PHYSIOLOGY

Sex-dependent effects of chronic intermittent ethanol treatment in Wistar rats

Mariia Dorofeikova[1,2], Irina Antonova[1], Galina Gromova[1], Anastasia Veraksa[1], Elena Filatova[1], and Alexei Egorov[1]

[1]Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, pr. Toreza, 44, Saint Petersburg, 194223, Russian Federation
[2]Center for Molecular Neurobiology Hamburg, Falkenried, 94, Hamburg, 20251, Germany

Address correspondence and requests for materials to Mariia Dorofeikova, mvdorofeykova@gmail.com

Abstract

Multiple behavioral parameters are associated with vulnerability to alcohol dependence, but the contribution of such factors as cognitive flexibility and sex differences still require further clarification. Our goal was to assess how sex differences mediate relationships between cognitive flexibility, affective state, hierarchy and ethanol preference before and after chronic intermittent ethanol (CIE) exposure in Wistar rats. The results of our study revealed sex-dependent changes in ethanol preference and locomotor activity throughout the time. Anxiety levels were sex-dependently affected by ethanol exposure with a dramatic decrease in ethanol-exposed females. Similarly, striatal tyrosine hydroxylase expression was affected by CIE in a sex-dependent manner, with ethanol-exposed females having higher expression than males. Baseline cognitive flexibility was better in rats with higher locomotor activity and lower anxiety levels but did not correlate with ethanol preference in rats. Taken together, these results are important for understanding sex-dependent alterations in brain and behavior induced by alcohol. This provides important insights into understanding sex differences in the risk for mental disorders.

Keywords: chronic intermittent ethanol exposure, ethanol preference, alcohol, sex differences, anxiety, cognitive flexibility, tyrosine hydroxylase, striatum, rat.

Citation: Dorofeikova, M., Antonova, I., Gromova, G., Veraksa, A., Filatova, E., and Egorov, A. 2023. Sex-dependent effects of chronic intermittent ethanol treatment in Wistar rats. Bio. Comm. 68(3): 164–172. https://doi.org/10.21638/spbu03.2023.304

Authors' information: Mariia Dorofeikova, PhD, Researcher, orcid.org/0000-0002-2181-2281; Irina Antonova, Junior Researcher; Galina Gromova, Junior Researcher; Anastasia Veraksa, Junior Researcher; Elena Filatova, PhD, Leading Researcher, orcid.org/0000-0003-1321-1053; Alexei Egorov, PhD, Professor, Head of Laboratory, orcid.org/0000-0002-5737-8560

Manuscript Editor: Anna Malashicheva, Laboratory of Regenerative Biomedicine, Institute of Cytology, Russian Academy of Sciences, Saint Petersburg, Russia; Laboratory of Molecular Cardiology, Almazov National Medical Research Centre, Saint Petersburg, Russia

Received: March 15, 2023;

Revised: July 30, 2023;

Accepted: August 31, 2023.

Funding: The work was carried out with the support of the State Program, topic No. 075-0152-22-00.

Ethics statement: All animal procedures were performed in accordance with institutional guidelines approved by the Bioethical Committee of the I. M. Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences (No. 4-2 of 28.04.2022) and based on the European Community Directive on the humane treatment of Experimental Animals.

Supplementary information: Supplemental material to the article is available at https://doi.org/10.21638/spbu03.2023.304. Supplementary files are published as submitted by the authors, and are not copyedited.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Substance use disorders are a major public health concern, with alcohol abuse and dependence affecting about 8 % of the world's population, causing significant social problems and financial costs. Given the clear relationship between alcohol consumption and its adverse effects, identifying its determinants, including social, demographic, genetic and other determinants, is an important task. These factors interact with each other to determine consumption patterns and negative consequences associated with alcohol (Collins, 2016). Although increasing attention has been paid in recent decades to understanding the neurobiological underpinnings of abuse and improving the pharmacological treatments for some addictions, prevention and treatment methods remain limited. Research is needed to understand the processes responsible for the development of ethanol preference and alcohol dependence.

Cognitive flexibility, the ability to adjust behavior in the face of changing circumstances, is impaired in many psychiatric and neurological disorders. In the context of addiction, cognitive flexibility may be associated with the transition from recreational to compulsive drug use. While drugs produce strong pleasurable effects that lead to initial use, at some point these pleasurable effects are balanced and overwhelmed by the negative effects of addiction (Koob and Volkow, 2010). Therefore, people with higher cognitive flexibility can be expected to stop using drugs when the negative effects outweigh the positive effects.

Research shows that a significant number of substance addicts have severe cognitive deficits; neurocognitive deficits in chronic alcohol use are also well documented and include impairments in memory, visuospatial processing, problem solving, and executive function (Glass et al., 2009). Studies in people suffering from alcohol dependence have shown that certain cognitive areas, in particular the executive functions regulated by the prefrontal cortex (PFC), are impaired in the early stages of abstinence and then slowly recover with prolonged abstinence. Alcohol abusers show lower scores in response inhibition and cognitive flexibility (Bickel et al., 2012). Chronic intermittent exposure to ethanol has previously been shown to impair cognitive flexibility in mice. These changes correlate with changes in N-methyl-D-aspartate receptor (NMDAR) function, causing impairment of synaptic plasticity in PFC (Kroener et al., 2012). In particular, the orbitofrontal cortex may be particularly sensitive to alcohol and play a role in alcohol-related cognitive impairment.

Sex differences may play a critical role in modulating how alcohol use impacts the brain to cause the development of alcohol use disorder (AUD). On the behavioral level, female rats show more appetitive approach to obtain the alcohol reward, consuming more alcohol relative to body weight and engaging in higher levels of cue-mediated alcohol-seeking behaviors compared to males (Aguirre et al., 2020; Flores-Bonilla and Richardson, 2020). Female, but not male, mice exhibit compulsive-like ethanol seeking in a modified conditioned place preference paradigm in which a previously ethanol-paired chamber is also associated with a foot shock (Xie, Buck, Bryant, and Baker, 2019). Further, this compulsive-like ethanol seeking that persists despite an aversive experience was attenuated in females with a history of chronic ethanol exposure (Xie, Buck, Bryant, and Baker, 2019), suggesting that chronic ethanol exposure enhanced sensitivity to aversive experiences in females.

On anatomical level, the anterior cingulate cortex is more negatively impacted by alcohol drinking in adolescent male rodents and young men compared to adolescent female rodents and young women. Also, binge drinking is associated with lower cortical volume and thickness in adolescent boys. On the other hand, alcohol impairs cell proliferation and reduces the number of granule neurons in the dentate gyrus of the hippocampus to a greater extent in females (Flores-Bonilla and Richardson, 2020). Recent transcriptional studies suggest that some sex differences of ethanol consumption may be due to female-dependent remodeling of the primary cilium (Hitzemann et al., 2022). Sex-specific binding of alcohol to choline carriers in hippocampus of male adolescent rats can contribute to the inhibition of acetylcholine synthesis and to neurotoxic effects of alcohol specifically in young males (Kristofiková, Platilová, and Klaschka, 2003).

The effects of gonadal hormones may provide an insight into the molecular mechanisms underlying sex differences in the rewarding properties of alcohol. Alcohol increased excitation of ventral tegmental area dopamine neurons in brain slices from mice of all hormonal conditions, but the effects were most robust when estradiol levels were moderate or high, which enhanced the rewarding effects of alcohol. Male rats and mice show a more pronounced display of negative affective-like behaviors and neural activity after withdrawal from chronic alcohol exposure compared to females. Alterations in glutamate signaling from the stria terminalis projecting into the basolateral amygdala are thought to mediate these behavioral differences. Men in particular exhibit higher levels of alcohol craving than women do, and cravings are associated with an increased activity in the striatum in men, but not in women. Like humans, male rodents appear more susceptible to relapse than females (Flores-Bonilla and Richardson, 2020). Many sex differences were found in the glutamatergic receptor expression. For example, female, but not male rats exhibit increased expression of the NR1 subunit of the NMDA receptor in the cerebral cortex as early as 3 days following exposure to a liquid diet containing ethanol. In contrast, males exhibit an increase in NR1 subunit expression in the hippocampus. Ethanol withdrawn female rats exhibit a significant increase in NR1 in the cerebral cortex and hypothalamus compared to male rats and control females. NR2B expression increases in the cortex of ethanol-withdrawn female, but not male rats (Giacometti and Barker, 2020).

In humans, there is some evidence of increased alcohol intake in females during the premenstrual stage of the menstrual cycle and increased withdrawal during the luteal phase. In rodents, progesterone decreases drug-seeking behavior and estradiol increases drug-seeking behavior. The decreased alcohol withdrawal response in females appears to be directly linked to the presence of progesterone and its metabolites in females and their actions on GABAergic neurotransmission, since they have higher CRF and dynorphin responses (Becker and Koob, 2016).

Speaking about the intersection of alcohol use, anxiety and sex differences, women exhibit greater sensitivity to stress-induced alcohol craving than men (Giacometti and Barker, 2020). Adolescent girls are more likely than boys to report drinking alcohol to alleviate stress, social isolation, and psychological distress. Similarly, female mice are more sensitive to the anxiolytic effects of experimenter-administered alcohol compared to males (Flores-Bonilla and Richardson, 2020). Women show higher rates of both depression and anxiety than men among those with alcoholic cardiomyopathy. Depression and anxiety could stem from alcohol use or be a factor that promotes it, further highlighting the complex interaction between sex and other factors that

drive and sustain alcohol use (Zachry, Johnson, and Calipari, 2019). Alcohol consumption has been found to differentially modulate oscillatory states in basolateral amygdala in male and female mice, a process that involves δ-GABAARs, the expression of which depends on ovarian hormones (DiLeo, Antonoudiou, and Maguire, 2022). BNST CRF neurons could also be the source of high extrahypothalamic CRF tone that predispose females to the co-expression of alcohol use and anxiety disorders (Pleil and Skelly, 2018).

Both clinical and preclinical studies demonstrate heightened susceptibility to stress-induced drinking in females. As reviewed by Mineur et al. (2022), several mechanisms may contribute to estrogen effects on alcohol consumption. Estrogen heightens ethanol sensitivity of dopamine neurons, with higher response of dopamine neurons to ethanol in ovariectomized mice following estradiol replacement. Also, stress-related increase in GABAergic transmission may occur in CeA in females and males through different mechanisms. Unlike male neurons, cells from female rats displayed reduced sensitivity to alcohol's inhibitory effects, and there are sex differences in the response of amygdala subnuclei to corticosterone which may promote sex differences in stress-induced alcohol intake (Logrip, Oleata, and Roberto, 2017).

Many researchers have focused on the basics of cognitive flexibility, but few have used in vivo methods to assess neuronal activity, especially in models of alcohol use disorders. Using patch clamp electrophysiology, W. Hu et al. have shown that chronic ethanol use increases presynaptic neurotransmitter release and enhances the postsynaptic function of the N-methyl-D-aspartate receptor in the medial PFC (Hu, Morris, Carrasco, and Kroener, 2015). S. Kroener used a mouse model of chronic intermittent ethanol exposure (CIE) to study how it alters plasticity in the medial PFC and showed that there was an increase in the NMDA/AMPA ratio in excitatory postsynaptic currents in layer V pyramidal neurons and altered expression of long-term potentiation. The authors demonstrated impairments in the executive function that may be associated with changes in synaptic plasticity in the medial PFC (Kroener et al., 2012).

Anxiety and stress disorders are highly associated with alcohol abuse, suggesting the possibility of common pathophysiological mechanisms or associated dysfunction of neural networks. There are conflicting data on the effect of chronic ethanol exposure on the basal function of the cortico-limbic areas of the brain; both excessive activation of the amygdala in alcohol-dependent rats and humans due to a loss of downstream modulation of PFC has been demonstrated, as well as an inverse effect in a mouse study (a decrease in central amygdala activity and an increase in PFC excitation) (Pleil et al., 2015).

The question of the relationship between a social rank and cognitive status in rats has been little studied. High working memory scores at 9–11 weeks of age, when social hierarchy is being formed, have been shown to predict higher social status in the future; later, after a period of permanent residence in social conditions, the working memory of dominant and subdominant animals decreases, while the subordinate, on the contrary, increases and begins to exceed the performance of dominant rats (Jaafari Suha, Hosseinmardi, and Janahmadi, 2022). There is evidence that the social rank does not affect alcohol preference (Scott, Tjernström, and Roman, 2020), but the authors of this article did not use forced drinking (only provided alcohol and water of choice for 3 days a week for 4 weeks).

Thus, multiple parameters are associated with vulnerability to alcohol dependence, but the contribution of such factors as cognitive flexibility and sex differences still require further clarification. Our goal was to assess how the sex differences mediate relationships between cognitive flexibility, hierarchy and ethanol preference before and after chronic intermittent ethanol exposure. Additionally, we aimed to determine the effects of ethanol exposure protocol on striatal expression of tyrosine hydroxylase (TH) and its possible role in the sex differences.

Materials and methods

Animals

20 male and 20 female Wistar rats were obtained from the the I. M. Sechenov Institute of Evolutionary Physiology and Biochemistry breeding facility and housed on a 12 h light/dark cycle with ad libitum access to water and chow under standard laboratory conditions ($545 \times 395 \times 200$ mm cages with sawdust shavings, relative humidity of 60–70 % and a temperature of 23 ± 2 °C). Rats were pair-housed 3 weeks before the start and all throughout the experiments. Experiments were performed during the light phase. Although there is some evidence of estrous phase-dependent ethanol intake (Becker and Koob, 2016), several recent papers demonstrated that individual variations between animals are much more important than estrous state (Levy et al., 2023; Lovick and Zangrossi, 2021). Because of this and also the long-term ethanol treatment that greatly surpassed estrous cycle length, we did not track the estrous state of females. 3 male and 3 female control rats for histological analysis were kept in the same conditions in the animal facility, but were not subjected to any behavioral experiments.

Experimental design

At the beginning of the experiment, 13-week-old rats were subjected to behavioral testing in the elevated plus maze and then in the Morris water maze with the reversal learning. After a two-bottle ethanol preference test, chronic intermittent ethanol treatment was started in half

of the animals. After 3 weeks, two-bottle test was performed again to assess the level of ethanol preference, and rats were retested in the elevated plus maze and then in the Morris water maze. After decapitation of the animals, nucleus accumbens was isolated to assess the levels of tyrosine hydroxylase. As an additional control group for behavioral manipulations, we used 3 male and 3 female rats to analyze their tyrosine hydroxylase levels.

Chronic intermittent ethanol (CIE) treatment

Rats were exposed to a CIE exposure regimen consisting of 10 doses of 5 g/kg ethanol (35 % v/v in saline at 18.12 mL/kg) or isovolumetric saline administered by intragastric gavage using a 2-days-on, 2-days-off intermittent schedule for 20 days followed by a 20-day washout period (Risher et al., 2013).

Behavioral tests

To assess the level of anxiety, the elevated plus maze (EPM) test was used (Walf and Frye, 2007). The maze consists of a cross-shaped platform raised 1 m above the floor level with a central area of 10 × 10 cm and four arms with a length of 50 and a width of 10 cm each. While two arms are open, the other two are closed with high side walls. The animal was placed in the center of the maze, and the time spent in the center, open and closed arms of the maze, as well as the length of the path traveled in different compartments were recorded for 3 minutes.

Spatial navigation and cognitive flexibility were assessed using the Morris water maze (MWM) (Menezes et al., 2020). The maze consisted of a white circular pool 2 m in diameter divided into four equal quadrants with four visual stimuli hanging on the walls to provide spatial cues. A white circular escape platform 12 cm in diameter was placed 2 cm beneath the surface of the 22–24 °C water colored opaque with powdered non-fat milk in a specific quadrant. The swimming path of the animals was recorded using a video camera mounted above the center of the pool and analyzed using a video tracking and analysis system (Microsoft LifeCam HD-3000 camera, Bandicam software, custom tracking app). Training was carried out during 5 successive days, 8 trials per day. A different starting location was used on each trial, which consisted of a swim followed by a 30 s platform sit. Any rat that did not find the platform within 60 s was guided to it by the experimenter. 24 h after the last training session rats were submitted to eight 60 s reversal training trials, in which the platform was placed in the opposite quadrant of the pool. This procedure requires cognitive flexibility from the animals, since they need to learn that the platform is moved to a different place. Cognitive flexibility/memory retention was evaluated in a probe test carried out 24 h after the last reversal trial. Escape latencies were averaged across the trials of each day.

Food competition test adapted from Costa, Moita, and Márquez (2021) was performed in the home-cage of non-food restricted pairs of animals. Before the test, rats were habituated to chocolate-flavored pellets for 4 days to reduce neophobic responses to the food. Food competition in a social context was performed for two consecutive days. Rats had access to 10 pellets for 2 min, and after a 1-min inter-trial interval 10 new pellets were delivered. The procedure was repeated for 5 trials and a total session of 15 min and 50 pellets. Rats which consumed more pellets were considered dominant.

To assess the level of ethanol preference, a two-bottle test was carried out. Rats had access to tap water and a 10 % alcohol solution for 10 min. Total intake of ethanol solution and water was measured. Ethanol preference was calculated as a ratio of ethanol consumption volume to the total volume of consumed liquids.

Histology

Expression of tyrosine hydroxylase enzyme in dopaminergic neurons of nucleus accumbens was assessed in randomly chosen rats (4 CIE+ males, 4 CIE+ females, 3 control males and 4 control females). Fixed brains were cut on a cryostat (Leica CM-1520, Germany) in 20 μm coronal slices. 6 slices per animal were analyzed. Sections were incubated in primary anti-TH antibodies (1 : 1500, Sigma, USA), and the secondary antibody was goat anti-mouse IgG conjugated with biotin (1 : 600, VectorLabs, UK). Images were obtained using a slide-scanning Carl Zeiss Imager A1 microscope (Germany) with an integrated Axiocam 712 video camera and Zen 3.4 (blue edition) software. Tyrosine hydroxylase optical density was quantified in arbitrary units using Image J program (NIH, USA).

Statistical analysis

Data were analyzed using Prism 9 (GraphPad Software, San Diego, CA). The definition of statistical significance was $P \leq 0.05$. To assess the interaction of factors, a 2- or 3-way repeated measures ANOVA, or linear mixed effects modeling for histological data was used. If a significant interaction was detected, Sidak's multiple comparisons test was used, because it assumes independent comparisons and has more power than the Bonferroni method. All statistical tests were two-tailed tests.

Results

A 3-way repeated-measures ANOVA revealed that there was no statistically significant interaction between the effects of sex, time point and ethanol exposure on the ethanol

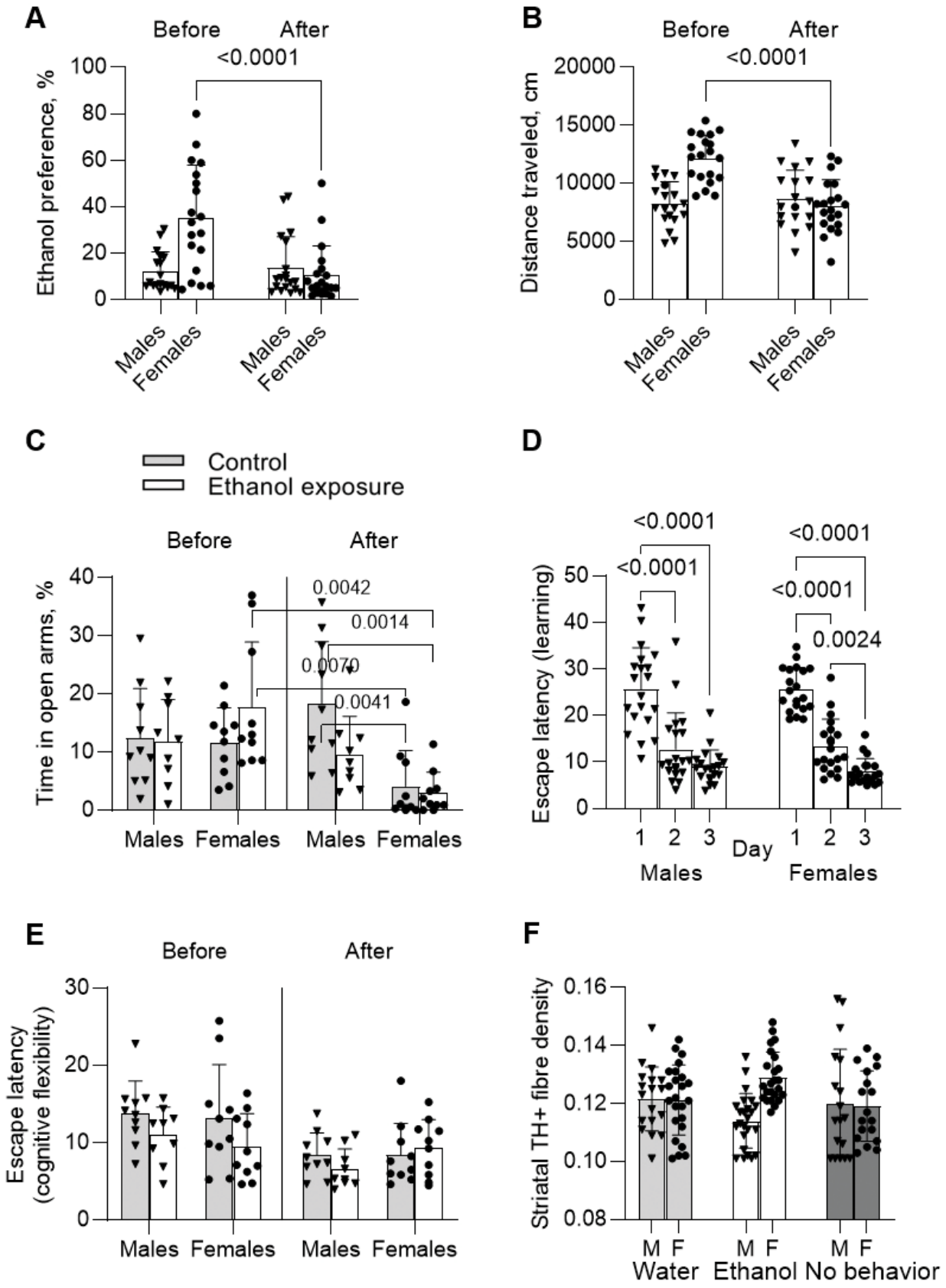

Fig. 1. Changes in ethanol preference, locomotion, anxiety-like behavior, learning and striatal TH+ fibre density during the experiment: *A* — ethanol preference was reduced in the female rats with time; *B* — females' locomotor activity decreased with time; *C* — anxiety-like behavior significantly increased in ethanol-exposed females, and differed significantly between control males and females by the end of experiment, females spending less time in open arms; *D* — rats of both sexes successfully learned to find the hidden platform; *E* — ethanol exposure did not affect cognitive flexibility; *F* — striatal TH+ fibre density was affected by CIE in a sex-dependent manner; example picture from CIE+ female. Data are presented as means ± SEM. Circles — females, triangles — males.

preference measured using the two-bottle test, but it was significantly affected by sex and time point (Suppl. Figure, A; Sex × Time point × Ethanol exposure: F (1, 35) = 0.5917, P = 0.4469; Sex × Time point: F (1, 35) = 14.76, P = 0.0005; Sex: F (1, 35) = 8.584, P = 0.0059; Time point: F (1, 35) = 11.85, P = 0.0015). After combining CIE exposure and control groups, Sex × Time point interaction was found to be significant at F (1, 37) = 15.50, P = 0.0004 (Suppl. Figure, A; 2-way repeated-measures ANOVA), Sex: F (1, 37) = 8.266, P = 0.0067, Time point: F (1, 37) = 12.21, P = 0.0013. Post-hoc Šídák's multiple comparisons test revealed that the change in ethanol preference was significant for the female group of rats (P < 0.0001). During the two-bottle test, female mice consumed 0.56 ± 0.35 ml of ethanol during the initial test, and males consumed 0.71 ± 0.47 ml. The second time, ethanol consumption was at 0.84 ± 1.07 for the female CIE group and 0.32 ± 0.10 for control females, and 0.78 ± 0.94 for the male CIE group and 0.77 ± 0.61 ml for control males.

The distance traveled in the EPM changed similarly, with sex and time interaction effect significant at F (1, 37) = 22.93, P < 0.0001; Sex: F (1, 37) = 10.23, P = 0.0028; Time point: F (1, 37) = 14.63, P = 0.0005 (Suppl. Figure, B). Females' locomotor activity significantly decreased with time (p < 0.0001).

Ethanol exposure significantly affected an anxiety measure of the EPM test, time spent in the open arms (Suppl. Figure, C; 3-way repeated measures ANOVA, Time point × Ethanol exposure: F (1, 35) = 4.683, P = 0.0374). Sex also affected the change in anxiety (it decreased only in females; Sex × Time point: F (1, 35) = 13.80, P = 0.0007), as well as sex (F (1, 35) = 4.561, P = 0.0398) and time (F (1, 35) = 7.034, P = 0.0119) independently. Multiple comparisons showed significant differences between ethanol exposed females before and after CIE procedure (P = 0.004), control groups of rats of different sex at the 2nd time point (P = 0.004), control males and ethanol exposed females at the 2nd time point (P = 0.001), and ethanol exposed females before CIE and control females at the 2nd time point (P = 0.007).

There were no sex differences in the learning in the Morris water maze (Suppl. Figure, D; 2-way repeated measures ANOVA, Training day × Sex: F (2, 76) = 0.311, P = 0.7333), and the performance significantly improved towards training day 3 (main effects analysis, Training day: F (2, 76) = 124, P < 0.0001). Results of a cognitive flexibility test showed only differences between the two time points, pointing towards successful learning and memory in the reversal task (Suppl. Figure, E, 3-way repeated measures ANOVA, Sex × Time point × Ethanol exposure: F (1, 35) = 1.032, P = 0.3165; Time point: F (1, 35) = 17.68, P = 0.0002). No significant differences were found for rats with different social ranks.

Spearman's correlation coefficient was used to assess the relationships between behavioral variables. These correlation analyses included the data from mice from all groups used in this study. Baseline cognitive flexibility was better in rats with higher locomotor activity and lower anxiety levels (escape latency during the reversal × distance traveled: r = –0.433, p=0.005; escape latency during the reversal × time spent in open arms: r = –0.465, p = 0.002). Rats with higher baseline ethanol preference had higher locomotor activity (r = 0.333, p = 0.036). Also, higher velocity was correlated with more time spent in the open arms of the EPM (test 1: r = 0.469, p = 0.002; test 2: r = 0.372, p = 0.020).

Striatal TH+ fibre density was affected by CIE in a sex-dependent manner, with ethanol-exposed females having higher expression than males (Suppl. Figure, F, linear mixed effects model, Sex × Ethanol exposure × Subject: F (1, 21) = 1.334, P = 0.174). Sex × Ethanol exposure interaction was significant at F (1, 5) = 3.608, P = 0.005 (sex main effect: F (1, 1) = 5.977, P = 0.016, ethanol exposure: F (1, 2) = 0.274, P = 0.761; subject: F (1, 17) = 1.186, P = 0.289).

Discussion

The present experiment examined the relationship between ethanol preference, CIE exposure, cognitive flexibility and affective state. The results of our study revealed sex-dependent changes in ethanol preference and locomotor activity throughout the time. Females had higher ethanol preference during the initial contact with ethanol in the two-bottle test, as shown before in the conditions of a free-choice access to alcohol and water (Juárez and Barrios de Tomasi, 1999) and during operant self-administration (Sneddon, Ramsey Thomas, and Radke, 2020). This could be due to estradiol-dependent increase in excitation of ventral tegmental area dopamine neurons which enhances the rewarding effects of alcohol in females (Flores-Bonilla and Richardson, 2020). Faster novelty habituation in females could also contribute to those differences (Hughes, 1990). Anxiety levels were sex-dependently affected by ethanol exposure with a significant decrease in ethanol-exposed females. Similar sex differences were shown by Healey et al. (2022) after adolescent intermittent ethanol exposure, which increased EPM open arm time in females but not in male Sprague Dawley rats. At the same time, Long Evans rats of all sexes demonstrated an increase in anxiety-like behaviors in the successive alleys test but not the EPM (Bach et al., 2021), so this effect may be strain- and test-dependent. To avoid any effects of a repeated assessment of behavior in an elevated plus maze, we performed the test only twice with a recommended time lag longer than 28 days (Schneider, Ho, Spanagel, and Pawlak, 2011), and had a control group of mice which were not exposed to CIE (Schrader, Taylor, Lowery-Gionta, and Moore, 2018).

The results of this study showed that rats successfully performed in the cognitive flexibility test, but it was not correlated with the initial or post-CIE ethanol preference. While recently it has been shown in C57BL/6J mice that individual variability in behavioral flexibility predicts future alcohol intake (Rodberg and Vazey, 2022), Wistar rats may be less suitable model animals to study this relationship. No effect of ethanol exposure on the ethanol preference means that adult Wistar rats were resistant to this regimen that has been successfully used to induce behavioral changes and pharmacokinetic tolerance to ethanol in Sprague Dawley rats (Risher et al., 2013).

In the experiments on rhesus monkeys, it was shown that lower levels of cognitive flexibility correlate with higher later alcohol consumption under conditions of free choice (Shnitko, Gonzales and Grant, 2019). Our goal was to attempt to confirm the presence of this relationship in rats, including assessing the dynamics of ethanol preference after a period of forced drinking. Previously, our research showed that rats after a chronic ethanol exposure demonstrated a delayed search for a platform during learning (Filatova et al., 2022). In the current study, rats were trained before CIE and demonstrated successful learning and performance in the reversal task independent of the CIE exposure.

The nucleus accumbens (NAc) is a major part of the ventral striatum that plays an important role in mediating emotional and motivation processing, and modulating reward. Ethanol activates the projections from the ventral tegmental area to NAc and induces striatal dopamine release, which is crucial for alcohol reinforcement, and dopamine D2 receptors in the dorsolateral striatum are involved in alcohol seeking with the reduction of their levels demonstrated after long-term alcohol drinking (Feltmann et al., 2018). Striatal TH-expressing GABAergic interneurons (THINs) exert widespread GABAergic inhibition onto direct and indirect spiny neurons throughout the striatum, and deliver dopamine to the cerebrospinal fluid via projections to the lateral ventricles (Xenias, Ibáñez-Sandoval, Koós, and Tepper, 2015). Ibáñez-Sandoval et al. (2015) showed that striatal THINs may play a role during the acquisition of associative learning. They are implicated in sex differences since THINs also express G-protein coupled estrogen receptors that affect morphogenesis of striatal neurons in the perinatal period, which leads to sexual differentiation of the striatum, and in adults, striatal GABAergic neurons express estrogen and progesterone receptor genes that control the release of dopamine (Cao, Willett, Dorris, and Meitzen, 2018; Troshev et al., 2022). In line with those data about sex differences, we found sex-dependent CIE-induced changes in striatal TH+ fibre density. Overall, our results suggest that CIE induces behavioral and neurochemical alterations in a sex-dependent manner in rats. Future studies that look into the factors that play a role in initiation and maintenance of alcohol misuse will hopefully lead to the development of specialized treatment for women with AUD.

Author contributions

MD, EF, AE designed the study. IA, GG performed the experiments. IA performed tissue processing. MD performed statistical analysis and wrote the manuscript. MD, IA, GG, EF, AE edited the manuscript.

References

Aguirre, C. G., Stolyarova, A., Das, K., Kolli, S., Marty, V., Ray, L., Spigelman, I., and Izquierdo, A. 2020. Sex-dependent effects of chronic intermittent voluntary alcohol consumption on attentional, not motivational, measures during probabilistic learning and reversal. *PloS one* 15(6):e0234729. https://doi.org/10.1371/journal.pone.0234729

Bach, E. C., Morgan, J. W., Ewin, S. E., Barth, S. H., Raab-Graham, K. F., and Weiner, J. L. 2021. Chronic ethanol exposures leads to a negative affective state in female rats that is accompanied by a paradoxical decrease in ventral hippocampus excitability. *Frontiers in Neuroscience* 15:669075. https://doi.org/10.3389/fnins.2021.669075

Becker, J. B. and Koob, G. F. 2016. Sex differences in animal models: focus on addiction. *Pharmacological Reviews* 68(2):242–263. https://doi.org/10.1124/pr.115.011163

Bickel, W. K., Jarmolowicz, D. P., Mueller, E. T., Gatchalian, K. M., and McClure, S. M. 2012. Are executive function and impulsivity antipodes? A conceptual reconstruction with special reference to addiction. *Psychopharmacology* 221(3):361–387. https://doi.org/10.1007/s00213-012-2689-x

Cao, J., Willett, J. A., Dorris, D. M., and Meitzen, J. 2018. Sex differences in medium spiny neuron excitability and glutamatergic synaptic input: heterogeneity across striatal regions and evidence for estradiol-dependent sexual differentiation. *Frontiers in Endocrinology* 9:173. https://doi.org/10.3389/fendo.2018.00173

Collins, S. E. 2016. Associations between socioeconomic factors and alcohol outcomes. *Alcohol Research: Current Reviews* 38(1):83–94.

Costa, D. F., Moita, M. A., and Márquez, C. 2021. Novel competition test for food rewards reveals stable dominance status in adult male rats. *Scientific Reports* 11(1):14599. https://doi.org/10.1038/s41598-021-93818-0

DiLeo, A., Antonoudiou, P., Ha, S., and Maguire, J. L. 2022. Sex differences in the alcohol-mediated modulation of BLA network states. *eNeuro* 9(4):ENEURO. 0010-22.2022. https://doi.org/10.1523/ENEURO. 0010-22.2022

Feltmann, K., Borroto-Escuela, D. O., Rüegg, J., Pinton, L., de Oliveira Sergio, T., Narváez, M., Jimenez-Beristain, A., Ekström, T. J., Fuxe, K., and Steensland, P. 2018. Effects of long-term alcohol drinking on the dopamine D2 receptor: gene expression and heteroreceptor complexes in the striatum in rats. *Alcoholism, Clinical and Experimental Research* 42(2):338–351. https://doi.org/10.1111/acer.13568

Filatova, E. V., Gromova, G. E., Dorofeikova, M. V., Antonova, I. V., and Egorov, A. Yu. 2022. Comparative study of the behavior of Wistar rats, dopamine transporter heterozygous rats and rats after long-term ethanol consumption in the Morris water maze. *Journal of Evolutionary Biochemistry and Physiology* 58:1653–1663. https://doi.org/10.1134/S0022093022050337

Flores-Bonilla, A. and Richardson, H. N. 2020. Sex differences in the neurobiology of alcohol use disorder. *Alcohol Research: Current Reviews* 40(2):04. https://doi.org/10.35946/arcr.v40.2.04

Giacometti, L. L. and Barker, J. M. 2020. Sex differences in the glutamate system: Implications for addiction. *Neuroscience and Biobehavioral Reviews* 113:157–168. https://doi.org/10.1016/j.neubiorev.2020.03.010

Glass, J. M., Buu, A., Adams, K. M., Nigg, J. T., Puttler, L. I., Jester, J. M., and Zucker, R. A. 2009. Effects of alcoholism severity and smoking on executive neurocognitive function. *Addiction* 104(1):38–48. https://doi.org/10.1111/j.1360-0443.2008.02415.x

Healey, K. L., Kibble, S. A., Bell, A., Kramer, G., Maldonado-Devincci, A., and Swartzwelder, H. S. 2022. Sex differences in the effects of adolescent intermittent ethanol exposure on exploratory and anxiety-like behavior in adult rats. *Alcohol* 98:43–50. https://doi.org/10.1016/j.alcohol.2021.11.002

Hitzemann, R., Bergeson, S. E., Berman, A. E., Bubier, J. A., Chesler, E. J., Finn, D. A., Hein, M., Hoffman, P., Holmes, A., Kisby, B. R., Lockwood, D., Lodowski, K. H., McManus, M., Owen, J. A., Ozburn, A. R., Panthagani, P., Ponomarev, I., Saba, L., Tabakoff, B., Walchale, A., Williams, R. W., and Phillips, T. J. 2022. Sex differences in the brain transcriptome related to alcohol effects and alcohol use disorder. *Biological Psychiatry* 91(1):43–52. https://doi.org/10.1016/j.biopsych.2021.04.016

Hu, W., Morris, B., Carrasco, A., and Kroener, S. 2015. Effects of acamprosate on attentional set-shifting and cellular function in the prefrontal cortex of chronic alcohol-exposed mice. *Alcoholism, Clinical and Experimental Research* 39(6):953–961. https://doi.org/10.1111/acer.12722

Hughes, R. N. 1990. Sex-dependent habituation to novelty in rats. *Current Psychology* 9:277–284. https://doi.org/10.1007/BF02686865

Ibáñez-Sandoval, O., Xenias, H. S., Tepper, J. M., and Koós, T. 2015. Dopaminergic and cholinergic modulation of striatal tyrosine hydroxylase interneurons. *Neuropharmacology* 95:468–476. https://doi.org/10.1016/j.neuropharm.2015.03.036

Jaafari Suha, A., Hosseinmardi, N., and Janahmadi, M. 2022. Spatial working memory is disparately interrelated with social status through different developmental stages in rats. *Behavioural Brain Research* 416:113547. https://doi.org/10.1016/j.bbr.2021.113547

Juárez, J. and Barrios de Tomasi, E. 1999. Sex differences in alcohol drinking patterns during forced and voluntary consumption in rats. *Alcohol* 19(1):15–22. https://doi.org/10.1016/s0741-8329(99)00010-5

Koob, G. F. and Volkow, N. D. 2010. Neurocircuitry of Addiction. *Neuropsychopharmacology* 35(1):217-238. https://doi.org/10.1038/npp.2009.110

Kristofiková, Z., Platilová, V., and Klaschka, J. 2003. Age- and sex-dependent effects of ethanol on hippocampal hemicholinium-3 sensitive choline carriers during postnatal development of rats. *Neurochemical Research* 28(3–4):397–405. https://doi.org/10.1023/a:1022832214475

Kroener, S., Mulholland, P. J., New, N. N., Gass, J. T., Becker, H. C., and Chandler, L. J. 2012. Chronic alcohol exposure alters behavioral and synaptic plasticity of the rodent prefrontal cortex. *PLoS ONE.* 7(5):e37541. https://doi.org/10.1371/journal.pone.0037541

Levy, D. R., Hunter, N., Lin, S., Robinson, E. M., Gillis, W., Conlin, E. B., Anyoha, R., Shansky, R. M., and Datta, S. R. 2023. Mouse spontaneous behavior reflects individual variation rather than estrous state. *Current Biology* 33(7):1358–1364.e4. https://doi.org/10.1016/j.cub.2023.02.035

Logrip, M. L., Oleata, C., and Roberto, M. 2017. Sex differences in responses of the basolateral-central amygdala circuit to alcohol, corticosterone and their interaction. *Neuropharmacology* 114:123–134. https://doi.org/10.1016/j.neuropharm.2016.11.021

Lovick, T. A. and Zangrossi, H., Jr. 2021. Effect of estrous cycle on behavior of females in rodent tests of anxiety. *Frontiers in Psychiatry* 12:711065. https://doi.org/10.3389/fpsyt.2021.711065

Menezes, J., Souto das Neves, B. H., Gonçalves, R., Benetti, F., and Mello-Carpes, P. B. 2020. Maternal deprivation impairs memory and cognitive flexibility, effect that is avoided by environmental enrichment. *Behavioural Brain Research* 381:112468. https://doi.org/10.1016/j.bbr.2020.112468

Mineur, Y. S., Garcia-Rivas, V., Thomas, M. A., Soares, A. R., McKee, S. A., and Picciotto, M. R. 2022. Sex differences in stress-induced alcohol intake: a review of preclinical studies focused on amygdala and inflammatory pathways. *Psychopharmacology* 239(7):2041–2061. https://doi.org/10.1007/s00213-022-06120-w

Pleil, K. E., Lowery-Gionta, E. G., Crowley, N. A., Li, C., Marcinkiewcz, C. A., Rose, J. H., McCall, N. M., Maldonado-Devincci, A. M., Morrow, A. L., Jones, S. R., and Kash, T. L. 2015. Effects of chronic ethanol exposure on neuronal function in the prefrontal cortex and extended amygdala. *Neuropharmacology* 99:735–749. https://doi.org/10.1016/j.neuropharm.2015.06.017

Pleil, K. E. and Skelly, M. J. 2018. CRF modulation of central monoaminergic function: Implications for sex differences in alcohol drinking and anxiety. *Alcohol* 72:33–47. https://doi.org/10.1016/j.alcohol.2018.01.007

Risher, M. L., Fleming, R. L., Boutros, N., Semenova, S., Wilson, W. A., Levin, E. D., Markou, A., Swartzwelder, H. S., and Acheson, S. K. 2013. Long-term effects of chronic intermittent ethanol exposure in adolescent and adult rats: radial-arm maze performance and operant food reinforced responding. *PloS one 8*(5):e62940. https://doi.org/10.1371/journal.pone.0062940

Rodberg, E. M. and Vazey, E. M. 2022. Individual differences in behavioral flexibility predict future volitional ethanol consumption in mice. *Alcohol* 101:37–43. https://doi.org/10.1016/j.alcohol.2022.03.003

Scott, H., Tjernström, N., and Roman, E. 2020. Effects of pair housing on voluntary alcohol intake in male and female Wistar rats. *Alcohol* 86:121–128. https://doi.org/10.1016/j.alcohol.2019.12.005

Schneider, P., Ho, Y. J., Spanagel, R., and Pawlak, C. R. 2011. A novel elevated plus-maze procedure to avoid the one-trial tolerance problem. *Frontiers in Behavioral Neuroscience* 5:43. https://doi.org/10.3389/fnbeh.2011.00043

Schrader, A. J., Taylor, R. M., Lowery-Gionta, E. G., and Moore, N. L. T. 2018. Repeated elevated plus maze trials as a measure for tracking within-subjects behavioral performance in rats (*Rattus norvegicus*). *PloS one* 13(11):e0207804. https://doi.org/10.1371/journal.pone.0207804

Shnitko, T. A., Gonzales, S. W., and Grant, K. A. 2019. Low cognitive flexibility as a risk for heavy alcohol drinking in non-human primates. *Alcohol* 74:95–104. https://doi.org/10.1016/j.alcohol.2018.04.007

Sneddon, E. A., Ramsey, O. R., Thomas, A., and Radke, A. K. 2020. Increased responding for alcohol and resistance to aversion in female mice. *Alcoholism, Clinical and Experimental Research* 44(7):1400–1409. https://doi.org/10.1111/acer.14384

Troshev, D., Bannikova, A., Blokhin, V., Kolacheva, A., Pronina, T., and Ugrumov, M. 2022. Striatal neurons partially expressing a dopaminergic phenotype: functional significance and regulation. *International Journal of Molecular Sciences* 23:11054. https://doi.org/10.3390/ijms231911054

Walf, A. A. and Frye, C. A. 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols* 2(2):322–328. https://doi.org/10.1038/nprot.2007.44

Xenias, H. S., Ibáñez-Sandoval, O., Koós, T., and Tepper, J. M. 2015. Are striatal tyrosine hydroxylase interneurons dopaminergic? *Journal of Neuroscience* 35(16):6584–6599. https://doi.org/10.1523/JNEUROSCI. 0195-15.2015

Xie, Q., Buck, L. A., Bryant, K. G., and Barker, J. M. 2019. Sex differences in ethanol reward seeking under conflict in mice. *Alcoholism, Clinical and Experimental Research* 43(7):1556–1566. https://doi.org/10.1111/acer.14070

Zachry, J. E., Johnson, A. R., and Calipari, E. S. 2019. Sex differences in value-based decision making underlie substance use disorders in females. *Alcohol and Alcoholism* 54(4):339–341. https://doi.org/10.1093/alcalc/agz052