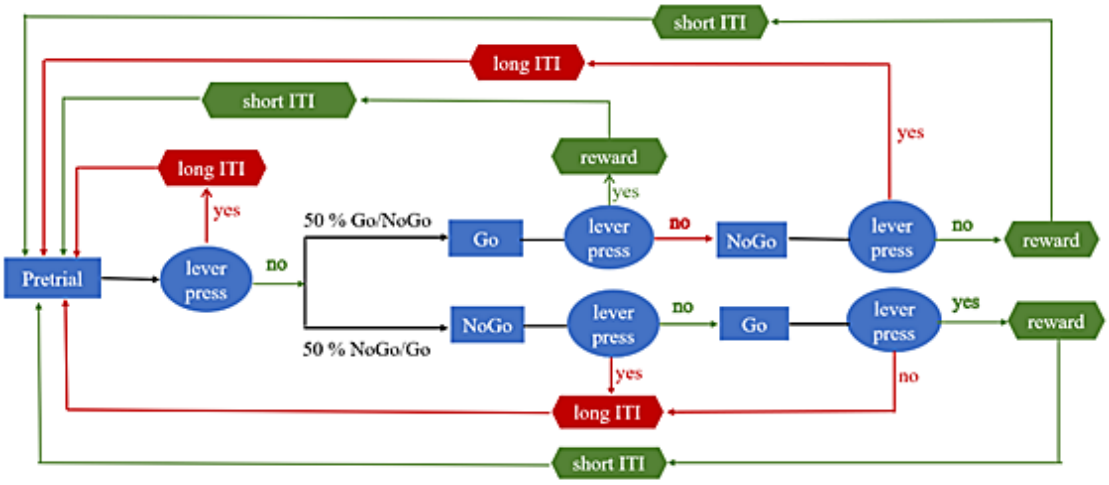

Figure 2. Within-trial Go/NoGo workflow.

Based on these outcomes, we extrapolated three behavioral variables:

1. number of premature responses (total outcome a)
2. missed NoGo ratio = total wrong NoGo / total NoGo phase = (f + g) / (e+f+g+h)
3. missed Go ratio = total wrong Go / total Go phase = (d+i) / (c+d+i+j)

Importantly, “missed ratio” measures are sufficient to fully describe animal behavior, as they correspond to “1- correct ratio” measures.

The inactive lever was presented and retracted contingently with the active lever. Inactive responding had no scheduled consequences and was recorded in every phase.

Model validation. The predictive validity of the within-trial Go/NoGo model was assessed with Atomoxetine as described in Experiment 1.

Experiment 3: Comparison of Wistar vs MsP Performance in the Within-Trials Go/NoGo Model.

Eleven msP rats (5 females, 6 males) were trained to self-administer 10% (w/v) sucrose according to the within-trial Go/NoGo model of impulsivity, as described in Experiment 2. When phase 4 of training reached a plateau, premature responses, missed Go and NoGo ratios, and inactive lever presses were averaged over the last three days and compared to the Wistars from Experiment 2

Experiment 4: Effect of Atomoxetine on Alcohol Self-Administration in MsP rats

Ten female and five male msP rats were trained to self-administer 10% alcohol (v/v) in 30-minute daily sessions. Pressing the active lever according to a fixed-ratio 1 schedule of reinforcement delivered 0.1 ml of alcohol solution into the drinking receptacle. Reward delivery was followed by a 5-second time-out period during which active lever presses were not reinforced. Inactive lever presses were recorded but had no scheduled consequences. After establishing a stable baseline, rats were treated intraperitoneally with atomoxetine-HCl (0, 1 mg/kg, and 5 mg/kg) 30 minutes before the session, following the same schedule described in Experiment 1.

STATISTICAL ANALYSIS

Samples size was based on our previous works on NPS effects on ASA and anxiety (Cannella et al. 2016; Cannella et al, 2009).

Data were analyzed using appropriate between- and within-subjects ANOVAs. The approximation to normality of the distributions was verified using Q-Q plots of the residuals before conducting the tests. Statistical significance was set at the conventional threshold of $p < 0.05$. ANOVA were followed by Dunnett’s post-hoc for treatment vs. control comparisons or Tukey’s post-hoc for pairwise comparisons when appropriate.

RESULTS

Experiment 1: Development and Validation of a Between-Trials Go/NoGo Model of Impulsivity.

Acquisition of active lever pressing (Phase1). A two-way ANOVA of the number of reinforced active lever presses during Phase 1 of training found an overall effect of time [$F(7, 154) = 33.37$; $p < 0.0001$; $\eta^2 = 0.374$], but no effect of sex [$F(1, 22) = 0.84$; $p = 0.37$; $\eta^2 = 0.014$] or a sex-by-time interaction [$F(7, 154) = 0.6$; $p = 0.75$; $\eta^2 = 0.007$]. This is consistent with the acquisition of sucrose self-administration in both male and female rats (**Figure 3A**).

Acquisition of Go-trial response (Phase 2-3). A two-way ANOVA of the missed Go ratio found an overall effect of time [$F(14, 308) = 5.08$; $p < 0.0001$; $\eta^2 = 0.132$], sex [$F(1, 22) = 11.58$; $p = 0.0025$; $\eta^2 = 0.075$], and a time-by-sex interaction [$F(14, 308) = 2.97$; $p = 0.0003$; $\eta^2 = 0.077$]. Females showed a low missed-Go ratio from the beginning of *Phase 2*. Males showed a higher ratio than females initially, but they improved their performance over time and performed stably at the female level (missed-Go < 10%) after 7–10 sessions (**Figure 3B**).

Training to Pre-trial (Phase 3-4). A two-way ANOVA found an overall effect of time [$F(17, 374) = 63.18$; $p < 0.0001$; $\eta^2 = 0.537$], but there was no effect of sex [$F(1, 22) = 0.87$; $p = 0.36$; $\eta^2 = 0.010$], and no significant time-by-sex interaction [$F(17, 374) = 1.62$; $p = 0.056$; $\eta^2 = 0.014$]. Both males and females decreased the number of premature responses over time (**Figure 3C**).

Random between- trial Go/NoGo training (Phase 4). A three-way ANOVA of missed response ratios found an overall effect of time [$F(11, 242) = 17.81$; $p < 0.0001$; $\eta^2 = 0.049$], trial (Go vs. NoGo) [$F(1, 22) = 70.96$; $p < 0.0001$; $\eta^2 = 0.476$], and a significant time-by-trial interaction [$F(11, 242) = 17.87$; $p < 0.0001$; $\eta^2 = 0.050$]. This is consistent with a higher number of missed NoGo trials compared to Go trials, and an improvement in NoGo trial performance (i.e., fewer missed NoGo trials) over time, although NoGo performance remained significantly worse (i.e., higher missed trial ratio) than Go performance (**Figure 3D**). There was no effect of sex [$F(1, 22) = 0.25$; $p = 0.62$; $\eta^2 = 0.002$], but there was a significant sex-by-time interaction [$F(11, 242) = 1.84$; $p = 0.048$; $\eta^2 = 0.005$] and a sex-by-time-by-trial interaction [$F(11, 242) = 2.15$; $p = 0.018$; $\eta^2 = 0.006$]. However, post-hoc analyses found no differences within the same day between sexes in either the Missed Go or Missed NoGo ratios (**Figure 3D**).

Inactive lever presses were low throughout all four phases of training, but they tended to increase in females during *Phase 4* (**Supplementary Figure S1**).

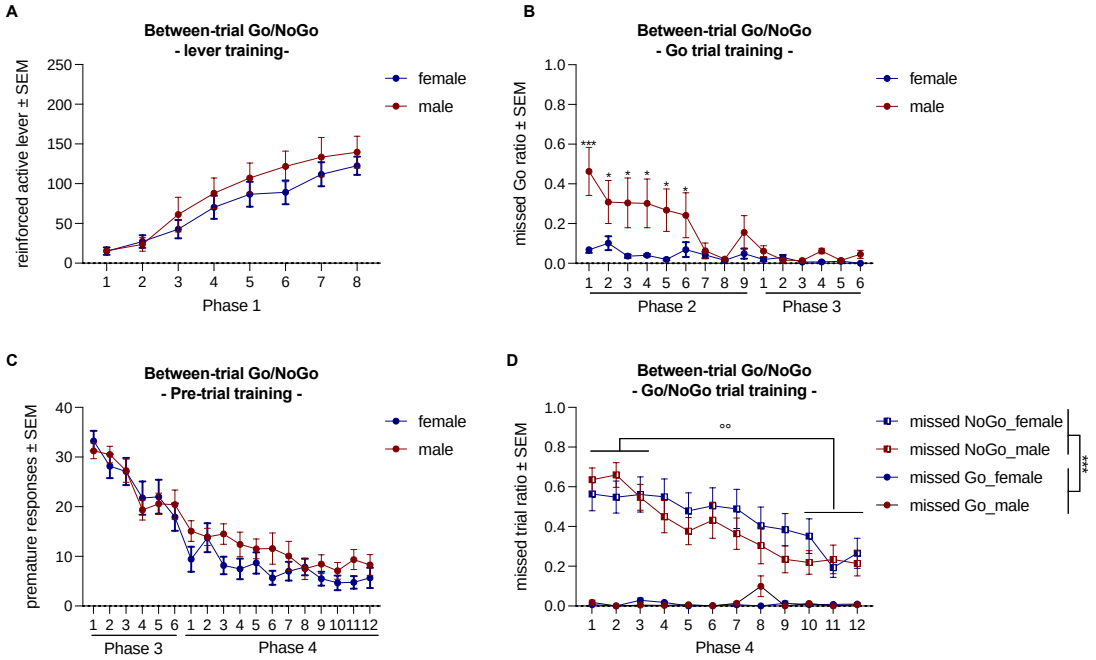

Figure 3. Development of the between-trials Go/NoGo model. A) During the first phase of training, rats acquired lever pressing reinforced by a sucrose solution. **B**) When Go-trials were introduced, missed go ratio in female was very low since the beginning. Males showed a higher missed Go ratio at the beginning, which fell to female level after 7 sessions. **C**) When the Pre-trial period was introduced, rats made a high number of premature responses that decreased over time. **D**) When rats were subjected to both Go and NoGo trials, missed Go ratio remained very low (<10%). Missed NoGo ratio decreased over time, but it remained constantly higher than missed Go. Data are presented as means ± SEM. Statistical significance: **A, C**) not shown; **B**) **p<0.01 and *p<0.05 vs female same day; **D**) °°p<0.01 between time points indicated by brackets independent of sex; ***p<0.001 between trial independent of sex.

Model validation with atomoxetine. A two-way ANOVA of premature responses during the pre-trial found an overall effect of atomoxetine dose [$F(2, 44) = 5.95$; $p = 0.005$; $\eta^2 = 0.059$], no effect of sex [$F(1, 22) = 1.5$; $p = 0.23$; $\eta^2 = 0.046$], and no sex-by-dose interaction [$F(2, 44) = 1.08$; $p = 0.35$; $\eta^2 = 0.011$]. Dunnett's post-hoc analysis of the main dose effect (i.e., males and females pooled) indicated that the highest dose of atomoxetine significantly reduced the number of premature responses (**Figure 4A**). Male and female rats showed similar missed NoGo ratios [$F(1, 22) = 0.15$; $p = 0.71$; $\eta^2 = 0.005$], which were affected by atomoxetine [$F(2, 44) = 8.23$; $p = 0.0009$; $\eta^2 = 0.050$], independently of sex [$F(2, 44) = 0.35$; $p = 0.7$; $\eta^2 = 0.002$]. Dunnett's post-hoc analysis of the main dose effect indicated that both doses of atomoxetine decreased the missed NoGo ratio (**Figure 4B**). The missed Go ratio was very low and was not affected by sex [$F(1, 22) = 0.25$; $p = 0.6$; $\eta^2 = 0.003$], atomoxetine [$F(2, 44) = 0.74$; $p = 0.48$; $\eta^2 = 0.021$], or their interaction [$F(2, 44) = 1.75$; $p = 0.18$; $\eta^2 = 0.051$] (**Figure 4C**). Male and female rats showed different numbers of inactive lever presses [$F(1, 22) = 11.87$; $p = 0.002$; $\eta^2 = 0.221$], which were affected by atomoxetine [$F(2, 44) = 4.09$; $p = 0.02$; $\eta^2 =$

0.055], independently of sex [$F(2, 44) = 1.32$; $p = 0.28$; $\eta^2 = 0.018$]. Dunnett's post-hoc analysis of the main dose effect indicated that the highest dose of atomoxetine decreased inactive lever presses (**Figure 4D**).

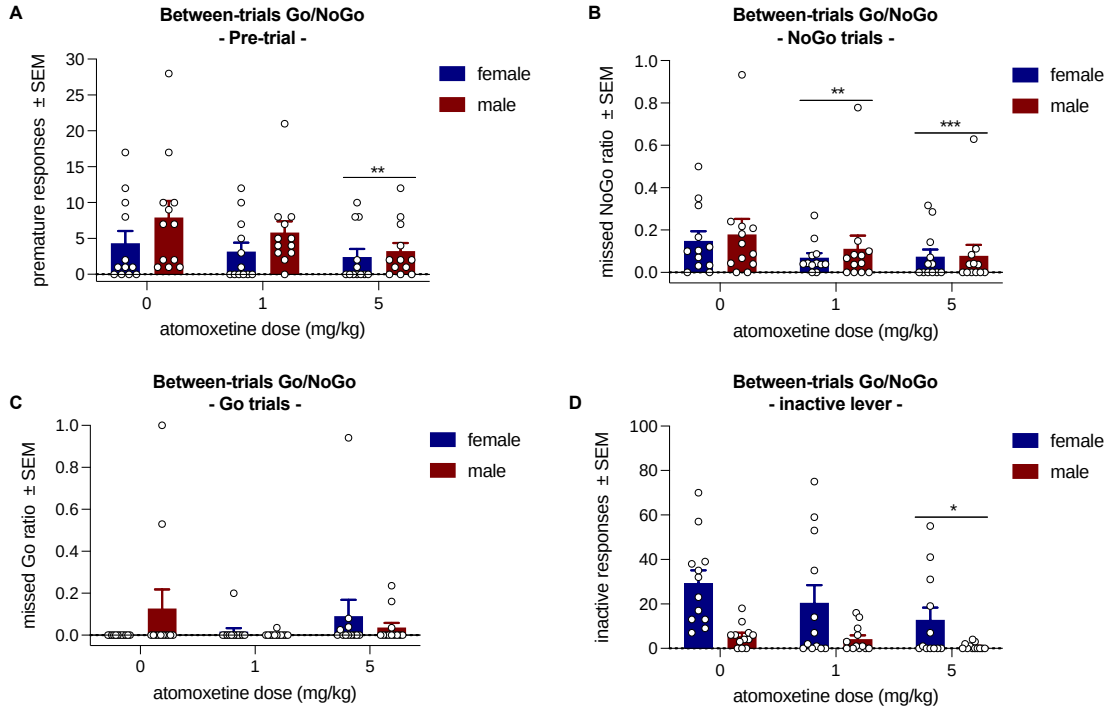

Figure 4. Effect of atomoxetine on between-trials Go/NoGo preformance. A) 5 mg/kg of Atomoxetine decreased the number of premature responses during Pre-trials periods. **B**) Both doses of Atomoxetine decreased missed NoGo ratio. **C**) Atomoxetine did not affect missed Go ratio. **D**) 5 mg/kg of Atomoxetine decreased the number of inactive lever presses. Data are presented as means ± SEM. Statistical significance: *p<0.05, **p<0.01, ***p<0.001 vs vehicle independent of sex.

Experiment 2: Development and Validation of a Within-Trials Go/NoGo Model of Impulsivity.

Acquisition of active lever pressing (Phase1). Rats acquired active lever presses over time [$F(10, 140) = 26.3$; $p < 0.0001$; $\eta^2 = 0.473$]. Males and females showed a similar acquisition curve (sex [$F(1, 14) = 1.39$; $p = 0.26$; $\eta^2 = 0.022$], sex-by-time interaction [$F(10, 140) = 1.6$; $p = 0.11$; $\eta^2 = 0.029$]; **Figure 5A**).

Acquisition of Go-trial response (Phase 2-3). When self-administration shifted to the go-trial schedule, rats reduced the number of missed Go responses over time [$F(11, 154) = 1.94$; $p = 0.038$; $\eta^2 = 0.073$]. Males and females showed a similar level of missed Go ratio throughout training [$F(1, 14) = 0.06$; $p = 0.81$; $\eta^2 = 0.002$], with no significant effect over time [$F(11, 154) = 0.78$; $p = 0.65$; $\eta^2 = 0.029$] (**Figure 5B**).

Training to Pre-trial (Phase 3-4). When a pre-trial period was introduced, rats

performed a high number of premature responses that decreased over time [F(16, 224) = 22.31; $p < 0.0001$; $\eta^2 = 0.542$]. A similar level of premature responses was maintained between males and females throughout training [F(1, 14) = 3.71; p = 0.074; $\eta^2 = 0.020$], with no significant effect over time [F(16, 224) = 0.92; p = 0.54; 0.022] (**Figure 5C**).

Random within-trial Go/NoGo training (Phase 4). A three-way ANOVA of missed response ratios found an overall effect of time [F(11, 154) = 10.09; $p < 0.0001$; $\eta^2 = 0.124$], trial (Go vs. NoGo) [F(1, 14) = 17.07; p = 0.001; $\eta^2 = 0.117$], and a significant time-by-trial interaction [F(11, 154) = 4.33; $p < 0.0001$; $\eta^2 = 0.088$]. There was neither an overall effect of sex [F(1, 14) = 0.5; p = 0.48; $\eta^2 = 0.003$], nor a sex-by-time-by-trial interaction [F(11, 154) = 1.32; p = 0.22; $\eta^2 = 0.012$]. This is consistent with a higher number of missed NoGo trials compared to Go trials, independent of sex at the beginning of training, which, however, tended to reach the missed Go ratio level over time (**Figure 5D**).

Inactive lever presses were low throughout all four phases of training, but they tended to increase during *Phase 4* (**Figure S2**).

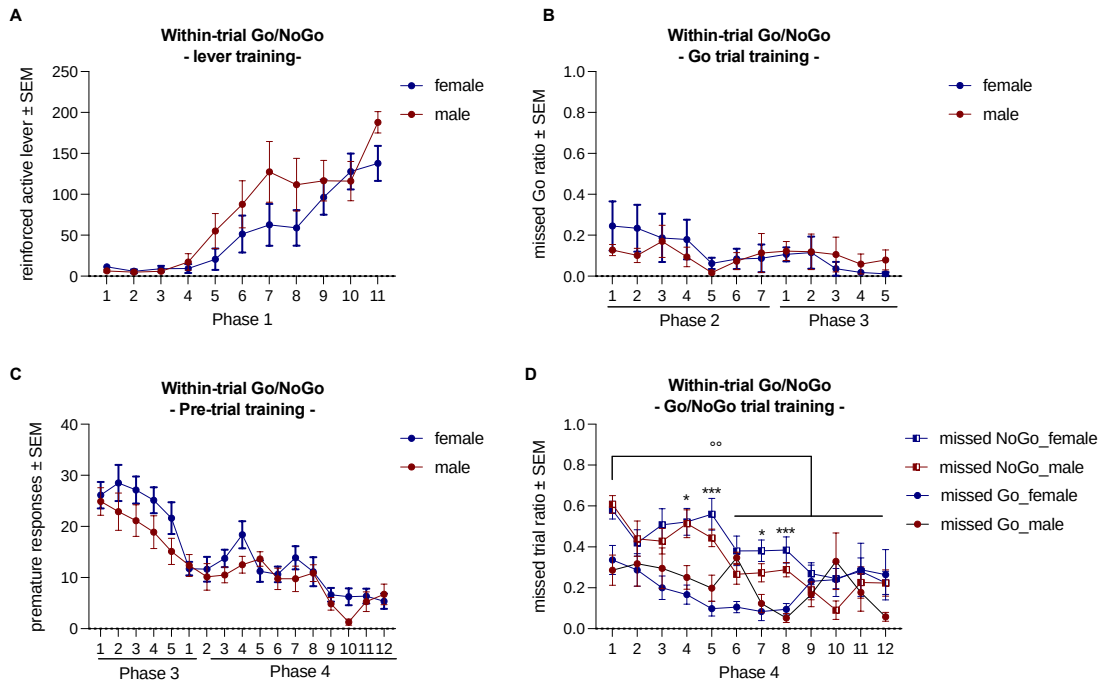

Figure 5. Development of the within-trial Go/NoGo model. A) During the first phase of training, rats acquired active lever pressing reinforced by 0.1 ml of sucrose solution. **B**) When Go-trials were introduced, missed go ratio was very low since the beginning. **C**) When the Pre-trial period was introduced, rats made a high number of premature responses that decreased over time. **D**) When rats were subjected to within-trial Go/NoGo trials, missed Go ratio increased compared to go-trial trainings. Missed NoGo ratio decreased over time, until missed Go and missed NoGo ratio were no longer statistically different. Data are presented as means ± SEM. Statistical significance: **A, C**) not shown; **D**) °°p<0.01 between time points indicated by

brackets independent of sex; *p<0.05 and ***p<0.001 between missed Go and missed NoGo independent of sex.

Model validation with atomoxetine. A two-way ANOVA of premature responses during the pre-trial period found an overall effect of atomoxetine dose [$F(2, 28) = 14.00$; $p < 0.0001$; $\eta^2 = 0.288$], no effect of sex [$F(1, 14) = 0.01$; $p = 0.92$; $\eta^2 = 2.955 \times 10^{-4}$], and no sex-by-dose interaction [$F(2, 28) = 0.27$; $p = 0.77$; $\eta^2 = 0.006$]. Dunnett's post-hoc analysis of the main dose effect indicated that both doses of atomoxetine significantly reduced the number of premature responses (**Figure 6A**). Male and female rats showed similar missed NoGo ratios [$F(1, 14) = 0.05$; $p = 0.82$; $\eta^2 = 0.002$], which were affected by atomoxetine [$F(2, 28) = 2.75$; $p = 0.001$; $\eta^2 = 0.205$], independently of sex [$F(2, 28) = 0.37$; $p = 0.69$; $\eta^2 = 0.009$]. Dunnett's post-hoc analysis of the main dose effect indicated that both doses of atomoxetine decreased the missed NoGo ratio (**Figure 6B**). The missed Go ratio was similar between sexes [$F(1, 14) = 0.12$; $p = 0.73$; $\eta^2 = 0.003$], and it was affected by atomoxetine [$F(2, 28) = 7.34$; $p = 0.003$; $\eta^2 = 0.246$], independently of sex [$F(2, 28) = 0.06$; $p = 0.94$; $\eta^2 = 0.002$]. Post-hoc analysis indicated that the highest dose of atomoxetine significantly increased the missed Go ratio. (**Figure 6C**). Similarly, analysis of inactive lever presses found an overall effect of dose [$F(2, 28) = 5.32$; $p = 0.01$; $\eta^2 = 0.184$], no effect of sex [$F(1, 14) = 2.97$; $p = 0.11$; $\eta^2 = 0.050$], and no sex-by-dose interaction [$F(2, 28) = 1.4$; $p = 0.26$; $\eta^2 = 0.048$]. Dunnett's post-hoc analysis of the main dose effect indicated that the highest dose of atomoxetine decreased inactive lever presses (**Figure 6D**).

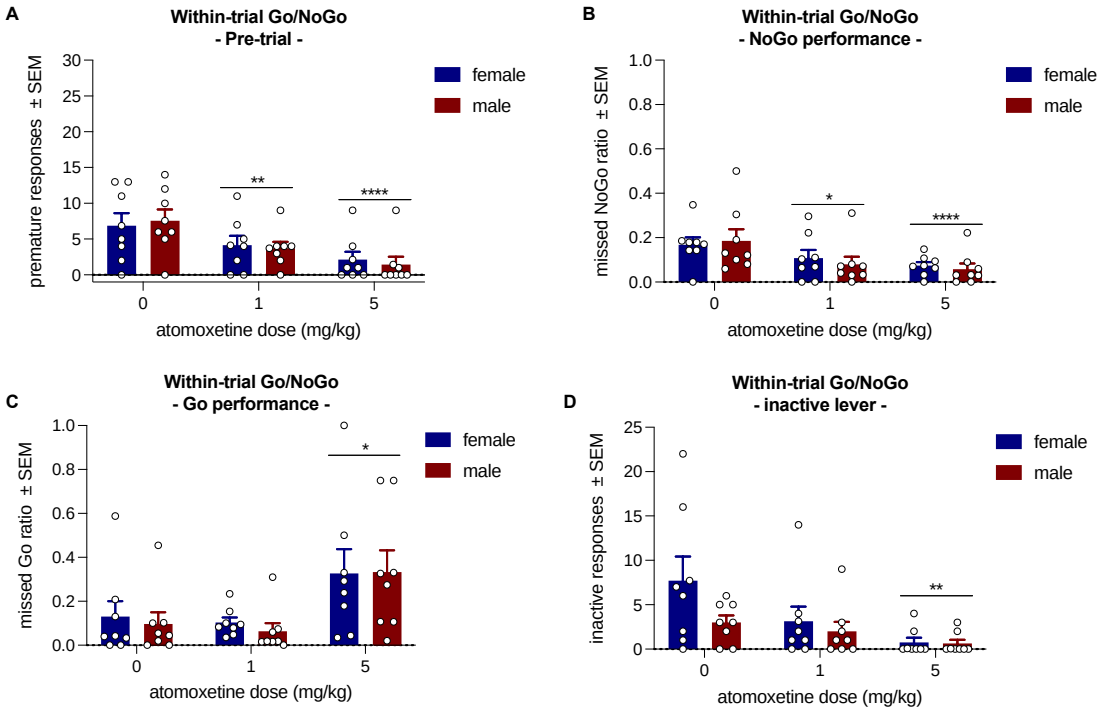

Figure 6. Effect of atomoxetine on within-trials Go/NoGo performance. A) Both doses of Atomoxetine decreased the number of premature responses during Pre-trials periods. **B**) Both

doses of Atomoxetine decreased missed NoGo ratio. **C**) The highest dose of Atomoxetine increased missed Go ratio. **D**) The highest dose of Atomoxetine decreased the number of inactive lever presses. Data are presented as means ± SEM. Statistical significance: *p<0.05, **p<0.01, ****p<0.0001 vs vehicle independent of sex.

In a control locomotor activity test, 5 mg/kg of atomoxetine reduced the distance traveled (**Figure S3**). This suggests that the effects observed at this dose could be secondary to a sedative effect of the drug. However, and more importantly, the 1 mg/kg dose of atomoxetine showed that premature responses and the missed NoGo ratio are valid indicators of impulsivity.

Therefore, we next compared the rats' performance in the two models. As no overall effect of sex was observed in the Go/NoGo performance of either models, males and females were pooled for this analysis. Premature responses (model [$F(1, 38) = 0.18$; $p = 0.67$; $\eta^2 = 0.002$], time [$F(11, 418) = 13.45$; $p < 0.0001$; $\eta^2 = 0.133$] time by model [$F(11, 418) = 2.88$; $p = 0.001$; $\eta^2 = 0.029$]; **Figure 7A**) and missed NoGo ratio (model [$F(1, 38) = 0.71$; $p = 0.4$; $\eta^2 = 0.009$], time [$F(11, 418) = 27.74$; $p < 0.0001$; $\eta^2 = 0.212$] time by model [$F(11, 418) = 2.46$; $p = 0.005$; $\eta^2 = 0.019$]; **Figure 7B**) did not differ between the two models. The time by model interaction observed in both analyses did not correspond to any difference between the two models on the same day. Conversely, missed Go ratio changed over time [$F(11, 418) = 2.90$; $p = 0.001$; $\eta^2 = 0.035$], and it was higher in the within-trial model [$F(1, 38) = 83.06$; $p < 0.0001$; $\eta^2 = 0.309$] in all time points except two [$F(11, 418) = 4.11$; $p < 0.0001$; $\eta^2 = 0.050$] (**Figure 7C**). Inactive lever presses were not affected by model [$F(1, 38) = 1.81$; $p = 0.19$; ; $\eta^2 = 0.024$], time [$F(11, 418) = 0.36$; $p = 0.97$; ; $\eta^2 = 0.004$] or their interaction [$F(11, 418) = 1.68$; $p = 0.075$; $\eta^2 = 0.019$]; **Figure 7D**).

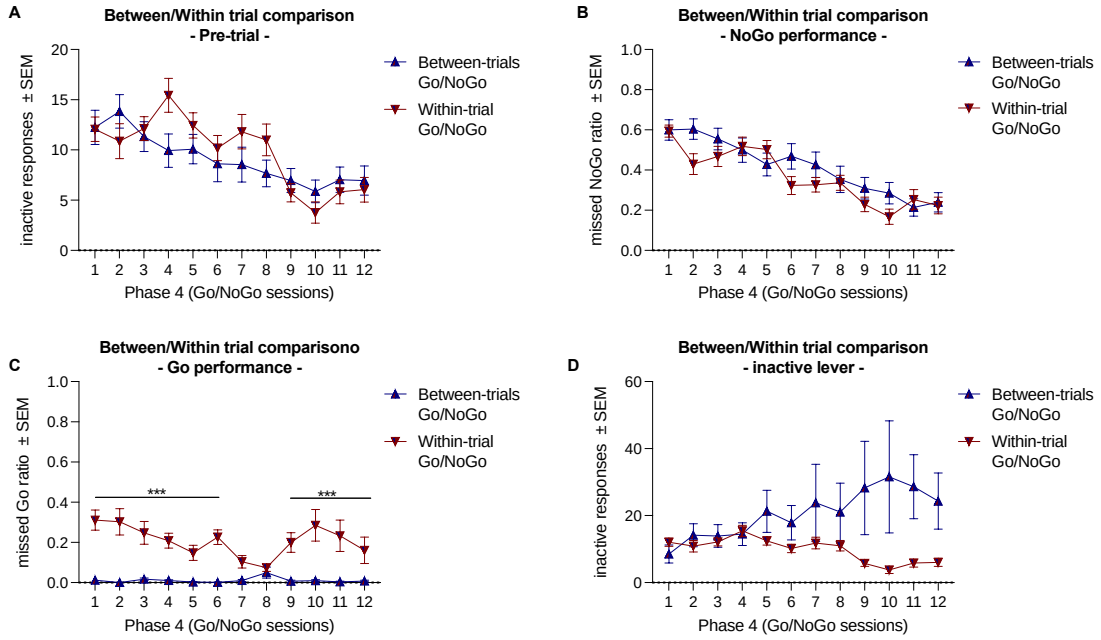

Figure 7. Comparison of between-trials and within-trials Go/NoGo model performance. **A**) Premature responses were similar between the two models. **B**) Missed NoGo ratio was similar between the two models. **C**) Missed Go ratio was higher in the within-trial model except on day 7 and 8. **D**) Inactive lever presses were similar between the two models. Data are presented as means ± SEM. Statistical significance: **A - B**) not shown; **D**) ***p<0.001 within-trial vs between-trials on the same day.

These results indicated that the two models are equivalent in terms of premature and NoGo responses. However, the within-trial Go/NoGo model induces a higher number of mistakes in the Go response, and the non-sedative dose of atomoxetine reduced premature responses only in the within-trial Go/NoGo model. Therefore, we chose the within-trial Go/NoGo model to characterize impulsive behavior in msP rats in the next experiment

Experiment 3: Comparison of Wistar vs msP Performance in the Within-Trials Go/NoGo Model.

Number of premature responses did not differ between male and female [$F(1, 23) = 0.62$; $p = 0.44$; $\eta^2 = 0.026$], and Wistar and msP [$F(1, 23) = 0.006$; $p = 0.94$, $\eta^2 = 2.436 \times 10^{-4}$], rats. Strain by sex interaction was also not significant [$F(1, 23) = 0.015$; $p = 0.9$; $\eta^2 = 6.418 \times 10^{-4}$] (**Figure 8A**). MsP showed overall higher missed NoGo ratio than Wistars [$F(1, 23) = 5.53$; $p = 0.028$; $\eta^2 = 0.187$], but there was no sex [$F(1, 23) = 0.58$; $p = 0.45$; $\eta^2 = 0.020$] and sex by strain interaction [$F(1, 23) = 0.58$; $p = 0.52$; $\eta^2 = 0.014$] (**Figure 8B**). Missed Go ratio significantly differed between sex [$F(1, 23) = 12.62$; $p = 0.002$; $\eta^2 = 0.278$], and strain [$F(1, 23) = 5.75$; $p = 0.025$; $\eta^2 = 0.127$]. Strain by sex interaction approached but did not reach significance threshold [$F(1, 23) = 4.05$; $p = 0.056$; $\eta^2 = 0.089$]. Post-hoc analysis indicated that male msP rats showed higher missed

Go ratio than male Wistars and female msPs.

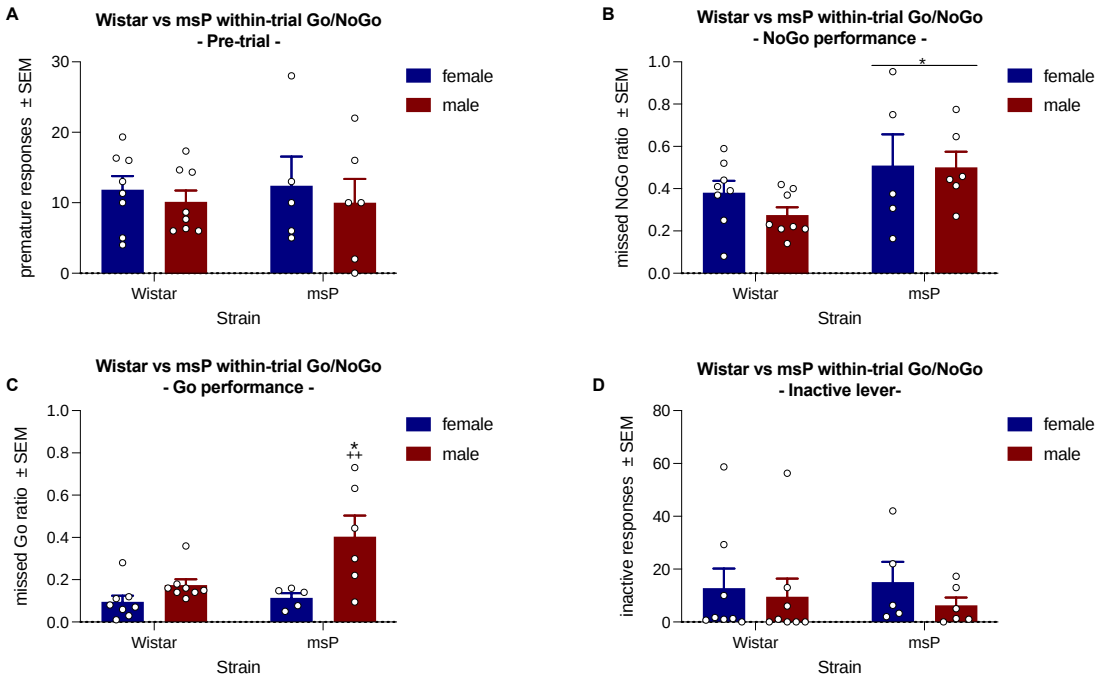

Figure 8. Comparison of msP and wistar performance in the within-trial Go/NoGo model. **A**) Male and female Wistar and msP rats showed similar premature responses independent of sex. **B**) Both male and female msP rats showed higher missed NoGo ratio than Wistars. **C**) Male msP rats showed higher missed Go ratio compared to male Wistars and female msPs. **D**) Inactive lever presses were similar between strains and sexes. Data are presented as means ± SEM. Statistical significance: * $p<0.05$ vs Wistars same sex, $^{++}p<0.01$ vs female msP.

These data indicated that msP rats exhibited higher impulsive-like behavior than Wistar rats. To explore whether this trait may contribute to the high alcohol preference of this strain, we tested the effect of atomoxetine on alcohol self-administration in msP rats in the next experiment.

Experiment 4: Effect of Atomoxetine on Alcohol Self-Administration in MsP Rats

When the effect of atomoxetine on alcohol self-administration was analyzed, a two-way ANOVA revealed an overall effect of atomoxetine dose [$F(2, 26) = 12.63$; $p = 0.0001$; $\eta^2 = 0.309$]. There was no overall effect of sex [$F(1, 13) = 1.15$; $p = 0.3$; $\eta^2 = 0.023$], but a significant dose × sex interaction [$F(2, 26) = 3.69$; $p = 0.039$; $\eta^2 = 0.090$]. Dunnett's post-hoc test indicated that both doses of atomoxetine decreased alcohol rewards earned by male rats, while only the lowest dose was effective in females (**Figure 9A**). Inactive lever responses were always low and unaffected by atomoxetine treatment (dose [$F(2, 26) = 1.42$; $p = 0.26$; $\eta^2 = 0.063$], sex [$F(1,13) = 0.42$; $p = 0.53$; $\eta^2 = 0.009$], interaction [$F(2, 26) = 1.62$; $p = 0.22$; $\eta^2 = 0.072$]; **Figure 9B**).

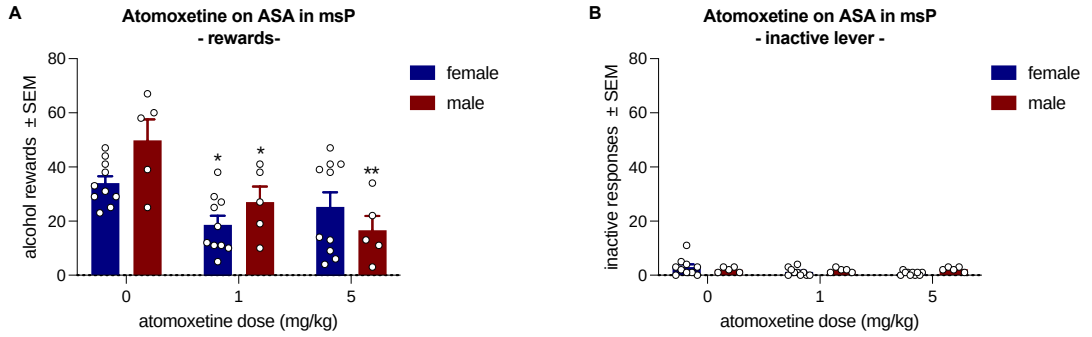

Figure 9. Effect of atomoxetine on alcohol self-administration by msP rats. A) The number of 10% alcohol rewards earned was reduced by both doses of Atomoxetine in male rats, and by the 1 mg/kg dose in females. **B**) Inactive lever response rate was not altered by atomoxetine treatment. Data are presented as means ± SEM. Statistical significance: * p<0.05 and **p<0.01 vs vehicle dose, same sex.

DISCUSSION

In this study, we developed two Go/NoGo models of impulsivity and began to explore its role in alcohol preference in msP rats. Specifically, we designed a between-trials and a within-trial Go/NoGo model.

Atomoxetine validation indicated that in both models premature responses and missed NoGo ratio are predictive of impulsive -like behavior. This is in line with the view according to which impulsivity in the Go/NoGo task is typically linked to failures to withhold responses in NoGo trials (Carter & Shieh, 2015),

We also observed an increase in missed Go ratio, which however occurred at a dose of atomoxetine that resulted to be sedative. Indeed, rats treated with 5 mg/kg atomoxetine exhibited significantly reduced locomotor activity compared to the vehicle group. The observed increase in missed Go responses at sedative doses suggests that this effect may stem from reduced motor activity or arousal rather than increased impulsivity. That is, atomoxetine-induced sedation may underlie the increased missed Go responses rather than a true deficit in response inhibition.

Compared to the between-trials model, the within-trial model showed a higher missed Go ratio. This could stem from a higher complexity of the model, which therefore might capture cognitive aspects of behavior beside pure motor impulsivity. Therefore, we selected the within-trial Go/NoGo model for further experiments.

Compared to Wistar rats, msP rats exhibited a higher missed NoGo ratio in both sexes, suggesting increased impulsivity in this line. We also observed an increased missed Go ratio in male msP rats, which may result from attentional impairment. Additionally, the sex-specific increase in missed Go responses in male msP rats, but not females,

highlights potential differences in the underlying mechanisms driving alcohol-seeking behavior between sexes.

Next, we explored whether the impulsive trait observed in msP contribute to alcohol self-administration in this line. To investigate this link, we hypothesized that in this case atomoxetine, a noradrenaline reuptake inhibitor known to reduce impulsivity, would reduce alcohol-seeking behavior in msP rats. As expected, atomoxetine reduced alcohol reward in both sexes at a 1 nmole dose but only in males at a 5 nmole dose. These results suggest that impulsivity may contribute to changes in alcohol consumption in msP rats.

The differential dose-response effect of atomoxetine on alcohol-seeking behavior in male and female msP rats suggests significant sex-dependent neurobiological mechanisms.

1. Atomoxetine's impact at higher doses varies between sexes. In male msP rats, the 5 mg/kg dose continued to reduce alcohol intake, indicating a dose-dependent effect where increased noradrenaline levels further suppress consumption. In contrast, this dose had no effect on females, indicating a leftward shift of dose-response curve in this sex.
2. The sex differences in atomoxetine's dose-response suggest that male and female msP rats have distinct noradrenergic sensitivity or baseline activity during impulsivity and alcohol-seeking behavior.

In short, these findings indicate that noradrenergic regulation of alcohol-seeking behavior differs between sexes, potentially due to stress-related mechanisms, baseline neurochemical differences, or distinct impulsivity-alcohol relationships. This underscores the importance of considering sex as a biological variable in AUD research.

Study Limitations and Perspectives

We have demonstrated that premature responses during pre-trials and missed NoGo ratio reflect impulsive behavior. However, our results on missed Go responses may reflect cognitive aspects also modelled by our within-trial model. Understanding how reward-paired cues influence different types of impulsive behavior across contexts could provide valuable insights (Winstanley et al., 2010).

It is crucial to examine the factors driving impulsive behavior across different impulse-control assessments, especially when responses are triggered by external cues. For example, deficits in inhibitory function in the Stop-Signal Reaction Time Task (SSRTT) and Go/NoGo task may stem from impaired processing of stop/no-go signals. Conversely, premature responding in the 5CSRT is considered self-generated, as it lacks an explicit inhibitory signal (Winstanley et al., 2010).

Since our study did not distinguish between these impulsivity subtypes, future research should employ behavioral paradigms that separately measure impulsive action (e.g., 5CSRTT) and cognitive impulsivity (e.g., Delay Discounting Task). This approach may help clarify the distinct neurobiological underpinnings of impulsivity in alcohol-seeking behavior.

Overall, we have developed two models of impulsivity and demonstrated that msP rats show impulsive-like behavior compared to Wistar controls, which may contributes to their motivation for alcohol.

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SUPPLEMENTARY INFORMATION TO CHAPTER 3

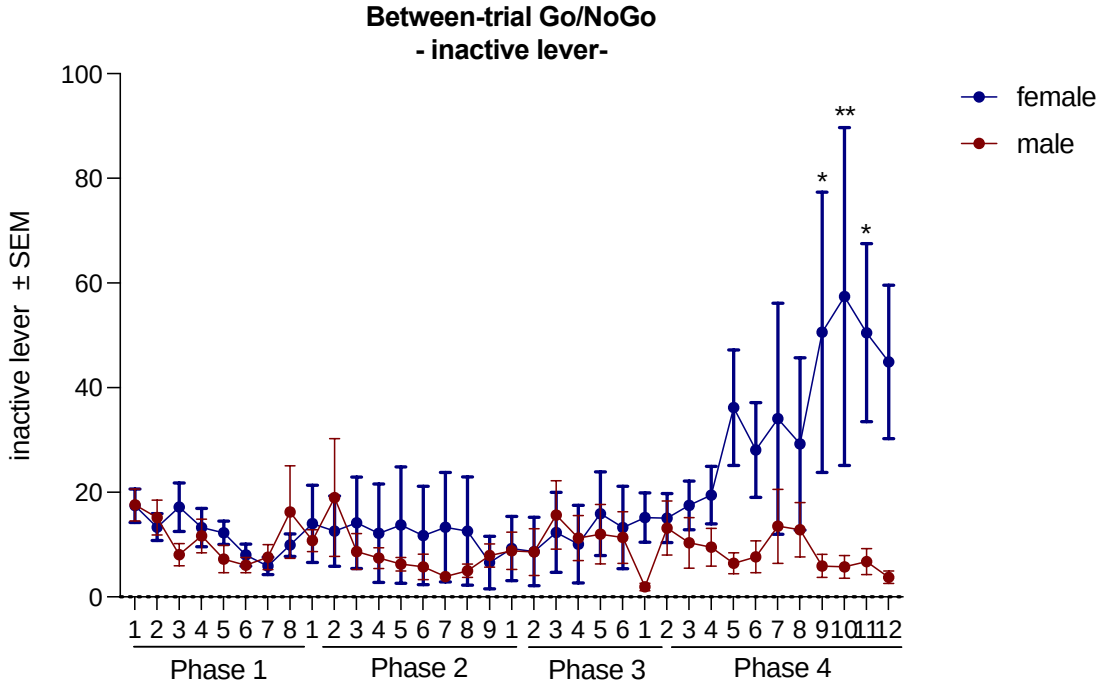

Figure S1. Analyses of inactive lever responses during the four phases of the Between-trials Go/NoGo model found an overall effect of time [$F(34, 748) = 1.5$; $p = 0.032$; $\eta^2 = 0.041$], no effect of sex [$F(1, 22) = 2.84$; $p = 0.11$; $\eta^2 = 0.046$], and a significant sex-by-time interaction [$F(34, 748) = 2.01$; $p = 0.00072$; $\eta^2 = 0.049$]. Data are presented as means ± SEM. Statistical significance: *$p<0.05$ and **$p<0.001$ vs day 1 of phase 1

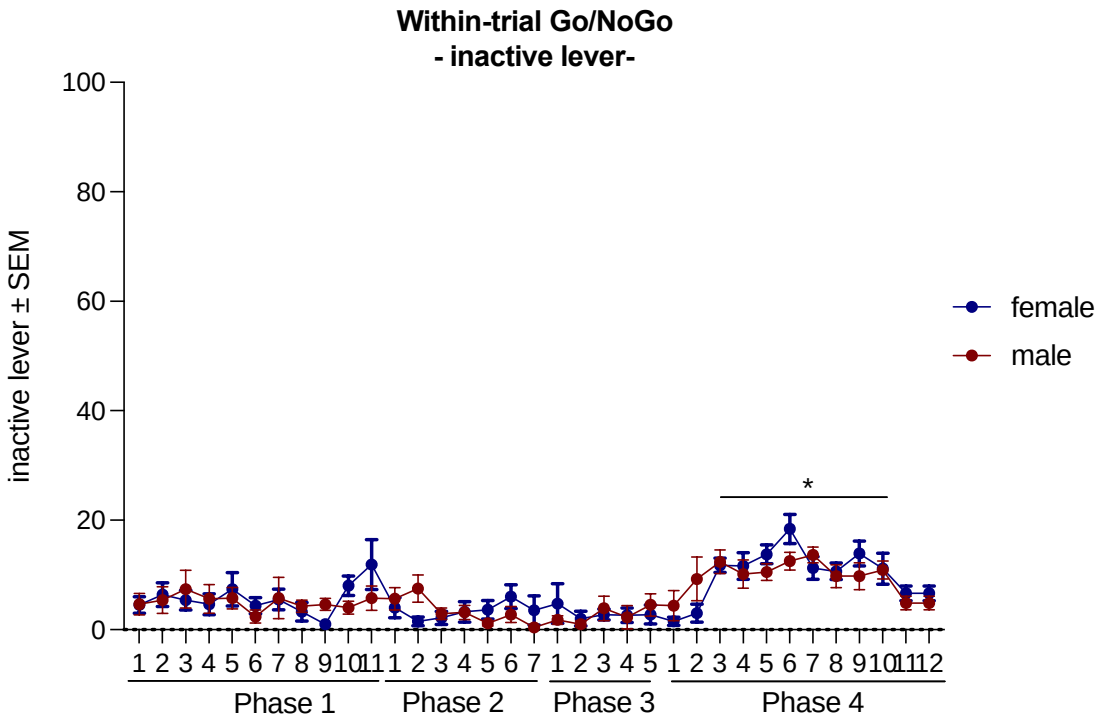

Figure S2. Analyses of inactive lever responses during the four phases of the Within-trials Go/NoGo model found an overall effect of time [$F(34, 476) = 7.238$; $p < 0.0001$; $\eta^2 = 0.297$], no effect of sex [$F(1, 14) = 0.2$; $p = 0.66$; $\eta^2 = 0.001$], and no significant sex-by-time interaction [$F(34, 476) = 1.\ 1$; $p = 0.32$; $\eta^2 = 0.045$]. Data are presented as means ± SEM. Statistical significance: *p<0.05 vs day 1 of phase 1 independent of sex.

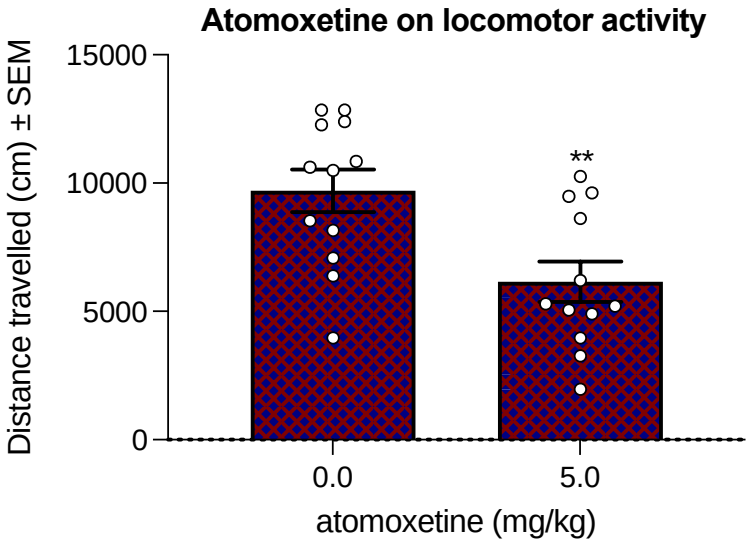

Figure S3. Effect of 5 mg/kg atomoxetine on 60-minutes locomotor activity. Wistar rats (n=12/group) were treated with 5 mg/kg or its vehicle and, 30 minutes later, placed in an open field arena. Rats were allowed to explore the arena for 1hour. Rats treated with atomoxetine covered a significantly shorter distance than vehicle. Data are presented as means ± SEM. Statistical significance: **p<0.01 vs 0.0.

CHAPTER 4

EFFECT OF NPS ON IMPULSIVE BEHAVIOR IN WISTAR AND MSP RATS

ABSTRACT

Background: The NPSR1 A/T polymorphism (Asn107Ile) has been associated with impulsivity, though the precise role of NPS in this regard remains unclear. This chapter aims to explore the impact of NPS on impulsivity and the role of NPS receptor signaling pathways.

Methods: First, we tested the effect of NPS on impulsivity using the within-trial Go/NoGo model in Wistar and msP rats. We then examined the effect of the tetrapeptide RTI263 on the same model in Wistar rats to investigate the role of NPS receptor signaling pathways in impulsivity.

Results: NPS treatment in the within-trial Go/NoGo model decreased premature and missed NoGo ratios and increased missed Go ratios in both Wistar and msP rats. In Wistar rats, RTI263 showed weaker or less consistent effects compared to NPS.

Conclusions: Our study reveals a novel anti-impulsive function of NPS and highlights a potential mechanism linking impulsivity and NPS. However, additional analyses are needed to assess sex differences and explore a wider dose range of RTI263, as 10 nmoles showed weak effects. This will help clarify the role of NPS receptor signaling pathways in impulsivity.

INTRODUCTION

Impulsivity is a multifaceted behavior, generally defined as a tendency toward premature, poorly planned, unduly risky, or inappropriate actions in response to internal or external stimuli, without regard for the negative consequences to oneself or others, often leading to maladaptive outcomes (Daruma & Barnes, 1993; McCown et al., 1993; Moeller et al., 2001; Bakhshani, 2014; Hamilton et al., 2015). The contribution of various brain regions, circuits, neurotransmitter systems, and genes results in different manifestations of impulsive behavior, as discussed in Chapter 3.

Laas and colleagues investigated the functional polymorphism of the NPSR1 gene, Asn107Ile (rs324981, A > T), in two cohorts from the Estonian Longitudinal Study: a younger cohort and an older cohort (Laas et al., 2014). They concluded that the NPSR1 A/T polymorphism is associated with impulsivity. The NPSR1 AA genotype was found to have a beneficial effect on impulsivity, particularly in males and within a favorable family environment. Conversely, the NPSR1 TT genotype had a detrimental effect,

especially in females. This suggests that T-allele carriers of the NPSR1 rs324981 polymorphism exhibit increased impulsivity traits, particularly in males (Laas et al., 2014). Given that individuals with the NPSR1 A/T polymorphism carrying the T allele show poor impulse control, the role of NPS in impulsivity remains unclear. Consequently, in this chapter, we test the role of NPS in impulsivity using the within-trial Go/NoGo model developed in chapter 3 as primary model of impulsivity.

Aim of This Chapter

As demonstrated in Chapter 3, msP rats exhibit higher impulsivity traits than Wistar rats. Here, we tested the effect of NPS on both Wistar and msP rats, considering the msP line as a genetically predisposed, high-impulsivity rat model, and the Wistar rats as their non-impulsive parental control. Additionally, since NPS has both anxiolytic and stimulatory effects, we tested whether both these traits are necessary for NPS's anti-impulsive properties.

Clark and colleagues demonstrated that NPS activates both cAMP and calcium-mediated NPSR signaling to simultaneously produce pro-arousal and anxiolytic effects (Clark et al., 2017). Their experiment showed that the cAMP-biased NPSR agonist RTI263 produced anxiolytic and learning effects similar to NPS but did not significantly enhance locomotor activity (Clark et al., 2017).

Therefore, we tested the effect of RTI263 on the within-trial Go/NoGo model of impulsivity in Wistar rats in order to explore the role of NPS receptor signaling pathways in impulsivity. Since RTI263 mimics some effects of NPS, such as anxiolysis and learning enhancement, without substantially increasing locomotor activity, it offers a method to distinguish the behavioral effects of NPSR activation. By testing RTI263 in the Go/NoGo model of impulsivity, we aim to determine whether impulsivity is more closely associated with cAMP-mediated signaling (which RTI263 may preferentially activate) or whether it requires full NPS-induced receptor activity, including calcium-mediated pathways. This could provide valuable insights into the neurochemical basis of impulsivity and inform the development of targeted therapies for disorders associated with impulsive behavior.

MATERIALS AND METHODS

Animals

Wistar and msP rats, bred at the University of Camerino, were 9 weeks old at the start of experimental procedures. The rats were housed in groups of four in a room with an artificial 12 h/12 h light/dark cycle (lights off at 8:00 am), constant temperature (20-22°C) and humidity (45-55%). Food (4RF18, Mucedola, Italy) and tap water were available *ad libitum*. All procedures were conducted during the dark phase of the

light/dark cycle and adhered to the *European Council Directive for the Care and Use of Laboratory Animals* and the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Surgeries

Rats were surgically implanted with an intracerebroventricular (ICV) cannula under isoflurane anaesthesia (1.5-2.5%). Guide cannulae were stereotaxically implanted and cemented to the skull. Coordinates referred to bregma were as follows: antero-posterior: -1.0 mm; medio-lateral: -1.8 mm; dorso-ventral: 2.0. To prevent postoperative pain, rats were treated subcutaneously with 0.1 ml of meloxicam (5 mg/ml). Surgeries were followed by a one-week recovery period, during which rats were left undisturbed in their home cages. The antibiotic Enrofloxacin (Baytril ®) was diluted in the drinking water (50 mg/100ml) for five days following surgery.

Cannula placement was verified before experiments by ICV injection of 100 ng of angiotensin II; only animals showing a clear dipsogenic response (consumption of at least 5 ml of water within 5 minutes) were used for further experimentation.

Drug Injection

NPS was a generous gift from Prof. R. Guerrini (University of Ferrara, Italy). RTI263 was a generous gift from Prof. Stewart Clark (University of Ferrara, Italy). Drugs were dissolved in sterile isotonic saline and administered ICV in a volume of 1 µl/rat using a stainless-steel injector 2.5 mm longer than the guide cannula, connected to a 10-µL Hamilton syringe. Once the injector was inserted into the guide cannula, the drug solution (or vehicle) was infused by gently pressing the syringe's plunger. After infusion, the injector was left in place for 10 seconds before removal to prevent fluid backflow.

Operant Training of Within-Trials Go/NoGo Model of Impulsivity.

The within-trial Go/NoGo model developed and described in Chapter 3 was used for this study.

Operant training consisted of four phases:

Phase 1; Self-administration training. The right and left levers were designated as active and inactive, respectively. Rats were trained to self-administer 0.1 mL of a 10% (w/v) sucrose solution in daily 30-minute sessions according to a fixed ratio 1 (FR1) schedule of reinforcement on the active lever. The cue lights above the active levers remained on throughout the session.

Phase 2; Go-Trial training. After self-administration SA training, they entered the Go Trial Training Phase. Session duration was reduced to 20 minutes and consisted of 20-second Go Trials spaced out by Inter-Trial-Intervals (ITI). Go Trials were identical to Phase 1: the levers were extracted, the active cue light - functioning as Go-signal - illuminated, and FR1 presses on the active lever were reinforced with 0.1 ml of 10% sucrose. A Go Trial ended, and the ITI began, if either a reward was delivered or 20 seconds elapsed before the rat pressed the active lever. In the first case, the trial was recorded as a "Correct-Go" trial, and in the second case, as a "Missed-Go" trial. During the ITI, the levers were retracted, and no cue was presented. The ITI lasted 5 seconds (short) if it followed a Correct-Go trial and 20 seconds (long) if it followed a Missed-Go trial; the longer ITI functioned as punishment for the missed response.

Phase 3; Introduction of a Pre-trial period. In this phase, a 3-second Pre-Trial period preceding the Go-Trial was introduced. During Pre-trials, levers were extracted, and the house light on the opposite wall was illuminated. If the rat did not press the active lever, the Pre-Trial was followed immediately by a Go Trial, as described in Phase 2. If the rat pressed the active lever during the Pre-Trial, the levers were retracted, and a long ITI began. Active lever presses during Pre-Trials were recorded as "Premature Responses."

Phase 4; Full within-trial Go-NoGo model. Each trial started with a Pre-trial as described in Phase 3, which — unless a rat produced a Premature Response — was randomly (50:50) followed by either a Go-NoGo trial or a NoGo-Go trial. Specifically, each trial included both a 5-second Go phase and a 15-second NoGo phase. Go-NoGo trials started with a 5-second Go phase, during which the rat could press the active lever to obtain a reward. If the rat did not press the active lever, the trial would move into the 15-second NoGo phase, during which the rat had to abstain from pressing the active lever to obtain the reward. In NoGo-Go trials, the order of Go and NoGo phases was inverted. Pressing the active lever during a Go phase and abstaining from it during a NoGo phase were recorded as a "correct response". Pressing the active lever during a NoGo phase and failing to press it during a Go phase were recorded as a "wrong response".

Premature, Go, and NoGo cueing were identical to the between-trials GoNoGo model described above.

This model yielded the following possible outcomes:

a- Pre-trial → active lever pressed → "Premature response" recorded → rat punished with long ITI
b- Pre-trial → active lever not pressed → random Go-NoGo / NoGo-Go trial begin

c- Go-NoGo (Go phase)→ active lever pressed → "correct Go" recorded →

sucrose reward followed by short ITI.

d- Go-NoGo (Go phase)→ active lever not pressed → "wrong Go" recorded → NoGo phase begin

e- Go-NoGo (NoGo phase)→ active lever not pressed → "correct NoGo" recorded → sucrose reward followed by short ITI.

f- Go-NoGo (NoGo phase)→ active lever pressed → "wrong NoGo" recorded → rat punished with long ITI

g- NoGo-Go (NoGo phase)→ active lever pressed → "wrong NoGo" recorded → rat punished with long ITI

h- NoGo-Go (NoGo phase)→ active lever not pressed → "correct NoGo" recorded → Go phase begin

i- NoGo-Go (Go phase)→ active lever not pressed → "wrong Go" recorded → rat punished with long ITI

j- NoGo-Go (Go phase)→ active lever pressed → "correct NoGo" recorded → sucrose reward followed by short ITI.

The within-trial Go/NoGo workflow is schematized in **Figure 1**

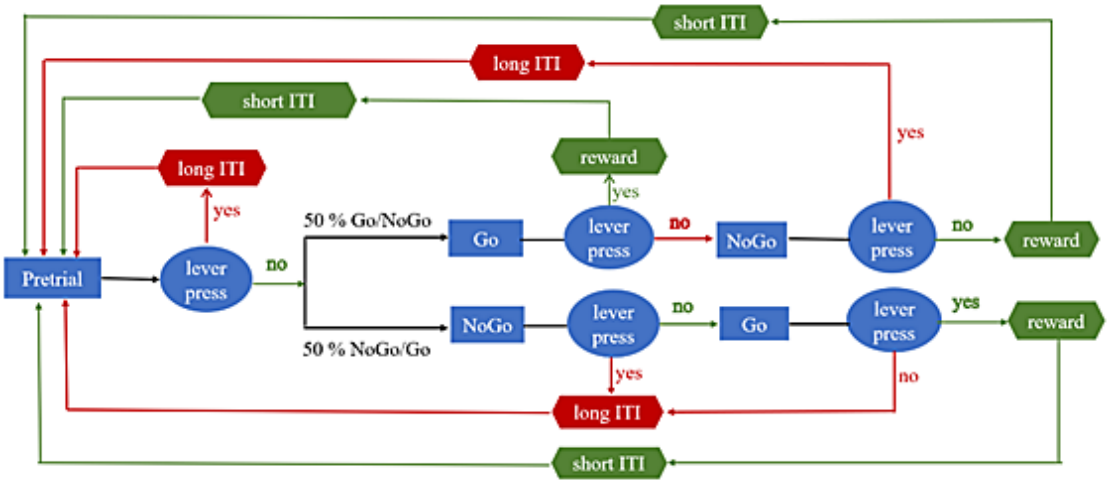

Figure 1. Within-trial Go/NoGo workflow.

Based on these outcomes, we extrapolated three behavioral variables:

1. number of premature responses (total outcome a)
2. missed NoGo ratio = total wrong NoGo / total NoGo phase = (f + g) / (e+f+g+h)
3. missed Go ratio = total wrong Go / total Go phase = (d+i) / (c+d+i+j)

Importantly, "missed ratio" measures are sufficient to fully describe animal behavior,

as they correspond to “1- correct ratio” measures.

The inactive lever was presented and retracted contingently with the active lever. Inactive responding had no scheduled consequences and was recorded in every phase.

EXPERIMENTAL PROCEDURES

Experiment 1: Effect of NPS on Within-Trials Go/NoGo Performance in Wistar and MsP Rats.

Thirteen Wistar (6♀+ 7♂) and 9 msP (4♀+ 5♂) rats were trained to self-administer sucrose according to the Within-Trials Go/NoGo model of impulsivity described above.

Rats were implanted ICV after eight sessions into the *Phase 4* of training. Sucrose self-administration was re-baselined before treatment started. Rats received ICV saline injections for two consecutive days to acclimate them to the treatment procedures. On test days, rats received ICV injection of NPS (0.1, 1.0 or 2.0 nmol/rat) or its vehicle 5 minutes before session. Treatment doses were delivered in a within-subjects counterbalanced order; test sessions were repeated every fourth day until each rat had received all doses. The first day after treatment rats remained in their home cages, the second and third day they underwent ASA baseline sessions.

The number of premature responses, Missed NoGo ratio, and Missed Go ratio were analyzed.

Experiment 2: Effect of RTI263 on Within-Trials Go/NoGo Performance in Wistar Rats.

Fourteen Wistar rats (6♀+ 8♂) were trained to self-administer sucrose according to the Within-Trial Go/NoGo model of impulsivity. This test was identical to Experiment 1 except that rats were treated with the tetrapeptide RTI263 (1.0 or 10.0 nmol/ICV), the reference compound NPS (2 nmol dose), and their vehicle.

STATISTICAL ANALYSIS

Samples size was based on our previous works on NPS effects on ASA and anxiety (Cannella et al. 2016; Cannella et al, 2009).

Due to the low number of subjects available in each sex, male and female data were pooled for analyses. Data were analyzed using appropriate between- and within-subjects ANOVAs. The approximation to normality of the distributions was verified using Q-Q plots of the residuals before conducting the tests. Statistical significance was set at the conventional threshold of $p < 0.05$. ANOVA were followed by Dunnett’s post-

hoc for treatment vs. control comparisons or Tukey's post-hoc for pairwise comparisons when appropriate.

RESULTS

Experiment 1: Effect of NPS on Within-Trials Go/NoGo Performance in Wistar and MsP rats.

Two-way ANOVA of premature responses found an overall effect of rat strain [$F(1, 20) = 14.48$; $p = 0.0011$; $\eta^2 = 0.137$] and NPS dose [$F(3, 60) = 8.09$; $p = 0.0001$; $\eta^2 = 0.146$], but no dose-by-strain interaction [$F(3, 60) = 0.17$; $p = 0.91$; $\eta^2 = 0.003$]. Dunnett's post-hoc analysis of main dose effect (i.e. strain pooled) revealed that 1 and 2 nmol of NPS decreased premature responses (**Figure 2A**).

Analysis of missed NoGo ratio found an overall effect of NPS doses [$F(3, 60) = 5.84$; $p = 0.0035$; $\eta^2 = 0.033$] and strain [$F(1, 20) = 16.78$; $p = 0.0006$; $\eta^2 = 0.270$], but no strain by dose interaction [$F(3, 60) = 0.61$; $p = 0.61$; $\eta^2 = 0.003$]. Dunnett's post-hoc analysis of main dose effect revealed that 1 and 2 nmol of NPS decreased missed NoGo ratio (**Figure 2B**).

Analysis of missed Go ratio found an overall effect of NPS doses [$F(3, 60) = 3.84$; $p = 0.014$; $\eta^2 = 0.109$] but no effect of strain [$F(1, 20) = 0.17$; $p = 0.68$; $\eta^2 = 0.002$] or strain by dose interaction [$F(3, 60) = 1.29$; $p = 0.28$; $\eta^2 = 0.0037$]. Dunnett's post-hoc analysis of main dose effect revealed that 1 and 2 nmol of NPS increased missed Go ratio (**Figure 2C**).

Response to the inactive lever was not affected by NPS [$F(3, 60) = 0.56$; $p = 0.64$; $\eta^2 = 0.020$], strain [$F(1, 20) = 0.05$; $p = 0.82$; $\eta^2 = 0.006$], or their interaction [$F(3, 60) = 0.58$; $p = 0.63$; $\eta^2 = 0.021$] (**Figure 2D**).

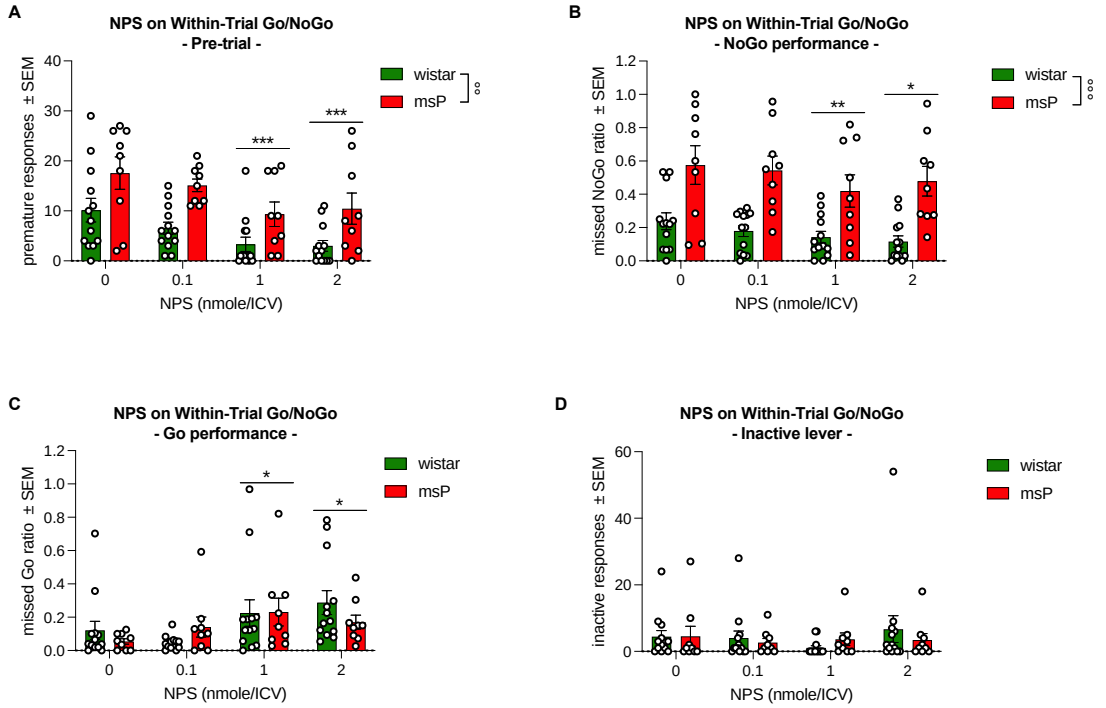

Figure 2. Effect of NPS on Within-trials Go/NoGo performance in Wistar and msP rats. A) 1 and 2 nmol/ICV of NPS decreased premature responses in both rat strains. MsP showed higher number of premature responses than Wistars. **B**) 1 and 2 nmol/ICV of NPS decreased missed NoGo ratio in both rat strains. MsP showed higher missed NoGo ratio than Wistars. **C**) 1 and 2 nmol/ICV of NPS increased missed Go ratio in both rat strains. **D**) NPS did not affect inactive lever responses. Data are presented as means ± SEM. Statistical significance: ***p<0.0001, **p<0.01 and *p<0.05 vs vehicle dose independent of strain; °°p<0.01 and °°°p<0.0001 between strains.

The results on premature response and missed NoGo ratio indicated that NPS showed anti-impulsive effects (see predictive validity of the model in Chapter 3). However, we also observed an increase in missed Go ratio. Although in chapter 3 we demonstrated that this measure is not predictive of impulsive behavior, we run a set of secondary analyses to explore the possible causes of this results. First of all, as NPS can decrease food intake (Cifani et al. 2011) (Fedeli et al. 2009), we verified that the effect was not secondary to a general decrease in sucrose self-administration. ANOVA of rewards earned indicated an overall effect of strain [$F(1, 20) = 3.5$; $p<0.0001$; $\eta^2 = 0.175$], no effect of dose [$F(3, 60) = 0.93$; $p = 0.43$; $\eta^2 = 0.122$], but a significant dose by strain interaction [$F(3, 60) = 4.28$; $p = 0.0084$; $\eta^2 = 0.037$]. Dunnett's post-hoc analysis indicated that 0.1 nmol of NPS increased the number of sucrose rewards in Wistar rats (**Figure 3**).

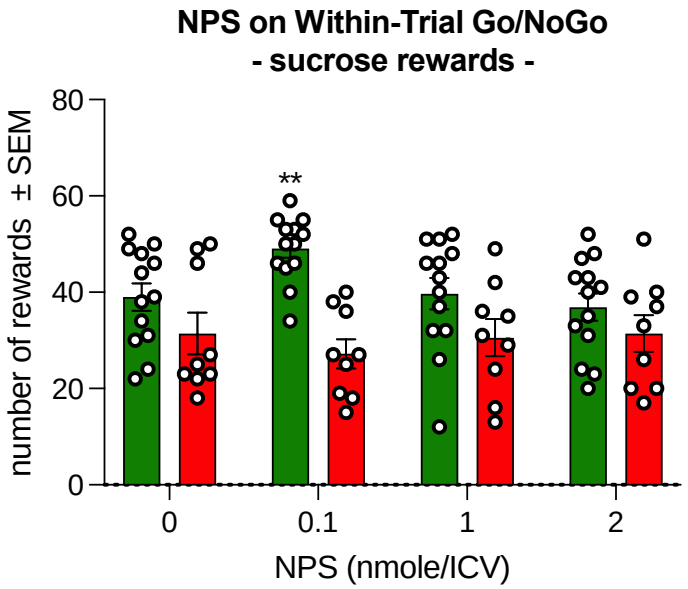

Figure 3. Effect of NPS on the sucrose rewards earned in the Within-trial Go/NoGo model in Wistar and msP rats. 0.1 nmol/ICV of NPS increased the number of sucrose rewards earned in Wistar rats. Data are presented as means ± SEM. Statistical significance: **p<0.01 vs vehicle dose.

Next, to further explore the possibility that the increase in missed Go ratio was secondary to an earlier satiation induced by NPS, we analyzed data expressed in 5-minute time bins. Three-way ANOVA confirmed an overall effect of doses [$F(3, 60) = 3.07$; $p = 0.034$; $\eta^2 = 0.025$] that however did not interact with time bins [$F(9, 180) = 0.73$; $p = 0.68$; $\eta^2 = 0.012$] (time bin [$F(3, 60) = 2.8$; $p = 0.048$; $\eta^2 = 0.018$], strain [$F(1, 20) = 0.41$; $p = 0.53$; $\eta^2 = 0.005$], dose by time bin by strain [$F(9, 180) = 0.55$; $p = 0.83$; $\eta^2 = 0.01$]; **Figure 4**).

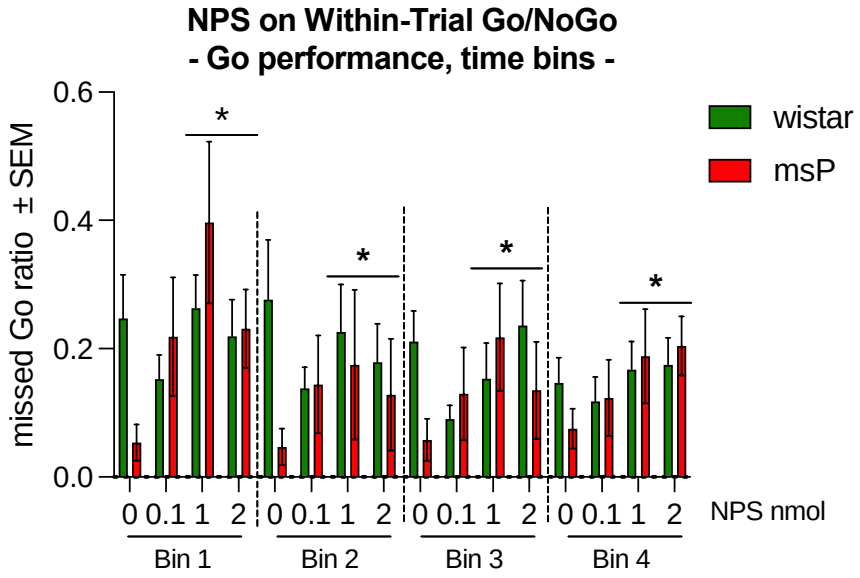

Figure 4. Effect of NPS on the Go performance analyzed in 5-minute time bins. The increase in missed GO ratio induced by 1 and 2 nmol/ICV of NPS was observed in every 5-minute bin. Data are presented as means ± SEM; scatter plot is omitted for clarity. Statistical significance: *p<0.05 vs vehicle within the same time bin and independent of strain.

Then, we reasoned that in the Go-NoGo trial a possible strategy to obtain a reward is to wait the end of the trial (outcomes d and e in the method section) and an increase in this strategy induced by NPS would reflect in a higher missed Go ratio. Therefore, we re-analyzed missed Go ratio considering exclusively NoGo-Go trials (outcome i in the method section). However, the increase of missed Go ratio by NPS was confirmed again (dose [$F(3, 60) = 6.95$; $p = 0.0004$; $\eta^2 = 0.198$], Strain [$F(1, 20) = 0.008$; $p = 0.455$; $\eta^2 = 7.881 \times 10^{-5}$], dose-by-strain [$F(3, 60) = 1.33$; $p = 0.27$; $\eta^2 = 0.038$] **Figure 5**).

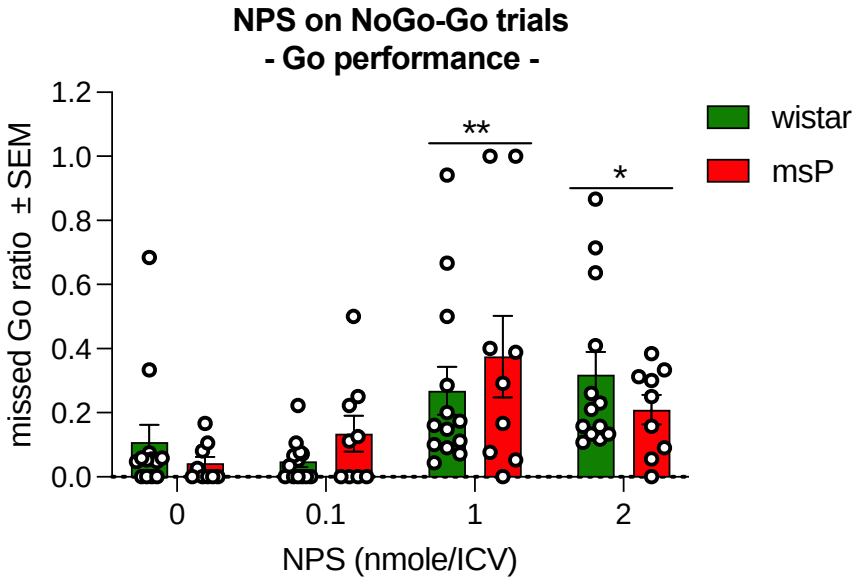

Figure 5. Effect of NPS on the Go phase of a NoGo-Go trial in the Within-trial Go/NoGo model in Wistar and msP rats. 1 and 2 nmol/ICV of NPS increased missed Go ratio in both rat strains. Data are presented as means ± SEM. Statistical significance: **p<0.01 and *p<0.05 vs vehicle dose independent of strain.

None of these tests explained the increase in missed Go ratio observed.

Finally, we also tested the effect of NPS on the Between-trials Go/NoGo impulsivity model that we developed in Chapter 3. Interestingly, the effect of NPS on measures of impulsivity (i.e. premature response and Missed NoGo ratio), but not that on missed Go ratio, were confirmed. (**Supplementary Figure S1**).

Experiment 2: Effect of RTI263 on Within-Trials Go/NoGo Performance in Wistar Rats.

When the effect of RTI263 and its reference compound NPS on premature response was analyzed, one-way ANOVA showed no overall effect of treatment [$F(3, 39) = 1.39$; $p = 0.26$; $\eta^2 = 0.96$] (**Figure 6A**).

When the effect of RTI263 and its reference compound NPS on missed NoGo ratio was analyzed, ANOVA found an overall effect treatment [$F(3, 39) = 3.86$; $p = 0.016$; $\eta^2 = 0.229$]. Dunnett's post-hoc analysis revealed that the reference treatment NPS decreased missed NoGo ratio (**Figure 6B**).

When the effect of RTI263 and its reference compound NPS on missed Go ratio was analyzed, ANOVA found no overall effect of treatment [$F(3, 39) = 1.02$; $p = 0.023$; η^2

= 0.073] (**Figure 6C**).

When the effect of RTI263 and its reference compound NPS on inactive lever presses was analyzed, ANOVA found an overall effect treatment [$F(3, 39) = 3.39$; $p = 0.027$; $\eta^2 = 0.207$]. Dunnett's post-hoc analysis revealed that the reference treatment NPS decreased the number of lever presses (**Figure 6D**).

These results indicated that RTI did not match the anti-impulsive effects of NPS. However, observation of scatterplots suggested that a possible effect of RTI263 10 nmol dose might have been diluted by the other groups in ANOVA's F-test. This prompted us to run secondary t-test analyses. Interestingly, in t-test 10 nmol of RTI263 decreased premature responses [$t(13) = 2.7$; $p = 0.018$; $d = 0.348$] and missed NoGo ratio [$t(13) = 2.18$; $p = 0.048$; $d = 0.226$], but did not significantly affect missed Go ratio [$t(13) = 1.32$; $p = 0.21$; $d = 0.073$]

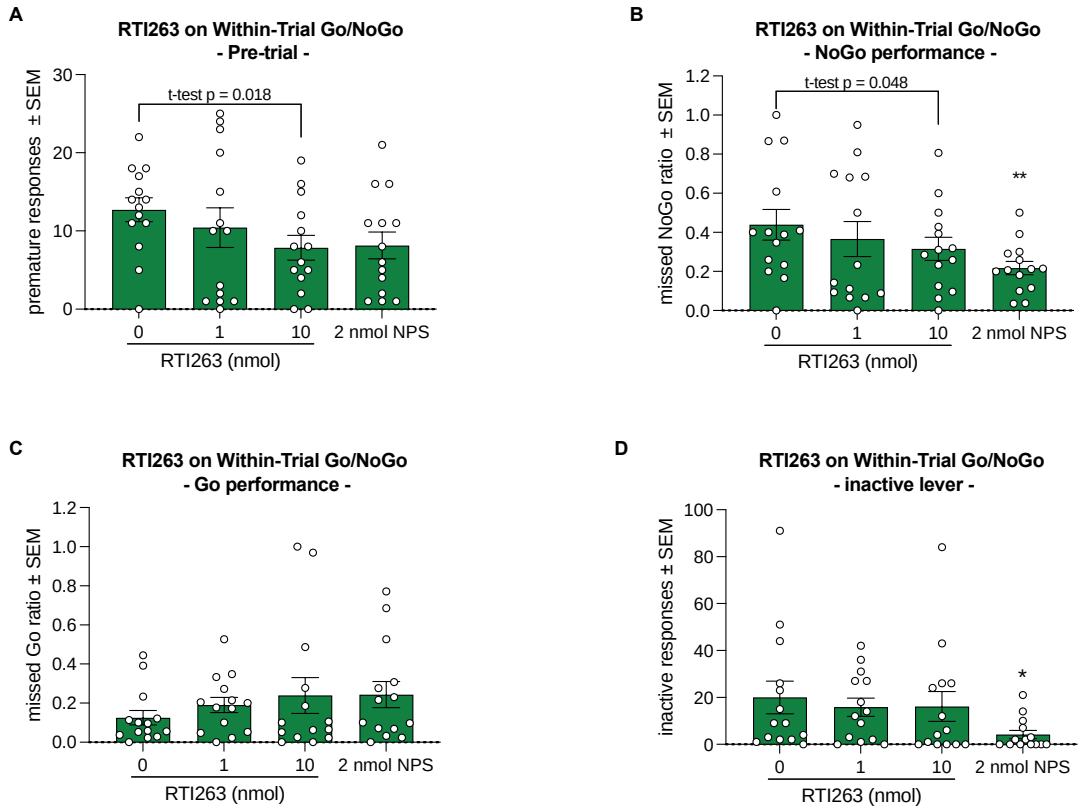

Figure 6. Effect of RTI263 and its reference compound NPS on Within-trial Go/NoGo performance in Wistar rats. A) 10 nmol of RTI263 showed a trend to reduce the number of premature responses that was confirmed by secondary t-test analysis. **B**) The reference drug NPS decreased missed NoGo ratio. A trend by 10 nmol of RTI263 was confirmed by secondary t-test analysis. **C)** Neither RTI263 nor NPS significantly affected missed Go ratio. **D**) NPS reduced inactive lever responses. Data are presented as means ± SEM. Statistical significance: **p<0.01 and *p<0.05 vs vehicle dose.

DISCUSSION

Our study reveals a novel function of NPS in impulsivity, showing that NPS has anti-impulsive effects and sheds light on the mechanism linking impulsivity and NPS. In the within-trial Go/NoGo model, NPS treatment decreased premature responses, reduced missed NoGo ratios, and increased missed Go ratios in both Wistar and msP rats, with no significant differences observed across time bins.

The decrease in premature responses suggests that NPS reduces impulsive actions, indicating that NPS may affect neural circuits associated with impulsivity. The reduction in missed NoGo ratios indicates improved response inhibition, meaning the animals were more successful at withholding responses during NoGo trials. This suggests that NPS enhances inhibitory control and cognitive flexibility, potentially by modulating arousal (Xu et al., 2004), making the animals more attuned to task contingencies.

The increase in missed Go ratio indicates that animals were more likely to fail to respond to Go cues. This suggests that while NPS improves inhibitory control, it may impair proactive motor readiness or response execution. This could reflect a trade-off between enhanced inhibition and reduced action initiation, or a shift in response strategy. It's also possible that NPS induces a more cautious, conservative approach.

Importantly, the effects of NPS observed here cannot be secondary to its effect of locomotion, as NPS increases locomotion, and this would result in a higher number of lever presses, i.e. in increased premature responses and missed NoGo ratio.

This finding suggests that pharmacological agents enhancing inhibitory control may sometimes reduce motor readiness, resulting in fewer responses in Go trials. The stability of NPS's effects across time bins indicates that its impact is not transient or due to fatigue, but rather represents a sustained effect during the trial. Interestingly, in the between-trial Go/NoGo model, NPS did not significantly alter the missed Go ratio, suggesting that NPS's effect on motor execution might be task-dependent.

The observed effects in both Wistar and msP rats suggest that NPS's influence on impulsivity is not strain-specific, despite differences in baseline impulsivity levels between the strains. This points to a generalized mechanism through which NPS modulates impulsive behavior, regardless of inherent impulsivity predispositions.

In summary, NPS appears to reduce impulsivity by decreasing premature responses and improving NoGo accuracy, though it may increase missed Go trials, possibly due to a more cautious response approach. The stability of these effects over time and across both strains supports the idea that NPS plays a significant role in impulsivity. Further studies are needed to explore the underlying neurobiological pathways involved in

impulsivity, particularly to clarify the cellular and molecular changes in brain circuitry during NPS's effects.

The effect of RTI263 and its reference NPS on within-trial Go/NoGo performance in Wistar rats showed that while NPS exhibited clear anti-impulsivity effects, RTI263 had weaker or less consistent effects. These findings suggest that RTI263 does not fully replicate the effects of NPS, and that the anti-impulsivity effect of this neurotransmitter require both the anxiolitic and stimulatory traits. Analyses should investigate sex differences and explore a wider dose range of RTI263, as 10 nmoles showed weak effects. This will help further clarify how NPS receptor signaling pathways are involved in impulsivity.

Study Limitations and Perspectives

In this study we pooled male and female rats. It is therefore possible that the high variability observed in the response to NPS was due to the sex pooling. Future studies should verify if NPS exerts a sex-specific effect on impulsive behavior.

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Supporting Information to Chapter 4

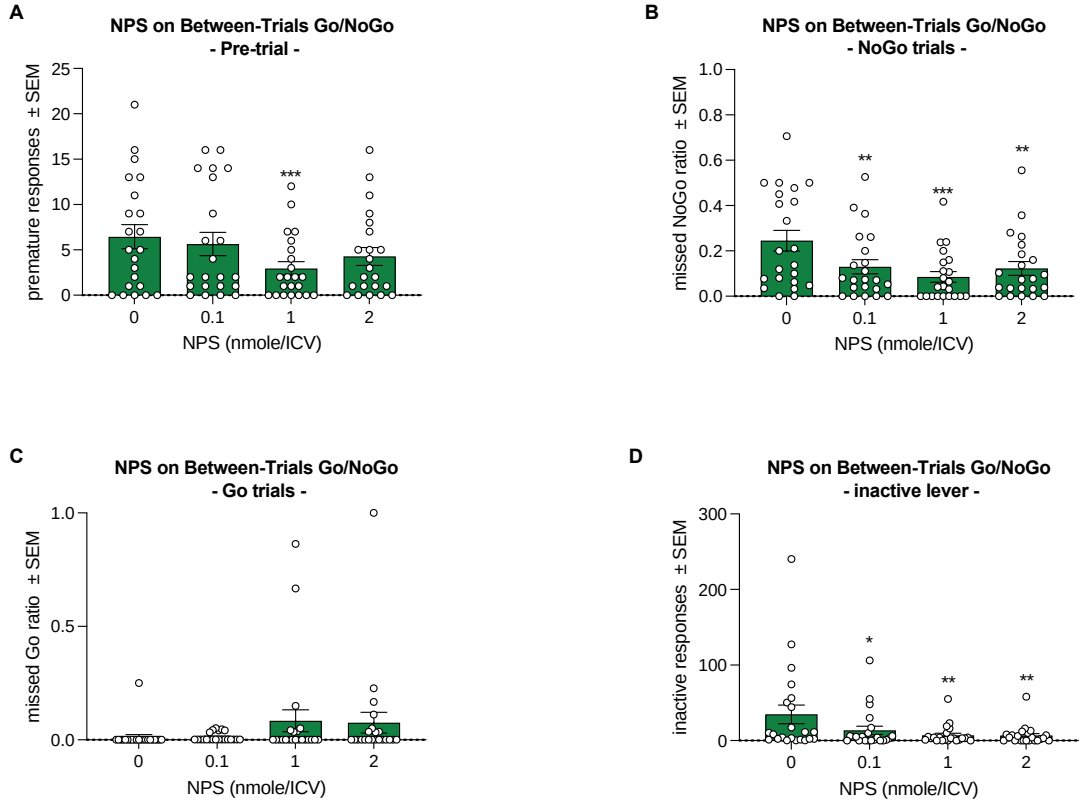

Figure S1. Effect of NPS on Between-trials Go/NoGo performance in Wistar rats. Rats were trained to the between-trial Go/NoGo model described in chapter 2 (i.e. each trial included exclusively a Go or a NoGo phase). **A**) 1 nmol/ICV of NPS decreased the number of premature responses. **B**) All three doses of NPS decreased missed NoGo ratio. **C**) NPS did not affect missed Go ratio. **D**) All three doses of NPS decreased inactive lever responses. Data are presented as means ± SEM. Statistical significance: ***p<0.001, **p<0.01 and *p<0.05 vs vehicle.

CHAPTER 5

OVERALL CONCLUSIONS

Overview

The overall aim of this thesis was to investigate the role of the NPS system in ASA and impulsivity in laboratory rats. The rationale behind this research is that both AUD and impulsivity are multifaceted behaviors that exert a substantial influence on our lives, both directly and indirectly. The association between impulsivity and AUD, as well as the relationship between the NPS receptor and both AUD and impulsivity, was further confirmed.

The specific experimental aims of this dissertation were to:

1. Verify the stimulatory, anxiolytic, and stress-coping roles of NPS in msP rats; assess NPS's ability to reduce alcohol ASA in msP rats; determine whether the anxiolytic effect of NPS alone is sufficient to reduce alcohol seeking; and evaluate whether NPS can suppress stress-induced relapse.
2. Develop a rat operant self-administration apparatus model to study impulsivity and investigate whether there is a relationship between alcohol abuse and impulsivity in rats.
3. Explore the impact of NPS on impulsivity and the role of NPS receptor signaling pathways in impulsivity.

Main Findings

The major findings of this research can be summarized as follows:

We verified that NPS retains its stimulatory role in msP rats. NPS reduced ASA in both male and female msP rats. The effect of NPS is specific to AUD, as it reduces ASA in female msP rats by facilitating stress coping, and in males through its anxiolytic effect. The anxiolytic effect of NPS alone is sufficient to reduce alcohol seeking in female rats, but not in male rats. NPS is more efficacious in modulating innate anxiety and stress-related behaviors than in modulating stress-induced relapse.

We developed both a between-trials Go/NoGo model and a within-trials Go/NoGo model to study impulsivity, which were validated using atomoxetine. MsP rats exhibited impulsive-like behavior compared to Wistar controls, contributing to their heightened motivation for alcohol. The underlying mechanism driving alcohol-drinking behavior in msP rats shows sex differences.

Our study revealed a novel function of NPS in impulsivity. It was demonstrated that NPS exerts an anti-impulsive effect, shedding light on the mechanism linking

impulsivity and NPS.

Our findings contribute to the growing body of literature on the NPS system's role in AUD and impulsivity. The results highlight that the NPS system is a potential therapeutic target for AUD and impulsivity. Additionally, the correlation between NPS and impulsivity suggests broader applications for disorders characterized by impaired impulse control, such as addiction and psychiatric conditions like attention-deficit/hyperactivity disorder.

Numerous studies have been conducted to examine the causal relationship between AUD and related symptoms. However, due to the complex and dynamic nature of the brain network, differences of opinion are inevitable, making it difficult to untangle the causal relationship between network changes and behavioral alterations (J. J. Anker et al., 2019; Song et al., 2024). As the theoretical framework progresses, AUD has been shown to interact extensively with stress, anxiety, fear conditioning, and impulsivity, whether bidirectionally or unidirectionally (Müller et al., 2023; Kushner, 2000; Dick et al., 2010). Clinical studies have shown that individuals with alcoholism often consume alcohol to self-medicate negative affective states and alleviate stress and anxiety (Turner et al., 2018). Borruto and colleagues demonstrated that alcohol has distinct effects in msP rats, with males drinking to reduce anxiety and females drinking to cope with stress (Borruto et al., 2021). Although alcohol is known to alter multiple aspects of human behavior, the potentiation of AUD by stress and anxiety appears to be particularly significant, as suppressing stress and anxiety has been shown to reduce alcohol consumption (J. Anker, 2019; Smith and Randall, 2012). To mimic these behaviors, we tested the effect of NPS on ASA in msP rats. The results indicated that NPS can reduce ASA in female msP rats by facilitating stress coping and in males through its anxiolytic effect. This suggests that NPS is a viable target for alcoholism and provides evidence supporting the self-medication hypothesis. Moreover, it emphasizes the importance of NPS as a potential target for AUD, stress, and anxiety.

In the experiment examining the effect of NPS on impulsivity, our study revealed a novel function of NPS in impulsivity. NPS was shown to exert an anti-impulsive effect, shedding light on the mechanism linking impulsivity and NPS.

Based on the experimental results of this thesis, NPS’s therapeutic potential is promising, as a dose of 1 nmole/icv could reduce ASA while simultaneously alleviating impulsivity, and moderating stress or anxiety in rats. However, uncertainties still remain on the interpretation of the increased missed Go ratio, which demands further tests.

In the light of the significant insights gained, the NPS system remains a useful target for treating AUD and impulsivity. However, although several NPS receptor agonists have been studied for their effects on AUD in rodents, none have progressed to clinical trials (Cannella et al., 2022).

To enhance the efficacy of pharmacotherapy for AUD and impulsivity-related conditions, there is a need to develop more effective medications (Ryan et al., 2014). Furthermore, electrophysiological recordings could help investigate potential correlations between NPS and the monoaminergic transmitter system, which has been implicated in the neurochemistry of arousal, stress, anxiety, AUD, and impulsivity (Xu et al., 2004). Advances in neuroscience research techniques have provided effective methodologies to investigate the detailed neurobiological mechanisms and therapeutic interventions underlying the multifaceted effects of NPS.

Implication of the Findings and Future Directions

From a practical perspective, therapeutic modulation based on the NPS system may offer a promising approach to the treatment of AUD and impulsivity-related behaviors. Future research should focus on the following:

1. More explicit tests are required to ascertain if increasing the dose of RTI263 may influence impulsivity in the Go/NoGo model. This will help further understand how NPS receptor signaling pathways are involved in impulsivity. Additionally, it is important to conduct further analyses to check for sex differences in the effects of NPS and RTI263 on impulsivity.
2. Examine the long-term effects of NPS modulation on AUD and impulsivity-related behaviors.
3. Explore the cellular, molecular, and neurocircuit changes underlying the function of the NPS system. For example, the application of electrophysiological studies, and investigation NPS's effects on ventral tegmental area dopamine neuron firing or prefrontal cortex inhibitory circuits using in vivo or ex vivo recordings could elucidate the neural mechanisms underlying its anti-impulsive effects. This could be a potential future direction to bridge behavioral findings with cellular insights.
4. Conduct translational studies to assess the therapeutic potential of NPS-based interventions in human populations suffering from AUD and impulsivity.

In conclusion, this study provides novel insights into the role of the NPS system in AUD and impulsivity, highlight the potential of the NPS system as a valuable target for future therapeutic interventions.

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LIST of PUBLICATIONS

- Sara De Carlo, Hela Mrizak, Andrea Della Valle, Veronica Lunerti, Alessandra Mammone, Manthoula Kyratzi, Antonio Lacorte, Adana Keshishian, **Min Li**, Di Qin, Leah Solberg Woods, Laura Soverchia, Esi Domi, Massimo Ubaldi, Roberto Ciccocioppo, and Nazzareno Cannella. Predicting individual treatment response in alcohol use disorders: a reverse translational proof-of-concept study. ***Translational Psychiatry*** [***Accepted***; the *merged PDF of the accepted manuscript is pasted below*]

- **Min Li**, Sara De Carlo, Laura Soverchia, Stewart Clark, Carolina Haass-Koffler, Roberto Ciccocioppo, Douglas Sheffler, and Nazzareno Cannella. Sex-dependent role of Neuropeptide-S on anxiety, fear conditioning, and alcohol seeking in alcohol preferring rats. [***Submitted to Neuropharmacology***]

Predicting individual treatment response in alcohol use disorders: a reverse translational proof-of-concept study

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ABSTRACT

The development of medications for alcohol use disorders (AUD) faces stagnation, as promising drugs failed to translate in clinic. Screening on homogeneous groups of animals drugs later tested on heterogeneous clinical cohorts may contribute to the translational gap. We hypothesized that a preclinical model of AUD accounting for inter-individual heterogeneity would predict the lack of efficacy of a drug that failed clinical trials (Memantine) and the efficacy of an approved AUD medication (Naltrexone).

Baseline alcohol drinking, motivation, and cued reinstatement were screened in NIH genetically heterogeneous-stock rats before testing the effect of Memantine and Naltrexone on alcohol (ASA) and saccharin self-administration (SSA).

Based on the individual effect of Memantine and Naltrexone on ASA, rats were allocated into independent clusters of responders and non-responders to each drug. The same doses of Memantine reduced both ASA and SSA in both clusters, while Naltrexone selectively reduced ASA in responder rats. Naltrexone responders were in majority males, while non-responders were mostly females. Naltrexone responders and non-responders showed similar alcohol drinking and motivation, but non-responders did not show cued reinstatement of alcohol seeking.

In line with clinical observations, in a model accounting for individual heterogeneity Memantine failed to selectively reduce ASA, the population could be unbiasedly clustered in responders and non-responders, and cued reactivity associated with Naltrexone response in males. These results advocate the use of inter-individual heterogeneity for preclinical prediction of drug efficacy in AUD before clinical trials. In addition, we observed sex differences in response to Naltrexone that can be back-translated in clinic.

INTRODUCTION

Alcohol use disorders (AUD) represent a worldwide unmet medical emergency accounting for 3 million yearly deaths globally. The AUD prevalence in Europe and in the Americas is estimated at 8-12% across genders, with peaks of 17% in the USA and >22% in eastern Europe; similar prevalence is found in developing and westernizing countries [1].

Alcohol dependence has long been recognized as a clinical syndrome [2, 3]. However, notwithstanding the bulk of time and resources dedicated to study this condition, and the fairly advanced biological characterization achieved [4], approved AUD medications show limited and heterogeneous efficacy [5-7]. In addition, in spite of the high expectancy and effort devoted, promising targets have failed to translate into clinical use [8-11]. These failures brought despondency in professionals working in the field of psychiatric disorders in general, and of addiction in particular, and led major pharmaceutical companies to abandon drug research and development (R&D) in this field as it is perceived as at high risk of business [12]. Indeed, psychiatric drugs are the longest and more expensive to develop, and those with the lowest clinical approval success rate [13, 14].

To address this stagnation, AUD experts are now highlighting the need for AUD personalized diagnosis and treatment approaches [15, 16]. Noteworthy, AUD develops only in 10-to-20% of the subjects (vulnerable users) consuming excessive amounts of alcohol [17]. Moreover, AUD diagnosis is a combination of two to eleven diagnostic criteria, therefore patients population is a heterogeneous group of individuals that cannot be expected to develop the same disease trajectory and to equally respond to treatments. There is, therefore, an increasing awareness that treatments should be tailored toward subgroups of patients rather than targeting the disease as a whole [6, 15, 16, 18]. While this perspective is widely acknowledged at the clinical level, at the preclinical level new groundworks are needed to rethink the contribution of animal models to drug R&D by the implementation of individual heterogeneity approaches.

AUD preclinical studies have traditionally adopted a group-based approach and focused on primary symptoms, like relapse, consumption, and craving [19]. Conversely, little attention has been paid to the heterogeneity in vulnerability traits of AUD patients [20-23]. In a classical group-based experiment, different doses of drugs are usually tested on behaviorally homogeneous groups of animals. Here, individual subjects are required to meet two basic inclusion criteria: (i) show

voluntary drug self-administration/seeking, and (ii) show homogeneous level of self-administration/seeking. This approach offers two unquestionable advantages: first, it guarantees the high construct validity of the test by optimizing the conditions for the experimental drug to show its efficacy and facilitating the sorting between compounds with and without therapeutic potential; second, it is cost-effective, it requires a small number of animals, and it is relatively quick. On the other hand, group-based approaches are blind to individual variability. Single animals that do not respond to the treatment are often perceived as outliers, laying on the right tail of an otherwise left-shifted distribution, and thus often excluded from the analysis. However, when preclinically successful compounds enter clinical trials, even those trials with stricter inclusion criteria, they will be tested on cohorts of patients showing a heterogeneity that was not accounted for at preclinical level.

Here, we propose that implementing individual variability approaches in preclinical drug screening would help refining the prediction of clinically successful new treatments for AUD. To substantiate our idea, we adopted a proof-of-concept reversed translational pharmacology approach in which two drugs were investigated. One of these drugs, (Memantine) has failed to reduce alcohol consumption in clinical settings [24-26]. The other drug (Naltrexone) is FDA/EMA approved for AUD, and it reduces alcohol consumption in patients [27, 28]. These drugs were tested on alcohol self-administration in a population of NIH genetically heterogeneous stock rats (HS) subjected to a multi-symptomatic screening of alcohol consumption and seeking. We chose the HS rats because this diverse outbred line was created to reflect the genetic heterogeneity of the human population [29-31], and it exhibits greater diversity than other outbred lines [31]. Moreover, HS rats were already demonstrated to show heterogeneity in addiction-like behaviors [32-35] and response to pharmacological treatments in addiction models [36]

Our primary working hypothesis was that Naltrexone, but not Memantine, would selectively reduce alcohol self-administration in HS rats. Then, as in clinical practice subpopulations of treatment responder and non-responder individuals do exist, our secondary hypothesis was that our individual-based approach would enable us to identify subgroups of rats that respond (treatment-responder) and do not respond (non-responder) to treatment. Finally, we run a retrospective analysis to profile alcohol seeking behavior in treatment-responder and non-responder rats and seek for features that can be back-translated in clinic.

MATERIALS AND METHODS

Animals

One hundred NIH-HS male and female rats (n=50/sex); Wake Forest University, (North Carolina, USA) weighed 325-375 and 175-225 grams respectively at the beginning of the experimental procedures. Rats were housed 4 per cage according to their sex in a room with reversed 12:12 h light/dark cycle and controlled temperature (20-22°) and humidity (45-50%). Food (4RF18, Mucedola, Italy) and tap water were provided *ad libitum*. After a week of acclimatation to the new environment, rats were handled 5 min a day for an additional week before the beginning of behavioral screening. All procedures were conducted during the dark phase of the light/dark cycle and were in adherence with the *European Community Council Directive for the Care and Use of Laboratory Animals* and the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*; Italian Ministry of Health approval 1D580.47.

Drugs

Alcohol solutions were prepared diluting 95 %v/v alcohol (Carsetti, Italy) with tap water. Saccharin (Sigma-Aldrich) was dissolved in tap water.

Naltrexone hydrochloride (Sigma-Aldrich) was dissolved in saline solution.

Memantine 20mg coated tablets (Memantina Mylan, Mylan Italia S.r.l.) were purchased from the local pharmacy store and suspended in tap water.

Self-administration apparatus

Operant training and testing were performed in self-administration (SA) stations (Med Associates, St Albans, VT, USA) equipped with two retractable levers located in the front panel of the chamber, and a house light on the opposite wall. Pressing on the lever designated as “active” according to the programmed reinforcement schedule activated a syringe pump delivering 0.1 ml of solution in a drinking reservoir (volume capacity 0.3 ml) located between the levers. Pressing on the other lever, designated as “inactive”, was recorded but had no scheduled consequences. Each SA chamber was enclosed in sound-attenuating ventilated cubicles. Behavioral sessions were controlled and recorded by a windows compatible PC equipped with Med-PC-5 software (Med Associates).

Experimental timeline

The experimental timeline (**Fig 1A**) is composed by three consecutive phases: 1) screening for alcohol related behaviors, 2) effect of Memantine and Naltrexone on alcohol self-administration, 3) effect of Memantine and Naltrexone on saccharin SA.

Phase 1: screening of alcohol related behaviors

This phase is composed of three consecutive tests, 3-bottle choice (3BC) drinking, motivation and cued reinstatement of alcohol seeking.

Three-bottle choice alcohol drinking

Alcohol naïve rats were given *ad libitum* access to three bottles containing water, 5 and 10 %v/v alcohol solutions respectively. The 3BC screening lasted for fifteen days. To acclimate rats to alcohol taste, in the first five days the bottles were provided in the common home cages. The following ten days rats were housed in single cages to monitor their individual liquid intake. To avoid the development of side preference, the position of the three bottles was changed every day. Bottles weight was recorded every 24h. At the end of the 3BC screening, rats were housed back into common cages with their original cage mates, and alcohol solutions were no longer provided in the home cage.

Motivation for alcohol expressed under progressive ratio contingency.

Following 3BC screening, rats were trained to self-administer 10% alcohol (v/v) in 30-min daily sessions under FR1 schedule of reinforcement. Sessions were run daily for five days a week. Each active lever response resulted in the delivery of 0.1 ml of 10% alcohol solution followed by a 5s time out (TO) during which further lever presses were not reinforced. The house light was illuminated contingently with the reinforcement delivery and remained on during the TO.

After seventeen sessions of FR1 training the motivation for alcohol was tested in three consecutive sessions run with a progressive ratio (PR) schedule of reinforcement in which the number of active lever presses required to obtain a single reward increased according to the following order: 1, 2, 3, 4, 6, 8, 10, 12, 16, PR+4 [21]. Session stopped when more than 30 minutes had elapsed from the last reward earned. The last ratio completed was defined as the break point (BP) and used as a measure of motivation for alcohol.

Cued reinstatement of alcohol seeking.

Following PR tests, rats were subjected to six additional alcohol SA (ASA) baseline sessions under FR1 contingency before entering extinction of alcohol seeking. During daily 30-min extinction sessions both levers were extracted but lever pressing was not reinforced by alcohol delivery, house light illumination, and pump activation. When responding at the previously active lever dropped in average below ten responses for three consecutive days, the cued reinstatement test began.

The cued reinstatement test was run the day after the last extinction session. In this test, the first lever press delivered one reward and illuminated the house light like in a standard ASA session. For the remainder of the session, active lever presses illuminated the house light but did not result in alcohol delivery.

Phase 2: effect of Memantine and Naltrexone on ASA

At the end of the cued reinstatement test, FR1 self-administration of 10% alcohol was re-baselined and the effect of Memantine and Naltrexone on ASA was evaluated in two separate tests. All rats were subjected to both treatment tests. The drug (either Memantine or Naltrexone) administered in the first test was counterbalanced between rats. A new ASA baseline was established between the first and the second treatment tests.

Group allocation and blinding: Memantine and Naltrexone starting groups were equal in size and balanced in sex prevalence, with no further arbitrary constrains in group allocation. Group allocation, treatment delivery, data collection, and data analysis were performed by independent operators.

Effect of Memantine on ASA.

On test days, rats received oral administration of Memantine (0.0, 6.0, 12.0 and 25.0 mg/kg) in a volume of 4 ml/kg, one hour before SA session [37]. Higher doses of Memantine were excluded because in preliminary studies they completely abolished ASA (**Supplementary Fig. S1**), preventing the observation of a dose/response relationship. Each rat received all Memantine doses or its vehicle in a within-subject counterbalanced order; size and sex ratio was balanced between the latin-square subgroups. Test sessions were run every fourth day until each rat had received the

whole dose range. The first day after the test, rats remained in their home cage, while the second and third days they were subjected to ASA baseline.

Effect of Naltrexone on ASA.

This test was identical to Memantine test except that the rats received subcutaneous administration of NTX (0.0 0.3 or 1.0 mg/kg) in a volume of 1ml/kg, 30 minutes before SA session [38].

Phase 3: effect of Memantine and Naltrexone on saccharin self-administration (SSA)

At the end of the Phase 2, rats were trained to self-administer 0.2%w/v of saccharin under FR1 schedule of reinforcement before the effect of Memantine and Naltrexone on SSA was tested in conditions identical to that described for ASA on Phase 2.

Effect of Memantine on SSA.

This test was run in the same condition described for Memantine in Phase 2.

Effect of Naltrexone on SSA.

This test was run in the same condition described for Naltrexone in Phase 2.

Statistical analysis

Data were analyzed by one-way, two-way, or three-way ANOVA with factors for the respective analysis indicated in conjunction with its results. Dunnett's or Sidak post-hoc analysis followed ANOVA when appropriate.

Power Analyses: initial sample size was estimated to allow detecting small Cohen's *f* effect size for both treatments using conventional power = 0.8 and $\alpha = 0.05$ as parameters (**Fig. S2**). Observed Cohen's *f* effect size were calculated a posteriori using ANOVA results and conventional power = 0.8 as parameters.

Data analysis structure

Step1, Inclusion/exclusion criterion: The ultimate goal of this study was to identify and characterize subjects not responding to treatments. However, subjects showing very low ASA level under vehicle condition could be identified as false non-responder due to a floor effect. To prevent having false non-responders we applied the following inclusion criterion: the number of rewards earned under vehicle treatment being higher than the "ASA baseline average minus one Standard Deviation" threshold.

Step 2: The effect of Memantine and Naltrexone on ASA was analyzed at whole population level.
Step 3: For each drug rats were allocated into two groups, later identified as Responders and Non-Responders. To this purpose, we used the difference in rewards between the vehicle and each treatment dose to allocate individual rats into clusters with different sensitivity to drug effects using a k-mean approach. The k number of clusters was determined for each drug using cluster silhouette analysis as described in **supplemental material**.
Step 4: we compared the effect of Memantine and Naltrexone on ASA and SSA between the clusters identified by k-means analyses. Only Naltrexone demonstrated a specific efficacy in reducing ASA, thus all subsequent analyses were retrospectively performed exclusively on the Naltrexone group (see results and discussion for the rationale to do so).
Step 5: The difference between the observed and expected prevalence of male and female subjects in the k clusters showing different sensitivity to Naltrexone were verified by Pearson's χ^2 crosstabulation analysis.
Step 6: A factor analysis of 3BC, motivation and cued reinstatement was performed using principal component extraction followed by normalized varimax rotation. Finally, we compared the performance of the k Naltrexone clusters in 3BC, motivation and cued reinstatement tests.

Statistical significance was set at conventional $p = 0.05$.

RESULTS

Naltrexone, but not Memantine, selectively reduced ASA in HS rats

One male rat was excluded from drug treatment tests due to health issues. Filtering rats for the inclusion criterion (**Fig. S3**) left 83 rats (41 males) in the Memantine experiment and 82 rats (40 males) in the Naltrexone experiment. Males and females showed similar responses to treatments (**Fig. S4**), therefore they were pooled to analyze the effect of Memantine and Naltrexone on ASA. We set out analyzing drug treatments at population level. Memantine significantly affected ASA [$F(3, 246) = 42.6$; $p<0.0001$; $f=0.262$], specifically 12 mg/kg ($p<0.05$) and 25 mg/kg ($p<0.0001$) of Memantine significantly reduced the number of alcohol rewards earned (**Fig. 1B**). Similarly, Naltrexone significantly reduced ASA [$F(2, 162) = 48.84$; $p<0.0001$; $f=0.232$] at both 0.3 mg/kg

(p<0.0001) and 1.0 mg/kg (p<0.0001) doses (**Fig. 1C**). Neither drug affected the responses at the inactive control lever (**Fig. S5**).

Next, we used the difference in rewards between the vehicle and each treatment dose to allocate individual rats into clusters with different sensitivity to drug effects using a k-mean approach; based on the cluster silhouette (**Fig. S6**), k=2 was applied to Memantine and Naltrexone data separately to allocate rats into clusters MEM1 or MEM2 (**Fig 2A**) and NTX1 or NTX2 (**Fig 2B**) respectively. Importantly, to validate the robustness of the k-mean clusters, we also applied hierarchical clustering for both drugs. Memantine and naltrexone hierarchical clusters were embedded for 92.8% and 87.8% respectively into k-means clusters (**Fig. S7**), confirming the robustness of the k-mean's clustering approach adopted here.

When we compared the effect of Memantine treatment between the two Memantine response clusters, we observed no significant effect of clusters [$F(1, 81) = 0.29$; $p>0.05$] but there was a significant effect of dose [$F(3, 243) = 62.8$; $p<0.0001$] and dose by cluster interaction [$F(3, 243) = 26.98$; $p<0.0001$; $f=0.264$]. Dunnett's post-hoc analysis revealed that all doses of Memantine reduced ASA in cluster MEM1, while only the highest dose was efficacious in cluster MEM2 (**Fig 2C**). Similarly, when Naltrexone data were analyzed, we found no effect of cluster [$F(1, 80) = 0.06$; $p>0.05$] but there was an overall effect of dose [$F(2, 160) = 56.3$; $p<0.0001$] and dose by cluster interaction [$F(2, 160) = 36.0$; $p<0.0001$; $f=0.259$]. All Naltrexone doses decreased ASA in cluster NTX1 while only the highest dose was efficacious in cluster NTX2 (**Fig 2D**).

These results indicated that clusters MEM1 and NTX1 included subjects showing high sensitivity, while MEM2 and NTX2 subjects showed low sensitivity, to Memantine and Naltrexone respectively.

To verify whether the effects observed were specific to alcohol or generalized to natural rewards, the same doses of Memantine and Naltrexone were tested on SSA. In thirty-four rats we had a partial data loss because of a power cut during a Memantine on SSA test session. Therefore, this test was analyzed by mixed-effect ANOVA. Mixed-effect two-way ANOVA found an overall effect of dose [$F(3, 172) = 77.11$; $p<0.0001$] but no effect of cluster [$F(1, 80) = 0.93$; $p>0.05$] or dose by cluster interaction [$F(3, 172) = 0.37$; $p>0.05$]. This result indicated that Memantine affected SSA in both MEM1 and MEM2 clusters. However, to check whether the two-way analysis was blind to shifts in D/R curves between the two clusters, we run secondary analyses on MEM1

and MEM2 data separately. One-way mixed-effect ANOVAs confirmed an overall effect of Memantine on SSA in both MEM1 [$F(3, 77) = 33.8$; $p<0.0001$; $f=0.226$] and MEM2 [$F(3, 95) = 45.4$; $p<0.0001$; $f=0.173$] clusters, and Dunnett's post-hoc analyses confirmed that the three doses of Memantine decreased SSA in both MEM1 and MEM2 clusters (**Fig 3A-B**). When we analyzed the effect of Naltrexone on SSA, we found an overall effect of dose [$F(2, 138) = 17.5$; $p<0.0001$] and of cluster [$F(1, 69) = 5.4$; $p<0.05$] but no dose by cluster interaction [$F(2, 138) = 0.7$; $p>0.05$]. Similarly to Memantine, we run secondary analyses on NTX1 and NTX2 data separately. One-way ANOVAs confirmed an overall effect of NTX on SSA in both NTX1 [$F(2, 82) = 9.2$; $p<0.001$; $f=0.29$] and NTX2 [$F(2, 56) = 9.2$; $p<0.001$; $f=0.286$] clusters. However, in this case Dunnett's post hoc revealed that in cluster NTX1 only the highest dose of NTX significantly reduced SSA (**Fig 3C**), whereas in cluster NTX2 both doses resulted efficacious (**Fig 3D**).

Altogether, these results indicated that Naltrexone but not Memantine selectively reduced alcohol seeking, specifically in cluster NTX1. To rule out the possibility that Memantine failed to show selectivity toward alcohol because the doses tested were too high, we also tested a lower Memantine dose (2.0 mg/kg) that, however, did not reduce ASA neither in cluster MEM1 nor in cluster MEM2 (**Fig. S8**) confirming that the drug lacked selective efficacy towards alcohol.

Importantly, repeating the analyses excluding the thirty-four rats affected by the power cut issue, both Memantine (**Fig. S9**) and Naltrexone (**Fig. S10**) results were confirmed, corroborating their robustness.

These results are in line with the heterogeneous clinical efficacy of Naltrexone and the lack of clinical efficacy of Memantine. Specifically, while Memantine failed to show alcohol selective efficacy in both Memantine clusters (i.e. neither cluster can be characterized as Memantine responder), the NTX1 and NTX2 clusters corresponded to Naltrexone Responders (NTX-R) and Non-Responders (NTX-NR) patient respectively and were accordingly renamed. Further analyses were therefore conducted exclusively on Naltrexone clusters to (i) explore the extent to which the behavioral profile distinguishing NTX-R to NTX-NR also reverse translates from clinic and (ii) provide novel insights to back translate to clinic.

Male and female subjects show different propensity to fall into Naltrexone response clusters

The number of males in the NTX-R cluster was 1.78-fold the number of females, conversely, the number of females in the NTX-NR cluster was 2.5-fold the number of males (**Fig 4A**). The observed count in the sex by Naltrexone cluster crosstabulation (**Fig 4B**) significantly deviated from the expected count ($\chi^2 = 9.98$; $p<0.01$); indicating that males were more likely than females to show response to Naltrexone treatment and *vice versa*.

Alcohol paired cues failed to reinstate alcohol seeking in male NTX-NR

Next, we run retrospective analyses to compare the performance of male and female NTX-R and NTX-NR rats in three tests of alcohol seeking that were acquired before any treatment: alcohol intake in 3BC drinking, motivation to obtain alcohol in three consecutive PR sessions, and cued reinstatement of alcohol seeking. The three behaviors laid on separate components **(Fig 5A)**, indicating that they represented three different subdimensions of alcohol seeking. No differences between Naltrexone response clusters were observed in 3BC drinking (alcohol concentration [$F(1, 78) = 0.7$, $p>0.05$]; sex [$F(1, 78) = 10.7$, $p<0.01$]; cluster [$F(1, 78) = 0.02$, $p>0.05$]; alcohol concentration by sex by cluster [$F(1, 78) = 0.0006$, $p>0.05$]; **Fig 5B**), and in break point for alcohol (session [$F(2, 156) = 7.6$, $p<0.001$]; sex [$F(1, 78) = 0.5$, $p>0.05$]; cluster [$F(1, 78) = 1.5$, $p>0.05$]; session by sex by cluster [$F(2, 156) = 1.4$, $p>0.05$]; **Fig 5C**).

Analysis of cued reinstatement found an overall effect of session (extinction vs cue) [$F(1, 78) = 38.8$, $p<0.0001$], no overall effect of sex [$F(1, 78) = 0.1$, $p>0.05$] and cluster [$F(1, 78) = 0.1$, $p>0.05$], but a significant session by cluster [$F(1, 78) = 4.8$, $p<0.05$] and session by sex by cluster [$F(1, 78) = 6.9$, $p=0.01$; $f=0.385$] interaction. Sidak post hoc analysis showed that alcohol paired cues reinstated alcohol seeking in all groups except males belonging to cluster NTX-NR (**Fig 5D**). Inactive lever response was not affected by any factor (**Fig. S11**).

DISCUSSION

In this work we conducted a proof-of-concept study to test the hypothesis that the implementation of an individual variability approach in a preclinical setting can help predicting the clinical efficacy of potential treatments for drug abuse. More specifically, we hypothesized that a drug that showed

efficacy in cross-sectional preclinical tests of ASA, but then failed to reduce alcohol consumption in clinical settings, would also fail to show efficacy in a preclinical test of ASA accounting for individual variability and genetic heterogeneity. As a positive control we predicted that, under the same conditions, a drug FDA/EMA approved for the treatment of AUD would confirm its efficacy on ASA. To this purpose, we chose Memantine as a test drug because of its lack of efficacy on alcohol drinking in clinical tests [24-26] while showing efficacy in preclinical studies [39-42]. Naltrexone was chosen as positive control drug due to its efficacy in reducing alcohol drinking both in the clinical practice [27, 28] and in preclinical settings [43, 44]. Our choice fell on Memantine over other drugs that failed to reduce alcohol drinking in patients because Memantine is currently prescribed in humans for diseases other than alcohol dependence [45]. Therefore, its failure to reduce alcohol consumption cannot be attributed to a lack of pharmacological activity in humans or to safety issues. Similarly, we tested the two drugs on ASA rather than alcohol craving and relapse prevention because Memantine showed efficacy in reducing alcohol craving in humans [24, 46, 47] and therefore the lack-of-efficacy assumption of our proof-of-concept study was not met by relapse tests. Finally, while a choice had to be made and we selected Memantine and Naltrexone as negative and positive drug in our test, we recognize that alternative options, both in terms of positive control in lieu of Naltrexone (e.g., acamprosate[28]) and negative controls in lieu of Memantine (e.g., quetiapine[10] or levetiracetam[11]), were available and should be the topic of future studies aimed at further validating the generalizability of the hypothesis tested here.

- **Memantine but not Naltrexone lacked selectivity in reducing alcohol consumption**

Our data indicated that Memantine reduced ASA, in the 6–25 mg/kg range in the cluster showing higher sensitivity to Memantine (cluster MEM1) and at the highest dose in the cluster showing lower sensitivity to the drug (cluster MEM2). However, in neither case this effect was selective for alcohol as the same doses also reduced self-administration of the natural reinforcer saccharin. In addition, when we completed the dose/response curve with 2.0 mg/kg of Memantine we found a lack of efficacy toward ASA, demonstrating that the dose range tested was enough to completely characterize Memantine's pharmacological profile. Our results are in contrast with studies adopting a homogeneous group-based approach, in which Memantine showed selectivity toward

alcohol over natural rewards [41, 42], thus confirming our hypothesis and not supporting the use of Memantine by itself to treat alcohol drinking. Conversely, as expected we observed an alcohol-selective effect of the positive control drug Naltrexone. Here, both drug doses reduced ASA in cluster NTX1, with the lowest dose resulting alcohol selective. Conversely, in cluster NTX2 only the highest dose of naltrexone reduced ASA, but this dose was not alcohol selective. While we already had an inefficacious dose for cluster NTX2, expanding the naltrexone dose range to lower doses would have allowed finding the inefficacious dose of Naltrexone also for cluster NTX1. However, this would not provide additional information on naltrexone selectivity and was therefore beyond the scope our study.

Altogether, and in the context of the published literature, our reverse translational pharmacology study indicates that preclinical experimental settings accounting for individual variability show a finer sensitivity than group-based studies in predicting clinical outcomes.

To check out the robustness and representativeness of the k-mean clusters, we also run a hierarchical clustering, in which the number of clusters were not set *a priori*. Hierarchical clustering of memantine efficacy yielded five clusters. Noteworthy, more than 90% of rats fell into two large clusters that corresponded *de facto* to k-mean cluster MEM1 and MEM2. Hierarchical clustering of Naltrexone efficacy yielded seven clusters. In this case, 90% of the population fell into two large clusters and one intermediate-size cluster. Interestingly, one large cluster included exclusively rats that k-mean identified as NTX2, the intermediate cluster included exclusively rats that k-mean identified as NTX1, and the second large cluster was for 77.5% composed of rats identified as NTX1 by k-mean. This brings two important information: first, hierarchical clustering separated rats into two families of clusters that corresponded the k=2 k-mean clusters, confirming the robustness and reliability of the k=2 k-mean approach for Naltrexone as well; second, the fact that the k-mean cluster NTX1 corresponded to two hierarchical clusters could indicate that NTX1 might be further separated into subgroups.

It is important to note that Memantine and Naltrexone were intended here as tools to proof a concept rather than being the primary focus of the study. In this view, we purposedly chose to administer Memantine alone and not in combination with other treatments because in this condition the drug met the clinical lack-of-efficacy assumption of our study. However, it is worth noting that in clinical settings, Memantine has proven efficacious toward alcohol craving [24, 46, 47]

and that the combination of Memantine and Naltrexone increases the efficacy of Naltrexone alone [48].

- **Male NTX-R showed enhanced cued reinstatement of alcohol seeking**

In view of the selectivity toward alcohol shown by Naltrexone in the NTX1 and NTX2 clusters, we renamed these clusters as NTX-R (Naltrexone responder) and NTX-NR (Naltrexone non-responder) respectively. To further validate the reverse translational efficacy of our individual based approach, we sought to characterize the AUD-like behavioral features of these two clusters.

The attempt to profile Naltrexone responder and non-responder patients has been traditionally conducted through hypothesis driven approaches. Clinical studies stratified patients cohorts based on different factors such as genotype [49-51], severity of symptomatology [52], preference for sweet tastes [53], alcohol reward/relief seeking [54, 55], and alcohol cues reactivity [56]. Then, the effect of Naltrexone on alcohol drinking outcomes was compared between these *a priori*-stratified groups. In other words, the research question common to all these clinical studies can be summarized as: do group A and group B differ in their response to Naltrexone? This approach can be easily modelled by cross-sectional group-based animal studies, as it stems from an *a priori* hypothesis, the grouping factor is a specific behavioral or biological feature, and the response to Naltrexone is the outcome measure upon which the groups are compared. Conversely, here we adopted an individual variability model in which rats were stratified based on their response to Naltrexone (i.e. Naltrexone response was the grouping factor and not the outcome measure) that allowed us to look for Naltrexone response endophenotypes in a hypothesis-free approach. To this purpose, we compared the behavioral performance of NTX-R and NTX-NR in three subdimensions of alcohol dependence: alcohol drinking, motivation to pursue alcohol, and cued alcohol craving. The three subdimensions were modelled by alcohol intake, breakpoint, and cued reinstatement scores respectively; three behaviors that loaded on three separate principal components, confirming that they represented distinct constructs of alcohol seeking. NTX-R and NTX-NR did not differ in the amount of alcohol consumed, or the breakpoint reached during PR ASA sessions. On the contrary, alcohol visual, olfactory and taste cues reinstated alcohol seeking in male NTX-R but not in NTX-NR clusters. These results align with clinical data indicating that the reactivity to alcohol associated cues predicts Naltrexone response. Mann and colleagues [56] median split their

Naltrexone treated patients in groups with high and low alcohol cues-induced ventral striatum activation and reported a better survival rate in time to first relapse in high activation groups. Schacht and co-workers [50] genotyped their patients for the A118G SNP of the *oprm1* gene, and Naltrexone selectively reduced the percentage of heavy drinking days in patients with A/A genotype. The same patients showed a higher ventral striatum activation induced by alcohol cues that was reduced by Naltrexone. Similarly, in an independent work, Naltrexone decreased cortical activation induced by alcohol olfactory and visual cues [57]. Additionally, two meta-analyses reported that alcohol cue reactivity directly correlated with self-reported craving [58, 59]. In rodent operant models, drug craving is typically assessed by the response at the drug-paired lever induced by drug-paired cues or by a drug priming dose in the absence of the reinforcer [60, 61]. Thus, our cued reinstatement data can be interpreted as the ability of alcohol paired cues to elicit craving in NTX-R but not in NTX-NR rats. This is also in agreement with human data in which alcohol craving has been shown to predict the efficacy of Naltrexone [53, 62-64].

Altogether, these studies have proposed that the efficacy of Naltrexone derives from its ability to decrease alcohol craving and alcohol craving is an endophenotype enabling the prediction of Naltrexone efficacy [50, 53, 56, 57, 62-64]. It should be mentioned, however, that in few studies Naltrexone was effective in patient with a less severe symptomatology [52], and in reward- but not in relief-seekers [54, 55]. Although craving was not analyzed as a predictor of treatment efficacy, in these cases the Naltrexone responder group was the one showing a lower craving rate. However, in these case craving was scored by the obsessive-compulsive drinking scale, while in the studies discussed above craving was scored through analogue-assisted scale or Penn Alcohol Craving Scale.

In summary, our data obtained using Naltrexone-response as grouping factor sustain the interpretation of clinical studies proposing cue reactivity and craving as predictors of Naltrexone response. Interestingly, the association to cued reinstatement and Naltrexone response was specific to male rats and the prevalence of male and female rats differed between NTX-R and NTX-NR groups. Perhaps because of the paucity of sex-specific studies on Naltrexone response, these observations do not find correspondence in the clinical literature, and therefore they represent a novel set of information awaiting translation into clinic.

- **Novel insights to back-translate into clinic**

χ^2 analysis indicated that the NTX-R cluster predominantly consisted of males while the NTX-NR was composed mainly by females. These data indicate that Naltrexone was more likely to selectively prevent alcohol drinking in males rather than females. Whether this result correlates with clinical prevalence is presently unclear. To the best of our knowledge, clinical studies that focused on sex difference did not categorize their patient cohorts into Naltrexone responder and non-responder groups [65-67] and when groups with different sensitivity to Naltrexone were investigated, the relative frequency of women and men was not reported [49-55]. Interestingly, the consistency of Naltrexone efficacy on alcohol drinking across studies is stronger in men than women. While Greenfield and co-workers [66] found no sex difference, Baros and colleagues [65] reported a similar effect size in women and men but a significant difference between placebo and Naltrexone only in men, which they attributed to the smaller women group size. Finally, Kranzler and co-worker [67] reported that Naltrexone was effective in men but not in women. Based on these results one could speculate that the heterogeneous results observed in women may stem from a higher number of non-responders in this gender.

In addition, in our study the difference between NTX-R and NTX-NR in cue reactivity was specific to males. However, the extent to which the lack of difference in females translates to humans is presently unclear, as the interaction between gender and cue reactivity or craving on Naltrexone response has not been analyzed in clinical studies.

Altogether, our prevalence and cued-reinstatement analyses provide the rational for dedicated clinical studies or meta-analyses exploring the prevalence of men and women in Naltrexone responder and non-responder groups of patients, and the difference in predictive endophenotypes between the two genders.

- **Study limitations and future developments**

Our results stimulate a number of considerations that would need future attention.

As discussed above, our work provides new insights on sex-differences that should be back translated in dedicated trials.

The individual variability and sex-difference in drug-response may derive from different genetic factors and consequently from differences in the pharmacokinetic and/or pharmacodynamics of

the drug. Addressing this point in future studies may uncover translational genetic and molecular biomarkers to help highlighting the subpopulation of patient more suitable to receive Naltrexone. Cue-reactivity is a key element that our work highlights as a translational behavioral marker of Naltrexone response, specifically in the male population. However, while humans do not normally go through an extinction training, our cue reactivity test was preceded by an extinction training in the absence of alcohol cues. In future studies, testing cue reactivity in the absence of extinction training and after different abstinence period should be taken into consideration.

Finally, it would be important to study the stability of responder and non-responder groups after a chronic treatment. Our results and related data bank can be the bases to design future between-subjects chronic treatment studies that would better mimic human treatment conditions.

- **Conclusions**

In conclusion, using a reverse translational approach, we demonstrated that an experimental design accounting for individual variability would have accurately predicted the clinical lack of efficacy of Memantine on alcohol drinking, as well as the presence of Naltrexone responder and non-responder subjects. In a wider perspective, our work advocates for the implementation of individual-based approaches in drug screening prior to entering clinical trials. While the classical group-based experiment maintains its primary importance as initial step to assess the therapeutic potential and characterize the toxicology of the experimental drugs, the individual based approach would complement the screening of highly promising compounds before entering clinical trials. If successful, this would address a significant unmet medical need in the development of treatments for psychiatric disorders.

Moreover, this approach would enable the prediction and profiling of drug responder and non-responder patients, thereby enhancing the efficiency and effectiveness of drug development processes. In this regard, we provided evidence that male and female show different propensity to fall into Naltrexone responder and non-responder clusters, and that endophenotypes predicting Naltrexone response in males may not be valid in females. The extent to which these observations translate into clinic is presently unknown, and we encourage clinical scholars to verify it.

AUTHORS CONTRIBUTION

Investigation: SDC, HM, VL, MOK, AM, AL, AK, ML, DQ, LS, MU. Data acquisition: SDC, HM. Data Analysis: ADV and NC. Access to HS colony: LSW. Visualization: ED. Manuscript draft: SDC and HM. Manuscript writing: NC. Funding acquisition: NC and RC. Conceptualization and supervision: NC.

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CONFLICT of INTEREST

Authors declare no competing interests.

DATA AVAILABILITY

Data are available upon reasonable request to the corresponding author.

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FIGURE LEGENDS

Figure 1. A) Schematic representation of experimental timeline. Abbreviation: 3BC, 3-bottle choice; ASA, Alcohol self-administration; PR, Progressive Ratio; Cued Reinst, cued reinstatement; MEM, Memantine; NTX, Naltrexone; SSA, saccharin self-administration. **B**) Effect of Memantine (n=83) on alcohol self-administration at whole population level. The intermediate and highest dose of Memantine significantly reduced alcohol self-administration. Groups mean ± 95%CI: 0.0 mg/kg, 17.99 ± 1.65; 6 mg/kg, 16.64 ± 2.3; 12.0 mg/kg, 12.27 ± 1.81; 25.0 mg/kg, 6.024 ± 1.445. **C**) Effect of Naltrexone (n=82) on alcohol self-administration at whole population level. Both doses of Naltrexone significantly reduced alcohol self-administration. Groups mean ± 95%CI: 0.0 mg/kg, 18.87 ± 1.59; 0.3 mg/kg, 13.21 ± 1.61; 1.0 mg/kg, 10.67 ± 1.35. Bars represent the Mean ± 95%CI of number of rewards earned in a 30 min session. Statistical significance: *p<0.05 and ***p<0.001 vs vehicle.

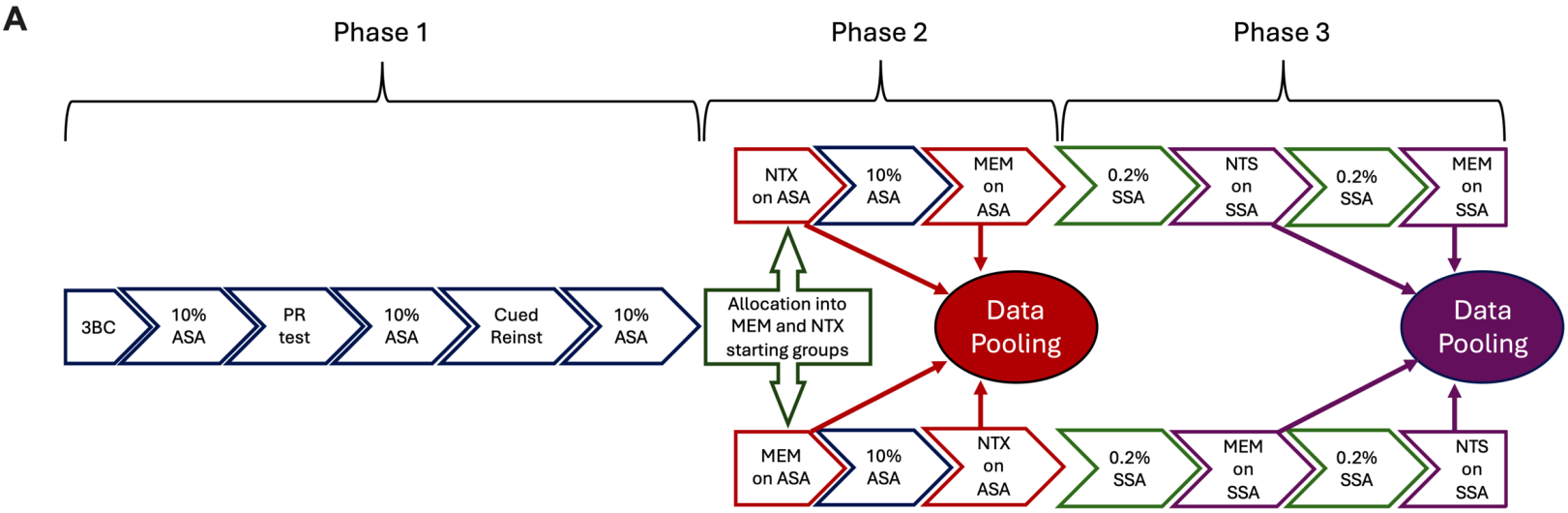
Figure 2. Effect of Memantine and Naltrexone treatment on alcohol self-administration in clusters based on individual effect of the drugs on ASA. **A, B**) Silhouette plot of K=2 clustering of individual response to (**A**) Memantine and (**B**) Naltrexone on alcohol self-administration. Horizontal bars represent individual silhouette coefficient, the vertical dashed line indicates the k=2 cluster silhouette score. **C**) Memantine reduced alcohol self-administration in cluster MEM1 (n=35) at all doses tested and in cluster MEM2 (n=48) only at the highest dose. Groups mean ± 95%CI: MEM1 0.0 mg/kg, 23.89 ± 2.45; MEM1 6 mg/kg, 15.57 ± 4.12; MEM1 12.0 mg/kg, 8.06 ± 2.65; MEM1 25.0 mg/kg, 3.943 ± 1.779; MEM2 0.0 mg/kg, 13.69 ± 1.24; MEM2 6 mg/kg, 17.42 ± 2.71; MEM2 12.0 mg/kg, 15.33 ± 2.11; MEM2 25.0 mg/kg, 7.542 ± 2.082. **D**) Both doses of Naltrexone reduced alcohol self-administration in both NTX1 (n=47) and NTX2 (n=35) clusters. Groups mean ± 95%CI: NTX1 0.0 mg/kg, 21.7 ± 2.09; NTX1 0.3 mg/kg, 11.17 ± 1.95; NTX1 1.0 mg/kg, 9.511 ± 1.787; NTX2 0.0 mg/kg, 15.06 ± 1.84; NTX2 0.3 mg/kg, 15.94 ± 2.51; NTX2 1.0 mg/kg, 12.23 ± 2.06. Bars represent the Mean ± 95% CI of number of rewards earned in a 30 min session. Statistical significance: *p<0.05 and ****p<0.0001 vs vehicle.

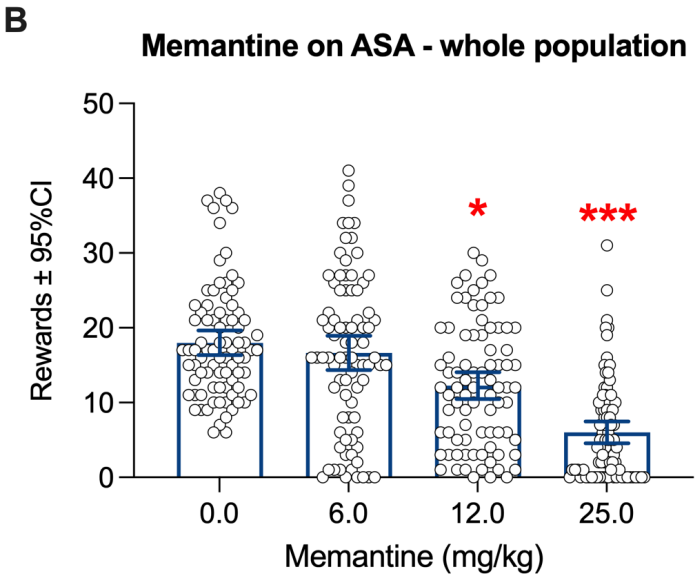
Figure 3. Effect of Memantine and Naltrexone treatment on saccharin self-administration in clusters based on individual effect of the drugs on ASA. **A**) All doses of Memantine reduced saccharin self-administration in MEM1 cluster. Groups mean ± 95%CI: 0.0 mg/kg, 54.18 ± 10.69; 6 mg/kg, 28.17 ± 12.8; 12.0 mg/kg, 17.61 ± 7.22; 25.0 mg/kg, 8.912 ± 3.94. **B**) All doses of Memantine reduced saccharin self-administration in and MEM2 cluster. Groups mean ± 95%CI: 0.0 mg/kg, 54.83 ± 8.6; 6 mg/kg, 37.48 ± 11.74; 12.0 mg/kg, 24.76 ± 9.07; 25.0 mg/kg, 11.17 ± 5.076. **C**) Only by the highest dose of Naltrexone reduced Saccharin self-administration in cluster NTX1. Groups mean ± 95%CI: 0.0 mg/kg, 53.24 ± 9.42; 0.3 mg/kg, 45.93 ± 8.97; 1.0 mg/kg, 36.67 ± 7.1. **D**) Saccharin self-administration was reduced by both Naltrexone doses in cluster NTX2. Groups mean ± 95%CI: 0.0 mg/kg, 70.48 ± 14.11; 0.3 mg/kg, 56.41 ± 11.64; 1.0 mg/kg,

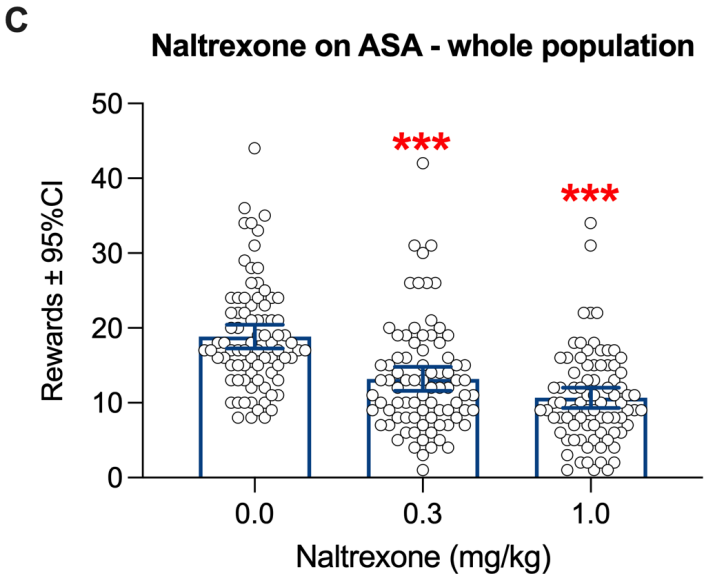
52.07 ± 9.41. Bars represent the Mean ± 95% CI of number of rewards earned in a 30 min session. Statistical significance: **p<0.01, ***p<0.001, and ****p<0.0001 vs vehicle.

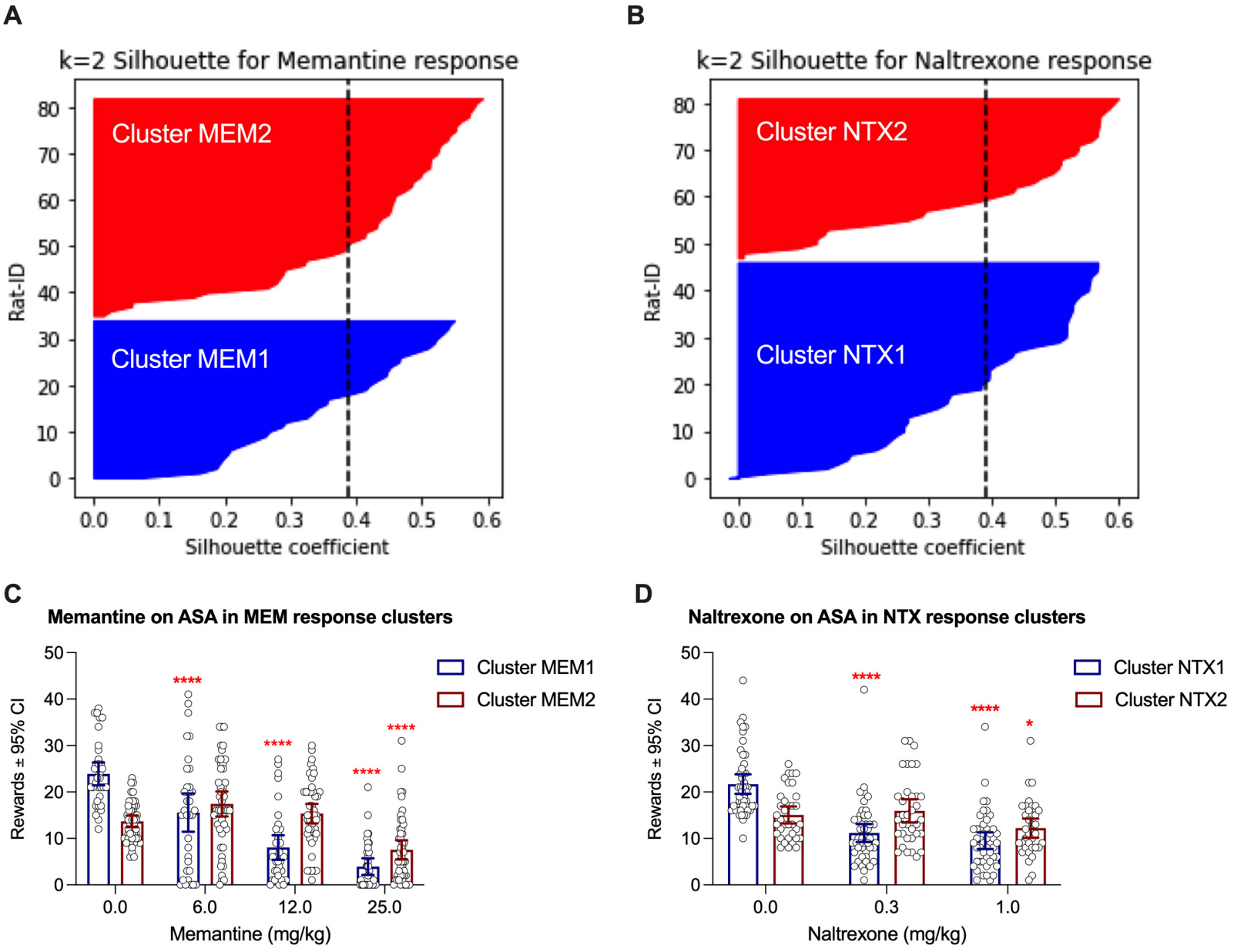
Figure 4. Prevalence of male and female rats in NTX-R and NTX-NR clusters. **A**) Relative (y-axis) and absolute (numbers within bars) frequencies of male and female rats in NTX-R and NTX-NR clusters. **B**) Sex by Naltrexone clusters crosstabulation showing the difference between observed and expected count for each sex by cluster combination.

Figure 5. Comparison of alcohol drinking, motivation and cued reinstatement between NTX-R (female n=17. male n=30) and NTX-NR (female n=25. male n=10) cluster. **A**) Factor analysis using principal component extraction followed by varimax normalized rotation of alcohol drinking in three-bottle choice test (3BC), break point in progressive ratio test (PR) and cued reinstatement test (Cue). **B**) Male and female NTX-R and NTX-NR rats showed similar level of daily alcohol intake at both 5% and 10% alcohol concentration in three-bottle choice test. Groups mean ± 95%CI: Female alcohol 5%, NTX-R 2.098 ± 0.947, NTX-NR 1.661 ± 0.603; Female alcohol 10%, NTX-R 1.25 ± 0.451, NTX-NR 1.461 ± 0.561; Male alcohol 5%, NTX-R 0.988 ± 0.277, NTX-NR 0.84 ± 0.495; Male alcohol 10%, NTX-R 0.842 ± 0.235, NTX-NR 1.321 ± 1.162. **C**) Male and female NTX-R and NTX-NR rats showed similar level of motivation expressed by the break point reached under PR contingency over three consecutive PR sessions. Groups mean ± 95%CI: Female session1, NTX-R 8.353 ± 2.596, NTX-NR 8.6 ± 1.39; Female session2, NTX-R 7.294 ± 2.403, NTX-NR 6.04 ± 1.127; Female session3, NTX-R 6.118 ± 2.418, NTX-NR 4.96 ± 0.964; Male session1, NTX-R 8.667 ± 1.722, NTX-NR 6.9 ± 2.79; Male session2, NTX-R 7.7 ± 1.157, NTX-NR 6.8 ± 1.679; Male session3, NTX-R 7.2 ± 1.191, NTX-NR 7.0 ± 1.686. **D**) Alcohol olfactory, taste and visual cues reinstated alcohol seeking in both NTX-R and NTX-NR female rats and in NTX-R male rats but not in NTX-NR male rats. Groups mean ± 95%CI: Female Ext, NTX-R 8.176 ± 2.392, NTX-NR 8.72 ± 2.163; Female Cue, NTX-R 17.47 ± 5.7, NTX-NR 19.36 ± 3.1; Male Ext, NTX-R 7.433 ± 2.375, NTX-NR 12.1 ± 12.86; Male Cue, NTX-R 23.43 ± 6.685, NTX-NR 13.6 ± 6.025. Bars represent the Mean ± 95%CI of respectively **B**) average 24h alcohol intake, **C**) break point, and **D**) number active lever presses produced in a 30 min session on the last day of extinction (Ext) and on cued reinstatement test (Cue). Statistical significance: *p<0.05, ***p<0.001, and ****p<0.0001 vs Ext same group.

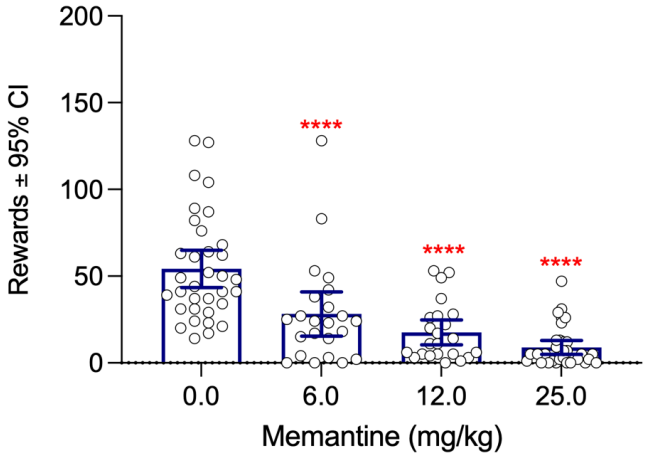

A
Phase 1
Phase 2
Phase 3
3BC
10% ASA
PR test
10% ASA
Cued Reinst
10% ASA
Allocation into MEM and NTX starting groups
NTX on ASA
10% ASA
MEM on ASA
MEM on ASA
10% ASA
NTX on ASA
Data Pooling
0.2% SSA
NTS on SSA
0.2% SSA
MEM on SSA
0.2% SSA
MEM on SSA
0.2% SSA
NTS on SSA
Data Pooling


B
Memantine on ASA - whole population
Rewards ± 95%CI
50
40
30
20
10
0
*
***
0.0
6.0
12.0
25.0
Memantine (mg/kg)

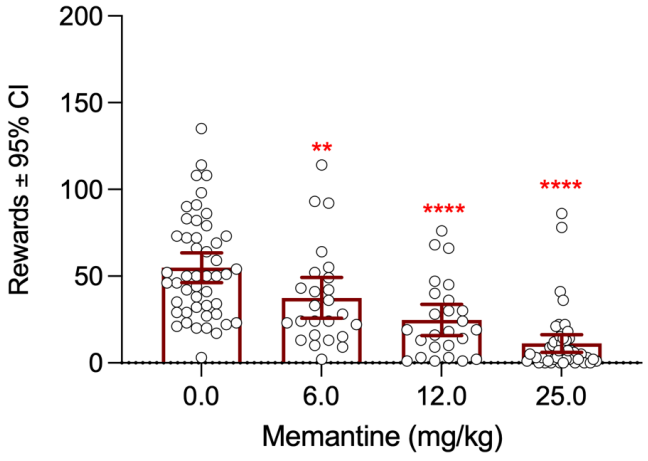

C
Naltrexone on ASA - whole population
Rewards ± 95%CI
50
40
30
20
10
0
***
***
0.0
0.3
1.0
Naltrexone (mg/kg)


A
k=2 Silhouette for Memantine response
Cluster MEM2
Cluster MEM1
Rat-ID
Silhouette coefficient
B
k=2 Silhouette for Naltrexone response
Cluster NTX2
Cluster NTX1
Rat-ID
Silhouette coefficient
C
Memantine on ASA in MEM response clusters
Cluster MEM1
Cluster MEM2
Rewards ± 95% CI
Memantine (mg/kg)
D
Naltrexone on ASA in NTX response clusters
Cluster NTX1
Cluster NTX2
Rewards ± 95% CI
Naltrexone (mg/kg)

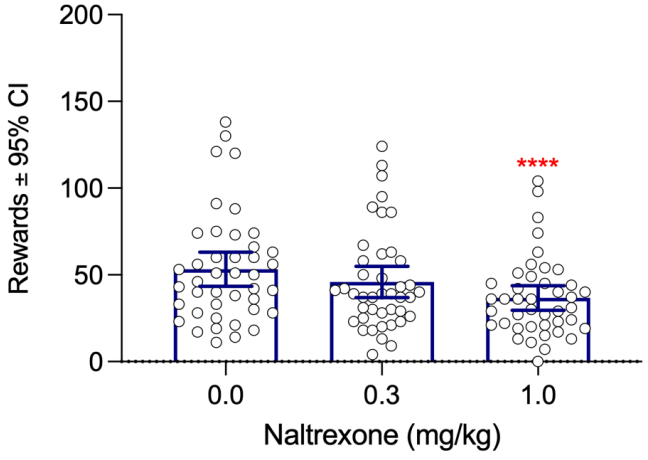
A


Memantine on Saccharin SA in Cluster MEM1
Rewards ± 95% CI
200
150
100
50
0
****
****
****
0.0
6.0
12.0
25.0
Memantine (mg/kg)

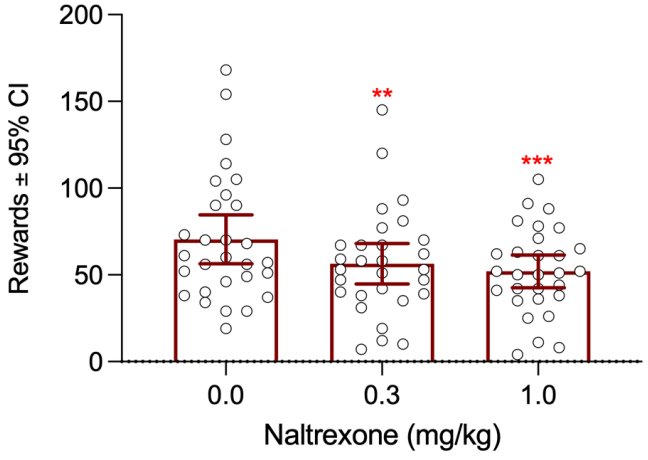
B


Memantine on Saccharin SA in Cluster MEM2
Rewards ± 95% CI
200
150
100
50
0
**
****
****
0.0
6.0
12.0
25.0
Memantine (mg/kg)

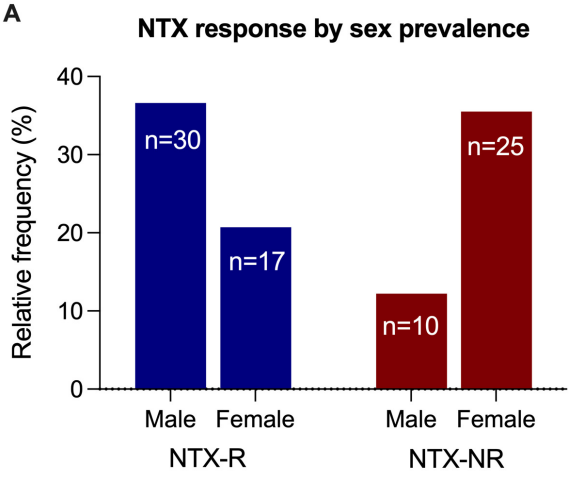
C


Naltrexone on Saccharin SA in Cluster NTX1
Rewards ± 95% CI
200
150
100
50
0
****
0.0
0.3
1.0
Naltrexone (mg/kg)

D


Naltrexone on Saccharin SA in Cluster NTX2
Rewards ± 95% CI
200
150
100
50
0
**
***
0.0
0.3
1.0
Naltrexone (mg/kg)

A

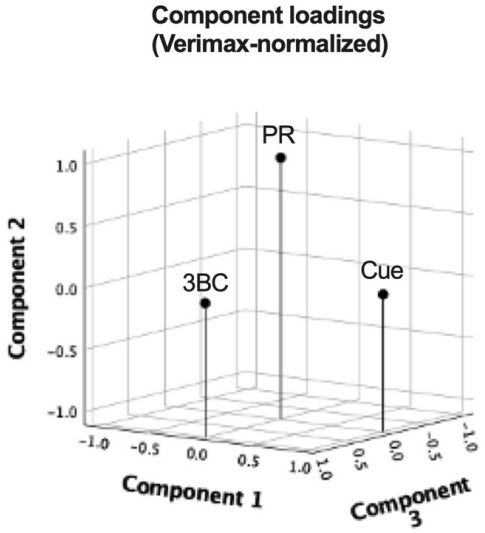


B

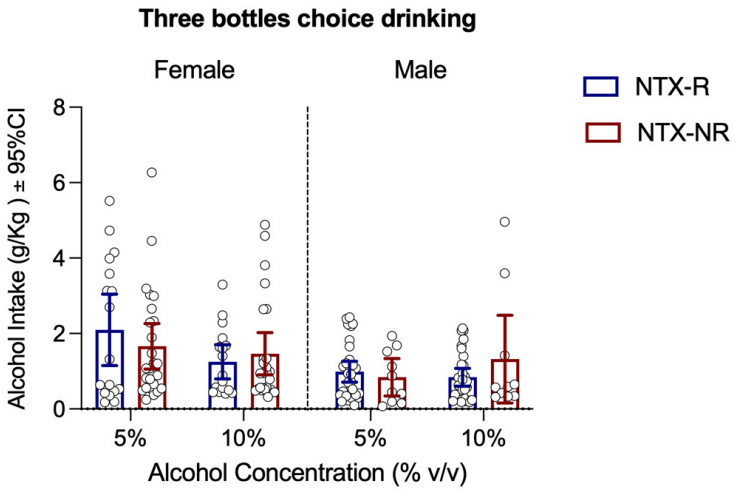
Sex * NTX Response Crosstabulation

			NTX (K=2) Cluster NTX-R	NTX-NR	Total
Sex	female	Count	17	25	42
		Expected Count	24.1	17.9	42.0
	male	Count	30	10	40
		Expected Count	22.9	17.1	40.0
Total		Count	47	35	82
		Expected Count	47.0	35.0	82.0

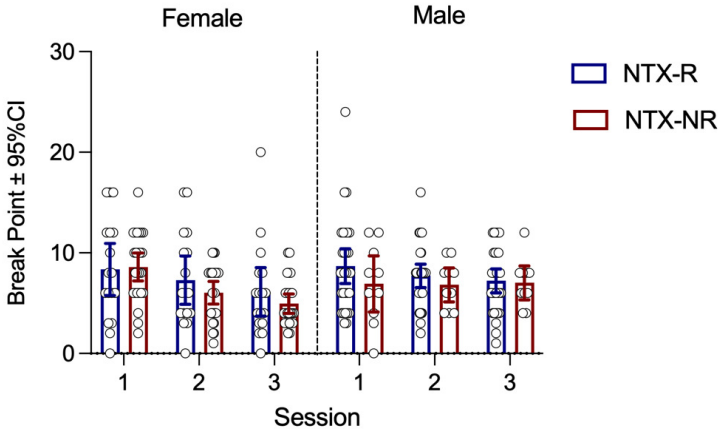
A


Component loadings
(Verimax-normalized)
PR
3BC
Cue
Component 2
Component 1
Component 3

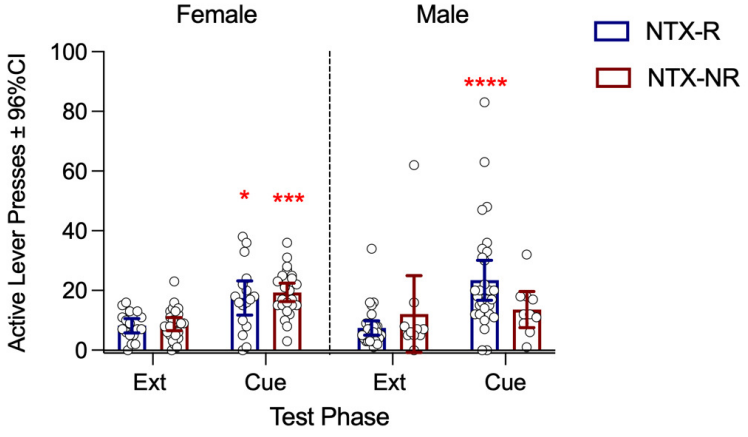
B


Three bottles choice drinking
Female
Male
NTX-R
NTX-NR
Alcohol Intake (g/Kg) ± 95%CI
5%
10%
5%
10%
Alcohol Concentration (% v/v)

C


Progressive Ratio
Female
Male
NTX-R
NTX-NR
Break Point ± 95%CI
Session

D


Cued Reinstatement of Alcohol Seeking
Female
Male
NTX-R
NTX-NR
Active Lever Presses ± 96%CI
*
***
****
Ext
Cue
Ext
Cue
Test Phase