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THE ROLE OF STRESS, SEX AND EARLY-LIFE ENVIRONMENT IN SHAPING ALCOHOL VULNERABILITY

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Chapter 1: Introduction

1. Alcohol Use Disorder

Alcohol use disorder (AUD) is a major public health problem and disease burden for affected patients and society, due to its immense economic cost. According to a recent report by the World Health Organization (WHO), AUD is one of the most common psychiatric disorders and alcohol is one of the most widely used addictive drugs in the world, only second to tobacco, responsible for 3 million deaths per year (Witkiewitz, 2019; American Psychiatric Association, 2013). This placed AUD as the fifth major risk factor for premature death and disability worldwide. At the global level, an estimated 237 million men and 46 million women suffer from alcohol use disorder AUD, with the highest prevalence among men and women in Europe (14.8% and 3.5%, respectively) and the United States (11.5% and 5.1%, respectively) (American Psychiatric Association, 2013). It is the most prevalent mental disorder at the global level (Disney et al. 1999; Slutske et al., 1999). Additionally, the rapid emergence of Covid-19 pandemic has led to an extraordinary upheaval throughout the world and has increased alcoholism rate. Individuals have raised their alcohol intake to cope with emotional stress and chronic uncertainty related to prolonged social isolation and economic precariousness (Killgore, 2021). Historical data indicate that the prevalence of AUD is greater in men than in women. However, this gap is progressively reducing (White et al., 2015). In recent years, the rate of AUD in women has increased by 84%, relative to a 35% increase in men (Grant et al., 2017). Much evidence indicates significant sex differences in the reasons for initiating alcohol use and for the trajectory of AUD (Peltier et al., 2019; Schulte et al., 2009). More frequently than men, women initiate alcohol consumption as a coping strategy to attenuate negative affective states (e.g., anxiety, depression, stress, and feelings of isolation). In men, drinking is often initiated for recreational purposes, especially among young people (Peltier et al., 2019; Oscar-Berman et al., 2014; Crutzen et al., 2013; Buchmann et al., 2010). Interestingly, the increased alcohol use coinciding with the onset of Covid-19 pandemia was higher in women than in men (Tucker JS et al., 2022). There was a significant 14% increase overall, and 19% increase among women, in the frequency of past month alcohol use from pre-pandemic (April-June 2019) to the early months of the pandemic (May-June, 2020). Among women, there was also a significant increase of 39% in alcohol problems (Pollard et al., 2020).

AUD is also characterized by a considerable degree of variability in the individual vulnerability to develop the disorder. That is, most individuals consume alcohol in a controlled manner, but only a subpopulation of individuals (3–5%) who regularly consume alcohol develop AUD

(World Health Organization, 2011). Alcohol dependence is a multifactorial disorder and various factors, including the age at which people start drinking, the presence of comorbid psychiatric conditions, the use of other substances of abuse, environmental factors and mental, social, and cultural status can contribute to the development and progression of AUD (Sloboda et al., 2012). Nevertheless, genetic factors are key elements in shaping the vulnerability to AUD. Indeed, genetic predisposition is estimated to contribute to approximately 50–60% of the vulnerability to AUD and well-designed human and animal studies have clearly shown that individuals may be genetically predisposed to AUD, although this does not exclude the importance of environmental factors (Bierut et al., 2002; Edenberg, 2002). Several environmental factors may be associated with the propensity to take drugs including proximal factors (e.g., parental drug use and dependence, parental depression and peer influences) and structural factors (e.g., socioeconomic status) (Sloboda et al., 2012). Importantly, stress also increases the risk for drug addiction and relapse (Koob and Volkow, 2016). Considering that treatment strategies for addiction are currently limited in number and efficacy (Pierce et al., 2012; van den Brink, 2012; Koob et al., 2009), understanding the factors and mechanisms that define the individual variability in the propensity for AUD is essential to prevent and treat this disorder.

Many definitions have been proposed for alcoholism that can shift from a social to a psychiatric framework. Alcoholism, and more generally drug addiction, can be defined as a chronically relapsing disorder characterized by excessive drug (alcohol) use, loss of control over its consumption and emergence of a negative emotional state (dysphoria, anxiety, irritability) when access to the drug is prevented (Koob, 2013). As we stated above, in most regions of the world, adults consume alcohol at least occasionally and in a controlled manner, but only a subpopulation of individuals (3–5%) who have experienced alcohol develop the pathology (World Health Organization, 2011). In fact, clinical and preclinical evidence suggests that the occasional but limited use of alcohol is distinct from escalated alcohol intake that characterize alcohol dependence. Generally, drug addiction starts with the recreational use of a drug of abuse in a social context. The initial decision to take drugs is voluntary for the majority of people, but repeated drug use leads to multiple and substantial neuroadaptations that alter the individual ability to self-control and persist beyond acute withdrawal triggering relapse and defining the alcohol dependent state (Koob, 2014; Koob, 2013). Alcohol addiction can be characterized by daily episodes or prolonged days of heavy drinking to constant drinking for fear of withdrawal, similar to opioids. Many individuals with AUD continue with a binge/withdrawal pattern for extended periods; for others, the pattern evolves into an opioid-like substance use disorder, in

which they must always have alcohol available to avoid the consequences of abstinence (Koob et al., 2019).

Using animal models, research has made clear that AUD as well as drug addiction is based on pathological changes in brain function, specifically within the mesocorticolimbic dopaminergic pathway. Alcohol and drugs effects require dopamine (DA) signaling in the mesolimbic pathway, specifically in neurons of the ventral tegmental area (VTA) that project to the the nucleus accumbens (NAcc) (Yang et al., 2018; Lammel et al., 2012; Di Chiara and Imperato, 1988). This mesolimbic pathway has also long been implicated in the mediation of both the anticipation of and the reinforcing effects of many natural reinforcers, including food (Kelley and Berridge, 2002). Alcohol dependence also involves brain regions related to the control of stress and emotionality, including the basal ganglia, prefrontal cortex (PFC) and extended amygdala (Koob and Volkow, 2016). Different mechanisms (genetic, epigenetic, cellular), psychological constructs and psychiatric symptoms (like craving and dysphoria) associated with drug addiction have been investigated (Koob and Le Moal, 1997).

Drug addiction can be conceptualized as a three-stage (*binge/intoxication*, *withdrawal/negative affect* and *preoccupation/anticipation*), recurring cycle that worsens over time and involves neuroplastic changes in the brain reward, stress, and executive function systems and interacts with each other, becoming more intense and ultimately leading to the pathological state known as addiction (Koob, 2013). In the *binge/intoxication*, the individual consumes drugs and experiences its pleasurable effects. Activation of the brain reward system establishes the emotional attachment of the subject to the drug (Molina and Nelson, 2018). In *withdrawal/negative affect state* the individual stops taking drugs and negative feelings, such as irritability, anxiety, dysphoria, stress and loss of motivation for natural rewards (eg, food, sex, reading, social relationships) begin to appear (Koob and Volkow, 2010). The term *hyperkatifeia* (derived from the Greek word *katifeia* for dejection, sadness, or negative emotional state) refers to the emotional distress associated with withdrawal from abused drugs and is defined as the increased intensity of negative emotional/motivational symptoms and signs observed during withdrawal. Hyperkatifeia reflects a pathological change in the emotional "set point" of addicted individuals and is analogous to the term *hyperalgesia* (Shurman et al., 2010). This negative emotional state brings the subject to fall into the third stage of the addiction cycle, the *preoccupation/anticipation*, in which the addicted person faces up with the craving of seeking-taking the drug again (Sliedrecht et al., 2019; Koob and Volkow, 2010). Drug craving is defined as the desire for previously experienced effects of the substance. This desire can become imperative and can increase in the presence of both internal and external cues,

particularly with perceived substance availability. Craving can be divided in *craving for reward* that is induced by drug priming or stimuli that have been paired with drug intake, such as environmental cues, and c*raving for relief* is triggered by an acute stressor or a negative affective state that produces relapses to excessive drug intake (Koob, 2013).

Alcoholism and more generally drug addiction is a reward deficit disorder and the emergence of a negative emotional state plays an important role in defining and exacerbating the disorder (Koob, 2014). The motivation to drink alcohol is initially driven by positive reinforcement mechanisms and alcohol is consumed for its pleasurable effects. Reinforcers were defined by Skinner as any stimulus that can increase the probability of a response and may produce an increase in frequency or duration of responding or a decrease in latency to respond (Skinner, 1938). Reinforcements can be positive or negative (Edwards, 2016). *Positive reinforcement* means that a situation has beneficial outcomes such as pleasure or reward. *Negative reinforcement* can be defined as a process by which removal of an aversive stimulus, such as negative emotional state during drug withdrawal, increases the probability of a response. As individuals progress into AUD, alcohol consumption is driven by negative reinforcement and less by its euphoric effects (positive reinforcement) with alcohol providing relief from uncomfortable affective states. This transition is facilitated in a certain subpopulation of individuals, in which genetics, developmental and environmental factors define the individual vulnerability to develop alcoholism (Edwards, 2016; Koob, 2013). At the neurochemical level, the positive reinforcing effects of alcohol are primarily mediated by dopamine, opioid peptides, serotonin, γ-aminobutyric acid (GABA), and endocannabinoids, while negative reinforcement involves increased recruitment of corticotropin-releasing factor (CRF) and glutamatergic systems and down-regulation of GABA transmission. Long-term exposure to alcohol causes adaptive changes in several neurotransmitters, including GABA, glutamate, and norepinephrine, among many others. Discontinuation of alcohol ingestion results in the nervous system hyperactivity and dysfunction that characterizes alcohol withdrawal (Koob and Volkow, 2016).

Alcohol and drug addiction also has aspects of both impulse control disorders and compulsive disorders. Notably, *impulsivity* is defined behaviorally as a predisposition toward rapid, unplanned reactions to internal and external stimuli without regard for the negative consequences of these reactions to themselves or others (Moeller et al., 2001). Impulsivity is often measured in two domains: the choice of a smaller, immediate reward over a larger, delayed reward (Rachlin and Green, 1972) or the inability to inhibit behavior by changing the course of action or to stop a response once it is initiated (Logan et al., 1997). Impulse control

disorders are characterized by an increasing sense of tension or arousal before committing an impulsive act and pleasure, gratification, or relief at the time of committing the act. Thus, impulse control disorders are largely associated with positive reinforcement mechanisms (Koob, 2013). Conversely, *compulsivity* includes elements of behavior that result in perseveration in responding in the face of adverse consequences or in the face of incorrect responses in choice situations or persistent reinitiation of habitual acts (Everitt and Robbins, 2005). Compulsive disorders are characterized by anxiety and stress before committing a compulsive repetitive behavior and relief from stress by performing the compulsive behavior. As such, compulsive disorders are largely associated with negative reinforcement mechanisms and automaticity (Koob, 2013). In this regard, in the composite addiction cycle impulsivity often dominates at the early stages whereas impulsivity combined with compulsivity governs at the later stages. As an individual moves from impulsivity to compulsivity, a shift occurs from positive reinforcement driving the motivated behavior to negative reinforcement (Koob, 2004). Lastly, as we stated above, the transition from initial alcohol use to dependence is associated with multiple adaptations that occur in the brain resulting in diminished brain reward circuitry (*within-system* neuroadaptations) and a recruitment and potentiation of hypothalamic and extrahypothalamic stress neurocircuitry (*between-system* neuroadaptations). Particularly, the extended amygdala represents a core anatomical substrate for alcohol dependence which integrates brain arousal-stress systems with hedonic processing systems contributing to the preponderant role of emotional distress and negative affect that characterize withdrawal contributing to the perpetuation of the pathology (Koob, 2013; Edwards and Koob, 2010).

2. Stress

Stress is a common experience in our daily lives which is responsible for causing or exacerbating many diseases, including AUD and substance use disorder. The term stress has an ambiguous meaning that can lead to misunderstanding and it is generally regarded with a negative light. Stress normally refers to those experiences that are challenging emotionally and physiologically, that push us beyond our ability to successfully cope with and result in physiological and/or behavioral responses (McEwen, 2006; McEwen, 2007). The state of stress, specifically the condition generated by the experience of the stressor and the stress response together, is essential for survival and the organism needs the normal stress response to face such threatening situations (De Kloet, 2019). The common definition of stress generally focuses on acute challenges as well as on the classical “fight or flight response”. Indeed, the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the “fight-or-flight” response is the classical

way of envisioning the behavioral and physiological response to a threat of a dangerous situation (McEwen, 2007). However, stress represents a more complex and wider concept.

One way to classify stress is as "good stress", "tolerable stress" and "toxic stress". We generally refer to "good stress" or "eustress" to those experiences that are limited in duration and that a person can master. Good stress includes experiences of rising to a challenge, taking a risk and feeling rewarded by a positive outcome. "Tolerable stress" refers to those situations where bad things happen, but the individual with healthy brain architecture is able to cope. Finally, "bad stress" or "toxic stress" refers to experiences where a sense of control and mastery is lacking and which are prolonged or recurrent, emotionally irritating and physically exhausting. In this situation the degree and/or duration of stress might be greater (McEwen, 2017; McEwen, 2007). Protection and damage as well as *allostasis* and *allostatic load* represent the two contrasting sides of the physiology of the stress response. All living organisms tend towards a dynamic equilibrium which is called homeostasis. In the classical stress concept, the equilibrium is threatened by certain physical and psychological events that are known as stressors (De Kloet, 2005; De Kloet, 1998; Chrousos and Gold, 1992). Homeostasis means the physiological state which the body maintains to keep us alive. In a changing social and physical environment, the brain and the body respond physiologically in order to adapt. In order to maintain homeostasis, our body activates hormone secretion and turns on our autonomic and central nervous system to help us to adapt (McEwen, 2019). *Allostasis* is an essential component in maintaining homeostasis and its concept was introduced by Sterling and Eyer in 1988 applied to the cardiovascular system (Sterling and Eyer, 1988). The term allostasis refers to the process of maintaining stability (homeostasis) by active means, specifically through the secretion of stress hormones and other mediators (McEwen, 1998). Adaptation is promoted by HPA axis activation, autonomic nervous system, metabolic system and the immune system and the physiological responses of all these systems lead to adaptation and protection of the organism to challenges. In the allostatic process a host of mediators (e.g., cortisol, noradrenaline) are released in a coordinated manner in order to promote adaptation and survival. They operate nonlinearly and influence each other, so that abnormal activity of one mediator might perturb the rest of the network (McEwen, 1998; McEwen, 2019). However, the same mediators have biphasic effects and can also promote pathological states. Indeed, when they are overused or when their activity is out of balance or when they do not respond adequately, they can cause damage leading to the condition called *allostatic load* (McEwen, 1998). The concept of *allostatic load* refers to the wear and tear of the body that results from the chronic overuse and imbalance of the mediators and can lead to disease over long periods (McEwen, 1998; McEwen,

2019). Allostatic load refers to an imbalance in systems that promote adaptation. This imbalance can simply be the result of too much repeated stress, but it can also be the result of adaptive systems that are out of balance and fail to shut down or systems that fail to turn on adequately. The shut-off of the stress-response is particularly important, because when systems do not shut off in time, they can cause damage promoting pathology. On the contrary, when systems do not respond adequately, there are other systems whose activity is elevated because they are normally counter regulated. (McEwen, 1998).

Allostasis and allostatic load recognize the central role of the brain in the response to stress and the resulting health-promoting and health-damaging behaviors. The brain is the organ that interprets such experiences as threatening or nonthreatening and which determines the behavioral and physiological responses to each situation. Besides the hypothalamus and brainstem, which are essential for autonomic and neuroendocrine responses to stressors, higher cognitive areas of the brain play a key role in memory, anxiety, and decision making. These brain areas are targets of stress and stress hormones, and the acute and chronic effects of stressful experiences influence how they respond. This is particularly evident over the life course, where early life experiences, combined with genetic factors, exert an important influence on adult stress responsiveness and the aging process (McEwen, 2007; McEwen, 1998). The elucidation of the underlying mechanisms of plasticity and vulnerability of the brain provides a basis for understanding the efficacy of interventions for stress-related disorders.

3. Stress and AUD

Stress has long been proposed as an important contributing factor for uncontrolled alcohol drinking and relapse following a period of abstinence (Stephens and Wand, 2012; Becker, 2012). Large epidemiological studies have reported that a variety of stressor are associated with increased alcohol consumption, binge drinking and AUD diagnosis (Keyes et al., 2012). Stress plays a key role in the transition from recreational alcohol use to dependence and represents one of the main triggers for relapse. Indeed, the transition from moderate to compulsive alcohol drinking is associated with the occurrence of several neuroadaptations within the mesocorticolimbic dopaminergic and stress systems resulting in diminished brain reward function and recruitment and potentiation of extrahypothalamic stress systems. Consequently, when individuals progress into AUD, alcohol consumption is more driven by negative reinforcement, with alcohol providing relief from uncomfortable affective states (Edwards and Koob, 2010). In fact, the brain stress response system is hypothesized to be activated by acute

excessive alcohol intake and to be sensitized during repeated withdrawal contributing to the development of the disease (Koob, 2013).

Although stress is known to be an important contributing factor to alcohol abuse, the relationship between stress exposure and alcohol drinking is complex, bidirectional and difficult to determine. Alcohol has potent anxiolytic effects and motivation for drinking is related to its ability to alleviate stress, including stress associated with periods of abstinence (Becker, 2012). It is generally acknowledged that stress increases alcohol drinking and the probability to relapse (Barchiesi et al., 2021; Mantsch et al., 2016; Norman et al., 2015; Lê et al., 2005). On the other hand, alcohol is also a potent stressor since it can activate the hypothalamic-pituitary-adrenal (HPA) axis, which represents the primary neuroendocrine network involved in the stress response (Richardson et al., 2008; Smith and Vale 2006). Additionally, this reciprocal interaction is complicated by an interplay between numerous biological (e.g., sex, age, genetics) and environmental variables that defines the subjective aspects of stress (e.g., perception of a stressful event) and how it impacts the susceptibility to develop alcoholism (Becker, 2012). For instance, clear sex differences exist in sensitivity to, perception of and responsiveness to stress and alcohol (Flores-Bonilla and Richardson, 2020). Particularly, studies suggest greater susceptibility of females to stress-induced potentiation of alcohol consumption with traumatic experiences more often preceding AUD development and associated with craving and relapse in females vs males (Guinle and Sinha, 2020). Indeed, women are more likely to drink alcohol as a stress-coping mechanism than men with the same diagnosis to attenuate negative affective states (e.g., anxiety, depression, post-traumatic stress disorder) (Guinle and Sinha, 2020; Peltier et al., 2019; Crutzen et al., 2013). Moreover, women are more likely to relapse in response to stressful events (Hyman et al., 2008; Greenfield et al., 2007).

Despite the complex reciprocal relationship existing between stress and alcohol, it is generally acknowledged that stressful life events influence alcohol drinking and relapse. The next sections will examine the HPA axis, one of the main biological stress systems involved in stress-alcohol interaction, summarizing the main findings from animal studies. It is well acknowledged that stressful life events, especially experienced during childhood and adolescence, a developmental period characterized by extensive reorganization of the brain, may increase the susceptibility to develop psychiatric disorders later in life, including alcoholism (Enoch, 2012; Enoch, 2011; Young-Wolff et al., 2012). Then, animal data concerning the impact of adverse early life experiences on adult ethanol intake will be presented.

3.1 Role of hypothalamic-pituitary-adrenal axis and glucocorticoids in alcohol dependence

The hypothalamic-pituitary-adrenal (HPA) axis is one of the main physiological stress response pathways and has been studied extensively in relation to alcohol dependence. Stress, dysregulation of the HPA axis and alteration in glucocorticoid receptor function have been linked to transition from recreational alcohol use to alcohol dependence (Stephens and Wand, 2012; Edwards et al., 2015). Exposure to stressors initiates the activation of the HPA axis, which results in the release of corticotropin releasing factor (CRF) from neurons in the paraventricular nucleus (PVN) of the hypothalamus, which leads to increased release of adrenocorticotropic hormone (ACTH) in the pituitary gland. The ACTH, in turn, induces glucocorticoids synthesis and secretion from the adrenal glands. Cortisol in humans and corticosterone (CORT) in rodents are the primary hormones released by the adrenal cortex in response to ACTH. Upon release, they bind to high-affinity mineralocorticoid receptors (MR) and low-affinity glucocorticoid receptors (GR) and mediate an array of physiological effects in order to restore homeostasis. The stress response is then terminated through a negative feedback mechanism on the HPA axis when glucocorticoids bind to GR. The HPA system is carefully modulated through negative-feedback mechanisms designed to maintain predetermined hormone levels and homeostasis and to prevent adverse consequences to health (Herman and Cullinan, 1997; Reul and De Kloet, 1985).

The motivation to drink alcohol is initially driven by positive reinforcement mechanisms and its consumption is usually linked to recreational purposes. Both clinical and preclinical evidence have demonstrated that glucocorticoids are reinforcing themselves. Indeed, elevated glucocorticoid levels resulting from HPA axis activation may interact with the mesocorticolimbic dopaminergic system in NAcc to give incentive value to drugs and increasing drug consumption in early stages of addiction (Piazza and Le Moal 1997; Deroche et al., 1997). Consistently, studies in rodents have demonstrated that central and systemic administration of CORT has been shown to increase alcohol drinking, whereas adrenalectomy or administration of the corticosteroid synthesis inhibitor metyrapone decreased alcohol intake in rodents. Noteworthy, alcohol drinking was recovered by corticosterone replacement suggesting that glucocorticoids facilitate alcohol reinforcement in the early stages (Fahkle, 1999; Fahkle et al., 1995; Fahkle et al., 1994a; Fahkle et al., 1994b). Likewise, alcohol acts as a stressor activating the body's stress response systems. Indeed, rodent studies have demonstrated that acute alcohol exposure (experimenter-administered or self-administered) stimulates corticosterone release, mimicking a stressor (Richardson et al., 2008; Lee et al.,

2001). Excessive alcohol use produces stress-related neuroadaptations primarily at the level of the HPA axis, but also in extrahypothalamic brain regions, such as central amygdala (CeA) and PFC, contributing to disease progression and to the onset of dependence (Edwards et al., 2015; Tunstall et al., 2017; Richardson et al., 2008). Earlier studies have demonstrated that alcohol dependent rats exhibited significant downregulation of GR during acute withdrawal and GR upregulation during protracted abstinence in several stress/reward related brain areas, suggesting that GR system may contribute to the progression of AUD. Importantly, administration of mifepristone, a non-selective GR and progesterone antagonist, systemically or within the CeA, reduced escalation of alcohol drinking in alcohol dependent rats (Vendruscolo et al., 2015; Vendruscolo et al., 2012). Likewise, intra-CeA infusion of mifepristone has been reported to attenuate stress-induced reinstatement of alcohol seeking, suggesting that glucocorticoids act on specific brain regions to modulate alcohol relapse-like behavior (Simms et al., 2012). The transition from social drinking to dependence is associated with disruption of the HPA axis resulting in a dampened capacity to cope with stress. Consistently, Richardson et al. demonstrated that long-term exposure to alcohol causes significant impairment of HPA axis function in adult male Wistar rats. Acute alcohol stimulated the release of CORT and its upstream regulator (ACTH), but chronic alcohol exposure produced dependence leading to a dampened neuroendocrine state (Richardson et a., 2008). Similar findings were observed in humans (Blaine and Sinha, 2017). A number of studies have reported that binge/heavy drinkers show a blunted cortisol response to an alcohol challenge, compared to moderate social drinkers (Allen et al., 2011; Mick et al., 2013). Additionally, acute withdrawal from chronic intermittent ethanol vapor exposure produced robust GR alterations signaling within PFC and enhanced relapse vulnerability indicating that transition to alcohol dependence is accompanied by alterations in PFC stress-related pathways that may intensify negative emotional symptoms and increase the vulnerability to relapse (Somkuwar et al., 2017). Circulating glucocorticoids also appear to sensitize signaling in central stress circuitry, apparently in opposition to its effects to dampen neuroendocrine mechanisms. In contrast to reducing CRF expression in the PVN, high corticosterone levels increase CRF gene expression in the CeA (Tunstall et al., 2017). Several findings have demonstrated that CRF receptor neurotransmission is dysregulated in alcohol dependence and may directly and/or by mediating withdrawal-related anxiety and stress/dysphoria responses influence the motivation to increase alcohol consumption. Indeed, it has been reported that increases in extrahypothalamic CRF signaling drives compulsive drinking (Makino et al., 1994; Shepard et al., 2000). The CRF1 receptor antagonists antalarmin, MJL-1-109-2, R121919, and MPZP selectively decreased

alcohol drinking in dependent rats (Funk et al., 2007; Richardson et al., 2007). Selective blockade of alcohol drinking in dependent rats was also reported using intracerebroventricular (Valdez et al., 2002) or direct injections of the CRF1/2 receptor antagonist D-Phe-CRF12–41 in the CeA (Funk et al., 2006). Here, intra-CeA but not intra-NAC or intra-bed nucleus of the stria terminalis (BNST) injections of CRF antagonists decreased the escalation of alcohol drinking in dependent but not in nondependent rats (Funk et al., 2006).

Collectively, all these findings suggest that multiple neuroadaptations occur within the stress regulatory systems by excessive alcohol drinking contributing to the negative affective state experienced during withdrawal and exacerbating the pathology. Moreover, impairment of HPA axis function could negatively impact overall health by decreasing the ability to respond appropriately to internal or environmental challenges.

3.2 Role of early-life stress and maternal separation on vulnerability to develop alcoholism

Since stress can influence ethanol addiction processes, early stressful experiences during the postnatal period represent a critical predictor of excessive alcohol consumption later in life. AUD is a multifactorial disorder and environmental influences, particularly early in life, are crucial for the development of the individual by shaping the behavior and affecting brain function and have a profound impact on the risk to develop psychopathology and alcohol dependence across the life course (Branchi and Cirulli, 2014; Nylander and Roman, 2013). In humans, early-life stress can be grouped into childhood maltreatment (childhood sexual abuse, physical and emotional abuse, physical and emotional neglect) and stressful life events and these two types of stressors often co-occur (Green et al., 2010). Exposure to stressful life events is common in the general population. It has been reported that 53% of adults had experienced some kind of stressor before age 18, most commonly parental divorce or death, family violence, economic adversity, or mental illness (Green et al., 2010). Although half of adults have been exposed to some kind of childhood stressor, only 26% of them developed substance use disorders (Green et al., 2010). Further, numerous studies have shown that the effect of early life stress and childhood maltreatment is greater in women who are more susceptible to develop AUD in adulthood (Anda et al., 2002; Dinwiddie et al., 2000).

Multiple environmental factors through the prenatal period, childhood and adolescence interact with genetic factors through epigenetic mechanisms and shape the brain. Interference with these vital processes, for example by emotional and social stress, can cause long-term neurobiological and behavioral changes, affect alcohol-induced reward and addiction processes and thereby result in enhanced vulnerability for AUD (Nylander and Roman, 2013). In the early stages of

life, the brain is extremely receptive to external stimuli and it adapts to the surrounding environment in order to best tune the developing individual to it (Cirulli et al., 2010). The impact of the environmental stimuli could have either benefits, allowing the individual to correctly and properly respond to its surroundings, or harmful consequences, like the increased risk to develop psychiatric disorders in adulthood associated with the exposure to stressful situations in early-life (Marco et al., 2013).

Maternal separation (MS) of rodent pups is a widely used paradigm to examine the long-term consequences of early-life stress on brain development and resilience to psychopathology. MS has demonstrated to affect brain growth and development and to evoke anxiety-like and depressive behaviors, to decrease social interactions as well as affect drug-taking behaviors, including ethanol intake (Bonapersona et al., 2019; Nishi et al., 2014; Nylander and Roman, 2013; Moffett et al., 2007; Lippmann et al., 2007). The relationship between the mother and the offspring is an important environmental factor during early development in mammals and newborn mammals are depending on their mothers for survival and normal development. Indeed, it has been shown that maternal behavior is important for epigenetic programming, a mechanism that can explain the long-lasting effects of maternal care on gene-expression of the offspring (Roman and Nylander, 2005). During postnatal days, rodents exhibit a Stress Hyporesponsive Period (SHRP), during which the activity of the HPA axis is lower than normal. The maintenance of the SHRP is guaranteed by the contact with the mother in order to encourage a correct development of the central nervous system (CNS) (Levine, 2001; Roman and Nylander, 2005). When this relationship is interrupted by repeated MSs, the HPA axis may be reactivated, increasing the blood levels of glucocorticoids in the brain and then stimulating the brain GRs with potential long-lasting effects in neurobiology and behavior (Nishi, 2020). As a result, isolating a developing pup from its mother causes not only emotional stress, but also results in physical stress, such as thermal stress, malnourishment and inability to express bodily waste. Moreover, interruption of normal mother-pup interaction has been reported to induce persistent changes in the neurobiology, physiology and emotional behavior in adolescent and adult animals, due to dysregulated programming of HPA axis responsiveness (Nishi, 2020). Additionally, over development, the dependence of the pups on their dam for emotional and physical support also changes. As pups become independent, their source of nutrition changes, they become able to independently thermoregulate, urinate, and defecate, and their social circle expands beyond their primary caregiver (Thompson et al., 2020).

Several different protocols of MS have been proposed and their outcomes depend on developmental stage, duration (e.g., 15min to 24h), and number of days (e.g.,1 to 21 days) of

the separation experience (Nishi, 2020; Roman and Nylander, 2005; Nylander and Roman, 2013). Briefly, two main protocols of MS are currently in use, including short and prolonged MS. A separation for a short period of time (15min), also referred as "early handling", can be viewed as mimicking the behavior of wild rats where the mother has to leave the nest to collect food. Upon reunion, after a short period of MS, the mother shows an increased intensity of maternal behavior, with more liking and grooming of her pups (Macrì et al., 2008; Macrì et al., 2004; Roman and Nylander, 2005). This paradigm might be a more ethological parenting behavior and seems to exert a protective effect on the offspring (Nishi, 2020). As a result, handling has been demonstrated to reduce depression, anxiety-like behaviors and stress responses and to promote greater ability to cope with stress throughout life (Bondar et al., 2018; Rana et al., 2015; Levine, 2005; Plotsky et al., 2005). Conversely, repeated prolonged periods of MS involve separations of >60 min, most often >180 min, at very early time points during the first two postnatal weeks and are used to stimulate a risk environment and result in effects that are opposite from those observed with the handling procedure (Nishi, 2020; Nylander and Roman, 2013; Roman and Nylander, 2005; Kuhn and Schanberg, 1998). Notably, many studies using the prolonged MS procedure have reported that separation of pups from their dam during postnatal period exacerbates depressive-like and/or anxiety-like behaviors in adulthood (Huot et al., 2004; Huot et al., 2001; Menard et al., 2004). Several lines of evidence have shown that postnatal environmental influences are essential for normal development of many neurotransmitters including those involved in brain stress/reward function and disruption of mother-infant interaction has been directly correlated with increased risk toward drug dependence and alcoholism (Roman and Nylander, 2005). Indeed, many long-term neurochemical alterations in brain reward and stress systems after MS procedures have been described and might be linked to higher predisposition for ethanol dependence later in life (Gondré-Lewis et al., 2016; Whitaker et al., 2013; Plotsky et al., 2005). For instance, early-life stress in rats has been demonstrated to result in profound and lasting changes in the responsiveness of dopamine neurons to stress and drugs in adulthood (Brake et al., 2004; Meaney et al., 2002).

A number of studies has assessed the impact of different protocols for MS during the postnatal period on later voluntary ethanol intake in rats. Generally, short daily MS (15min) seem to provide a protective environment and animals show low propensity to start drinking ethanol in a two-bottle choice (2BC) paradigm as an adult (Gustafsson and Nylander, 2006). A prolonged MS (360 min) might provide an emotionally disturbed environment, which is indicated by the finding that most rats respond to this environmental manipulation with an enhanced propensity

to drink alcohol (Amancio-Belmont et al., 2020; De Almeida Magalhães et al., 2017; Odeon et al., 2017; García-Gutiérrez et al., 2016; Palm et al., 2013; Nylander and Roman, 2013; Moffett et al., 2007). However, the results from currently used MS protocols are not uniform and no differences in alcohol consumption or inconsistent results have been reported in some studies that used different MS paradigms (litter-wise vs individual pup), other controls, different alcohol-drinking models or low ethanol concentrations as well as the species, sex and age of the experimental animals (Nylander and Roman, 2013).

Collectively, all these findings indicate that adverse early life experiences affect mental health, elevating the risk for stress-related disorder as well as alcohol dependence later in life. Numerous rodent studies have been conducted to investigate the influence of early life stress on brain function and to elucidate the underlying mechanisms of increased risk to psychopathology. It is well recognized that prolonged MS is associated with increased ethanol intake and preference in rodents. However, given some contradictory results, it is important to establish robust experimental models to overcome the limitations of the rodent MS paradigm and to identify and characterize the neurobiological mechanisms associated with enhanced vulnerability to drug and alcohol abuse.

4. Marchigian Sardinian alcohol-preferring rats

Genetically-selected Marchigian Sardinian alcohol-preferring (msP) rats have long been proposed as a unique animal model of innate excessive alcohol drinking and preference and anxiety disorder and have been used to study the neurobiology of alcoholism (Borruto et al., 2021; Ciccocioppo et al., 2006). They have been selectively bred for high alcohol preference and consumption at the University of Camerino (Marche, Italy) beginning from the 13th generation of sP rats that were originally developed at the University of Cagliari, Sardinia, Italy. Several studies have been conducted in order to characterize their behavior and to explore their neurobiology, genetics and response to drug treatment. Firstly, it has been shown that msP rats consume pharmacologically relevant daily doses of alcohol (7-8 g/kg) when exposed to a standard home-cage two-bottle choice (2BC) paradigm. Drinking is organized in three main episodes and occurs mostly during the dark phase (active phase) of the light/dark cycle, during which they consume around 80% of their daily alcohol reaching blood alcohol levels (BALs) around 70–80 mg/dl but can peak over 100 mg/dl to produce pharmacologically meaningful effects (Ciccocioppo et al., 2006). Msp rats, differently from other animals, do not show spontaneous aversion to alcohol and voluntarily drink large amounts of alcohol from the very first day of home cage presentation (Ciccocioppo et al., 2006). When they are subjected to

operant alcohol self-administration, msP rats display a higher rate of ethanol responding under both fixed ratio 1 (FR-1) that is acquired spontaneously and much faster than unselected Wistar controls (Domi et al., 2019; Cannell et al., 2016; Ayanwuyi et al., 2013). Moreover, under a progressivo ratio (PR) schedule of reinforcement, msP rats reach a significantly higher breakpoint for alcohol, indicating higher motivation for alcohol (Ciccocioppo et al., 2006; Domi et al., 2019). Additionally, msP rats have higher vulnerability to resume extinguished ethanol seeking when exposed to stimuli that predict alcohol availability or in response to stress. Indeed, they showed higher magnitude and persistence of the reinstating effect of alcohol-associated cues compared to heterogeneous Wistar rats (Cannella et al., 2016; Ayanwuyi et al., 2013; Ciccocioppo, 2013; Stopponi et al., 2013). The msP rat line is also characterized by high sensitivity to stress, which may contribute to their excessive alcohol drinking phenotype (Hansson et al., 2006). Notably, they show an anxious phenotype and depressive-like symptoms that are attenuated after ethanol consumption (Ciccocioppo et al., 2006). Altogether, these findings suggest that ethanol drinking in msP rats is likely motivated by negative reinforcement, specifically mimicking a large subpopulation of alcoholic patients in which alcohol is consumed to ameliorate negative affective symptoms that are associated with anxiety and depression (Koob and Le Moal, 2005).

Over the years, several studies have investigated genetic factors that are responsible for the high alcohol drinking phenotype of msP rats (Ciccocioppo, 2013; Hansson et al., 2007; Ciccocioppo et al., 2006; Hansson et al., 2006). MsPs carries two single-nucleotide polymorphisms at CRF1 receptor locus leading to CRF1 receptor overexpression in brain areas associated with negative affect, such as the amygdala (Ayanwuyi et al., 2013; Cippitelli et al., 2015; Hansson et al., 2006; Logrip et al., 2018). As a consequence of this overexpression, msP rats exhibited a high anxious-like phenotype, were sensitive to stress, and had depression-like symptoms that were all improved by alcohol drinking (Ciccocioppo, 2013; Ciccocioppo et al., 2006). Importantly, voluntary alcohol consumption attenuated the overexpression of CRF1 receptors in various brain areas, suggesting the possibility that these animals drink to alleviate negative symptoms that are associated with an overactivity of the stress system (Hansson et al., 2007). Consistently, the CRF1 receptors antagonist antalarmin reduced alcohol self-administration in msP rats but not in unselected Wistar controls (Hansson et al., 2006).

Recently, given the importance of studying sex differences in animal models of AUD, the behaviour and neurobiology of female msP rats was extensively characterized (Borruto et al., 2021; Vozella et al., 2021; Benvenuti et al., 2021; Natividad et al., 2021). Indeed, Borruto et al. found that both naive male and female msP rats exhibited higher anxiety and depressive-like

behavior compared to Wistar rats in the elevated plus maze test and forced swim test. However, alcohol drinking reduced anxiety-like behavior only in males but not in females in the elevated plus maze test and reduced immobility time in msP rats in both sexes (Borruto et al., 2021). Lastly, msP rats of both sexes showed longer freezing times in response to foot-shock stress compared to unselected Wistar rats and alcohol consumption attenuated the freezing time in both in male and female msP rats (Borruto et al., 2021). Given these observations, we suppose that male and female msP rats are both characterized by innate negative affect traits that co-segregated with alcohol drinking during the selection process. However, the motivation for alcohol in males is probably linked to its ability to attenuate anxiety, while in female rats, alcohol drinking appears to be linked to its antidepressant (and possibly anti-stress) properties (Borruto et al., 2021). This hypothesis is consistent with human data that show that psychiatric comorbidity is different between male and female AUD patients. For example, female alcohol abusers are more likely than men who abuse substances to be diagnosed with post-traumatic stress disorder (Cottler et al., 1992; Kessler et al., 1995). Although further investigations are needed to better characterize the impact of sex differences on alcohol abuse-related behaviors of msP rats, these preliminary findings corroborate the observation that alcohol-related sex differences present in AUD patients can be detected also in msP rats, supporting the translational value of this animal model.

5. Classic hallucinogens for AUD and stress-related disorders therapy

"Classic hallucinogens" are powerful psychoactive substances that alter perception and mood and affect numerous cognitive processes and include mescaline, LSD and psilocybin (Nichols, 2004). The psychoactive effects of all classic hallucinogens appear to depend primarily on their agonist or partial agonist activity at 5-hydroxytryptamine 2A (5HT2A) receptors (Vollenweider and Kometer, 2010; Nichols, 2004). Although classic hallucinogens have been used by humans for at least 5 millennia and they were employed by early cultures in many sociocultural and ritual contexts, they are known to science for over a century, when mescaline was isolated and its effects described by Arthur Heffter. In 1943, Albert Hofmann discovered the psychoactive effects of lysergic acid diethylamide (LSD) when he accidentally ingested traces of an ergot derivative he had synthesized and confirmed the effects through self-experimentation. Following Gordon Wasson's report of hallucinogenic mushroom use by the Mazatec tribe in Mexico, Hofmann isolated psilocybin from samples of the mushrooms and synthesized it in the laboratory (Bogenschutz and Ross, 2018). After an interruption of almost forty years, there have

been numerous developments in serotonergic psychedelics' research and then in the clinical field in the past decades (Nichols et al., 2004). One of the main classic psychedelics that has been the focus of recent research is psilocybin, a psychoactive alkaloid contained in hallucinogenic Psilocybe mushrooms dated back to 3000 years in Mexico (Carod-Artal, 2015). Psilocybin (O-phosphoryl-4-hydroxy-N,N-dimethyltryptamine) and its active dephosphorylated metabolite psilocin (N,N-dimetyltryptamine) structurally belong to the group of tryptamine/indolamine hallucinogens and are structurally related to serotonin (Hasler et al., 1997; Horita and Weber, 1961). From a therapeutic point of view, in the past 10–15 years several FDA-approved clinical studies have indicated potential medical value for psilocybin-assisted psychotherapy in treating depression and anxiety disorders (Griffiths et al., 2016; Carhart-Harris et al., 2016). Specifically, psilocybin has been shown to elicit positive changes in attitudes about life, self, mood, relationships, with increased well-being or life satisfaction (Griffiths et al., 2016). These positive effects may reflect in an overall improved quality of life, reduced odds of suicidal tendencies and a rise of treatment adherence (Davis et al., 2021). A recent small pilot study tested the effect of psilocybin in treatment-resistant major depression (Carhart-Harris et al., 2016). Patients received 10 mg oral psilocybin in the first session, and 25 mg in a second session one week later. Depressive symptoms, as measured by Quick Inventory of Depressive Symptoms (BDI), were significantly decreased at 1 week and 3 months post-treatment, when compared with baseline scores (Carhart-Harris et al., 2016). As a consequence, psilocybin-assisted therapy may provide a promising alternative for patients unresponsive to traditional antidepressant treatments (primarily selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs)).

Furthermore, recent developments in the study of classic hallucinogens, combined with a re-appraisal of the older literature, have led to a renewal of interest in possible therapeutic applications for these drugs in the treatment of addiction (Bogenschutz, 2013). Specifically, a recent study investigating psilocybin in tobacco dependence demonstrated that 80% of the participants had quit smoking after a 6-month follow up, thereby substantially exceeding success rates for other behavioral and/or pharmacological therapies (Johnson et al., 2014; Garcia-Romeu et al., 2015). Additionally, promising results have been observed in alcohol addiction, where participants typically receive 0.3-0.4 mg/kg of psilocybin in two to three sessions. A recent pilot study on the efficacy of psilocybin in alcohol dependence in alcohol-dependent patients showed a significant reduction in both percentage of drinking days and heavy drinking days with large effect sizes (Bogenschutz et al., 2015).

Currently, pharmacological therapy for AUD is based on three drugs FDA approved for AUD therapy: the acetaldehyde dehydrogenase inhibitor disulfiram, acamprosate and the opioid receptor antagonist naltrexone (Witkiewitz, 2019). However, these pharmacological treatments for alcohol dependence are limited in their effectiveness to only certain subgroups of patients (Litten et al., 2018) and, therefore, new therapies are urgently needed. The hallucinogen psilocybin may provide a new treatment option for AUD patients, given the beneficial results observed in this recent evidence.

6. Summary, Research Objectives and Significance:

AUD represents a major public health problem and results in significant disability and morbidity among individuals. Despite the extensive disease burden with which it is associated, alcohol use continues to be widespread. The actions of alcohol on the nervous systems are complex and the reasons for using alcohol may differ between individuals and situations. Different factors influence alcohol abuse vulnerability, including sex, age, genetics/family history, environment, copresence of other psychiatric disorders and stress. Several lines of evidence have shown that stress can influence ethanol addiction processes including acquisition as well as maintenance and relapse. Clinical and preclinical evidence reported that stress represents a primary trigger of alcohol craving and relapse. Stress is a physiological mechanism that allows individuals to adapt and survive. However, when stress exposure is prolonged or inadequate, the stress response becomes pathological, exacerbating many diseases, including alcoholism. Alcohol is often consumed to alleviate stress and it is a potent stressor itself. Stress and chronic alcohol consumption have been demonstrated to alter the activity of brain stress system and dysregulation of these systems have effects on alcohol consumption. Numerous mechanisms have been suggested to be involved in the complex interaction between stress and alcohol dependence. Although stress is known to be an important contributing factor to alcohol abuse and alcoholism, the interaction between stress and alcohol drinking behavior, as well as the mechanisms underlying this interaction, in the context of dependence are complex and not fully understood.

The aim of the present dissertation is to provide further knowledge on the influence of stress in perpetuating alcoholism and correlated stress-related disorders by examining this complex interaction at various levels and considering different aspects of stress that might shape individual vulnerability to develop alcoholism.

First (**Chapter II**), I investigated the effect of glucocorticoid receptor antagonism on alcohol self-administration and anxiety-related behaviors in Marchigian-Sardinian alcohol preferring

(msP) rats, which represent a unique animal model of excessive alcohol drinking and anxiety disorder. HPA axis dysfunction and exposure to stress are critical components that interact to convey risk for developing AUD and stress-related comorbidities. Currently, it is unknown if the GR system might contribute to the excessive alcohol drinking and anxious phenotype of msP rats. Considering the crucial role of GR in the development of alcohol dependence and in modulating both adaptive and maladaptive stress-associated behaviors, I will explore the effects of pharmacological blockade of GR on alcohol self-administration and in a battery of tests that captures different anxiety-related features comparing the msP rat line with its Wistar counterpart. The present study will also explore sex differences.

Second (**Chapter III**), I expanded current knowledge of the influence of early-life stress on the later susceptibility to develop AUD. Environmental influences experienced during the postnatal period are crucial in shaping brain development and MS is the most widely studied stressor in the context of alcohol seeking. However, the majority of studies have conducted maternal separation at very early time points, starting at PND1. Therefore, it is unclear how stressors experienced by rodents during this very early developmental period effectively model similar stressors experienced by humans at comparatively later developmental stages. Given the importance of having appropriate animal models to overcome the limitation of preclinical studies and to reliably study the mechanisms and the mediators of early-life impact, the present study will provide a novel paradigm of environmental manipulation, that differs from the canonical procedures, both in terms of the psychological stress involved and the developmental window in which the stressor is applied. I will evaluate the effect of early life stress on later susceptibility to develop alcohol-related behaviors in male and female msP and Wistar rats.

Third (**Chapter IV**), I investigated the efficacy of the psychedelic drug psilocybin as potential therapy for AUD and stress-related disorders (e.g., post-traumatic stress disorder) in msP rats. Scientific interest in the classic psychedelic psilocybin has rapidly risen within the last decade because of clinical preliminary evidence of robust efficacy in treating anxiety and depression, as well as alcohol dependence and PTSD. Despite these promising clinical findings, scientific literature is missing preclinical studies and animal models are necessary to further understand the psychological and neurobiological mechanisms underlying psilocybin putative therapeutic effects. In light of this, I investigate the efficacy of psilocybin on alcohol-related behavior in msP rats. Notably, recent evidence suggests that msP rats also exhibit PTSD-like symptomatology. Particularly, I explore if psilocybin administration might disrupt alcohol-related memories, a treatment strategy useful to prevent alcohol relapse after a period of

abstinence or to impair trauma-related cues. Finally, the main findings and possible implications of this research are summarized and discussed in **Chapter V**.

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Chapter 2: Effect of glucocorticoid receptor antagonism on alcohol-self-administration and on anxiety-related behaviors in genetically-selected Marchigian Sardinian alcohol preferring and non-preferring Wistar rats.

ABSTRACT

Background: Genetically-selected Marchigian Sardinian alcohol-preferring (msP) rats represent a unique animal model of excessive alcohol drinking and anxiety disorder. They show innate upregulation of the corticotropin-releasing factor receptor 1 (CRF1) which correlates with heightened ethanol consumption and stress sensitivity. Stress, dysregulation of the hypothalamic-pituitary-adrenal axis (HPA) and alterations in glucocorticoid receptor (GR) function have been linked to transition from recreational alcohol use to dependence and GR is considered to play an essential role in modulating stress-associated behaviors Here, we investigated the effect of pharmacological blockade of GR on alcohol self-administration (SA) using male and female msP and Wistar rats. Furthermore, we evaluated if GR antagonism might decrease innate symptoms of anxiety in msPs.

Methods: Animals were trained to self-administer 10% (v/v) alcohol. Once stable alcohol SA baseline was reached, we tested the effect of the GR antagonists mifepristone (0.0, 10, 30 and 60 mg/kg) and CORT113176 (0.0, 10, 30 and 60 mg/kg) on alcohol SA. To evaluate whether the effects of the two compounds were specific for alcohol, the two drugs were tested on a similar saccharin SA regimen. Basal blood corticosterone (CORT) levels before and after alcohol SA were determined. Finally, we evaluated the effect of mifepristone injection (0.0 and 60 mg/kg) on novelty-induced hypophagia (NIH) assay, comprehensive lab monitoring system (CLAMS) and stress sensitivity using acoustic startle measures.

Results: Systemic injection with mifepristone dose-dependently reduced alcohol SA in male and female Wistars but not in msPs. Administration of CORT113176 decreased alcohol SA in male and female Wistars as well as in female msPs but not in male msP rats. At the highest dose, mifepristone also reduced saccharin SA in male Wistars and female msPs, suggesting the occurrence of some nonspecific effects at 60 mg/kg of the drug. Similarly, the highest dose of CORT113176 (60 mg/kg) decreased saccharin intake in male Wistars. Analysis of CORT levels revealed that females of both rat lines had higher blood levels of CORT compared to males. Additionally, we found that male and female msPs display greater anxiety-like behaviors as well as enhanced acoustic startle responses compared to Wistar counterparts. Importantly, the

enhanced anxiety-like behavior and startle responses were not ameliorated by mifepristone administration.

Conclusions: Overall, these findings indicate that selective blockade of GR selectively reduces alcohol SA and genetically selected msP rats are less sensitive to this pharmacological manipulation compared to heterogeneous Wistars. Results also suggest sex differences in the ability of alcohol to regulate GR transmission. Moreover, the increased expression of stress-related behaviors in msPs are not mediated by activation of GR system.

1. Introduction

Alcohol use disorder (AUD) is a complex psychiatric condition characterized by excessive drug use, loss of control over its consumption, and emergence of a negative emotional state during withdrawal which contribute to excessive drinking and susceptibility to relapse (Koob, 2013). AUD is a major public health problem and alcohol represents a significant disability and morbidity factor responsible for about 3 million deaths per year (Witkiewitz et al., 2019). Stress and dysregulation of related hormones of the hypothalamic-pituitary-adrenal (HPA) axis have been proposed as important factors affecting disease progression (Stephens and Wand, 2012; Edwards et al., 2015). The HPA axis represents the major component controlling stress response and its activation in response to external or internal challenges culminates in the production and release of cortisol in humans and corticosterone (CORT) in rodents (Herman and Cullinan, 1997). Once released, glucocorticoids act through either the low affinity mineralcorticoid or the high affinity glucocorticoid receptor (GR). GR are well expressed in several brain regions of the limbic system, in the paraventricular nucleus (PVN) of the hypothalamus and the anterior pituitary gland (Reul and De Kloet, 1985). Once released, glucocorticoids produce an array of physiological effects to adjust the organism to stressor exposure, and one of their roles is to terminate the stress response via negative feedback inhibitions at HPA level (Herman and Cullinan, 1997).

The motivation to drink alcohol is initially driven by positive reinforcement mechanisms and its consumption is usually linked to recreational purposes. Studies in rodent models mimicking early stages of alcohol consumption demonstrated that CORT administration increased alcohol self-administration (SA) (Fahkle et al., 1994a; Fahkle et al., 1994b; Fahkle and Hansen, 1999), whereas adrenalectomy decreased it (Fahkle et al., 1995). Noteworthy, alcohol drinking was recovered by corticosterone replacement suggesting that glucocorticoids facilitate alcohol reinforcement (Fahkle et al., 1995). As a result of chronic alcohol drinking the excessive and protracted activation of the HPA axis may lead to its dysregulation. This contributes to the surge

of compulsive alcohol drinking motivated by the need to self-medicate to attenuate the negative symptoms associated with drug withdrawal (Edwards et al., 2015; Tunstall et al., 2017; Richardson et al., 2008). Earlier studies demonstrated that alcohol dependent rats exhibited significant downregulation of GR during acute withdrawal and GR upregulation during protracted abstinence in several stress/reward related brain areas, suggesting that GR system may contribute to the progression of AUD (Vendruscolo et al., 2012).

Genetically-selected Marchigian Sardinian alcohol-preferring (msP) rats have been extensively characterized as a model of both enhanced alcohol preference and negative affective phenotypes. Ethanol drinking in msP rats is likely motivated by negative reinforcement, specifically mimicking the subpopulation of individuals who drink for tension relief and self-medication purposes (Ciccocioppo et al., 2006; Borruto et al., 2021). MsP rats carry two single nucleotide polymorphisms in the promoter region of the CRF1 receptor (CRF1-R) leading to CRF1 receptor overexpression in areas of the brain associated with negative affect such as the amygdala (Ayanwuyi et al., 2013; Cippitelli et al., 2015; Hansson et al., 2006; Logrip et al., 2018; Herman et al., 2013; Herman et al., 2016). This mutation causes innate hyperactivity of the CRF/CRF1 system, which correlates with excessive alcohol drinking and a decreased threshold for stress-induced alcohol-seeking (Hansson et al., 2006), heightened stress sensitivity and behavioral alterations that possibly resemble post-traumatic stress disorder (PTSD) traits (Cippitelli et al., 2015) and higher sensitivity to CRF1-R antagonists (Hansson et al., 2006; Ayanwuyi et al., 2013; Cippitelli et al., 2015). Noteworthy, these gene polymorphisms are conserved in the human CRF system and have been correlated with the diagnosis of AUD (Blomeyer et al., 2008; Treutlein et al., 2006). It has also been reported that male msP rats displayed dysregulated GABA and glutamate signaling (Herman et al., 2013; Herman et al., 2016; Natividad et al., 2017).

Recently, it has been found that male msP rats displayed diminished stress-induced GR phosphorylation at the serine site 232, a site that is functionally associated with higher transcriptional activity, in the PVN and a constitutive increase in phosphorylated GR levels in the central nucleus of the amygdala (CeA) (Natividad et al., 2021). Similarly, alcohol-dependent rats during acute withdrawal showed increased GR phosphorylation in the CeA (Vendruscolo et al., 2015). In postdependent rats, systemic and intra-CeA administration of mifepristone, a potent GR and progesterone receptor (PR) antagonist with a higher binding affinity than the endogenous ligands (Sitruk-Ware and Spitz, 2003), reliably reduced alcohol intake and yohimbine-induced reinstatement of alcohol seeking (Vendruscolo et al., 2015; Vendruscolo et al., 2012; Simms et al., 2012). Furthermore, mifepristone has also been used as

a pharmacological tool to test the role of GR in several models of stress-induced anxiety-like behaviors. For instance, intracerebroventricular infusion of mifepristone prior to a restraint procedure abolished stress-induced anxiety-like behavior (Calvo and Volosin, 2001). Moreover, systemic administration of mifepristone also decreased negative affect produced by chronic stress in mice with high-trait anxiety (Jakovcevski et al., 2011). The functional effects of mifepristone as an anxiety-alleviating agent are mixed since other reports demonstrate that this drug produces no restorative changes to stress in a mouse model that lacks stress coping mechanisms (Kim et Han, 2009).

Currently, it is unknown whether the constitutive alteration of GR levels of msP rats might contribute to their excessive alcohol-drinking and highly stressed and anxious phenotype. Considering that this rat line shows features resembling postdependent rats, we tested the hypothesis that GR signaling would play a key role in the regulation of these behaviors by examining the efficacy of the GR antagonism on alcohol self-administration and on a battery of anxiety-related behavioral tests and comparing the msP rat line with its Wistar counterpart. Importantly, sex differences in alcohol consumption and dependence have been extensively described, and evidence shows that the prevalence of alcohol consumption as a coping strategy to attenuate negative affective states is higher in women than men (Guinle and Sinha, 2020; Peltier et al., 2019; Crutzen et al., 2013). Similarly, preclinical studies have reported that female msP rats consume higher amounts of alcohol when compared to males (Borruto et al., 2021; Borruto et al., 2020). Furthermore, several sex differences have been also described in response to stress indicating that HPA axis function is greater in female rats (Peltier et al., 2019; Heck and Handa, 2019; Hudson and Stamp, 2011). Based on these observations, the effects of GR antagonism were assessed in both sexes.

2. Materials and Methods

2.1 Animals

A total of N = 250 rats were used in this study. Experiments were carried out in two geographically distinct locations: University of Camerino (Italy) and Scripps Research Institute (La Jolla, CA, USA). Experiments concerning the effect of GR antagonism on alcohol drinking were conducted at the University of Camerino, Italy. For these experiments we used male (N = 25-26/line) and female (N = 28/line) msP and Wistar rats. They were bred at the animal facility of the University of Camerino, Italy and weighed 250-300g (male) and 160-200g (female) at the beginning of the experiments. Rats were housed 4 per cage in a temperature (20-22°C) and humidity (45-50%) controlled room with a reverse 12h light/dark cycle (lights off at 8 AM).

During the entire permanence in the facility, animals were offered free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy).

Experiments concerning the effect of GR antagonism on anxiety-related behaviors were conducted at the Scripps Research Institute (La Jolla, CA, USA). Adult male (N = 40, ~450 g) and female (N = 34, ~250 g) msP rats were bred at The Scripps Research Institute (La Jolla, CA, USA) from a colony obtained from the University of Camerino, while adult male (N = 35, ~450 g) and female (N = 34, ~250 g) Wistar rats were purchased from Envigo (Indianapolis, IN, USA). Rats were housed 2 per cage on a 12h reverse light/dark cycle (lights off at 8 AM.), with food and water available ad libitum.

Before the beginning of behavioral procedures, for three days rats were handled 5 minutes daily by the same operators who performed the experiments. Experiments were conducted during the dark phase of the light/dark cycle.

All the procedures were conducted in adherence with the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Italy) and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (USA) and with The Scripps Research Institute Institutional Animal Care and Use Committee policies.

2.2 Drugs

The alcohol drinking solution 10% (v/v) was prepared by diluting 95% alcohol (F.L.Carsetti, Camerino, Italy) with tap water. Saccharin (Sigma-Aldrich, Milan, Italy) was diluted to 0.2% (w/v) with tap water. The glucocorticoid and progesterone receptors antagonist mifepristone (Cayman Chemical, USA) was dissolved in propylene glycol (Sigma-Aldrich, Milan, Italy). Mifepristone was administered intraperitoneally (i.p.) at the doses of 0.0, 10, 30 and 60 mg/kg in a volume of 1 mL/kg, 90min before drug tests. For anxiety tests, a single dose of mifepristone (60mg/kg) was used, according to previous published reports demonstrating that doses ranging between 20 mg/kg and 120 mg/kg had an anxiolytic effect in male rats (Boero et al., 2018; Fox et al., 2016). The selective glucocorticoid receptor antagonist CORT113176 (Corcept Therapeutics Incorporated, Menlo Park, CA, USA) was suspended in a vehicle containing 10% dimethylformamide (Sigma-Aldrich, Milano, Italy), 10% Cremophor EL (Sigma-Aldrich, Milano, Italy) and 80% saline. The drug was administered at the doses of 0.0, 10, 30 and 60 mg/kg (i.p). in a volume of 3 mL/kg, 90min prior the test session. For alcohol drinking experiments, drug doses for both mifepristone and CORT113176 and 90 min pretreatment were

chosen based on published data (Vendruscolo et al., 2015). Before starting drug tests, animals from each experiment were i.p. injected three times with physiological saline for habituation to the injection procedure.

2.3 Self-administration apparatus

Self-administration (SA) sessions were conducted in standard operant conditioning chambers (Med Associates, St Albans, VT, USA) enclosed in ventilated sound-attenuating cubicles. Each chamber was equipped with two retractable levers, located in the front panel of the chamber, with a drinking reservoir placed in between, connected with a syringe pump. A house-light was located on the wall opposite to the levers. Behavioral sessions were controlled and recorded by a windows compatible PC equipped with Med-PC-5 software (Med Associates).

2.4 Self-administration training

Animals were trained to self-administer 10% (v/v) alcohol or saccharin 0.2% (w/v) for five days a week, in 30min daily sessions under a fixed-ratio 1 (FR1) schedule of reinforcement. Operant sessions started with lever insertion and ended with levers retraction. Responses at the right (active) lever were reinforced with 0.1 ml of fluid (alcohol or saccharin solution) delivered in the drinking reservoir. Rats were trained to alcohol SA using a saccharin-fading procedure (Samson, 1986). Briefly, the first 5 days of training, active lever responses were reinforced with 0.2% (w/v) saccharin. Next, 8% (v/v) alcohol was added to saccharin to familiarize rats with alcohol, and then alcohol concentration was stepwise increased to 10% (v/v) and saccharin removed. Starting with alcohol 10% (v/v) SA, reinforcement delivery was followed by 5sec time-out (TO), during which the house light was contingently illuminated. During the TO active lever responses were recorded but not reinforced. Throughout the sessions, responses at the left (inactive) lever were recorded but had no scheduled consequences. Drug treatments began once a stable SA baseline was established.

2.5 Novelty-Induced Hypophagia (NIH) test

Anxiety like behavior was monitored by using hypophagia procedures as previously published (Dulawa and Hen, 2005). The animals received home cage exposure to a novel palatable food (50% sucrose, chocolate flavored pellets, 45 mg, 5TUL, Test Diets, St. Louis, MO, USA) 24h before being tested. The rats were acclimated to their housing room during their dark phase (red lights). Following exposure to the novel palatable food, rats were monitored by the experimenter to confirm that each rat tasted the novel food. The following day, the latency for

the rats to consume the chocolate pellets and the total intake in an unfamiliar environment over a 10min trial were recorded under novel testing conditions (white lights on, unfamiliar double-size cage, white noise).

2.6 Comprehensive Lab Monitoring System (CLAMS)

Sleep patterns were assessed in a comprehensive lab animal monitoring system (CLAMS, Columbus Instruments, Columbus, OH, USA). Such noninvasive, activity-based measurements correlate well with EEG-defined sleep (Fox et al., 2016; Steinman et al., 2021). We used OXYmax–CLAMS units to interpret each rat's sleep parameters from photocell-defined motor activity across the first 11h of their 12h light phase. Rats were placed in the single units (32 x 20 x 19 cm) 12h prior to testing to allow them to acclimatize. Each CLAMS chamber was equipped with a water sipper and tray that provided ad libitum access to food. Twenty-four photobeams were used to detect horizontal or vertical movement and were located 2.5 cm apart, at 9 cm and 14 cm above the floor. CLAMS sleep detection works in time periods called "epochs". Sleeping epochs were defined as no more than one photocell interruption during a 60sec epoch, and sleep bouts were defined as successive strings of such epochs. We extracted and analyzed the average duration of the sleeping bouts (min), the number of sleeping bouts and the total sleeping time (min) throughout the inactive phase.

2.7 Acoustic Startle response test

Startle reflexes were measured in four identical startle response systems (SR-LAB, San Diego Instruments, San Diego, CA, USA) consisting of a nonrestrictive Plexiglas cylinder (13 cm inner diameter, 25 cm length for males; 9 cm inner diameter, 20 cm length for females) mounted on a Plexiglas platform and placed in a dark, ventilated, sound-attenuated chamber. The movements were detected and measured by a piezoelectric element mounted under each cylinder. A dynamic calibration system was used to ensure comparable startle magnitudes across the four devices. Throughout the session, the startle system delivered a constant background white noise of 68 dB. Startle stimuli were presented through a high-frequency speaker located above the startle chambers and lasted for 30 msec. Startle magnitudes were sampled each millisecond during a period of 100 msec, beginning at the onset of the startle stimulus. Startle response was defined as the peak response during this 100 msec period. During a 30min session, 75 trials were presented in a pseudorandom order. The SR-LAB startle response system measured startle responses to acoustic stimuli (80–120 dB) and no-stimulus control trials. The test session began with a 5 min acclimation period followed by four

consecutive blocks of test trials. Block 1 and 4 consisted of six startle 120 dB stimulus-alone trials. Prepulse inhibition was tested in block 2 by interspersing non-prepulsed 120 dB trials with six 120 dB trials that were prepulsed with an 80 dB tone by 70 msec. Block 3 consisted of trials of varying intensity (80, 85, 90, 95, 100, 105 dB), each one presented six times in a randomized order. Between each block, three no-stimulus trials were included, during which only the background noise was presented.

2..8 Experimental procedures

2.8.1 EXPERIMENT 1: *Effect of mifepristone on alcohol self-administration in male and female msP and Wistar rats.*

On test days, male and female msP (N=10/sex) and Wistar (N=9-10/sex) rats were injected with mifepristone (10, 30 and 60 mg/kg, i.p.) or its vehicle 90min before the SA session in a within subject counterbalanced design. Tests were conducted every fourth day until each rat had received all doses of mifepristone. The first of the three intervening days, rats remained in their home cage whereas the second and third days they performed baseline alcohol SA sessions. The number of operant responses at both the active and inactive levers and the number of reinforcers received in each drug test were recorded.

2.8.2 EXPERIMENT 2: *Effect of mifepristone on saccharin self-administration in male and female msP and Wistar rats.*

This experiment was conducted on male and female msP (N=6-8/sex) and Wistar (N=7-8/sex) rats. The procedure was identical to experiment 1 except that the SA fluid was saccharin 0.2% (w/v).

2.8.3 EXPERIMENT 3: *Effect of CORT113176 on alcohol self-administration in male and female msP and Wistar rats.*

This experiment was conducted on male and female msP (N=9-10/sex) and Wistar (N=10/sex) rats. The procedure was identical to experiment 1 except that the selective GR antagonist CORT113176 (0.0, 10, 30 and 60 mg/kg) was used.

2.8.4 EXPERIMENT 4: *Effect of CORT113176 on saccharin self-administration in male and female msP and Wistar rats.*

This experiment was conducted on male and female msP (N=9-10/sex) and Wistar (N=9 10/sex) rats. The procedure was identical to experiment 3 except that the SA fluid was saccharin 0.2% (w/v).

2.8.5 EXPERIMENT 5: *Blood corticosterone levels under basal conditions and following alcohol self-administration in male and female msP and Wistar rats.*

The effect of alcohol SA on blood corticosterone levels in male and female msP (N = 6/sex) and Wistar (N=7-8/sex) rats was evaluated. Rats were trained to self-administer alcohol as described above. When a stable alcohol SA baseline was established, blood for corticosterone analysis was collected under basal alcohol-free condition and immediately after the alcohol self-administration session. The experiment was conducted in a within-subject design and animals were subjected to two blood samplings, one under basal condition and the other immediately after the self-administration session. At least three days passed between the two blood samplings and sampling order was counterbalanced. Blood was collected by tail nicking. The hypothalamic stress response induced by this sampling procedure is detectable after 3min (Vahl et al.2005); to avoid this confounding factor, we completed sampling within 2min. Blood was sampled in lithium-heparinized tubes (Sars EDT, Nümbrecht, Germany). Samples were centrifuged at 1500 x rcf for 10min at 4 °C and plasma was collected, aliquoted and stored at −20 °C until further use. Plasma corticosterone levels were determined using enzyme-linked immunosorbent assay (ELISA) (RE52211, IBL International GmbH, Hamburg, Germany) following manufacturer instructions.

2.8.6 EXPERIMENT 6: *Effect of mifepristone on Novelty-Induced Hypophagia test.*

Exposure to novel environments elicits a stressful reaction in rodents that can interfere with normal behavior, including food consumption (Bechtholt et al., 2007; Bluett et al., 2014). The experimental groups consisted of rats that were randomly selected, and simple randomization for treatment groups condition occurred prior to the start of the experiments via a number labeling system for each rat. Then, the rats were arbitrarily assigned to different treatment groups regardless of body weight. Male and female msP (N=25-18/sex) and Wistar (N=19-18/sex) rats were tested for anxiety in a NIH test as described above. 24h after receiving home cage exposure to a novel palatable food, the rats were treated with mifepristone (0.0 and 60 mg/kg, i.p.) 90min prior test evaluation under novel testing conditions. The latency for the rats

to consume the chocolate pellets and the total food intake in an unfamiliar environment over a 10min trial were recorded.

2.8.7 EXPERIMENT 7: *Effect of mifepristone on sleep disturbances.*

Since stress and anxiety are often accompanied by sleep disturbances (Ross et al., 1989), we assessed sleep patterns in CLAMS. Male and female msP (N=15-16/sex) and Wistar (N=16/sex) rats were used in this experiment. Animals were arbitrarily assigned to different treatment groups regardless of body weight as described above. Mifepristone (0.0 and 60mg/kg) was i.p. injected and rats were subjected to the CLAMS test after 90min. The average duration of the sleeping bouts (min), the number of sleeping bouts and the total sleeping time (min) throughout the inactive phase were measured.

2.8.8 EXPERIMENT 8: *Effect of mifepristone on Acoustic Startle response test.*

Exaggerated acoustic startle responses are present in patients with PTSD and indicate hyperarousal (Steinman et al., 2021). Acoustic startle response was used to capture startle reflexive responses following sound stimuli across various intensity trials. Male and female msP (N=14/sex) and Wistar (N=16/sex) rats were used in this experiment. Animals were arbitrarily assigned to different treatment groups regardless of body weight as described above. Mifepristone (0.0 and 60mg/kg) was i.p. injected and rats were subjected to acoustic startle response after 90min. The test was conducted as previously described. 120 dB trial 1, 120 dB trials 2-6, 120 dB, average prepulse inhibition and 80-105 dB startle responses were measured.

2.9 Statistical analysis

All alcohol drinking experiments were analyzed by three-way analysis of variance (ANOVA) with “treatment” as a repeated measure and “genotype” and “sex” as between-subject factors. Active and inactive lever responses were analyzed separately. Behavioral performance of each independent strain/sex group was further analyzed by one-way ANOVA with “treatment” as a repeated measure. ANOVAs were followed by Dunnet’s *post-hoc* analysis when appropriate. Significance was conventionally set at $p < 0.05$.

CORT ELISA standards were used to generate an optimalfit 4-parameter standard curve from which sample values were extrapolated. CORT data were analyzed via three-way ANOVA, with “conditions” (basal vs alcohol condition) as within-subject factor and “genotype” and “sex” as between-subject factors. Significant effects were explored with Newman-Keuls multiple comparison test. Significance was conventionally set at $p < 0.05$.

NIH, CLAMS, and acoustic startle were analyzed using separate two-way ANOVAs with "treatment" and "genotype" as between-subject factors. Significant interaction effects were followed by Fisher's LSD protected *post-hoc* tests. For acoustic startle data containing repeated stimulus intensities, a mixed model three-way ANOVA was used with "genotype" and "treatment" as between-subjects factors and "levels of intensity" as within-subjects factor. To investigate the role of sex differences, a separate level of analysis using similar two-way ANOVAs was included with "sex" and "genotype" as between-subject factors. Significance level was determined at $p < 0.05$.

3. Results

3.1 Experiment 1: *Effect of mifepristone on alcohol self-administration in male and female msP and Wistar rats.*

We tested the effect of mifepristone on alcohol SA under Fixed Ratio 1 (FR1) schedule of reinforcement in male and female msP (N = 10/sex) and Wistar (N = 9-10/sex) rats. Experimental subjects received mifepristone (10, 30 and 60mg/kg) or its vehicle in a counterbalanced within subject Latin square design. A three-way ANOVA revealed an overall effect of treatment [$F_{(3,35)} = 7.5$; $p < 0.001$], sex [$F_{(1,35)} = 55.8$; $p < 0.0001$] and strain [$F_{(1,35)} = 41.2$; $p < 0.0001$]. There was a significant sex x strain interaction [$F_{(1,35)} = 10.2$; $p < 0.01$], but no other significant interactions. These results reflect a higher SA level in msP, a higher number of rewards by male msP rats, and a general reduction of alcohol SA induced by mifepristone. To further evaluate the effect of mifepristone, we carried out single ANOVAs to independently analyze the drug effect on male and female msPs as well as on male and female Wistars. In msP rats no overall effect of treatment in males [$F_{(3,9)} = 0.4$; $p > 0.05$] or in females [$F_{(3,9)} = 1.1$; $p > 0.05$] was detected. Conversely, an overall significant effect of treatment was detected in male [$F_{(3,8)} = 4.0$; $p < 0.05$] and female [$F_{(3,9)} = 7.5$; $p < 0.01$] Wistars. Dunnett's *post-hoc* analysis showed a significant decrease in the number of alcohol reinforced responding at the doses of 30 mg/kg and 60 mg/kg of mifepristone in both male and female Wistar rats ($p < 0.05$) (**Figure 1, upper panel**).

A three-way ANOVA applied to inactive lever responding showed no overall effect of treatment [$F_{(3,35)} = 0.9$; $p > 0.05$], sex [$F_{(1,35)} = 0.05$; $p > 0.05$] or strain [$F_{(1,35)} = 2.3$; $p > 0.05$]. Neither interactions were detected (**Figure 1, lower panel**).

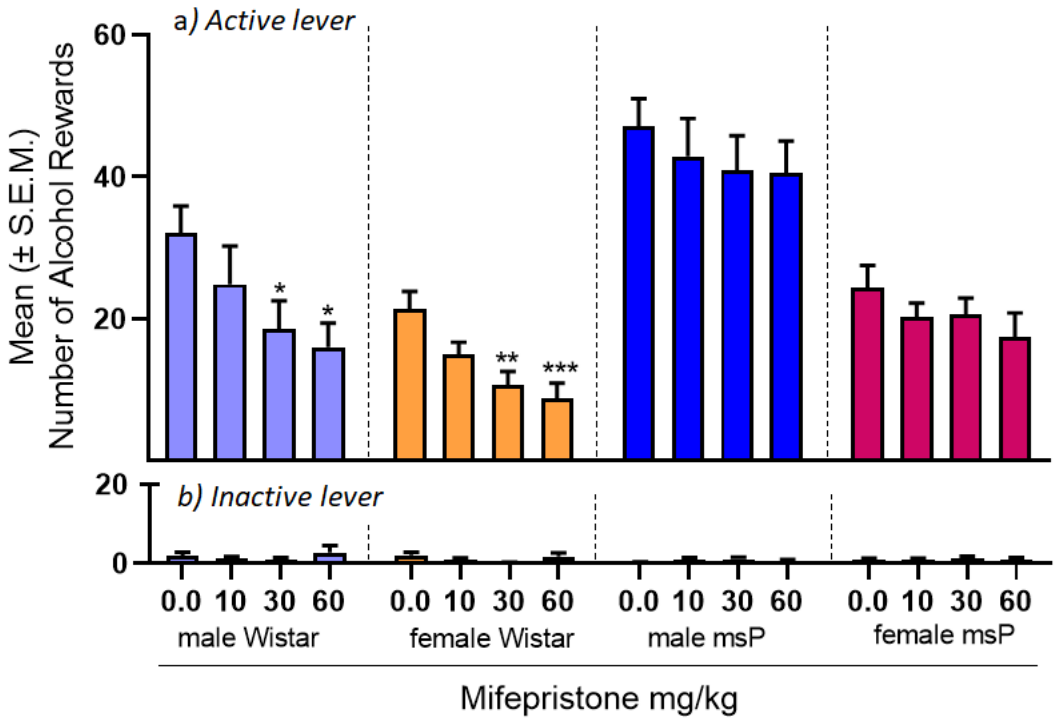

Figure 1. Effect of mifepristone on alcohol self-administration in male and female msP and Wistar rats. Male and female msP and Wistar rats were treated with mifepristone (0.0, 10, 30 and 60 mg/kg) i.p., 90min prior to test session. Mifepristone treatment significantly reduced the number of alcohol rewards in male and female Wistars. Drug treatment did not decrease alcohol SA in male and female msPs. Data are expressed as the mean ±SEM of number of: a) reinforced responses (rewards) at the active lever and b) total responses at the inactive lever. Significant difference from vehicle (0.0 mg/kg): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.2 Experiment 2: *Effect of mifepristone on saccharin self-administration in male and female msP and Wistar rats.*

To control for the selectivity of mifepristone effect on alcohol SA, other groups of male and female msP (N = 6-8/sex) and Wistar (N = 7-8/sex) rats were tested for the effect of mifepristone (10, 30 and 60 mg/kg) or its vehicle on saccharin SA. A three-way ANOVA found a significant effect of treatment [$F_{(3,25)} = 8.8$; $p = 0.0001$], no effect of sex [$F_{(1,25)} = 0.3$; $p > 0.05$], no effect of strain [$F_{(1,25)} = 0.4$; $p > 0.05$] and no interactions. To further explore the effect of mifepristone, data from male and female msPs and male and female Wistars were analyzed separately by single ANOVAs. Results revealed an overall effect of treatment in male Wistars [$F_{(3,6)} = 4.7$; $p < 0.05$] and female msPs [$F_{(3,7)} = 6.4$; $p < 0.01$]. Conversely, no overall effect was found in female Wistars [$F_{(3,7)} = 1.4$; $p > 0.05$] and male msPs [$F_{(3,5)} = 1.2$; $p > 0.05$]. Dunnet's

post-hoc tests showed that 60 mg/kg of mifepristone reduced saccharin SA in both male Wistars and female msPs ($p < 0.05$) (**Figure 2, upper panel**).

Analysis of inactive lever responding found no significant overall effect of treatment [$F_{(3,25)} = 0.7$; $p > 0.05$], sex [$F_{(1,25)} = 1.4$; $p > 0.05$], strain [$F_{(1,25)} = 0.005$; $p > 0.05$] and no interactions (**Figure 2, lower panel**).

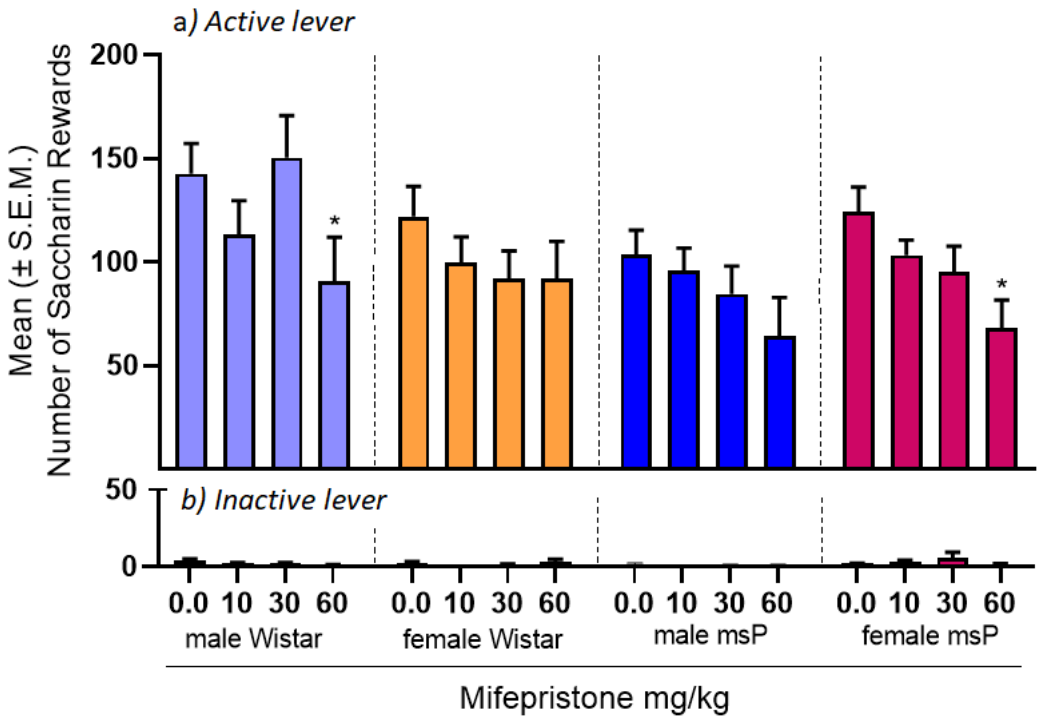

Figure 2. Effect of mifepristone on saccharin self-administration in male and female msP and Wistar rats. Male and female msP and Wistar rats were treated with mifepristone (0.0, 10, 30 and 60 mg/kg) i.p., 90min prior to test session. At the dose of 60 mg/kg mifepristone significantly reduced saccharin SA in male Wistars and in female msPs. Data are expressed as the mean ±SEM of number of: a) reinforced responses (rewards) at the active lever and b) total responses at the inactive lever. Significant difference from vehicle (0.0 mg/kg): * $p < 0.05$.

3.3 EXPERIMENT 3: *Effect of CORT113176 on alcohol self-administration in male and female msP and Wistar rats.*

Mifepristone is a GR antagonist that has residual activity also on progesterone receptor. To confirm that effects observed were specifically mediated by GR blockade, we tested CORT113176, that is another more selective GR antagonist (Vendruscolo et., 2015). Once stable baseline of alcohol SA was reached, male and female msP (N = 9-10/sex) and Wistar (N = 10/sex) rats were treated with CORT113176 (10, 30, 60 mg/kg) or its vehicle. A three-way

ANOVA revealed an overall effect of treatment [$F_{(3,35)} = 11.1$; $p < 0.0001$], sex [$F_{(1,35)} = 16.04$; $p < 0.001$], strain [$F_{(1,35)} = 24.6$; $p < 0.0001$] and sex x strain interaction [$F_{(1,35)} = 6.3$; $p < 0.05$], but no other significant interactions. At this point we conducted single ANOVAs to further determine the effect of CORT113176 on male and female msPs and male and female Wistars. Results showed an overall effect of treatment in male Wistars [$F_{(3,9)} = 4.4$; $p < 0.05$], female Wistars [$F_{(3,9)} = 4.5$; $p < 0.05$] and female msPs [$F_{(3,9)} = 4.0$, $p < 0.05$]. No effect was found in male msP [$F_{(3,8)} = 1.9$; $p > 0.05$] rats. Dunnet's *post-hoc* tests revealed that at 60 mg/kg CORT113176 decreased alcohol SA in male ($p < 0.01$) and female Wistars ($p < 0.05$) as well as female msPs ($p < 0.01$) (**Figure 3, upper panel**).

Analysis of the inactive lever found no significant overall effect of treatment [$F_{(3,35)} = 0.8$; $p > 0.05$] and strain [$F_{(1,35)} = 3.7$; $p > 0.05$] but an overall effect of sex [$F_{(1,35)} = 7.7$; $p < 0.01$], treatment x strain [$F_{(1,35)} = 5.6$; $p < 0.01$] and sex x strain interaction [$F_{(1,35)} = 5.5$; $p < 0.05$] was observed (**Figure 3, lower panel**).

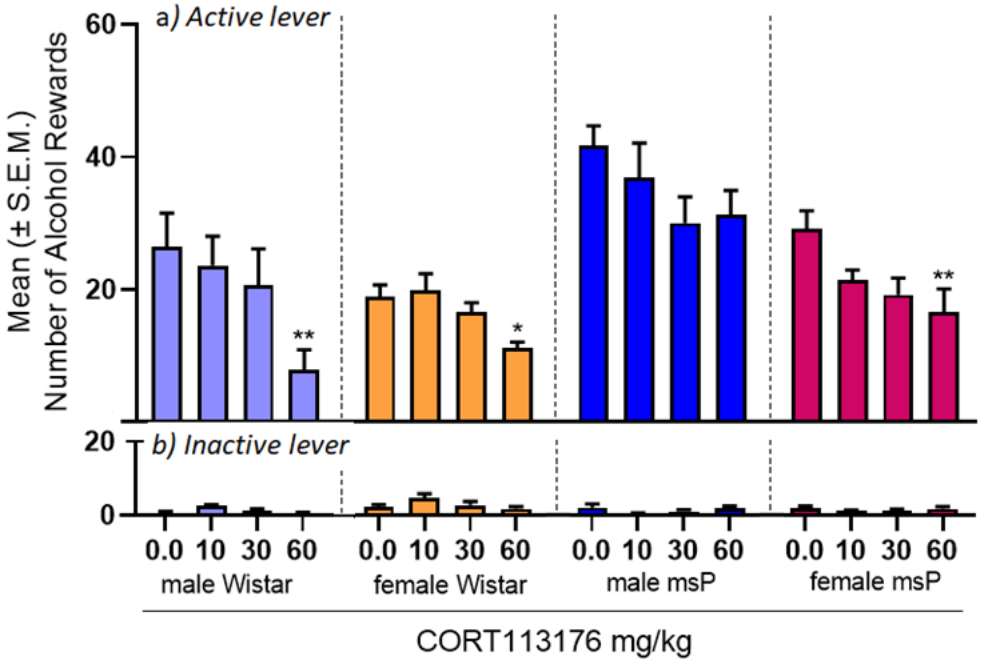

Figure 3. Effect of CORT113176 on alcohol self-administration in male and female msP and Wistar rats. Male and female msP and Wistar rats were treated with CORT113176 (0.0, 10, 30 and 60mg/kg) i.p., 90min prior to test session. CORT113176 treatment significantly reduced the number of alcohol rewards in male and female Wistars and in female msP rats. Data are expressed as the mean ± SEM of number of: a) reinforced responses at the active and b) total responses at inactive lever. Significant difference from vehicle (0.0 mg/kg): ** $p < 0.01$; * $p < 0.05$.

3.4 EXPERIMENT 4: *Effect of CORT113176 on saccharin self-administration in male and female msP and Wistar rats.*

We next verified the specificity of action of CORT113176 by testing its effect on saccharin SA in male and female msP (N = 9-10/sex) and Wistar (N = 9-10/sex) rats. Three-way ANOVA demonstrated a significant effect of treatment [$F_{(3,34)} = 5.2$; $p < 0.01$], strain [$F_{(1,34)} = 10.3$; $p < 0.01$] and treatment x sex interaction [$F_{(3,102)} = 3,4$; $p < 0.05$]. When single ANOVAs were carried out we found an overall effect of CORT113176 on saccharin SA only in male Wistar rats [$F_{(3,8)} = 4.5$; $p < 0.05$]. No drug effect was detected in female Wistars [$F_{(3,9)} = 0.7$; $p > 0.05$], male msPs [$F_{(3,8)} = 1.7$; $p > 0.05$] and in female msPs [$F_{(3,9)} = 0.4$; $p > 0.05$] (**Figure 4, upper panel)**.

Analysis of the inactive lever found no significant overall effect of treatment [$F_{(3,34)} = 2.7$; $p > 0.05$], but there was a significant effect of sex [$F_{(1,34)} = 9.3$; $p < 0.01$], strain [$F_{(1,34)} = 15.4$; $p < 0.01$] and treatment x strain interaction [$F_{(3,102)} = 2.9$; $p < 0.05$] (**Figure 4, lower panel**).

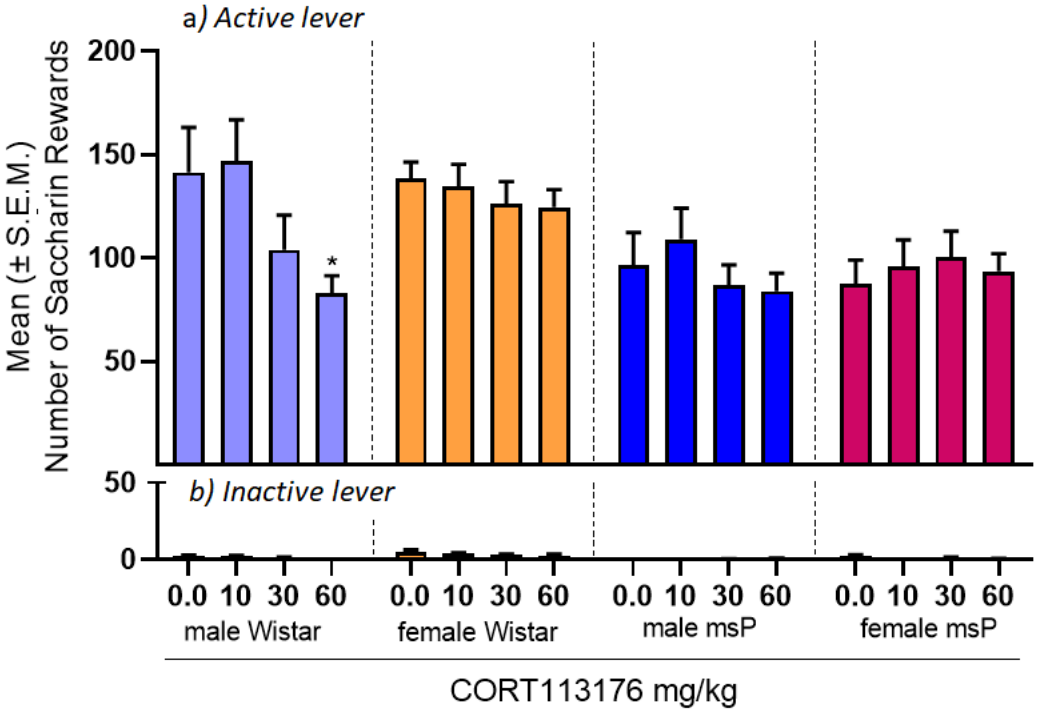

Figure 4. Effect of CORT113176 on saccharin self-administration in male and female msP and Wistar rats. Male and female msP and Wistar rats were treated with CORT113176 (0.0, 10, 30 and 60mg/kg) i.p., 90min prior to test session. CORT113176 at the dose of 60 mg/kg significantly reduced saccharin SA in male Wistar rats only. Data are expressed as the mean ± SEM of number of: a) reinforced responses at the active and b) total responses at inactive lever. Significant difference from vehicle (0.0 mg/kg): * $p < 0.05$.

3.5 Experiment 5: *Blood CORT levels under basal conditions and following alcohol self-administration in male and female msP and Wistar rats.*

Finally, we assessed the blood CORT levels under basal conditions and after alcohol SA in male and female msP (N = 6/sex) and Wistar rats (N = 8-7/sex). Three-way ANOVA revealed a main effect of sex [$F_{(1,23)} = 84.5$; $p < 0.0001$], alcohol condition [$F_{(1,23)} = 19.5$; $p < 0.001$], strain [$F_{(1,23)} = 13.8$; $p < 0.01$], sex x alcohol condition interaction [$F_{(1,23)} = 18.3$; $p < 0.001$] and sex x strain interaction [$F_{(1,23)} = 4.4$; $p < 0.05$]. Female rats from both genotypes displayed persistently higher levels of CORT compared to males in both conditions. Female Wistar rats

showed higher CORT levels than female msPs ($p < 0.001$). Alcohol consumption in a SA session decreased CORT levels only in female animals ($p < 0.001$). In male rats, blood CORT concentrations were not affected by alcohol SA (**Figure 5**).

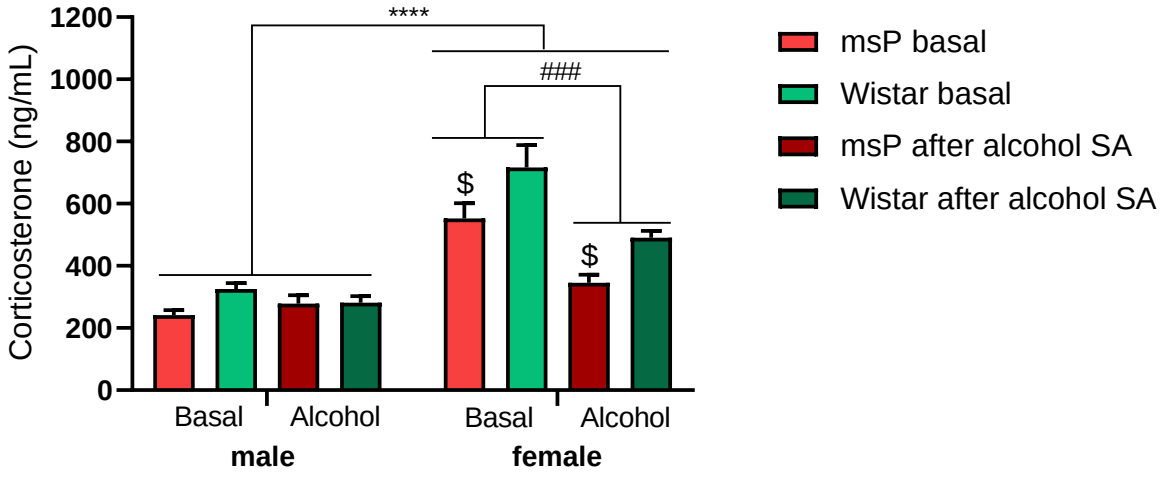

Figure 5. Blood corticosterone (CORT) levels under basal conditions and after alcohol SA session in male and female msP and Wistar rats. Females displayed significantly higher blood CORT levels than males independently of rat strain. Female Wistars had higher CORT levels than female msPs. Alcohol consumption decreased basal CORT levels in female animals only. In both rat lines, CORT levels of males remained unchanged following alcohol SA. Data are presented as mean ±SEM. Main effect of sex: **** $p < 0.0001$; main effect of sex x alcohol condition: ### $p < 0.001$; $ $p < 0.05$ vs msP same condition and sex (sex x strain interaction).

3.6 EXPERIMENT 6: *Effect of mifepristone on Novelty-Induced Hypophagia test.*

Mifepristone (0.0 and 60mg/kg) was i.p. injected to male and female msP and Wistar rats and they were tested under novel environmental conditions using the NIH procedure. The latency to eat the chocolate pellets and the total food intake were recorded. Two-way ANOVA of the effect of mifepristone on latency to eat in males revealed an overall effect of genotype [$F_{(1,40)} = 45.5$, $p = 0.001$], but no significant effect of treatment [$F_{(1,40)} = 0.07$, $p > 0.05$] and treatment x genotype interaction [$F_{(1,40)} = 1.7$, $p > 0.05$] (**Figure 6A**). Two-way ANOVA of the effect of mifepristone on food intake in males revealed a main effect of genotype [$F_{(1,40)} = 49.5$; $p = 0.0001$], but no effect of treatment [$F_{(1,40)} = 0.5$; $p > 0.05$] and treatment x genotype interaction [$F_{(1,40)} = 0.3$; $p > 0.05$] (**Figure 6B**). Similar to males, two-way AOVA of the effect of mifepristone in female animals found an overall effect of genotype [$F_{(1,32)} = 12.1$, $p = 0.001$], but no significant effect of treatment [$F_{(1,32)} = 0.195$, $p > 0.05$] and treatment x genotype

interaction [$F_{(1,32)} = 0.3$, $p > 0.05$] (**Figure 6C**). Similarly, two-way ANOVA of the effect of mifepristone on pellet intake found a main effect of genotype [$F_{(1,32)} = 16.7$, $p = 0.0001$], but no significant effect of treatment [$F_{(1,32)} = 0.2$, $p > 0.05$] and treatment x genotype interaction [$F_{(1,32)} = 1.4$, $p > 0.05$] (**Figure 6D**). These data suggest tha both male and female msPs displayed an increase in latency to eat chocolate pellets and lower overall food intake under novelty stress conditions relative to their Wistar counterparts Importantly, a single systemic mifepristone administration did not affect the latency to eat or intake of the chocolate pellets in male and female msPs.

To further examine the contribution of sex differences in promoting anxiety-like behavior, we also compared male versus female rats within each genotype as a function of sex, regardless of mifepristone treatment. Two-way ANOVA relative to the latency to eat revealed a main effect of genotype [$F_{(1,35)} = 34.7$, $p = 0.0001$], but no significant effect of sex [$F_{(1,35)} = 0.3$, $p > 0.05$] an sex x genotype interaction [$F_{(1,35)} = 0.9$, $p > 0.05$] (**Figure 6E**). Two-way ANOVA relative to the food intake revealed a main effect of genotype [$F_{(1,35)} = 10.9$, $p = 0.002$] and sex [$F_{(1,35)} = 4.9$, $p < 0.05$], but no sex x genotype interaction [$F_{(1,35)} = 0.01$, $p < 0.05$] (**Figure 6F**). We found that males displayed lower levels of food intake as compared to females regardless of genotype, suggesting that they displayed greater vulnerability produced by novelty stress.

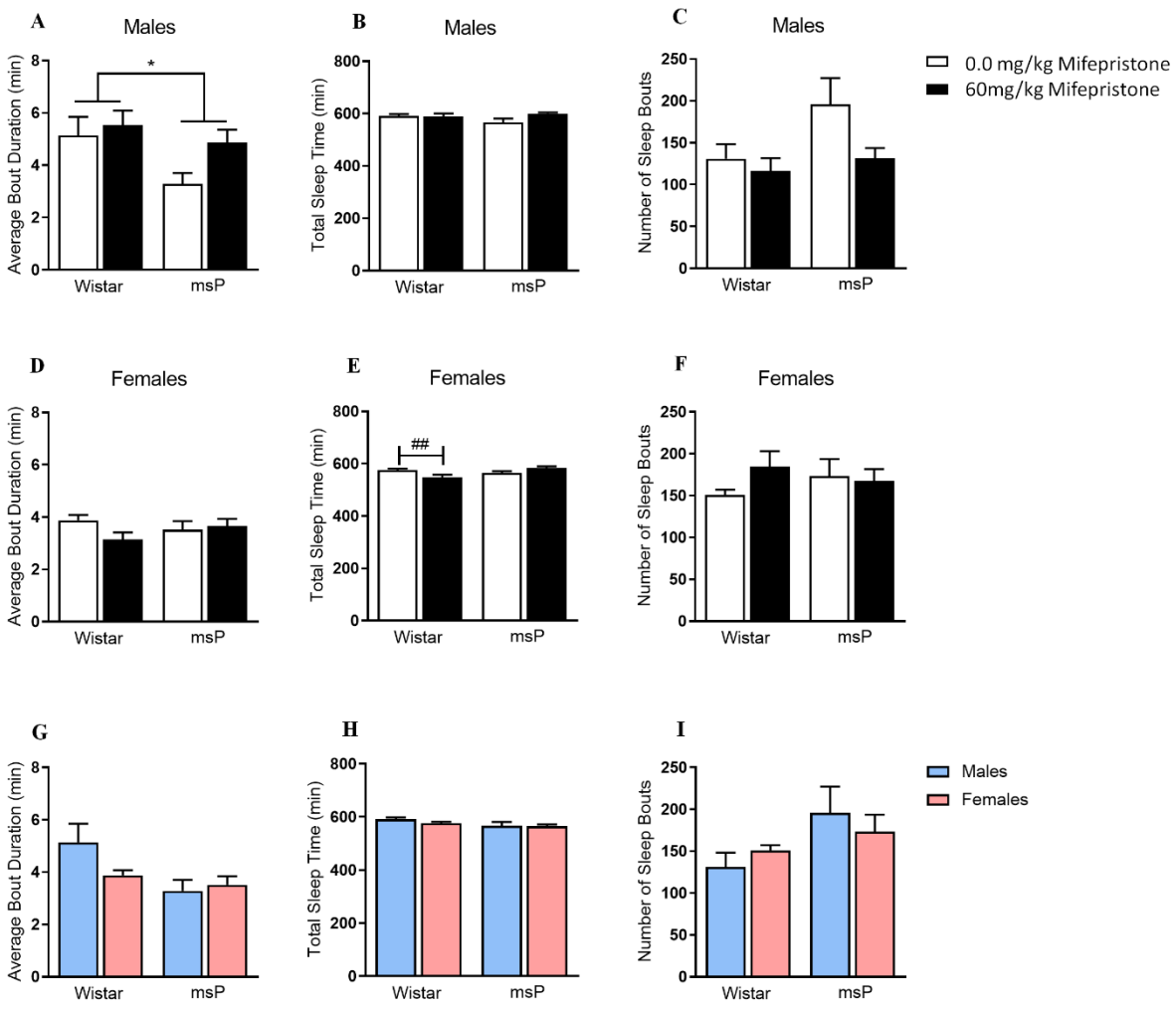

Figure 6. Effect of mifepristone on novelty-induced hypophagia test in male and female msP and Wistar rats. Mifepristone (60 mg/kg) was i.p. injected, and rats were subjected to NIH test after 90-minutes. Male and female msP rats displayed higher anxiety-like behavior during NIH that was not reduced by mifepristone. (**A**) latency to eat chocolate pellets, (**B)** pellet intake in vehicle (N = 9) or mifepristone-treated (N = 10) male Wistar rats and vehicle (N = 13) or mifepristone-treated (N = 12) male msP rats. (**C)** latency to eat chocolate pellets, (**D)** pellet intake in vehicle (N = 9) or mifepristone-treated (N = 9) female Wistar rats and vehicle (N = 8) or mifepristone-treated (N = 10) female msP rats. (**E)** latency to eat chocolate pellets, (**F)** pellet intake in male Wistar (N = 9) and male msP (N = 13) rats, female Wistar (N = 9) and female msP (N = 8) rats. Data are expressed as the mean ± SEM. Main effect of genotype: *$p < 0.05$; main effect of sex, $^{@}p < 0.05$.

3.7 EXPERIMENT 7: *Effect of mifepristone on sleep disturbances.*

We examined whether mifepristone administration might restore sleep disturbances produced by heightened stress in male and female msP and Wistar rats. The average duration of the sleeping bouts (min), the number of sleeping bouts and the total sleeping time (min) throughout the inactive phase were measured. Two-way ANOVA of the effect of mifepristone on average bout duration in males revealed an overall effect of genotype [$F_{(1,27)} = 4.9$, $p < 0.05$], but no significant effect of treatment [$F_{(1,27)} = 3.06$, $p > 0.05$] and treatment x genotype interaction [$F_{(1,27)} = 1.1$, $p > 0.05$] (**Figure 7A**). No genotype differences or acute mifepristone effect were observed in total sleep time (treatment [$F_{(1,27)} = 2.4$, $p > 0.05$], genotype [$F_{(1,27)} = 0.5$, $p > 0.05$], treatment x genotype interaction [$F_{(1,27)} = 3.1$, $p > 0.05$]) (**Figure 7B**) and number of sleep bouts (treatment [$F_{(1,27)} = 4.08$, $p > 0.05$], genotype [$F_{(1,27)} = 4.1$, $p > 0.05$], treatment x genotype interaction [$F_{(1,27)} = 1.1$, $p > 0.05$]) (**Figure 7C**) in male animals. To sum up, male msPs, in general, displayed a shorter average bout duration when compared to male Wistar rats, which is not restored by mifepristone administration, suggesting that the interrupted sleep observed in male msPs is not ameliorated by a single administration of GR antagonist.

Two-way ANOVA of the effect of mifepristone on average bout duration in females revealed no significant effect of genotype [$F_{(1,28)} = 1.2$, $p > 0.05$], treatment [$F_{(1,28)} = 0.08$, $p > 0.05$] and treatment x genotype interaction [$F_{(1,28)} = 2.5$, $p > 0.05$] (**Figure 7D**). Two-way ANOVA of the effect of mifepristone on total sleep time in females revealed no significant effect of genotype [$F_{(1,28)} = 2.2$, $p > 0.05$] and treatment [$F_{(1,28)} = 0.4$, $p > 0.05$], but a significant treatment x genotype interaction [$F_{(1,28)} = 9.4$, $p = 0.005$]. Specifically, Wistar rats treated with mifepristone displayed a significant reduction in total sleep time as compared to vehicle-treated controls ($p = 0.015$). The latter effect was only observed in female Wistar rats suggesting that mifepristone does not influence sleep patterns in female msPs (**Figure 7E**). Importantly, there were no changes observed in number of sleep bouts (treatment [$F_{(1,28)} = 0.8$, $p > 0.05$], genotype [$F_{(1,28)} = 0.02$, $p > 0.05$], treatment x genotype interaction [$F_{(1,28)} = 1.6$, $p > 0.05$]) (**Figure 7F**).

To further delineate the contribution of sex in promoting sleep disturbances, we compared male versus female rats within each genotype as a function of sex, regardless of mifepristone treatment. Here, we found no sex-dependent changes in average bout duration (sex [$F_{(1,27)} = 1.2$, $p > 0.05$], genotype [$F_{(1,27)} = 5.7$, $p > 0.05$], sex x genotype interaction [$F_{(1,27)} = 2.6$, $p > 0.05$]) (**Figure 7G**), total sleep time (sex [$F_{(1,27)} = 0.9$, $p > 0.05$], genotype [$F_{(1,27)} = 4.2$, $p = 0.049$], sex x genotype interaction [$F_{(1,27)} = 0.5$, $p > 0.05$]) (**Figure 2H**), or number of sleep bouts (sex [$F_{(1,27)} = 0.003$, $p > 0.05$], genotype [$F_{(1,27)} = 4.7$, $p < 0.05$], sex x genotype interaction [$F_{(1,27)} = 1.1$, $p > 0.05$]) (**Figure 2I**).

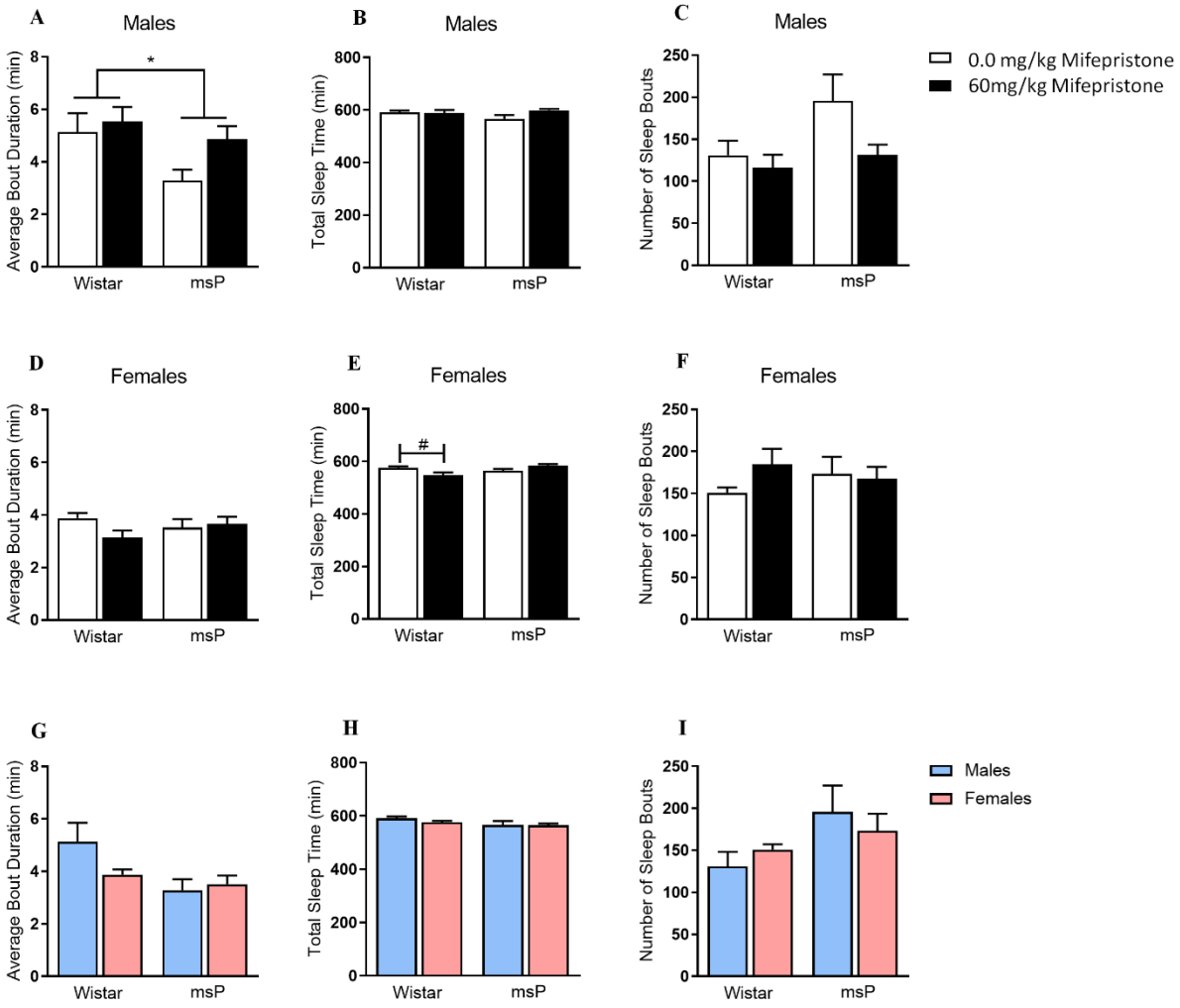

Figure 7. Effect of mifepristone on sleep disturbances in male and female msP and Wistar rats. Mifepristone (60 mg/kg) was i.p. injected and rats were subjected to CLAMS after 90-minutes. Mifepristone had no effect on ameliorating sleep disturbances. (**A)** average bout duration, (**B)** total sleep time, (**C)** number of sleep bouts in vehicle (N = 8) or mifepristone-treated (N = 8) male Wistar rats and vehicle (N = 7) or mifepristone-treated (N = 8) male msP rats. (**D)** average bout duration, (**E)** total sleep time, (**F)** number of sleep bouts in vehicle (N = 8) or mifepristone-treated (n = 8) female Wistar rats and vehicle (N = 8) or mifepristone-treated (N = 8) female msP rats. (**G)** average bout duration, (**H)** total sleep time, (**I)** number of sleep bouts in male Wistar (N = 8) and male msP (N = 7) rats, female Wistar (N = 8) and female msP (N = 8) rats. Data are expressed as the mean ± SEM. Main effect of genotype: *$p < 0.05$; Post hoc test revealed significant differences between treatment: # $p < 0.05$.

3.8 EXPERIMENT 8: *Effect of mifepristone on Acoustic Startle response test.*

We examined if mifepristone (0.0 and 60mg/kg) might reduce acoustic startle reflexive responses across various intensity trials in male and female msP and Wistar rats. In males, two-way ANOVA revealed that msPs in general displayed significantly higher startle response when compared to male Wistar rats during the 120 dB trials 2-6 (significant effect of genotype [$F_{(1,26)}$ = 10.6, p = 0.003]) (**Figure 8B**) and 120 dB final block (significant effect of genotype [$F_{(1,26)}$ = 14.5, p = 0.001] (**Figure 8C**). Furthermore, male msPs also displayed higher average prepulse inhibition when compared to male Wistar rats (significant effect of genotype [$F_{(1,26)}$ = 9.3, p = 0.005] (**Figure 8D**). A three-way ANOVA across the sequence of various levels of intensities revealed an intensity x genotype interaction [$F_{(5,130)}$ = 9.3, p = 0.001]. Specifically, male msPs displayed a higher startle response at 105 dB stimulus when compared to male Wistar rats ($p < 0.05$) (**Figure 8E**). Mifepristone administration did not reduce the genotypic differences in the enhanced startle response or prepulse inhibition behavior across various intensity trials (120dB trial 1 [$F_{(1,26)}$ = 1.6, p > 0.05]; 120dB trials 2-6 [$F_{(1,26)}$ = 0.6, p > 0.05]; 120dB final block [$F_{(1,26)}$ = 0.1, p > 0.05]; prepulse inhibition [$F_{(1,26)}$ = 1.2, p > 0.05]; 80-105dB [$F_{(5,130)}$ = 1.08, p > 0.05] (**Figure 8A-B-C-D-E**).

In females, we observed that msPs in general displayed significantly higher startle responses when compared to female Wistar rats during the 120 dB trial 1 (significant effect of genotype [$F_{(1,28)}$ = 8.9, p = 0.006]) (**Figure 8F**) and 120 dB final block (significant effect of genotype [$F_{(1,28)}$ = 5.4, p > 0.05]) (**Figure 8H**). Female msPs also displayed higher average prepulse inhibition when compared to their counterpart Wistar rats (significant effect of genotype [$F_{(1,28)}$ = 7.1, p > 0.05]) (**Figure 3I**). Furthermore, a three-way ANOVA across the various sound intensities revealed an intensity x genotype interaction [$F_{(5,140)}$ = 4.8, p < 0.05] (**Figure 3J**). Specifically, female msPs displayed higher startle responses at both the 100 dB ($p = 0.01$) and 105 dB stimuli ($p < 0.01$) when compared to female Wistar rats. Importantly, mifepristone administration did not reduce the enhanced startle response or prepulse inhibition across various intensity trials (120dB trial 1 [$F_{(1,28)}$ = 2.2, p > 0.05]; 120dB trials 2-6 [$F_{(1,28)}$ = 4.1, p = 0.052]; 120dB final block [$F_{(1,28)}$ = 0.5, p > 0.05]; prepulse inhibition [$F_{(1,28)}$ = 0.8, p > 0.05]; 80-105dB [$F_{(5,140)}$ = 0.6, p > 0.05] (**Figure 8F-G-H-I-J**).

To examine the unique role of sex in promoting heightened stress sensitivity regardless of glucocorticoid blockade, we compared male versus female rats within each genotype as a function of sex. We found that female msPs displayed an increase in startle response during the 120 dB trial 1 when compared to female Wistar rats (**Figure 8K**). The two-way ANOVA

revealed a sex x genotype interaction [$F_{(1,26)}$ = 4.8, p < 0.05], and the post hoc analysis showed that these effects were attributable to the different genotypes in a within females comparison (p < 0.01) and to the sex in a within msPs comparison (p = 0.001) (**Figure 8K**).

Interestingly, regardless of genotype, females overall displayed higher startle responses when compared to males during the 120 dB trials 2–6 (significant effect of sex [$F_{(1,26)}$ = 18.1, p = 0.0001] (**Figure 8L**) and during 120 dB final block (significant effect of sex $F_{(1,26)}$ = 18.2, p = 0.0001] (**Figure 8M**), as well as higher average prepulse inhibition (significant effect of sex [$F_{(1,26)}$ = 5.6, p < 0.05]) (**Figure 8N**). Lastly, females in general displayed higher startle responses across 80–105 dB series of intensities when compared to males (significant effect of sex [$F_{(1,26)}$ = 20.02, p = 0.0001] and intensity x sex interaction $F_{(5,130)}$ = 12.7, p = 0.0001] (**Figure 8O**). Taken together, these data suggest that females generally show higher startle susceptibility to sound stress than male rats.

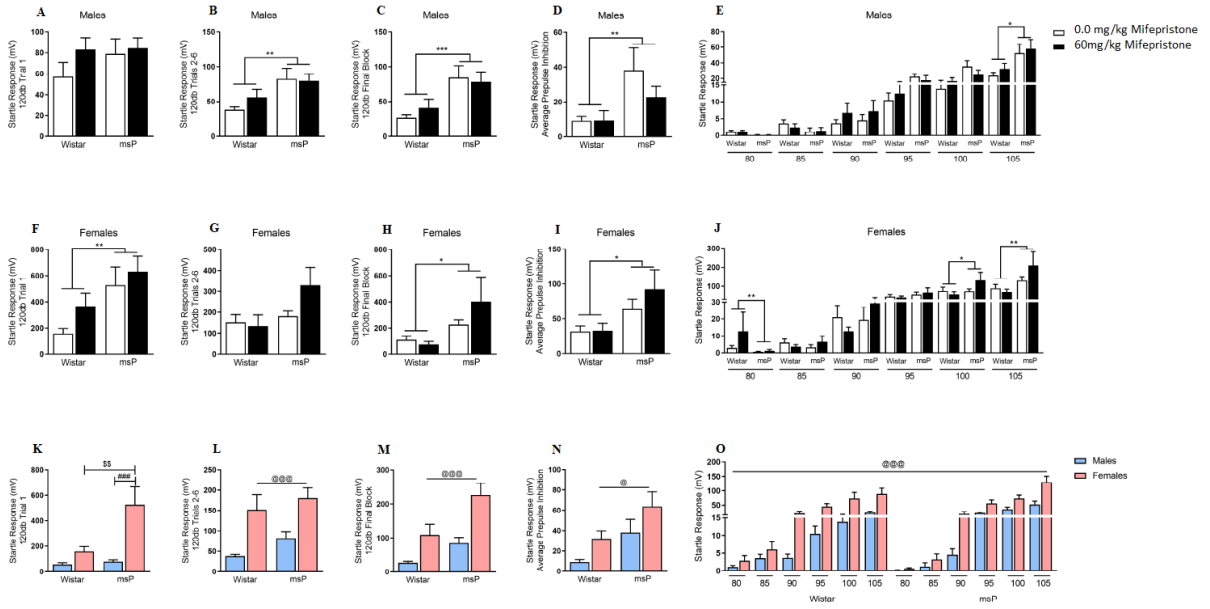

Figure 8. Effect of mifepristone on hyperarousal states in male and female msP and Wistar rats. Mifepristone (60 mg/kg) was i.p. injected and rats were subjected to acoustic startle response test after 90min. Mifepristone had no effect on ameliorating the startle response to acoustic stimuli. (**A)** 120 dB trial 1 startle response, (**B)** 120 dB trials 2–6 startle response, (**C)** 120 dB final block startle response, (**D)** average prepulse inhibition startle response, (**E**) 80–105 dB startle responses in vehicle (N = 8) or mifepristone-treated (N = 8) male Wistar rats and vehicle (N = 6) or mifepristone-treated (N = 8) male msP rats. (**F)** 120 dB trial 1 startle response, (**G)** 120 dB trials 2–6 startle response, (**H)** 120 dB final block startle response, (**I)** average prepulse inhibition startle response, (**J)** startle responses in vehicle (N = 8) or mifepristone-treated (N = 8) female Wistar rats and vehicle (N = 6) or mifepristone treated (N

= 8) female msP rats. (**K)** 120 dB trial 1 startle response, (**L)** 120 dB trials 2–6 startle response, (**M)** 120 dB final block startle response, (**N)** average prepulse inhibition startle response, (**O)** 80–105 dB startle responses in male Wistar (N= 8) and male msP (N = 6) rats, female Wistar (N = 8) and female msP (N = 8) rats. Data are expressed as the mean ± SEM. Main effect of genotype: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; main effect of sex: @@@ $p < 0.001$, @ $p < 0.05$. Post hoc test revealed significant differences between genotypes, $$ < p 0.01. Post hoc test revealed significant differences between sexes, ### $p < 0.001$.

4. Discussion

The present study investigated the effect of GR antagonism on alcohol drinking and anxiety-related behaviors in genetically-selected msP rats in comparison with non-preferring Wistar rats. To summarize, we report that systemic injection with mifepristone dose-dependently reduced alcohol SA in male and female Wistars but not in msPs. Administration of CORT113176 decreased alcohol SA in male and female Wistars as well as in female msPs but not in male msP rats. At the highest dose, mifepristone also reduced saccharin SA in male Wistars and female msPs, suggesting the occurrence of nonspecific effects. Similarly, the highest dose of CORT113176 (60 mg/kg) decreased saccharin intake in male Wistars. Analysis of CORT levels revealed that females of both rat lines had higher blood levels of CORT compared to males. Additionally, we found that male and female msPs display greater anxiety-like behaviors as well as enhanced acoustic startle responses compared to Wistar counterparts. Importantly, the enhanced anxiety-like behavior was not ameliorated by mifepristone administration. Our findings provide a further step in understanding the biological mechanisms that mediate excessive alcohol drinking and anxiety-like states in an animal model that display innate ethanol preference and sensitivity to negative affect.

We found that mifepristone administration reduced alcohol SA in both male and female Wistar rats, but not msPs, at similar dose ranges effective in decreasing alcohol-related behaviors in dependent Wistar rats (Vendruscolo et al., 2015). The ability of mifepristone to reduce alcohol SA was apparent at the intermediate dose of 30 mg/kg, while the higher dose (60 mg/kg) appeared to produce nonselective reductions of saccharin SA, suggesting the occurrence of nonspecific effects. Given the non-selectivity of mifepristone in antagonizing progesterone receptors also, we tested the selective CORT113176 compound that targets GR to confirm whether reducing alcohol SA requires specificity for the GR. Consistent with results with mifepristone, CORT113176 significantly reduced alcohol SA in male and female Wistars as

well as female msP rats. As for mifepristone, male msPs did not respond to CORT113176 treatment. Furthermore, administration of CORT113176 at the highest dose reduced saccharin SA only in male Wistar rats. Taken together, we suggest that our drug regimen is specific to alcohol SA, since the number of saccharin rewards was not modified in the other groups of rats. However, at high doses, nonspecific inhibition of motivated behavior may emerge. Several reports have provided evidence of GR antagonism efficacy in reducing alcohol-related behaviors and the potential of mifepristone for AUD treatment. In preclinical models of alcohol dependence across different species, mifepristone reduced alcohol consumption (Repunte-Canonigo et al., 2015; Vendruscolo et al., 2015), prevented the development of alcohol dependence-induced escalation of alcohol drinking (Somkuwar et al., 2017; Vendruscolo et al., 2012), reduced escalated alcohol SA during protracted alcohol abstinence selectively in male alcohol-dependent but not in nondependent rats (Vendruscolo et al., 2012) and also reduced heavy alcohol drinking in non-human primates (Jimenez et al., 2020). Notably, mifepristone has also been shown to decrease alcohol consumption and craving for alcohol in humans with AUD (Vendruscolo et al., 2015). Additionally, it has been reported that it decreased alcohol intake in a limited-access two-bottle choice paradigm (Koenig and Olive, 2004) and intra-CeA infusion of the non-selective GR antagonist reduced alcohol-seeking behavior following a yohimbine challenge (Simms et al., 2012). Moreover, both mifepristone and CORT113176 selectively reduced binge-like ethanol intake in mice selectively bred for high ethanol concentration using drinking in the dark procedures (Savarese et al., 2020). Finally, it was shown that in non-dependent Wistar rats, GR antagonism was more efficacious in female than in male rats (Logrip and Gainey, 2020). Our results are consistent with these latest findings and confirmed that GR antagonists also reduced alcohol intake in nondependent animals, an effect more robust in female versus male rats (Savarese et al., 2020; Logrip and Gainey, 2020).

We assessed anxiety-like behaviors in msPs versus non-selected Wistar counterpart in an array of novel behavioral paradigms that are closely associated with stress disorders. Specifically, we found that male and female msPs display greater anxiety-like behaviors as compared to Wistars when tested in the NIH paradigm. Since sleep disturbances and enhanced startle response are hallmark symptoms in the etiology of stress disorders, the present study examined for the first time whether genotypic differences may underlie changes during diurnal sleep maintenance and hyperarousal states in msPs. CLAMS well correlates with EEG-defined sleep studies (Zeng et al., 2012), while startle responses can capture exaggerated hyperarousal similarly observed in human PTSD patients (Golub et al., 2009). We did not find genotype-dependent changes in sleep maintenance as defined by average bout duration, total sleep time, and number of sleep

bouts. Specifically, we observed that male but not female msPs displayed lower average bout duration during their sleeping phase. Additionally, msPs displayed enhanced acoustic startle responses as compared to their non-selected Wistar counterparts, suggesting that innate disrupted stress systems in msPs resulted in increased hyperarousal. Collectively, these data suggest that msPs are more vulnerable to stressful stimuli. These findings are consistent with previous works from our laboratory demonstrating that msPs display enhanced anxiety-like behavior in the NIH and EPM (Natividad et al., 2017) and marble-burying tasks (Natividad et al., 2021). Prior reports also have revealed that msPs display greater anxiety and depressive-like behaviors in numerous behavioral paradigms involving stress and anxiety (Borruto et al., 2021; Ayanwuyi et al., 2013; Cippitelli et al., 2015; Natividad et al., 2017; Stopponi et al., 2018). To explore the role of the GR in mediating anxiety-like behavior system in msPs, we employed acute systemic administration of mifepristone to reduce the levels of stress and anxiety across our battery of behavioral tests. We found that mifepristone administration produced no beneficial effects in anxiety-like behavior in the NIH paradigm. Also, mifepristone did not significantly restore sleep disturbances and hyperarousal to the level of non-selected Wistar controls. These findings are surprising since it has been well documented that a similar dose range of mifepristone have anxiety alleviating behavioral effects in male rats (Boero et al., 2018; Fox et a., 2016). Taken together, these observations suggest that heightened magnitude of anxiety-related behaviors in msPs does not depend upon acute GR activation.

Msp rats have long been proposed as an innate phenocopy of a subpopulation of patients that drink excessive amounts of alcohol for tension relief and self-medicating purposes (Ciccocioppo et al., 2006). Earlier studies have demonstrated that this rat line is characterized by two single-nucleotide polymorphisms at the CRF1-R receptor locus, leading to an enhanced expression of CRF1R in different brain regions (Hansson et al., 2006). Because of this overexpression, they are highly sensitive to stress and show anxious and depressive-like symptoms that are relieved by alcohol consumption (Ciccocioppo et al., 2006; Hansson et al., 2007; Hansson et al., 2006). Recent findings have proved that negative feedback processes regulating HPA responsiveness are impaired in msP versus Wistar rats. Notably, male msP rats showed an innate increase in phosphorylation at the serine site 232 in the CeA, a marker of GR nuclear localization and transactivation (Natividad et al., 2021). Given these constitutive alterations in their stress system and the role of GR in the progression to alcohol dependence and stress-associated behaviors, we initially hypothesized that administration of GR antagonists would attenuate alcohol SA and innate anxiety-like symptoms more efficaciously in msP rats versus Wistar controls. In fact, msP rats have long been proposed as a phenocopy of

postdependent animals, since they display comorbid symptoms of alcohol preference, high anxiety-like traits and hypersensitivity to stress. However, contrary to our expectations, GR antagonists appeared more efficacious in attenuating alcohol drinking in Wistars than in msP rats and did not alleviate innate negative affect in msP rats.

There are few possibilities to explain the limited efficacy of GR antagonists in msPs. For instance, in an earlier study we found that male msPs had higher adrenocorticotropic hormone levels but lower circulating CORT, whereas in females, msP rats displayed larger elevation of CORT levels in response to restraint stress versus Wistars. In line with this observation, in response to a dexamethasone challenge, msP rats showed a lower reduction in CORT compared to Wistar controls suggesting that msPs contain a blunted stress response that is innate and the negative feedback processes modulating HPA axis responsiveness are diminished in this rat line (Natividad et al., 2021). Hence, it is possible that an acute injection of GR antagonist is not sufficient to ameliorate the heightened levels of stress and to prevent the high alcohol drinking of msP rats. Indeed, repeated daily administration of mifepristone restores depressive-like behavior following chronic defeat stress in mice (Wang et al., 2019), while chronic mifepristone treatment also prevents escalation of alcohol self-administration over time in dependent rats (Vendruscolo et al., 2012). In addition, acute versus chronic mifepristone treatment would impact non-genomic versus genomic GR signaling respectively, likely representing differential mechanisms of action (Groeneweg et al., 2011). As such, future studies are needed to evaluate the effects of GR antagonists following chronic administration. A second possibility is that the higher innate GR phosphorylation observed in msP rats may lead to a differential regulation of the intracellular signaling pathways associated with the GR and prevent the therapeutic effects of GR antagonists. Thus, it is important to examine if transcriptional changes associated with GR activation are different in msPs versus Wistars.

In anxiety-related behavioral tests, we evaluated only the effect of mifepristone, which may exert non-GR-associated actions. Mifepristone is known to be a competitive progesterone receptor antagonist, and progesterone may also serve to modulate anxiety-like behavior (Silva et al., 2016; Galeeva et al., 2007). Thus, a possible explanation is that the effects of progesterone may contribute to the innate anxiety phenotype in msPs in a manner that prevents the therapeutic effects of mifepristone. Indeed, there is evidence suggesting that low levels of progesterone are correlated with greater anxiety-like behavior and corticosterone plasma levels (Flores et al., 2020). Future work is needed to study the effects of mifepristone during specific phases of the estrous cycle, particularly in females who are more vulnerable to stress, and of selective GR antagonists on anxiety-like behaviors.

The present study also assessed the contribution of sex differences in promoting stress-related behaviors in a genotype-dependent manner. While extensive work has focused on male msPs, there are only a few published reports studying the role of sex differences in modulating the anxiety-like predisposition in msPs (Borruto et al., 2021; Natividad et al., 2021; Kirson et al., 2018). We found that males, regardless of genotype, generally display a suppression of food intake as compared to females during novelty stress. In addition, female msPs display a significant increase in startle response during the earliest and most intense audible trial as compared to their respective non-selected Wistar counterparts as well as to male msPs. Consistent with these findings, female msPs display higher amounts of alcohol consumption as compared to their respective Wistars counterparts as well as male msPs (Borruto et al., 2021). However, this report also revealed that both male and female msPs display similar levels of stress and anxiety in tasks involving forced swim and footshock procedures following chronic alcohol exposure (Borruto et al., 2021). This discrepancy between the latter report and our findings may be due to differences in the subjective effects of alcohol relative to alcohol-naïve states.

In this study, we also measured plasma CORT levels prior to and after alcohol SA. Consistent with the results of earlier work, we found higher basal CORT levels in females compared to male rats (Natividad et al., 2021; Oyola and Handa, 2017). The highest concentration was detected in female Wistars followed by female msPs. Moreover, we observed that alcohol SA markedly reduced CORT levels in females of both strains, whereas no changes were observed in males. These data are consistent with earlier studies showing that females displayed enhanced glucocorticoids secretion both at baseline and following stress, and after an alcohol challenge (Oyola and Handa, 2017; Ogilvie and Rivier, 1997; Rivier, 1993). The motivational factors contributing to drinking in males and females may be different, and whether circulating corticosteroid levels may contribute to these discrepancies is unclear. However, it is worth noting that our results indicate that the higher the basal circulating CORT levels, the stronger the inhibitory effect of GR antagonists on alcohol drinking. Since stress enhances the motivation for alcohol, particularly in female rats, we speculate that their drinking is reduced by GR antagonists via processes that suppress HPA axis function and possibly reduce negative mood associated with steroid hormones dysregulation (Solomon et al., 2014; Wulsin et al., 2010).

In summary, our results showed that GR antagonism attenuates alcohol SA, particularly in female rats. Moreover, despite the observation that msPs are more vulnerable to stress and are highly motivated to drink alcohol for tension relieving purposes, they showed a poorer response

to GR antagonists. The present study also revealed that both male and female msPs display elevated anxiety-like behavior versus their non-selected Wistar counterparts in a battery of behavioral tests that are closely associated with stress disorders. In particular, female msPs display greater startle responses versus male msPs, suggesting that responses to stressful stimuli are sex-dependent. Our findings also indicate that mifepristone does not alleviate the innate anxiety-like profile of this genetically-selected rat model.

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Chapter 3: Effect of early social isolation on the vulnerability to develop alcohol-related behaviors in genetically-selected Marchigian Sardinian alcohol preferring and non-preferring Wistar rats.

ABSTRACT

Background: Adverse early life experiences during the postnatal development induce long-lasting neurobiological effects on the stress/reward system and affect subsequent behaviors. Such changes persist throughout life making individuals more vulnerable to suffer from psychiatric disorders, including alcoholism and drug addiction. Maternal separation (MS) in rodent pups represents the most widely used paradigm to examine the long-term effects of early life stress on brain functions and behaviors. However, the majority of prior studies have used MS at very early life time points and stressors experienced by rodents during this developmental period do not properly mimic similar stressors experienced by humans at comparatively developmental stages. Here, to enhance the translational value of our research to understand the long-term consequences of early life stress exposure on later vulnerability to develop alcohol-related behaviors, using a novel experimental protocol, we exposed preweaning rats to mild repeated social deprivations.

Methods: Male and female Marchigian Sardinian alcohol preferring (msP) and Wistar rats were used. From postnatal day (PND)14 to PND21 half of the pups from each experimental group were socially isolated for 30 min a day. Control animals were left undisturbed in their home cage with their mothers and littermates. Operant responding for alcohol under fixed ratio 1 (FR1) and progressive ratio (PR) schedule of reinforcement were then determined at adolescence starting from PND35. The effect of the pharmacological stressor yohimbine in increasing alcohol self-administration (SA) as well as the vulnerability to relapse after stress exposure (yohimbine) were also evaluated.

Results: Operant responding and motivation for alcohol were not altered by our environmental manipulation either in Wistars or in msP rats. Administration of the pharmacological stressor yohimbine (0.0, 0.312, 0.625 and 1.25mg/kg) increased alcohol SA in both rat lines independently from early social isolation (ESI). Following extinction, yohimbine (0.625 mg/kg) significantly reinstated alcohol seeking in female rats only, where ESI resulted in a higher level of reinstatement in adult female msPs.

Conclusions: Overall, results indicate that repeated mild social deprivation experiences during the third week of postnatal life did not affect later susceptibility to increase the motivation for alcohol in male and female msP and Wistar rats. However, in female msP rats, known for having higher anxiety-like traits and stress sensitivity, ESI increased alcohol seeking triggered by the pharmacological stressor yohimbine.

1. Introduction

Alcohol dependence is a multifactorial disorder and genetic and environmental factors interact to determine the individual vulnerability or resilience to develop it (Ducci and Goldman, 2008; Enoch, 2011; Young-Wolff et al., 2011). Stress experienced during the postnatal period represents one of the main negative environmental factors enhancing the risk to develop alcohol use disorder (AUD) later in life (Enoch, 2012; Enoch, 2011; Young-Wolff et al., 2012). Experiencing maltreatment and cumulative stressful life events prior to puberty and particularly in the first few years of life has been reported to be associated with early onset of problem drinking in adolescence and alcohol and drug addiction in early adulthood (Hyman et al., 2006; De Bellis, 2002; Pilowsky et al., 2009; Nelson et al., 2006). Further, numerous studies have shown an association between AUD in women and a history of childhood maltreatment (Anda et al., 2002; Dinwiddie et al., 2000). Therefore, it is of interest to examine how early life factors can influence individual trajectory to develop stress vulnerability and alcohol abuse-related behaviors.

Maternal separation (MS) of rodent pups is considered one of the most potent naturally occurring stressors to which rat pups can be exposed during the neonatal period and has been used to model stressful events during childhood and to study long-lasting brain plastic changes (De Almeida Magalhães et al., 2017; Odeon et al., 2017; García-Gutiérrez et al., 2016; Nishi, 2014; Nylander and Roman, 2013; Palm et al., 2013; Kuhn and Schanberg, 1998). In this procedure, neonatal rats are subjected to repeated separations from the mother for long periods of time, commonly 180-360min per day, during the first two or three weeks of life. This procedure is used to disrupt the mother-pup interaction, which is considered essential for normal neuronal and behavioral development (Nishi, 2014; Nylander and Roman, 2013: Levine, 2002). Several lines of evidence support the notion that early-life rearing conditions have long-term consequences for ethanol consumption (Nylander and Roman, 2013; Becker et al., 2011; Miczek et al., 2008; Roman and Nylander, 2005). As reported in many studies, repeated prolonged separations between the dam and the litters result in a propensity for higher ethanol consumption in adolescent and adult offspring (Amancio-Belmont et al., 2020; De Almeida

Magalhães et al., 2017; Odeon et al., 2017; García-Gutiérrez et al., 2016; Palm et al., 2013; Nylander and Roman, 2013; Moffett et al., 2007).

The isolation of a developing pup from its mother results in a significant emotional stress, but it acts also as a physical stressor (thermal stress, malnourishment, inability to express bodily waste (Thompson et al., 2020). In addition, in the majority of studies MS was applied at very early time points, generally starting at postnatal day (PND) 1, making unclear whether the stress experience at this very early stage of life of the rodent effectively mimic stressful experience in humans at comparatively later developmental stages. It is known in fact that the impact and the modality of MS also changes over development (Thompson et al., 2020). Given the importance to overcome these limitations in the animal models used, and to enhance the translational value of our findings, here we applied a novel protocol of early social isolation (ESI), that differs from the canonical procedures, both in terms of the psychological stress involved and the developmental window in which the stressor was applied. To attenuate the physical stress associated with MS we isolated the rats for brief daily periods (30 min). Moreover, we applied the ESI protocol starting from PND14 through PND-21, an age range that in laboratory rat is characterized by the maturation of crucial social, sensory, motor and cognitive abilities (Rice and Barone, 2000) and in which reconfiguration of the neuronal epigenome and extensive synaptogenesis occur in the brain (Lister et al., 2013). In preliminary experiments we have validated this ESI procedure by showing that Wistar rats briefly isolated during the third postnatal week display altered social behavior at adolescence and a depressive phenotype at adulthood (unpublished data). We explored the long-term consequences of this adverse early social experience on later vulnerability to develop alcoholism between PND35 and PND75, an age span particularly vulnerable to the development of psychiatric disorders characterized by aberrant reward processing (Sale et a., 2014). To assess if environmental stress interacts with heritable factors, we used unselected Wistar rats and genetically-selected Marchigian Sardinian alcohol-preferring (msP) rats, a rodent line characterized by heightened ethanol consumption and stress sensitivity (Ciccocioppo et al., 2006; Borruto et al., 2021). Clinical evidence has reported a link between a history of child maltreatment and a higher risk to develop drug abuse and psychiatric disorders in women than in men, both in adolescence and adulthood, (Anda et al., 2002; Dinwiddie et al., 2000; Osofsky et al., 2021). Moreover, it is known that more frequently than men, women initiate alcohol consumption as a coping strategy to attenuate negative affective states (e.g., anxiety, depression, post-traumatic stress disorder) (Guinle and Sinha, 2020; Peltier et al., 2019; Crutzen et al., 2013). Women are also more likely to relapse in response to stressful events (Hudson and Stamp, 2011; Hyman et al., 2008; Greenfield et al.,

2007). Noteworthy, the majority of prior studies have focused their investigations on male rats only, and very few reports examined the consequence of early life stress on females (Lundberg et al., 2017). Since it is well acknowledged that the vulnerability for several psychopathological conditions display sex differences also in experimental animals (Palanza and Parmigiani, 2017; Zagni et al., 2016), here we decided to investigate the impact of MS in both sexes

3.2 Materials and Methods

3.2.1 Animals and breeding

Male (N = 16-16/line) and female (N = 15-13/line) msP and Wistar rats were used in the study. All subjects were bred in-house at the animal facility of the University of Camerino, Italy. MsP and Wistar dams were single-mated with an individual male rat of the same genotype for 21 days until pregnancy could be verified. At this point, the male rat was removed from the cage and pregnant females were single housed until delivery. The breeding colony was kept on a 12-h light/dark cycle (light on at 7 AM). All animals were disturbed as little as possible during the breeding process and had ad libitum access to food pellets (4RF18, Mucedola, Settimo Milanese, Italy) and tap water. The day of birth was considered postnatal day (PND) 0. At birth, litters were left undisturbed with their mothers until PND14, at which half of the pups from each nest was subjected to the protocol of early social isolation (ESI) (described below). At PND21 all pups were weaned and housed in groups belonging to the same sex and environmental condition in a new room on a 12-h light/dark cycle (light off 8 AM). They were housed in same sex-environmental condition groups of 3 or 4 per cage and had ad libitum access to food and water. All subsequent experiments started on PND35. They weighed approximately 150g (male) and 130g (female) at the beginning of the behavioral experiments. Before starting alcohol self-administration (SA) training, rats were handled for three days 5 minutes daily by the same operators who performed the experiments. Experiments were conducted during the dark phase of the light/dark cycle.

All efforts were made to minimize animal suffering and to reduce the number of animals used.

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

3.2.2 Early social isolation (ESI) protocol

Early social isolation (ESI) occurred between PND14–21. Half of the pups from each nest was singly removed from the nest and placed in a cage with clean bedding for 30 min/day. After the 30 min separation time, they were returned to their home cages. Control pups were left undisturbed with their mothers in their home cages.

3.2.3 Drugs

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

3.2.4 Self-administration apparatus

Operant alcohol self-administration (SA) training and drug testing were conducted in standard operant conditioning chambers (Med Associates, St Albans, VT, USA) enclosed in ventilated sound-attenuating cubicles. Each chamber was equipped with two retractable levers, located in the front panel and positioned laterally to a drinking reservoir, connected with a syringe pump. A house-light was located on the wall opposite to the levers. A windows compatible PC equipped with Med-PC-5 software (Med Associates) controlled the delivery of the fluid, presentation of visual stimuli and recording of the behavioral data.

3.2.5 Experimental procedures

3.2.5.1 EXPERIMENT 1: *Effect of early social isolation on alcohol self-administration.*

Behavioral tests began on PND35. Male (N = 8/environmental condition) and female (N = 8-7/environmental condition) msP rats and male (N = 8/environmental condition) and female (N = 6-7/environmental condition) Wistar rats were used in this experiment. Before the start of operant training, rats were given intermittent access to 10% (v/v) alcohol in an additional water bottle in their home cage for one week. The purpose of this procedure was to avoid neophobic response to alcohol in operant chambers. It has been previously demonstrated that water deprivation, saccharin fading procedure or extended access training are not required to initiate oral ethanol operant responding in rodent strains that do not show natural preference for ethanol (Puaud et al., 2018). On the first day of operant training, rats were given 15 hours access to a

single lever (right lever) that produced 0.1 ml deliveries of water on a fixed-ratio 1 (FR1) schedule of reinforcement with ad libitum food available on the floor of the operant chamber. Afterwards, animals were trained to respond for 10% (v/v) alcohol, in 30 minutes daily sessions under a FR1 schedule of reinforcement. Operant sessions started with lever insertion and ended with levers retraction. Responses at the right (active) lever was reinforced with 0.1 ml of 10% (v/v) alcohol delivered in the drinking reservoir. Reinforcement delivery was followed by 5 seconds time-out (TO), during which the house light was contingently illuminated. During the TO active lever responses were recorded but not reinforced. Throughout the sessions, responses at the left (inactive) lever had no scheduled consequences. The number of operant responses at both the active and inactive levers and the number of reinforcers received were recorded. Alcohol SA training was performed 5 days a week until successful acquisition of a stable baseline of operant responding (18 sessions).

3.2.5.2 EXPERIMENT 2: *Effect of early social isolation on alcohol self-administration on a progressive ratio schedule of reinforcement.*

After successful acquisition of operant responding under FR1 schedule of reinforcement, the same cohort of animals of the previous experiment was switched to a progressive ratio (PR) schedule in order to evaluate their motivation for alcohol (Kallupi et al., 2017; Ayanwuyi et al., 2013). The PR is an operant schedule that allows measuring the maximum amount of work an animal is willing to carry out to obtain the reward, reflecting its motivation for it (Richardson and Roberts, 1996). The break point, defined as the last ratio completed by the animals to obtain one dose of 10% alcohol, was used as a measure of motivation. Under PR contingency, the response requirement necessary to receive one dose of 10% (v/v) alcohol was increased according to the following scale: 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104. Each alcohol-reinforced response resulted in the house light being turned on for 5 seconds, whereas sessions were terminated when more than 30 min had elapsed since the last reinforced response.

3.2.5.3 EXPERIMENT 3: *Effect of early social isolation on alcohol self-administration following yohimbine administration.*

The same animals of the previous experiments were switched to a FR1 schedule for 5 days in order to restore alcohol SA baseline. Once stable self-administration responding was obtained under this reinforcement schedule, the experiment was started. Stress exposure consisted of the challenge with the pharmacological stressor yohimbine at doses previously shown to increase

alcohol-reinforced lever pressing in unselected Wistar animals (Marinelli et al., 2007). To habituate animals to the injection procedure physiological saline was injected i.p. three times prior to drug testing. Yohimbine (0.312, 0.625, 1.25mg/kg) or its vehicle was i.p. injected 30 min before the SA session using a within subject counterbalanced Latin square design. Drug tests were conducted every fourth day. Following each test day, animals were allowed one day off, and a new baseline was then established over the following two days. The number of operant responses at both the active and inactive levers and the number of reinforcers received were recorded.

3.2.5.4 EXPERIMENT 4: *Effect of early social isolation on yohimbine-induced reinstatement of alcohol seeking.*

The experimental procedure consisted of three phases: operant training, extinction and reinstatement. Briefly, the same cohort of animals used in previous experiments was subjected to 5 days of alcohol SA in order to restore a stable baseline of operant responding. Rats were then subjected to 16 daily 30 min extinction sessions, during which lever presses were no longer associated with alcohol delivery, but house light was still presented to allow for its concomitant extinction. Stress exposure consisted of a challenge with a single dose (0.625mg/kg) of the pharmacological stressor yohimbine. Since yohimbine administration increased alcohol SA level in all groups (described below), an intermediate dose of the drug was used for stress-induced reinstatement experiment. Additionally, 0.625mg/kg of yohimbine was previously shown to produce reinstatement to alcohol seeking in msP and Wistar rats (Ayanwuyi et al., 2013). Yohimbine 0.625mg/kg was administered i.p. 30 min prior the 30 min reinstatement session that was conducted under identical conditions to the extinction training. Total responses at the active lever were recorded and used to evaluate alcohol-seeking behavior. Inactive lever responses were also measured.

3.2.6 Statistical analysis

Data of Wistar and msP rats were analyzed independently. For evaluation of operant self-administration training data, the number of alcohol rewards was analyzed by three-way analysis of variance (ANOVA) with “sessions” as a repeated measure and “ESI” and “sex” as between-subject factors.

The break point for alcohol SA under PR schedule of reinforcement was evaluated two-way ANOVAs with “sex” and “ESI” as between subject factors.

The effect of yohimbine on alcohol SA was analyzed by three-way ANOVA with "treatment" as a repeated measure and "sex" and "ESI" as between-subject factors. Active and inactive lever responses were analyzed separately.

Results from yohimbine-induced reinstatement were analyzed by two-way ANOVA with "reinstatement" as a repeated measure and ESI as between-subject factor. When appropriate the Newman-Keuls test was used for post hoc analysis. Significance was conventionally set at $p < 0.05$.

3.3 Results

3.3.1 EXPERIMENT 1: *Effect of early social isolation on alcohol self-administration.*

We set out to test the effect of ESI on acquisition of alcohol SA and pattern of responding for alcohol. Male (N = 8/environmental condition) and female (N = 8-7/environmental condition) msP and male (N = 8/environmental condition) and female (N = 6-7/environmental condition) Wistar rats were trained to self-administer 10% (v/v) alcohol under FR1 schedule of reinforcement. In msP rats a three-way ANOVA revealed an overall effect of sessions [$F_{(17,486)} = 9.3$; $p < 0.0001$] and sex [$F_{(1,486)} = 78.5$; $p < 0.0001$], but no significant effect of ESI [$F_{(1,486)} = 3.5$; $p > 0.05$] and interactions. These results reflect a higher number of lever presses in male msP throughout training (**Figure 1A, left panel**) compared to females (**Figure 1A, right panel**). Similarly, a three-way ANOVA of self-administration data in male (**Figure 1B, left panel**) and female (**Figure 1B, right panel**) Wistar rats found a significant effect of sessions [$F_{(17,450)} = 5.2$; $p < 0.0001$] and sex [$F_{(1,450)} = 28.9$; $p < 0.0001$] and sessions x sex interactions [$F_{(17,450)} = 3.5$; $p < 0.0001$], but no significant effect of ESI [$F_{(1,450)} = 0.6$; $p > 0.05$] and other interactions. Overall, these data suggest that ESI procedure did not alter acquisition and responding for alcohol under FR1 schedule of reinforcement in all groups tested, independently from sex and rat strain.

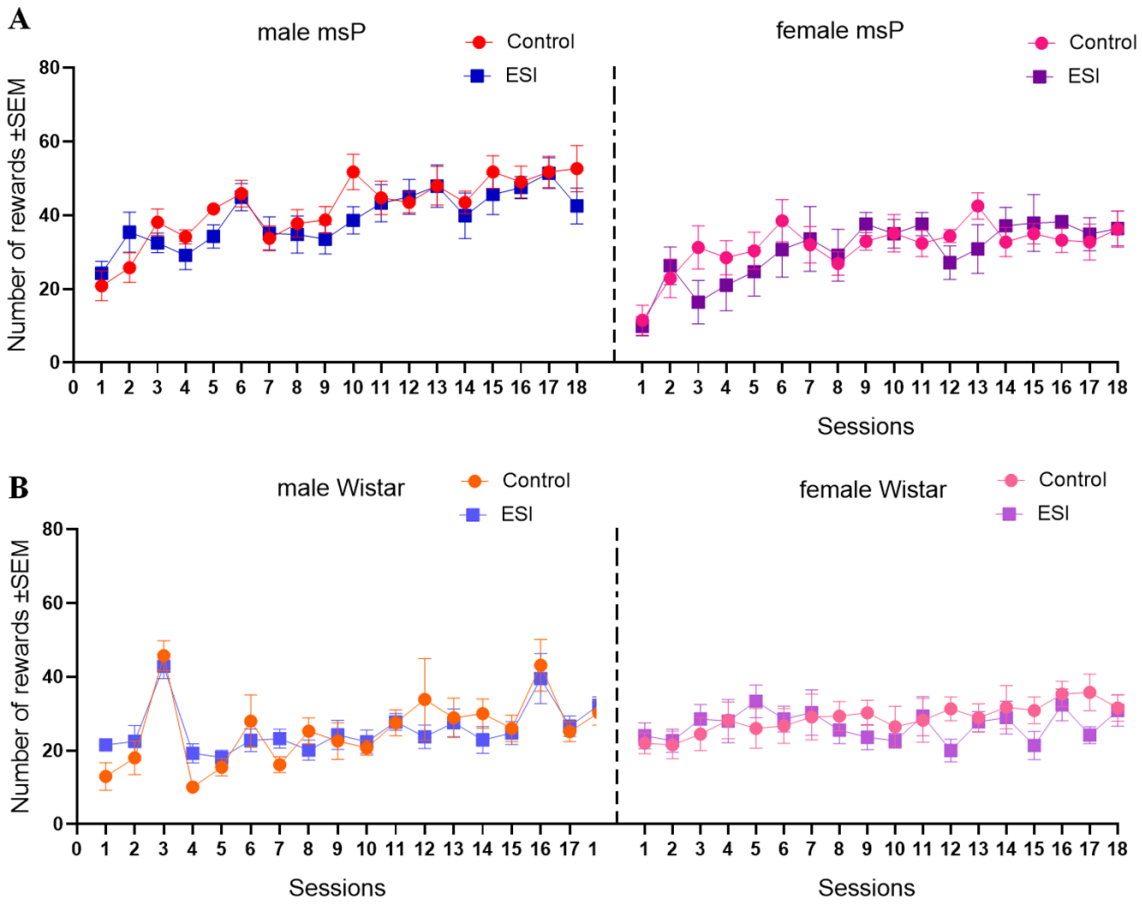

Figure 1. Effect of early social isolation on alcohol self-administration in male and female msP and Wistar rats. Acquisition pattern of alcohol SA in male and female msP and Wistar rats. Early social isolation (ESI) did not affect alcohol reinforcement under FR1 schedule either in male (N = 8 control; N = 8 ESI) (**A, left panel**) and in female (N = 8 control; N = 7 ESI) msP (**A, right panel**) or in male (N = 8 control; N = 8 ESI) (**B, left panel**) and female (N = 6 control; N = 7 ESI) (**B, right panel**) Wistar rats. Data are presented as mean ±SEM of number of rewards (0.1 ml 10% ethanol) gained by animals.

3.3.2 EXPERIMENT 2: *Effect of early social isolation on alcohol self-administration on a progressive ratio schedule of reinforcement.*

After acquisition of a stable baseline of alcohol SA under FR1 contingency, animals were tested in a PR schedule of reinforcement to evaluate their motivation for alcohol. In male and female msP rats a two-way ANOVA showed a main effect of sex [$F_{(1,27)} = 4.9$; $p < 0.05$], but no significant effect of ESI [$F_{(1,27)} = 0.6$; $p > 0.05$] and sex x ESI interaction [$F_{(1,27)} = 0.7$; $p > 0.05$] (**Figure 2A**). These results suggest that male msPs, independently from ESI, showed a higher

motivation for ethanol. In male and female Wistar rats ANOVA revealed no significant effects of sex [$F_{(1,25)} = 0.3$; $p > 0.05$], ESI [$F_{(1,25)} = 0.02$; $p > 0.05$] and sex x ESI interaction [$F_{(1,25)} = 2.4$; $p > 0.05$] (**Figure 2B**).

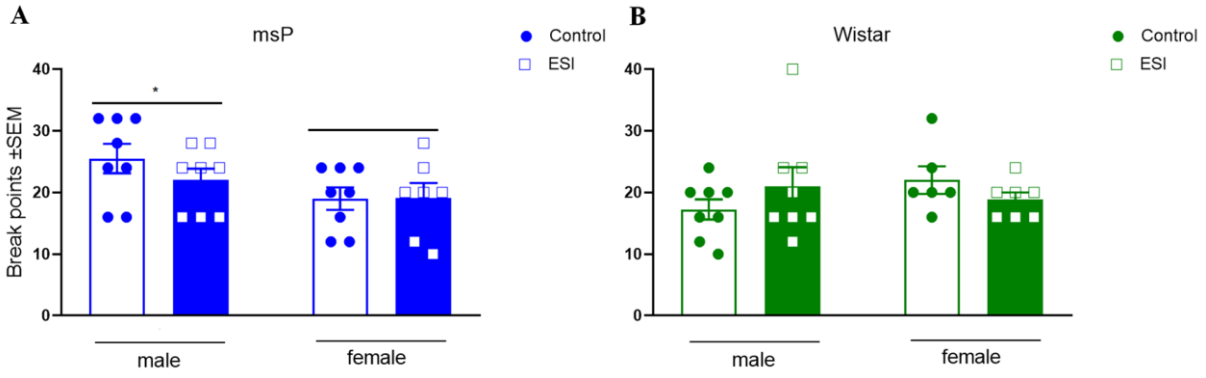

Figure 2. Effect of early social isolation on alcohol self-administration on a progressive ratio schedule of reinforcement in male and female msP and Wistar rats. Early social isolation did not affect the motivation for alcohol under PR contingency in male (N = 8 control; N = 8 ESI) and female (N = 8 control; N = 7 ESI) msP (**A**) as well as in male (N = 8 control; N = 8 ESI) and female (N = 6 control; N = 7 ESI) Wistar rats (**B**). Data are presented as mean ±SEM number of the break points achieved by animals during alcohol SA under a PR schedule of reinforcement. Main effect of sex: * $p < 0.05$. Where not indicated differences from controls were not statistically significant.

3.3.3 EXPERIMENT 3: *Effect of early social isolation on alcohol self-administration following yohimbine administration.*

In this experiment, we tested the hypothesis that administration of the pharmacological stressor yohimbine (0.0, 0.312, 0.625 and 1.25mg/kg) might elicit a further increase of alcohol SA in animals subjected to ESI compared to non-isolated controls and that, being msP more sensitive to stress than Wistars, they might have responded more to yohimbine. In msP rats, a three-way ANOVA revealed an overall effect of treatment [$F_{(3,108)} = 11.02$; $p < 0.0001$], sex [$F_{(1,108)} = 7.2$; $p < 0.01$], but no significant effect of ESI [$F_{(1,108)} = 0.9$; $p > 0.05$] and no interactions indicating that yohimbine enhanced alcohol-reinforced lever pressing both in male and females equally, and that its effect was not affected by ESI (**Figure 3A, upper panel**). ANOVA applied to inactive lever responding showed a significant effect of sex [$F_{(1,108)} = 7.7$; $p < 0.01$] and sex x

ESI interaction [$F_{(1,108)} = 6.2$; $p < 0.05$], but no effect of treatment [$F_{(3,108)} = 2$; $p > 0.05$], ESI [$F_{(1,108)} = 3.8$; $p > 0.05$] and other interactions (**Figure 3A, lower panel**).

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

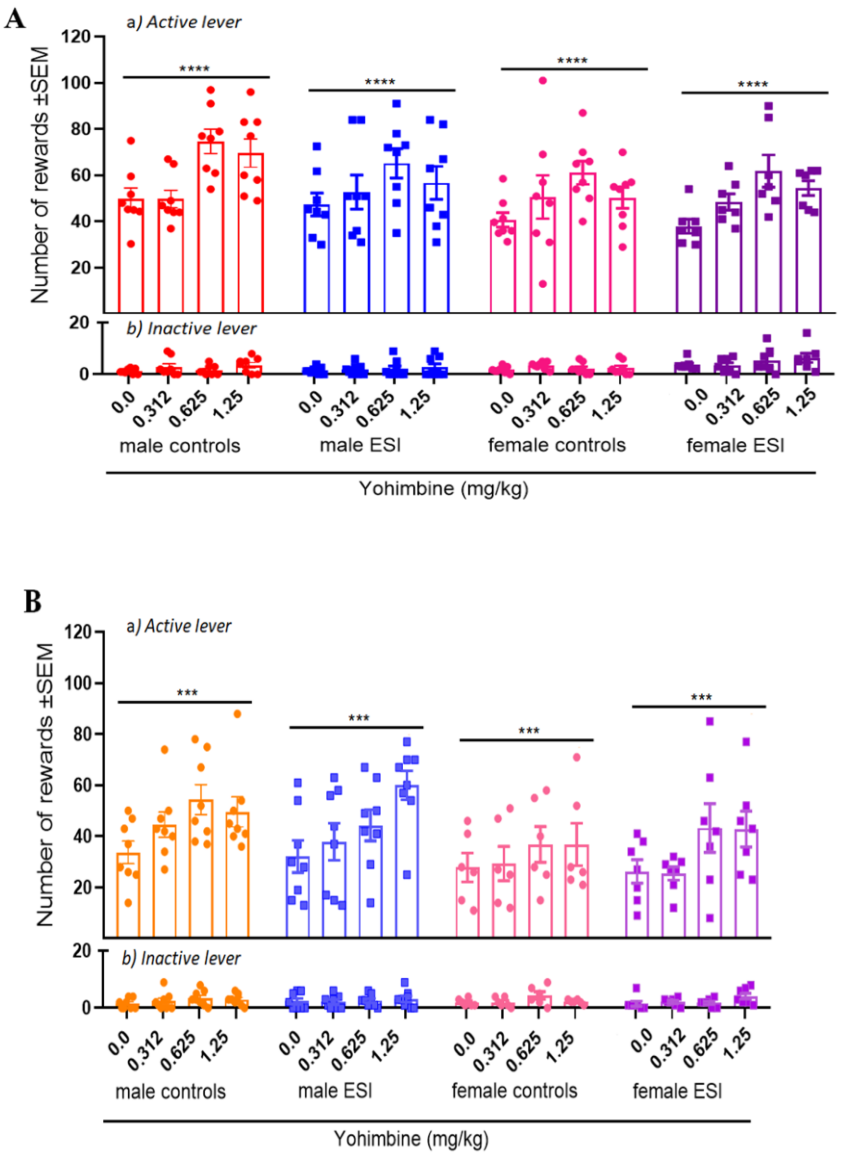

Figure 3. Effect of early social isolation on alcohol self-administration following yohimbine administration in male and female msP and Wistar rats. Male and female msP and Wistar rats were treated with the pharmacological stressor yohimbine (0.0, 0.312, 0.625 and 1.25mg/kg) i.p., 30 min prior to test sessions. Independently from ESI, yohimbine injection increased operant alcohol SA in male (N = 8 control; N = 8 ESI) and female (N = 8 control; N = 7 ESI) msP (**A**) and in male (N = 8 control; N = 8 ESI) and female (N = 6 control; N = 7 ESI) Wistar rats (**B**) Data are expressed as the mean ± SEM of number of: a) reinforced responses (rewards) at the active lever and b) responses at the inactive lever. Main effect of yohimbine treatment: *** $p < 0.001$; **** $p < 0.0001$. Where not indicated differences from controls were not statistically significant.

3.3.4 EXPERIMENT 4: *Effect of early social isolation on yohimbine-induced reinstatement of alcohol seeking.*

Rat trained to operant alcohol self-administration were subjected to an extinction phase during which lever pressing progressively decreased and then were tested for yohimbine-induced reinstatement. During training the mean value of active lever presses relative to the last 3 days of alcohol SA was: male msp controls 77.5 ± 6.6, male msPs ESI 68.6 ± 7.8, female msP controls 45.8 ± 4, female msPs ESI 57.4 ± 6.4, male Wistar controls 44.5 ± 4, male Wistars ESI 42.2 ± 5.4, female Wistar controls 43.4 ± 1.2, female Wistars ESI 31.4 ± 2.6. During the extinction phase responding at active lever progressively decreased. The mean number of lever pressing during the last 3 days of extinction were: male msp controls 12.5 ± 1.8, male msPs ESI 9.5 ± 1.8, female msP controls 9.5 ± 1.9, female msPs ESI 19.5 ± 6.6, male Wistar controls 8.9 ± 1.4, male Wistars ESI 11.4 ± 1.5, female Wistars controls 19.2 ± 3.5, female Wistars ESI 11.5 ± 2.3. In msP rats a three-way ANOVA showed an overall effect of treatment [$F_{(1,27)} = 17.7$, $p < 0.001$], sex [$F_{(1,27)} = 10.3$, $p < 0.01$] and ESI [$F_{(1,27)} = 5.8$, $p < 0.05$] (**Figure 4A)**. Overall ANOVA also revealed a significant treatment x sex interaction [$F_{(1,27)} = 11.9$, $p < 0.01$], treatment x ESI [$F_{(1,27)} = 4.4$, $p < 0.05$], sex x ESI [$F_{(1,27)} = 9.6$, $p < 0.01$], but lack of the treatment x sex x ESI interaction [$F_{(1,27)} = 3.06$, NS]. Post hoc analysis revealed that yohimbine (0.625mg/kg) robustly reinstated alcohol seeking behavior in female rats ($p<0.01$). Moreover it was found that in female msP yohimbine-induced reinstatement was higher ($p<0.05$) in ESI compaered to controls (**Figure 4A, upper panel**). Inactive lever responding was unaffected and ANOVA revealed no effect of treatment [$F_{(1,27)} = 2.2$; $p > 0.05$], sex [$F_{(1,27)} = 0.2$; $p > 0.05$] or ESI [$F_{(1,8)} = 2.3$; $p > 0.05$] and no interactions (**Figure 4A, lower panel**).

In Wistars, ANOVA revealed an overall effect of treatment [$F_{(1,25)} = 29.5$, $p < 0.0001$], sex [$F_{(1,25)} = 12.3$, $p < 0.01$], but no significant effect of ESI [$F_{(1,25)} = 0.9$, $p > 0.05$]. Overall ANOVA also revealed a significant treatment x sex interaction [$F_{(1,27)} = 6.9$, $p < 0.05$], but no significant treatment x ESI [$F_{(1,25)} = 0.2$, $p > 0.05$], sex x ESI [$F_{(1,25)} = 0.9$, $p > 0.05$] and treatment x sex x ESI interactions [$F_{(1,25)} = 0.07$, $p > 0.05$]. Post hoc analysis demonstrated that yohimbine (0.625mg/kg) reinstated extinguished operant responding for alcohol in female rats that was not influenced by ESI (**Figure 4B, upper panel**). Inactive lever presses were negligible and not significantly affected by yohimbine treatment ([$F_{(1,25)} = 4.01$, $p > 0.05$], sex [$F_{(1,25)} = 6.7$, $p < 0.05$], ESI [$F_{(1,25)} = 0.3$, $p > 0.05$], treatment x sex [$F_{(1,25)} = 14.3$, $p < 0.001$], treatment x ESI [$F_{(1,25)} = 0.003$, $p > 0.05$], sex x ESI [$F_{(1,25)} = 7.7$, $p < 0.01$], treatment x sex x ESI [$F_{(1,25)} = 0.008$, $p > 0.05$] (**Figure 4B, lower panel**).

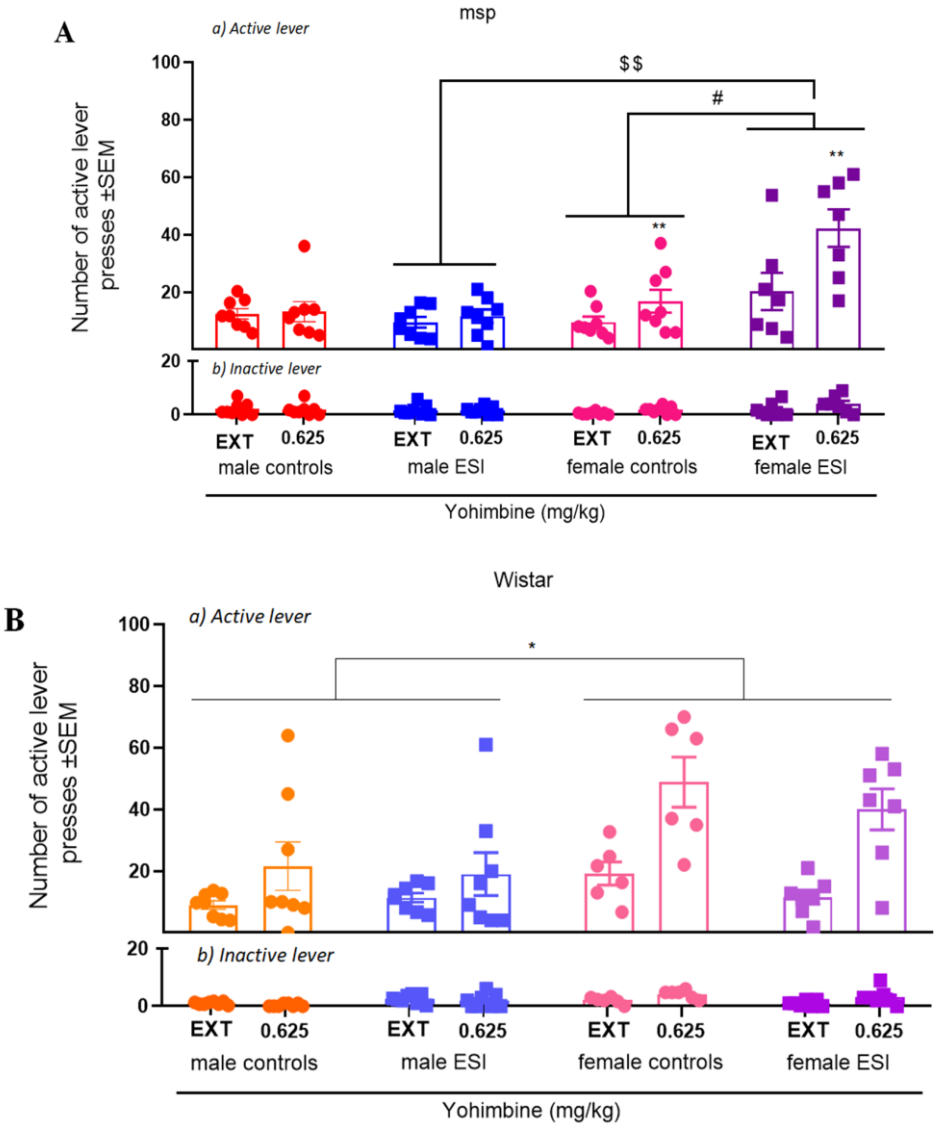

Figure 4. Effect of early social isolation on yohimbine-induced reinstatement of alcohol seeking in male and female msP and Wistar rats. Following alcohol self-administration training, male and female msP and Wistar rats were subjected to an extinction phase and then were treated with yohimbine (0.625mg/kg, i.p.). 30min later reinstatement of responding was evaluated. Extinction (EXT): mean number of lever pressing during the last 3 days of extinction. Yohimbine injection elicited a significant reinstatement of responding in female msP rats (N = 8 control; N = 7 ESI) but not in male msP rats (N = 8 control; N = 8 ESI). ESI increased the level of reinstatement in female msP only (**A**). Similarly, yohimbine reinstated alcohol seeking in female (N = 6 control; N = 7 ESI) but not in males Wistars (N = 8 control; N = 8 ESI). ESI did not potentiate the effect of yohimbine (**B**). Data are expressed as mean ± SEM of: a) total responses at the active; b) inactive levers. Main effect of sex x treatment interaction: * p <

0.05, ** $p < 0.01$; main effect of treatment x ESI interaction: # $p < 0.001$; main effect of sex x ESI interaction $$ $p < 0.01$. Where not indicated differences from controls were not statistically significant.

4. Discussion

The present study investigated the effect of repeated mild early life social isolation on later susceptibility to develop excessive alcohol drinking and yohimbine-stress susceptibility in male and female msP and Wistar rats. Main findings were that ESI during the third postnatal week did not increase the motivation for alcohol either in Wistar rats or in animals genetically selected for high ethanol preference and intake. Independently from ESI exposure administration of yohimbine increased alcohol SA both in msPs and Wistars. In an extinction reinstatement paradigm, yohimbine administration significantly reinstated alcohol seeking in female rats. Interestingly, ESI resulted in a higher level of reinstatement in female msPs but not Wistars. Overall, these data indicate that female rats are more vulnerable to yohimbine-stress induced reinstatement of alcohol-seeking and that female msPs are more sensitive to ESI compared to female Wistars.

Maternal separation in rodent pups has been extensively used as an experimental procedure to study the impact of early life stress on later susceptibility to develop psychiatric disorders and alcohol abuse related behaviors (Nishi, 2020; Nylander and Roman, 2013; Becker et al., 2011; Miczek et al., 2008; Roman and Nylander, 2005). A large body of evidence from rodent studies has shown that prolonged and repeated MS during the first two weeks of postnatal life resulted in higher ethanol consumption at adolescence and adulthood (Amancio-Belmont et al., 2020; De Almeida Magalhães et al., 2017; Odeon et al., 2017; García-Gutiérrez et al., 2016; Palm et al., 2013; Nylander and Roman, 2013; Moffett et al., 2007). MS negatively affects brain areas involved in stress/reward process and several studies have been conducted in order to identify the underlying mechanisms and changes that support the increased vulnerability to alcohol consumption after stress exposure (Gondré-Lewis et al., 2016; Whitaker et al., 2013; Plotsky et al., 2005). However, the results from currently used MS protocols are not uniform and no differences in alcohol consumption or inconsistent results have been reported in some studies that used different MS paradigms (i.e., litter-wise vs individual pup), other controls, low ethanol concentrations or examined adult females (Nylander and Roman, 2013). Here, we used a novel environmental manipulation that differs from canonical MS procedures. The developmental timing at which the stressor is applied and its duration are critical variables that may differently affect neuronal responses and plasticity. Here, we decided to apply brief repeated social

isolations during PND14-21, an age range that is characterized by the maturation of crucial social, sensory, motor and cognitive abilities (Rice and Barone, 2000) and in which reconfiguration of the neuronal epigenome and extensive synaptogenesis occur in the rodent brain (Lister et al., 2013). The time point that we used may more closely map into human toddlerhood and may therefore be more translational than earlier timepoints (Watson et al., 2006). To our knowledge, only one study conducted 1h MS at PND13-21, which resulted in increased adolescent alcohol consumption (Thompson et al., 2020). In addition, our protocol differs from more conventional procedures also for the brief duration of maternal separation. 30min/day compared to more commonly used separations lasting up to 3h per day or even longer (6h). Long maternal separation protocols expose the pups not only to psychological but also to physical (e.g., thermal stress and malnourishment) stress (Thompson et al., 2020) making difficult to disentangle these two stress components.

Results demonstrated that, differently from previous study, our ESI protocol applied during the third postnatal week did not alter the acquisition and the pattern of operant responding for alcohol. It is possible that manipulating the postnatal environment for 30 min/day is not sufficient to increase the motivation for alcohol later in life. Further work is needed to better characterize our social deprivation paradigm in other behavioral tests to examine if it provides a protective or risk environment for the offspring.

Another significant difference is that here we used operant alcohol SA, whereas in most of published papers on MS free choice home cage drinking was employed (Amancio-Belmont et al., 2020; Odeon et al., 2017; De Almeida Magalhães et al., 2017). Nevertheless, few studies in mice reported that MS increased operant alcohol self-administration (Bertagna et al., 2021; García-Gutiérrez et al., 2016). However, species differences can make difficult any direct comparison with the present study. Notably, Lesscher et al. observed that social isolation from PND21-42, a developmental period in which social play is highly abundant, enhanced alcohol intake in the two-bottle choice (2BC) paradigm, whereas did not alter operant responding for alcohol under FR and PR schedule of reinforcement (Lesscher et al., 2015). As such, future studies will be aimed at examining the consequences of our ESI protocol on home cage alcohol drinking.

In our study we systematically evaluated impact of social isolation in female compared to male rats. To study sex differeces is particularly important; in fact, vulnerability for, and severity of, several psychopathological conditions display differences between males and females, in humans as well as in experimental animals (Palanza and Parmigiani, 2017; Zagni et al., 2016). The majority of prior studies have focused their investigations on male rats only with very little

work has been carried out to examine the consequences of early life stress on female animals, particularly in the context of alcohol-related behaviors (Lundberg et a., 2017; Gustafsson et al., 2005; Roman et al., 2004). In one study in mice it was demonstrated that females subjected to 180 min MS consumed more ethanol, showed increased motivation for it and were more resistant to bitter quinine taste than males (Bertagna et al., 2021). Here we observed that female msP and Wistar rats subjected to our ESI protocol did not differ in alcohol intake compared to their respective control groups.

To examine if stressful stimuli later in life interact with ESI to affect ethanol intake, we tested the effect of the pharmacological stress yohimbine on alcohol self-administration. Yohimbine is an alpha-2 adrenoceptor antagonist that increases noradrenaline cell firing (Aghajanian and VanderMaelen, 1982) and enhances noradrenaline release in terminal areas (Abercrombie et al., 1988; Pacak et al., 1992). Yohimbine induces anxiety-like responses both in humans (Holmberg and Gershon, 1961; Bremner et al., 1996b) and laboratory animals (Bremner et al., 1996a), and induces craving in alcohol-dependent patients (Umhau et al., 2011). Results of the present study demonstrated that yohimbine increased alcohol-reinforced lever pressing in all experimental groups, independently from the rearing conditions, genotype and sex indicating that the early social isolation does not alter the response to this pharmacological stressor. Few studies have examined the effect of subsequent stressors different from yohimbine on ethanol intake revealing no difference (Thompson et al., 2020) or an increase in ethanol intake (García-Gutiérrez et al., 2016; Peñasco et a., 2015; Roman et al., 2004).

To the best of our knowledge, no prior studies have examined the consequences of early life stress on later susceptibility to relapse in response to a yohimbine challenge. We found that following extinction, yohimbine significantly reinstated alcohol seeking in female rats and that ESI resulted in a higher level of reinstatement in female msPs only. Yohimbine has demonstrated to reinstate, at least in part, alcohol seeking via activation of extrahypothalamic corticotropin-releasing factor (CRF) 1 receptors mechanisms (Marinelli et al., 2007; Lê et al., 2005). Although previous studies have shown that yohimbine reinstates alcohol seeking in male Wistar (Marinelli et al., 2007; Lê et al., 2005) and msP rats (Ayanwuyi et al., 2013; Hansson et al., 2006), here we did not replicate this finding even though in male Wistars a marginal increase of lever pressing was observed. Noteworthy, here in the attempt to evaluate if ESI enhanced the sensitivity to yohimbine a lower dose of drug (0.625mg/kg) was used compared to earlier work in which it was used at higher (1.25 m/kg) concentration (Marinelli et al., 2007; Lê et al., 2005; Ayanwuyi et al., 2013; Hansson et al., 2006)). Hence, it is highly possible that this low dose is not sufficient to reinstate alcohol seeking in male rats. Overall, our results suggests that females

are more sensitive to yohimbine and this is consistently with previous findings showing higher yohimbine-induced reinstatement of cocaine (Anker et al., 2010) and EtOH (Bertholomey et al., 2016) seeking in females compared to males. This finding is also in line with clinical work indicating that women who abuse cocaine or alcohol are more likely to relapse in response to stressful events (Guinle and Sinha, 2020; Fox and Sinha 2009). Interestingly, we observed that the effect of ESI on yohimbine induced reinstatement was observed only female msP rats. Recently, we carried out an extensive characterization of female msP rats in relation to alcohol drinking and anxiety-related behaviors (Borruto et al., 2021; Vozella et al., 2021). Results showed that female msP rats exhibited increased anxiety-like behaviors and higher freezing in response to footshock stress compared to Wistar rats (Borruto et al., 2021; Vozella et al., 2021). Given the innate heightened stress phenotype of female msP and their higher tendency to reinstate after stress exposure, it is possible that they are more susceptible to the effects of a mild stressor applied in early life compared to their Wistar counterpart. However, the specific mechanism and the neurobiological changes underlying the heightened propensity to relapse of female msPs subjected to early social isolation are still unknown and worthy of further investigations. The noradrenergic system might represent one potential candidate since it was found that alcohol drinking rats with a history of early-life stress had lower Adra2a expression in the hypothalamus than drinking rats not exposed to stress (Comasco et al., 2015).

In summary, data showed that mild ESI during the third postnatal week did not affect the propensity and the motivation to consume alcohol in adolescence and adulthood, but females showed increased sensitivity to a stress challenge which is selectively increased by ESI in female msPs. Given the fact that women that reported childhood adverse experiences are more vulnerable to develop AUD (Anda et al., 2002; Dinwiddie et al., 2000) and women are more likely to relapse in response to stressful events (Hudson and Stamp, 2011; Hyman et al., 2008; Greenfield et al., 2007), our model seems to possess translational value when applied to rats in which excessive alcohol drinking is genetically determined. Future studies will be aimed at better investigating the epigenetic and neurobiological changes that support the increased vulnerability to reinstate after a yohimbine challenge in female msP rats and to further characterize the long-term effects of our ESI protocol on the risk to develop substance use and stress-related disorders.

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Chapter 4: Effect of psilocybin on alcohol-related behaviors in genetically-selected Marchigian Sardinian alcohol preferring rats: a new pharmacological strategy for AUD and stress-related disorder therapy.

ABSTRACT

Background: For most psychiatric conditions, including alcohol use disorder (AUD), FDA approved pharmacological treatments are limited and their efficacy is restricted to only certain subgroups of patients. Scientific interest in the potential of psychedelic drugs has dramatically increased because of clinical preliminary evidence of robust efficacy in treating various psychiatric disorders, including alcoholism. One of the most promising compounds belonging to this class of molecules is psilocybin. Here, to elucidate the therapeutic potential and treatment modalities with this drug, we investigated the effect of psilocybin on alcohol drinking and seeking.

Methods: Male and female genetically selected Marchigian Sardinian alcohol preferring (msP) rats, a well validated model of AUD, were used in the study. In the first experiment we investigated whether psilocybin administration prior to any exposure to alcohol would reduce the propensity of this rat line to drink alcohol in a two-bottles free choice (2BC) paradigm. In the second experiment, we studied the effect of psilocybin on voluntary alcohol consumption in a 2BC paradigm. We also tested the effect of psilocybin in an alcohol deprivation effect (ADE) model of relapse and in a cue-induced reinstatement of alcohol seeking after a period of abstinence. Finally, we investigated if psilocybin may disrupt the reconsolidation process of alcohol-related memory.

Results: Psilocybin given prior to acquisition of alcohol drinking did not change the propensity of msP rats to drink. The drug did to reduce alcohol consumption, nor it prevented increased alcohol drinking after a period of forced abstinence and cue-induced reinstatement of alcohol-seeking. Noteworthy, in a memory retrieval-reconsolidation paradigm, psilocybin markedly attenuated reinstatement of alcohol seeking.

Conclusions: Altogether these data suggest that, despite psilocybin does not affect alcohol drinking and relapse, it may be highly effective if used to block the reconsolidation process of alcohol-related memories. This opens to the possibility of using this psychedelic drug in clinical settings in which AUD patients undergo procedures to recall the memory of alcohol and are then treated with psilocybin during the memory reconsolidation phase.

1. Introduction

Alcohol use disorder (AUD) is one of the leading causes of preventable mortality, responsible for 3 million deaths per year (Witkiewitz, 2019) and represents a significant burden for affected patients and society due to its huge economic costs (Grant et al., 2017). Currently, the acetaldehyde dehydrogenase inhibitor disulfiram, acamprosate and the opioid receptor antagonist naltrexone are the only three drugs FDA approved for AUD therapy (Witkiewitz, 2019). However, these pharmacological treatments for alcohol dependence are limited in their effectiveness to only certain subgroups of patients (Litten et al., 2018) and new more efficacious therapies are desirable.

Recent developments in the study of classic hallucinogens, combined with a re-appraisal of older literature, have led to a renewal of interest in possible therapeutic applications for these drugs, specifically in the treatment of addictions. Psilocybin (3-[2-(dimethylamino) ethyl]-1H-indol-4-yl] dihydrogen phosphate), is one of these hallucinogens, that thanks to some promising clinical preliminary results has gained a lot of attention (De Veen et al., 2017; Bogenschutz, 2013). Psilocybin is a hallucinogenic substance contained in magic mushrooms and has been shown to increase trait openness (MacLean et al., 2011), cognitive and behavioral flexibility (Gallimore, 2015) and to decrease depressive symptoms in terminally ill cancer patients (Grob et al., 2011). These findings suggest that psilocybin might be a valuable compound for the treatment of psychiatric conditions and promising findings have been observed in addiction research (Bogenschutz et al., 2014; Johnson et al., 2014). A recent proof-of-concept study on the efficacy of psilocybin in alcohol dependence, in which participants received 0.3-0.4 mg/kg of psilocybin in two or three sessions, showed a significant reduction in both percentage of drinking days and heavy drinking days, with large effect sizes (Bogenschutz et al., 2014). Although these preliminary results are promising, very little is known on its mechanism of action and on how to use it to maximize its effectiveness.

It is well acknowledged that relapse contributes considerably to the maintenance of alcohol dependence and a major challenge in the treatment of alcoholism and drug addiction is the ability of environmental cues to evoke drug-seeking even after long periods of abstinence (Martin-Fardon and Weiss, 2013; Cooney et al., 1997; O'Brien et al., 1992; Monti et al., 1987). Indeed, environmental cues are one of the most important risk factors driving alcohol seeking, maintenance and relapse (Sinha, 2001). Consistent with the long-lasting risk for relapse, it has been demonstrated that memory formation and drug addiction share common neural circuitries and molecular mechanisms (Hyman et al., 2006; Kelley, 2004). Memory has been found to

exist in two states: a labile or active state and a consolidated or inactive state. Within the first minutes to hours, memory is sensitive to disruption that can lead to its impairment or enhancement. Once consolidation process is completed and memory is stored into stable long-term memory and become resistant to interference (Mcgaugh, 2000). Misanin et al. proposed that, following reactivation of a consolidated memory, it returns to an unstable state again, after which a restabilization process takes place (Misanin et al., 1968). Consistent with this view, infusion of protein synthesis inhibitor anisomycin after reactivation of fear memories during retrieval produced amnesia (Nader et al., 2000). Similarly, several reports demonstrated that appetitive drug memories undergo reconsolidation in a manner similar to aversive memories and that this reconsolidation process can be disrupted to reduce the impact of drug cues on drug seeking (Xue et al., 2012; Bernardi et al., 2007; Robinson and Franklin, 2007; Hellemans et al., 2006; Lee et al, 2006; Lee et al., 2005; Miller and Marshall, 2005). Consistently, it has been found that ethanol-associated memories can also become unstable and liable to disruption after their reactivation (Von der Goltz et al., 2009). Human studies have shown that single hallucinogenic doses of psychedelics can produce long-lasting changes in mood and behavior (Carhart-Harris and Goodwin, 2017; Griffiths et al., 2011; Griffiths et al., 2006). Similarly, rodent studies have found that a single hallucinogenic dose of psychedelics can change the brain structure and behavior long after the drug has been cleared from the body (Cameron et al., 2018; Ly et al. 2018). Additionally, psychedelics and psilocybin have been demonstrated to induce molecular and cellular adaptations related to neuroplasticity (De Vos et al., 2021; Jefsen et al., 2021). Given the ability of psychedelics to alter brain structure, it is possible that they might interfere with the reconsolidation process of alcohol-associated memory after a retrieval procedure.

The present study aimed to investigate the therapeutic potential of the psychedelic drug psilocybin in reducing alcohol-related behaviors in Marchigian Sardinian alcohol preferring (msP) rats, a rat line with excessive alcohol drinking and heightened stress sensitivity. Previous studies conducted in msP rats demonstrated that these animals are highly responsive to approved pharmacological treatment agents of AUD (e.g., naltrexone) (Ciccocioppo et al., 2006). Additionally, we also investigated whether psilocybin, administered shortly after a memory retrieval procedure, might block the reconsolidation of alcohol associated cues preventing cue-induced reinstatement of alcohol seeking.

2. Materials and Methods

2.1 Animals

Male (N = 33) and female msP rats (N = 35) were used in the study. They were bred at the Department of Experimental Medicine of University of Camerino, Italy. For alcohol drinking experiments, they were individually housed on a reverse 12h light/dark cycle (lights off at 9 AM) in a temperature (20-22°C) and humidity (45-50%) controlled room. For the self-administration experiments, they were housed four per cage in a different room with a reverse 12h light/dark cycle (lights off at 8 AM). During the experiments, animals were offered free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy). All the animals were repeatedly handled for 5 minutes a day by same operators who performed the experiments to familiarize them to human contact. All efforts were made to minimize animal suffering and to reduce the number of animals used.

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

2.2 Drugs

The alcohol drinking solution 10% (v/v) was prepared by diluting 95% alcohol (F.L.Carsetti, Camerino, Italy) with tap water. Psilocybin (3-[2-(dimethylamino) ethyl]-1H-indol-4-yl] dihydrogen phosphate, THC Pharm, Frankfurt am Main, Germany) was dissolved in sterile physiological saline and administered intraperitoneally (i.p.) at the volume of 1ml/kg. A single dose of psilocybin (5mg/kg) was used, according to previous published reports on rodent psilocybin using doses ranging between 2mg/kg and 10mg/kg (Meinhardt et al. 2020, Jefsen et al., 2019). Route of administration for each single experiment is described in detail below. To habituate animals to drug administration procedures, rats were injected i.p. three times with physiological saline prior to experiments.

2.3 Two-bottle choice paradigm

The two-bottle free-choice (2BC) paradigm (choice between water and 10% v/v alcohol) was used to measure alcohol drinking and preference (Koob et al., 2003; Tabakoff and Hoffman, 2000). Rats were given continuous access to 10% (v/v) alcohol and water under free access conditions. Fluids were delivered in two graduated drinking tubes with metallic drinking spouts.

Consumption was measured by reading the volume that was consumed after 2, 8 and 24h after alcohol was offered to animals. The tubes were switched daily to avoid the development of side preference. Food intake was measured by weighing the containers. Alcohol, water and food intake was calculated as absolute values at each time interval and are expressed as grams per kilogram (g/kg) to control for the influence of body weight differences (Becker and Lopez, 2004; Finn et al., 2007; Rimondini, Sommer, Heilig, 2003).

2.4 Self-administration apparatus

Self-administration (SA) sessions were conducted in standard operant conditioning chambers (Med Associates, St Albans, VT, USA) enclosed in ventilated sound-attenuating cubicles. Each chamber was equipped with two retractable levers, located in the front panel of the chamber, with a drinking reservoir placed in between, connected with a syringe pump. A house-light was located on the wall opposite to the levers. Behavioral sessions were controlled and recorded by a windows compatible PC equipped with Med-PC-5 software (Med Associates).

2.5 Self-administration training

Animals were trained to self-administer 10% (v/v) alcohol for five days a week, in 30 min daily sessions under a fixed-ratio 1 (FR1) schedule of reinforcement. Before the start of operant training, rats were given intermittent access to 10% (v/v) alcohol in an additional water bottle in their home cage for one week. The purpose of this procedure was to avoid neophobic response to alcohol in operant chambers. On the first day of operant training, rats were given 15 hours access to a single lever (right lever) that produced 0.1 ml deliveries of water on a fixed-ratio 1 (FR1) schedule of reinforcement with ad libitum food available on the floor of the operant chamber. Afterwards, animals were trained to respond for 10% (v/v) alcohol, in 30 minutes daily sessions under a FR1 schedule of reinforcement. Operant sessions started with lever insertion and ended with levers retraction. Responses at the right (active) lever were reinforced with 0.1 ml of fluid of alcohol solution delivered in the drinking reservoir. Reinforcement delivery was followed by 5sec time-out (TO). Discriminative stimuli predictive of ethanol availability (S) consisted in an orange flavor extract deposited on the bedding of the operant chamber before every session. Additionally, each active lever press resulting in ethanol delivery was accompanied by illumination of a house light stimulus (CS). During the TO active lever responses were recorded but not reinforced. Throughout the sessions, responses at the left (inactive) lever were recorded but had no scheduled consequences. All training sessions were

performed during the dark phase of their light/dark cycle and were carried out for 5 days a week for 4 weeks.

2.6 Experimental procedures

2.6.1 EXPERIMENT 1: *Effect of three doses of psilocybin on alcohol consumption in a two-bottle choice paradigm in adolescent msP rats.*

Experiments in rodents have demonstrated that a single hallucinogenic dose of psychedelics can change the brain structure and behavior long after the drug has been cleared from the body (Cameron et al., 2018; Ly et al. 2018). Here, we tested the hypothesis that administration of psilocybin during adolescence, a period characterized by extensive neuroanatomical and functional reorganization of the brain (Schneider, 2013; Fuhrmann et al., 2015), would have long-lasting beneficial effects on alcohol intake. Male (N = 10) and female (N = 10) msP rats were used. Rats were 30 days old at the beginning of the experiments and weighed ~80g (both males and females). They were assigned to different treatment (psilocybin and vehicle) groups consisting of 50% males and 50% females. From postnatal day (PND) 34 animals were injected i.p. three times with psilocybin (5mg/kg) or saline. An interval of 3 days intercurred between injections. 48 hours after the last injection rats were given continuous access to 10% alcohol and water; fluids and food intake were recorded at 2, 8 and 24h from the beginning of the dark phase for five consecutive days.

2.6.2 EXPERIMENT 2: *Effect of psilocybin on Alcohol Deprivation Effect in msP rats.*

Animals from experiment 1 (male (N=10) and female (N=10)) were subjected to an alcohol deprivation phase of three weeks. At the end of this period, animals were divided into two groups (psilocybin and vehicle; consisting of 50% males and 50% females) according to their alcohol intake during the alcohol drinking phase. Animals were injected i.p. with psilocybin (5mg/kg) or saline 12h before the beginning of the dark cycle to ensure that any behavioral effects would be due to persistent changes and not to the acute effects of the drug. The day after, when the light switched off, 10% alcohol was reintroduced and animals were allowed to drink. Alcohol, water and food intake were measured at 2, 8 and 24h and the occurrence of an Alcohol Deprivation Effect (ADE) was determined.

2.6.3 EXPERIMENT 3: *Effect of psilocybin on alcohol consumption in a two-bottle choice paradigm in msP rats.*

Adult male (N=12, ~400g) and female (N=14, ~250g) msP rats were used. They were single housed under a reverse 12h/12h light/dark cycle (lights off at 9 AM). For 12 days, they were given continuous access to 10% alcohol and water under free access conditions to achieve a stable baseline of drinking and high preference for alcohol. Once baseline drinking was reached, rats were divided into two groups with similar alcohol intake during the last 3 days of 2BC. Each group consisted of male and female animals 50% each. They were i.p. injected with psilocybin (5mg/kg) or saline 12h before the beginning of the dark phase to avoid the acute hallucinogenic effects of the drug. Alcohol, water and food intake were recorded the day after at 2, 8 and 24h.

2.6.4 EXPERIMENT 4: *Effect of psilocybin on a cue-induced reinstatement of alcohol-seeking behavior.*

A new cohort of msP rats (male (N=8, ~400g) and female (N=8, ~250g)) was trained to self-administer 10% (v/v) alcohol solution as described above. All training sessions were performed during the dark phase of their light/dark cycle until achieving a stable baseline of operant responding. Subsequently, all animals were subjected to a period of forced abstinence of 21 days during which they were left undisturbed in their home cages. On the 21st day of abstinence, animals were divided into two groups, consisted of males and females 50% each, balanced for alcohol rewards obtained during the last 3 days of operant training. Rats were injected i.p. with psilocybin (5mg/kg) or vehicle 12h before the beginning of the dark phase and cue-induced reinstatement test was performed the following day. During the reinstatement test rats were exposed to the same conditions of the alcohol SA session, except that ethanol was not delivered. Total responses at both the active and inactive levers were recorded.

2.6.5 EXPERIMENT 5: *Effect of psilocybin on reconsolidation of alcohol-related memory.*

Since a single hallucinogenic dose of psychedelics can change the brain structure and behavior (Cameron et al., 2018; Ly et al. 2018), we tested the hypothesis that an injection of psilocybin might impair the reconsolidation of alcohol related-associations after a memory retrieval procedure, by preventing cue-induced reinstatement of alcohol seeking. Additional male (N=8, ~400g) and female (N=8, ~250g) msP rats were trained to self-administer 10% (v/v) alcohol in 30 min daily sessions under FR1 schedule of reinforcement in the presence of conditioned

stimuli, as described above. Once stable SA responding was obtained, animals were left undisturbed in their home cages for the following 3 weeks. Subsequently, animals were assigned into different treatment groups on the basis of the number of active levers presses relative to the last three training sessions. They were split into two treatment groups, consisting of males and females 50% each. Drug test consisted of three cycles, each composed of a memory retrieval session the first day, and a cue-induced reinstatement test performed 24h later; retrieval/reinstatement cycles were repeated once a week for three times. The 5 min memory retrieval session was identical to alcohol SA session, except that ethanol was not made available except for the first two lever presses that were reinforced with 0.1 ml of ethanol, which served as an additional olfactory/gustatory ethanol cue (Vengeliene et al., 2007). Immediately after the memory reactivation session, rats received an i.p. injection of psilocybin (5 mg/kg) or its vehicle. 24h later, animals were returned to the operant box and a 30 min self-administration/reinstatement session in the presence of cues predictive of alcohol availability started. The reinstatement session was identical to the 5-min retrieval session of the day before. Responses at both the active and inactive levers were recorded. For confirmation of the results, at completion of this experiment, rats were retrained to alcohol self-administration for two weeks and the memory retrieval/reconsolidation experiment was replicated. For this second experiment the group of rats previously treated with psilocybin receive vehicle, while animals previously treated with saline were injected with psilocybin.

2.7 Statistical analysis

In the 2BC experiments the effects of psilocybin on alcohol, water, and food intake were analyzed by a two-way analysis of variance (ANOVA), with “treatment” as between-subject factor and “time” as within-subject factor.

The effect of psilocybin on ADE was analyzed by two-way ANOVA with “treatment” as between-subject factor and “ADE” as within-subject factor. Drug effect on water and food intakes was analyzed by two-way ANOVA with “treatment” as between-subject factor and “time” as within-subject factor.

An unpaired Student’s t-test was used to analyze the effect of psilocybin in preventing cue-induced reinstatement of alcohol-seeking. Active and inactive lever responses were analyzed separately.

Data from memory reactivation derived from the two tests were pooled and analyzed by two-way ANOVA with both “treatment” and “time” as within-subject factors. To further explore the contribute of sex, data from male and female msP rats were analyzed separately using a

three-way ANOVA with “time” and “treatment” as within-subject factors and “sex” as between-subject factor.

3. Results

3.1 EXPERIMENT 1: *Effect of three doses of psilocybin on alcohol consumption in a two-bottle choice paradigm in adolescent msP rats.*

Two-way ANOVA on alcohol intake revealed a main effect of time, but no significant effect of psilocybin treatment and time x treatment interaction at each time point at which alcohol intake was recorded (2h: time [$F_{(4,18)} = 4.9$; $p < 0.05$], treatment [$F_{(1,18)} = 0.2$; $p > 0.05$], time x treatment interaction [$F_{(4,72)} = 0.6647$; $p > 0.05$] **Figure 1A**; 8h: time [$F_{(4,18)} = 4,2$; $p < 0.05$], treatment [$F_{(1,18)} = 0.05$; $p > 0.05$], time x treatment interaction [$F_{(4,72)} = 0.6$; $p > 0.05$] **Figure 1B**; 24h: time [$F_{(4,18)} = 9.4$, $p < 0.001$], treatment [$F_{(1,18)} = 0.05$, $p > 0.05$], time x treatment interaction [$F_{(4,72)} = 0.8$, $p > 0.05$] **Figure 1C**). Two-way ANOVA of the effect of psilocybin on water intake at 2h revealed no significant effect of time [$F_{(4,18)} = 1.8$; $p > 0.05$], treatment [$F_{(1,18)} = 0.09$; $p > 0.05$] and time x treatment interaction [$F_{(4,72)} = 0.6$; $p > 0.05$] **Figure 1D**. At the other time points two-way ANOVA of the effect of psilocybin on water intake found a main effect of time, but no significant effect of treatment and time x treatment interaction (8h: time [$F_{(4,18)} = 3.5$; $p < 0.05$], treatment [$F_{(1,18)} = 0.04$; $p > 0.05$], time x treatment interaction [$F_{(4,72)} = 1.9$; $p > 0.05$] **Figure 1E**; 24h: time [$F_{(4,18)} = 2.5$; $p < 0.05$], treatment [$F_{(1,18)} = 0.04$; $p > 0.05$], time x treatment interaction [$F_{(4,72)} = 0.9$; $p > 0.05$] **Figure 1F**. Two-way ANOVA of the effect of psilocybin on food intake at 8h revealed no significant effect of time time [$F_{(4,18)} = 2.5$; $p > 0.05$], treatment [$F_{(1,18)} = 0.9$; $p > 0.05$], time x treatment interaction [$F_{(4,72)} = 0.5$; $p > 0.05$] **Figure 1H**. Two-way ANOVA of the effect of psilocybin on food intake at the other time points found a main effect of time, but no significant effect of time and time x treatment interaction (2h: time [$F_{(4,18)} = 5.7$; $p < 0.05$], treatment [$F_{(1,18)} = 0.7$; $p > 0.05$], time x treatment interaction [$F_{(4,72)} = 0.7$; $p > 0.05$] **Figure 1G**; 24h: time [$F_{(4,18)} = 8.4$; $p < 0.0001$], treatment [$F_{(1,18)} = 1.1$; $p > 0.05$], time x treatment interaction [$F_{(4,72)} = 0.8$; $p > 0.05$] **Figure 1I**). Overall, these data indicate that administration of three doses of psilocybin during adolescence did not reduce alcohol drinking in msP rats and water and food intakes were unaffected by psilocybin treatment.

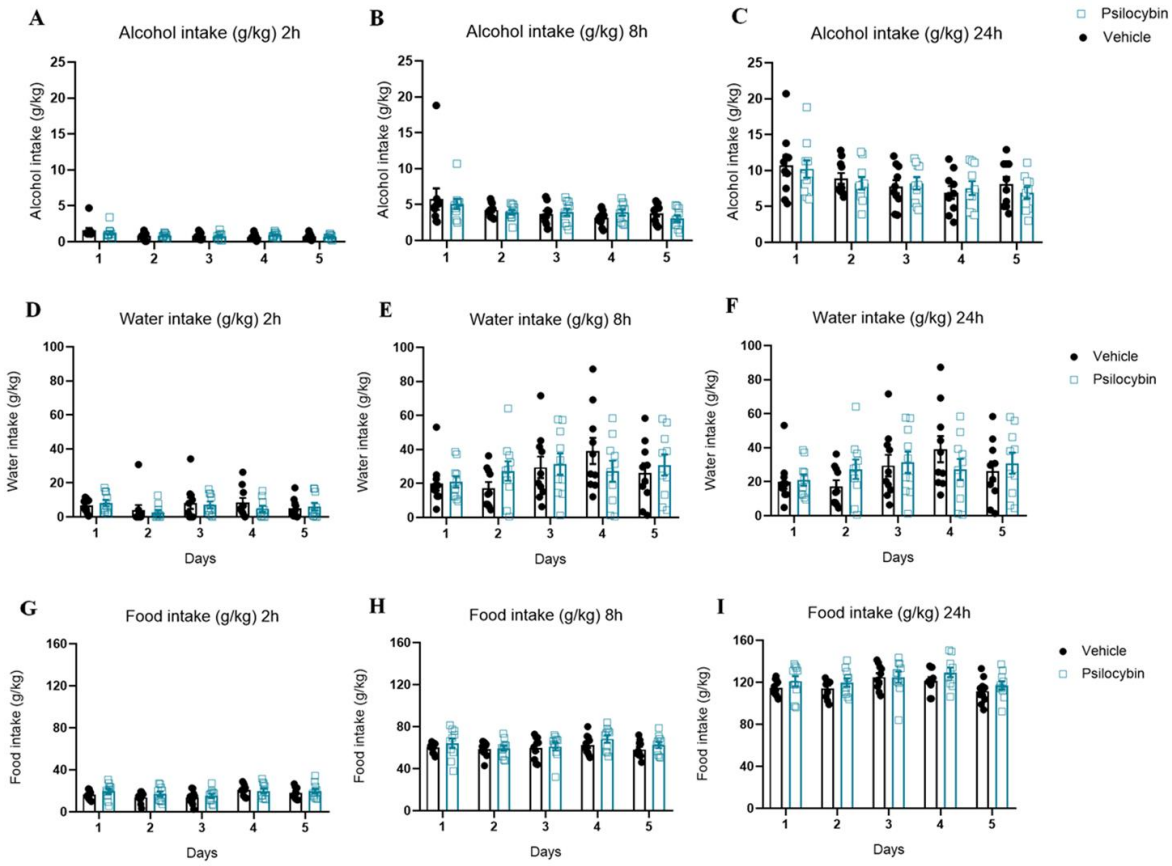


Figure 1. Effect of three injections of psilocybin (5mg/kg/injection) during adolescence on alcohol intake in a two-bottle choice paradigm. After receiving three injections of psilocybin (5mg/kg) or vehicle (N = 10, males and females 50% each/group) during adolescence, 10%(v/v) alcohol was offered to animals and alcohol, water and food intakes were recorded at 2, 8, and 24 h for five consecutive days. Administration of psilocybin did not reduce alcohol drinking in msP rats at 2h (**A**), 8h (**B**) and 24h (**C**). Psilocybin treatment did not affect water intake at 2h (**C**), 8h (**D**) and 24h (**F**) and food intake at 2h (**G**), 8h (**H**) and 24h (**I**) during the five days of observation. Data are expressed as mean ± SEM. Where not indicated, differences from vehicles were not statistically significant.

3.2 EXPERIMENT 2: Effect of psilocybin on alcohol deprivation effect in msP rats.

After alcohol exposure, the same cohort of rats of the previous experiment (N = 10/sex) was subjected to a period of alcohol deprivation lasting 3 weeks, after which psilocybin (5mg/kg) or vehicle were injected 12h prior alcohol reintroduction and the occurrence of an ADE was determined. Alcohol, water and food intake were monitored at 2, 8 and 24h. The mean baseline values for alcohol intake (g/kg) relative to the last 3 day of alcohol exposure were: vehicle group: 7.8 ± 0.8; psilocybin group: 7.2 ± 0.9 (N = 10, males and females 50%, each group). Two-way ANOVA of alcohol intake revealed no significant effect of ADE [$F_{(1,18)} = 1.5$; $p > 0.05$], treatment [$F_{(1,18)} = 0.02$; $p > 0.05$], ADE x treatment interaction [$F_{(1,18)} = 0.004$; $p > 0.05$] at 2h (**Figure 2A**). ANOVA of alcohol intake at 8h revealed no significant effect of ADE [$F_{(1,18)} = 3.9$; $p > 0.05$], treatment [$F_{(1,18)} = 0.7$; $p > 0.05$], ADE x treatment interaction [$F_{(1,18)} = 0.2$; $p > 0.05$] (**Figure 2B**). Finally, ANOVA of alcohol intake at 24h revealed an overall effect of ADE [$F_{(1,18)} = 7$; $p < 0.05$], but no significant effect of treatment [$F_{(1,18)} = 1.1$; $p > 0.05$], ADE x treatment interaction [$F_{(1,18)} = 0.5$; $p > 0.05$] (**Figure 2C**) suggesting that both groups increased their alcohol consumption after a period of deprivation. Water and food intakes were recorded at 2, 8 and 24h to confirm the specificity of drug effect on alcohol intake. Two-way ANOVA of the effect of psilocybin on water and food intake found a main effect of time, but no significant effect of treatment and time x treatment interaction (water: time [$F_{(2,36)} = 14.9$; $p < 0.0001$], treatment [$F_{(1,18)} = 2.1$; $p > 0.05$], time x treatment interaction [$F_{(2,36)} = 1.3$; $p > 0.05$] **Figure 1D**; food: time [$F_{(2,36)} = 565.5$; $p < 0.0001$], treatment [$F_{(1,18)} = 0.03$; $p > 0.05$], time x treatment interaction [$F_{(2,36)} = 0.5$; $p > 0.05$] **Figure 1D**).

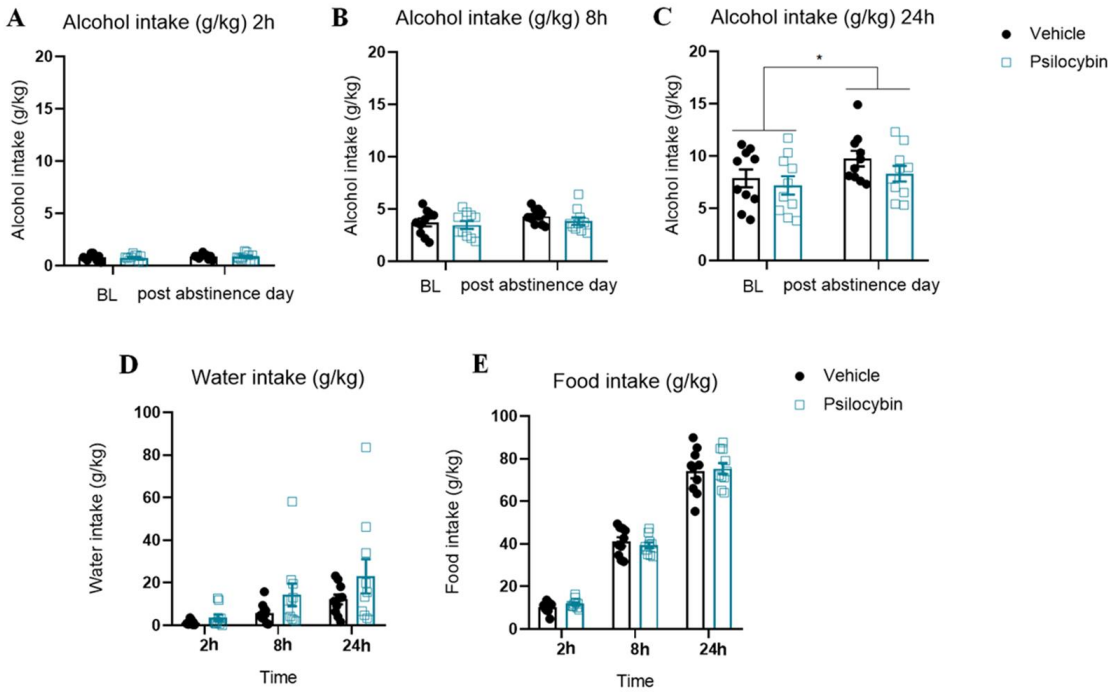


Fig.2 Effect of psilocybin (5mg/kg) on alcohol deprivation effect. The mean of the last three days of measurements of ethanol intake is given as baseline (BL) drinking. After three weeks of alcohol abstinence, psilocybin (5mg/kg) or vehicle were administered to animals (N = 10, males and females 50% each/group) and voluntary alcohol intake was recorded at 2h (**A**), 8h (**B**) and 24h (**C**). Psilocybin did not prevent the occurence of ADE after 24h of alcohol reintroduction (**C**). Psilocybin treatment did not affect water (**D**) and food intake (**E**) at each time point. Data are expressed as mean ± SEM. * $p < 0.05$ significant difference from baseline drinking.

3.3 EXPERIMENT 3: *Effect of psilocybin on alcohol consumption in a two-bottle choice paradigm in msP rats.*

After achieving a stable baseline of drinking and high preference for alcohol, male and female were injected with psilocybin (5mg/kg) or vehicle (N = 13/group; males N=6/group females N = 7/group) and alcohol, water and food intake were recorded at 2, 8 and 24 from the beginning of the dark phase. Two-way ANOVA revealed an overall effect of time [$F_{(2,24)} = 212.7$; $p < 0.0001$], but no significant effect of treatment [$F_{(1,24)} = 1.3$; $p > 0.05$] and time x treatment interaction [$F_{(2,48)} = 1.4$; $p > 0.05$] (**Figure 3A**). Considering that we had sufficiently large male and female groups size, we also analyzed male and female data separately. Overall ANOVA of

alcohol intake of male msP rats revealed a significant effect of time [$F_{(2,10)} = 202.1$, $p < 0.0001$], but no main effect of treatment [$F_{(1,10)} = 4$; $p > 0.05$] and time x treatment interaction [$F_{(2,20)} = 2.4$; $p > 0.05$] (**Figure 3B**). As regard female msP rats, two-way ANOVA showed a significant effect of time [$F_{(2,12)} = 215.6$, $p < 0.0001$], but no significant effect of treatment [$F_{(1,12)} = 0.8$; $p > 0.05$] and time x treatment interaction [$F_{(2,24)} = 1.3$; $p > 0.05$] (**Figure 3C**). Analysis of water consumption revealed a significant effect of time [$F_{(2,24)} = 22.5$; $p < 0.0001$] and treatment [$F_{(1,24)} = 4.3$; $p < 0.05$], but not time x treatment interaction [$F_{(2,48)} = 1.8$; $p > 0.05$] (**Figure 3D**). Food consumption was unaffected by psilocybin administration at each time point at which food intake was recorded. ANOVA showed an overall effect of time [$F_{(2,24)} = 622.6$; $p < 0.0001$], but not treatment [$F_{(1,24)} = 0.05$; $p > 0.05$] and time x treatment interaction [$F_{(2,48)} = 0.2$; $p > 0.05$] (**Figure 3G**). Again, data were also analyzed separately for male and female animals. In male msP rats, overall ANOVA of water intake revealed a significant effect of time [$F_{(2,10)} = 7.9$; $p < 0.05$], but not treatment [$F_{(1,10)} = 1.2$; $p > 0.05$] and no time x treatment interaction [$F_{(2,20)} = 1.2$; $p > 0.05$] for water consumption of male msP rats (**Figure 3E)**. As regard of food intake in male msP rats, ANOVA showed a significant effect of time [$F_{(2,10)} = 972.0$; $p < 0.0001$], but not treatment [$F_{(1,10)} = 0.06$; $p > 0.05$] and time x treatment interaction [$F_{(2,20)} = 0.6429$; $p > 0.05$] (**Figure 3H**). In female msP rats, ANOVA of water intake revealed a significant effect of time [$F_{(2,12)} = 24.4$; $p < 0.0001$] and treatment [$F_{(1,12)} = 4.9$; $p < 0.05$], but not time x treatment interaction [$F_{(2,24)} = 2.2$; $p > 0.05$] for water intake (**Figure 3F**). ANOVA of food intake in female msP rats showed an overall effect of time [$F_{(2,12)} = 389.8$; $p < 0.0001$], but not treatment [$F_{(1,12)} = 0.3$; $p > 0.05$] and time x treatment interaction [$F_{(2,24)} = 0.1$; $p > 0.05$] (**Figure 3I**).

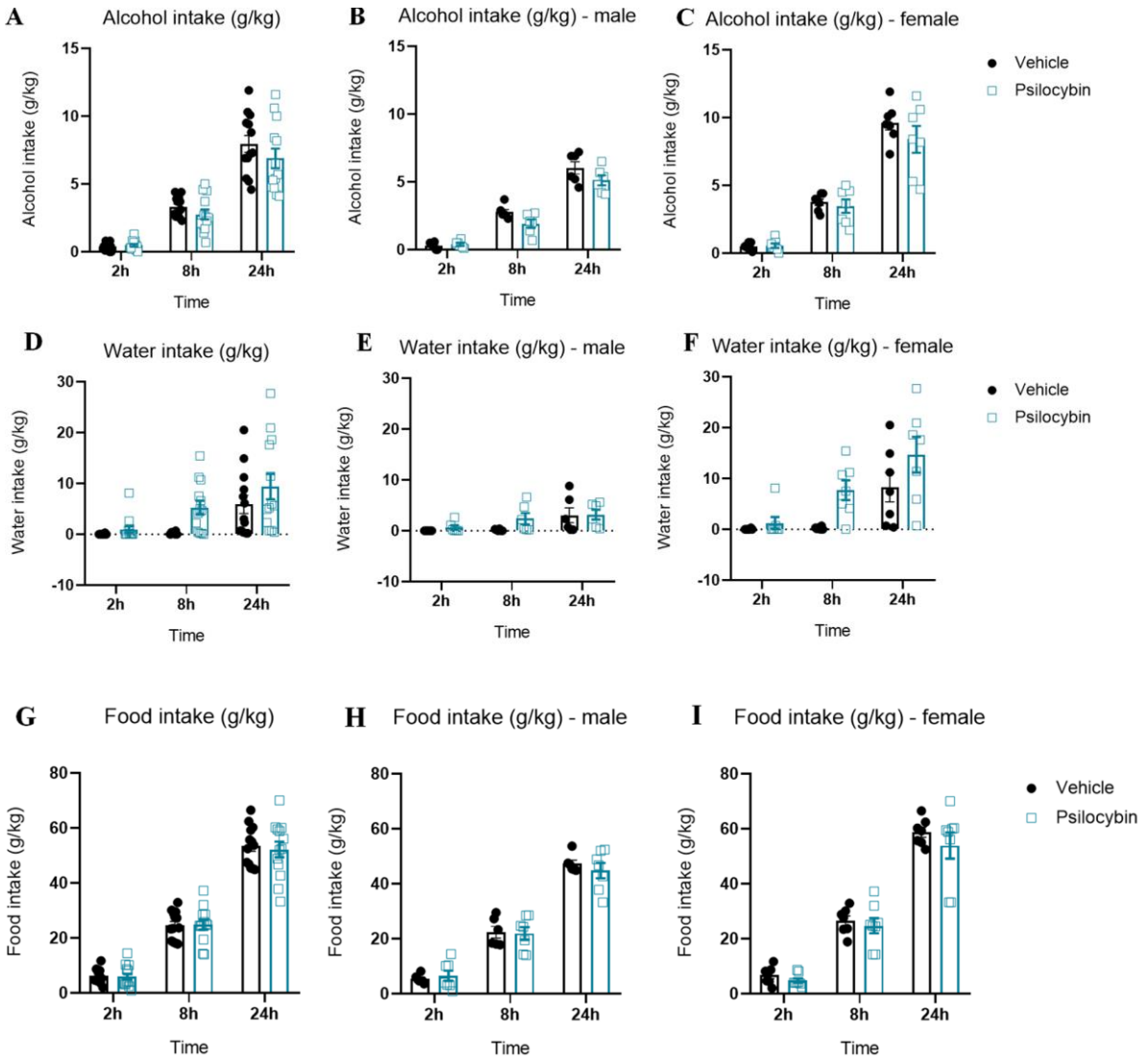


Figure 3. Effect of psilocybin (5mg/kg) on alcohol consumption in a two-bottle choice paradigm. Rats were injected with psilocybin (5mg/kg) or vehicle (N = 13, males N=6, females N = 7/group), 10% (v/v) alcohol was offered to animals and voluntary alcohol, water and food intakes were recorded at 2, 8, and 24h from the beginning of the dark phase. Administration of psilocybin did not reduce alcohol drinking in msP rats at each time point (**A**). Psilocybin did not show sex specific effects when data were analyzed separately for males (**B**) and females (**C**). Drug treatment did not affect water (**C**), (**D**),(**F**) and food intakes (**G**), (**H**), (**I**). Data are expressed as mean ± SEM. Where not indicated, differences from vehicles were not statistically significant.

3.4 EXPERIMENT 4: *Effect of psilocybin on a cue-induced reinstatement of alcohol-seeking behavior.*

Animals were divided into two different groups with similar alcohol SA levels relative to the last 3 days of training (vehicle: 51.5 ± 4.9; psilocybin: 51 ±3.8). After 21 days of forced abstinence, animals were injected with psilocybin (5mg/kg) or vehicle 12h before performing cue-induced reinstatement test of alcohol seeking. Data analysis by means of unpaired student's t-test revealed no significant difference between psilocybin and vehicle groups ($t(14) = 0.04$; $p > 0.4$) (**Figure 4, upper panel**). Statistical analysis of inactive lever responses by unpaired student's t-test revealed no significant effect of psilocybin ($t(14) = 1.05$; $p > 0.05$) (**Figure 4, lower panel**).

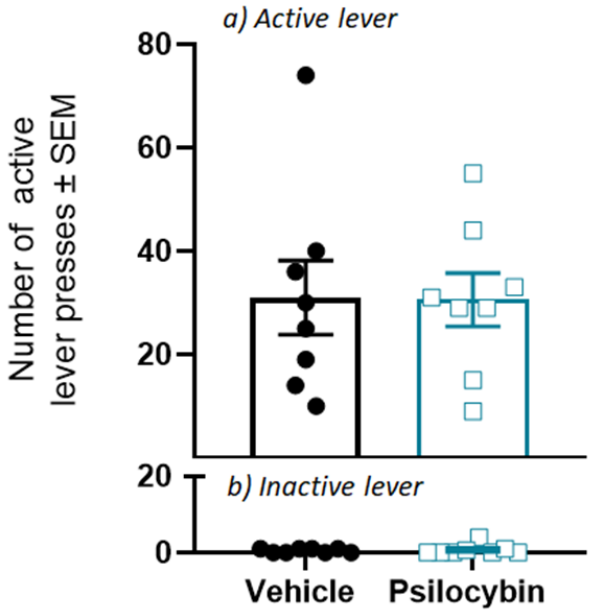
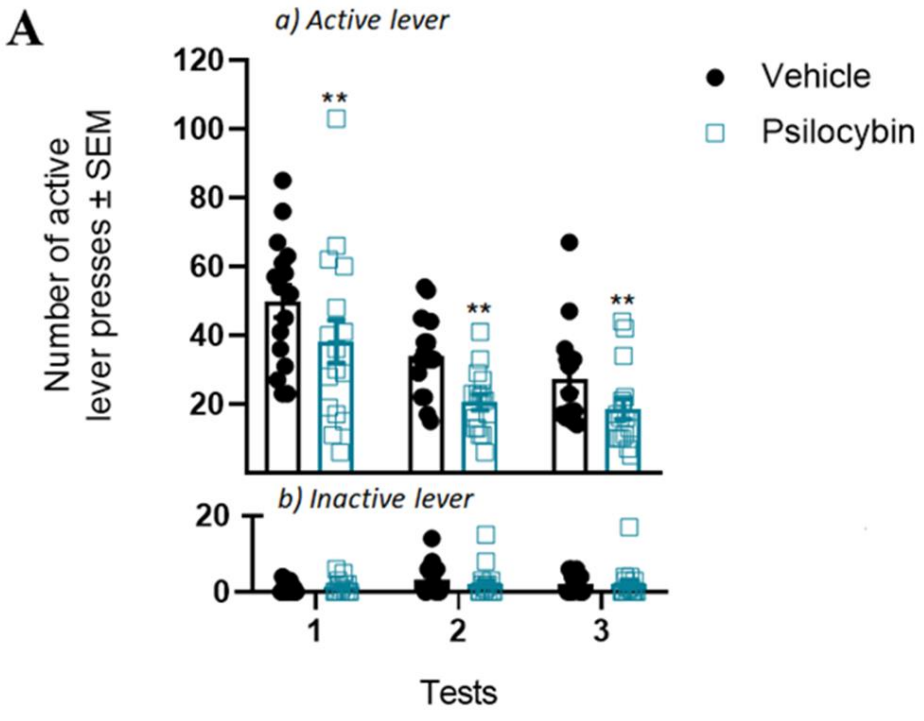


Figure 4. Effect of psilocybin (5mg/kg) on cue-induced reinstatement of alcohol-seeking behavior. MsP rats were treated with psilocybin (5mg/kg) or vehicle (N = 8, males and females 50% each/group) 12h before the reinstatement session. Data are expressed as mean ± SEM of a) total responses at the active and b) total responses at inactive lever. Where not indicated, differences from vehicles were not statistically significant.

3.5 EXPERIMENT 5: *Effect of psilocybin on reconsolidation of alcohol-related memory.*

Animals were divided into two different groups with similar levels of active lever presses relative to the last 3 days of operant training (vehicle: 58 ± 7; psilocybin: 57 ± 5). Data were first analyzed with males and females pooled in the same group. Two-way ANOVA for repeated measures relative to the effect of psilocybin (5mg/kg) revealed an overall effect of time [$F_{(2,30)} = 17.05$; $p < 0.0001$] and treatment [$F_{(1,15)} = 12.9$; $p < 0.001$] but no significant time x treatment interaction [$F_{(2,30)} = 0.3$; $p > 0.05$] (**Figure 5A, upper panel**). A two-way ANOVA applied to inactive lever responding showed no overall effect of time [$F_{(2,30)} = 0.03$; $p > 0.05$], treatment [$F_{(1,15)} = 0.8$; $p > 0.05$] or time x treatment interaction [$F_{(1,35)} = 1.7$; $p > 0.05$] (**Figure 5A, lower panel**). Then, we investigated the contribution of sex differences by analyzing data by means of three-way ANOVA. Three-way ANOVA revealed a significant effect of time [$F_{(2,28)} = 16.9$; $p < 0.0001$] and treatment [$F_{(1,14)} = 0.2$; $p > 0.05$] but no overall effect of sex [$F(1,7) = 2.599$, $p > 0.05$] and interactions (**Figure 5B, upper panel**). A three-way ANOVA applied to inactive lever responding showed a significant effect of time [$F_{(2,28)} = 3.0$; $p < 0.05$], but no significant effect of treatment [$F_{(1,14)} = 0.06$; $p > 0.05$] or sex [$F_{(1,14)} = 1$; $p > 0.05$] and interactions (**Figure 5B, lower panel**). Altogether these data indicate that psilocybin significantly reduced alcohol seeking behavior 24h after memory retrieval process.



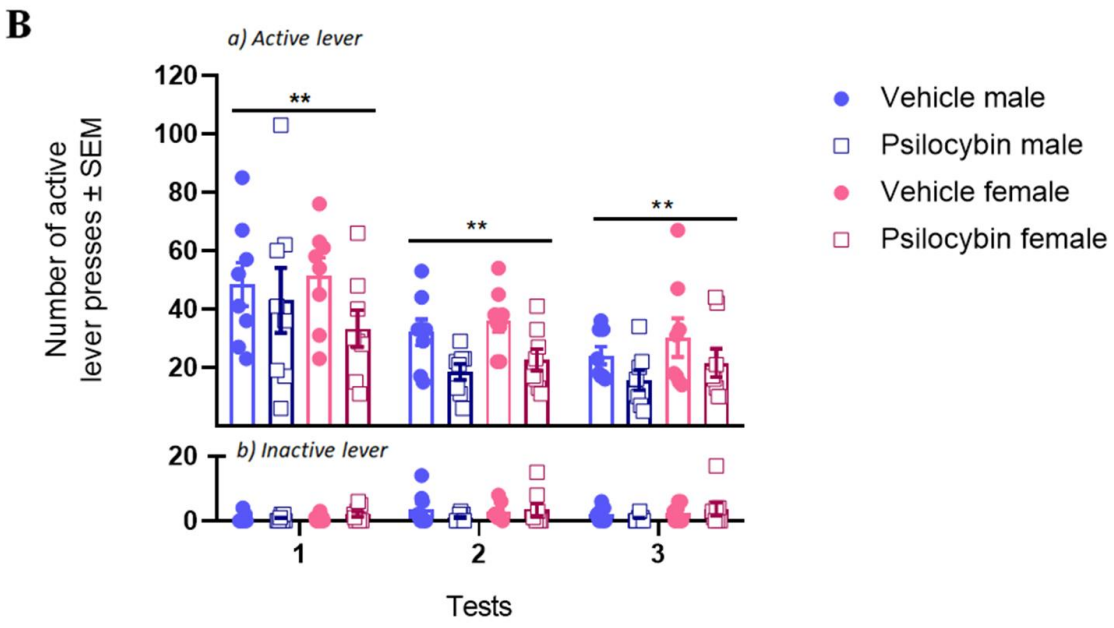


Figure 5. Effect of psilocybin (5mg/kg) on the reconsolidation of alcohol-related memory. At the end of a memory reactivation session, msP rats were treated with psilocybin (5mg/kg) or vehicle (N = 16, males and females 50% each/group). 24h after drug injection, a single cue-induced alcohol-seeking behavior test was performed. Psilocybin significantly reduced active lever presses at each time point tested (**A**). No sex differences contributed to psilocybin effect (**B**). Data are expressed as mean ± SEM of a) total responses at the active and b) total responses at inactive lever. * *p < 0.01 significant difference from baseline drinking.

4. Discussion

The present study investigated the therapeutic potential of psilocybin in a well-established behavioral paradigms of alcohol drinking and relapse. Specifically, we evaluated psilocybin effects on alcohol consumption using a 2BC paradigm, in the ADE model of relapse and on cue-induced reinstatement of alcohol seeking in male and female msP rats. We also investigated if psilocybin interferes with the reconsolidation process of alcohol-related memories preventing cue-induced reinstatement of alcohol seeking. Results showed that three injections of psilocybin given in adolescence prior to alcohol exposure failed to prevent or decrease alcohol consumption. Similarly, a single injection of psilocybin did not reduce voluntary alcohol intake in a 2BC paradigm in adult male and female msP rats. Additionally, psilocybin did not prevent the increase of alcohol drinking after a period of forced abstinence as well as cue-induced reinstatement of alcohol-seeking. Importantly, we observed that psilocybin, administered immediately after the memory retrieval session, significantly reduced alcohol seeking behavior.

Overall, we provide evidence for a new therapeutic application of psilocybin for alcohol dependence in which reactivity to environmental cues is reduced by interfering with memory reconsolidation processes. By inference this treatment approach could be useful also for the treatment of other psychiatric disorders such as post-traumatic stress.

Since studies conducted in rodents have demonstrated that a single hallucinogenic dose of psychedelics can change the brain structure and behavior long after the drug has been cleared from the body (Cameron et al., 2018; Ly et al. 2018) and that adolescence is a developmental period characterized by extensive neuroanatomical and functional reorganization of the brain (Schneider, 2013; Fuhrmann et al., 2015), we tested the hypothesis that administration of three doses of psilocybin during adolescence would produce long-lasting beneficial effects on alcohol intake. However, contrary to our expectations, psilocybin had no long-lasting effects on alcohol consumption in adolescent msP rats as well as failed to reduce alcohol intake in the other drinking paradigms and to prevent relapse in adult msP rats. Few studies in rodents have examined the effect of psilocybin on alcohol-related behaviors. A recent report found that administration of psilocybin (1 and 2,5mg/kg) was effective in restoring mGluR2 expression and preventing alcohol relapse in rats in which infralimbic cortex mGluR2 knockdown generated a phenotype with reduced cognitive flexibility and excessive alcohol seeking (Meinhardt et al., 2021). In another study, Meinhardt et al. investigated the effects of LSD and psilocybin in the alcohol deprivation effect (ADE) model of relapse. Particularly, they tested the hypothesis that repeated sub-acute treatment with psychedelics might reduce the occurrence of the ADE (Meinhardt et al., 2020). Results revealed that only sub-chronic treatment with psilocybin produced a significant anti-relapse effect 24h after ADE, which anyway faded away starting from 48h after ADE, while psychedelics had no long-lasting effects on the ADE in male and female rats when administered in a high dosage regime nor in a chronic microdosing scheme (Meinhardt et al., 2020). One possible explanation for the discrepancy between these latter findings and our results lies in the fact that we conducted our experiments in msP rats while in previous studies Wistars were used. Additionally, in the study from Meinhardt et al. psilocybin was effective in decreasing cue-induced reinstatement of alcohol seeking by restoring mGluR2 function in infralimbic cortex (Meinhardt et al., 2021). This finding suggests a strong interaction between the 5-HT2AR and the mGluR2 and indicates that mGluR2 modulation can counteract and enhance the behavioral effects of hallucinogenic drugs, respectively. At present, there is no evidence of altered mGluR2 in msP rats that may contribute to their high alcohol drinking phenotype. In summary, our negative data do not support the results of a recent pilot study in which psilocybin reduced heavy alcohol drinking in AUD

(Bogenschutz et al., 2014). It is possible that the lack of psilocybin efficacy on alcohol drinking in msP rats may be associated with the fact that this drug, and in general psychedelics, might exert their therapeutic effects on alcohol addiction only in conjunction with psychotherapy. Indeed, studies involving therapeutic applications of classic hallucinogens considered the pharmacological treatment and the psychosocial elements of treatment as an integrated approach (Bogenschutz, 2013). Particularly, the psychosocial treatment might help to establish the purpose or therapeutic goals of the session, promote certain kinds of drug experience, provide additional therapeutic elements not directly related to the drug administration in order to increase the probability of obtaining positive findings (Johnson et al., 2008).

Importantly, we showed that psilocybin (5mg/kg), given right after re-exposure of animals to conditioned stimuli, impaired the ability of drug-related cues to induce alcohol-seeking behavior in subsequent test sessions. Drug use and relapse has demonstrated to involve learned associations between drug-associated environmental cues and drug effects. Several studies have demonstrated that long-term memory formation and development of drug addiction shared common molecular mechanisms and neuronal circuitries (Hyman et al., 2006; Kelley, 2004). Earlier studies have shown that newly acquired memories are initially labile but then are stabilized through a memory consolidation process. Once consolidation is completed, memory is thought to be stored in a fixed and stable manner (McGaugh, 2000; Dudai, 2004). Previous studies have demonstrated that retrieval of cocaine, opioids, alcohol-related memories underwent a reconsolidation process and that administration of protein synthesis inhibitor anysomicin or NMDA receptor antagonists interfered with this process reducing the risk of relapse (Xue et al., 2012; Von der Goltz, 2009; Bernardi et al., 2007; Robinson and Franklin, 2007; Hellemans et al., 2006; Lee et al, 2006; Lee et al., 2005; Miller and Marshall, 2005). To our knowledge, this is the first study demonstrating the efficacy of psilocybin efficacy in impairing the reconsolidation of drug-related memory in animals. However, the neurobiological mechanism underlying psilocybin efficacy in disrupting drug-related memory is unknown and further investigations are needed to elucidate it. Previous reports on the effects of psilocin/psilocybin on cognition in animals are very sparse, use different paradigms and did not bring uniform results, which make difficult to formulate hypotheses. One potential mechanism might involve 5HT2A receptors since the hallucinogenic drug psilocin has demonstrated to disrupt memory retrieval in the Morris water maze (Rambousek et al., 2014). However, this interpretation is speculative and has to be tested in further experiments focusing on the underlying neurobiological mechanism.

In conclusion, the classic psychedelic psilocybin was not effective in reducing alcohol consumption and in preventing relapse in msP rats. However, when it was administered right after the memory reconsolidation process, psilocybin successfully prevented cue-induced reinstatement of alcohol seeking. The specific mechanism by which psilocybin could impair alcohol-related memories is still unknown and worthy of further investigations. This latter finding has important clinical implications since the disruption of alcohol-related memory induced by psilocybin may represent an effective treatment strategy for preventing relapse in abstinent alcoholics. Moreover, this therapeutic approach might be effective in also treating those pathologies, in which the presentation of environmental cues exacerbates the disease (e.g., post-traumatic stress disorders).

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Chapter 5: Concluding remarks

Stress, defined as any stimulus that disrupts body homeostasis, has long been proposed as an important correlate for uncontrolled alcohol drinking and relapse (Edwards et al., 2015; Stephens and Wand, 2012). Indeed, alcohol has potent anxiolytic properties and can relieve stress, but at the same time can act as a stressor and activates the body stress response system. Moreover, the consequent HPA axis dysfunction could negatively impact overall health because of decreased ability to respond appropriately to internal or environmental challenges (Becker, 2012). A large body of evidence from clinical and preclinical studies has investigated how stress and alcohol interact with each other and contribute to the onset and perpetuation of the disorder. Different neurobiological systems (e.g, glucocorticoid, CRF, neuropeptide Y, dynorphine, norepinephrine systems) as well as different types of stressors (e.g., early life stress) have been proposed to modulate stress-alcohol interactions and have been extensively investigated. Although the bidirectional relationship between stress and alcohol dependence is well established, it is complex and needs broader investigation. The aim of the present research was to provide further understanding on the influence of stress in perpetuating alcohol abuse and correlated stress-related disorders by examining this complex interaction at various levels and considering different aspects of stress that might shape individual vulnerability to develop alcoholism.

To summarize, at first, the present research extended current knowledge on msP rats. This rat line has long been proposed as an innate phenocopy of alcohol dependence characterized by high alcohol preference and excessive drinking linked to self-medication from negative affect (Ciccocioppo et al., 2006; Borruto et al., 2021). These animals in fact, are characterized by an innate overactivity of CRF/CRF1 system, which correlates with excessive alcohol drinking and a decreased threshold for stress-induced alcohol-seeking (Hansson et al., 2006; Ayanwuyi et al., 2013; Cippitelli et al., 2015). So far it was unclear whether the GR system would contribute to their excessive alcohol drinking and highly stressed and anxious phenotype. Given the role of this system in modulating alcohol dependence and stress-related disorders (Vendruscolo et al., 2015; Vendruscolo et al., 2012; Calvo and Volosin, 2001; Jakovcevski et al., 2011), we explored if GR signaling would play a role in the regulation of these behaviors. For this reason, we evaluated the effect of GR antagonism on alcohol self-administration and on a battery of anxiety-related behavioral tests. Contrary to our expectations, msP rats showed a poor response to GR antagonists suggesting that excessive alcohol drinking and anxiety-related behaviors of msP rats are not dependent upon a hyperactivation of the GR system. A corollary finding was that GR antagonism is more efficacious in attenuating drinking in female rats.

A second achievement was the establishment of a novel protocol to study the impact of early life social isolation on alcohol abuse and stress response later in life. Our paradigm differs from more commonly used maternal separation procedures targeting different developmental periods (PND14-21 vs PND1-14/21) and characterized by much longer duration of the separation (30min vs 3-6h). The time point that we proposed may more closely map into human toddlerhood and may therefore have more translational value than earlier applied procedures (Watson et al., 2006). Our environmental manipulation did not affect alcohol intake, motivation for alcohol and alcohol consumption after yohimbine challenge in contrast to maternal separation that classically increases alcohol intake in adulthood (Becker et al., 2011; Miczek et al., 2008; Roman and Nylander, 2005. Interestingly, we found that female msP rats subjected to early social isolation are more vulnerable to reinstatement of alcohol seeking following administration of the pharmacological stressor yohimbine. This suggests that early social isolation induced persistent changes in female rats with an innate high stressed phenotype that become more vulnerable to stressors later in life. Since early trauma and negative affect have been associated with heavy drinking in women (Guinle and Sinha, 2020) and women are more likely to relapse in response to stressful events (Hudson and Stamp, 2011; Hyman et al., 2008; Greenfield et al., 2007), our model appears to have significant translational value.

The present research assessed also the contribution of sex differences in promoting stress and alcohol-related behaviors. Results showed that GR antagonism was more effective in female rats which showed a higher levels of corticosterone than males in general, and in msP rats display greater startle responses. Furthermore, females are more vulnerable to stress exposure displaying higher levels of reinstatement after a yohimbine challenge and adult female msPs are more sensitive to early social isolation applied during postnatal life. All these findings support the observation that alcohol and stress-related sex differences observed among AUD patients can be detected also at preclinical level underlying the importance of integrating the study of sex differences in preclinical research.

Finally, we provided evidence for a novel and promising treatment strategy for AUD therapy and relapse prevention using psilocybin. Recent developments in the study of classic hallucinogens combined with re-appraisal of old literature, have led to a renewal of interest in therapeutic applications of this class of drugs, specifically in the treatment of alcohol dependence (Bogenschutz et al., 2013). Despite these encouraging clinical findings, scientific literature is missing preclinical studies to elucidate the psychological and neurobiological mechanisms underlying psilocybin therapeutic effects. Importantly, since reinstatement to alcohol seeking evoked by environmental cues represents the main reason for failure for current

therapies, we investigated the effect of psilocybin on the reinstatement of alcohol seeking. Results demonstrated that if psilocybin is administered immediately after the reactivation of alcohol-related memory is able to blunt seeking response elicited by them. This opens the possibility of using psilocybin in human laboratory settings in conjunction with specific psychotherapy practices aimed at recalling drug-related memories. By inference, it is possible to hypothesize that the use of this psychedelic can be extended to other psychiatric disorders in which presentation of environmental cues exacerbates the disease. One possible application is the treatment of post-traumatic stress disorder (PTSD). PTSD can develop after exposure to a traumatic event that is more extreme than a typical daily stressor. It is a highly debilitating disease, with poor treatment response and high comorbidity with other pathologies, including AUD (National Institute of Mental Health, 2017). According to our observation, PTSD patients could be brought into a clinical setting, presented with a stimulus that retrieves the fearful memory and given psilocybin, and the fear memory would be weakened. The observation that psychedelics can disrupt alcohol and possibly fear memories opens to the possibility of using this drug to treat patients with comorbid alcohol abuse and post-traumatic stress.

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Appendix: Scientific contributions

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ARTICLE

Activation of peroxisome proliferator-activated receptor γ reduces alcohol drinking and seeking by modulating multiple mesocorticolimbic regions in rats

Yannick Fotio[1,2], Anna Maria Borruto[1], Federica Benvenuti[1], Gregory Demopulos[3], George Gaitanaris[3], Marisa Roberto[4] and Roberto Ciccocioppo[1]

Peroxisome proliferator-activated receptor γ (PPARγ) is an intracellular transcription factor whose signaling activation by the selective agonist pioglitazone reduces alcohol drinking and alcohol-seeking behavior in rats. The present study utilized the two-bottle choice and operant self-administration procedures to investigate neuroanatomical substrates that mediate the effects of PPARγ agonism on alcohol drinking and seeking in msP rats. Bilateral infusions of pioglitazone (0, 5, and 10 µg/µl) in the rostromedial tegmental nucleus (RMTg) decreased voluntary alcohol drinking and alcohol self-administration. Microinjections of pioglitazone in the ventral tegmental area (VTA), central amygdala (CeA), and nucleus accumbens (NAc) shell had no such effect. Notably, water, food, and saccharin consumption was unaltered by either treatment. The yohimbine-induced reinstatement of alcohol seeking was prevented by infusions of pioglitazone (0, 2.5, 5, and 10 µg/µl) in the CeA, VTA, and RMTg but not in the NAc shell. These results emphasize the involvement of mesocorticolimbic circuitries in mediating the effects of PPARγ agonists on alcohol drinking and seeking. These results will facilitate future studies that investigate the pathophysiological role of PPARγ in alcohol use disorder and help clarify the mechanisms by which the activation of this receptor decreases the motivation for drinking.

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INTRODUCTION

Alcohol use disorder (AUD) is a chronic brain disease that is characterized by compulsive alcohol drinking and withdrawal symptoms when access to alcohol is prevented, thus heightening the risk of relapse to pathological drinking [1]. AUD is considered the fifth highest risk factor for premature death and disability worldwide. In 2016 alone, more than 3 million deaths and 132.6 million disability-adjusted life years at the global level were attributable to AUD. The neurobiological mechanisms that underlie AUD are still only partially understood but are thought to be associated with profound counteradaptive alterations of reward and stress neurocircuitries [2, 3]. Untangling these neuroadaptations is complex but essential to develop more efficacious therapies.

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor that belongs to a large group of nuclear receptors. Upon activation, PPARγ regulates gene expression by translocating to the nucleus and binding to a selective DNA sequence called PPAR response element [4]. Although PPARγ is mainly expressed in adipose tissue and macrophages where it controls metabolism and the immune response [5, 6], recent studies showed that this nuclear factor is also densely expressed in the central nervous system. PPARγ is highly expressed in neurons and glial cells where it is involved in neuroprotection, cell repair, and antiinflammatory responses [7–10]. Earlier studies showed that PPARγ is expressed on dopaminergic cells in the ventral tegmental area (VTA), suggesting that this receptor could be involved in modulating the reinforcing effects of drugs of abuse [10]. Consistent with this hypothesis, research in our laboratory showed that the systemic administration of two selective PPARγ agonists, pioglitazone, and rosiglitazone, significantly reduced alcohol drinking and seeking in alcohol-preferring rats [11, 12]. However, the neurocircuitries and putative mechanisms that subserve such effects are still unknown. The present study investigated the neuroanatomical substrates that mediate the effects of PPARγ agonists on alcohol drinking and seeking to facilitate future characterizations of their molecular and cellular mechanisms.

MATERIALS AND METHODS

Animals

In total, 10–11-week-old male Marchigian Sardinian alcohol-preferring (msP) rats ($N_{total} = 135$), weighing 250–280 g, were employed in this study. They were bred and housed under a reverse 12 h/12 h light/dark cycle (light on at 8 p.m.) in the vivarium of the University of Camerino and controlled temperature (22 °C) and humidity (55%). Food (4RF18, Mucedola, Settimo Milanese, Italy) and water were provided ad libitum. Before starting the experiments, the rats were pair housed in conventional clear plastic cages with standard bedding. The experiments were conducted during the dark phase of the light/dark cycle, and

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the procedures were conducted in accordance with directives on the care and use of laboratory animals of the European Community Council and National Institutes of Health. Formal approval was obtained from the Italian Ministry of Health and Internal Ethical Committee for Laboratory Animal Protection and Use of the University of Camerino. All efforts were made to minimize the rats' suffering and distress.

Chemicals and treatments

Saccharin (Sigma, Italy) was dissolved in tap water to obtain a 0.2% (w/v) solution. Alcohol (Carsetti, Camerino, Italy) was diluted with tap water to obtain a 10% concentration. The selective PPARγ agonist pioglitazone ($ED_{50} = 0.2–0.6$ μM at PPARγ inactive at PPARα and PPARδ at 10^{-3}) [13–15] was purchased from Molcan Corporation (Richmond Hill, ON, Canada) and dissolved in vehicle that consisted of 10% dimethylsulfoxide, 3% Tween 80, and 87% distilled water. To evaluate the effects of intracranial pioglitazone administration on alcohol drinking and seeking, the rats were treated twice with the compound: at the onset of the light cycle (8:00 p.m.) and 15 min before the dark cycle began, when alcohol was made available. The pioglitazone administration schedule was based on previous studies [11, 12]. Yohimbine (Sigma, Milano, Italy) was dissolved in saline and was used to evoke the reinstatement of alcohol seeking [16]. It was administered intraperitoneally (i.p.) at a dose of 1.25 mg/kg, 15 min after the second injection of pioglitazone and corresponding to the beginning of the dark phase (8:00 a.m.). Reinstatement testing was performed 30 min after the yohimbine injection. To minimize the diffusion of pioglitazone from the injection site, it was administered in a volume of 0.3 μl per site in the rostromedial tegmental nucleus (RMTg) and VTA. In the nucleus accumbens (NAc) shell and central amygdala (CeA), the injection volume was 0.5 μl per site. All of the treatments were administered in a counterbalanced Latin-square design to limit the number of rats used.

Intracranial surgery

The rats were anesthetized by an intramuscular injection (100–150 μl) of a solution that contained tiletamine (58.17 mg/ml) and zolazepam (7.5 mg/ml). Bilateral guide cannulas (0.65 mm outer diameter) that were aimed at the CeA, VTA, RMTg, and NAc shell were implanted and cemented to the skull. We used the following stereotaxic coordinates (from bregma) according to previous reports [17, 18]: CeA (anterior/posterior, −1.8 mm; dorsal/lateral, ±4.3 mm; medial/ventral, −7.0 mm), VTA (anterior/posterior, −5.8 mm; dorsal/lateral, ±2.2 mm; medial/lateral, −7.4 mm; 12° angle), RMTg (anterior/posterior, −6.7 mm; dorsal/lateral, ±2.2 mm; medial/ventral, −7.4 mm; 12° angle), NAc shell (anterior/posterior, +1.4 mm; dorsal/lateral, ±0.9 mm; medial/ventral, −6.1 mm). After surgery, the rats received a single subcutaneous injection of ketoprofen (2.5 mg/kg) and allowed to recover for 1 week in their home cage. During this period, the rats were handled daily and habituated to the injection procedure, consisting of inserting a stainless-steel injector into the guide cannulas, for at least 3 days before the tests began. The injector was 1.5 mm longer than the guide cannula and left in place for an additional 20 s after the injection to allow diffusion of the solution. Upon completion of the experiments, the rats were anesthetized with isoflurane, and black India ink (0.5 μl per site) was injected into the studied brain areas. The rats were then immediately euthanized to remove the brain and histologically analyze the cannula placements.

Two-bottle choice procedure

The two-bottle choice (2-BC) procedure (free choice between water and 10% alcohol) was used to measure voluntary alcohol drinking and preference [19]. The rats were single housed in experimental chambers (30 cm length × 30 cm width × 30 cm height) for 1 week of habituation before beginning the 2-BC test. They were given free access to water and 10% alcohol (v/v) for the next 15 days to establish a stable baseline and preference for alcohol. Preference was defined as 80–90% preference for alcohol vs. water. The fluids were offered through graduated drinking tubes that were equipped with metal spouts. Fluid intake was measured by reading the volume that was consumed at specific time points (2, 8, and 24 h) following initiation of the active (dark) phase of the light/dark cycle. The drinking tubes were switched daily to avoid the development of side preference. The rats also had free access to food. Food consumption was measured by weighing the food container while considering the spillage weight. Alcohol, water, and food intakes were calculated as absolute values of consumption at each time-point and are expressed as g/kg body weight [20].

Operant alcohol and saccharin self-administration

Operant chambers were used in daily 30-min sessions to establish alcohol and saccharin self-administration under fixed-ratio 1 (FR1) schedule of reinforcement [21, 22]. Each chamber was equipped with an active lever and an inactive lever that were symmetrically centered on the side panel. Responding at the active lever activated the infusion pump and released 0.1 ml of 10% alcohol (v/v) or 0.2% saccharin (w/v) in a liquid receptacle that was located between the two levers. Presses at the inactive lever were recorded but did not activate the infusion pump. During the infusion, a stimulus light that was located above the active lever was turned on for a 5 s timeout period. Lever pressing during the timeout period was recorded but did not lead to further infusions. When the rats achieved a stable baseline of self-administration for both alcohol and saccharin over the last 3 days of training, we evaluated the effects of microinfusions of pioglitazone in the RMTg every 4 days using a counterbalance Latin-square design.

Yohimbine-induced reinstatement of alcohol seeking

The reinstatement experiments consisted of three phases: training for alcohol self-administration, extinction (during which alcohol was no longer available), and reinstatement tests.

In the training phase, alcohol self-administration was performed as described previously (see "Operant alcohol and saccharin self-administration" section above). Lever responding under the FR1 schedule was maintained for 10 days (sessions) before and after surgery to reestablish baseline alcohol self-administration.

In the extinction phase, after the last alcohol self-administration session, the rats underwent 15 days of extinction sessions, during which they were placed under environmental conditions that were similar to the alcohol training phase, with the exception that responding at the active lever did not result in alcohol deliveries. During the last 3 days of extinction, the rats were habituated to the intracranial treatment procedures.

In the reinstatement phase, the experimental conditions were identical to the extinction phase, but the rats were subjected to a reinstatement test. In separate experiments, pioglitazone (2.5, 5, and 10 μg/μl) or its vehicle was injected in the CeA, VTA, RMTg, and NAc shell. The experiment was conducted in a counterbalanced Latin-square design, with a 4-day interval between test sessions. During this interval, the rats were subjected to extinction sessions. The dose of yohimbine and experimental design were based on previous studies [11, 23, 24].

Statistical analyses

The data were analyzed using analysis of variance (ANOVA) followed by the Newman–Keuls multiple-comparison post-hoc test when appropriate. The effects of intracranial injections of pioglitazone in the CeA, VTA, RMTg, and NAc shell on alcohol, water, and food intake were analyzed using two-way repeated-measures ANOVA, with time and treatment as within-subjects factors. The effects of microinfusions of pioglitazone in the RMTg on alcohol and saccharin self-administration were analyzed using one-way repeated-measures ANOVA, with treatment as the

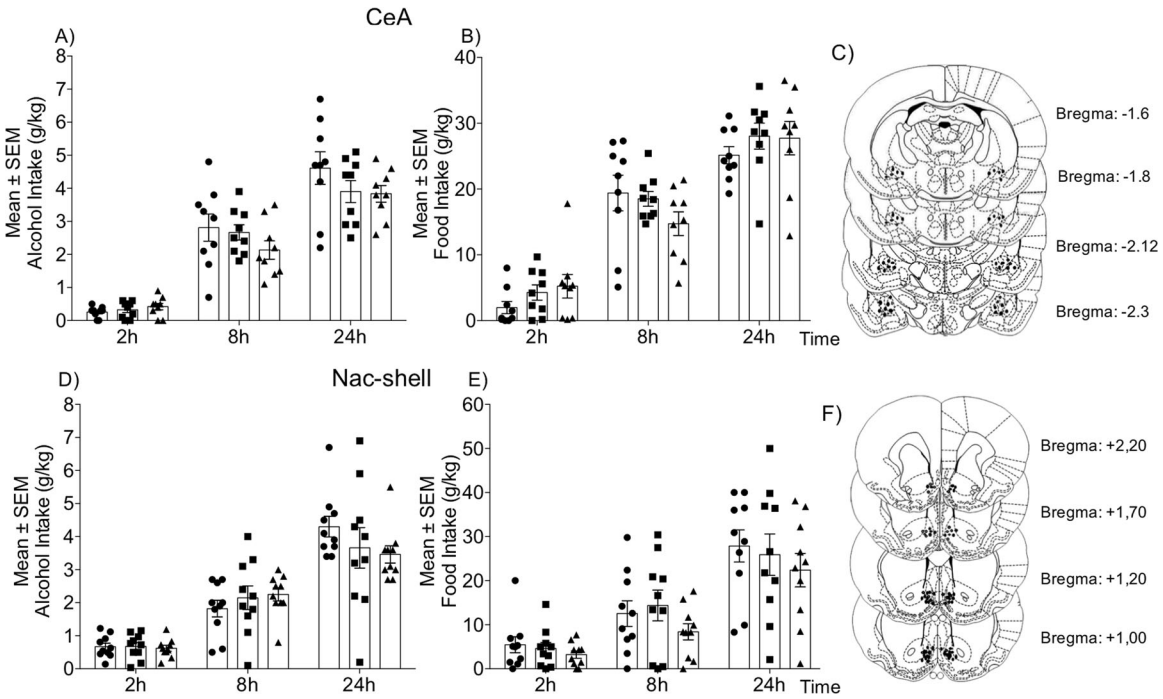

Fig. 1 Effect of intra-CeA and intra-NAc shell pioglitazone administration on alcohol and food intake in msP rats. a, **d** Time-course of alcohol drinking following pioglitazone administration in the CeA and NAc shell, respectively. **b**, **e** Changes in food intake following treatment. Schematic illustration of vehicle and pioglitazone injection sites (dots) in the CeA (**c**) and NAc shell (**f**). The data are expressed as mean (±SEM) intake. $n = 9$ for CeA. $n = 11$ for NAc shell.

within-subjects factor. The effects of microinfusions of pioglitazone in the CeA, VTA, RMTg, and NAc shell on the yohimbine-induced reinstatement of alcohol seeking were analyzed using one-way repeated-measures ANOVA, with treatment as the within-subjects factor. For the reinstatement experiments, differences between lever responding during the extinction and reinstatement sessions were analyzed using paired Student's *t* test. The 2-BC data are expressed as the mean (±SEM) of intake (g/kg of body weight). For operant self-administration, the data are expressed as the mean (±SEM) of the number of responses at the active and inactive levers. Only data from rats with correct cannula placements were included in the statistical analyses. The following numbers of rats were included in the statistical analyses: voluntary alcohol drinking (CeA, $n = 9$; VTA, $n = 11$; RMTg, $n = 11$; NAc shell, $n = 10$), alcohol self-administration (RMTg, $n = 8$), saccharin self-administration (RMTg, $n = 16$), reinstatement of alcohol seeking (CeA, $n = 12$; VTA, $n = 10$, RMTg, $n = 15$: NAc shell, $n = 13$). The statistical analyses were performed using Prism 8.0 software (GraphPad, La Jolla, CA, USA). Values of $p < 0.05$ vs. the vehicle control were considered statistically significant.

RESULTS

Effect of intra-CeA activation of PPARγ on voluntary 2-BC alcohol drinking

Pioglitazone (5 and 10 µg/µl) was microinfused in the CeA in msP rats in a counterbalanced Latin-square design ($n = 9$). As shown in Fig. 1a, voluntary alcohol drinking was monitored at 2, 8, and 24 h. The overall ANOVA revealed no difference in the amount of alcohol consumption between the pioglitazone- and vehicle-treated groups at any time-point (time: $F_{2,16} = 33.91$, $p < 0.0001$; treatment: $F_{2,16} = 2.492$, $p = 0.344$; time × treatment interaction: $F_{4,32} = 0.7949$, $p = 0.5373$). Similarly, no difference in the amount of water (time: $F_{2,16} = 8.685$, $p = 0.0028$; treatment: $F_{2,16} = 1.311$, $p = 0.2970$; time × treatment interaction: $F_{4,32} = 8834$, $p = 0.4849$; Table S1) or food (time: $F_{2,16} = 64.11$, $p < 0.0001$; treatment: $F_{2,16} = 6025$, $p = 0.5594$; time × treatment interaction: $F_{4,32} = 2.674$, $p = 0.0946$; Fig. 1b) consumption was found between the pioglitazone- and vehicle-treated groups.

Effect of intra-NAc shell activation of PPARγ on voluntary 2-BC alcohol drinking

The ANOVA revealed that alcohol consumption was detectable 2 h after treatment and progressively increased in the following hours (time: $F_{2,18} = 78.76$, $p < 0.0001$; Fig. 1d). The ANOVA also revealed that intake were unaffected by treatment, although a slight reduction was observed at 24 h ($F_{2,18} = 0.2135$, $p = 0.8098$). No time × treatment interaction was detected ($F_{4,36} = 2.067$, $p = 0.1055$). Intra-NAc shell pioglitazone administration did not alter the consumption of water (time: $F_{2,18} = 11.89$, $p < 0.001$; treatment: $F_{2,18} = 0.073$, $p = 0.9298$; time × treatment interaction: $F_{4,36} = 0.3109$, $p = 0.8688$; Table S1) or food (time: $F_{2,18} = 54.39$, $p < 0.0001$; treatment: $F_{2,18} = 0.9515$, $p = 0.4048$; time × treatment interaction: $F_{4,36} = 0.5584$, $p = 0.6942$; Fig. 1e).

Effect of intra-RMTg activation of PPARγ on voluntary 2-BC alcohol drinking

Pioglitazone (5 and 10 µg/µl) was microinfused in the RMTg in msP rats ($n = 11$). The ANOVA revealed significant effects of time ($F_{2,20} = 104.7$, $p < 0.0001$) and treatment ($F_{2,20} = 21.27$, $p < 0.0001$) and a significant time × treatment interaction ($F_{4,40} = 8.701$, $p < 0.0001$). As shown in Fig. 2a, voluntary alcohol consumption was detectable but not significantly affected by intra-RMTg pioglitazone administration 2 h after treatment. However, at 8 and 24 h post treatment, alcohol intake dose-dependently decreased. Interestingly, intra-RMTg pioglitazone administration did not alter water (time: $F_{2,20} = 5.106$, $p = 0.0162$; treatment: $F_{2,20} = 1.593$, $p = 0.2280$; time × treatment interaction: $F_{4,40} = 0.2922$, $p = 0.8813$; Table S1) or food (time: $F_{2,20} = 45.21$, $p < 0.0001$; treatment: $F_{2,20} = 0.3759$, $p = 0.6914$; time × treatment interaction: $F_{4,40} = 0.1251$, $p = 0.3051$; Fig. 2b) consumption.

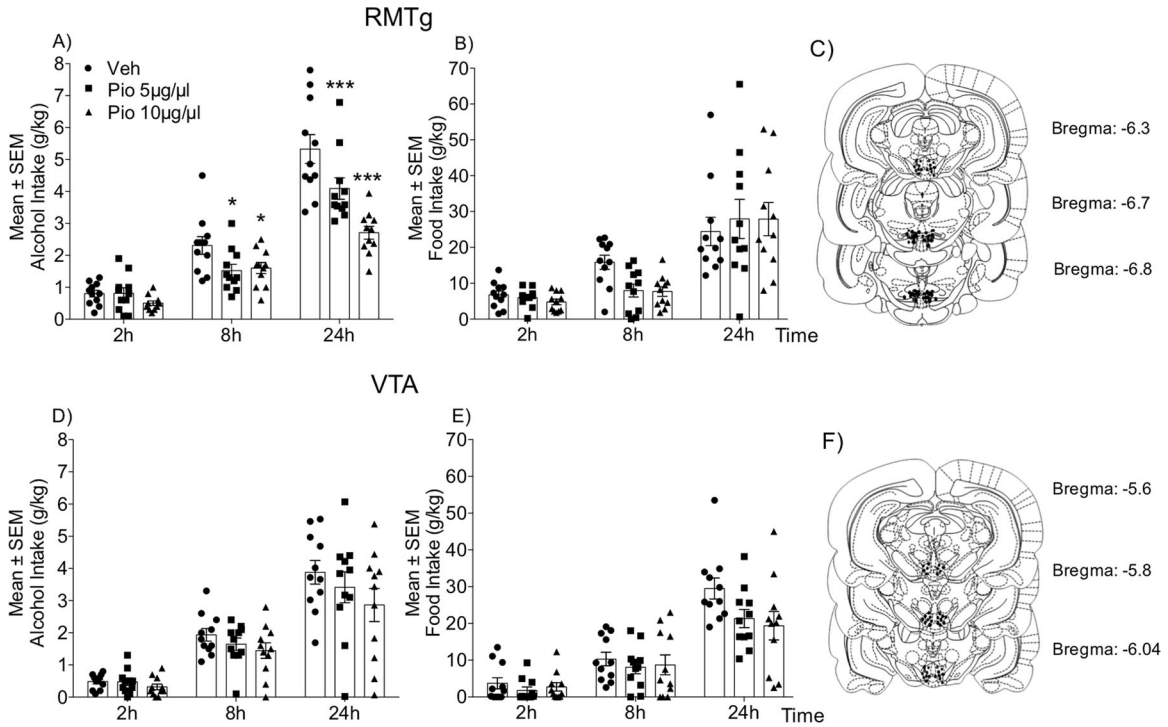

Fig. 2 Effect of intra-RMTg and intra-VTA pioglitazone administration on alcohol, water, and food intake in msP rats. a, **d** Time-course of alcohol drinking following pioglitazone administration in the RMTg and VTA, respectively. **b**, **e** Changes in food intake following treatment. Schematic illustration of vehicle and pioglitazone injection sites (dots) in the RMTg (**c**) and VTA (**f**). The data are expressed as mean (±SEM) intake. $n = 11$ for RMTg. $n = 11$ for VTA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, vs. vehicle-treated control.

Effect of intra-VTA activation of PPARγ on voluntary 2-BC alcohol drinking

Pioglitazone (5 and 10 µg/µl) was microinfused in the VTA in msP rats ($n = 11$). Alcohol intake was detectable 2 h after initiation of the dark phase. The ANOVA revealed a significant effect of time on alcohol intake ($F_{2,20} = 80.74$, $p < 0.0001$; Fig. 2d) but no effect of treatment ($F_{2,20} = 2.425$, $p = 0.114$) and no time × treatment interaction ($F_{4,40} = 0.8606$, $p = 0.4959$). The intra-VTA administration of pioglitazone or its vehicle did not alter the absolute amount of alcohol consumption at any time-point (2, 8, and 24 h). Treatment did not affect water (time: $F_{2,20} = 6.38$, $p = 0.0096$; treatment: $F_{2,20} = 0.7005$, $p = 0.5081$; time × treatment interaction: $F_{4,40} = 0.7241$, $p = 0.5807$; Table S1) or food (time: $F_{2,20} = 76.40$, $p < 0.0001$; treatment: $F_{2,20} = 2.178$, $p = 0.1394$; time × treatment interaction: $F_{4,40} = 1.895$, $p = 0.074$; Fig. 2e) consumption.

Effect of intra-RMTg activation of PPARγ on alcohol and saccharin self-administration

To further investigate the role of the RMTg in modulating alcohol intake through PPARγ, msP rats ($n = 8$) underwent operant alcohol (10%, v/v) self-administration training. When they reached a stable mean number of reinforcements earned, pioglitazone (5 and 10 µg/µl) or its vehicle were administered in the RMTg, and their effects on operant responding were evaluated. As expected, the ANOVA showed that pioglitazone dose-dependently decreased the number of reinforced lever presses ($F_{2,14} = 6.361$, $p = 0.006$; Fig. 3a). The number of responses at the inactive lever was negligible and did not changed throughout the experiment (Fig. 3b).

To test whether the observed effect of intra-RMTg PPARγ activation is selective for alcohol, rats ($n = 16$) were trained to self-administer saccharin (0.2%, w/v) under an FR1 schedule until they reached a stable baseline of reinforcements obtained. Pioglitazone (5 and 10 µg/µl) was then microinfused in the RMTg. The ANOVA revealed that this treatment did not alter saccharin self-administration ($F_{2,30} = 0.3996$, $p = 0.6748$; Fig. 3c). Responding at the inactive lever was negligible and did not changed throughout the experiment (Fig. 3d).

Effect of intra-CeA activation of PPARγ on yohimbine-induced reinstatement of alcohol seeking

Pioglitazone (2.5, 5, and 10 µg/µl) or its vehicle were microinfused in the CeA in msP rats ($n = 12$) to evaluate its effect on the yohimbine-induced reinstatement of alcohol seeking. During the training phase, the mean number of responses at the active lever was 68.73 ± 5.95, which sharply decreased during extinction (21.41 ± 1.97). Paired Student's t test (vehicle vs. extinction) revealed that yohimbine administration (1.25 mg/kg, i.p.) significantly reinstated operant alcohol-seeking behavior ($t_{11} = 3.8$, $p = 0.0029$; Fig. 4a), which was prevented by intra-CeA infusions of pioglitazone ($F_{3,33} = 16.12$, $p < 0.0001$). Responding at the inactive lever was low (1.79 ± 0.49) and not significantly affected by the treatment (Fig. 4b).

Effect of intra-NAc shell activation of PPARγ on yohimbine-induced reinstatement of alcohol seeking

In msP rats ($n = 13$) with cannula implants in the NAc shell during the training phase, the mean number of responses at the active lever was 65.33 ± 5.54, which significantly decreased during extinction (19.12 ± 4.95) and was reinstated ($t_{12} = 5.096$, $p = 0.0003$) by yohimbine treatment (1.25 mg/kg, i.p.). However, intra-NAc shell pioglitazone administration did not alter the yohimbine-induced reinstatement of alcohol seeking ($F_{3,36} = 1.838$, $p = 0.1578$; Fig. 4d). Responding at the inactive lever was low and unchanged by the treatments (Fig. 4e).

Effect of intra-VTA activation of PPARγ on yohimbine-induced reinstatement of alcohol seeking

During the training phase in msP rats ($n = 10$), the mean number of responses at the active lever was 67.87 ± 6.52. Operant responding

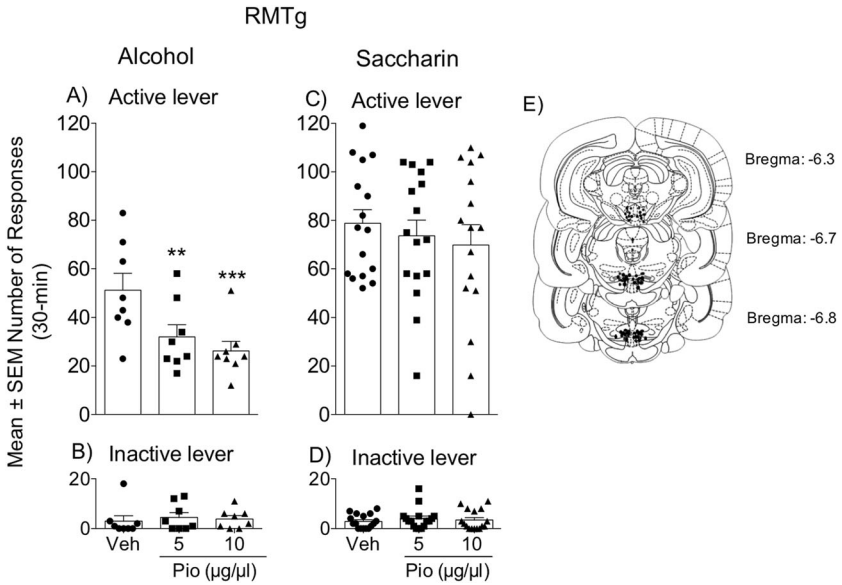

Fig. 3 Effect of intra-RMTg pioglitazone administration on operant alcohol and saccharin self-administration. a, **c** Number of alcohol and saccharin reinforcements earned following pioglitazone administration in the RMTg. **b**, **d** Number of responses at the inactive lever. **e** Schematic illustration of vehicle and pioglitazone injection sites (dots) in the RMTg. The data are expressed as the mean ± SEM. $n = 8$ for alcohol. $n = 16$ for saccharin. *$p < 0.05$; **$p < 0.01$, vs. vehicle-treated control.

markedly decreased during extinction (13.63 ± 1.51 lever presses). As shown in Fig. 5a, treatment with yohimbine (1.25 mg/kg, i.p.) significantly reinstated ($t_9 = 6.552$, $p < 0.0001$) operant responding for alcohol. This effect was dose-dependently prevented by intra-VTA pioglitazone administration ($F_{3,27} = 8.87$, $p = 0.0003$). Responding at the inactive lever was negligible (4.86 ± 1.66) and not significantly affected by the treatments (Fig. 5b).

Effect of intra-RMTg activation of PPARγ on yohimbine-induced reinstatement of alcohol seeking

In msP rats ($n = 15$) with cannula implants in the RMTg, the mean number of responses at the active lever was 72.35 ± 4.81 during the training phase, which rapidly decreased during extinction (19.22 ± 1.7). Yohimbine (1.25 mg/kg, i.p.) significantly increased the number of responses at the active lever ($t_{14} = 4.460$, $p = 0.0005$; Fig. 5d). This effect was dose-dependently decreased by intra-RMTg pioglitazone administration ($F_{3,42} = 74.54$, $p < 0.0001$; Fig. 5d). Responding at the inactive lever (Fig. 5e) was negligible and unaffected by the treatments.

DISCUSSION

Administration of pioglitazone in the RMTg decreased alcohol intake

The mesocorticolimbic dopamine system which originates in the VTA and projects to the NAc, CeA, and prefrontal cortex. This system plays a key role in controlling the reinforcing properties of drugs of abuse, including alcohol [25–31]. The majority of afferent connections to VTA dopaminergic cells are γ-aminobutyric acid (GABA)ergic and inhibitory [32–34]. Emerging evidence indicates that the tail of the VTA, also known as the RMTg, provides important GABAergic inputs to VTA dopaminergic cells [32, 35–37]. Therefore, the RMTg is a key structure in the development and maintenance of drug addiction. PPARγ expression has been detected on VTA dopaminergic neurons [10] and RMTg GABAergic cells [18]. Thus, we investigated whether the effect of pioglitazone on alcohol drinking involves PPARγ-dependent signaling in these two adjacent areas. We infused pioglitazone in the VTA and RMTg and evaluated its effect in the 2-BC procedure. We found that PPARγ activation in the RMTg but not the VTA significantly attenuated alcohol drinking compared with vehicle-treated rats. Moreover, water and food consumption were unaltered by pioglitazone treatment, indicating that its effect in the RMTg is specific to alcohol and does not generalize to water or food. To confirm this finding, we subsequently administered pioglitazone in the RMTg in two groups of rats that were trained to self-administer alcohol or saccharin. As expected, pioglitazone significantly attenuated alcohol but not saccharin intake, suggesting that PPARγ activation may specifically reduce the motivation for alcohol. Notably, the VTA and RMTg are in anatomical contiguity. Hence, the fact that pioglitazone was efficacious only when injected in the RMTg demonstrated that it did not diffuse to neighboring regions at the dose and volume tested. A corollary to this finding is that the RMTg is the sole neuroanatomical substrate for the alcohol-suppressing effect of PPARγ agonists. This hypothesis was supported by findings that showed that pioglitazone microinfusions in other brain areas of the mesocorticolimbic system where PPARγ is expressed (e.g., CeA and NAc shell) did not affect alcohol drinking [8, 38]. Such a specific role for PPARγ activation in the RMTg in controlling the reinforcing effects of drugs of abuse has also been observed in opioid self-administration studies in our laboratory [18]. In this earlier study, we found that the effect of pioglitazone in the RMTg was linked to its ability to increase the inhibitory tone of RMTg GABAergic cells, thereby inhibiting dopamine neuron activation in the VTA [18]. Although more studies are needed to support this hypothesis, we speculate that a similar mechanism may be involved in the alcohol-suppressing effects of PPARγ agonists.

An interesting observation in the present study was that the effect of pioglitazone in the 2-BC test was observed at 8 and 24 h but not at 2 h. In the operant self-administration experiments, this effect was observed at 30 min. Two possibilities may explain this apparent discrepancy. First, in the operant self-administration session, the rats consumed ~1.25 g/kg alcohol in 30 min. In the 2-BC test, the rats had to drink for more than 2 h to reach this level of consumption. This may result in different pharmacokinetics of the drug (i.e., peak levels in the brain) that in turn may influence the response to pioglitazone. Second, motivation of the animals may be more effectively captured in operant self-administration experiments than in 2-BC experiments. If pioglitazone acts by

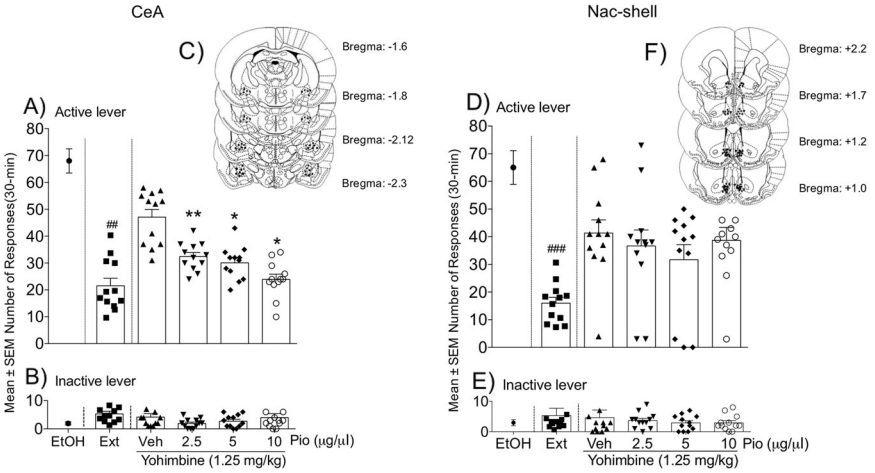

Fig. 4 Effect of intra-CeA and intra-NAc shell pioglitazone administration on the yohimbine-induced reinstatement of alcohol seeking. a, **d** Number of responses at the active lever following pioglitazone administration in the CeA and NAc shell, respectively. **b**, **e** Number of responses at the inactive lever following treatment. Schematic illustration of vehicle and pioglitazone injection sites (dots) in the CeA (**c**) and NAc shell (**f**). The data are expressed as mean (±SEM) intake. $n = 12$ for CeA. $n = 13$ for NAc shell. $^{\#\#}p < 0.01$, vehicle vs. extinction; $^{*}p < 0.05$; $^{**}p < 0.01$, vehicle- vs. pioglitazone-treated rats.

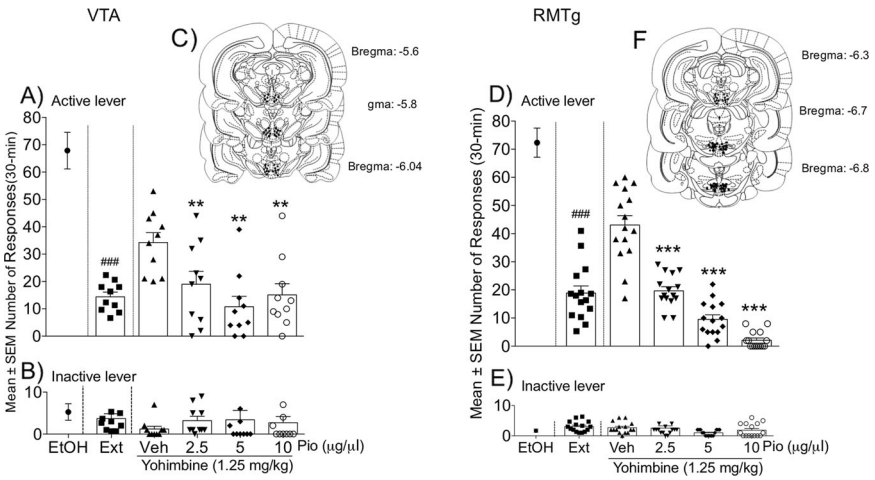

Fig. 5 Effect of intra-RMTg and intra-VTA pioglitazone administration on the yohimbine-induced reinstatement of alcohol seeking. a, **d** Number of responses at the active lever following pioglitazone administration in the VTA and RMTg, respectively. **b**, **e** Number of responses at the inactive lever following treatment. Schematic illustration of vehicle and pioglitazone injection sites (dots) in the VTA (**c**) and RMTg (**f**). The data are expressed as mean (±SEM) intake. $n = 10$ for VTA. $n = 10$ for RMTg. $^{\#\#\#}p < 0.001$, vehicle vs. extinction; $^{**}p < 0.01$; $^{***}p < 0.001$, vehicle- vs. pioglitazone-treated animals.

attenuating the motivation for alcohol, then a more pronounced effect may be observed under operant contingencies rather than under free-drinking conditions.

Administration of pioglitazone in the VTA and RMTg reduced the yohimbine-induced reinstatement of alcohol seeking

The high rate of relapse among individuals with alcohol and substance use disorders is a major clinical problem [39, 40]. Studies that utilized well-validated animal models of drug reinstatement demonstrated that the α_2-adrenergic receptor antagonist yohimbine increased drug craving in humans [41, 42] and reinstated extinguished alcohol-seeking behavior in rats that were trained to self-administer alcohol [43–45]. Yohimbine reinstates drug seeking through complex mechanisms that partially involve activation of the stress system and the potentiation of responding to sensory cues [46–49]. Consistent with these mechanisms, earlier studies showed that the yohimbine-induced reinstatement of drug seeking was reduced by corticotropin-releasing factor-1 receptor antagonists and the blockade of dopamine transmission [45, 47, 49–52]. Previous reports from our laboratory showed that systemic PPARγ agonist administration prevented the yohimbine- but cue-induced reinstatement of alcohol seeking in msP rats [11, 12]. Here, under identical experimental conditions, we found that PPARγ activation in the RMTg profoundly and dose-dependently decreased the yohimbine-induced reinstatement of alcohol seeking. A similar but less marked effect was also observed following pioglitazone administration in the VTA. PPARγ agonists may engage intra-RMTg GABAergic signaling to reduce the firing of VTA dopaminergic neurons [18]. This hypothesis is supported by previous studies that showed that stress strongly activated VTA dopaminergic neurons to induce the reinstatement of drug seeking [53, 54]. This effect of stress was prevented by intra-VTA administration of the $GABA_B$ receptor agonist baclofen [55]. Moreover, yohimbine-induced reinstatement was blocked by both systemic and intra-medial prefrontal cortex (i.e., a region that receives dopaminergic afferents from the VTA) administration of dopamine receptor antagonists [50–52]. The

present results demonstrate that the RMTg might play a critical role in the stress-induced reinstatement of alcohol seeking. However, because of the tight apposition of the RMTg and VTA, one possibility is that the effect of pioglitazone on yohimbine-induced alcohol seeking is at least partially attributable to spread of the drug into the nearby VTA. This possibility cannot be excluded, but appears to be unlikely because the effect of pioglitazone was much more pronounced when it was injected directly in the RMTg rather in the VTA. An opposite effect would be expected if the VTA was the main site of action of the drug.

Administration of pioglitazone in the RMTg did not affect operant responding for saccharin

GABAergic neurons in the RMTg are also known to strongly inhibit dopaminergic cells in the substantia nigra compacta, thereby controlling motor coordination and motor learning [56, 57]. Based on evidence that RMTg GABAergic signaling is the main neurocircuitry that mediates the PPARγ agonist-induced reduction of alcohol intake, we considered the possibility that the effects of pioglitazone on lever pressing for alcohol may have been influenced by an influence on locomotor activity. However, when we microinjected pioglitazone in the RMTg in rats that were trained to self-administer saccharin, we found that the number of reinforcements earned was unaffected by the drug. These results indicate that PPARγ activation in the RMTg selectively controls alcohol intake and the yohimbine-induced reinstatement of alcohol seeking by modulating the mesocorticolimbic system without altering transmission of the nigrostriatal pathway.

Administration of pioglitazone in the CeA but not NAc shell attenuated the reinstatement of alcohol seeking

Finally, we found that the yohimbine-induced reinstatement of alcohol seeking was attenuated by intra-CeA but not intra-NAc shell (pioglitazone administration). These results suggest that neurocircuitry in the CeA may also be recruited by PPARγ agonists to attenuate the reinstatement of alcohol seeking. This intra-CeA effect of pioglitazone may be secondary to anxiolytic properties of the compound [38]. In fact, it has been demonstrated that the CeA plays an important role in the expression of excessive anxiety linked to stress exposure [17, 58, 59]. Moreover, the pharmacological and genetic blockade of PPARγ signaling in the CeA exacerbated basal anxiety-like behavior and increased the vulnerability to stress [38]. Therefore, a tempting speculation is that the anxiolytic properties of pioglitazone may be partially responsible for the protective effects of PPARγ agonists against the stress-induced reinstatement of alcohol seeking.

In conclusion, the present findings filled a gap in the literature by revealing brain areas that modulate the effect of PPARγ activation on alcohol-seeking behavior. The results also demonstrate an important role for RMTg in modulating the yohimbine stress-induced reinstatement of alcohol seeking. Pioglitazone is clinically used for the treatment of insulin resistance in patients with type 2 diabetes, and its tolerability has been largely demonstrated [60–62]. Hence, the ability of pioglitazone to decrease alcohol seeking may open new avenues for further clinical investigation of its efficacy.

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AUTHOR CONTRIBUTIONS

YF, RC, and MR designed the project. YF designed and performed the experiments, analyzed the data, and wrote the paper. AMB and FB performed the experiments and analyzed the data. RC supervised the project and contributed to writing the paper. MR, GD, and GG provided critical comments, helped interpret the data, and contributed to writing the paper. All of the authors reviewed the paper.

ADDITIONAL INFORMATION

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Glucocorticoid Receptor Antagonist Mifepristone Does Not Alter Innate Anxiety-Like Behavior in Genetically-Selected Marchigian Sardinian (msP) Rats

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Abstract: Marchigian Sardinian alcohol-preferring (msP) rats serve as a unique model of heightened alcohol preference and anxiety disorders. Their innate enhanced stress and poor stress-coping strategies are driven by a genetic polymorphism of the corticotropin-releasing factor receptor 1 (*CRF1*) in brain areas involved in glucocorticoid signaling. The activation of glucocorticoid receptors (GRs) regulates the stress response, making GRs a candidate target to treat stress and anxiety. Here, we examined whether mifepristone, a GR antagonist known to reduce alcohol drinking in dependent rats, decreases innate symptoms of anxiety in msPs. Male and female msPs were compared to non-selected Wistar counterparts across three separate behavioral tests. We assessed anxiety-like behavior via the novelty-induced hypophagia (NIH) assay. Since sleep disturbances and hyperarousal are common features of stress-related disorders, we measured sleeping patterns using the comprehensive lab monitoring system (CLAMS) and stress sensitivity using acoustic startle measures. Rats received an acute administration of vehicle or mifepristone (60 mg/kg) 90 min prior to testing on NIH, acoustic startle response, and CLAMS. Our results revealed that both male and female msPs display greater anxiety-like behaviors as well as enhanced acoustic startle responses compared to Wistar counterparts. Male msPs also displayed reduced sleeping bout duration versus Wistars, and female msPs displayed greater acoustic startle responses versus male msPs. Importantly, the enhanced anxiety-like behavior and startle responses were not reduced by mifepristone. Together, these findings suggest that increased expression of stress-related behaviors in msPs are not solely mediated by acute activation of GRs.

Keywords: anxiety; stress; sleep disturbances; hyperarousal; mifepristone; glucocorticoid receptor antagonist; alcohol-preferring rats

1. Introduction

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is a core feature of alcohol use disorder (AUD) and stress-related comorbidities [1]. Exposure to stressors initiates the activation of the HPA axis, which results in an increased release of corticotropin-releasing factor (CRF) from specific subnuclei of the hypothalamus, such as the paraventricular nucleus (PVN) [2]. Activation of corticotropin-releasing factor receptor 1 (CRF1) through CRF stimulates the release of adrenocorticotropic hormone from the anterior pituitary and subsequent glucocorticoid secretion from the adrenal glands. This

systemic stress response is then terminated through a negative feedback process on the HPA axis when glucocorticoids bind to glucocorticoid receptors (GRs) [3], which are enriched in the hypothalamic PVN [4]. Furthermore, GR activity also impacts central brain stress circuitry, including major regulation of the amygdala and prefrontal cortex [5], to modulate cognition and negative affective states [6–9]. Thus, GR is considered to play an essential role in modulating both adaptive and maladaptive stress-associated behaviors [10].

Genetically-selected Marchigian Sardinian alcohol-preferring (msP) rats have been extensively characterized as a model of both enhanced alcohol preference and negative affective phenotypes [11,12]. The msP rats carry a unique mutation driven by two single nucleotide polymorphisms at the *CRF1* locus, leading to CRF1 receptor overexpression in areas of the brain associated with negative affect such as the amygdala [13–18]. This mutation causes innate hyperactivity of the CRF/CRF1 system, which correlates with excessive alcohol drinking [15], heightened stress sensitivity, potentiated negative affect, and behavioral alterations that possibly resemble post-traumatic stress disorder (PTSD) traits [14]. Ethanol drinking in msP rats is thought to be motivated by negative reinforcement, modeling the drinking behavior of a subpopulation of individuals who drink for tension relief and self-medication purposes [16,19].

Recently, we found that male msP rats display increased GR phosphorylation at serine 232, a site that is functionally associated with higher transcriptional activity, in the central nucleus of the amygdala (CeA) [20]. This is consistent with several reports demonstrating that GR phosphorylation is also increased in the CeA of alcohol-dependent rats during acute withdrawal [21]. Emerging evidence has shown that mifepristone, a potent GR and progesterone receptor (PR) antagonist with a higher binding affinity than the endogenous ligands [22], reliably reduces alcohol self-administration in dependent rats [21,23,24] and suppresses yohimbine stress-induced reinstatement of alcohol-seeking [25]. Furthermore, mifepristone has been clinically validated in a human laboratory model of craving and found to reduce the number of drinks per week in abstinent alcohol-dependent volunteers [21].

Mifepristone has also been used as a pharmacological tool to test the role of GR in several models of stress-induced anxiety-like behaviors. For instance, intracerebroventricular infusion of mifepristone prior to a restraint procedure abolished stress-induced anxiety-like behavior [26]. Systemic administration of mifepristone also decreased negative affect produced by chronic stress in mice with high-trait anxiety [27]. The functional effects of mifepristone as an anxiety-alleviating agent are mixed since other reports demonstrate that this drug produces no restorative changes to stress in a mouse model that lacks stress coping mechanisms [28].

Emerging clinical research suggests that there are sex differences in alcohol consumption and dependence, and evidence shows that the prevalence of alcohol consumption as a coping strategy to attenuate negative affective states (e.g., anxiety, depression, stress, and isolation) is higher in women than men. Similarly, preclinical studies have reported that female msP rats consume higher amounts of alcohol when compared to males [12]. Noteworthy, Borruto et al. [12] demonstrated that voluntary 10% alcohol drinking reduced elevated plus maze (EPM) anxiety-like behavior in male, but not in female msP rats. These observations point to the possibility that alcohol drinking in male and female msPs is motivated by different forms of anxiety (i.e., generalized anxiety versus stress-induced inability to engage in stress coping). Based on these observations, it is crucial to assess the potential anxiolytic effects of GR antagonism in both sexes.

In the present study, we explored whether acute systemic administration of the non-selective glucocorticoid receptor antagonist mifepristone reduces the inherited high stress responses and anxiety-like behaviors in msPs versus non-selected control Wistar rats. Specifically, we assessed male and female rats in a battery of tests that captures different anxiety-related features. We employed novelty-induced hypophagia (NIH) to test anxiety-like behaviors under novel environmental conditions. Since sleep disturbances are a hallmark of anxiety and stress-related disorders (e.g., PTSD), we examined whether

genotypic and sex differences may alter diurnal sleep maintenance. Finally, we utilized stronger stress-sensitive measures such as high-intensity acoustic signals to assess startle responses. Moreover, we tested the hypothesis that GR signaling would play a key role in the regulation of these behaviors by examining the efficacy of the GR antagonist mifepristone.

2. Results

2.1. Effects of Mifepristone on Anxiety-Like Behavior

To determine whether acute mifepristone administration reduces anxiety-like behavior in a genotype-specific manner, male and female Wistar and msP rats were pre-exposed to palatable chocolate pellets and then tested under novel environmental conditions using the novelty-induced hypophagia procedure. We found that male msPs in general displayed a greater latency to eat under novelty stress conditions as compared to male Wistar rats, main effect of genotype $F_{(1,40)} = 42.52$, $p = 0.001$ (Figure 1A). Male msPs also displayed lower overall pellet intake during novelty stress as compared to male Wistar rats, main effect of genotype $F_{(1,40)} = 49.48$, $p = 0.0001$ (Figure 1B). Interestingly, a single systemic mifepristone administration did not affect the latency to eat or the intake of chocolate pellets in male msPs, suggesting that the enhanced anxiety-like phenotype in msPs is not ameliorated by an acute administration of GR antagonist mifepristone. Similar to males, we found that female msPs displayed an increase in the latency to eat chocolate pellets under novelty stress conditions as compared to Wistar rats, main effect of genotype $F_{(1,32)} = 12.10$, $p = 0.001$ (Figure 1C). Female msPs also displayed lower overall pellet intake under novelty stress conditions relative to their Wistar counterparts, main effect of genotype $F_{(1,32)} = 16.69$, $p = 0.0001$ (Figure 1D). Importantly, mifepristone administration did not affect the latency to eat or intake of chocolate pellets in female msPs, suggesting that the enhanced anxiety-like phenotype observed in female msPs also is not ameliorated by acute exposure to the GR antagonist.

To further examine the contribution of sex differences in promoting anxiety-like behavior (Figure 1E,F), we also compared male versus female rats within each genotype as a function of sex, regardless of mifepristone treatment. We found that males displayed lower levels of pellet intake as compared to females regardless of genotype, suggesting that males displayed greater vulnerability produced by novelty stress, main effect of sex $F_{(1,35)} = 4.91$, $p = 0.048$ (Figure 1F).

2.2. Effects of Mifepristone on Sleep Disturbances

To examine whether mifepristone administration restores sleep disturbances produced by heightened stress, we first assessed diurnal sleeping patterns in male and female Wistar and msP rats. We found that male msPs, in general, displayed a shorter average bout duration when compared to male Wistar rats, main effect of genotype $F_{(1,27)} = 4.92$, $p = 0.035$ (Figure 2A). Interestingly, mifepristone administration did not restore the reduced average bouts' duration in male msPs suggesting that the interrupted sleep observed in male geneticallyselected msPs is not ameliorated by a single administration of GR antagonist. Importantly, no genotype differences or acute mifepristone effects were observed in total sleep time (Figure 2B) or number of sleep bouts (Figure 2C).

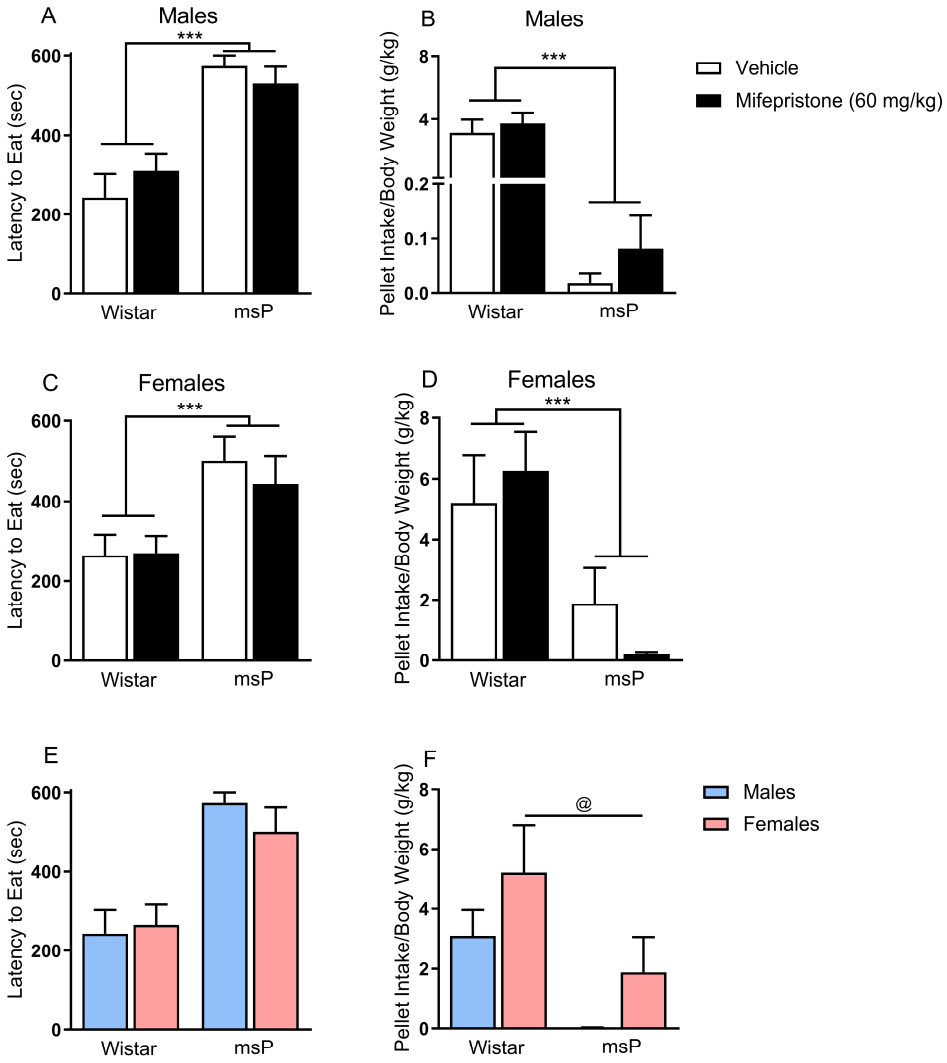

Figure 1. Effect of glucocorticoid receptor (GR) antagonist mifepristone on novelty-induced hypophagia (NIH) in both male and female Wistar and msP rats. Mifepristone (60 mg/kg) was injected intraperitoneally, and rats were subjected to the NIH test after 90 min. Male and female msP rats displayed higher anxiety-like behavior during NIH that was not reduced by mifepristone. (**A**) latency to eat chocolate pellets, (**B**) pellet intake in vehicle (n = 9) or mifepristone-treated (n = 10) male Wistar rats and vehicle (n = 13) or mifepristone-treated (n = 12) male msP rats. (**C**) latency to eat chocolate pellets, (**D**) pellet intake in vehicle (n = 9) or mifepristone-treated (n = 9) female Wistar rats and vehicle (n = 8) or mifepristone-treated (n = 10) female msP rats. (**E**) latency to eat chocolate pellets, (**F**) pellet intake in male Wistar (n = 9) and male msP (n = 13) rats, female Wistar (n = 9) and female msP (n = 8) rats. Results are expressed as mean ± SEM. Two-way ANOVA followed by Fisher's LSD protected post hoc tests when an interaction between variables occurred. Main effect of genotype, *** $p \leq 0.001$. Main effect of sex, $^{@}$ $p \leq 0.05$.

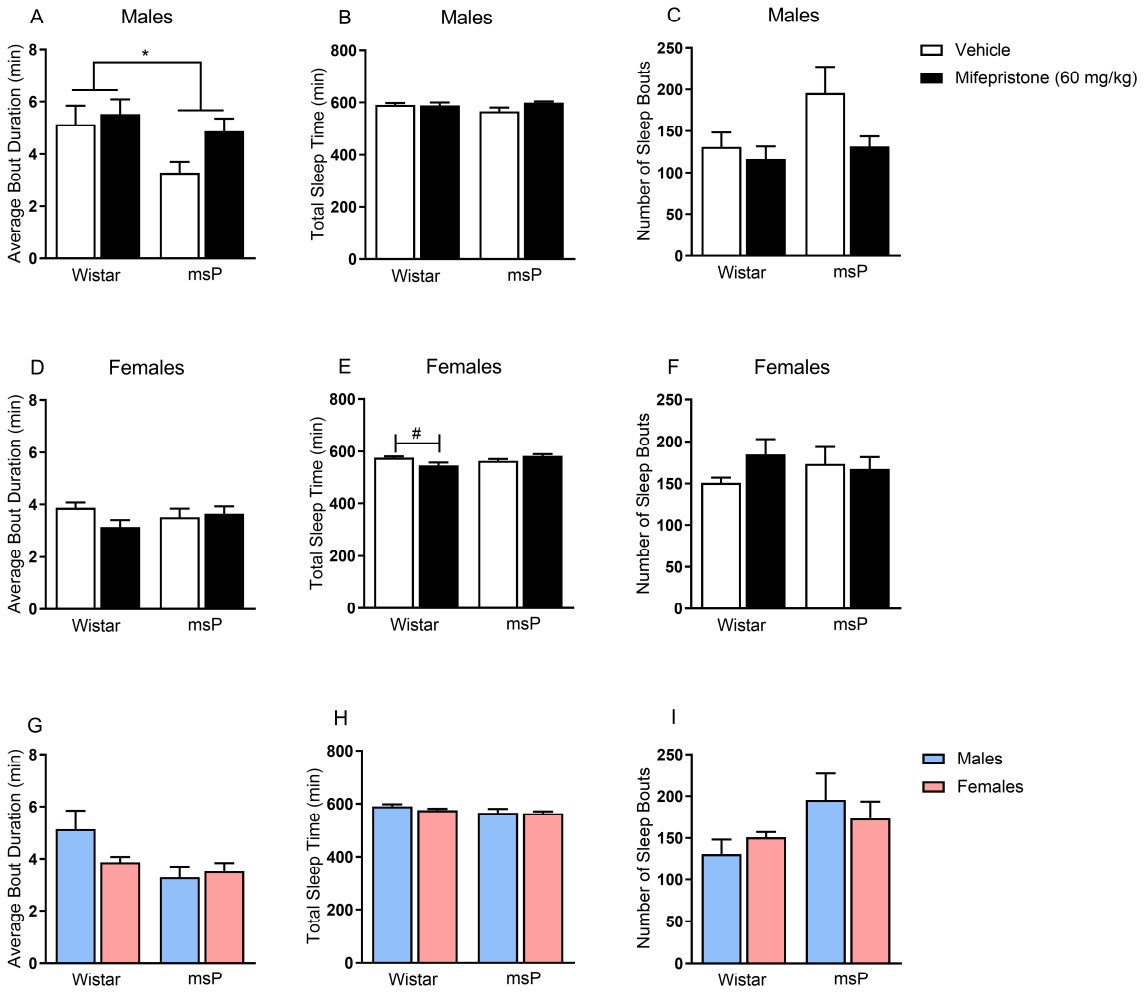

Figure 2. Effect of glucocorticoid receptor (GR) antagonist mifepristone on sleep disturbances in both male and female Wistar and msP rats. Mifepristone (60 mg/kg) was injected intraperitoneally, and rats were subjected to the comprehensive lab animal monitoring system (CLAMS) test after 90 min. Mifepristone had no effect on ameliorating sleep disturbances. (**A**) average bout duration, (**B**) total sleep time, (**C**) number of sleep bouts in vehicle (n = 8) or mifepristone-treated (n = 8) male Wistar rats and vehicle (n = 7) or mifepristone-treated (n = 8) male msP rats. (**D**) average bout duration, (**E**) total sleep time, (**F**) number of sleep bouts in vehicle (n = 8) or mifepristone-treated (n = 8) female Wistar rats and vehicle (n = 8) or mifepristone-treated (n = 8) female msP rats. (**G**) average bout duration, (**H**) total sleep time, (**I**) number of sleep bouts in male Wistar (n = 8) and male msP (n = 7) rats, female Wistar (n = 8) and female msP (n = 8) rats. Results are expressed as mean ± SEM. Two-way ANOVA followed by Fisher's LSD protected post hoc tests when the interaction between variables occurred. Main effect of genotype, * $p \leq 0.05$. Post hoc test revealed significant differences between treatments, $^{\#}$ $p \leq 0.05$.

In females, we observed that mifepristone administration decreased total sleep time in a genotype-specific manner, drug × genotype interaction $F_{(1,28)} = 9.36$, $p = 0.005$ (Figure 2E). Specifically, Wistar rats that received mifepristone displayed a significant reduction in total sleep time as compared to vehicle-treated controls ($p = 0.015$). The latter effect was only observed in non-selected Wistar rats suggesting that mifepristone does not influence sleep patterns in genetically-selected msP rats. Importantly, there were no changes observed in average bout duration (Figure 2D) or number of sleep bouts (Figure 2F).

To further delineate the contribution of sex in promoting sleep disturbances, we compared male versus female rats within each genotype as a function of sex, regardless of mifepristone treatment. Here, we found no sex-dependent changes in average bout duration (Figure 2G), total sleep time (Figure 2H), or number of sleep bouts (Figure 2I).

2.3. Effects of Mifepristone on Hyperarousal States

Since mifepristone did not reduce the heightened anxiety-like behavior observed in genetically-selected msPs (Figure 1A–D), we employed stronger, more stress-sensitive measures often necessary for this drug to induce changes [25]. We used stress-sensitive procedures that capture startle reflexive responses following sound stimuli across various intensity trials. In males, we found that msPs in general displayed higher startle responses when compared to male Wistar rats during the 120 dB trials 2–6, main effect of genotype $F_{(1,26)} = 10.56$, $p = 0.003$ (Figure 3B) and 120 dB final block, main effect of genotype $F_{(1,26)} = 14.51$, $p = 0.001$ (Figure 3C). Furthermore, male msPs also displayed higher average prepulse inhibition when compared to male Wistar rats, main effect of genotype $F_{(1,26)} = 9.32$, $p = 0.005$ (Figure 3D). A three-way ANOVA across the sequence of various levels of intensities revealed an intensity × genotype interaction, $F_{(5,130)} = 9.25$, $p = 0.001$ (Figure 3E). Specifically, male msPs displayed a higher startle response at 105 dB stimulus when compared to male Wistar rats ($p = 0.027$) (Figure 3E). Interestingly, mifepristone administration did not reduce the genotypic differences in the enhanced startle response or prepulse inhibition behavior, suggesting that GR antagonism does not mitigate the msP sensitivity to these stress phenotypes.

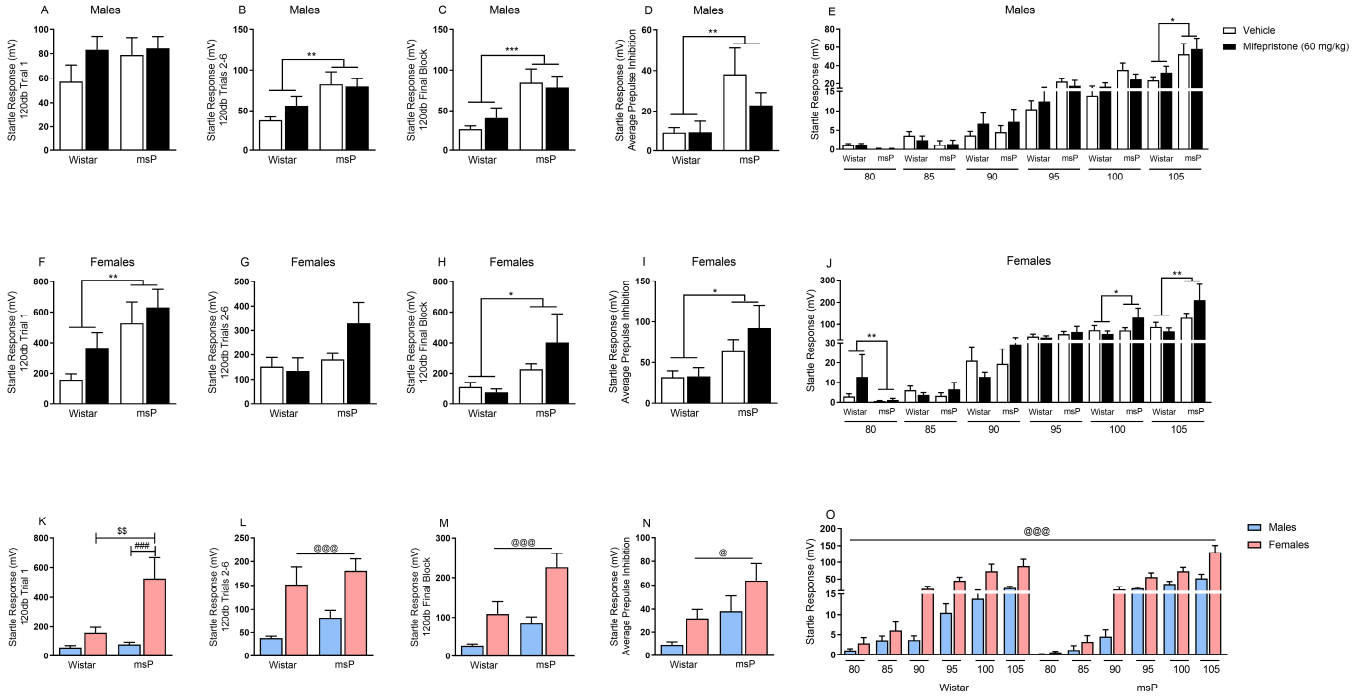

Figure 3. Effect of glucocorticoid receptor (GR) antagonist mifepristone on hyperarousal states in both male and female Wistar and msP rats. Mifepristone (60 mg/kg) was injected intraperitoneally, and rats were subjected to the acoustic startle response test after 90 min. Mifepristone had no effect on ameliorating the startle response to acoustic stimuli. (**A**) 120 dB trial 1 startle response, (**B**) 120 dB trials 2–6 startle response, (**C**) 120 dB final block startle response, (**D**) average prepulse inhibition startle response, (**E**) 80–105 dB startle responses in vehicle ($n = 8$) or mifepristone-treated ($n = 8$) male Wistar rats and vehicle ($n = 6$) or mifepristone-treated ($n = 8$) male msP rats. (**F**) 120 dB trial 1 startle response, (**G**) 120 dB trials 2–6 startle response, (**H**) 120 dB final block startle response, (**I**) average prepulse inhibition startle response, (**J**) 80–105 dB startle responses in vehicle ($n = 8$) or mifepristone-treated ($n = 8$) female Wistar rats and vehicle ($n = 6$) or mifepristone-treated ($n = 8$) female msP rats. (**K**) 120 dB trial 1 startle response, (**L**) 120 dB trials 2–6 startle response, (**M**) 120 dB final block startle response, (**N**) average prepulse inhibition startle response, (**O**) 80–105 dB startle responses in male Wistar ($n = 8$) and male msP ($n = 6$) rats, female Wistar ($n = 8$) and female msP ($n = 8$) rats. Results are expressed as mean ± SEM. Two-way ANOVA followed by Fisher's LSD protected post hoc tests when interaction between variables occurred. For repeated stimulus intensities (**E,J,O**), a mixed model three-way ANOVA was used. Main effect of genotype, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Main effect of sex, $^{@@@}$ $p \leq 0.001$, $^{@}$ $p < 0.05$. Post hoc test revealed significant differences between genotypes, $^{\$\$}$ $p \leq 0.01$. Post hoc test revealed significant differences between sexes, $^{\#\#\#}$ $p \leq 0.001$.

In females, we observed that msPs in general displayed higher startle responses when compared to female Wistar rats during the 120 dB trial 1, main effect of genotype $F_{(1,28)} = 8.89$, $p = 0.006$ (Figure 3F) and 120 dB final block, main effect of genotype $F_{(1,28)} = 5.37$, $p = 0.028$ (Figure 3H). Female msPs also displayed higher average prepulse inhibition when compared to their counterpart Wistar rats, main effect of genotype $F_{(1,28)} = 7.14$, $p = 0.012$ (Figure 3I). Furthermore, a three-way ANOVA across the various sound intensities (i.e., 80–105 dB), revealed an intensity × genotype interaction $F_{(5,140)} = 4.88$, $p = 0.026$ (Figure 3J). Specifically, female msPs displayed higher startle responses at both the 100 dB ($p = 0.011$) and 105 dB stimuli ($p = 0.006$) when compared to female Wistar rats. Importantly, mifepristone administration did not reduce the enhanced startle response or prepulse inhibition suggesting that the heightened sensitivity phenotype observed within female genetically-selected msPs is not acutely ameliorated by the GR antagonist.

To examine the unique role of sex in promoting heightened stress sensitivity regardless of glucocorticoid blockade, we compared male versus female rats within each genotype as a function of sex. We found that female msPs displayed an increase in startle response during the 120 dB trial 1 when compared to female Wistar rats (Figure 3K). The two-way ANOVA revealed a sex × genotype interaction $F_{(1,26)} = 4.81$, $p = 0.037$, and the post hoc analysis showed that these effects were attributable to the different genotypes in a within females comparison ($p = 0.002$) and to the sex in a within msPs comparison ($p = 0.001$; Figure 3K). Interestingly, regardless of genotype, females overall displayed higher startle responses when compared to males during the 120 dB trials 2–6, main effect of sex $F_{(1,26)} = 18.14$, $p = 0.0001$ (Figure 3L) and during 120 dB final block, main effect of sex $F_{(1,26)} = 18.20$, $p = 0.0001$ (Figure 3M), as well as higher average prepulse inhibition, main effect of sex $F_{(1,26)} = 5.61$, $p = 0.025$ (Figure 3N). Lastly, females in general displayed higher startle responses across 80–105 dB series of intensities when compared to males, main effect of sex $F_{(1,26)} = 20.02$, $p = 0.0001$ and intensity × sex interaction $F_{(5,130)} = 12.66$, $p = 0.0001$ (Figure 3O). Taken together, these data suggest that females generally show higher startle susceptibility to sound stress than male rats.

3. Discussion

Increased anxiety, stress sensitivity, and an impaired ability to cope with stress are comorbid and, in some cases, promote the development of AUD, whereas in other cases, they are the consequences of excessive drinking and a reflection of alcohol dependence. Efficacious therapeutic interventions to alleviate these comorbid pathologies still lack [29], particularly for drugs that normalize glucocorticoid and other stress-related systems. Prior work has revealed that mifepristone, a non-selective GR antagonist, reliably reduces alcohol drinking and seeking in alcohol-dependent Wistar rats and humans [21,25]. As a rodent line that displays enhanced motivation for alcohol drinking and innate negative affect, msPs were tested on a battery of anxiety-related behavioral tests. We hypothesized that mifepristone would alleviate innate anxiety-related behaviors in msP rats versus non-selected Wistar counterparts. In summary, we found that male and female msPs display greater anxiety-like behaviors as compared to Wistars when tested in the NIH paradigm. In addition, while male msPs but not female msPs displayed lower average bout duration during their sleeping phase, both male and female msPs showed amplified acoustic startle responses versus non-selected Wistar counterparts. Furthermore, we observed that the level of startle responses was sex-dependent within the msP group, with female msPs displaying greater acoustic startle responses versus male msPs. Importantly, the enhanced anxiety-like behavior, lower average bout duration, and enhanced startle responses were not ameliorated by a single dose of mifepristone. Together, these findings suggest that the heightened magnitude of anxiety-related behaviors in msPs does not depend upon acute GR activation (see below for further discussion). Our findings provide a further step in understanding the role of the GR system in mediating anxiety-like states, particularly in models that display an innate sensitivity to negative affect.

A major finding of this study was that msPs display enhanced anxiety-like behaviors versus non-selected Wistar counterparts in an array of novel behavioral paradigms that are closely associated with stress disorders (e.g., PTSD). The latter finding is consistent with prior work from our laboratory demonstrating that msPs display enhanced anxiety-like behavior in the NIH and EPM [30] and marble-burying tasks [20]. Prior reports also have revealed that msPs display greater anxiety and depressive-like behaviors on numerous behavioral paradigms involving stress and anxiety. For example, msPs display more immobility on the forced swim test and more time on the corner zones of an open field arena, an indication that the magnitude of stress and anxiety is greater in msPs versus non-selected Wistars [12–14,30,31]. More recent studies in our laboratory have revealed that the high magnitude of stress and anxiety in msPs may relate to diminished HPA axis function, an effect that is unique to genetically-selected msPs [20]. The msP rats display impaired constraint of the HPA axis that normally curbs the stress response, and this effect is likely mediated in part by glucocorticoid signaling in the PVN [20]. Together these findings suggest that dysregulation of glucocorticoid signaling alters biological/brain systems in a manner that may contribute to the anxiety-like phenotype in msPs.

Since sleep disturbances and enhanced startle response are hallmark symptoms in the etiology of stress disorders, the present study examined for the first time whether genotypic differences may underlie changes during diurnal sleep maintenance and hyperarousal in msPs. These noninvasive and activity-based techniques correlate well with EEG-defined sleep studies [32], while startle responses can capture exaggerated hyperarousal similarly observed in human PTSD patients [33]. Importantly, we did not find genotype-dependent changes in sleep maintenance as defined by average bout duration, total sleep time, and number of sleep bouts. Notably, in the present study, the animals were not exposed to alcohol, which might be an important factor affecting the development of sleep disturbances that are often associated with AUD and anxiety disorders or PTSD symptoms. Indeed, msPs displayed enhanced acoustic startle responses as compared to their non-selected Wistar counterparts, suggesting that innate disrupted stress systems in msPs resulted in increased hyperarousal. Collectively, these data suggest that msPs are more vulnerable to stressful stimuli.

To explore the role of the glucocorticoid system in msPs, we employed acute systemic administration of mifepristone to reduce the levels of stress and anxiety across our battery of behavioral tests. We found that mifepristone administration produced no beneficial effects in anxiety-like behavior in the NIH paradigm. Also, mifepristone did not significantly restore sleep disturbances and hyperarousal to the level of healthy, non-selected Wistar controls. These findings are surprising since it has been well documented that a similar dose range of mifepristone reliably decreases alcohol-related behaviors in dependent Wistar rats (60 mg/kg) [21,34] and have anxiety alleviating behavioral effects in male rats (20 mg/kg, [35]; 120 mg/kg, [36]). There are likely a few possibilities to explain these findings. First, much work has revealed that prior exposure to physical (i.e., social defeat; [37]) or pharmacological (i.e., yohimbine; [25]) induction of stress is required for mifepristone to produce alleviating effects on anxiety in rats. This stress induction approach prior to mifepristone administration increases circulating stress hormone release that may increase the effectiveness of mifepristone binding activity to ameliorate anxiety. Second, since msPs contain a blunted stress response that is innate, an acute administration of mifepristone may not be sufficient to ameliorate the heightened levels of stress and anxiety. Indeed, repeated daily administration of mifepristone restores depressive-like behavior following chronic defeat stress in mice [37], while chronic mifepristone treatment also prevents escalation of alcohol self-administration over time in dependent rats [23]. In addition, acute versus chronic mifepristone treatment would impact non-genomic versus genomic GR signaling (respectively), likely representing differential mechanisms of action [38]. It should also be noted that mifepristone has demonstrated clinical efficacy in patients suffering from psychotic depression (reviewed in [39]). As such, to detect beneficial effects of mifepristone, future studies may involve chronic treatment regimens prior to behavioral assessments,

as well as additional measures of negative affect. Lastly, prior work has revealed that msPs biochemically display impaired activation of the stress response, an effect largely mediated via glucocorticoid signaling in the CeA [20]. Specifically, phosphorylation of glucocorticoid receptor within the CeA is increased in male msPs versus non-selected Wistar rats and decreased in females [20]. The latter finding suggests that the regulation of GR signaling is compromised in the brains of msP rats, a neuroadaptation that may prevent the therapeutic effects of mifepristone administration in our experimental animal groups. Future studies will examine the mechanistic underpinnings of GR signaling on synaptic functions in the CeA.

While extensive work has focused on male msPs, there are only a few published reports studying the role of sex differences in modulating the anxiety-like predisposition in msPs [12,20,40]. Thus, the present study assessed the contribution of sex differences in promoting stress-related behaviors in a genotype-dependent manner. We found that males, regardless of genotype, generally display a suppression of food intake as compared to females during novelty stress. In addition, female msPs display a significant increase in startle response during the earliest and most intense audible trial as compared to their respective non-selected Wistar counterparts as well as to male msPs. Consistent with these findings, female msPs display higher amounts of alcohol consumption as compared to their respective Wistars counterparts as well as male msPs [12]. However, the latter report also revealed that both male and female msPs display similar levels of stress and anxiety in tasks involving forced swim and footshock procedures following chronic alcohol exposure [12]. This discrepancy between the latter report and our findings may be due to differences in the subjective effects of alcohol relative to alcohol-naïve states.

It is important to note that non-selectivity of mifepristone may exert several non-GR-associated actions. Mifepristone is known to be a competitive progesterone receptor antagonist, and progesterone may also serve to modulate anxiety-like behavior [41,42]. Thus, a possible explanation is that the effects of progesterone may contribute to the innate anxiety phenotype in msPs in a manner that prevents the therapeutic effects of mifepristone. Indeed, there is evidence suggesting that low levels of progesterone are correlated with greater anxiety-like behavior and corticosterone plasma levels [43]. Future work is needed to study the effects of mifepristone during specific phases of the estrous cycle, particularly in females who are more vulnerable to stress.

Overall, the present study revealed that both male and female msPs display elevated anxiety-like behavior versus their non-selected Wistar counterparts. In particular, female msPs display greater startle responses versus male msPs, suggesting that responses to stressful stimuli are sex-dependent. Our findings also indicate that mifepristone does not alleviate the innate anxiety-like profile of this genetically-selected rat model. One might expect that GR receptor antagonism would alleviate pre-existing anxiety-like traits in msPs. However, this drug may instead function to normalize the recovery after acute stress versus inherited stress-related traits. Furthermore, the purpose of this study was to determine the acute, non-genomic actions of mifepristone on anxiety-like behaviors since previous reports found that acute mifepristone reduced alcohol self-administration in dependent rats [21], suppressed yohimbine stress-induced reinstatement of alcohol-seeking [25], as well as acutely decreased negative affect produced by chronic stress in mice with high-trait anxiety [27]. Thus, future studies are required to examine the delayed genomic effects following chronic mifepristone administration. Future work is also needed to fully understand the stress systems in relation to stress hormone fluctuations as well as changes in circadian rhythms, in addition to potential biomarkers that interact with glucocorticoid-GR systems for optimal administration of mifepristone.

4. Materials and Methods

4.1. Animals

A total of N = 143 rats were used in this study. Adult male (n = 40, ~450 g) and female (n = 34, ~250 g) msP rats were bred at The Scripps Research Institute (La Jolla, CA, USA)

from a colony obtained from the University of Camerino (Camerino, Italy). For genotypic comparisons, we used adult male (n = 35, ~450 g) and female (n = 34, ~250 g) Wistar rats (Envigo, Indianapolis, IN, USA) from which the msP line was generated. Rats were housed on a 12 h reverse light/dark cycle (lights off at 8:00 a.m.), with food and water available *ad libitum*. The rats were pair-housed, separated by a perforated clear plexiglass divider to habituate them to the behavioral test conditions while also reducing isolation stress [44]. The experimental groups consisted of rats that were randomly selected, and simple randomization for treatment groups condition occurred prior to the start of the experiments via a number labeling system for each rat. Then, the rats were arbitrarily assigned to different treatment groups regardless of body weight. We conducted all procedures in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with The Scripps Research Institute Institutional Animal Care and Use Committee policies.

4.2. Drug Preparation and Treatment

Rats were injected intraperitoneally (i.p.) with a single dose (60 mg/kg; 1 mL/kg) of mifepristone (Cayman Chemical, Ann Arbor, MI, USA) or vehicle (propylene glycol) 90 min prior to each behavioral test. At the used dose and injection volume, mifepristone is characterized by poor solubility in aqueous vehicles. To improve the solubility, 100% propylene glycol [45] was slowly added to the compound while mixing with a magnetic stir bar. The 60 mg/kg dose and 90 min pretreatment were chosen based on previously published reports demonstrating that the acute systemic administration of this dose of mifepristone reduces alcohol reinforcement [21], and doses ranging between 20 mg/kg and 120 mg/kg have an anxiolytic effect in male rats [35,36].

4.3. Novelty-Induced Hypophagia (NIH)

Exposure to novel environments elicits a stressful reaction in rodents that can interfere with normal behavior, including food consumption [46,47]. Here rats were monitored for anxiety-like behavior using hypophagia procedures as previously published [48]. The animals received home cage exposure to a novel palatable food (50% sucrose, chocolate-flavored pellets, 45 mg, 5TUL, Test Diets, St. Louis, MO, USA) 24 h before being tested. The rats were acclimated to their housing room during their dark phase (red lights). Following exposure to the novel palatable food, rats were monitored by the experimenter to confirm that each rat tasted the novel food. The following day, the rats were treated with mifepristone (60 mg/kg, 1 mL/kg, i.p.) or vehicle and were left undisturbed for 90 min prior to evaluation under novel testing conditions that are perceived to be stressful (i.e., white lights on, unfamiliar double-size cage, white noise). We measured the latency for the rats to consume the chocolate pellets and the total intake in an unfamiliar environment over a 10 min trial.

4.4. Comprehensive Lab Animal Monitoring System (CLAMS)

Since stress and anxiety are often accompanied by sleep disturbances [49], we assessed sleep patterns in a comprehensive lab animal monitoring system (CLAMS, Columbus Instruments, Columbus, OH, USA). Such noninvasive, activity-based measurements correlate well with EEG-defined sleep [32,44]. We used OXYmax–CLAMS units to interpret each rat's sleep parameters from photocell-defined motor activity across the first 11 h of their 12 h light phase. Rats were placed in the single units (32 × 20 × 19 cm) 12 h prior to testing to allow them to acclimatize. Each CLAMS chamber was equipped with a water sipper and tray that provided *ad libitum* access to food. Twenty-four photobeams were used to detect horizontal or vertical movement and were located 2.5 cm apart, at 9 cm and 14 cm above the floor. CLAMS sleep detection works in time periods called "epochs". Sleeping epochs were defined as no more than one photocell interruption during a 60 sec epoch, and sleep bouts were defined as successive strings of such epochs. We extracted and analyzed

the average duration of the sleeping bouts (min), the number of sleeping bouts and the total sleeping time (min) throughout the inactive phase.

4.5. Acoustic Startle

Exaggerated acoustic startle responses are present in patients with PTSD and indicate hyperarousal [44]. Startle reflexes were measured in four identical startle response systems (SR-LAB, San Diego Instruments, San Diego, CA, USA) consisting of a nonrestrictive Plexiglas cylinder (13 cm inner diameter, 25 cm length for males; 9 cm inner diameter, 20 cm length for females) mounted on a Plexiglas platform and placed in a dark, ventilated, sound-attenuated chamber. The movements were detected and measured by a piezoelectric element mounted under each cylinder. A dynamic calibration system was used to ensure comparable startle magnitudes across the four devices. Throughout the session, the startle system delivered a constant background white noise of 68 dB. Startle stimuli were presented through a high-frequency speaker located above the startle chambers and lasted for 30 msec. Startle magnitudes were sampled each millisecond during a period of 100 msec, beginning at the onset of the startle stimulus. Startle response was defined as the peak response during this 100 msec period. During a 30 min session, 75 trials were presented in a pseudorandom order. The SR-LAB startle response system measured startle responses to acoustic stimuli (80–120 dB) and no-stimulus control trials. The test session began with a 5 min acclimation period followed by four consecutive blocks of test trials. Block 1 and 4 consisted of six startle 120 dB stimulus-alone trials. Prepulse inhibition was tested in block 2 by interspersing non-prepulsed 120 dB trials with six 120 dB trials that were prepulsed with an 80 dB tone by 70 msec. Block 3 consisted of trials of varying intensity (80, 85, 90, 95, 100, 105 dB), each one presented six times in a randomized order. Between each block, three no-stimulus trials were included, during which only the background noise was presented.

4.6. Statistical Analyses

NIH, CLAMS, and acoustic startle were analyzed using separate two-way analyses of variance (ANOVA) with treatment (vehicle versus mifepristone) and genotype (Wistar versus msP) as between-subject factors. Significant interaction effects were followed by Fisher's LSD-protected post hoc tests. For acoustic startle data containing repeated stimulus intensities, a mixed model three-way ANOVA was used with genotype and drug treatment as between subjects' factors and levels of intensity as within-subject factor. To investigate the role of sex differences, a separate level of analysis using similar two-way ANOVAs was included with sex (male versus female) and genotype (Wistar versus msP) as between-subject factors. The rationale for this analytical approach is based on our hypothesis regarding drug treatment effects separately within each sex and based on prior work [20]. Each sex was tested on separate days with experimenters blind to the subjects' treatment condition. All data are presented as mean ± standard error of the mean (SEM). The significance level was determined at $p < 0.05$. All statistical analyses were performed on SPSS V26 (IBM Corporation, Armonk, NY, USA), and all graphs were generated using Prism V8 (GraphPad, San Diego, CA, USA). For a complete description of the individual experiments' statistical analysis, please see Supplementary Tables S1–S4.

Supplementary Materials: The following are available online at https://www.mdpi.com/1422-0067/22/6/3095/s1.

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Effect of Glucocorticoid Receptor Antagonism on Alcohol Self-Administration in Genetically-Selected Marchigian Sardinian Alcohol-Preferring and Non-Preferring Wistar Rats

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Abstract: Alcoholism is a chronically relapsing disorder characterized by high alcohol intake and a negative emotional state during abstinence, which contributes to excessive drinking and susceptibility to relapse. Stress, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and alterations in glucocorticoid receptor (GR) function have been linked to transition from recreational consumption to alcohol use disorder (AUD). Here, we investigated the effect of pharmacological antagonisms of GR on alcohol self-administration (SA) using male and female Wistar and Marchigian Sardinian alcohol-preferring (msP) rats, a rodent line genetically selected for excessive alcohol drinking and highly sensitive to stress. Animals were trained to self-administer 10% (*v*/*v*) alcohol. Once a stable alcohol SA baseline was reached, we tested the effect of the GR antagonists mifepristone (0.0, 10, 30 and 60 mg/kg; i.p.) and CORT113176 (0.0, 10, 30 and 60 mg/kg) on alcohol SA. To evaluate whether the effects of the two compounds were specific for alcohol, the two drugs were tested on a similar saccharin SA regimen. Finally, basal blood corticosterone (CORT) levels before and after alcohol SA were determined. Systemic injection with mifepristone dose-dependently reduced alcohol SA in male and female Wistars but not in msPs. Administration of CORT113176 decreased alcohol SA in male and female Wistars as well as in female msPs but not in male msP rats. At the highest dose, mifepristone also reduced saccharin SA in male Wistars and female msPs, suggesting the occurrence of some nonspecific effects at 60 mg/kg of the drug. Similarly, the highest dose of CORT113176 (60 mg/kg) decreased saccharin intake in male Wistars. Analysis of CORT levels revealed that females of both rat lines had higher blood levels of CORT compared to males. Alcohol consumption reduced CORT in females but not in males. Overall, these findings indicate that selective blockade of GR selectively reduces alcohol SA, and genetically selected msP rats are less sensitive to this pharmacological manipulation compared to heterogeneous Wistars. Moreover, results suggest sex differences in response to GR antagonism and the ability of alcohol to regulate GR transmission.

Keywords: alcohol use disorder; stress; alcohol preferring rats; glucocorticoids; mifepristone; alcohol self-administration

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1. Introduction

Alcohol use disorder (AUD) is a complex psychiatric condition characterized by excessive drug use, loss of control over its consumption and emergence of a negative emotional state during withdrawal that contribute to relapse [1]. AUD is a major public health problem, and alcohol represents a significant disability and morbidity factor responsible of about three million deaths per year [2].

Stress and dysregulation of related hormones of the hypothalamic-pituitary-adrenal (HPA) axis have been proposed as important factors affecting disease progression [3,4].

The HPA axis represents the primary neuroendocrine network controlling stress response, and its activation in response to external or internal perturbation culminates in the production and release of cortisol in humans and corticosterone (CORT) in rodents [5]. Once released, glucocorticoids act through either the high affinity mineralocorticoid or the low affinity glucocorticoid receptor (GR). GR is highly expressed in several brain regions of the limbic system, in the paraventricular nucleus (PVN) of the hypothalamus and the anterior pituitary gland [6]. Once released, glucocorticoids produce an array of physiological effects to adjust the organism to stressor exposure and are also responsible for termination of their actions via negative feedback inhibition at HPA level [5].

The motivation to drink alcohol is initially driven by positive reinforcement mechanisms, and its consumption is usually linked to recreational purposes. Studies in rodent models mimicking the early stages of alcohol consumption demonstrated that CORT administration increased alcohol self-administration (SA) [7–9] whereas adrenalectomy decreased it [10]. Noteworthy is that alcohol drinking was recovered by corticosterone replacement, suggesting that glucocorticoids facilitate alcohol reinforcement [10]. As a result of chronic alcohol drinking, the excessive and protracted activation of the HPA axis may lead to its dysregulation. This contributes to the surge of compulsive alcohol drinking motivated by the need to self-medicate to attenuate the negative symptoms associated with alcohol withdrawal [4,11,12]. Earlier studies demonstrated that alcohol-dependent rats exhibited significant downregulation of GR during acute withdrawal, and GR upregulation during protracted abstinence in several stress/reward related brain areas, suggesting that the GR system may contribute to the progression of AUD [13].

The genetically selected Marchigian Sardinian alcohol-preferring (msP) rat line is a well consolidated animal model to study AUD. In this rat line, anxiety and depressive-like traits have been cosegregated with high alcohol preference during the selection process. Hence, it is possible that their innate propensity to consume high amounts of alcohol is driven by the attempt to self-medicate from an innate negative affect, specifically mimicking the subpopulation of humans with alcoholism that consume alcohol for tension relief purposes [14,15]. Consistent with this view, earlier studies showed that msP rats carry two single nucleotide polymorphisms in the promoter region of the CRF1 receptor (CRF1-R) leading to hyperactivation of the system that is attenuated by voluntary alcohol consumption [16–18]. These mutations have been also associated with a decreased threshold for stress-induced alcohol-seeking and conferred to msP rats higher sensitivity to CRF1-R antagonists [16,19,20]. Noteworthy is that these gene polymorphisms are conserved in the human CRF system and have been correlated with the diagnosis of AUD [21,22]. We also reported that male msP rats displayed dysregulated GABA and glutamate signaling [23–25]. Recently, it has been found that male msP rats displayed diminished stress-induced GR phosphorylation at the serine site 232 in the PVN and a constitutive increase in phosphorylated GR levels in the central nucleus of the amygdala (CeA) [26]. The elevation of GR phosphorylation was also observed in the CeA of alcohol-dependent rats during acute withdrawal [27]. In postdependent rats, systemic and intra-CeA administration of mifepristone, a nonselective glucocorticoid and progesterone receptor antagonist, reduced alcohol intake and yohimbine-induced reinstatement of alcohol seeking [27,28].

Currently, it is unknown whether the constitutive alteration of GR levels of msP rats might contribute to their excessive alcohol-drinking phenotype. However, considering that this rat line shows features resembling postdependent rats, we thought it important to explore the effect of pharmacological antagonism of GR on alcohol self-administration by comparing the msP rat line with its Wistar counterpart. Moreover, considering that several sex differences have been described in response to stress and to alcohol, and that the HPA axis function is greater in female rats, in the present study we tested males and females separately [15,29–33].

2. Results

2.1. Experiment 1.1: Effect of Mifepristone on Alcohol Self-Administration in Male and Female msP and Wistar Rats

We tested the effect of mifepristone on alcohol SA under Fixed Ratio 1 (FR1) schedule of reinforcement in male and female msP (N = 10/sex) and Wistar (N = 9–10/sex) rats. Experimental subjects received mifepristone (10, 30 and 60 mg/kg) or its vehicle in a counterbalanced within subject Latin square design. A three-way ANOVA revealed an overall effect of treatment [$F_{(3,35)} = 7.5$; $p < 0.001$], sex [$F_{(1,35)} = 55.8$; $p < 0.0001$] and strain [$F_{(1,35)} = 41.2$; $p < 0.0001$]. There was a significant sex x strain interaction [$F_{(1,35)} = 10.2$; $p < 0.01$], but no other significant interactions. These results reflect higher SA levels in msP, a higher number of rewards by male msP rats and a general reduction of alcohol SA induced by mifepristone. To further evaluate the effect of mifepristone, we carried out single ANOVAs to independently analyze the drug effect on male and female msPs as well as on male and female Wistars. In msP rats no overall effect of treatment in male [$F_{(3,9)} = 0.4$; $p > 0.05$] or in female rats [$F_{(3,9)} = 1.1$; $p > 0.05$] was detected. Conversely, an overall significant effect of treatment was detected in male [$F_{(3,8)} = 4.0$; $p < 0.05$] and female [$F_{(3,9)} = 7.5$; $p < 0.01$] Wistars. Dunnett's post hoc analysis showed a significant decrease in the number of alcohol-reinforced responding at doses of 30 mg/kg and 60 mg/kg of mifepristone in both male and female Wistar rats ($p < 0.05$; Figure 1A, upper panel).

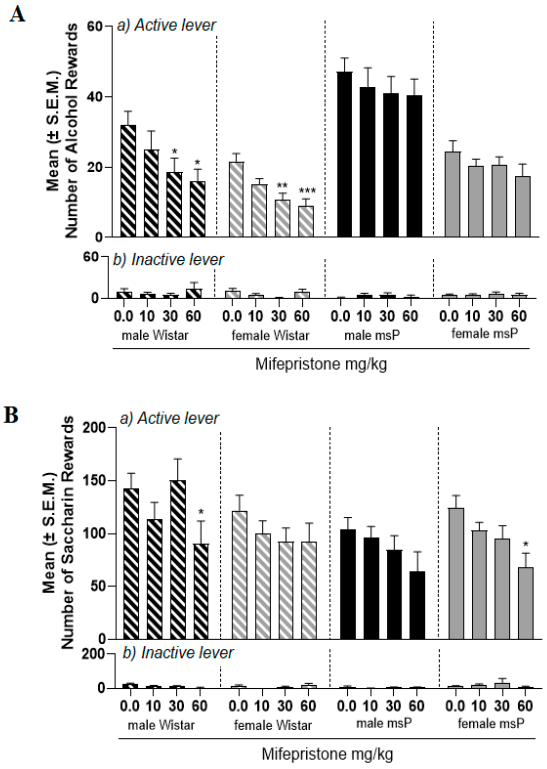

Figure 1. Effect of mifepristone on alcohol and saccharin self-administration in male and female msP and Wistar rats. Male and female msP and Wistar rats were treated with mifepristone (0.0, 10, 30 and 60 mg/kg) i.p., 90 min prior to test session. (**A**) Mifepristone treatment significantly reduced the number of alcohol rewards in male and female Wistars. Drug treatment did not decrease alcohol SA in male and female msPs. (**B**) At the dose of 60 mg/kg, mifepristone significantly reduced saccharin SA in male Wistars and in female msPs. Data are expressed as the mean ± SEM of number of: (**a**) reinforced responses (rewards) at the active lever and (**b**) total responses at the inactive lever. Significant difference from vehicle (0.0 mg/kg): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

A three-way ANOVA applied to inactive lever responding showed no overall effect of treatment [$F_{(3,35)} = 0.9$; $p > 0.05$], sex [$F_{(1,35)} = 0.05$; $p > 0.05$] or strain [$F_{(1,35)} = 2.3$; $p > 0.05$]. Neither interaction was detected (Figure 1A, lower panel).

2.2. Experiment 1.2: Effect of Mifepristone on Saccharin Self-Administration in Male and Female msP and Wistar Rats

To control for the selectivity of mifepristone effect on alcohol SA, other groups of male and female msP (N = 6–8/sex) and Wistar (N = 7–8/sex) rats were tested for the effect of mifepristone (10, 30 and 60 mg/kg) or its vehicle on saccharin SA. A three-way ANOVA found a significant effect of treatment [$F_{(3,25)} = 8.8$; $p = 0.0001$], no effect of sex [$F_{(1,25)} = 0.3$; $p > 0.05$], no effect of strain [$F_{(1,25)} = 0.4$; $p > 0.05$] and no interactions. To further explore the effect of mifepristone, data from male and female msPs and male and female Wistars were analyzed separately by single ANOVAs. Results revealed an overall effect of treatment in male Wistars [$F_{(3,6)} = 4.7$; $p < 0.05$] and female msPs [$F_{(3,7)} = 6.4$; $p < 0.01$]. Conversely, no overall effect was found in female Wistars [$F_{(3,7)} = 1.4$; $p > 0.05$] and male msPs [$F_{(3,5)} = 1.2$; $p > 0.05$]. Dunnet's post hoc tests showed that 60 mg/kg of mifepristone reduced saccharin SA in both male Wistars and female msPs ($p < 0.05$) (Figure 1B, upper panel).

Analysis of inactive lever responding found no significant overall effect of treatment [$F_{(3,25)} = 0.7$; $p > 0.05$], sex [$F_{(1,25)} = 1.4$; $p > 0.05$], strain [$F_{(1,25)} = 0.005$; $p > 0.05$] and no interactions (Figure 1B, upper panel).

2.3. Experiment 2.1: Effect of CORT113176 on Alcohol Self-Administration in Male and Female msP and Wistar Rats

Mifepristone is a GR antagonist that also has activity on the progesterone receptor. To confirm that effects observed were specifically mediated by GR antagonism, we tested CORT113176, which is another more selective GR antagonist [27]. Once a stable baseline of alcohol SA was reached, male and female msP (N = 9–10/sex) and Wistar (N = 10/sex) rats were treated with CORT113176 (10, 30, 60 mg/kg) or its vehicle. A three-way ANOVA revealed an overall effect of treatment [$F_{(3,35)} = 11.1$; $p < 0.0001$], sex [$F_{(1,35)} = 16.04$; $p < 0.001$], strain [$F_{(1,35)} = 24.6$; $p < 0.0001$] and sex x strain interaction [$F_{(1,35)} = 6.3$; $p < 0.05$], but no other significant interactions (Figure 2A, upper panel). At this point we conducted single ANOVAs to further determine the effect of CORT113176 on male and female msPs and male and female Wistars. Results showed an overall effect of treatment in male Wistars [$F_{(3,9)} = 4.4$; $p < 0.05$], female Wistars [$F_{(3,9)} = 4.5$; $p < 0.05$] and female msPs [$F_{(3,9)} = 4.0$, $p < 0.05$]. No effect was found in male msP [$F_{(3,8)} = 1.9$; $p > 0.05$] rats. Dunnet's post hoc tests revealed that at 60 mg/kg, CORT113176 decreased alcohol SA in male ($p < 0.01$) and female Wistars ($p < 0.05$) as well as female msPs ($p < 0.01$).

Analysis of the inactive lever found no significant overall effect of treatment [$F_{(3,35)} = 0.8$; $p > 0.05$] and strain [$F_{(1,35)} = 3.7$; $p > 0.05$] but an overall effect of sex [$F_{(1,35)} = 7.7$; $p < 0.01$], treatment x strain [$F_{(1,35)} = 5.6$; $p < 0.01$] and sex x strain interaction [$F_{(1,35)} = 5.5$; $p < 0.05$] was observed (Figure 2A, lower panel).

2.4. Experiment 2.2: Effect of CORT113176 on Saccharin Self-Administration in Male and Female msP and Wistar Rats

We next verified the specificity of action of CORT113176 by testing its effect on saccharin SA in male and female msP (N = 9–10/sex) and Wistar (N = 9–10/sex) rats. Three-way ANOVA demonstrated a significant effect of treatment [$F_{(3,34)} = 5.2$; $p < 0.01$], strain [$F_{(1,34)} = 10.3$; $p < 0.01$] and treatment x sex interaction [$F_{(3,102)} = 3,4$; $p < 0.05$] (Figure 2B, upper panel). When single ANOVAs were carried out, we found an overall effect of CORT113176 on saccharin SA only in male Wistar rats [$F_{(3,8)} = 4.5$; $p < 0.05$]. No drug effect was detected in female Wistars [$F_{(3,9)} = 0.7$; $p > 0.05$], male msPs [$F_{(3,8)} = 1.7$; $p > 0.05$] and in female msPs [$F_{(3,9)} = 0.4$; $p > 0.05$].

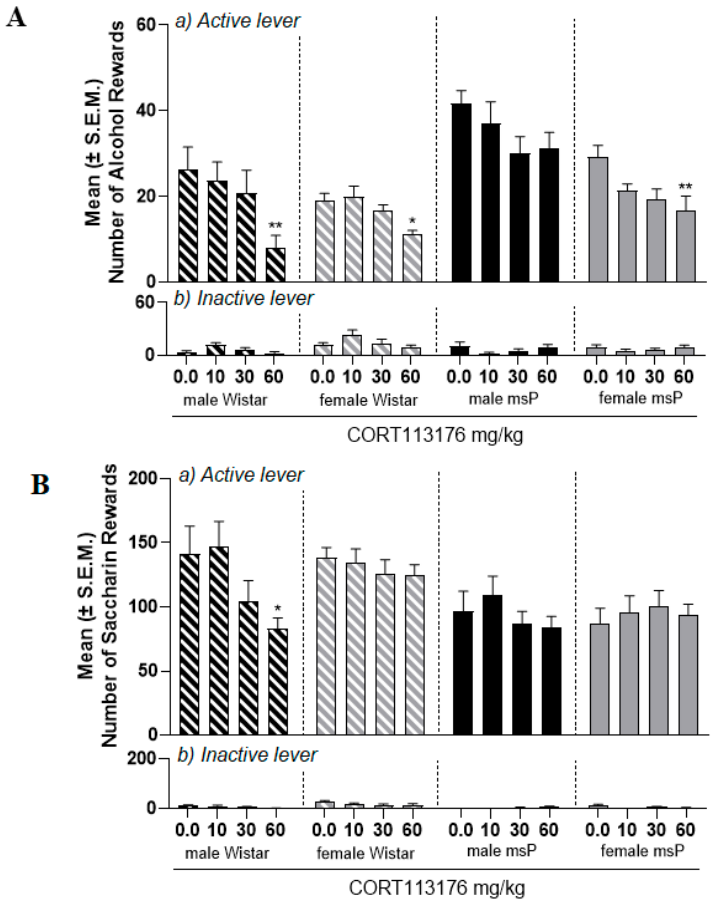

Figure 2. Effect of CORT113176 on alcohol and saccharin self-administration in male and female msP and Wistar rats. Male and female msP and Wistar rats were treated with CORT113176 (0.0, 10, 30 and 60 mg/kg) i.p., 90 min prior to test session. (**A**) CORT113176 treatment significantly reduced the number of alcohol rewards in male and female Wistars and in female msP rats. (**B**) CORT113176 at the dose of 60 mg/kg significantly reduced saccharin SA in male Wistar rats only. Data are expressed as the mean ± SEM of number of: (**a**) reinforced responses at the active and (**b**) total responses at inactive lever. Significant difference from vehicle (0.0 mg/kg): ** $p < 0.01$; * $p < 0.05$.

Analysis of the inactive lever found no significant overall effect of treatment [$F_{(3,34)} = 2.7$; $p > 0.05$], but there was a significant effect of sex [$F_{(1,34)} = 9.3$; $p < 0.01$], strain [$F_{(1,34)} = 15.4$; $p < 0.01$] and treatment x strain interaction [$F_{(3,102)} = 2.9$; $p < 0.05$] (Figure 2B, lower panel).

2.5. Experiment 3: Blood CORT Levels Following Alcohol Self-Administration in Male and Female msP and Wistar Rats

Finally, we assessed the blood CORT levels under basal conditions and after alcohol SA in male and female msP (N = 6/sex) and Wistar rats (N = 8–7/sex). Three-way ANOVA revealed a main effect of sex [$F_{(1,23)} = 84.5$; $p < 0.0001$], alcohol condition [$F_{(1,23)} = 19.5$; $p < 0.001$], strain [$F_{(1,23)} = 13.8$; $p < 0.01$], sex x alcohol condition interaction [$F_{(1,23)} = 18.3$; $p < 0.001$] and sex x strain interaction [$F_{(1,23)} = 4.4$; $p < 0.05$]. Female rats from both genotypes displayed persistently higher levels of CORT compared to male rats in both conditions. Female Wistar rats showed higher CORT levels than female msPs ($p < 0.001$). Alcohol consumption in a SA session decreased CORT levels only in female animals ($p < 0.001$). In male rats, blood CORT concentrations were not affected by alcohol SA (Figure 3).

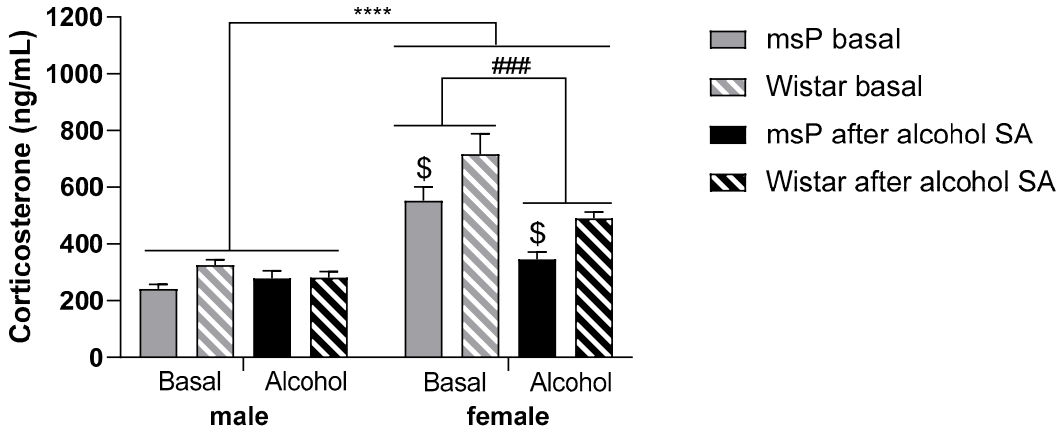

Figure 3. Blood corticosterone (CORT) levels under basal conditions and after alcohol SA sessions in male and female msP and Wistar rats. Females displayed significantly higher blood CORT levels than males independently of rat strain. Female Wistars had higher CORT levels than female msPs. Alcohol consumption decreased basal CORT levels in female animals only. In both rat lines, CORT levels of male rats remained unchanged following alcohol SA. Data are presented as mean ± SEM. Main effect of sex: **** $p < 0.0001$; main effect of sex x alcohol condition: ### $p < 0.001$; $ $p < 0.05$ vs. msP same condition and sex (sex x strain interaction).

3. Discussion

The present study investigated the effect of glucocorticoid receptor antagonism on alcohol drinking in genetically-selected msP rats in comparison with nonselected Wistar rats. To summarize, we found that mifepristone administration reduced alcohol SA in both male and female Wistar rats, but not msPs, at similar dose ranges utilized in previous studies measuring alcohol SA in dependent Wistar rats [27]. The ability of mifepristone to reduce alcohol SA was apparent at the intermediate dose of 30 mg/kg, while higher doses (60 mg/kg) appeared to produce nonselective reductions of saccharin SA, suggesting the occurrence of nonspecific effects. Given the nonselectivity of mifepristone in antagonizing progesterone receptors also, we tested the selective CORT113176 compound that targets GR to confirm whether reducing alcohol SA requires specificity for the GR. Consistent with results with mifepristone, CORT113176 significantly reduced alcohol SA in male and female Wistars as well as female msP rats. As for mifepristone, male msPs did not respond to CORT113176 treatment. Furthermore, administration of CORT113176 at the highest dose reduced saccharin SA only in male Wistar rats. Taken together, we suggest that our drug regimen is specific to alcohol SA, since the number of saccharin rewards was not modified in the other groups of rats. However, at high doses, nonspecific inhibition of motivated behavior may emerge. Earlier work demonstrated that mifepristone decreases alcohol consumption in a limited-access two-bottle choice paradigm [34], and intra-CeA infusion of mifepristone reduces alcohol-seeking behavior following a yohimbine challenge [28]. Noteworthy is that it has been also demonstrated that chronic administration of mifepristone in alcohol vapor-exposed rats prevented the escalation of alcohol intake [13]. Consistently, acute mifepristone administration selectively reduced alcohol intake in alcohol-dependent but not in nondependent rats [27]. Moreover, both mifepristone and CORT113176 selectively reduced binge-like ethanol intake in mice selectively bred for high ethanol concentration using drinking in the dark procedures [35]. Finally, it was shown that in nondependent Wistar rats, GR antagonism was more efficacious in female than in male rats [36]. Our results are consistent with these earlier works and confirmed that GR antagonists also reduced alcohol intake in nondependent animals, an effect more robust in female versus male rats [35,36].

Msp rats have long been proposed as an innate phenocopy of a subpopulation of patients that drink excessive amounts of alcohol for tension relief and self-medicating purposes [14]. Earlier studies have demonstrated that this rat line is characterized by two single-nucleotide polymorphisms at the CRF1-R receptor locus, leading to an enhanced

expression of CRF1R in different brain regions [16]. Because of this overexpression, they are highly sensitive to stress and show anxious and depressive-like symptoms that are relieved by alcohol consumption [14,16,17]. Recent findings have proved that negative feedback processes regulating HPA responsiveness are impaired in msP versus Wistar rats. Notably, male msP rats showed an innate increase in phosphorylation at the serine site 232 in the CeA, a marker of GR nuclear localization and transactivation [26]. Considering these constitutive alterations in their stress system, and the role of GR in the transition to alcohol dependence, we initially hypothesized that administration of GR antagonists would attenuate alcohol SA more efficaciously in msP rats versus Wistar controls.

In fact, msP rats have long been proposed as a phenocopy of postdependent animals, since they display comorbid symptoms of alcohol preference, high anxiety-like traits and hypersensitivity to stress. Consequently, we proposed that GR antagonism would attenuate the negative affect state that may drive their high alcohol consumption. However, contrary to our expectations, GR antagonists appeared more efficacious in Wistars than in msP rats. Furthermore, we recently reported the GR antagonism also does not alter the innate anxiety-like behaviors in msP rats [30].

There are few possibilities to explain the limited efficacy of GR antagonists in msPs. For instance, in an earlier study we found that male msPs had higher adrenocorticotropic hormone levels but lower circulating CORT, whereas in females, msP rats displayed larger elevation of CORT levels in response to restraint stress versus Wistars. In line with this suggestion, in response to a dexamethasone challenge, msP rats showed a lower reduction in CORT compared to Wistar controls [26]. These findings suggest that msP rats have a different regulation of the HPA axis, and the negative feedback processes modulating its responsiveness are diminished in this rat line. Hence it is possible that an acute injection of GR antagonist is not sufficient to normalize the hormonal equilibrium and to prevent the high alcohol drinking of msP rats. Future studies are needed to evaluate the effects of GR antagonists following chronic administration. A second possibility is that the higher innate GR phosphorylation observed in msP rats may lead to a differential regulation of the intracellular signaling pathways associated with the GR, an effect that may impair binding activity following mifepristone and CORT113176 administration. Thus, it is important to evaluate if transcriptional changes associated with GR activation are different in msPs versus Wistars.

In this study, we also measured plasma CORT levels prior to and after alcohol self-administration. Consistent with the results of earlier work, we found higher basal CORT levels in female compared to male rats [26,37,38]. The highest concentration was detected in female Wistars followed by female msPs. Moreover, we observed that alcohol SA markedly reduced CORT levels in females of both strains, whereas no changes were observed in males. These data are consistent with earlier studies showing that females displayed enhanced glucocorticoids secretion both at baseline and following stress, and after an alcohol challenge [37–40]. The motivational factors contributing to drinking in males and females may be different, and whether circulating corticosteroid levels may contribute to these discrepancies is unclear. However, it is worth noting that our results indicate that the higher the basal circulating CORT levels, the stronger the inhibitory effect of GR antagonists on alcohol drinking.

Since stress enhances the motivation for alcohol, particularly in female rats, we speculate that their drinking is reduced by GR antagonists via processes that suppress HPA axis function and possibly reduce negative mood associated with steroid hormones dysregulation [41,42].

In summary, our results showed that GR antagonism attenuates alcohol SA, particularly in female rats. Moreover, despite the observation that msPs are more vulnerable to stress and are highly motivated to drink alcohol for tension relieving purposes, they showed a poorer response to GR antagonists.

4. Materials and Methods

4.1. Animals

Male (N = 25–26/line) and female (N = 28/line) msP and Wistar rats, bred at the animal facility of the University of Camerino, Italy, weighed 250–300 g (male) and 160–200 g (female) at the beginning of the experiments. Rats were housed three per cage in a temperature (20–22 °C) and humidity (45–50%) controlled room with a reverse 12 h light/dark cycle (lights off at 8 AM). During the entire residence in the facility, animals were offered free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy). Before the beginning of training, for three days rats were handled 5 min daily by the same operators who performed the experiments. Experiments were conducted during the dark phase of the light/dark cycle. All the procedures were conducted in adherence with the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Italian Ministry of Health approval 1D580.24.

4.2. Drugs

The alcohol drinking solution 10% (*v*/*v*) was prepared by diluting 95% alcohol (F.L.Carsetti, Camerino, Italy) with tap water. Saccharin (Sigma-Aldrich, Milan, Italy) was diluted to 0.2% (*w*/*v*) with tap water. The glucocorticoid and progesterone receptors antagonist mifepristone (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in propylene glycol (Sigma-Aldrich, Milan, Italy). Mifepristone was administered intraperitoneally (i.p.) at the doses of 0.0, 10, 30 and 60 mg/kg in a volume of 1 mL/kg, 90 min before tests. The selective glucocorticoid receptor antagonist CORT113176 (Corcept Therapeutics Incorporated, Menlo Park, CA, USA) was suspended in a vehicle containing 10% dimethylformamide (Sigma-Aldrich, Milano, Italy), 10% Cremophor EL (Sigma-Aldrich, Milano, Italy) and 80% saline. The drug was administered at the doses of 0.0, 10, 30 and 60 mg/kg (i.p). in a volume of 3 mL/kg, 90 min prior the test session. Drug doses were chosen based on published data [24,30].

4.3. Self-Administration Apparatus

Self-administration (SA) sessions were conducted in standard operant conditioning chambers (Med Associates, St Albans, VT, USA) enclosed in ventilated sound-attenuating cubicles. Each chamber was equipped with two retractable levers located in the front panel of the chamber with a drinking reservoir placed in between and connected with a syringe pump. A house-light was located on the wall opposite to the levers. Behavioral sessions were controlled and recorded by a windows compatible PC equipped with Med-PC-5 software (Med Associates).

4.4. Self-Administration Training

Animals were trained to self-administer 10% (*v*/*v*) alcohol or saccharin 0.2% (*w*/*v*) for five days a week, in 30 min daily sessions under a fixed-ratio 1 (FR1) schedule of reinforcement. Operant sessions started with lever insertion and ended with lever retraction. Responses at the right (active) lever were reinforced with 0.1 mL of fluid (alcohol or saccharin solution) delivered in the drinking reservoir. Rats were trained to alcohol SA using a saccharin-fading procedure [43]. Briefly, during the first five days of training, active lever responses were reinforced with 0.2% (*w*/*v*) saccharin. Next, 8% (*v*/*v*) alcohol was added to saccharin to familiarize rats with alcohol and then alcohol concentration was stepwise increased to 10% (*v*/*v*) and saccharin removed. Starting with alcohol 10% (*v*/*v*) SA, reinforcement delivery was followed by a 5 s time-out (TO), during which the house light was contingently illuminated. During the TO, active lever responses were recorded but not reinforced. Throughout the sessions, responses at the left (inactive) lever were recorded but had no scheduled consequences.

Drug treatments began once a stable SA baseline was established. Approximately three weeks (five SA sessions per week) were necessary to reach a stable baseline of responding.

4.5. Experiment 1.1: Effect of Mifepristone on Alcohol Self-Administration in Male and Female msP and Wistar Rats

On test days, male and female msP (N = 10/sex) and Wistar (N = 9–10/sex) rats were injected with mifepristone (10, 30 and 60 mg/kg, i.p.) or its vehicle 90 min before the SA session in a within-subject counterbalanced design. Tests were conducted every fourth day until each rat had received all doses of mifepristone. During the first of the three intervening days, rats remained in their home cage, whereas during the second and third days they performed baseline alcohol SA sessions.

4.6. Experiment 1.2: Effect of Mifepristone on Saccharin Self-Administration in Male and Female msP and Wistar Rats

This experiment was conducted on male and female msP (N = 6–8/sex) and Wistar (N = 7–8/sex) rats. The procedure was identical to experiment 1.1 except that the SA fluid was saccharin 0.2% (*w*/*v*).

4.7. Experiment 2.1 Effect of CORT113176 on Alcohol Self-Administration in Male and Female msP and Wistar Rats

This experiment was conducted on male and female msP (N = 9–10/sex) and Wistar (N = 10/sex) rats. The procedure was identical to experiment 1.1 except that the selective GR antagonist CORT113176 (0.0, 10, 30 and 60 mg/kg) was used.

4.8. Experiment 2.2: Effect of CORT113176 on Saccharin Self-Administration in Male and Female msP and Wistar Rats

This experiment was conducted on male and female msP (N = 9–10/sex) and Wistar (N = 9–10/sex) rats. The procedure was identical to experiment 2.1 except that the SA fluid was saccharin 0.2% (*w*/*v*).

4.9. Experiment 3: Blood Corticosterone Levels Following Alcohol Self-Administration in Male and Female msP and Wistar Rats

The effect of alcohol SA on blood corticosterone levels in male and female msP (N = 6/sex) and Wistar (N = 7–8/sex) rats was evaluated. Rats were trained to self-administer alcohol as described above. When a stable alcohol SA baseline was established, blood for corticosterone analysis was collected under a basal alcohol-free condition and immediately after the alcohol self-administration session. The experiment was conducted in a within-subject design and animals were subjected to two blood samplings, one under the basal condition and the other immediately after the self-administration session. At least three days passed between the two blood samplings and sampling order was counterbalanced. Blood was collected by tail nicking. The hypothalamic stress response induced by this sampling procedure is detectable after 3 min [44]; to avoid this confounding factor, we completed sampling within 2 min. Blood was sampled in lithium-heparinized tubes (Sars EDT, Nümbrecht, Germany). Samples were centrifuged at 1500× rcf for 10 min at 4 °C and plasma was collected, aliquoted and stored at −20 °C until further use. Plasma corticosterone levels were determined using enzyme-linked immunosorbent assay (ELISA) (RE52211, IBL International GmbH, Hamburg, Germany) following manufacturer instructions.

4.10. Statistical Analysis

All behavioral experiments were analyzed by three-way analysis of variance (ANOVA) with treatment as a repeated measure, and strain and sex as between-subject factors. Active and inactive lever responses were analyzed separately. Behavioral performances of each independent strain/sex group were further analyzed by one-way with factor ANOVAs with treatment as a repeated measure. ANOVAs were followed by Dunnet's post hoc analysis when appropriate. Significance was conventionally set at $p < 0.05$.

CORT ELISA standards were used to generate an optimalfit 4-parameter standard curve from which sample values were extrapolated. CORT data were analyzed via three-way ANOVA with condition (basal vs. alcohol condition) as the within-subject factor

and strain and sex as between-subject factors. Significant effects were explored with Newman-Keuls multiple comparison test. Significance was conventionally set at $p < 0.05$.

All statistical analyses were performed using GraphPad Prism v8.

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Lethal and behavioural effects of a green insecticide against an invasive polyphagous fruit fly pest and its safety to mammals

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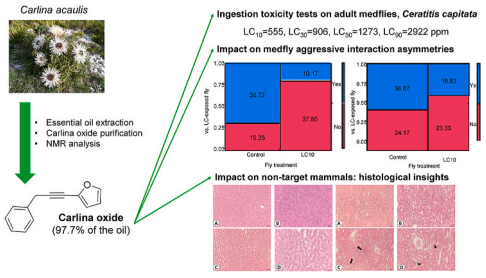
HIGHLIGHTS

- *Carlina acaulis* essential oil represents green insecticides for fruit fly control.
- Carlina oxide is the main constituent of the essential oil (>97%).
- Carlina oxide influences the aggression directionality of *C. capitata* adults.
- A "lure & kill" formulation based on carlina oxide has been developed against *C. capitata*.
- Oral toxicity tests on rats showed that the *C. acaulis* essential oil is safe for mammals.

GRAPHICAL ABSTRACT



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ABSTRACT

Plant essential oil-based insecticides, with special reference to those that may be obtained from largely available biomasses, represent a valuable tool for Integrated Pest Management. However, the sublethal effects and the potential effects on aggressive insect traits of these green insecticides are understudied. Herein, the lethal and sub-lethal effects of the carlina oxide, constituting more than 97% of the whole *Carlina acaulis* (Asteraceae) root essential oil (EO), were determined against an invasive polyphagous tephritid pest, *Ceratitis capitata* (medfly). The carlina oxide was formulated in a mucilaginous solution containing carboxymethylcellulose sodium salt, sucrose, and hydrolysed proteins, showing high ingestion toxicity on medfly adults. The behavioural effects of carlina oxide at LC_{10} and LC_{30} were evaluated on the medfly aggressive traits, which are crucial for securing reproductive success in both sexes. Insecticide exposure affected the directionality of aggressive actions, but not the aggression escalation intensity and duration. The EO safety to mammals was investigated by studying its acute toxicity on the stomach, liver, and kidney of rats after oral administration. Only the highest dose (1000 mg/kg) of the EO caused modest neurological signs and moderate effects on the stomach, liver, and kidney. The

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other doses, which are closer to the practical use of the EO when formulated in protein baits, did not cause side effects. Overall, *C. acaulis*-based products are effective and safe to non-target mammals, deserving further consideration for eco-friendly pesticide formulations.

1. Introduction

Growing concerns about climate change, biodiversity, animal welfare, and food security have pushed agriculture towards a more sustainable approach (Bijani et al., 2019; Karami et al., 2017; Valizadeh and Hayati, 2021). The overuse of synthetic insecticides affected human health (Singh et al., 2018), the environment (Cachada et al., 2016; Capowiez et al., 2005; Postigo et al., 2021; Senthil Rathi et al., 2021), especially non-target organisms (Bozhgani and Talebi, 2018; Forson and Storfer, 2006; Ricupero et al., 2020; Wu et al., 2018). In addition, synthetic insecticides are also responsible for the quick development of insecticide-resistant pest strains (Guillem-Amat et al., 2020; Hsu and Feng, 2006; Nwankwo, 2021; Horowitz et al., 2020). Given all the side effects of using synthetic insecticides, (Postigo et al., 2021; Singh et al., 2018; Wu et al., 2018), botanical pesticides, such as essential oils (EOs) and their main compounds, represent a valid alternative for the control of pests of agricultural interest (Benelli et al., 2021).

Polyacetylenes are a chemical class that includes all compounds with one or more carbon-carbon triple bond or alkynyl functional groups (Minto and Blacklock, 2008). These compounds are widely distributed and occurred in several botanical families among which Asteraceae, Apiaceae, Campanulaceae, and Pittosporaceae are the most representative ones (Negri, 2015). Polyacetylenes may act as phytoalexins, i.e., insect-induced defence compounds (Yactayo-Chang et al., 2020), and therefore they may represent an interesting prototype for the development of ecological insecticides. In particular, our research group recently focused on the insecticidal potential of carlina oxide (syn. 2-(3-phenylprop-1-ynyl)furan), an aromatic C_{13} polyacetylene bearing one carbon-carbon triple bond and a furan ring, which is the main component (>95%) of *Carlina acaulis* L. (Asteraceae) EO as well as other *Carlina* species (Mejdoub et al., 2020; Strzemski et al., 2017), and *Carthamus caeruleus* L. (Mami et al., 2020). The carlina oxide and its corresponding EOs, probably due to their phytotoxicity and ease of penetration into the insect cuticle (Champagne et al., 1986), are effective against a wide number of noxious insects, including mosquitoes (*Culex quinquefasciatus* Say), house flies (*Musca domestica* L.), tephritid flies (*Ceratitis capitata* (Wiedemann)), moths (*Lobesia botrana* (Denis & Schiffermüller)), and stored-product beetles (*Prostephanus truncatus* (Horn) and *Trogoderma granarium* Everts) (Benelli et al., 2019, 2020, 2021, 2020; Kavallieratos et al., 2020; Pavela et al., 2020).

To develop a proper pest management protocol, it is also essential to gain knowledge about the impact of EOs applied at low doses. Sublethal doses of botanical pesticides may impact the insect life span, development, sex ratio, fertility, fecundity, and behavioural traits (Lee, 2000). The large majority of sublethal effect research is focused on synthetic pesticides (Fernandes et al., 2016; Lin et al., 2020), while a lower number of efforts has been directed to understand the impact of new plant-borne insecticides (Izakmehri et al., 2013; Khosravi et al., 2010; Borzoui et al., 2016; Nouri-Ganbalani and Borzoui, 2017). Concerning the EO of *C. acaulis*, the exposure of *M. domestica* adults to a sublethal dose (LD_{30}) led to significant reductions in female longevity, fecundity, and F_1 vitality (Pavela et al., 2020). Recent studies investigated how exposure of *C. capitata* adults to *C. acaulis* essential oil also impacts insect intraspecific aggression dynamics (Benelli et al., 2021).

As a continuation of our studies on this interesting plant species, herein we evaluated the acute toxicity of the main compound of *C. acaulis* EO: carlina oxide (constituting >90% of the whole *C. acaulis* EO) towards adult of *C. capitata* in ingestion toxicity assays. Also known as medfly, *C. capitata* is a highly invasive polyphagous pest species causing both quantitative and qualitative damages to several crops (Schliserman et al., 2014). Besides its importance as a fruit pest, the medfly is a model organism for behavioural research, due to its complex aggressive and mating behaviour (Benelli et al., 2015a; Benelli and Romano, 2018; Briceño et al., 1999). Therefore, LC_{10} and LC_{30} of carlina oxide were evaluated for their potential impact on aggressive traits of medfly adults, which are crucial for securing reproductive success in both sexes (Benelli et al., 2015b). Lastly, to give new insights into the mammal safety of *C. acaulis* based products, herein we evaluated the acute toxicity of its EO, containing 97.7% of carlina oxide, on the stomach, liver, and kidney of rats after oral administration. These assays were performed with *C. acaulis* EO, for sake of practicality, yield, and costs related to carlina oxide purification. Our findings shed light on the possible utilization of the polyacetylene carlina oxide as an active ingredient to substitute insecticides in "attract and kill" formulations, and at the same time, we provided new important information about its mammal safety.

2. Materials and methods

2.1. Carlina acaulis EO extraction

Carlina acaulis dry roots were purchased from A. Minardi & Figli S.r.l. (Bagnacavallo, Ravenna, Italy, https://www.minardierbe.it). The roots were powdered with a grinder (Albrigi, Stallavena, Verona, Italy, mod. E0585) using a 1.5 mm sieve. One kg of the roots was soaked overnight in 6 L of distilled water into a 10 L Pyrex glass flask and hydrodistilled for 8 h with a Clevenger-type device, heated by a Falc MA mantle (Falc Instruments, Treviglio, Italy). The yellowish EO was isolated in 0.89% yield (w/w, dw), with a density of 1.063 g/mL.

2.2. Chemical analysis of C. acaulis EO and purification of carlina oxide

The chemical composition of the EO was investigated by an Agilent 6890 N gas chromatograph provided with a single quadrupole 5973 N mass spectrometer and an auto-sampler 7863 (Agilent, Wilmingotn, DE). The separation of EO chemical constituents was performed using an HP-5MS capillary column (30 m length, 0.25 mm i.d., 0.1 μm film thickness; 5% phenylmethylpolysiloxane) from Agilent (Folsom, CA, USA). The column was allowed to reach a temperature of 60 °C for 5 min, then of 220 °C at 4 °C/min, and finally of 280 °C at 11 °C/min held for 15 min. Injector and detector were thermostated at 280 °C. The mobile phase was constituted of 99.9% He, with a flow of 1 mL/min. Before the analysis, the EO was diluted 1:100 in *n*-hexane and then 2 μL were injected in split mode (1:50). Electron impact (EI, 70 eV) mode in the range of 29–400 *m/z* was used for peak acquisition. The analysis of chromatograms was performed using the MSD ChemStation software (Agilent, Version G1701DA D.01.00), while data analysis was performed using the NIST Mass Spectral Search Program for the NIST/EPA/NIH EI and NIST Tandem Mass Spectral Library v. 2.3. The identification of the EO components was achieved by the combination of the temperature programmed retention indices (RIs) and mass spectra (MS) in comparison with those of ADAMS, NIST 17, and FFNSC2 libraries (Adams, 2007; NIST, 2017; FFNSC, 2012). The RI were calculated using a mix of *n*-alkanes (C_8–C_{30}, Supelco, Bellefonte, CA, USA), according to the Van den Dool and Kratz (1963) formula. The EO was mainly characterized by carlina oxide (97.7%) and two minor components such as benzaldehyde (1.14%) and *ar*-curcumene (0.29%). The total of identified compounds was 99.13%. The EO (1.41 g) was chromatographed by silica gel column chromatography (70–230 mesh, 60 Å, Merck) using 100% of *n*-hexane as mobile phase, yielding 1.34 g of pure carlina oxide. NMR analysis

confirmed its chemical structure, through a Bruker Avance 400 Ultrashield spectrometer using tetramethyl silane (TMS) as an internal standard. The NMR spectrum was linear with the literature (Benelli et al., 2019).

2.3. Carlina oxide ingestion toxicity on medflies

C. capitata medflies were from a mass-rearing of the University of Pisa; they were reared as detailed by Canale and Benelli (2012) at 25 ± 1 °C and 45% R.H., 16:8 (L:D) photoperiod. Following Benelli et al. (2012, 2021), ingestion toxicity was evaluated on 30 medfly adults (both males and females), placing them in a plastic container (600 mL) covered with a thin mesh. *C. capitata* adults were fed for 96 h with 2 mL of viscous formulation containing 0.0039, 0.0078, 0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1% of carlina oxide. The viscous formulation was obtained emulsifying carlina oxide with dimethyl sulfoxide (DMSO) (1:1), then adding 2% of carboxymethylcellulose Na salt, 12.5% of sucrose, and 1% of protein bait (NuBait, Biogard, Italy). The formulation was provided in a bakelite cup (∅ = 30 mm). For the prevention of medfly drowning, a cotton disk (∅ = 30 mm) was used to cover the cup. The negative control for each test was performed testing the viscous carrier without carlina oxide.

During the experiments, medfly mortality was noted after 96 h. No less than 4 replicates were performed per carlina oxide concentration, over different days, under laboratory conditions (21 ± 1 °C, 45 ± 10% R.H., 16:8 (L:D) photoperiod).

2.4. Analysis of medfly aggressive behaviour

Carlina oxide at the LC_{10} and LC_{30} estimated on adult medflies as described above was evaluated for its potential impact on *C. capitata* aggressive behaviour, following the methodology recently proposed by Benelli et al. (2021). Either the mucilage with carlina oxide or the negative control was prepared and administered as described in paragraph 2.3. Prior observation, medfly sorted by sex were fed on the viscous formulation for 96 h, and the potential impact of feeding on carlina oxide on the fly aggressiveness was observed till 4 days post-feeding.

The behavioural assays were performed inside an arena (i.e., a plastic container, volume: 600 mL) covered by a piece of glass, where a *Citrus limon* (L.) Osbeck twig or leaf (~15 cm, 1 leaf/twig) was placed. Trials on a given LC and fly sex were repeated on 30 groups composed of 4 *C. capitata* adults, two earlier were fed on the mucilage containing carlina oxide, while the remaining were fed with the viscous control carrier. Each replicate lasted 30 min, with an initial adaptation phase of 10 min. At the end of each replica, the glass and the plastic container were washed with soap and hot water, new specimens and lemon twig were used. An aggression event occurred when a *C. capitata* adult (treated or control) approached the other and displayed an escalating aggressive behaviour as reported by Benelli et al. (2015a). The intensity of the aggression event was recorded in terms of aggression score, both during ♂-♂ and ♀-♀ aggressive interactions. The above-mentioned aggression score ranges from 0 to 12 and includes the following events: 1. Avoidance (one). 2. Wing waving (one). 3. Wing waving (both). 4. Chasing. 5. Fast head rocking. 6. Pouncing. 7. Labellar (one). 8. Labellar (both). 9. Wing strike. 10. Dive. 11. Boxing (one). 12. Boxing (both). Each event is defined in detail in Benelli et al. (2015a). We also noted which fly (i.e., control or treated) started attacking a conspecific, as well as the number of aggressive events per sex, and the length of each aggressive event (Benelli et al., 2021). Experiments were conducted from May to October 2020 in a room illuminated with fluorescent daylight tubes (Philips 30 W/33) (16:8 (L:D) photoperiod).

2.5. Non-target toxicity on rats

To have enough amount of sample to perform *in vivo* toxicological assays, we decided to test the *C. acaulis* EO containing 97.7% of carlina oxide instead of the purified compound (99%) which requires additional costs in terms of devices for chromatographic separations and consumption of organic solvent with relative waste disposal.

2.5.1. Animals

For the acute toxicity studies female Wistar rats weighting 250–300 g were employed. Each animal was single caged and kept in cycles of 12 h of the dark followed by 12 h of light at 20–22 °C and R.H. 44–45%. Water and food were available ad libitum. Housing and experiments were carried out following the guidelines of the European Community Council Directive Care and Use of Laboratory Animals (Ministry of Health Authorization n° 1D580.22).

2.5.2. Acute toxicity procedures

The doses of the *C. acaulis* EO were constituted by the EO dissolved in 2% Tween 80 vehicle. The administration was performed by gavage. For the acute toxicity studies, the animals were divided into four groups, each composed of four individuals. The first group received the vehicle; the second group 250 mg of *C. acaulis* EO per kg by oral administration; the third group 500 mg/kg; the fourth group 1000 mg/kg. After the oral administration, possible signs of toxicity were observed in each animal for the first 30 min, then periodically, for the remaining 48 h until the harvesting of the tissues. When occurring, the time of death was registered. Signs of toxicity noted focused on central nervous and autonomic system activities and were convulsions, tremors, ataxia, straub, ptosis, coma, cyanosis, lacrimation, piloerection, and salivation. 48 h after the dosing, the weight of each rat was recorded, and the rats were sacrificed. The organs were surgically removed, and their weight and characteristics were observed. Finally, the organs were placed in a fixative solution or frozen at – 80 °C.

The stomach, the liver, and the kidneys were removed, separated into small pieces, and fixed in Bouin's solution for 6 h. After fixation, samples were dehydrated in gradual ethanol from 70% to absolute and cleared in xylene for the paraffin embedding. 5 μm consecutive sections were stained with haematoxylin and eosin dye (H&E), observed under a light microscope Leica DMR (Germany) connected by a DS-R12 Nikon camera to the computer and estimated using a NIS Elements Nikon image analyser software. Sections were blindly analysed; at the level of the stomach, the presence of inflammatory aggregate, and the presence of ulcer and elements of necrosis were evaluated. In the sections of the liver, the following parameters were evaluated: inflammatory elements, degeneration of hepatocytes, vacuolization, presence of apoptotic cells or hepatic necrosis, dilated sinusoids. Signs of infiltration, glomerular and tubular alterations were examined in the kidney.

2.6. Data analysis

In *C. capitata* ingestion toxicity assays, control mortality values ranging from 1 to 20% were adopted to accurate experimental mortality rates through Abbott's formula (Abbott, 1925). Ingestion LC_{10}, LC_{30}, LC_{50}, and LC_{90} with associated 95% confidence intervals (CI), χ^2 values, and *p*-values were assessed using probit analysis (Finney, 1978).

Following Benelli et al. (2021), differences in the abundance of *C. capitata* adults, showing aggressive displays after feeding on a given carlina oxide LC, were analysed using a likelihood chi-square test with Yates' correction (Rohlf and Sokal, 1981). Differences among the aggression duration values characterizing carlina oxide-exposed or control *C. capitata* adults were analysed through a weighted generalized linear model (GLZ) described by Benelli et al. (2015a, 2021), i.e., $y = X\beta + \varepsilon$, where y is the vector of the observation (i.e., aggression duration), X is the incidence matrix, β is the vector of the fixed effect (i.e., exposure to a given carlina oxide LC), and ε is the vector of the random residual effects ($\alpha = 0.05$). Differences among the aggression scores characterizing carlina oxide-exposed or control *C. capitata* adults were assessed through the Kruskal-Wallis test ($p = 0.05$). JMP® 9 (SAS) and Minitab

Inc., State College, PA were used for these analyses.

In mammal safety experiments, the rat body weight data were analysed by one-way ANOVA as the main effects. When appropriate, Tukey's multiple test was used as post-hoc test ($\alpha = 0.05$). GraphPad Prism 8 software (San Diego, CA) was used for analysing these data.

3. Results

3.1. Carlina oxide ingestion toxicity on medfly

The toxicity of carlina oxide obtained in *C. capitata* adults was estimated by probit analysis and evaluated at different concentrations. Dose-response bioassays showed that the LC values of the carlina oxide were: $LC_{10} = 555$ ppm, $LC_{30} = 906$ ppm, $LC_{50} = 1273$ ppm, and $LC_{90} = 2922$ ppm. Overall, the carlina oxide was highly effective and proved to be a good candidate as an active ingredient in the "attract and kill" formulation tested adult medflies (Table 1).

3.2. Analysis of the medfly aggressive behaviour

3.2.1. Number of aggressive events

The potential impact of feeding on carlina oxide was firstly examined on *C. capitata* aggressive behaviour at a population level, i.e., impact on the overall abundance of medfly adults displaying aggressive behaviour, regardless of the intensity and length of the events. As a general trend, both male and female medflies fed on carlina oxide-based viscous formulations showed an aggressiveness comparable to control individuals, except for females exposed to LC_{10} (Fig. 1). Indeed, LC_{10}-fed females showed a significant difference in number of aggressive events, if compared with the control ones (47 vs. 73 aggressive events, respectively, $\chi^2 = 5.642$, *d.f.* $= 1$, $p = 0.017$), while the number of aggressive events performed by LC_{10}-fed males (85 vs. 92 aggressive events, $\chi^2 = 0.282$, *d.f.* $= 1$, $p = 0.598$), LC_{30}-fed males (86 vs. 94 aggressive events, $\chi^2 = 0.361$, *d.f.* $= 1$, $p = 0.551$), and LC_{30}-fed females (55 vs. 70 aggressive events, $\chi^2 = 1.808$, *d.f.* $= 1$, $p = 0.179$) did not differ from the respective controls (Fig. 1). Overall, control medflies performed a higher number of aggressive actions compared to medflies exposed to the carlina oxide, but a significant difference was found only between LC_{10}-fed females and control flies.

3.3. Asymmetries in aggressive interactions

Herein, we evaluated the directionality of the aggressions to discriminate between medflies carrying out the aggression and those suffering it. The tested LC significantly influenced the directionality of aggressive actions. Both medfly sexes fed on carlina oxide LC_{30} were more attacked by control flies (♂: $\chi^2 = 26.945$, *d.f.* $= 1$, $p < 0.0001$; ♀: $\chi^2 = 24.890$, *d.f.* $= 1$, $p < 0.0001$) (Fig. 2). Similarly, medflies fed on carlina oxide LC_{10} were more attacked by control flies (♂: $\chi^2 = 43.427$, *d.f.* $= 1$, $p < 0.0001$; ♀: $\chi^2 = 4.517$, *d.f.* $= 1$, $p = 0.033$) (Fig. 2). Overall, the exposure to both concentrations of the tested carlina oxide seemed to affect the willingness to receive attacks from control flies by the treated flies.

Table 1
Ingestion toxicity of carlina oxide in proteic baits on *Ceratitis capitata* adults.

Tested product	LC_{10} (ppm) (95% CI)	LC_{30} (ppm) (95% CI)	LC_{50} (ppm) (95% CI)	LC_{90} (ppm) (95% CI)	χ^2, (*df*), *p*-value
Carlina oxide	555 (394–698)	906 (725–1071)	1273 (1078–1480)	2922 (2413–3839)	5.860, (7), $p = 0.556$ ns

LC = lethal concentration.
95% CI = lower and upper limits of the 95% confidence interval.
ns = not significant ($p > 0.05$).

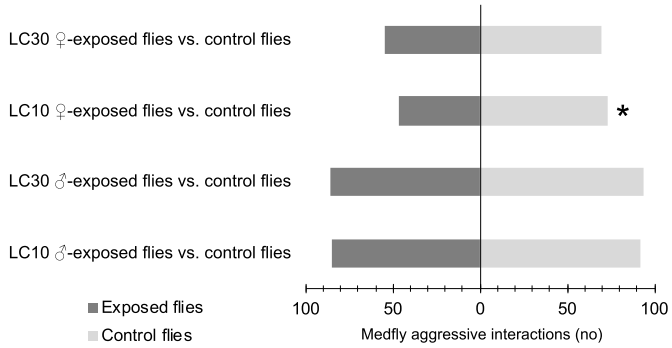

Fig. 1. Overall abundance of aggressive events performed by *Ceratitis capitata* adults fed on LC_{10} and LC_{30} of carlina oxide *vs.* control flies. The asterisk shows a significant difference over the control (χ^2 test with Yates' correction, $p < 0.05$).

3.4. Sex of the fighting flies

Aggressive interactions might vary according to the sex of the involved fly. Contingency analysis carried out between the fly treatment, i.e, LC-fed or LC-unfed fly, and the sex showed no significant differences between sexes for both sub-lethal doses (LC_{10}: $\chi^2 = 2.272$, *d.f.* $= 1$, $p = 0.131$; LC_{30}: $\chi^2 = 0.424$, *d.f.* $= 1$, $p = 0.515$) (Fig. 3), highlighting that there is no difference in terms of aggressiveness between males and females of *C. capitata*.

3.4.1. Aggression score

Ceratitis capitata fights are escalating and highly ritualized. Relying on the aggression score described in the Materials and Methods section, herein we quantified the intensity of the aggressions carried out by medflies. As regards aggression scores, no differences were found between *C. capitata* fed on distinct carlina oxide concentrations and the control insects (males fed on LC_{10}: $\chi^2 = 1.670$, *d.f.* $= 1$, $p = 0.196$; males fed on LC_{30}: $\chi^2 = 0.585$, *d.f.* $= 1$, $p = 0.444$; females fed on LC_{10}: $\chi^2 = 0.097$, *d.f.* $= 1$, $p = 0.754$; *female fed on* LC_{30}: $\chi^2 = 1.670$, *d.f.* $= 1$, $p = 0.196$) (Fig. 4). The carlina oxide did not influence the aggression score of exposed medflies.

3.4.2. Aggression duration

As for the aggression score, also the overall duration of the aggression was not affected by the ingestion of the carlina oxide. Indeed, our results showed that there was no significant difference in terms of aggression duration between *C. capitata* adults fed on carlina oxide and control flies (males fed on LC_{10}: $\chi^2 = 1.226$, *d.f.* $= 1$, $p = 0.268$; males fed on LC_{30}: $\chi^2 = 0.0076$, *d.f.* $= 1$, $p = 0.930$; females fed on LC_{10}: $\chi^2 = 0.482$, *d.f.* $= 1$, $p = 0.487$; females fed on LC_{30}: $\chi^2 = 0.419$, *d.f.* $= 1$, $p = 0.517$) (Fig. 5). The duration of an individual aggressive event ranged from a few seconds to a maximum of 30 s, i.e., wing waving.

3.5. Non-target acute toxicity on rats

Signs of toxicity consisting of tremors, sedation, ataxia, and ptosis were observed in the rat group administered with the dose of 1000 mg/kg which was slightly lower than the LD_{50} previously determined (Pavela et al., 2021). No evident signs of toxicity were instead observed for the lower doses. No significant alterations in the body weight and the organ's weight were evident after the acute administration of the different doses of the *C. acaulis* EO, even if a non-statistically significant decrease in the body weight was noticeable in animals treated with the highest dose (Table 2).

In the gross anatomy evaluation of the inner wall of the stomach, an area of hyperaemia with a sign of necrosis was evident at the level of the

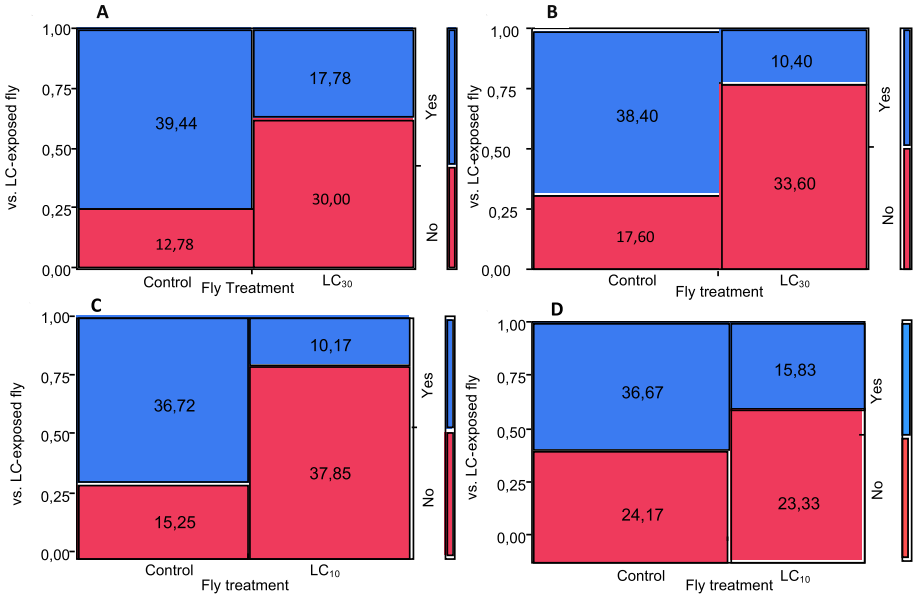

Fig. 2. Mosaic diagram of the aggressions in *Ceratitis capitata* adults after being fed on carlina oxide. The bar on the right (yes/no) shows the directionality of the action, i.e., whether it has been directed or not towards the target subject, namely: (A) males fed on carlina oxide LC_{30}; (B) females fed on carlina oxide LC_{30}; (C) males fed on carlina oxide LC_{10}; (D) females fed on carlina oxide LC_{10}. The value within each mosaic tile specifies the percentage of aggressions. The size of each various mosaic tile varies according to the number of individuals who have shown aggressive behaviour.

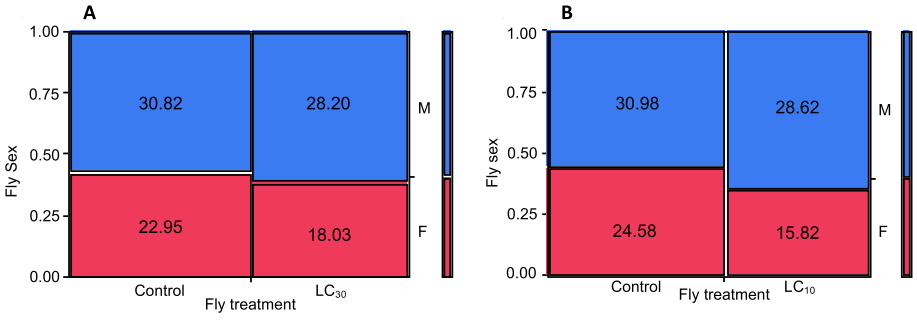

Fig. 3. Mosaic diagram of the aggressions in *Ceratitis capitata* adult males and females feeding on carlina oxide: (A) adults fed on LC_{30} of carlina oxide (B) adults fed on LC_{10} of carlina oxide. The bars on the right denote the percentage of males and females out of the total number of individuals tested. The numbers inside each box show the percentage of aggressions based on gender (red = female; blue = male). The size of each mosaic tile varies according to the number of adults that have shown aggressive behaviour. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

fundus, in animals treated with the highest dose of *C. acaulis* EO (Fig. 6). No evident signs of damage were present in the wall of the stomach of the animals of the other experimental groups (data not shown). Analysis of 5 μm sections of the organs stained with H&E allowed for comparison of structures and evaluation of the presence of damages resulting from the exposure to the EO. The stomachs of rats of groups "vehicle" and the rats treated with 250 and 500 mg/kg of *C. acaulis* EO did not show any significant damage (data not shown). The stomach of two out of four rats treated with 1000 mg/kg of *C. acaulis* EO showed mucosae necrosis (Fig. 6), especially at the level of the fundus. The morphological analysis at the level of gastric mucosae of the body revealed no morphological alterations in the animal treated with vehicle or 250 mg/kg and 500 mg/kg of *C. acaulis* EO (data not shown). Ulcers with the presence of inflammatory elements were evident in the animals treated with *C. acaulis* EO at the highest dose (Fig. 6). In the gross anatomical analysis of the liver, the hepatic parenchyma was not macroscopically affected by the different doses of *C. acaulis* EO, and we observed the occurrence of the normal capsule made of dense connective tissue (data not shown). The liver of the animals treated with an acute dose of 250 and 500 mg/kg of *C. acaulis* EO did not reveal damage in the parenchyma (data not shown). Differently in the liver of animals treated with the highest dose (1000 mg/kg) were evident signs of the dilated sinusoid, vacuolations in a perivascular central zone, without inflammatory aggregate, degenerated hepatic cord, and apoptotic cells (Fig. 7).

Finally, the kidney was histologically investigated in micrometers sections stained with haematoxylin and eosin. The section of the renal cortex showed, even at low magnification, normal renal corpuscles, and convoluted tubules (Fig. 8A and B). The images of kidney sections from an animal treated with 1000 mg/kg of *C. acaulis* EO showed that alterations of the renal interstitium were oedematous even with a lack of mononucleate cellular infiltrates such as macrophages and lymphocytes. Glomeruli were normal, but in some elements, capillary and Bowman's spaces were more dilated (arrowheads in Fig. 8D). The *C. acaulis* EO at the dose of 500 and 1000 mg/kg induced an increase of the luminal diameter of renal tubules compared with control ones, which was more evident with the highest dose in all animals (arrow in Fig. 8C).

4. Discussion

Several studies have been carried out to propose novel plant-borne insecticides effective against medfly adults, and various routes of applications of botanicals have been attempted, including contact, fumigation, and ingestion toxicity. For example, topical applications of *Melaleuca alternifolia* (Maiden and Betche) Cheel (Myrtaceae) EO were toxic to medfly, even at 0.117 μL/cm^2 (Benelli et al., 2013). *Baccharis spartioides* (Hook. and Arn. ex DC.) Remy (Asteraceae) and *Schinus polygama* (Cav.) Cabrera (Anacardiaceae) EOs also obtained a good level of toxicity when applied at 10–22 μg/fly (Barud et al., 2014). *B. spartioides* and *S. polygama* showed toxicity comparable to *Tagetes* species EOs (i.e., $LC_{50} \leq 20$ μg/fly) (López et al., 2011), as well as *Baccharis darwinii* Hook. & Arn. (Asteraceae) (i.e., $LC_{50} < 31$ μg/fly) (Kurdelas et al., 2012) and *Azorella cryptantha* (Clos) Reiche (Apiaceae) (i.e., $LC_{50} = 11$ μg/fly) (López et al., 2012).

While the impact of EO fumigation efficacy against a frugivorous pest

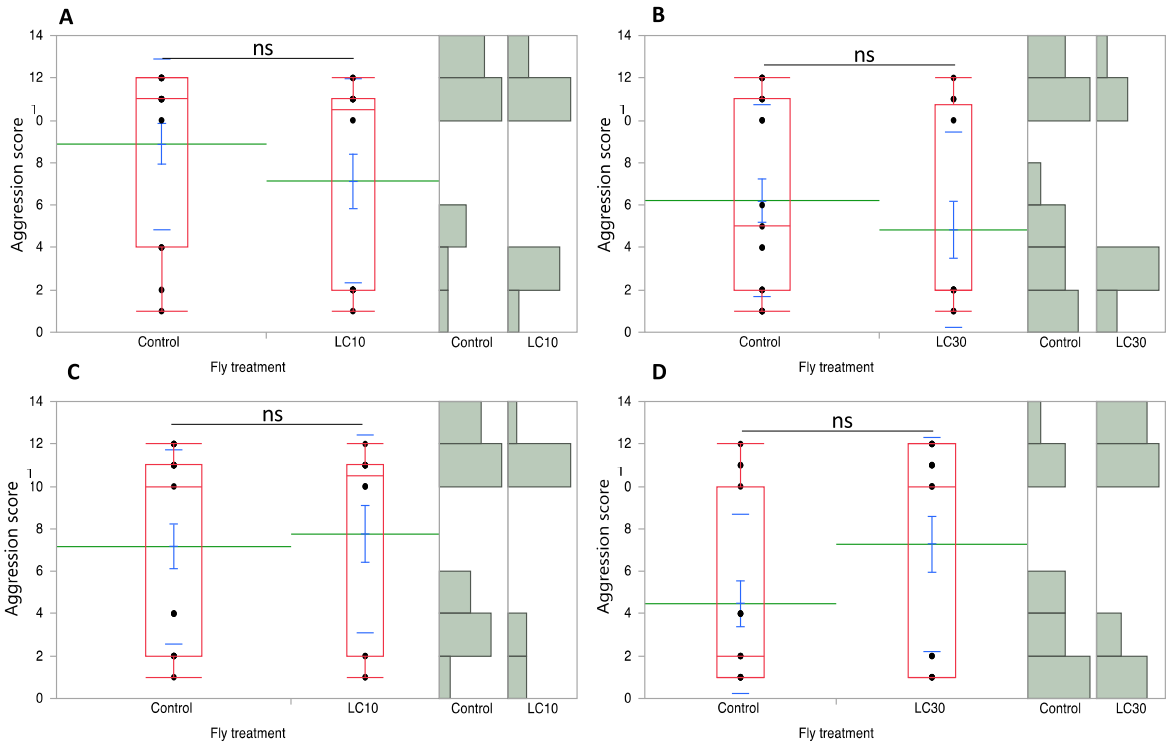

Fig. 4. Aggression score of *Ceratitis capitata* males and females fed or not to LC_{10} and LC_{30} of carlina oxide (A) male fed on LC_{10}, (B) male fed on LC_{30}, (C) female fed on LC_{10}, (D) female fed on LC_{30}. Each box plot indicates the median (red line) and its dispersion range (lower, upper quartile and extreme values, outliers). The mean is indicated by a green line, the standard error is a blue T-bar; ns = not significant (Kruskal-Wallis test, $p > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

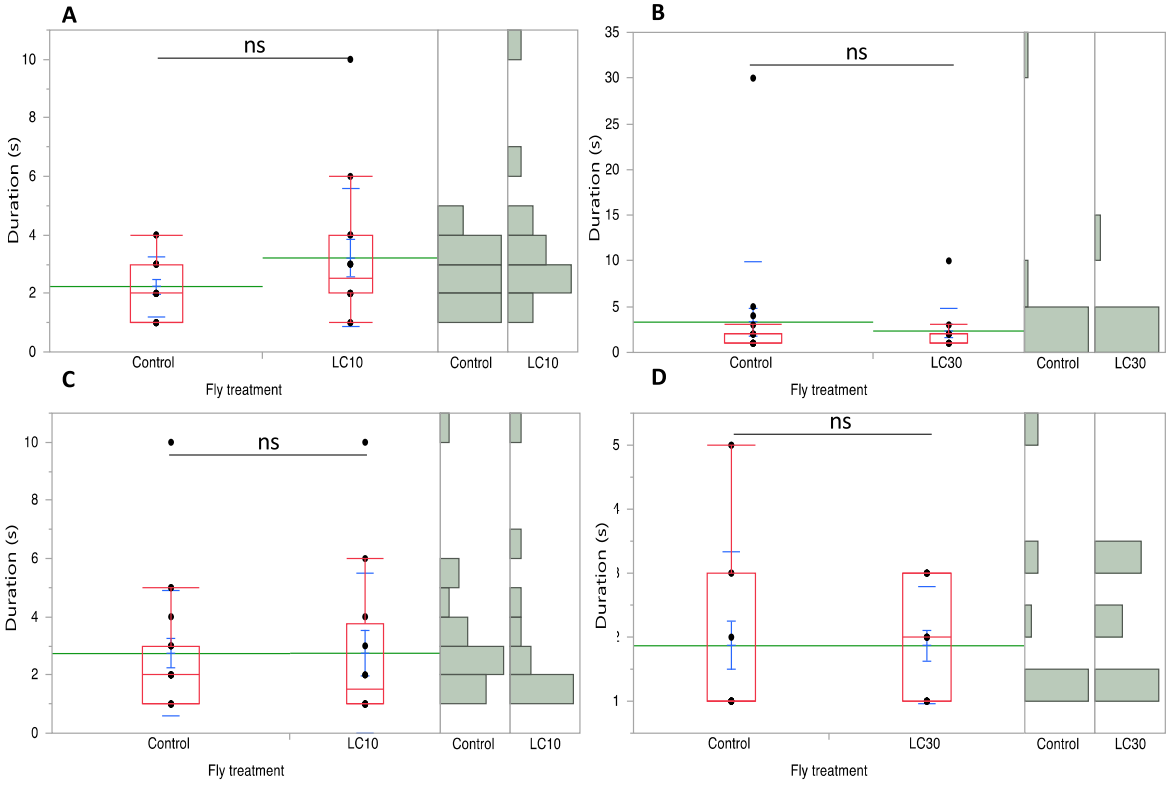

Fig. 5. Aggression duration in *Ceratitis capitata* males and females fed or not to LC_{10} and LC_{30} of carlina oxide: (A) males fed on LC_{10}, (B) males fed on LC_{30}, (C) females fed on LC_{10}, (D) females fed on LC_{30}. Each box plot indicates the median (red line) and its dispersion range (lower, upper quartile and extreme values, outliers). The mean is indicated with a green line, the standard error is a blue T-bar. ns = not significant (Kruskal-Wallis test, $p > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Non-target toxicity of *Carlina acaulis* essential oil on rats: body and organs weight in the different experimental groups.

Dose	Body weight		Liver weight	Kidney weight
	0 h	48 h	48 h	48 h
1000 mg/kg	223.25 ± 6.22	211.75 ± 8.68	10.58 ± 0.51	0.96 ± 0.04
500 mg/kg	212.50 ± 9.00	219.75 ± 11.02	11.70 ± 0.94	0.93 ± 0.05
250 mg/kg	237.50 ± 13.17	241.5 ± 15.19	13.52 ± 1.04	0.93 ± 0.06
Vehicle	223.75 ± 6.91	231.5 ± 10.07	10.13 ± 0.76	0.87 ± 0.02
		0 h *vs.* 48 h $F_{7,24} = 1.096$ $p = 0.396$	$F_{3,12} = 3.244$ $p = 0.060$	$F_{3,12} = 0.7463$ $p = 0.545$

Time 0: weight at the time of the essential oil administration. Time 48 h: weight at the moment of the sacrifice. Data are the mean ± SE. No significant differences between experimental groups were noted ($p > 0.05$).

like the medfly is of limited interest and can be disregarded, replacing synthetic insecticides with eco-friendly molecules in "attract and kill" programs is a valuable goal for modern tephritid research (Scolari et al., 2021). In this framework, the insecticidal efficacy of *C. acaulis* EO on *C. capitata*, has been recently highlighted by Benelli et al. (2021). The study showed high ingestion toxicity in both sexes. The EO, formulated in protein baits, showed an LC_{50} value of 1094 ppm, which is markedly lower if compared with the LC_{50} estimated for other EOs tested against the medfly, such as *Lavandula angustifolia* Mill. (Lamiaceae), *Hyptis suaveolens* (L.). Poit. (Lamiaceae), *Thuja occidentalis* L. (Cupressaceae), and *Rosmarinus officinalis* L. (Lamiaceae), all showing LC_{50} ranging from 3664 ppm (*R. officinalis*) to 13,041 ppm (*H. suaveolens*) (Benelli et al., 2012). The relevant insecticidal activity of *C. acaulis* EO seems to be linked to carlina oxide (>97% of the EO). Of note, carlina oxide has been recently reported as a highly effective insecticide on other key Diptera species, such as *C. quinquefasciatus* ($LC_{50} = 1.39$ μg mL $^{-1}$), and its mode of action seems to be moderately related to acetylcholinesterase (AChE) inhibition (Benelli et al., 2019). In the present study, carlina oxide showed a very promising LC_{50} (1273 ppm) against adult of medflies, outlining that the overall toxicity of *C. acaulis* EO is mostly linked to the bioactivity of this major constituent. Polyacetylenes, as carlina oxide, have insecticide, fungicide, and nematicide properties (Gorman et al., 1993). Their toxicity can be linked to several modes of action dictated by environmental conditions. Aromatic polyacetylenes, such as carlina oxide, can lead to phototoxicity in insects (Arnason and Bernards, 2010; Konovalov, 2014). In the absence of light, polyacetylenes are anti-feedant to insects, while in the presence of light the toxicity may be caused by a photocatalytic cycle of single oxygen generation and another excited state molecule that leads to rapid lipid peroxidation and cell death (Haouas et al., 2011). Furthermore, several polyacetylenes are responsible for modulating the gamma-aminobutyric acid-A (GABA-A) receptors (Lin et al., 2016). The compounds binding to GABA receptors related to chloride channels located on the membrane of postsynaptic neurons disrupt the functioning of the GABA synapse (Pavela and Benelli, 2016).

As reported by Benelli et al. (2019), the carlina oxide toxicity is partially attributable to cholinergic system blockage by AChE inhibition, which mediates nerve transmission splitting acetylcholine into choline and acetic acid. The insect dies of acetylcholine accumulation in the synaptic space (Boison, 2007). Furthermore, the high lipophilicity of carlina oxide leads to an easy entrance into the insect body (Benelli et al., 2019).

The aggressive behaviour of *C. capitata* plays a key role in routing the reproductive success of this species (Benelli et al., 2014, 2015a, b). The research carried out by Benelli et al. (2021) and this study, considered the influence of *C. acaulis* EO and carlina oxide, respectively, at low concentrations on the medfly aggressive behaviour dynamics. Our results showed a significant impact of feeding on the carlina oxide on the directionality of aggressive actions; at both tested concentrations (LC_{10} and LC_{30}), medfly males and females have received more aggressions by control flies, at variance with the results obtained testing the *C. acaulis* EO (Benelli et al., 2021). Surprisingly, our study displayed a substantial decrease of medfly aggressive interactions at the population level only for females fed on LC_{10} vs. control flies. However, by testing the whole EO an important reduction of medfly aggressive interaction at the population level was noted, along with a shorter time of aggressive events, in medflies treated with both EO LC_{10} and LC_{30} (Benelli et al., 2021). The differences exposed above may be due to a synergic action of the other minor components of EO, with special reference to benzaldehyde (1.14%) and *ar*-curcumene (0.29%), as synergistic and antagonistic interactions between EO constituents have been reported in earlier studies (Benelli et al., 2017a, b; Yuan et al., 2019). The two minor constituents mentioned above have insecticidal activity and can also act

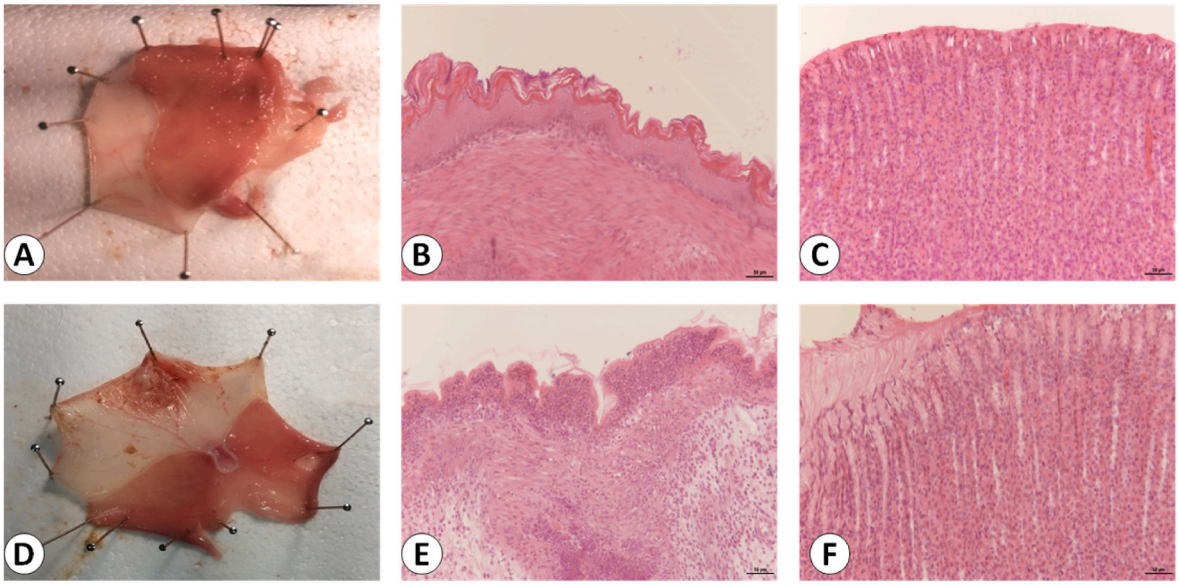

Fig. 6. Inner wall of the stomach (A, D) and sections staining with haematoxylin and eosin at the levels of the fundus (B, E) and body (C, F) of animal treated with vehicle (A–C) and *Carlina acaulis* essential oil (D, E, F) at the dose of 1000 mg/kg. B, C, E, F: calibration bar 50 μm (magnification 20X).

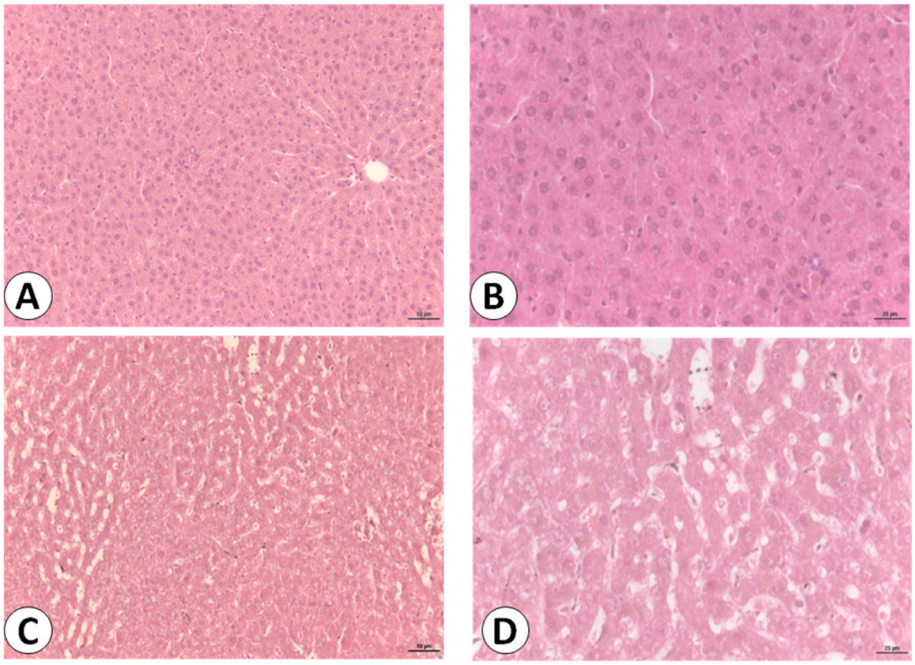

Fig. 7. Sections of the liver stained with haematoxylin and eosin of animal treated with vehicle (A, B) and 1000 mg/kg of *Carlina acaulis* essential oil (C,D). A, C: calibration bar 50 μm (magnification 20X). B, D: calibration bar 25 μm (magnification 40X).

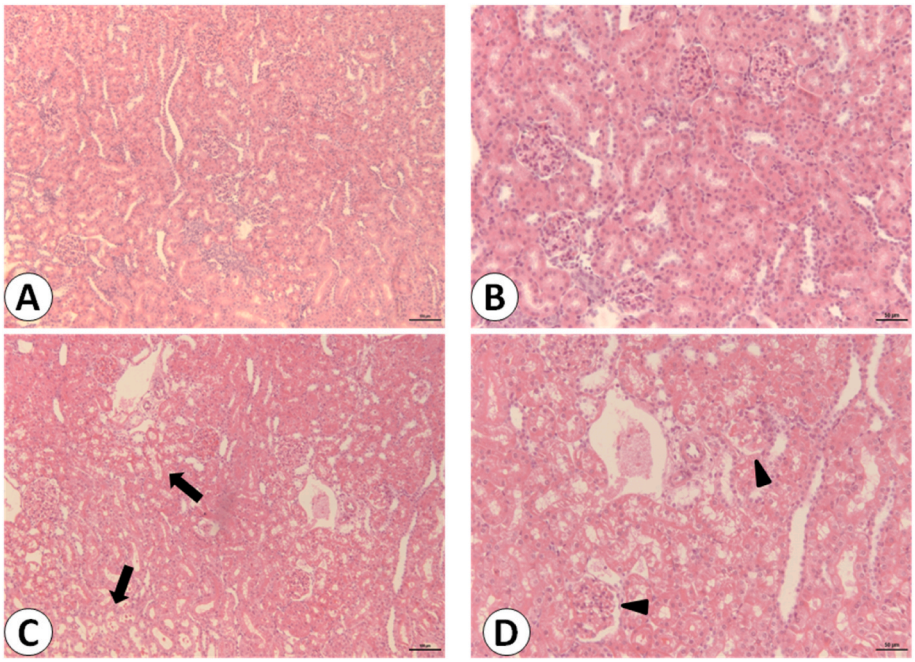

Fig. 8. Sections of the cortical layer of kidney stained with haematoxylin and eosin of animal treated with vehicle (A, B) and 1000 mg/kg of *Carlina acaulis* essential oil (C, D). A, C: calibration bar 100 μm (magnification 10X). B, D: calibration bar 50 μm (magnification 20X).

as carrier agents for carlina oxide to penetrate better into the insect cuticle. Indeed, as reported by Alshebly et al. (2016), *Hedychium larsenii* M. Dan & C. Sathish Kumar (Zingiberaceae) EO and its main components, *ar*-curcumene and epi-*β*-bisabolol showed sublethal effects on *Anopheles stephensi* Liston (Diptera: Culicidae), *Aedes aegypti* L. (Diptera: Culicidae) and *C. quinquefasciatus*. Low concentrations of *ar*-curcumene and epi-*β*-bisabolol negatively influenced the oviposition of the three species tested. Another study, carried out by Nattudurai et al. (2012), showed how the exposition of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) to low concentration (i.e., LC_{10}, LC_{20}, and LC_{30}) of benzaldehyde reduced the female fecundity (i.e., from 4.7 to 0.92 eggs/female). Further research on the bioactivity of these two molecules against adult medflies is required, with a special focus on synergistic toxicity tests.

Concerning mammal toxicity, changes in body and organs weight are valuable indicators of toxicity (Michael et al., 2007; OECD, 2008). Herein, we did not observe any change in the weights of the liver and kidney of rats due to the different dosages of *C. acaulis* EO. Similarly, the total body weight was unaffected by the treatments. Thus, our results showed that the treatment with the EO does not affect the body weight.

However, it should be considered that we carried out an acute administration, and the time of observation was limited. A trend in the reduction of body weight was observed after 48 h in the animals with the highest dosage compared with the vehicle and the lower doses. Symptoms of neurological toxicity were observed only after acute oral administration of the highest dose (1000 mg/kg), a little lower than the LD_{50}. Increasing signs of sedation, ataxia, ptosis, and tremors were detected over the 48 h of observation after the administration, indicating that the main component of the EO impacts the central nervous system. Neurotoxicity is not uncommon among the EOs that can pass the brain-blood barrier without difficulty due to their lipophilicity. For instance, it is well known that thujone can cause convulsion and excitation (PMID: 23201408). Similarly, 1,8-cineole and camphor, which are abundant compounds in the EO of eucalyptus and rosemary, are capable of inducing seizures (PMID: 19893077). These effects are probably due to the modulation of the GABAergic system. On the other hand, no signs of neurological toxicity were observed at the lower doses, suggesting a significant safe profile of *C. acaulis* EO since its dosage in the insecticidal formulations would be much lower than the ones tested here.

The assessment of histopathological alterations in organs represents one of the basic tests for the assessment safety of tested materials (Greaves, 2011). No abnormality was observed on gross anatomy evaluations of organs examined in this study. The histopathological findings on the liver, the stomach, and the kidney indicated a moderately toxic effect of *C. acaulis* EO, at the dose of 1000 mg/kg. On the other hand, the dose of 500 mg/kg did not cause any toxicity in the rats. Based on our evidence, the *C. acaulis* EO seemed to be slightly toxic to rats, with LD_{50} overlapping those of other industrially important EO elements such as thymol and cinnamaldehyde (Pavela and Benelli, 2016). Therefore the *in vivo* toxicological study reveals that the EO has low oral toxicity. Nevertheless, it should be appropriate to avoid high dosages to prevent possible harmful effects. The LD_{50} of *C. acaulis* EO was higher when compared with that of plant extracts containing polyacetylenes as active compounds. Polyacetylenes are widely distributed among the families Apiaceae, Araliaceae, and Asteraceae, and some of them showed antibacterial, antifungal, anti-inflammatory, anticancer, and antiplatelet aggregation properties (Christensen and Brandt, 2006; Hinds et al., 2017; Zaini et al., 2012). Some of these compounds have been considered undesirable due to their toxic properties. A study on the toxicity of *Bupleurum longiradiatum* Turcz. (Apiaceae) displayed that the CH_2Cl_2 fraction and the ethanol extract exhibited high toxicity, with LD_{50} values of 37.5 mg/kg (95% CI: 32.8–42.9 mg/kg) and 77.7 mg/kg (95% CI: 67.7–89.3 mg/kg), respectively, and toxicity correlated to the amount of polyacetylenes in this plant (You et al., 2002).

5. Conclusions

The chief contribution of carlina oxide to the overall bioactivity of *C. acaulis* EO for the development of "attract and kill" tools has been outlined in the present study. Concerning *C. acaulis* EO and carlina oxide, marked differences have been found about their impact through ingestion on medfly aggressive behaviour. Our results show an influence of carlina oxide on aggression directionality, with the actions of control flies directed mostly to medflies previously fed on carlina oxide concentrations. Further research is still needed to assess possible subtle interactions among EO minor constituents, as well as to assess toxicity and potential behavioural variations (e.g., impact on predation or parasitization activity) in invertebrates acting as biocontrol agents of *C. capitata* treated with a low concentration of carlina oxide.

Although the cultivation of *C. acaulis* and the consequent extraction of the EO and the carlina oxide is a feasible process, certain limitations related to the physical and chemical properties of the compounds, especially their photosensitivity, must be overcome. Indeed, from an application point of view, it should be stressed that nanotechnologies can be particularly useful to improve the effectiveness and stability of *C. acaulis* EO and carlina oxide and enable long-term effectiveness in the field (Pavoni et al., 2019). So, further studies are needed to analyse the lethal and sub-lethal effects of micro- or nanoemulsion of *C. acaulis* EO and carlina oxide. The *in vivo* toxicological assays displayed that the *C. acaulis* EO containing 97.7% of carlina oxide, can produce modest neurological signs and moderate effects on the stomach, liver, and kidney only at the highest dose (1000 mg/kg), slightly lower than the LD_{50}. The other tested doses, which are closer to the practical use of the EO when formulated in protein baits, did not cause side effects worthy of mention. Therefore, our study proved the safety of this natural product to be used in "attract and kill" approaches for controlling major agricultural pests.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The multitarget FAAH inhibitor/D3 partial agonist ARN15381 decreases nicotine self-administration in male rats.

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Abstract.

Tobacco use disorder is a worldwide health problem for which available medications show limited efficacy. Nicotine is the psychoactive component of tobacco responsible for its addictive liability. Similar to other addictive drugs, nicotine enhances mesolimbic dopamine transmission. Inhibition of the fatty acid amide hydrolase (FAAH), the enzyme responsible for the degradation of the endocannabinoid anandamide (AEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), reduces nicotine-enhanced dopamine transmission and acquisition of nicotine self-administration in rats. Down-regulation of dopamine transmission by antagonists or partial agonists of the dopamine D3 receptor (DRD3) also reduced nicotine self-administration and conditioned place preference. Based on these premises, we evaluated the effect of ARN15381, a multitarget compound showing FAAH inhibition and DRD3 partial agonist activity in the low nanomolar range, on nicotine self-administration in rats. Pretreatment with ARN15381 dose dependently decreased self-administration of a nicotine dose at the top of the nicotine dose/response (D/R) curve, while it did not affect self-administration of a nicotine dose laying on the descending limb of the D/R curve. Conversely, pretreatment with the selective FAAH inhibitor URB597 and the DRD3 partial agonist CJB090 failed to modify nicotine self-administration independent of the nicotine dose self-administered. Our data indicates that the concomitant FAAH inhibition and DRD3 partial agonism produced by ARN15381 is key to the observed reduction of nicotine self-administration, demonstrating that a multitarget approach may hold clinical importance for the treatment of tobacco use disorder.

Keywords: Tobacco use disorder, endocannabinoid, dopamine transmission, self-administration

1. Introduction

Tobacco use disorder (TUD) is an unmet medical emergency for which new and more efficacious therapies are needed due to the limited efficacy of approved medications (Garcia-Rivas et al., 2017; Xi et al., 2009). Nicotine is the main psychoactive component of tobacco, responsible for the development and maintenance of TUD (Garcia-Rivas and Deroche-Gamonet, 2019; Le Foll and Goldberg, 2006). In rats, nicotine reinforces self-administration behavior (Corrigall and Coen, 1989) and induces conditioned place preference (CPP) (Le Foll and Goldberg, 2005).

The endocannabinoid system plays a role in the reinforcing effects of nicotine, representing a potential pharmacological target for the treatment of TUD (Muldoon et al., 2013; Scherma et al., 2016). This system comprises two receptors - CB1 and CB2 - and their endogenous ligands - anandamide (AEA) and 2-arachidonoylglycerol (2-AG) - that are inactivated by the fatty acid amide hydrolase (FAAH) and the monoacylglycerol lipase respectively (Rodriguez de Fonseca et al., 2005). The selective FAAH inhibitor URB597 reduces nicotine-induced dopamine mesolimbic transmission (Melis et al., 2008; Scherma et al., 2008), and it blocks the acquisition of nicotine self-administration and CPP in rats (Forget et al., 2009; Scherma et al., 2008). Beside AEA, FAAH inhibition also increases the levels of palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) (Melis et al., 2008; Panlilio et al., 2013), two endogenous agonists at the α-subtype peroxisome proliferator-activated receptor (PPAR-α). The PPAR-α antagonist MK886 reduced the effects of FAAH inhibition on nicotine self-administration, but not the effects of URB597 on cued nicotine seeking in squirrel monkeys (Justinova et al., 2015), suggesting that FAAH may modulate the primary reinforcing effects of nicotine through mechanisms independent of the endocannabinoid system. As such, FAAH inhibition may represent a promising target to help smoking cessation.

The endocannabinoid system has shown to modulate the mesolimbic dopamine transmission (Melis and Pistis, 2012), and the inhibition of FAAH activity to counteract nicotine-induced dopaminergic release (Luchicchi et al., 2010; Melis et al., 2008; Scherma et al., 2008); except for Pavon et al. (2018). The dopamine D3 receptor (DRD3) is predominantly expressed within the mesolimbic system (Levesque et al., 1992), and striatal levels of DRD2/DRD3 negatively correlated with TUD (Okita et al., 2016). Selective antagonism at DRD3 attenuated nicotine self-administration (Ross et al., 2007), while the partial agonist BP-897 and the antagonist SB-277011A blocked the expression of nicotine induced CPP in rats (Le Foll et al., 2005; Pak et al., 2006). These findings suggest that molecules able to tone down DRD3 activity may have potential as treatments for TUD (Le Foll et al., 2007; Sokoloff and Le Foll, 2017).

Based on these premises, we hypothesized that the concurrent inhibition of FAAH activity and activation of DRD3 by a partial agonist would contrast the primary reinforcing effects of nicotine and we developed the novel dual FAAH inhibitor/DRD3 partial agonist ARN15381 accordingly (De Simone et al., 2017). To test our hypothesis, we compared the ability of ARN15381 to reduce nicotine self-administration in rats with that of the selective FAAH inhibitor URB597 and the DRD3 partial agonist CJB090.

2. Materials and Methods

2.1. Animals

Fortyeight male Wistar rats (Charles River, Calco, Italy), weighing 225-250g the day of their arrival, were housed two per cage on a 12-hour light/dark cycle (lights off at 8.00am) in a temperature (21-22°C) and humidity-controlled room (45-55%). During the experiments, animals were given *ad libitum* access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy). The week after their arrival, animals were

allowed to acclimate to the housing room. The second week they were handled daily for 5 minutes by the same operator who carried out the experiment. All behavioral procedures were performed during the dark phase of the light/dark cycle.

All procedures were conducted in adherence with the guidelines of the *European Community Council Directive for Care and Use of Laboratory Animals and National Institutes of Health, and were approved by the local ethical commission (1D580.21).*

2.2. Drugs

For self-administration training, (-)-nicotine hydrogen tartrate salt was dissolved in sterile physiological saline at the free-base dose of 30 µg/0.1ml and 15 µg/0.1ml per infusion (pH adjusted to 7.4 ± 0.1). The solutions were filtered through a 0.2 µm filter and given intravenously (i.v.). ARN15381, corresponding to the hydrochloride salt of compound **2** (**Figure 1**, derivative **33** in (De Simone et al., 2017)), was synthetized at the Italian Institute of Technology (IIT) (Genova, Italy) as described in supplementary information (SI). ARN15381 synthesis (**Figure 1)** was performed following the procedure reported by De Simone et al. (2017). ARN15381 was dissolved in a vehicle consisting of 5% DMSO, 20% PEG 400 and 75% distilled water. The FAAH inhibitor URB597 was dissolved in 20% DMSO and saline. The dopamine DRD3 receptor partial agonist CJB090 was dissolved in 5% β-cyclodextrin. Excluding ARN15381, all drugs and solvents were purchased from Sigma-Aldrich (Milan, Italy).

The molecular structures ARN15381, CJB090 and URB597 are represented in **Figure 2**.

2.3. Catheter implantation

Animals were anesthetized with isoflurane diluted in oxygen (5% for the induction of anesthesia and 2-2.5% for its maintenance). Incisions were made to expose the right jugular vein and in the intra-scapular region. A catheter made of micro-renathane tubing (I.D.=0.020 in., O.D.=0.037 in.) was subcutaneously positioned between the vein and the back. After insertion into the vein, the proximal end of the catheter was anchored to the muscles underlying the vein with surgical silk. The distal end of the catheter was attached to a stainless-steel cannula bent at a 90° angle that protruded from the back of the animal. The cannula was embedded in a support made by dental cement. One week of recovery was allowed before starting self-administration training. During this week, rats received the antibiotic enrofloxacin (Baytril®, Germany) dissolved in the drinking water (25 mg/100ml). For the duration of the experiments, catheters were flushed daily with 0.2 ml of heparinized saline solution containing 1 mg/ml of enrofloxacin. After experiments, catheter patency was confirmed by administration of 0.2 ml of a thiopental sodium (20 mg/ml) solution. Catheter patency was assumed when an immediate loss of reflexes was observed.

2.4. Self-administration apparatus

Experiments were performed in MedAssociate operant conditioning chambers (ENV-008CT) enclosed in sound attenuating and ventilated environmental cubicles. Each chamber was equipped with two retractable levers located on the front panel, a cue-light above each lever and a tone generator. Nicotine was delivered by a plastic tube that was connected to the catheter before the beginning of the session. An infusion pump was activated by the response on the active lever according to the programmed schedule, while responses on the inactive lever were recorded but had no scheduled consequence. Activation of the pump resulted in the

delivery of 0.1 ml of fluid. A Windows-compatible Med-PC-IV software controlled the delivery of nicotine solution and recorded behavioral data.

2.5. Nicotine self-administration training

Rats were trained to self-administer nicotine i.v. in two-hours daily sessions, five days a week. Half of the rats were trained to self-administer 30 µg/infusion and the other half 15 µg/infusion of nicotine (infusion volume was 0.1 ml delivered over 5 seconds).The first week, rats were trained under Fixed Ratio 1 (FR1) schedule of reinforcement. The second week, reinforcement contingency was increased to FR2 and the third week to FR3, which was maintained for the remainder of training and tests. A 20-second time out period (TO) started contigently with nicotine infusion. During the TO, the cue-light positioned above the active lever was illuminated and responses at the active lever were not reinforced. A 2.9 kHz intermittent beep-tone (1s ON/1s OFF) was generated by a SC628 tone generator (MedAssociate) and was presented throughout the session. Pharmacological tests started when a stable baseline of nicotine infusion was achieved (a minimum of ten infusions and ±20% variation over the last three days). For each nicotine dose, rats were divided into three groups (N = 7-9 each): one receiving ARN15381, one receiving CJB090 and the third receiving URB597.

2.6. Effect of ARN15381 on nicotine self-administration

2.6.1 Nicotine 30µg/infusion: The effect of ARN15381 (0.0, 3.0, 10.0 mg/kg (De Simone et al., 2017)) on voluntary nicotine (30µg/infusion) self-administration was tested in rats (N=8) trained as described above, using a within subject Latin-square design, in which each rat received all doses of ARN15381 in a counterbalanced order. ARN15381 was administered orally by gavage 60 minutes prior to the test session at

the volume of 1 ml/kg (De Simone et al., 2017). Animals were acclimated to the treatment procedure for three days before tests. Test sessions were carried out every fourth day. The first day between test sessions, rats remained in their home-cage, whereas the second and third day they were subjected to nicotine self-administration baseline.

2.6.2 Nicotine 15µg/infusion: The effect of ARN15381 (0.0, 3.0, 10.0 mg/kg) on voluntary nicotine (15µg/infusion) self-administration was tested in a separate group of rats (*N*=9) using the same protocol described for nicotine 30µg/infusion dose.

2.7 Effect of CJB090 on nicotine self-administration

2.7.1 Nicotine 30µg/infusion: The experimental design described in session 2.6 was also used to test the effect of CJB090 (0.0 and 3.0 mg/kg) on nicotine (30 µg/infusion) self-administration in a separate group of rats (*N*=7). CJB090 was administered i.v. through the same catheter used for self-administration at the volume of 1 ml/kg immediately before the test session. Considering the plasma volume estimated by our rats' body weights at the time of experiment (Bijsterbosch et al., 1981; Lee and Blaufox, 1985) and the brain/plasma concentration ratio of CJB090 (Mason et al., 2010), we estimated that 3.0 mg/kg i.v. dose of CJB090 would yield a brain concentration more than one thousand times higher than CJB090 EC_{50} (6.3 nM) (Newman et al., 2003) and therefore adequate to effectivelly engage the target.

2.7.2 Nicotine 15µg/infusion: The same protocol was used to test the effect of CJB090 (0.0 and 3.0 mg/kg) on nicotine (15 µg/infusion) self-administration in a separate group of rats (*N*=8).

173

174 *2.8 Effect of URB597 on nicotine self-administration*

175 2.8.1 Nicotine 30µg/infusion: The experimental design described in session 2.6 was used to test the effect of
176 URB597 (0.0, 0.3 and 1.0 mg/kg) on nicotine (30 µg/infusion) self-administration in an independent group of
177 rats (*N*=9). URB597 was administered intraperitoneally (i.p.) 30 minutes before the test session at the volume
178 of 1ml/kg. These doses, administration route, and time was previously demonstrated to fully inhibit FAAH
179 activity in the rat brain (Fegley et al., 2005).

180

181 2.8.2 Nicotine 15µg/infusion: To test the effect of URB597 (0.0, 0.3, and 1.0 mg/kg) on nicotine (15
182 µg/infusion) self-administration a separate group of rats (*N*=7) was trained as described above.

183

184 *2.9. Statistical analyses*

185 Acquisition of nicotine self-administration was analyzed independently for the two doses of nicotine by two-
186 ways ANOVA with both lever and time as repeated measures. The infusions earned after vehicle treatment of
187 the three drug tests for each nicotine dose were analyzed by two-tailed *t*-test for independent samples. The
188 effects of ARN15381 (0.0, 3.0 and 10.0 mg/kg), and URB597 (0.0, 0.3 and 1.0 mg/kg), on nicotine self-
189 administration were analyzed using a one-way within subjects analysis of variance (ANOVA) with dose as a
190 repeated measure. The effect of CJB090 (0.0 and 3.0 mg/kg) on nicotine self-administration was analyzed by
191 a two-tailed paired *t*-test. Statistical significance was conventionally set at $p<0.05$, ANOVAs were followed
192 by Dunnett's *post-hoc* test when appropriate. Experiments run on different doses of nicotine were analysed
193 separately. Infusions earned, active and inactive-lever responses were analyzed separately.

194

3. Results

Self-administration acquisition training of the rats subjected to ARN15381 treatment are presented as typical self-administration acquisition curve for the two doses of nicotine (30µg/infusions and 15µg/infusions; **Figure 3**)

Rats trained to self-administer 30µg/infusions of nicotine increased the number of infusion earned over time, which became stable starting from the fourth session. Rats responded at increasing reinforcement schedule (from FR1 to FR2 and finally to FR3) by increasing the number of active lever presses produced, in order to maintain a stable number of infusions; conversely, inactive lever responses remained very low and stable (**Figure 3A**; Lever [$F(1,7)=142.5$; $p<0.0001$], Time [$F(16,112)=27.7$; $p<0.0001$], Lever by Time interaction [$F(16,112)=25.0$; $p<0.0001$]).

Also rats trained to self-administer 15µg/infusions adapted their active lever responding to increasing reinforcement schedule while inactive lever responses remained very low and stable (**Figure 3B**; Lever [$F(1,8)=115.8$; $p<0.0001$], Time [$F(16,128)=8.4$; $p<0.0001$], Lever by Time interaction [$F(16,128)=13.1$; $p<0.0001$]).

Comparison of 15 and 30µg nicotine infusions earned after vehicle treatment in the three drug tests indicated that the 30µg nicotine dose laid on the descending limb of the nicotine D/R curve (**Supplementary Figure 1S**).

3.1. Effect of ARN15381 on nicotine self-administration

3.1.1 Nicotine 30 µg/infusion: ANOVA of nicotine 30µg-infusions earned after ARN15381 treatment found no overall effect of doses on nicotine self-administration [$F(2,14)=0.77$, $p=0.44$] (**Figure 4A**). Analysis of active lever responses was consistent with infusions and reported no overall effect of treatment [$F(2,14)=1.05$;

p=0.35] (**Figure 4B, upper panel**). Inactive lever responses were very low and also not affected by treatment [F(2,14)=1.56, p=0.25] (**Figure 4B, lower panel**).

To exclude the possibility that the 10 mg/kg ARN15381 was not sufficently high to observe an effect on nicotine 30µg/infusion self-administration, we tested the effect of 30 mg/kg of ARN15381 in an independent group of rats, but we found no increased efficacy ([t(6)=0.96; p=0.37]; **Figure 4A inset**). Inactive lever response was also not affected by ARN15381 30 mg/kg treatment [t(6)=0.3; p=0.77].

3.1.2 Nicotine 15µg/infusion: ANOVA of nicotine 15µg-infusions earned after ARN15381 treatment found an overall effect of doses on nicotine self-administration [F(2,16)=6.95, p=0.008]. Dunnett's *post-hoc* analysis revealed that the 10 mg/kg dose of ARN15381 decreased the number of infusions earned compared to vehicle (p<0.05; **Figure 4C**). ANOVA of active lever responses found an overall effect of ARN15381 treatment [F(2,16)=7.72; p=0.005]. Dunnet's *post-hoc* test indicated that both doses of ARN15381 decreased active lever presses compared to vehicle (**Figure 4D, upper panel**). Conversely, inactive lever responses was very low and not affected by treatment [F(2,16)=1.04, p=0.37] (**Figure 4D, lower panel**).

These results indicated that concomitant inhibition of FAAH activity and partial agonistic activity at DRD3 produced by ARN15381 selectively reduced 15µg/infusion nicotine self-administration.

Next, we wanted to test whether the partial agonism at DRD3 and the selective inhibition of FAAH activity alone would affect nicotine self-administration.

3.2. Effect of CJB090 on nicotine self-administration

One rat from the 30µg/infusion group and one rat from the 15µg/infusion group were excluded from analyses because of catheter failure.

3.2.1 Nicotine 30µg/infusion: 3.0 mg/kg of CJB090 did not affect nicotine 30µg/infusion self-administration [$t(5)=0.3$, $p=0.77$] (**Figure 5A**). Analysis of active lever responses was consistent with infusions and reported no overall effect of treatment [$t(5)=0.04$; $p=0.97$] (**Figure 5B, upper panel**). Inactive lever responses were very low and were also not affected by treatment [$t(5)=1.4$, $p=0.22$] (**Figure 5B, lower panel**).

3.2.2 Nicotine 15µg/infusion: CJB090 did not affect nicotine 15µg-infusions self-administration [$t(6)=0.23$, $p=0.82$] either (**Figure 5C**). Analysis of active lever responses was consistent with infusions and reported no effect of treatment [$t(6)=0.3$, $p=0.8$] (**Figure 5D, upper panel**). Inactive lever responses were very low and were also not affected by treatment [$t(6)=1.3$, $p=0.23$] (**Figure 5D, lower panel**).

3.3. Effect of URB597 on nicotine self-administration

3.3.1 Nicotine 30µg/infusion: When the effect of the FAAH inhibitor URB597 on nicotine 30µg-infusions earned was evaluated, ANOVA found no effect of URB597 doses on nicotine self-administration [$F(2,16)=1.29$, $p=0.3$] (**Figure 6A**). Analysis of active lever responses was consistent with infusions and reported no overall effect of treatment [$F(2,16)=1.08$; $p=0.35$] (**Figure 6B, upper panel**). Inactive lever responses were very low and also not affected by treatment [$F(2,16)=0.21$, $p=0.74$] (**Figure 6B, lower panel**).

3.3.2 Nicotine 15µg/infusion: When the effect of the FAAH inhibitor URB597 on nicotine 15µg-infusions earned was evaluated, ANOVA found no effect of URB597 doses on nicotine self-administration [$F(2,12)=1.42$, $p=0.28$] (**Figure 6C**). Analysis of active lever responses was consistent with infusions and

reported no overall effect of treatment [$F(2,12)=0.21$, $p=0.80$] (**Figure 6D, upper panel**). Inactive lever responses were very low and also not affected by treatment [$F(2,12)=0.20$, $p=0.77$] (**Figure 6D, lower panel**).

4. Discussion

Based on the hypothesis that a multitarget mechanism of action towards FAAH (inhibition) and DRD3 (down-regulation of transmission) would decrease the primary reinforcing properties of nicotine, we set out to evaluate the effect of the FAAH inhibitor/DRD3 partial agonist ARN15381 (De Simone et al., 2017), on nicotine self-administration in rats. ARN15381 selectively decreased self-administration of 15 µg/infusion nicotine dose. Whereas neither the selective inhibition of FAAH by URB597, nor DRD3 modulation by the partial agonist CJB090 significantly decreased nicotine self-administration. These findings demonstrate that the concomitant inhibition of FAAH activity and modulation of DRD3 transmission by partial agonism exerted by ARN15381 was efficacious where the two mechanisms alone failed.

ARN15381 decreased nicotine self-administration of 15 µg/infusion of nicotine but was not effective at 30 µg/infusion dose. Normalizing nicotine doses according to the rats' body weight at the time of the self-administration tests corresponded to doses of 35 and 61 µg/kg/infusions, falling on the top and on the descending limb of the nicotine dose/response (D/R) curve respectively (Watkins et al., 1999). In other words, ARN15381 decreased nicotine self-administration on the top of the D/R curve, and it failed to affect response for nicotine on the descending limb. Our results are consistent with previous reports in non-human primates using the FAAH inhibitors URB597 and URB694, in which both FAAH inhibitors decreased nicotine self-administration on the top but not on the descending limb of the nicotine D/R curve (Justinova et al., 2015).

The lack of ARN15381 effect on the higher dose of nicotine can be explained by three alternative interpretations. A first interpretation would be that the 30µg nicotine infusion dose (≃ 60 µg/kg) fell within the aversive range of the nicotine D/R curve, and rats were not sufficiently engaged to self-administer nicotine to observe a significant decrease. For instance, Markou and colleagues reported that rats could self-administer 60µg/kg/infusion of nicotine only after tolerance had developed (Watkins et al., 1999), suggesting an intrinsic aversive effect of this dose. However, in our experiments rats readily acquired self-administration of this dose of nicotine and they adapted their active lever response in order to maintain a stable level of infusion when FR contingency was increased from FR1 to FR3. This indicates that the 30µg/infusion dose of nicotine was devoid of aversive effects, and it was rather experienced by rats as a positive reinforcer, which prompts us to deem them unlikely that possible aversive effects of nicotine affected ARN15381 efficacy.

A second interpretation could be that the dose of ARN15381 was not sufficiently high. However, notwithstanding a 21% oral bioavailability, the brain concentration (391 nM) following oral administration of 10 mg/kg of ARN15381 is more than adequate to effectively engage the targets (FAAH IC_{50}=0.9 nM, DRD3 EC_{50}=18 nM) (De Simone et al., 2017). Yet, to exclude this possibility, and to generate a maximal dose response, in an independent group of rats, we tested the effect of 30 mg/kg of ARN15381 on nicotine 30µg/infusion self-administration and, as expected, there was no increased efficacy of ARN15381 with a dose three times as high.

A third and more appealing, though speculative at this time, mechanicistic interpretation can be proposed. ARN15381 exerts a double action by inhibiting FAAH activity, and therefore increasing AEA, PEA and OEA levels (Panlilio et al., 2013; Rodriguez de Fonseca et al., 2005), and down-toning DRD3 transmission. Inhibition of FAAH is expected to counteract dopamine release induced by nicotine (Imperato et al., 1986;

Melis et al., 2008), and the partial agonism at DRD3 to prevent the reinforcing effects of possible residual dopamine release. The inhibition of nicotine-induced dopaminergic firing could be obtained through a PPAR-α mediated inactivation of β2 nicotinic acetylcholine receptors (β2nAChR) promoted by the increase in OEA levels (Melis et al., 2010; Melis et al., 2008) induced by the FAAH inhibition (Panlilio et al., 2013). Indeed, activation of PPAR-α was reported to decrease self-administration of 30μg/kg of nicotine, a dose similar to the one on which ARN15381 was effective here (15μg/infusion = 35μg/kg/infusion). To reconcile the lack of effect on nicotine 30μg/infusion, one could consider that the reinforcing effects of nicotine are mediated by β2nAChR, as this subtype of nAChR promotes burst firing of DA neurons and thus the switching from tonic to phasic activity (Mameli-Engvall et al., 2006). Nicotine has been reported to increase the relative expression and membrane availability of β2nAChR in high affinity state (Moroni et al., 2006; Vallejo et al., 2005; Wecker et al., 2010). Therefore, the possibility exists that the dose of 30μg/infusion was sufficiently high to surmount the inactivation of nAChR induced by ARN15381 through OEA, thereby promoting enough DA release to displace ARN15381 from DRD3. As a consequence, 30μg/infusion would have maintained its reinforcing effects. Future studies should be directed to verify this hypothesis.

One target of ARN15381 is the inhibition of FAAH activity (De Simone et al., 2017). Justinova et al. (2015) investigated the effects of the FAAH inhibitors URB597 and URB694 on nicotine self-administration in squirrel monkeys and demonstrated that enzyme inhibition reduces nicotine self-administration for a dose of nicotine on the top, but not for doses on the descending limb, of the D/R curve. On the one hand, our results with ARN15381 are in line with those of Justinova et al. (2015), but on the other hand, we also demonstrated that URB597 only showed a trend to reduce nicotine self-administration in the rat. The lack of a significant

effect due to URB597 is not to be attributed to a weak pharmacological action because we chose doses that were previously demonstrated to fully inhibit FAAH activity in the rat (Fegley et al., 2005). In addition, Scherma et al. (2008) demonstrated that the FAAH inhibitor URB597 prevents the acquisition of nicotine self-administration at a dose within the range tested here. However, Scherma et al. (2008) administered URB597 throughout the acquisition phase of nicotine self-administration, starting from the first operant responding session, and an effect appeared only after twelve training sessions. On the contrary, to the best of our knowledge we are the first to report the effect of acute URB597 on nicotine self-administration under FR contingency. It should also be emphasized that, in line with our results, in the work by Forget et al. (2009) neither acute nor chronic URB597 modified nicotine self-administration under PR contingency. Altogether, these findings suggest that URB597 may reduce nicotine self-administration in the rat only if given chronically from the beginning of the acquisition phase. However, since Justinova and colleagues, like us, tested URB597 after the acquisition of operant responding (Justinova et al., 2015), the more likely explanation of the inconsistency with our results relies on inter-species differences between the two studies.

It is worth noting that the fact that URB597 induced a non-significant decrease of self-administration where ARN15381 was instead fully effective emphasizes the importance of the double action of ARN15381 on FAAH and DRD3. As proposed above, ARN15381 could have been efficacious where URB597 failed because the partial agonist action on DRD3 would have toned down possible residual dopamine transmission after inactivation of β2nAChR by the FAAH inhibitory activity.

The other target of ARN15381 is DRD3, which ARN15381 selectively activates as a partial agonist (65% efficacy, EC_{50} 18 nM (De Simone et al., 2017). Here, we report for the first time data on the effect of selective

DRD3 partial agonism either alone (CJB090) or in combination with FAAH inhibition (ARN15381) on operant nicotine self-administration under FR contingencies. Partial agonists have lower intrinsic activity at receptors than full agonists (Hoyer and Boddeke, 1993), allowing them to act as agonists or antagonists depending on the levels of the endogenous neurotransmission. Similar to other addictive drugs, the primary reinforcing effects of nicotine are mediated by the release of mesolimbic dopamine (Di Chiara and Imperato, 1988; Imperato et al., 1986). In fact, earlier studies showed that the DRD3 antagonist SB-277011A reduced nicotine self-administration under progressive ratio contingency (Ross et al., 2007). However, in our experiments, CJB090 did not reduce nicotine self-administration under FR contingency. This might indicate that to reduce self-administration by exclusively targeting DRD3 an antagonist rather than a partial agonist would be necessary, or in alternative that observations made on this target under PR contingency does not translate to FR contingency. Within the interpretation of the effect of ARN15381 on nicotine self-administration, the lack of effect of CJB090 alone further corroborates the view proposed above that the action on DRD3 co-operate with the inhibitory effects on FAAH to reduce nicotine self-administration.

This work was conducted exclusivelly in male rats. Female rats show differences in nicotine self-administration compared to males (Flores et al., 2019). In addition, estrogen has been demonstrated to modulate dopamine transmission (Peart et al., 2022; Yoest et al., 2014) and the activity of FAAH (Grimaldi et al., 2009). This suggests that females might respond differently to ARN15381 than males and dedicated studies should be conducted before generalizing the present results to both sexes.

In summary, we tested the effect of ARN15381, a compound exerting both FAAH inhibitory and DRD3 partial agonism activity on nicotine self-administration in male rats. Our results demonstrated that ARN15381 decreases nicotine self-administration. Importantly, the FAAH inhibitor URB597 and the DRD3 agonist CJB090 alone failed in significantly affecting nicotine seeking, demonstrating that the concomitance of the two mechanisms of action is the major advantage of ARN15381 and represents a novel promising line within TUD research and development. From a translational perspective, our preclinical results cast clinical importance as they suggest that targeting multi-pharmacological sites may represent a valuable approach to treat TUD.

Supplementary Materials: Chemicals, materials, and methods for the synthesis of ARN15381; Figure S1, self-administration training; Figure S2, effect of ARN15381 (30 mg/kg) on nicotine (30µg/infusion) self-administration.

Author Contributions: VL, HL, QS, FB, and AD, performed the experiments; LS assisted with the project implementation, RMCDM synthesized ARN15381; GB designed ARN15381; NC supervised the project and analyzed data; NC, CLH-K, FB, RMCDM, VL, and GB wrote the manuscript.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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FIGURE LEGENDS

Figure 1. ARN15381 synthesis scheme. i) dry CH_3CN, K_2CO_3, 85 °C, 6 h, yield: 99%; (ii) hydrazine monohydrate, CH_3OH, 80 °C, 2 h, then HCl (2 N), 1 h, yield: 90%; (iii) 4-phenylphenol, $(Boc)_2O$, DMAP, dry CH_3CN, rt, 23 h, yields: 23%; (d) HCl in CH_3OH (1.25 M), room temperature, 2 h, yield: 100%.

Figure 2. Chemical structures of the**:** selective FAAH inhibitor URB597, DRD3 partial agonist CJB090, and the FAAH inhibitor/ DRD3 partial agonist ARN15381.

Figure 3. Self-administration trainings of rats of rats subjected to ARN15381 treatment are presented as typical nicotine self-administration acquisition ratio obtained with our protocol. **A**) Acquisition of nicotine 30µg/infusion self-administration. The number of nicotine infusions increased over time and became stable starting from the forth session. When reinforcement schedule was increased from FR1 to FR2 and finally to FR3, rats increased their response at the active lever in order to maintain a stable nicotine self-administration, while inactive lever responses remained very low and stable. **B**) Acquisition of nicotine 15µg/infusion self-administration. the number of infusions of this dose of nicotine became readily stable. When reinforcement schedule was increased from FR1 to FR2 and finally to FR3, rats increased their response at the active lever in order to maintain a stable nicotine self-administration, while inactive lever responses remained very low and stable. Data are expressed as mean ± *SEM* of nicotine infusion (black dots), active lever responses (black squares) and inactive lever responses (white squares).

Figure 4. Effect of the FAAH inhibitor/DRD3 partial agonist ARN15381 on nicotine self-administration (SA) under FR3 schedule of reinforcement. A) Pretreatment with ARN15381 (0.0, 3.0 and 10.0 mg/kg) did not affect nicotine 30µg/infusion self-administration. *Inset*: 30.0 mg/kg of ARN15381 did not affect nicotine 30µg/infusion self-administration. **B)** Active (upper panel) and inactive (lower panel) lever responses were also not affected by ARN15381 treatment. **C**) ARN15381 (0.0, 3.0 and 10.0 mg/kg) dose dependently reduced nicotine 15µg/infusion self-administration. **D**) Active (upper panel) but not inactive (lower panel) lever responses were reduced by both doses of ARN15381. Data are expressed as mean ± *SEM* of number of nicotine infusions earned during a 2-hours SA session. Statistical significance: **p*<0.05 vs vehicle (0.0 mg/kg).

Figure 5. Effect of the DRD3 partial agonist CJB090 on nicotine self-administration under FR3 schedule of reinforcement. A) Pretreatment with CJB090 (0.0 and 3.0 mg/kg) did not affect nicotine 30µg/infusion SA. **B)** Active (upper panel) and inactive (lower panel) lever responses for nicotine 30µg/infusions were also not affected by CJB090 treatment. **C)** Pretreatment with CJB090 (0.0 and 3.0 mg/kg) did not affect nicotine 15µg/infusion SA. **D)** Active (upper panel) and inactive (lower panel) lever responses for nicotine 15µg/infusions were also not affected by CJB090 treatment. Data are expressed as mean ± *SEM* of number of nicotine infusions earned during a 2-hours SA session.

Figure 6. Effect of the FAAH inhibitor URB597 on nicotine self-administration under FR3 schedule of reinforcement. A) Pretreatment with URB597 (0.0, 0.3, and 1.0 mg/kg) did not significantly affect nicotine 30µg/infusion SA. **B)** Active (upper panel) and inactive (lower panel) lever responses for nicotine 30µg/infusions were also not affected by URB597 treatment. **C)** Pretreatment with URB597 (0.0, 0.3, and 1.0 mg/kg) did not affect

15µg/infusion nicotine SA. **D)** Active (upper panel) and inactive (lower panel) lever responses for nicotine 15µg/infusions were also not affected by URB597 treatment. Data are expressed as mean ± *SEM* of number of nicotine infusions earned during a 2-hours SA session.

Figure 1

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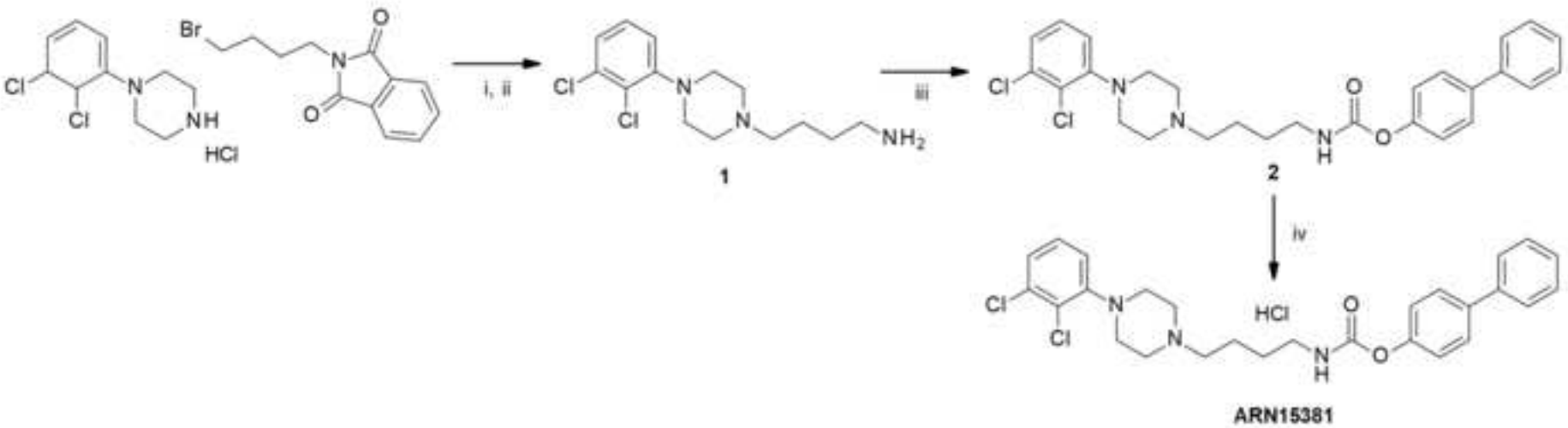
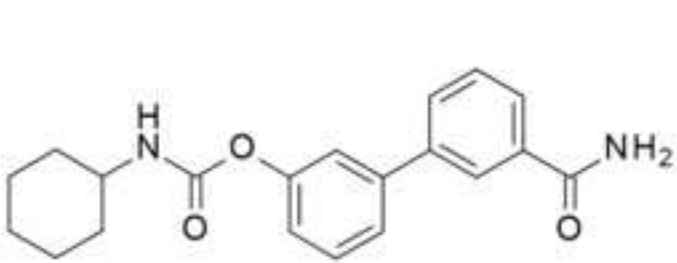
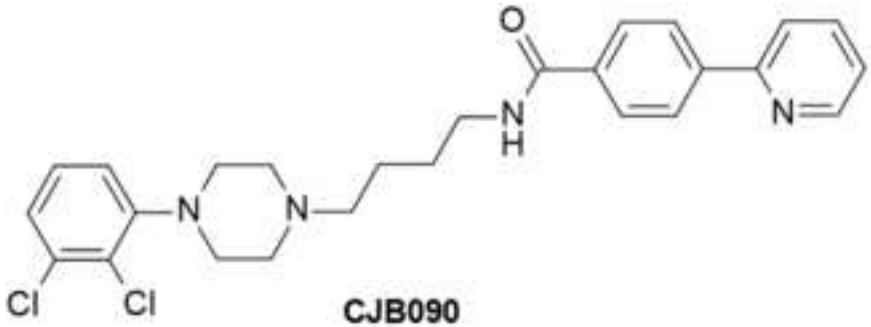


Figure 2



URB597



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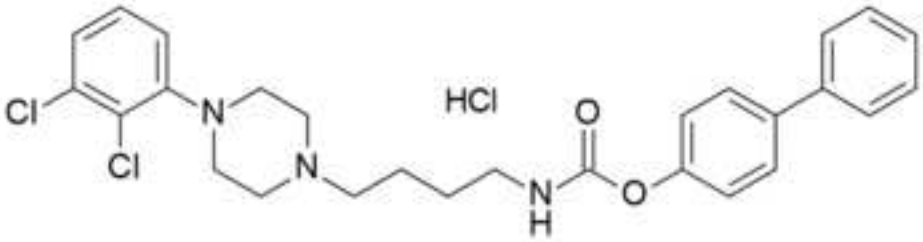

ARN15381

Figure 3

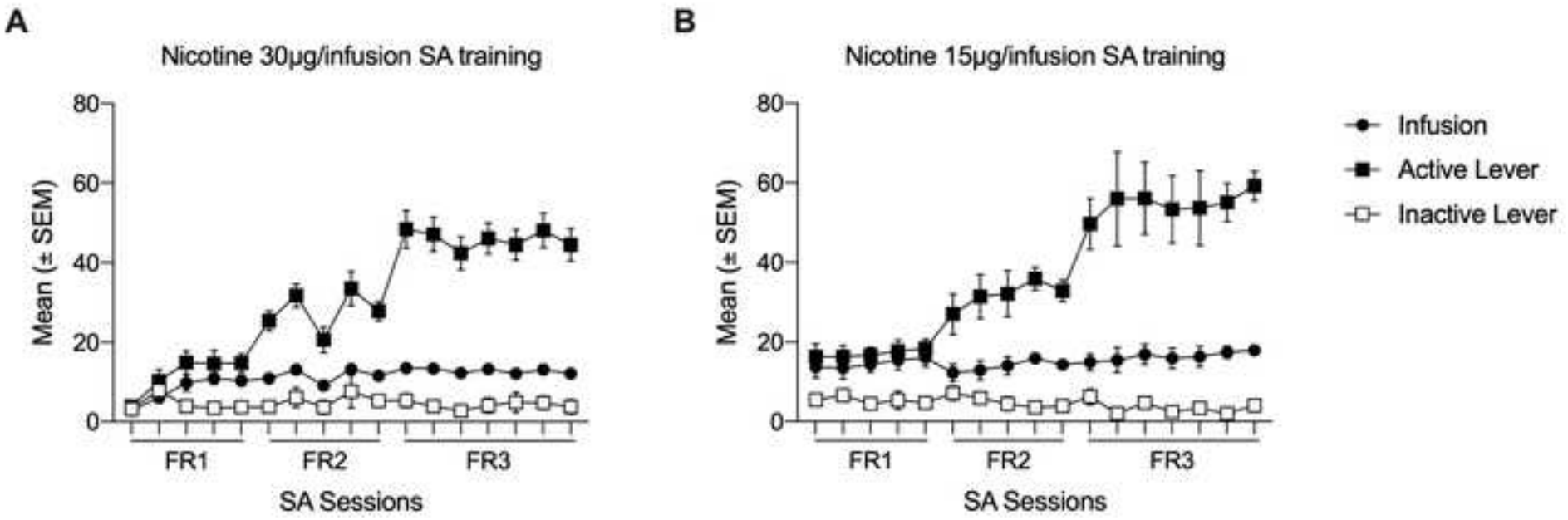

Figure 4

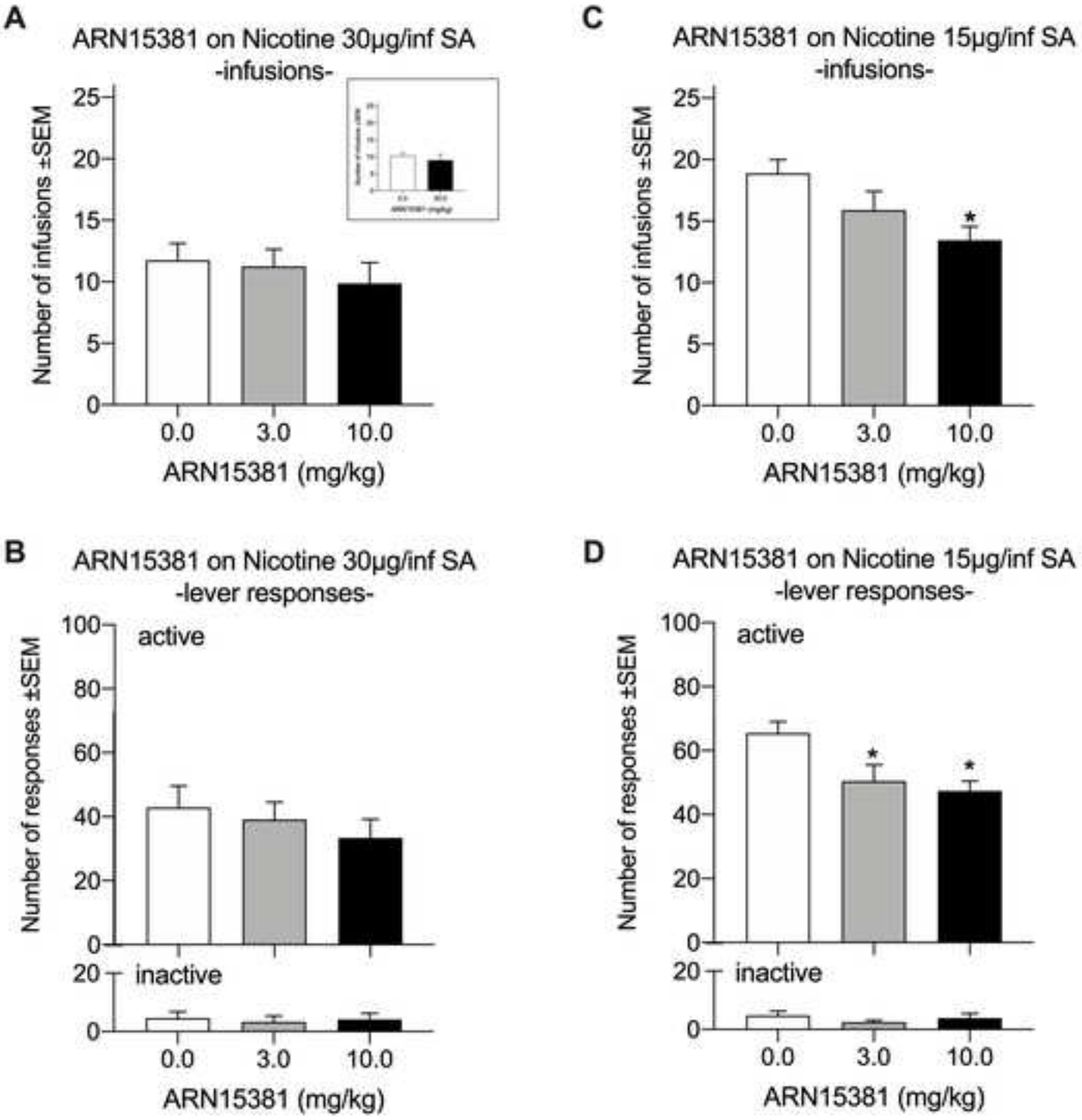

Figure 5

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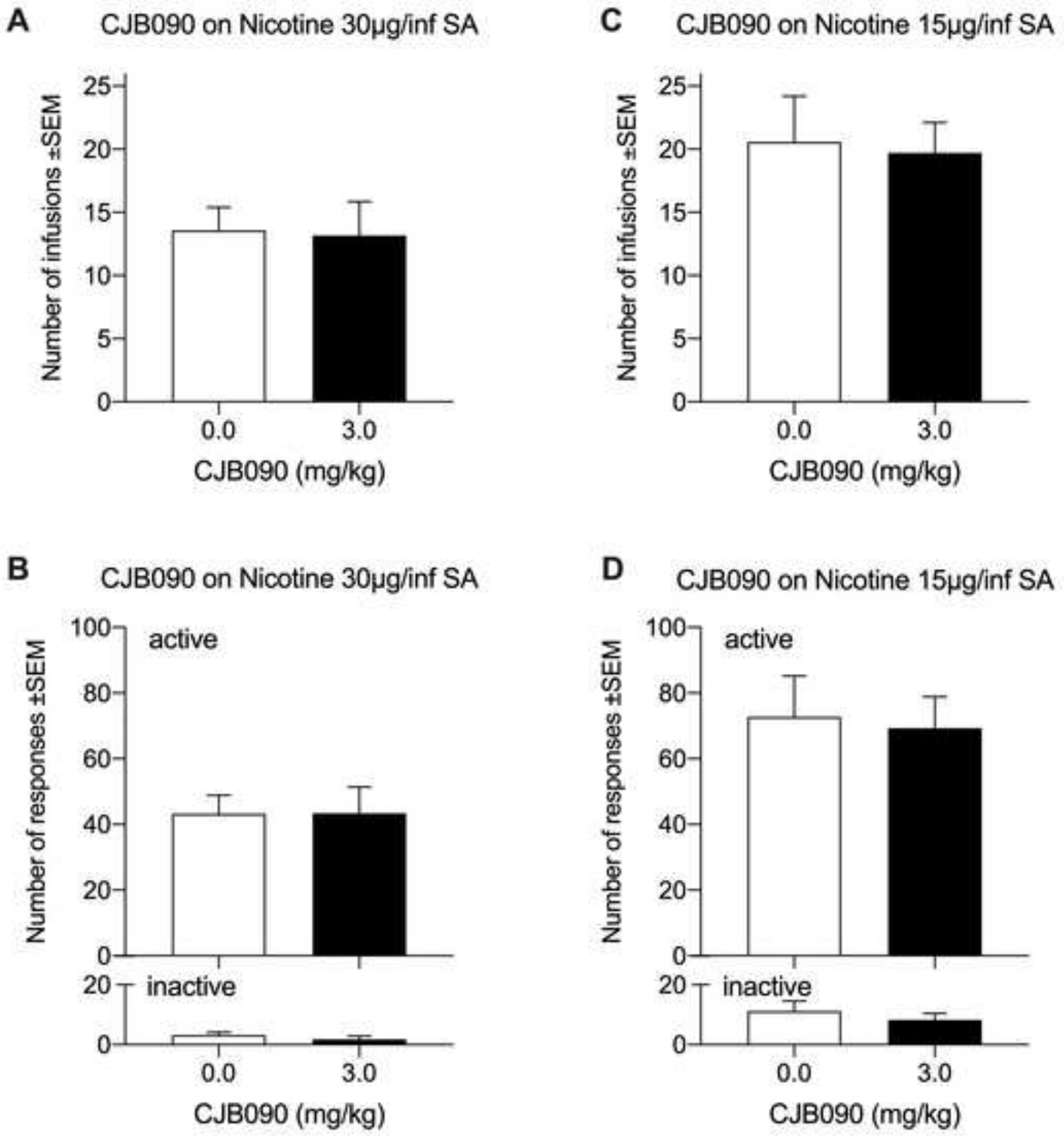

Figure 6

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