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Genetically selected alcohol-preferring msP rats to study alcohol use disorder: Anything lost in translation?

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Abstract

For several decades, genetically selected alcohol-preferring rats have been successfully used to mimic and study alcohol use disorders (AUD). These rat lines have been instrumental in advancing our understanding of the neurobiology of alcoholism and enabling pharmacological studies to evaluate drug efficacy on alcohol drinking and relapse. Moreover, the results of these studies have identified genetic variables that are linked to AUD vulnerability. This is an up-to-date review that focuses on genetically selected Marchigian Sardinian alcohol-preferring (msP) rats. To support the translational relevance of the findings that are obtained from msP rats and highlight important similarities to AUD patients, we also discuss the results of recent brain imaging studies. Finally, to demonstrate the importance of studying sex differences in animal models of AUD, we present original data that highlight behavioral differences in the response to alcohol in male and female rats. Female msP rats exhibited higher alcohol consumption compared with males. Furthermore, msP rats of both sexes exhibit higher anxiety- and depressive-like behaviors in the elevated plus maze and forced swim test, respectively, compared with unselected Wistar controls. Notably, voluntary alcohol drinking decreases foot-shock stress and depressive-like behavior in both sexes, whereas anxiety-like behavior in the elevated plus maze is attenuated only in males. These findings suggest that male and female msP rats both drink high amounts of alcohol to self-medicate negative affective symptoms. For females, this behavior may be driven by an attempt to treat stress and depressive-like conditions. For males, generalized anxiety appears to be an important additional factor in the motivation to drink alcohol.

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CRediT authorship contribution statement

Anna Maria Borruto: Writing: Original draft preparation, Writing: Reviewing and editing, Investigation, Formal Analysis. Serena Stopponi: Writing: Reviewing and editing. Hongwu Li, investigation, Friedbert Weiss, Marisa Roberto, Roberto Ciccocioppo: Conceptualization, Funding acquisition, Supervision, Writing: Reviewing and editing.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neuropharm.2020.108446>.

Keywords

Alcoholism; Sex differences; Genetic selection; Animal models; Relapse; Self-administration; fMRI; Brain imaging

1. Introduction

According to a recent report by the World Health Organization (WHO), alcohol is one of the most commonly abused psychotropic drugs in the world, second only to tobacco, leading to more than 3 million deaths as a result of its harmful use. 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). The *Diagnostic and Statistical Manual of Mental Disorders*, 5th edition (DSM-5), describes AUD as a chronic relapsing disorder with substantial heritability (American Psychiatric Association, 2013). It is the most prevalent mental disorder at the global level (Disney et al., 1999; Slutske et al., 1999). The chronicity and relapsing nature of AUD is caused by maladaptive changes in the brain that occur in response to prolonged exposure to alcohol (Costin and Miles, 2014). Furthermore, AUD is associated with many physical (e.g., liver and heart disease) and psychiatric (e.g., anxiety and depression) comorbidities, the loss of productivity, and impairments in interpersonal functioning (Grant et al., 2015; Li et al., 2004).

Alcohol use disorder is also characterized by individual variability. Not all people are equally vulnerable to the disease. Various factors, including the age at which people start drinking, comorbid psychiatric conditions, and mental, social, and cultural status, can contribute to the development and progression of AUD. Nevertheless, family history studies have shown that genetic factors are key elements in shaping the vulnerability to AUD.

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; Gianoulakis and de Waele, 1994). Genetic predisposition is estimated to contribute to approximately 50–60% of the vulnerability to AUD (Costin and Miles, 2014; Goodwin et al., 1974; Prescott and Kendler, 1999), as shown by adoption studies (Bakhireva et al., 2018; Bohman et al., 1981; Cloninger et al., 1981; Goodwin et al., 1977; Waaktaar et al., 2018) and twin-pair studies (Heath et al., 1997; Kendler et al., 1992, 1995). The multigenic nature of AUD hampers the identification of specific genes that confer either vulnerability or resilience to the disease, thus limiting successes in this field of study (Costin and Miles, 2014). Because of the complexity of AUD, preclinical research has encountered significant difficulties in successfully mimicking all characteristics of the disease in laboratory animals. However, the general consensus is that there are minimal criteria, such as predictive, face, and construct validity, that must be met to consider an animal model valid (for details, see Ciccocioppo (2013)).

Despite these difficulties, extensive work over the past few decades has been performed to develop new preclinical procedures and animal models that are able to mimic the human condition. One of the major contributions in this field has come from studies of

genetically selected alcohol-preferring rats. These models allow studies of genetic factors that are associated with excessive alcohol drinking, links to specific phenotypes, and the impact of the environment on disease progression. Furthermore, the establishment of *in vivo* brain imaging techniques and new experimental paradigms that promote excessive alcohol drinking and intoxication in rodents have further helped bridge the gap between preclinical and clinical research. Unfortunately, despite these advances, female rodents have been relatively understudied. Sex differences in the response to alcohol are a neglected area of research that deserve particular attention.

The present review provides an update on different rat lines that have been genetically selected for high alcohol preference, with a particular focus on Marchigian Sardinian alcohol-preferring (msP) rats. We describe major advances in the field that have been achieved through the use of this rat line. We also present original data that underscore the importance of studying sex differences in AUD. Furthermore, we provide a summary of *in vivo* brain imaging data that demonstrate the importance of experimental approaches to improve the translational impact of preclinical research. Lastly, we critically discuss the translational validity of genetically selected alcohol-preferring rat lines as model to fully mimic the human AUD conditions. In particular we analyze the case of corticotropin releasing factor one (CRF1) receptor antagonists that in several animal models, including the msP rat, demonstrated significant efficacy, but then failed in the clinic.

2. Historical overview of genetically selected alcohol-preferring rats

Genetically selected alcohol-preferring rat lines were originally developed to gain insights into genetic factors that affect voluntary alcohol intake and preference. Through genetic selection and selective breeding, animals with high alcohol preference and animals with low alcohol preference have been generated. As a result of this work, at least six different rat lines have been generated around the world.

In 1951, the first selective breeding program was initiated at Universidad de Chile (UCh), where high alcohol-drinking (UChB) and low alcohol-drinking (UChA) lines were developed (Mardones et al., 1953; Mardones and Segovia-Riquelme, 1984; Quintanilla et al., 2006). Eriksson and colleagues later initiated another selective breeding program at Alko Research Laboratories in Finland, where the Alko Alcohol high alcohol-preferring (AA) and Alko Non-Alcohol low alcohol-preferring (ANA) rat lines were generated (Eriksson, 1968). After the success of these two initiatives, additional selective breeding programs were initiated in several laboratories. These programs resulted in the development of alcohol-preferring (P) and non-preferring (NP) rat lines at Indiana University/Purdue University Indianapolis (Indianapolis, IN, USA; Li et al., 1979) and Sardinian alcohol-preferring (sP) and non-preferring (sNP) rats in Cagliari, Italy (Ciccocioppo et al., 2006; Colombo et al., 2006; Li et al., 1979). Years later, starting from selectively bred rats from the N/NIH founder stock (a cross of eight inbred rat strains with varying levels of alcohol intake) in Indianapolis, the same research team launched a new bidirectional breeding program that resulted in the generation of High Alcohol Drinking (HAD) and Low Alcohol Drinking (LAD) rat lines (Hansen and Spuhler, 1984; Murphy et al., 2002).

Over the years, some of these rat lines have been transferred to other laboratories, from which additional genetically selected alcohol-preferring rat lines were derived. For example, using P rats as founders, an inbred alcohol-preferring (iP) rat line was developed in Indianapolis. This new iP line was made available to colleagues at the Howard Florey Institute (University of Melbourne, Australia) that are now maintaining it. The iP line conserved the high alcohol-drinking phenotype of the founder stock. Over the years, it has been used in several genetic and pharmacological studies (Carr et al., 2007; Ciccocioppo et al., 2006; Cowen et al., 2005; Kimpel et al., 2007; Rodd et al., 2007).

In Italy, a few pairs of sP rats have been transferred from the University of Cagliari to the University of Camerino. Starting from this founder stock, a new line of alcohol-preferring rats was generated, namely Marchigian Sardinian alcohol-preferring (msP) rats (for details, see (Ciccocioppo et al., 2006; Ciccocioppo et al., 1999a,b,c). We have used this rat line in an extensive program to characterize these rats genetically, behaviorally, and pharmacologically. Moreover, in an attempt to obtain translationally meaningful information, msP rats have been used in various functional magnetic resonance imaging (fMRI) studies, some of which also involve cohorts of AUD patients (De Santis et al., 2019, 2020). Unfortunately, as for the other genetically selected rat lines, females msP rats have been rarely used in experiments. Thus, what we know about this rat line comes mostly from males. To start filling this gap, this review reports original data that show different behavioral responses to alcohol in male and female msP rats.

3. Genetically selected Marchigian Sardinian alcohol-preferring rats: a rodent model to study the neurobiology of alcohol use disorder

MsP rats were originally derived from Wistar rats. They have been selectively bred for high alcohol preference and consumption at the University of Camerino (Marche, Italy) for more than 80 generations, beginning from the 13th generation of sP rats that were originally developed at the University of Cagliari (Sardinia, Italy; (Colombo et al., 2006). The first publication on msP rats appeared in 1991 (Ciccocioppo et al., 2006). Since then, more than 80 studies have explored behavior, genetics, neurobiology, and responses to pharmacological manipulations in this rat line (see the key studies in Table 1).

3.1. Genetics of msP rats

Over the years, several studies have investigated genetic factors that are responsible for the high alcohol drinking phenotype in msP rats. For example, an extensive genetic mapping of the msP line was performed using microarrays and gene sequencing (Ciccocioppo, 2013; Ciccocioppo et al., 2006; Hansson et al., 2006, 2007). The results of these studies showed that several genes that encode aldehyde dehydrogenase (ADH) isoforms are altered in msP rats compared with unselected Wistar controls (Ciccocioppo et al., 2006). The *Aldh2* gene was downregulated, and *Aldh1a1*, *Aldh1a4*, *Aldh3a2*, and *Aldh5a1* were upregulated. Furthermore, the msP line exhibited overactivity of the corticotropin-releasing factor (CRF) system in different brain regions, driven by two single-nucleotide polymorphisms (SNPs) at CRF₁ receptor locus (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). In msP rats, a few days of voluntary alcohol drinking 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 overactive stress system (Hansson et al., 2007). Pharmacological studies provided direct support for this hypothesis. In fact, the blockade of CRF₁ receptors by antalarmin reduced alcohol self-administration in msP rats but not in unselected Wistar controls (Hansson et al., 2006). The same phenomenon was reported following treatment with nociceptin receptor (NOP) agonists, which acted as functional CRF₁ receptor antagonists in reduced drinking in msP rats but not in Wistar controls (Economidou et al., 2008; Ubaldi et al., 2016; Witkin et al., 2014). Interestingly, Wistar rats that were chronically exposed to intoxicating doses of alcohol through intermittent vapor exposure exhibited neuroadaptive changes in the CRF system that resembled its innate dysregulation in msP rats. At the behavioral level, this genetic trait was associated with higher anxiety-like behavior, greater sensitivity to stress, and greater sensitivity to CRF₁ receptor antagonists (Ciccocioppo et al., 2009; Gehlert et al., 2007; Herman et al., 2016; Kirson et al., 2018; Natividad et al., 2017; Sommer et al., 2008). At the cellular level, the dysregulation of γ -aminobutyric acid (GABA)ergic and glutamatergic synapses in the central nucleus of the amygdala (CeA) and greater sensitivity to CRF/CRF₁ and cannabinoid CB₁ receptor compounds was reported in msP rats compared with Wistar controls, supporting the role of these systems in the anxiety-like and excessive drinking phenotypes (Herman et al., 2016; Kirson et al., 2018; Natividad et al., 2017). Notably, CRF₁ receptor gene polymorphisms have also been identified in humans. Importantly, these polymorphisms appear to be linked to AUD (Quadros et al., 2016). Indeed, they correlate with the lifetime prevalence of drunkenness, high levels of alcohol drinking, and binge drinking episodes (Chen et al., 2010; Treutlein et al., 2006). Altogether, these findings indicate that humans and msP rats, at least to some extent, share common genetic factors that predispose them to AUD.

Differences between msP and Wistar rats have been detected at the level of expression of genes that are linked to glutamatergic and GABAergic transmission (Ciccocioppo et al., 2006). For example, alterations of the gene that encodes the metabotropic glutamate receptor (*Grm3*) and the solute carrier family 6 (*Slc6a1*) gene that encodes the GBA transporter have been linked to alcohol sensitivity in mice (Hu et al., 2004).

Other genes of interest that were found to be differentially expressed in msP rats encode the opioid receptor μ 1 (*Oprm1*) gene, opioid receptor-like (*Oprl-1*; referred to as the NOP receptor) gene, and neuropeptide Y receptor 5 gene, which have all been linked to alcohol abuse (Ciccocioppo et al., 2000, 2006; Heilig and Thorsell, 2002; Schroeder et al., 2005; Thorsell et al., 1999). Specifically, msP rats exhibited higher expression of nociceptin/orphanin FQ (N/OFQ) and NOP receptor mRNA in numerous brain regions compared with their Wistar counterparts, accompanied by a significant increase in NOP receptor binding in the CeA, the bed nucleus of the stria terminalis (BNST), the ventral tegmental area (VTA), and several cortical structures (Economidou et al., 2008). Notably, Wistar rats that were exposed to chronic alcohol exhibited neuroadaptive changes in the N/OFQ-NOP system that resembled innate dysregulation that is detected in msP rats. These observations indicate

an association between innate upregulation of the N/OFQ-NOP system and high alcohol preference. Supporting this possibility, NOP receptor blockade with selective antagonists markedly reduced alcohol intake in msP rats (Borruto et al., 2020). This effect, however, also extended to unselected lines of rats and mice (Brunori et al., 2019; Rorick-Kehn et al., 2016). In summary, consistent with the complex polygenic nature of AUD, genetic data indicate that the selection of msP rats has been accompanied by alterations of several genes that affect the pharmacokinetic and pharmacodynamic properties of alcohol.

3.2. Behavioral characterization of msP rats

If exposed to a standard home-cage two-bottle choice (2BC; 10%, v/v, alcohol vs. water) regimen with continuous access for 24 h/day, msP rats drink approximately 7–8 g/kg of alcohol daily (Ciccocioppo et al., 1999, 2006). Drinking occurs mostly during the dark phase of the light/dark cycle, during which they consume around 80% of their daily alcohol. Drinking is organized into bouts. The largest bout occurs within the first hour of the dark phase. The second large bout occurs in the middle of the dark phase, and a third bout usually occurs immediately before the new light phase begins. These drinking bouts produce 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). If subjected to operant alcohol self-administration, msP rats exhibit robust lever-responding for alcohol that is acquired spontaneously and much faster than unselected Wistar controls (Ayanwuyi et al., 2013; Cannella et al., 2016; Domi et al., 2019). Moreover, compared with Wistar rats, if subjected to a progressive-ratio (PR) schedule of reinforcement, msP rats reach a significantly higher breakpoint for alcohol, suggesting stronger motivation for alcohol (Ciccocioppo et al., 2006; Domi et al., 2019). In msP rats, the intragastric (IG) administration of 0.7–1.5 g/kg alcohol produces the expression of significant conditioned place preference (CPP; Ciccocioppo et al., 1999a,b,c). In Wistar rats, the administration of these doses of alcohol leads to conditioned aversive responses (Fidler et al., 2004).

In addition to a high alcohol drinking phenotype compared with Wistar controls, msP rats have a higher propensity to relapse when exposed to stimuli that predict alcohol availability or in response to stress (Ayanwuyi et al., 2013; Cannella et al., 2016; Ciccocioppo, 2013; Ciccocioppo et al., 2004, 2014; Cippitelli et al., 2008; Fotio et al., 2020; Stopponi et al., 2013).

Environmental contexts, such as alcohol-paired cues, are well known to be determining factors that can trigger relapse in alcoholic patients (Cooney et al., 1997; Martin-Fardon and Weiss, 2013). Likewise, msP rats that are trained to self-administer 10% alcohol in the presence of discriminative cues, following a withdrawal period during which lever pressing is extinguished, resume a marked level of responding when re-exposed to alcohol cues in the absence of the primary reinforcer (Ciccocioppo, 2013; Ciccocioppo et al., 2006). Similar results have also been reported in heterogeneous Wistar rats (Augier et al., 2016; Bachteler et al., 2005). However, the magnitude and persistence of the reinstating effect of alcohol-associated cues is much higher in msP rats than in heterogeneous Wistar rats (Ciccocioppo et al., 2006). A similar phenomenon has also been shown in other lines of

genetically selected alcohol-preferring rats, such as P and sP rats (Ayanwuyi et al., 2013; Ciccocioppo, 2013; Koob et al., 2005; Vengeliene et al., 2003).

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). For example, in a self-administration study with an implemented extinction-reinstatement paradigm, stress that was induced by intermittent footshock reinstated the alcohol-paired response in both msP and Wistar rats (Hansson et al., 2006). However, msP rats had the highest level of reinstatement after a 0.3 mA electric shock, whereas the same response in Wistar rats was achieved at a current intensity between 0.6 and 1.0 mA. At this current intensity, msP rats exhibited marked freezing behavior, thus demonstrating higher sensitivity to footshock stress compared with their Wistar counterparts. The high stress sensitivity in msP rats was also revealed in other behavioral paradigms, including the forced swimming test, elevated plus maze, open field test under unfamiliar conditions, and the defensive and marble burying tests (Ayanwuyi et al., 2013; Cippitelli et al., 2015; Natividad et al., 2017; Stopponi et al., 2018). Biochemical and electro-physiological data indicate that the hyper-anxious, high stress-sensitivity phenotype of msP rats depends on the dysregulation of endocannabinoid signaling in the CeA that is triggered by their innate hyperactivity of the CRF/CRF₁ receptor system (Natividad et al., 2017; Stopponi et al., 2018). Notably, anxiety- and depressive-like symptoms and relatively poor stress coping ability in msP rats are attenuated by voluntary alcohol consumption and repeated intragastric alcohol administration (Ciccocioppo et al., 1999a,b,c; Domi et al., 2019). Altogether, these findings reflect the results of several clinical studies that showed that a large subpopulation of alcoholic patients is characterized by a low ability to engage in adequate stress-coping responses, and alcohol is consumed to ameliorate negative affective symptoms that are associated with anxiety and depression (Koob and Le Moal, 2005).

Following abstinence episodes, AUD patients usually report a greater urge to drink that normally terminates in relapse, followed by severe alcohol intoxication episodes (Boening et al., 2001; McBride et al., 2002; Vengeliene et al., 2005). Resembling humans, rodents that are trained to chronically drink alcohol and subjected to periods of forced abstinence consume higher amounts of alcohol when re-exposed to it. This phenomenon is known as the alcohol deprivation effect (ADE), which has been described in both genetically selected alcohol-preferring rats and in unselected heterogeneous rats. Specifically, msP rats that were exposed to chronic alcohol exhibited a robust ADE when they were returned to alcohol after a forced abstinent period of 10 days (Perfumi et al., 2005). However, similar to unselected rodent lines, the increase in drinking in msP rats is transient and usually returns to baseline levels after a couple of days (Holter and Spanagel, 1999; Vengeliene et al., 2014). Genetically selected alcohol-preferring rats do not appear to differ from unselected heterogeneous stock rats in this regard.

One of the major criticisms of using alcohol-preferring rats, and animal models of AUD in general, is that they only minimally incorporate the diagnostic criteria of the DSM-IV and DSM-5, which are mostly based on interviews and self-report questionnaires that assess the quantity and frequency of drinking and perceived consequences. Another common critique of these models is that only a small proportion of human alcohol users develop

AUD. Laboratory animal experiments, including those that utilize genetically selected rats, are usually performed on the entire subject population without considering individual variability. In an attempt to address these important critiques, we recently developed a model to explore interindividual differences in the propensity to shift from controlled to compulsive alcohol intake between msP rats and unselected Wistar controls. This model was originally developed by Deroche-Gamonet and Piazza for cocaine and has been used for psychostimulants in different laboratories (Belin et al., 2011; Deroche-Gamonet and Piazza, 2004; Spanagel, 2017). This model was named the “0/3crit model of addiction,” based on the DSM-IV diagnostic criteria for addiction (American Psychiatric Association, 2000). It consists of a multidimensional experimental approach that seeks to identify subpopulations of rats that possess vulnerability (3crit) and resilience (0crit) to drug addiction-like behaviors by measuring three traits: (1) inability to refrain from drug seeking, (2) high motivation for the drug, and (3) maintenance of drug use despite negative consequences. By comparing msP and unselected Wistar rats, we adapted this experimental model to characterize their alcohol-addiction phenotype based on the 0/3 crit model (Domi et al., 2019). The results showed significant interindividual variability among both msP and Wistar rats. Only a subset of subjects (~13%) were positive for all three AUD criteria that were tested. Interestingly, the number of msP rats that could be classified as 3crit was three-times higher than Wistar rats (9.5% vs. 3.17%). Conversely, the 0crit group was enriched of Wistar rats. These findings are consistent with human data that show that only a proportion of subjects with a chronic alcohol drinking habit actually develop AUD, with genetic factors accounting for approximately 50% of this progression (Wagner and Anthony, 2002). A secondary finding of the study was that the amount of alcohol that was consumed positively correlated with the expression of anxiety-like behavior in msP rats but not in Wistar rats. This observation further supports the hypothesis that this alcohol-preferring rat line resembles a specific subgroup of AUD patients, in which drinking is motivated by tension-relief purposes. Future studies should assess the predictive validity of this model in pharmacological studies. The development of this model may represent an important advancement in the field because one of the most frequently debated issues in the alcohol research community is whether existing preclinical models effectively mimic AUD patients.

3.3. Sex-related behavioral differences in msP rats

Studies of sex differences in substance use disorders are a largely neglected area of research that is receiving growing attention (Becker and Chartoff, 2019; Becker et al., 2017; Perry et al., 2013). Historical data indicate that the rate 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 social reasons, especially in young people who are trying to be accepted by groups (Buchmann et al., 2010; Buckner et al., 2006; Crutzen et al., 2013; Oscar-Berman et al., 2014; Peltier et al., 2019).

Sex differences in the trajectory of AUD have also been documented in humans and to some extent have been replicated in laboratory animals. For example, women have a common tendency to experience a shift from recreational alcohol use to compulsive drinking more rapidly than men. Female rats are more prone to escalate alcohol consumption than males (Becker et al., 2012, 2017; Perry et al., 2013). Moreover, stress plays an important role in all phases of AUD, but its consequences are more pronounced in females than in males. Women are more likely to relapse in response to stressful events (Greenfield et al., 2007; Hudson and Stamp, 2011; Hyman et al., 2008; Sinha et al., 2006; Walitzer and Dearing, 2006). Moreover, females escalate their drug use more rapidly than males (Anglin et al., 1987; Becker et al., 2017; Bobzean et al., 2014). After a prolonged period of alcohol consumption, women have a higher risk of developing physical pathologies, such as breast cancer, cardiovascular problems, and liver inflammation, than men (Ashley et al., 1977; Smith-Warner et al., 1998; Urbano-Marquez et al., 1995).

Clinical research on AUD currently lacks a sufficient number of gender-related studies, but even more problematic is the situation in preclinical science. In fact, especially in rodents, most studies have been performed only in males. This has resulted in the generation of incomplete data to guide clinical trials (Landis et al., 2012; Zucker and Beery, 2010).

A few years ago, NIH issued a new series of guidelines to emphasize the need to study both males and females also at the preclinical level (Clayton and Collins, 2014; Collins and Tabak, 2014; Fattore and Melis, 2016b). In response to these recommendations, preclinical researchers in the addiction field made substantial efforts to include both sexes in their investigations (for review, see (Becker and Koob, 2016; Fattore and Melis, 2016a). However, the gap of knowledge in studies of sex differences remains large, and the research community should further commit to fill it (Lee, 2018; Sanchis-Segura and Becker, 2016). For this reason, in our laboratory, we recently began a research program to investigate sex differences in response to alcohol in msP rats and heterogeneous Wistar controls and evaluate alcohol-related behaviors in both males and females. Below we report the results of a series of experiments in which adult males and females of both strains were compared for voluntary alcohol drinking and the effects of alcohol on anxiety- and depressive-like responses (for details see Supplementary Materials).

The first series of experiments used the 2BC procedure (choice between water and 10% alcohol). Singly housed male and female msP and Wistar rats were given continuous (24 h/day) access to 10% alcohol and water under free-choice condition. The first phase of the experiment consisted of an acclimation period that continued until baseline drinking was stable. Parallel groups with access to water only were used as controls. At this point, we monitored voluntary fluid consumption at 2, 8, and 24 h by recording the volume of intake from graduated cylinders as previously described (Borruto et al., 2020). Under baseline condition, the level of drinking in Wistar rats was very low, and we could not distinguish any difference between males and females. Conversely, msP rats consumed a high volume of alcohol, and female msP rats took significantly higher amounts of alcohol compared with male msP rats (Fig. 1A–C), whereas preference for alcohol was very high and did not differ between sexes (Fig. 1D). Overall, these findings are consistent with the results of studies in other rat strains, in which higher alcohol consumption was reported in females than in males

(Cailhol and Mormede, 2001; Li et al., 2019); for review, see (Becker and Koob, 2016; Hilderbrand and Lasek, 2018).

In earlier experiments, compared with Wistar rats, we repeatedly observed that msP rats exhibited higher levels of anxiety- and depressive-like symptoms that were attenuated by alcohol drinking. These behaviors have never been systematically explored in female msP rats. Here, we report data from the elevated plus maze, forced swim test and footshock stress response in male and female msP and Wistar rats following exposure to 2BC 10% alcohol drinking or water only. The results showed that both naive male and female msP rats exhibited higher anxiety-like behavior compared with Wistar rats (Fig. 2A and B). However, alcohol drinking reduced anxiety-like behavior in males (Fig. 2A) but not in females (Fig. 2B) in the elevated plus maze test. When the rats were tested in the forced swim test, we found that both male and female msP rats exhibited significantly longer immobility time (a measure of depression) compared with their Wistar counterparts. Alcohol drinking reduced immobility time in msP rats of both sexes (Fig. 3A and B). Lastly, msP rats of both sexes showed higher freezing in response to foot-shock stress compared to unselected Wistar rats. Alcohol consumption attenuated the freezing time both in male and female msP rats (Fig. 4A and B). These latter results expand our earlier findings in male msP rats showing their higher sensitivity to foot-shock stress induced freezing compared to Wistar controls (Cippitelli et al., 2015; Hansson et al., 2006). Most important, as previously demonstrated, these differences are not due to a different pain/sensitivity threshold of these two rat lines (Cippitelli et al., 2015; Hansson et al., 2006). Based on these findings, we hypothesize that male and female msP rats are both characterized by traits that confer negative mood conditions that co-segregated with alcohol drinking during genetic selection. However, the motivation for alcohol in males is probably linked to its ability to attenuate anxiety. In female rats, alcohol drinking appears to be linked to its antidepressant (and possibly anti-stress) properties. Although speculative, this hypothesis is consistent with human data that show that psychiatric comorbidity is different between male and female AUD patients. For example, women alcohol abusers are more likely than men who abuse substances to be diagnosed with post-traumatic stress disorder (Cottier et al., 1992; Kessler et al., 1995). Numerous studies have shown an association between AUD in women and a history of child maltreatment (Anda et al., 2002; Dinwiddie et al., 2000; Fergusson et al., 1996; Wilsnack et al., 1997) and physical and sexual assault in adulthood (Kilpatrick et al., 1997, 2003). Further studies are needed to better characterize the impact of sex differences on alcohol abuse-related behaviors of msP rats. On the other hand, these preliminary findings corroborate the observation that alcohol-related sex differences present in AUD patient can be detected also in msP rats, which support the translational value of this type of investigation.

3.4. Limits and caveats in msP rats

Genetically selected alcohol-preferring rats have been successfully used to explore various aspects of AUD that are impossible to study in humans or unselected rodent lines. On the other hand, alcoholism is a heterogeneous disorder to which several genetic, environmental and personality factors can contribute (Cloninger, 1987; Cloninger et al., 1981; Heilig et al., 2011). Therefore, the complexity of this disease would be hardly represented by

a single animal model. For instance, the msP line, in addition to showing an innate preference for alcohol, is characterized by high-stress sensitivity and proclivity to negative affect, resembling a specific subpopulation of AUD patients that drink to self-medicate from negative mood and stress-relieving purposes. Hence, it is conceivable that msP rats more closely mick this cohort of individuals rather than other AUD subpopulations. If so, the generalization of the findings obtained in msP rats to AUD patients other than those that drink for self-medication and tension relieving purposes may be a mistake. Often, pharmacological experiments demonstrated that the use of this rat line is associated with good predictive validity. For example, acamprosate and naltrexone, two medications approved for AUD, are also effective in reducing the motivation for alcohol in msP rats (Bachteler et al., 2005; Perfumi et al., 2003). Whereas the 5-HT₂ receptor antagonist ritanserin that is not efficacious in humans failed to attenuate drinking also in msP rats (Johnson et al., 1996; Panocka et al., 1993). However, in other circumstances, the validity of the msP model appeared questionable. The most striking example is that of CRF1 receptor antagonists. In fact, genetic, pharmacological and, molecular data in msP rats converged to suggest the therapeutic potential of this class of molecules (Bachteler et al., 2005; Panocka et al., 1993; Perfumi et al., 2005). However, when tested in AUD patients they systematically failed (Kwako et al., 2015; Schwandt et al., 2016).

In msP rats the neurochemical effects of alcohol have never been investigated and the only paper published so far demonstrated that following an amphetamine challenge, msP rats revealed a higher peak in extracellular dopamine levels in the NAc shell compared to the Wistar counterpart (Bifone et al., 2019). In the absence of these data, it is hard to fully clarify the neurochemical mechanisms subserving the high motivation for alcohol of msP rats. In the past, few neurochemical studies have been carried out in the sP line from which the msPs have been derived (Fadda et al., 1999; Richter et al., 2000). Starting three decades ago, msP rats have been re-derived from sP progenitors and we are now at the 91st generation of separate breeding. It is conceivable that during such a long period of separate selection significant *genetic drift* might have occurred making msP and sP rats different at both genotypic and phenotypic levels (Crabbe et al., 2010). Hence, any generalization between msP and sP rats should be taken cautiously.

Clinical studies demonstrated that alcohol abuse during adolescence represents a critical risk factor in the development of alcoholism in adulthood (Amodeo et al., 2017; Bates and Labouvie, 1997; Gilpin et al., 2012; Spear, 2018). In msP rats, spontaneous preference for alcohol appears very early in life making this animal line ideal to investigate the consequences of adolescent drinking. Systematic studies to investigate this phenomenon have not been conducted yet, but considering its importance future efforts should be dedicated to the exploration of this research area.

4. Neuroimaging studies: structural and functional MRI in msP rats

In clinical research, fMRI techniques are widely used to investigate structural and functional brain properties in healthy and pathological states (i.e., AUD). Several developments in the field have allowed the possibility of performing fMRI also in rodents (Bifone and Gozzi, 2012; Gozzi et al., 2011; Zahr and Pfefferbaum, 2017). This technological advancement

allows preclinical researchers to generate data that can be directly translated to humans, thus providing a unique possibility to explore the face and construct validity of an animal model. Over the years, we have conducted a few fMRI experiments in msP rats, some of them directly comparing this rat line with AUD patients (De Santis et al., 2019, 2020).

The first fMRI study in alcohol-naïve msP rats performed basal cerebral blood volume (bCBV) mapping followed by voxel-based morphometry (VBM). We observed a reduction of gray matter (GM) volume in different thalamic and cortical regions in these animals compared with Wistar controls. Particularly striking was the reduction of bCBV, a marker of resting brain function, in cortical regions and the striatum (Gozzi et al., 2013). Consistent with these data, people with a high risk for alcohol abuse and AUD patients during the withdrawal phase exhibited lower metabolism in striatal regions and thalamo-cortical circuits (Kareken et al., 2004; Vollstadt-Klein et al., 2010). Moreover, loss of the GM signal in cortical regions in msP rats was compatible with their innate tendency to exhibit depressive- and anxiety-like symptoms. In fact, lower GM volume in the rostro-dorsal cingulate, which was reminiscent of msP rats, was also observed in patients who suffered from depression and anxiety (Spampinato et al., 2009; van Tol et al., 2010). Overall, these data suggest that some of the morphological and functional features that are observed in msP rats resemble characteristics of AUD patients. A tempting hypothesis is that these characteristics that are present before alcohol exposure may predispose individuals to AUD.

In addition to this innate condition, chronic alcohol consumption is well known to lead to numerous functional, neuro-morphological, and neuro-metabolic deficits in alcoholic patients (Buhler and Mann, 2011). For example, several studies reported lower GM and white matter volumes in heavy drinkers (Buhler and Mann, 2011; Demirakca et al., 2011; Fein et al., 2002; Mechtcheriakov et al., 2007; Pfefferbaum et al., 1995; Rando et al., 2011). These abnormalities are particularly pronounced in the frontal lobes, but conspicuous brain atrophy has also been reported in limbic areas and the cerebellum (Fein et al., 2006; Makris et al., 2008). In a recent translational study that used a diffusion tensor imaging (DTI) approach, we observed similar microstructural changes in white matter in AUD patients and msP rats following a period of voluntary alcohol drinking (De Santis et al., 2019). These changes continued to progress over the first 2–6 weeks of abstinence in both humans and msP rats, suggesting an underlying process that continues to evolve, even after alcohol cessation. In a subsequent imaging study, we found that chronic alcohol consumption produced a widespread increase in mean diffusivity (MD) in GM in both alcohol-preferring rats and alcoholic patients, which persisted into early abstinence (De Santis et al., 2020). We found that these alterations were associated with a marked decrease in extracellular space tortuosity that was linked to an increase in microglial reactivity in msP rats. A mathematical elaboration that was applied to the results of these imaging experiments indicated that such changes in MD can facilitate the extra synaptic release and extracellular propagation of neurotransmitters like dopamine. This may contribute to an increase in the rewarding effects of alcohol and the motivation for alcohol that is a common feature in chronic drinkers and genetically selected alcohol-preferring rats.

One socially and scientifically debated question is whether the predisposition to alcohol dependence confers vulnerability to addiction to other substances of abuse. To test

this hypothesis, we combined functional brain imaging, neurochemistry, and behavioral techniques in a group of msP rats and evaluated their response to psychostimulants (Bifone et al., 2019). The results revealed that msP rats that were challenged with an intravenous injection of D-amphetamine exhibited a higher level of activation of regions of the extended amygdala, detected by fMRI, compared with Wistar controls. This higher activation was associated with an increase in the extracellular release of dopamine in the nucleus accumbens shell and a higher propensity to escalate operant cocaine self-administration (Bifone et al., 2019). Altogether, these results support the hypothesis that there are some common genetic traits that predispose individuals to the development of substance use disorders, independent of the drug used. Considering the importance of this observation, it will be important to conduct further studies to confirm this initial observation.

5. Concluding remarks

Alcohol use disorder is one of the major burdens of disease at global level. Despite its impact on society, however, few Food and Drug Administration- or European Medicines Agency-approved pharmacotherapeutic options are available for its treatment. These include disulfiram, naltrexone, and acamprosate and in Europe also nalmefene. Moreover, clinical experience has demonstrated that their efficacy is limited to only certain subgroups of patients (Heilig and Egli, 2006). The successful development of these medications provides a proof-of-concept that supports the feasibility of drug development programs for AUD. This optimism, however, is tempered by numerous failures with several drugs that showed efficacy in laboratory animals but not in humans. The most striking negative experience involves CRF₁ receptor antagonists. In fact, a wealth of preclinical data that were generated over 30 years demonstrated remarkable efficacy of this class of molecules in reducing excessive drinking and relapse to alcohol seeking and preventing negative symptoms that are associated with alcohol withdrawal (Zorrilla et al., 2013).

Genetic and behavioral studies in msP rats have consistently supported the potential of CRF₁ receptors as a promising treatment target for AUD. For instance, msP rats overexpress CRF₁ receptors in the brain, triggered by two SNPs in the promoter region of the respective gene (Ayanwuyi et al., 2013; Cippitelli et al., 2015; Hansson et al., 2006; Logrip et al., 2018). Similar evidence was obtained in human genetic studies that showed that CRF₁ receptor gene polymorphisms are associated with binge drinking in AUD patients (Chen et al., 2010; Treutlein et al., 2006). Gene variations at the *Crh1R* locus was also reported in a Caucasian population with a diagnosis of AUD (Chen et al., 2010; Treutlein et al., 2006). Data suggest that these mutations conferred to msP rats higher sensitivity to the pharmacological blockade of CRF₁ receptors. For example, CRF₁ receptor antagonists are more effective in attenuating alcohol drinking, stress-induced relapse, and anxiety that is associated with alcohol abstinence in this rat line than in Wistar controls (Ciccocioppo, 2013; Ciccocioppo et al., 2006). This negative experience raises the general question of whether animal models of AUD, more specifically genetically selected alcohol-preferring rats, have adequate predictive validity. Importantly, AUD is a heterogeneous disorder to which multiple genetic factors, environmental conditions, and cultural experiences can contribute. It is unlikely, therefore, that a single animal model (e.g., genetically selected alcohol-preferring rats) can mimic all these complexities. More reasonably, animal models are useful for studying specific aspects

of AUD. For instance, msP rats may mimic conditions under which drinking is triggered by the necessity to self-medicate a negative affective state. To overcome these limitations, we believe that preclinical research should refine its approach to study AUD and other complex psychiatric pathologies. Preclinical experiments should incorporate key elements that have emerged from human studies, including individual vulnerability. In fact, among alcohol users, only a small proportion of individuals develop AUD over time. It is unlikely that this condition can be captured in preclinical studies in which experimental subjects are tested together as a homogeneous group without distinguishing them based on their individual response to alcohol. To overcome this limitation, we recently proposed the use of the 0/3crit model to study AUD based on DMS-5 criteria, including individual animals' propensity to exhibit (1) an inability to refrain from drug seeking, (2) high motivation for alcohol, and (3) the maintenance of drug use despite negative consequences.

Moreover, it is imperative to integrate preclinical research with experiments that explore sex differences in the response to alcohol and related pharmacological treatments. In fact, human studies have very clearly documented that men and women may initiate drinking for completely different reasons and may respond differently to alcohol and AUD medications (Buchmann et al., 2010; Buckner et al., 2006; Crutzen et al., 2013; Oscar-Berman et al., 2014; Peltier et al., 2019). Furthermore, not all individuals with AUD are the same. This disorder is a very heterogeneous condition and associated with multiple endophenotypes. A valid animal model should not be proposed as a phenocopy of AUD in general. Rather, it should simulate specific endophenotypic characteristics that resemble distinct subpopulations of patients. If such distinctions are not considered, then such preclinical models would not be endowed with sufficient predictive, face, or construct validity. Finally, future studies should expand the use of *in vivo* brain imaging in laboratory animals because these techniques allow direct comparisons between the results from animal models and humans.

Over the years, preclinical research on AUD has advanced substantially, and the use of alcohol-preferring rats has made important contributions to this effort. However, there is still much room for improvement to reduce the risks of failure when translating from preclinical studies to the human condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2BC	two-bottle choice
5-HT_{2A}	serotonin 5-hydroxytryptamine-2A

AA	Alko Alcohol-preferring rat line
ADE	alcohol deprivation effect
ADH	aldehyde dehydrogenase
AUD	alcohol use disorder
ANA	Alko Nonalcohol-preferring rat line
BAL:	blood alcohol level
bCBV	basal cerebral blood volume
BNST	bed nucleus of the stria terminalis
CeA	central nucleus of the amygdala
CPP	conditioned place preference
CRF	corticotropin-releasing factor
CRF₁	corticotropin-releasing factor 1 receptor
DTI	diffusion tensor imaging
DSM	Diagnostic and Statistical Manual of Mental Disorders
GABA	γ -aminobutyric acid
GM	gray matter
HAD	High Alcohol Drinking rat line
IG	intra-gastric
MD	mean diffusivity
LAD	Low Alcohol Drinking rat line
MRI	magnetic resonance imaging
msP	Marchigian Sardinian alcohol-preferring rat line
NIH	National institutes of Health
N/OFQ	nociceptin/orphanin FQ
NOP	nociception/orphanin FQ receptor
NP	Alcohol Non-preferring rat line
OPRM-1	opioid receptor mu 1
OPRL-1	opioid-related nociceptin receptor 1
P	alcohol-preferring rat line

PR	progressive ratio
sNP	Sardinian alcohol non-preferring rat line
SNP	single nucleotide polymorphism
sP	Sardinian alcohol-preferring rat line
UChA	Universidad de Chile low alcohol-drinking rat line
UChB	Universidad de Chile “Bebidores” high alcohol-drinking rat line
VTA	ventral tegmental area
WHO	World Health Organization

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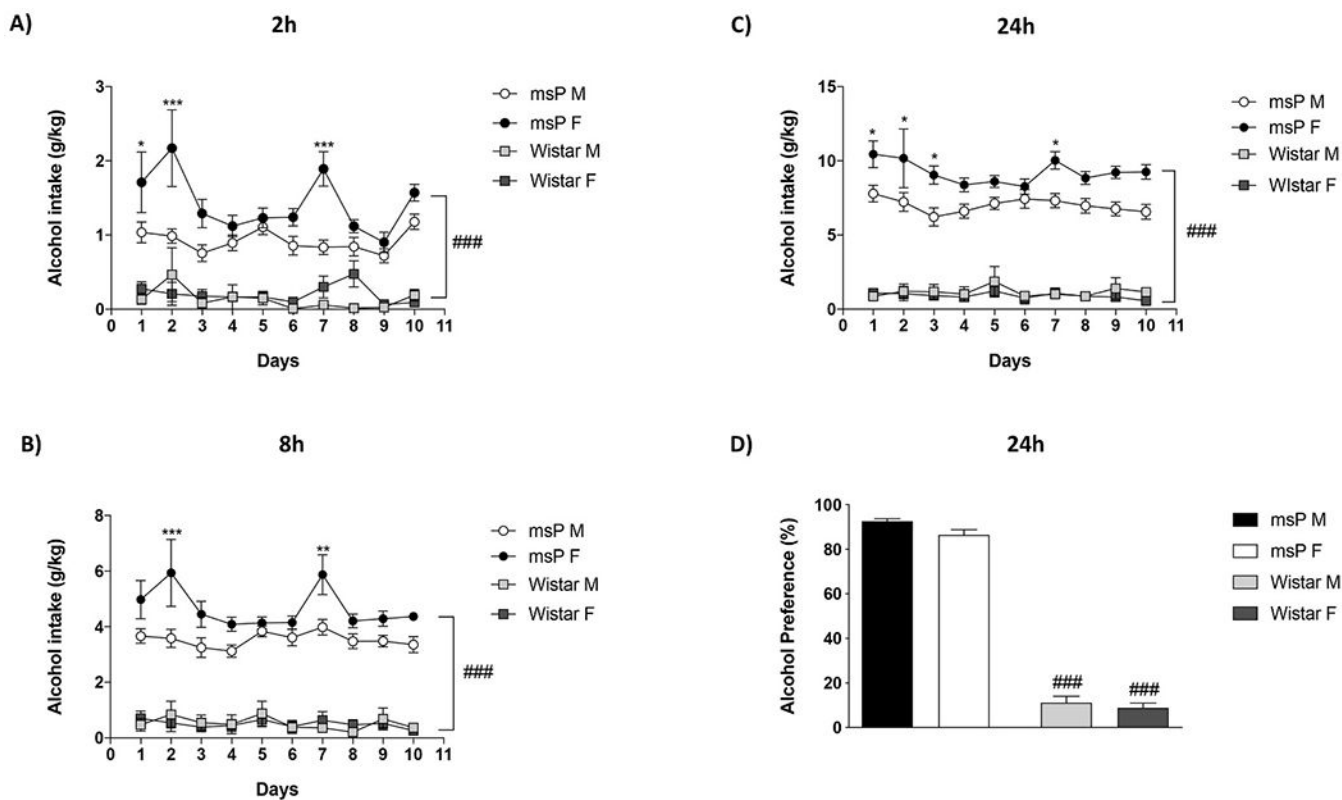
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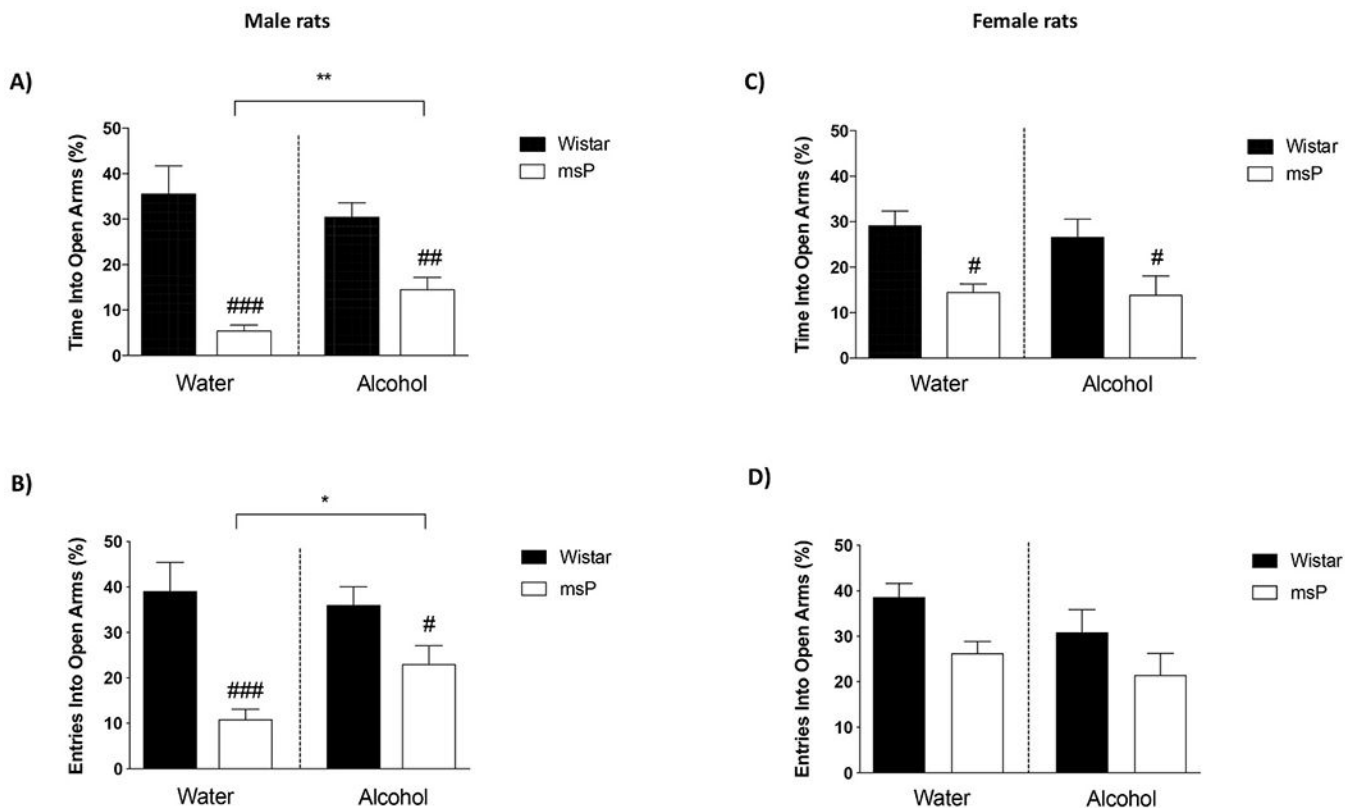
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**Fig. 1.**

Voluntary 10% alcohol intake in male msP ($n = 14/\text{group}$) and Wistar ($n = 12/\text{group}$) rats and female msP ($n = 10/\text{group}$) and Wistar ($n = 12/\text{group}$) rats at (A) 2 h (B) 8 h, and (C) 24 h. Alcohol intake is expressed as g/kg to reduce the influence of differences in body weight. Alcohol preference is expressed as the mean percentage \pm SEM of the last 3 days/24 h of alcohol intake over water (D). The data are expressed as mean \pm SEM. ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$, difference between msP and Wistar; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, difference between males and females (three-way ANOVA followed by Newman-Keuls *post hoc* test).

**Fig. 2.**

Anxiety-like behavior in (A–B) male msP ($n = 14/\text{group}$) and Wistar ($n = 12/\text{group}$) rats and (C–D) female msP ($n = 10/\text{group}$) and Wistar ($n = 12/\text{group}$) rats in the elevated plus maze. The data are expressed as the mean percentage \pm SEM of open arm time and open arm entries. ### $p < 0.001$, ## $p < 0.01$, difference between msP and Wistar; ** $p < 0.01$, * $p < 0.05$, difference between alcohol and water (two-way ANOVA followed by Newman-Keuls *post hoc* test).

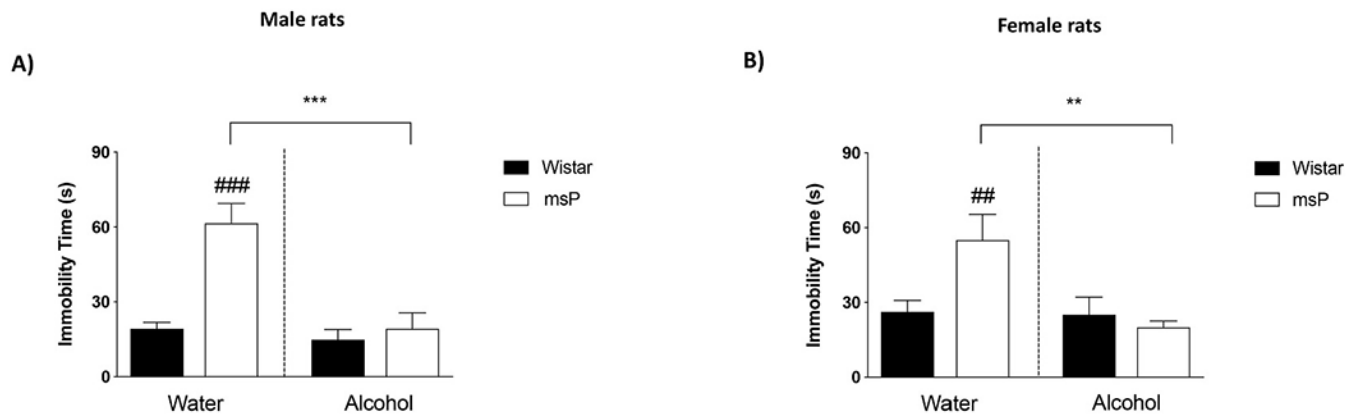


Fig. 3. Depressive-like behavior in (A) male msP ($n = 14/\text{group}$) and Wistar ($n = 12/\text{group}$) rats and (B) female msP ($n = 10/\text{group}$) and Wistar ($n = 12/\text{group}$) rats in the forced swim test. The data are expressed as mean \pm SEM. ### $p < 0.001$, ## $p < 0.01$, difference between msP and Wistar; *** $p < 0.001$, ** $p < 0.01$, difference between alcohol and water (two-way ANOVA followed by Newman-Keuls *post hoc* test).

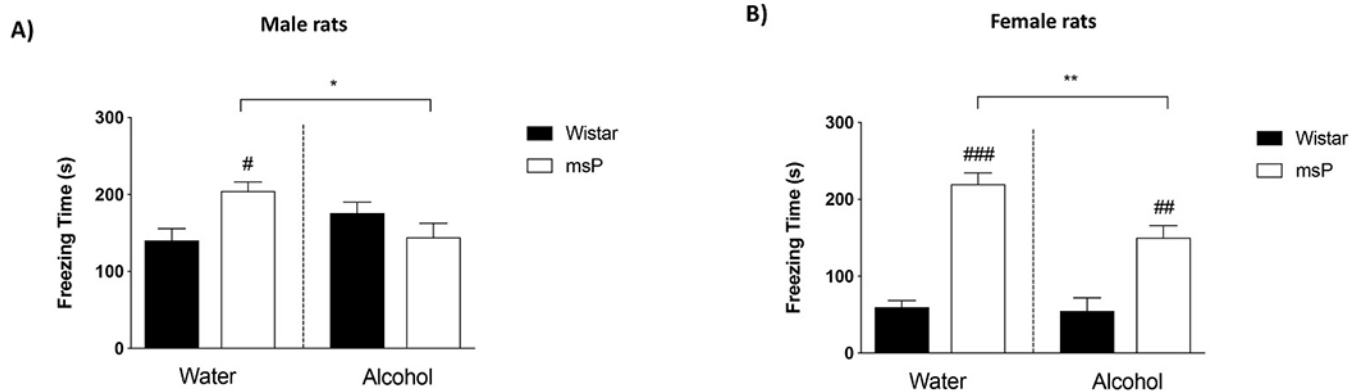


Fig. 4. Foot-shock induced freezing in (A) male msP ($n = 10/\text{group}$) and Wistar ($n = 10/\text{group}$) rats and (B) female msP ($n = 10/\text{group}$) and Wistar ($n = 10/\text{group}$) rats. The data are expressed as mean \pm SEM of the total time spent in freezing during 7 consecutive 1-min blocks. ### $p < 0.001$, # $p < 0.05$, difference between msP and Wistar; ** $p < 0.01$, * $p < 0.05$, difference between alcohol and water (two-way ANOVA followed by Newman-Keuls *post hoc* test).

Table 1

Summary of the key genetic, behavioral and neuroimaging studies on msP rats.

Type of study	Result	Reference
Genetic	msP rats show innate upregulation of CRF1-R expression and density in multiple corticolimbic regions.	Hansson et al. (2006)
Genetic	Voluntary alcohol consumption produces down-regulation of <i>Crhrl</i> transcript levels in multiple corticolimbic regions.	Hansson et al., 2007
Genetic	Genetic polymorphism in the CRF1-R promoter region is associated to an increased sensitivity to the effects of the pharmacological blockade of CRF1-R.	Ayanwuyi et al., 2013
Genetic	1 Polymorphism in the CRF1-R gene plays a role in shaping the high anxious phenotype of msP rats.	Cippitelli et al. (2015)
Genetic	msP rats show innate upregulation of N/OFQ and NOP mRNA expression in multiple corticolimbic regions.	Economidou et al., 2008
Behavior	Access to alcohol reduce the anxious and depressive phenotype of msP rats.	Ciccocioppo et al., 1999
Behavior	Alcohol induces conditioned place preference in msP rats.	Ciccocioppo et al., 1999
Behavior	msP rats are highly motivated to lever press for alcohol and show high level of seeking behavior in response to stress and environmental cues.	Ciccocioppo et al., 2004; Cippitelli et al., 2008
Behavior	msP rats exhibit a robust ADE when they are returned to alcohol after a forced abstinent period.	Perfumi et al., 2005
Behavior	Characterization of alcohol-addiction phenotype in msP rats based on the 0/3 crit model.	Domi et al. (2019)
Neuroimaging	Innate reduction of gray matter volume in different thalamic and cortical regions in msP rats.	Gozzi et al. (2013)
Neuroimaging	Microstructural changes in white matter after voluntary alcohol drinking associated to a decreased extracellular space tortuosity.	De Santis et al., 2019; De Santis et al., 2020
Neuroimaging	Amphetamine challenge induces strong functional responses in the extended amygdala of msP rats.	Bifone et al. (2019)