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Sex-specific effects of environment on behavioral outcomes across the lifespan

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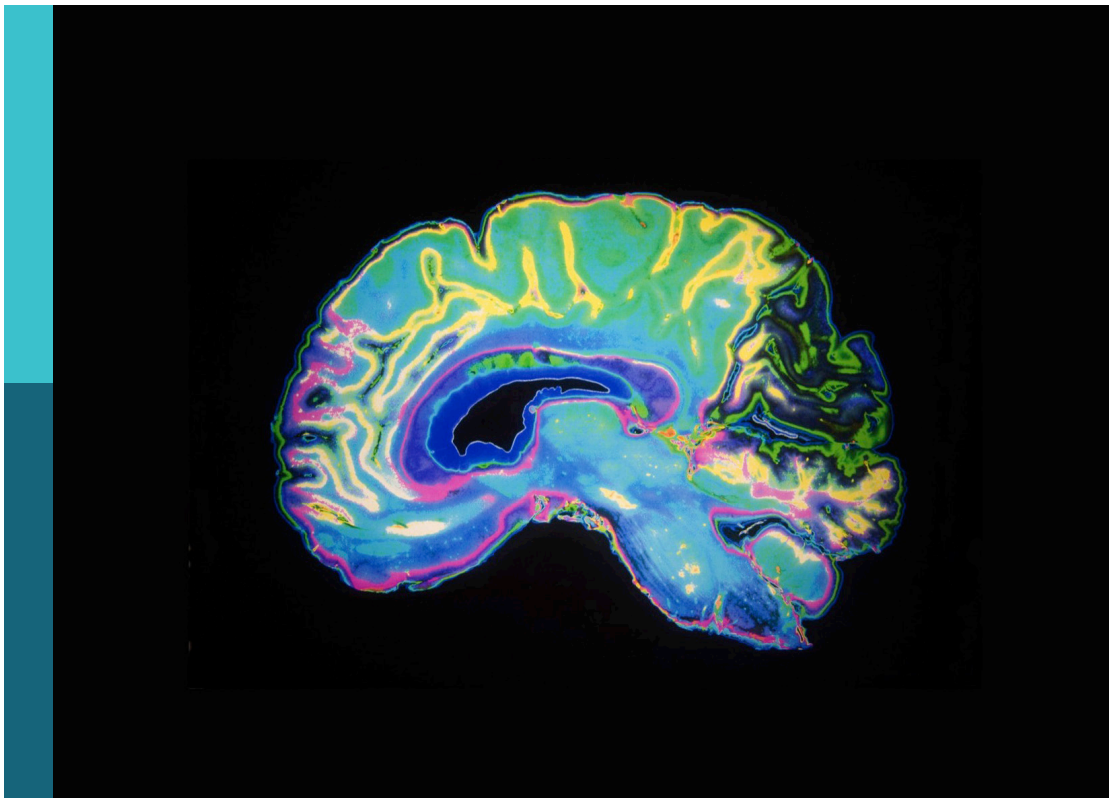

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Sex-specific effects of environment on behavioral outcomes across the lifespan

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ORIGINAL RESEARCH
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Anxiety-Induced Sleep Disturbance and Associated Lifestyle Behaviors According to Sex in Argentine Adolescents

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Purpose: The aim of the current study was twofold: first, to determine the prevalence of anxiety-induced sleep disturbances among Argentine adolescents according to sex, and second, to identify the association between these sleep disturbances and lifestyle behaviors in this population.

Methods: This is a cross-sectional study with data from the Global School-based Student Health Survey (GSHS) in Argentina (2018). A total of 32,393 adolescents (aged 12–17 years; 53.4% girls) were included in the final analysis. Anxiety-induced sleep disturbances were assessed with the question "During the past 12 months, how often have you been so worried about something that you could not sleep at night?"

Results: The prevalence of anxiety-induced sleep disturbances was higher in girls (17.4%) than in boys (7.9%) ($p < 0.001$). In boys, results indicated that those who used marijuana (cannabis) (odds ratio [OR] = 1.46, 95% confidence interval [CI] 1.08–1.98), used amphetamine or methamphetamine (OR = 2.19, 95% CI 1.28–3.77), walked or biked to or from school (OR = 1.53, 95% CI 1.19–1.96), and spent 3 h or more in sedentary behaviors (OR = 1.35, 95% CI 1.05–1.74) were more likely to report anxiety-induced sleep disturbances. In girls, those who ate from a fast-food restaurant (OR = 1.24, 95% CI 1.05–1.47), consumed alcoholic beverages (OR = 1.45, 95% CI 1.19–1.75), smoked cigarettes (OR = 2.09, 95%CI 1.05–4.14), consumed any tobacco product (OR = 1.47, 95% CI 1.19–1.82), used amphetamine or methamphetamine (OR = 2.08, 95% CI 1.33–3.26), and those who spent 3 h or more in sedentary behaviors (OR = 1.32, 95% CI 1.11–1.57) were more likely to report frequent anxiety-induced sleep disturbances.

Conclusion: In conclusion, considerable sex differences were observed with respect to the prevalence of anxiety-related sleep disturbances and associated lifestyle aspects.

Keywords: physical activity, screen time, eating healthy, adolescence, youths

INTRODUCTION

Anxiety is a leading cause of sleep disturbance in adolescents (Mullin and Simon, 2017), while sleep disturbance is associated with an increased risk of developing symptoms of anxiety (Armstrong et al., 2014; Mullin and Simon, 2017). Available data indicate that sleep disturbances are quite common in children with anxiety disorders (de Zambotti et al., 2018; Haugland et al., 2021). For instance, a recent study among Norwegian adolescents found that total anxiety symptoms were associated with several sleep variables (e.g., insomnia, short sleep duration, long sleep onset latency) among adolescents with and without a risk for depression, after adjusting for age and sex (Haugland et al., 2021). Both sleep problems and anxiety are commonly associated with other psychiatric disorders and are independent risk factors for substance use, cardiovascular diseases, and suicide in adolescence, suggesting that treatment of both in early adolescence may reduce the risk for adverse outcomes (de Zambotti et al., 2018). Difficulties falling asleep and awakenings during nights are often caused by anxiety disorders, and these disorders (anxiety and sleep) are bi-directionally associated, suggesting that each contributes to the development and is a consequence of one another (Alvaro et al., 2013). In this sense, Vancampfort et al. (2018) showed that the overall prevalence of anxiety-induced sleep disturbance, defined as being worried about something that keeps you awake at night, was 7.8% in their study involving 181,093 adolescents from 67 countries. Other authors have used a more comprehensive definition of anxiety-induced sleep disturbance, considering it as a condition of low sleep quality, lack of sleep or clinical insomnia as a consequence of loneliness (either social or emotional), worries, sleep disturbance, and disproportionate fear (Joyce-Beaulieu and Sulkowski, 2016; Ahinkorah et al., 2021b).

Adolescence is a major stage of risk for the development of anxiety symptoms and syndromes that can range from mild transient symptoms to complete anxiety disorders (Beesdo et al., 2009). Furthermore, adolescence is an age phase in which sleep is of critical importance (Owens and Weiss, 2017). Adequate sleep is another key factor in promoting healthy development in the young population, and the risk of health problems, such as mental disorders, is increased as a consequence of sleep deprivation and unstable sleep routines (Owens and Weiss, 2017; Schneider et al., 2018). Strikingly, although cognitive-behavioral treatments seem to be effective for anxiety and sleep disturbances in adolescents (Kendall and Peterman, 2015; de Zambotti et al., 2018), few prevention strategies are available (Cabral and Patel, 2020). From a public health perspective, knowing the potential risk factors associated with anxiety-induced sleep disturbances is crucial to developing interventions to prevent sleep disturbances in adolescents (Beesdo et al., 2009).

On the other hand, adolescence is also a critical phase for sex differences in sleep alterations. Whereas the prevalence of sleep problems is comparable between girls and boys during childhood (Fricke-Oerkermann et al., 2007; Zhang et al., 2009), these tend to be more common in girls during this age phase (Blank et al., 2015; Zhang et al., 2016). Additionally, some sex differences have been found regarding factors associated with sleep disturbances (Zhang et al., 2016).

Furthermore, lifestyle behaviors have been shown to affect sleep. For instance, Phillips et al. (1975) found in their experiment that dietary habits (e.g., type of foods and timing of consumption) influence sleep duration. On the other hand, a systematic review with meta-analysis performed by Córdova et al. (2018) showed an inverse cross-sectional relationship between sleep duration in children and unhealthy dietary habits, especially with higher consumption of energy-dense food (e.g., snacks, soda) and lower consumption of fruits and vegetables. In adolescents, a cross-sectional study performed in Italy showed that participants with shorter sleep duration were more likely to eat fewer vegetables and fruits and to eat away more often (Ferranti et al., 2016).

In addition, the relationship between sleep problems and movement-related behaviors has been studied. In a meta-analysis of cross-sectional studies, increased physical activity (PA) and reduced sedentary behavior were associated with greater psychosocial health in young people (Rodriguez-Ayllon et al., 2019). In youth, a systematic review and meta-analysis conducted by Lang et al. (2016) showed that adolescents with increased PA are more likely to have adequate sleep according to objective and subjective measures of both PA and sleep. Similarly, prolonged sedentary behavior has been cross-sectionally related to greater anxiety-induced sleep disturbances in the existing literature (Vancampfort et al., 2018; Werneck et al., 2020).

Similarly, the consumption of psychoactive substances such as alcohol, tobacco, and illicit drugs (e.g., marijuana, amphetamines, etc.) has been reported to have a harmful influence on sleep quality among adolescents (Bartel et al., 2015). Indeed, the consumption of some substances has been associated with poor sleep duration and increased nocturnal arousal (Kotagal and Pianosi, 2006). Likewise, one study conducted among US adolescents showed an association of sleep problems with substance use or smoking (Zhang et al., 2017). In summary, substance use disorders can cause or aggravate sleep problems, which in turn can lead to addictions to these and other psychoactive substances, thus closing the vicious circle (Reid-Varley et al., 2020).

To date, the role of lifestyle factors associated with anxiety-induced sleep disturbance among adolescents has been little explored. Understanding this relationship could be helpful for adolescents to adopt lifestyle changes to reduce anxiety-induced sleep disturbance, which could result in improved mental health. Considering that both anxiety disorders and sleep problems at young ages increased considerably during the COVID-19 pandemic (Wang et al., 2022), the relationship between the two conditions has gained increased attention and requires the expansion of the available body of scientific evidence. In addition, considering the sex differences found in both the prevalence of sleep disturbance (Blank et al., 2015; Zhang et al., 2016) and its associated factors (Zhang et al., 2016), it seems of relevance to know the specific lifestyle factors differences between sexes, in order to promote more appropriate prevention and intervention programs.

Therefore, this study examined data from a nationally representative sample of Argentine adolescents to address two

objectives: first, to determine the prevalence of anxiety-induced sleep disturbances and, second, to identify the association between these sleep disturbances and healthy lifestyle behaviors in this population. These objectives have been analyzed separately for boys and girls because the progression of pubertal maturation varies according to sex and may generate, for example, differences in the prevalence of insomnia symptoms (Zhang et al., 2016).

MATERIALS AND METHODS

Design and Sample

This is a cross-sectional study using data from the Global School-based Student Health Survey (GSHS) in Argentina (2018). The GSHS is a school-based survey that applies self-administered questionnaires to acquire information on people's health behavior and protective factors associated with the leading causes of morbidity and mortality worldwide.

A two-stage cluster sampling strategy was designed to obtain representative data for all students in eight first-grade primary/polymodal schools and 12 fifth-grade polymodal schools in Argentina. First, schools with probability proportional to entry size were chosen. Second, classes were randomly selected, and all students in these classes were eligible to participate. Further information on the specific methodological methods used in the GSHS can be obtained from the World Health Organization (WHO) website[1] and the Centers for Disease Control and Prevention (CDC) website.[2] A total of 56,981 students participated in the Argentina GSHS. Of these students, 21,201 (37.2%) were excluded because of missing body mass index data. Additionally, 3,387 (5.9%) were excluded due to missing data for other variables of interest (i.e., self-reported sleep problems, physical activity, and substance use). Finally, a total of 32,393 (56.8%) students were included in the final analysis.

Procedures

Anxiety-Induced Sleep Disturbance

For anxiety-induced sleep disturbances (dependent variable), the following question was used: "During the past 12 months, how often have you been so worried about something that you could not sleep at night?" The response options were 1-never, 2-rarely, 3-sometimes, 4-most of the time, and 5-always. For further analysis, this variable was dichotomized into no anxiety-induced sleep disturbance ("never," "rarely," and "sometimes") and anxiety-induced sleep disturbance ("most of time" and "always"). In the absence of a specific cut-off point to determine anxiety-induced sleep disturbance, this choice was based on the categorization made in previous studies (Vancampfort et al., 2018; Smith et al., 2020; Werneck et al., 2020; Ahinkorah et al., 2021a,b).

Lifestyle Behaviors

Table 1 describes the results of the measurement of lifestyle behaviors (independent variables) in the present study. Lifestyle behaviors were categorized into three groups: eating behaviors (fruit, vegetable, soft drink, and fast-food intake), movement-related behaviors (physical activity, sedentary behavior, and active commuting), and substance use behaviors (alcohol, cigarettes, any tobacco product, marijuana, and amphetamines/methamphetamines).

TABLE 1 | Global School-based Health Survey questions included in the analysis of lifestyle correlates related to anxiety-induced sleep disturbance.

Variable	Question	Codification
Eating behavior		
Fruit intake	During the past 7 days, how many times did you eat fruit?	0 = 0–1 time per day 1 = 2 or more times per day
Vegetable intake	During the past 7 days, how many times did you eat vegetables?	0 = 0–1 time per day 1 = 2 or more times per day
Soft drink intake	During the past 7 days, how many times did you drink a can, bottle, or glass of a carbonated soft drink?	0 = 0 times per day 1 = At least one time per day
Fast-food intake	During the past 7 days, on how many days did you eat food from a fast-food restaurant?	0 = 0 days per week 1 = At least 1 day per week
Movement behavior		
Physically active	During the past 7 days, on how many days were you physically active for a total of at least 60 min per day?	0 = 0–6 days per week 1 = 7 days per week
Sedentary behavior	How much time do you spend during a typical or usual day sitting and watching television, playing computer games, talking with friends, or doing other seated activities such as surfing the Internet?	0 = Less than 3 h per day 1 = 3 or more hours per day
Active commuting	During the last 7 days, on how many days did you walk or ride a bicycle to or from school?	0 = 0 days per week 1 = At least one time per week
Substance use behavior		
Alcohol consumption	During the past 30 days, on how many days did you have at least one drink containing alcohol?	0 = 0 days per month 1 = At least 1 day per month
Smoke cigarettes	During the past 30 days, on how many days did you smoke cigarettes?	0 = 0 days per month 1 = At least 1 day per month
Any tobacco products use	During the past 30 days, on how many days did you use any tobacco products other than cigarettes?	0 = 0 days per month 1 = At least 1 day per month
Marijuana use	During the past 30 days, how many times have you used marijuana?	0 = 0 times per month 1 = At least one time per month
Amphetamines and methamphetamines use	During your life, how many times have you used amphetamines or methamphetamines?	0 = 0 times per month 1 = At least one time per month

[1] http://www.who.int/ncds/surveillance/gshs/en/

[2] https://www.cdc.gov/gshs/index.htm

Potential Covariates

Sex and age were self-reported. Height and weight were assessed with the following questions: "How tall are you without your shoes on?" and "How much do you weigh without your shoes on?" respectively. Body mass index (BMI) was computed by dividing the weight (kg)/height (m^2) of the participants. BMI z-scores were determined following the World Health Organization (WHO) criteria (Onis et al., 2007). Perceived hunger (used as a proxy of socioeconomic status) was determined through the following question: "During the past 30 days, how often did you go hungry because there was not enough food in your home?" Responses varied from 1-never, 2-rarely, 3-sometimes, 4-most of the time, to 5-always. These covariates were selected according to scientific evidence on their potential confounding effect on the associations of interest (Rethorst et al., 2014; Vancampfort et al., 2018; Ahinkorah et al., 2021a).

Statistical Analysis

Data are shown as the mean and standard deviation for continuous variables and numbers and frequencies for categorical variables. Differences between continuous and categorical variables were examined by Student's *t*-test and the chi-square test, respectively. An exploratory analysis was conducted to define the percentage of adolescents reporting anxiety-induced sleep disturbance. In preliminary analyses, a statistically significant interaction between all analyzed lifestyle factors and sex in relation to anxiety-induced sleep disturbances ($p < 0.05$ for all) was observed. Consequently, all the analyses were stratified by sex. Backward logistic regression analyses were performed to investigate the association between lifestyle factors (independent variable) and anxiety-induced sleep disturbances (dependent variable). First, binary logistic regression analyses were performed including all the lifestyle variables. Second, the lifestyle variable with the highest *p*-value was excluded from the model for each step of the backward logistic regression. Thus, exclusion of lifestyle variables from the model was stopped once all lifestyle variables had a $p < 0.10$. Third, variables included in the last step with $p < 0.05$ were considered significantly associated with anxiety-induced sleep disturbance. All analyses were adjusted by age, BMI z-score, and perceived hunger (as a proxy of socioeconomic status). We used the survey functions in STATA 16.1 (StataCorp, College Station, TX, United States) to perform all analyses and account for weighting for each observation. Statistical significance was represented by a *p*-value < 0.05.

TABLE 2 | Characteristics of the study participants according to sex ($N = 32,393$).

Variables	Boys ($n = 15,095$)	Girls ($n = 17,298$)	
	M (SD)/n (%)	n (%)	*p*
Age (years)	15.1 (1.3)	15.0 (1.3)	0.012
Age group			
12	30 (0.2)	26 (0.2)	0.003
13	2,292 (15.2)	2,855 (16.5)	
14	3,260 (21.6)	3,601 (20.8)	
15	3,345 (22.2)	3,974 (23.0)	
16	3,569 (23.6)	3,987 (23.0)	
17	2,599 (17.2)	2,855 (16.5)	
Height (m)	1.69 (0.10)	1.60 (0.07)	<0.001
Weight (kg)	64.1 (15.3)	56.8 (13.3)	<0.001
BMI (z-score)	0.62 (1.19)	0.45 (1.07)	<0.001
Overweight/Obesity[a]	6,543 (43.3)	5,404 (31.2)	<0.001
Perceived hungry			
Never	10,567 (70.0)	11,849 (68.5)	<0.001
Anxiety-induced sleep disturbance			
Never	5,469 (36.4)	3,357 (19.5)	<0.001
Rarely	4,833 (32.1)	5,121 (29.7)	
Sometimes	3,547 (23.6)	5,746 (29.2)	
Most of the time	890 (5.9)	2,227 (12.9)	
Always	306 (2.0)	778 (4.5)	
Most of the time/Always[b]	1,196 (7.9)	3,005 (17.4)	<0.001

[a]*Prevalence of excess of weight according to the World Health Organization criteria (Onis et al., 2007).* [b]*Anxiety-induced sleep disturbance defined as the sum of adolescents who reported that they could not sleep "most of the time" and "always." BMI, Body mass index.*

Ethics

All GSHS surveys have been previously approved in each country by the corresponding national government agency and by an institutional ethics committee or board. The protocol was approved by an independent ethics committee in Argentina and by the Pan American Health Organization's Ethics Review Committee. Survey administration was authorized by the Ministry of Education, provincial ministries, and school authorities. Responses were completely confidential and anonymous in unnamed answer sheets, which were collected at the end of class. Students were required to provide their verbal consent before completing the survey.

RESULTS

Table 2 shows the characteristics of the adolescents included in this study by sex. The mean ± standard deviation of age was slightly higher in boys (15.1 ± 1.3) than in girls (15.0 ± 1.3) ($p = 0.012$). The prevalence of excess weight (overweight/obesity) was higher in boys (43.3%) than in girls (31.2%) ($p < 0.001$). Finally, the prevalence of anxiety-induced sleep disturbances was higher in girls (17.4%) than in boys (7.9%) ($p < 0.001$).

Figure 1 shows the prevalence of different lifestyle behaviors stratified by sex. Regarding movement-related behaviors, boys showed a higher prevalence of active commuting and being physically active ($p < 0.001$ for all), as well as less time spent in sedentary behaviors ($p < 0.001$). According to substance use behavior, the prevalence of consumption of alcohol and cigarettes was higher in girls ($p < 0.001$ for all). Conversely, the prevalence of amphetamine/methamphetamine use, marijuana (cannabis), and any tobacco product use were higher in boys than in girls ($p < 0.001$ for all). In the case of eating behavior, significant differences were only found for soft drink intake ($p < 0.001$), with a higher prevalence for boys.

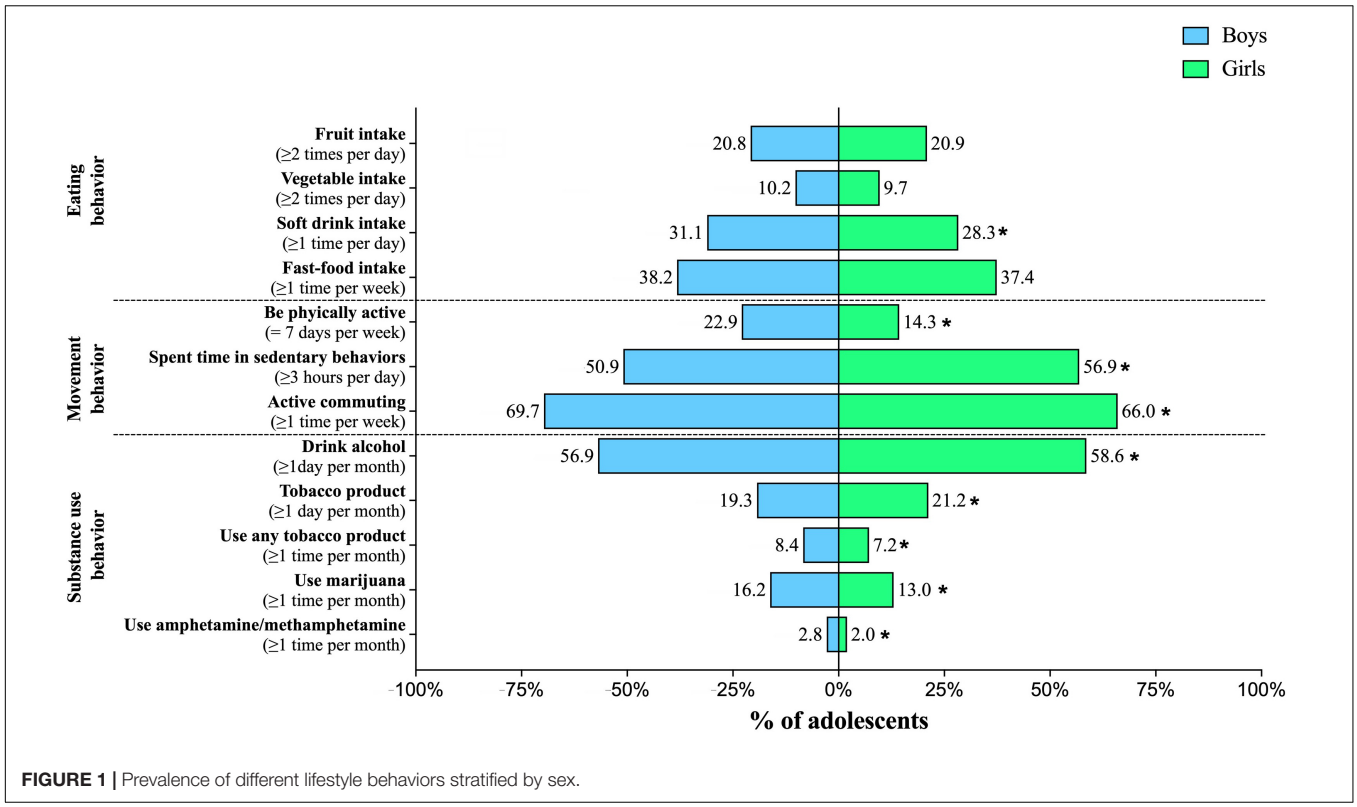

FIGURE 1 | Prevalence of different lifestyle behaviors stratified by sex.

Finally, **Table 3** depicts the lifestyle behaviors retained in the last step of the multivariate model. The backward logistic regression analysis included seven steps for boys and four for girls, in which the least statistically significant correlation was removed from the model at each step. In boys, the results indicated that those who consumed marijuana (cannabis) (odds ratio [OR] = 1.46, 95% confidence interval [CI] 1.08–1.98), consumed amphetamine or methamphetamine (OR = 2.19, 95% CI 1.28–3.77), walked or biked to or from school (OR = 1.53, 95% CI 1.19–1.96), and spent 3 h or more in sedentary behaviors (OR = 1.35, 95% CI 1.05–1.74) were more likely to report anxiety-induced sleep disturbance. In girls, those who ate at a fast-food restaurant (OR = 1.24, 95% CI 1.05–1.47), consumed alcoholic beverages (OR = 1.45, 95% CI 1.19–1.75), smoked cigarettes (OR = 2.09, 95% CI 1.05–4.14), consumed any tobacco products (OR = 1.47, 95% CI 1.19–1.82), consumed amphetamine or methamphetamine (OR = 2.08, 95% CI 1.33–3.26), and those who spent 3 h or more in sedentary behaviors (OR = 1.32, 95% CI 1.11–1.57) were more likely to report frequent anxiety-induced sleep disturbances.

DISCUSSION

The current study aimed to evaluate the prevalence of anxiety-induced sleep disturbance and lifestyle behaviors linked to this condition in a nationally representative sample of Argentine adolescents. A marked sex difference was found in this cross-sectional study with respect to the prevalence of anxiety-related sleep disturbances, which was more than twice as high in girls as in boys. This finding agrees with previous studies that found that anxiety-related sleep disturbances are more prevalent among girls (Pengpid and Peltzer, 2020, 2022; Werneck et al., 2020). Regarding the causes of inadequate sleep in adolescents, internal biological processes have been identified, such as the normal change (delay) in circadian rhythm that occurs in relation to puberty and a development-based slowing of the "sleep drive" (Owens and Weiss, 2017). Thus, sex differences in sleep problems (e.g., anxiety-induced sleep disturbances) during adolescence are especially recognized, due to biological, behavioral, or social factors. Regarding biological factors, sexual maturation has been suggested as a factor that could influence sleep problems, since it is influenced by the changing the hormone milieu (Marsella and Sharkey, 2020). Similarly, the menstrual cycle could influence sleep, due to variations in sex steroid hormones (Baker et al., 2020). In relation to social factors, it has been suggested that girls may be more likely to have anxiety-induced sleep disturbances because they experience adverse life events (e.g., domestic violence) more frequently than boys, which could exacerbate the development of anxiety disorders (Biaggi et al., 2016). Supporting this notion, exposure to violence (e.g., intentional injury, physical attack, physical fight) has been positively associated with higher odds of anxiety-induced insomnia among adolescents (Smith et al., 2020). Finally, the coexistence of specific anxiety disorders with substance use in adolescence (especially in girls) has also been identified as a relevant social factor (Wu et al., 2010).

Considerable sex differences were also observed with respect to lifestyle aspects associated with anxiety-related sleep

TABLE 3 | Final result of the univariate multilevel logistic regression analysis with anxiety-induced sleep disturbance as a dependent variable and potential lifestyle correlates as independent variables, among Argentine adolescents by sex.

Predictors	Boys (*n* = 14,516)			Girls (*n* = 16,872)		
	OR	95% CI	*p*	OR	95% CI	*p*
Eating habits						
Fast-food intake						
≥1 day per week		Variable excluded in step 4[a]		1.24	(1.05–1.47)	0.014
0 days per week				1		
Movement						
Active commuting						
≥1 time per week	1.53	(1.19–1.96)	0.001		Variable excluded in step 2[a]	
0 times per week	1					
Sedentary behavior						
≥3 or more hours per day	1.35	(1.05–1.74)	0.019	1.32	(1.11–1.57)	0.001
<3 h per day	1			1		
Drugs						
Alcohol consumption						
≥1 day per month		Variable excluded in step 2[a]		1.45	(1.19–1.75)	<0.001
0 days per month				1		
Smoke cigarettes						
≥1 day per month		Variable excluded in step 3[a]		1.47	(1.19–1.82)	<0.001
0 days per month				1		
Any tobacco product use						
≥1 day per month		Variable excluded in step 1[a]		1.59	(1.17–2.16)	0.003
0 days per month				1		
Marijuana use						
≥1 time per month	1.46	(1.08–1.98)	0.014		Variable excluded in step 1[a]	
0 times per month	1					
Amphetamine or methamphetamine use						
≥1 time per month	2.19	(1.28–3.77)	0.004	2.08	(1.33–3.26)	0.001
0 times per month	1			1		

Data indicated as odds ratio and 95% confident intervals. Adjusted by age, body mass index (z-score), and perceived hunger.
[a]Logistic regression models with backward stepwise variable selection method (statistical criterion $p > 0.20$).

disturbance. According to eating behavior, anxiety-induced sleep disturbance was associated with eating fast food from a restaurant at least once (or more) per week in girls. In this regard, one study showed that adolescents who had a high intake of fast food were more than three times more likely to report anxiety-induced sleep disturbance (Khan et al., 2017). Similarly, the consumption of ultra-processed foods, such as those available in fast food restaurants, has been associated with elevated anxiety-induced sleep disturbance among Brazilian adolescents (Werneck et al., 2020). The absence of a significant association in boys could be explained because this eating behavior possibly has less influence on sleep problems compared to others more frequent in boys than in girls, such as active commuting and marijuana use. There are some reasons that might explain the association between anxiety-induced sleep disturbances and fast food. First, sleep problems could affect dietary choices, which have previously been associated with shorter sleep duration and might play a crucial role in mediating the relationship between sleep and adolescent health (Kruger et al., 2014). Second, fast food often contains high glycemic index carbohydrates. In this regard, some authors have advised that hyperglycemia following a high glycemic index diet and subsequent compensatory hyperinsulinemia may stimulate the release of autonomic counter-regulatory hormones (e.g., cortisol, adrenaline, growth hormone, glucagon), which could exert an impact on sleep disturbances (Gais et al., 2003; Gangwisch et al., 2020). Similarly, foods with a high glycemic index have been shown to enhance inflammatory immune responses (Kim et al., 2018) and cause alterations in the gut microbiome, which could also deeply influence sleep quality (Gérard and Vidal, 2019). Third, fast food also tends to contain more saturated fatty acids. Supporting this notion, a higher intake of saturated fats during the day has been linked to a shorter duration of slow-wave sleep (Grandner et al., 2010; St-Onge et al., 2016) and more arousal episodes at night (St-Onge et al., 2016). However, caution is required to interpret these results, since studies on the role of saturated fatty acids on sleep are relatively scarce (Zhao et al., 2020).

Furthermore, we found associations between anxiety-induced sleep disturbances and movement-related behaviors. Thus, our results showed that spending three or more hours in sedentary behaviors was likely related to anxiety-induced sleep disturbances

in both sexes. In this regard, Vancampfort et al. (2018) provided strong multinational evidence on the relationship between anxiety-induced sleep disturbances and sedentary behaviors in adolescents. Possible reasons for these findings are that some sedentary behaviors, such as excessive media use and prolonged overnight exposure, may influence circadian rhythms and relocate sleep timing (Primack et al., 2009; Soundy et al., 2014). In addition, the literature has also suggested that inactivity could alter serum melatonin levels and lead to changes in nocturnal melatonin release (Vancampfort et al., 2018). Additionally, exposure to blue spectrum light through screens may also influence this relationship (LeBourgeois et al., 2017). Conversely, although physical activity has been found in the scientific literature to be associated with longer sleep durations (Lang et al., 2016), in this study, the association between physically active conditions and sleep disturbances was not statistically significant in either sex. This lack of association could be justified by the use of subjective measures applied to determine the level of physical activity in the GSHS surveys. Furthermore, these questions only ask about the number of days above 60 min of moderate-to-vigorous physical activity, an aspect that has been questioned in the new guidelines on physical activity in children and adolescents (Bull et al., 2020), which suggests that the counting of minutes of physical activity should be considered globally per week and not by isolated days.

In relation to substance use behavior, we found an association between some substances and anxiety-induced sleep disturbance in both sexes. An association with higher illicit substance use was found in girls (e.g., alcohol, cigarettes, any tobacco product) than in boys (e.g., marijuana), which is consistent with a previous study among US adolescents (Wu et al., 2010). However, the relatively lower prevalence of anxiety-induced sleep disturbance in boys than in girls could (at least partially) explain the lack of statistical significance. Adolescents are generally more vulnerable to tobacco, alcohol, and cannabis than adults (Spear, 2016). Our results are in agreement with the findings of Sivertsen et al. (2015) in their study of Norwegian adolescents, who found that all measured sleep indicators were associated with substance involvement. Similarly, one longitudinal study showed that trajectories of sleep health were linked to trajectories of cannabis and alcohol use during late adolescence and early adulthood (Troxel et al., 2021). Additionally, one study of adolescents from sub-Saharan Africa found that cannabis use was related to anxiety-induced sleep disturbances (Ahinkorah et al., 2021a). Regarding amphetamine or methamphetamine use, in adults, D-amphetamine caused a marked decrease in sleep drive and harmful effects on certain elements of recovery sleep (Waters et al., 2003). Likewise, amphetamine-type stimulants are the most potent agents that promote wakefulness by blocking dopamine reuptake and stimulating dopamine release (Wisor et al., 2001). Moreover, in another study it was observed that e-cigarette (as tobacco-related products) users were more predisposed to have shorter sleep durations than non-users of e-cigarette (Wiener et al., 2020). There are other reasons that could explain these results. First, many e-cigarettes have stimulants (e.g., nicotine) that are known to influence sleep (Wiener et al., 2020). For instance, nicotine influences the sleep-wake cycle through nicotine receptors, promoting wake time and decreasing total sleep time, as well as rapid eye movement sleep (Siqueira and Committee on Substance Use and Prevention, 2017). Second, concomitant changes in circadian rhythms and the reward function of the brain that is activated when engaging in risky behaviors could be an influential factor (Hasler et al., 2014). Furthermore, human circadian genetic variability is closely related to substance use (Blomeyer et al., 2013), and this association is likely to be bidirectional: adolescents with addiction problems often have altered circadian rhythms, and certain chronotypes have also been shown to increase the risk of substance abuse (Logan et al., 2014). Supporting this idea, circadian disruption and sleep problems have been associated with higher alcohol consumption (Hasler et al., 2015).

This study is not without limitations. First, because of the cross-sectional design, cause-and-effect relationships cannot be established from the present findings. Second, although previous studies have assessed anxiety-induced sleep disturbance by the same single-item question (Vancampfort et al., 2018; Werneck et al., 2020; Ahinkorah et al., 2021a), we cannot adequately determine the perseverance and specific type of anxiety-induced sleep disturbance reported by adolescents. This item did not provide in-depth data outcome data. However, it is complex to use more specific methodologies regarding psychological outcomes in national epidemiological studies. Future studies using validated questionnaires are required to acquire more detailed information about this outcome. Third, this study included self-reported information from adolescents, which may result in some bias. For instance, this fact may be especially relevant for the illicit substance use. However, to reduce this bias, information from GHSH did not include school or student identifiers in the public use data set (i.e., anonymous data). Furthermore, in this study height and weight were self-reported by adolescents which, in addition to the large loss of participants due to missing values or outliers, could also have introduced an information bias in the present findings (Gorber et al., 2007). Fourth, information about race/ethnicity was not assessed, which could influence the results obtained. Finally, it is important to note that the classification of behaviors and outcomes into only two categories, although it is a common procedure in epidemiological studies and facilitates comparison of the results with those of other authors, limits the possibility of analyzing the effect of different levels of adherence to certain lifestyle habits. In contrast, the present study has some strengths. For instance, this study included a large nationally representative sample of Argentine adolescents. This fact allowed us to obtain a high statistical power and to analyze the association between a large number of lifestyle variables and anxiety-induced sleep disturbances, stratified by sex. In this sense, although previous studies adjusted their analyses by sex (Haugland et al., 2021), our findings indicate that the study of factors associated with anxiety-induced sleep disturbances in adolescents requires separate analyses for boys and girls due to biological and behavioral differences between the sexes. Additionally, this study adds information to the scientific knowledge about the understudied relationship between lifestyle behaviors and anxiety-induced sleep disturbances among adolescents.

CONCLUSION

Our results indicate a high prevalence of anxiety-related sleep disturbances, specifically among girls, and point to sex-specific lifestyle issues related to these disorders. Lifestyle behaviors, such as eating habits, movement-related behavior, and substance use, are associated with the prevalence of anxiety-induced sleep disturbances, which could affect adolescent health. Programs and strategies to encourage a healthy lifestyle throughout adolescence and that take into account sex specificity are necessary and should focus on actions considering the importance of the noted lifestyle behaviors in decreasing the prevalence of anxiety-induced sleep disturbances.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://extranet.who.int/ncdsmicrodata/index.php/catalog/866.

ETHICS STATEMENT

All GSHS surveys have been previously approved in each country by the corresponding national government agency and by an institutional ethics committee or board. The protocol was approved by an independent ethics committee in Argentina and by the Pan American Health Organization's Ethics Review Committee. Survey administration was authorized by the Ministry of Education, provincial ministries, and school authorities. Responses were completely confidential and anonymous in unnamed answer sheets, which were collected at the end of class. Students were required to provide their verbal consent before completing the survey. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JL-G: conceptualization, methodology, software, validation, analysis, data curation, and writing—original draft preparation. AM and IC-R: supervision. AM, PT, EJ-L, AG, IS-D, and IC-R: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Sex Differences in the Association Between Poor Sleep Quality and Alcohol-Related Problems Among Heavy Drinkers With Insomnia

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Background: Alcohol Use Disorder (AUD) and insomnia are highly comorbid; at least 40% of individuals with AUD suffer from insomnia. Women are more likely to report insomnia than men and have seen a concerning rise in rates of AUD in recent years. As such, the association between AUD and insomnia could be particularly pronounced in women. However, currently little is known regarding sex differences in this association. Here we examined the degree to which relationships between alcohol use and sleep quality differ between women and men.

Methods: Heavy drinking women (n = 66) and men (n = 45) completed the Pittsburgh Sleep Quality Index (PSQI) to assess sleep quality and the Alcohol Use Disorders Identification Test (AUDIT) to assess alcohol use and alcohol-related problems. Hierarchical regression analyses were conducted to determine sex differences in the association between poor sleep quality and alcohol-related problems.

Results: After controlling for age, global subjective stress, and depression, sex significantly moderated the positive association between poor sleep quality and alcohol-related problems. Further analyses of the simple slopes for each sex revealed that poorer sleep quality (i.e., higher scores on the PSQI) were associated with greater alcohol-related problems (i.e., higher scores on the AUDIT) in women, but not in men.

Conclusion: These results suggest that in heavy drinkers with insomnia, poor sleep is more strongly associated with drinking problems in women than in men. Future research is needed to investigate potential mechanisms underlying this relationship. Specifically, it will be important to determine whether sleep problems in heavy drinking women are a cause or consequence, or both, of heavy drinking.

Keywords: AUD, women, sleep disturbances, risky drinking, stress, AUDIT, PSQI

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INTRODUCTION

At least 40% of individuals with alcohol use disorder (AUD) suffer from insomnia, suggesting these issues are highly comorbid (Zhabenko et al., 2012). This comorbidity is due in part to the bidirectional and feed-forward relationship between AUD and insomnia (Koob and Colrain, 2020). Excessive alcohol consumption, including both acute intoxication and withdrawal, negatively

impacts sleep. Specifically, acute intoxication leads to more awakenings throughout the night, disruption of REM sleep, and reductions in sleep stages N1 and N2 (MacLean and Cairns, 1982; Williams et al., 1983; Chan et al., 2013). Additionally, alcohol withdrawal leads to insomnia, which can persist for several months following the cessation of drinking (Brower et al., 2011). Specifically, withdrawal is associated with decreases in slow wave sleep, N2 sleep, and REM latency and increases in N1 sleep and REM sleep, and these effects can persist for several months (Williams and Rundell, 1981; Drummond et al., 1998). Conversely, disrupted sleep contributes substantially to alcohol-related problems, in part because alcohol is often used as a sleep aid in an effort to self-medicate sleep disruptions (Johnson et al., 1998; Brower et al., 2001). Given this pronounced interconnection between insomnia and AUD, a better understanding of this relationship could improve intervention and prevention efforts for both disorders.

Associations between alcohol and sleep have historically been assessed in predominately male samples, with women underrepresented in these studies (Baker et al., 2020; Inkelis et al., 2020; Koob and Colrain, 2020). The lack of data on sleep-alcohol associations in women is especially concerning given that both insomnia and AUD are particularly problematic and increasingly prevalent in women. Specifically, women have a higher insomnia risk and self-report greater sleep disturbances and poorer sleep quality than men (Lindberg et al., 1997; Zhang and Wing, 2006; Zhang et al., 2016). Likewise, rates of AUD are increasing much more quickly among women compared to men (Grant et al., 2017). Such high rates of both insomnia and AUD in women highlight the need for studies to directly investigate the reciprocal influence of these disorders in women.

To date, a handful of studies have examined sex differences in associations between poor sleep and alcohol-related problems, and results have been mixed. In some studies, the negative association between sleep and drinking is stronger in men. For example, a large-scale longitudinal study found a bidirectional relationship between heavy drinking and sleeplessness in men, but not women (Rognmo et al., 2019). Additionally, Jaussent et al. (2011) reported that moderate alcohol use was associated with fewer insomnia symptoms in elderly women, whereas no relationship was observed in elderly men. By contrast, other studies have reported findings in the opposite direction, with a stronger negative association between sleep and alcohol use in women. For instance, Haario et al. (2013) found that insomnia was associated with higher rates of heavy drinking and binge drinking in women but not men. Further, in a sample of social alcohol and cannabis users, the association between risky alcohol use and poor sleep quality was stronger in women than men (Ogeil et al., 2015). Taken together, these findings suggest that among heavy drinkers specifically, women may experience stronger associations between alcohol and poor sleep quality than men.

It is important to note that the studies to date examining sex differences in associations between alcohol use and sleep have included predominately healthy, community-dwelling adults with relatively low levels of problem drinking and/or insomnia. Indeed, to our knowledge, no studies to date have examined sex differences in associations between alcohol and sleep in individuals meeting clinical criteria for both insomnia and problematic drinking. Given that alcohol-sleep associations are likely strengthened with increased levels of comorbid AUD and insomnia, this is an important gap in the literature. In order to address this gap, the current study examined sex differences in associations between problematic drinking and poor sleep quality in a sample of heavy drinkers with insomnia. Problem drinking was assessed with the Alcohol Use Disorders Identification Test (AUDIT; Babor et al., 1989) and poor sleep quality was assessed with the Pittsburgh Sleep Quality Index (PSQI; Buysse et al., 1989). We hypothesized that alcohol-related problems would be positively related to sleep disturbances (i.e., individuals with greater alcohol-related problems would also report poorer sleep quality). Moreover, we hypothesized that the association between poor sleep and alcohol problems would be more pronounced in women.

MATERIALS AND METHODS

Participants

Male and female volunteers were recruited through online and printed advertisements for the parent study testing the efficacy of an insomnia intervention on sleep and drinking habits. The analyses presented here represent one of the aims of the larger project and were carried out with baseline data (collected prior to any intervention). Participants were required to be heavy drinkers [i.e., report weekly binge episodes (4/5 + drinks in one sitting for women/men) and an AUDIT score > 7] and to suffer from insomnia [i.e., self-report insomnia for three or more nights a week for the past 3 months and an Insomnia Severity Index (ISI) score > 14]. Additional inclusion criteria were: fluency in English, regular access to the internet, and 21–50 years old. Volunteers were excluded if they self-reported a previous diagnosis of AUD or substance use disorder, schizophrenia, bipolar disorder, or other psychotic spectrum disorder, sleep apnea, or if they were pregnant, lactating, or planning to become pregnant in the next 3 months.

Procedure

The study was completed entirely online. Study data were collected and managed using REDCap electronic data capture tools hosted at the University of Kentucky (Harris et al., 2009, 2019). Eligible participants were emailed an invitation to the study along with a link to the consent form and baseline surveys. After electronically giving informed consent and agreeing to participate in the study by selecting yes on the online consent form, participants completed a battery of baseline surveys. Questionnaires assessed alcohol use, sleep quality, stress, and depression, and took approximately 30 min to complete. The Institutional Review Board of the University of Kentucky approved the study, and it was carried out in accordance with the Declaration of Helsinki. Upon completion of all baseline requirements for the parent study, participants were compensated $40 via Amazon gift card.

Measures

Insomnia Severity Index (ISI; Bastien et al., 2001). This seven-item self-report questionnaire was included as a screening measure to assess eligibility based on severity of insomnia. Each item requires the subject to rate the severity of their symptoms on a Likert scale from 0 to 4. Scores range from 0 to 28 and are interpreted as follows: 0–7—no clinically significant insomnia; 8–14—subthreshold insomnia; 15–21—moderately severe clinical insomnia; 22–28—severe clinical insomnia.

Pittsburgh Sleep Quality Index (PSQI; Buysse et al., 1989). The PSQI is a 19-item self-report measure that provides a general index of sleep quality and sleep disturbances. It is composed of seven subscales assessing Sleep Quality, Sleep Latency, Sleep Duration, Sleep Efficiency, Sleep Disturbances, Use of Sleeping Medications, and Daytime Dysfunction. Each subscale is rated on a scale of 0–3 and then summed together, producing total scores ranging from 0 to 21. Scores of 6 or higher indicate poor sleep.

Alcohol Use Disorders Identification Test (AUDIT; Babor et al., 1989). The AUDIT was administered to assess alcohol-related problems. This is a 10-item self-report measure with scores ranging from 0 (no-alcohol related problems) to 40 (most severe alcohol-related problems). A score of 8 or higher typically indicates hazardous drinking.

Drinking Habits Questionnaire. Participants reported their typical number of drinking days and typical number of binge episodes (4/5+ drinks for women/men on one occasion) per month on the drinking habits questionnaire.

Perceived Stress Scale (PSS; Cohen et al., 1983). The PSS is a 10-item self-report measure used to gauge an individual's global stress level by asking non-specific questions about how overloaded, uncontrollable, or unpredictable one's life has been in the past month. All items are scored on a scale of 0 to 4, with higher scores indicating greater psychological distress.

Center for Epidemiologic Studies Depression Scale revised 10-item version (CESDR-10; Radloff, 1977). The CESDR-10 is a 10-item self-report measure that gauges a subject's symptoms of depression. Each item is scored on a scale of 0 to 3, with higher scores indicating more severe symptoms.

Analyses

Sex differences in all measures were analyzed by independent samples *t*-tests. We analyzed the degree to which impaired sleep was associated with alcohol-related problems by hierarchical regression analyses. Alcohol-related problems, as determined by AUDIT scores, served as the dependent variable. Age, PSS, and CESD-R scores were included as covariates and entered in Step 1. Age was included as a covariate due to marginal sex differences in our sample (see **Table 1**), and stress and depression scores were included as covariates due to well-established associations between these variables and both sleep [for reviews see Tsuno et al. (2005) and Kim and Dimsdale (2007)] and alcohol use [for reviews see Breese et al. (2011) and McHugh and Weiss (2019)]. Sex (male vs. female) and sleep quality, as determined by PSQI scores, were entered in Step 2. The interaction term (Sex × PSQI) was entered in Step 3 to determine whether the association between sleep quality and alcohol consumption differed by sex. Follow-up analyses tested the significance of the simple slopes for both men and women using the PROCESS extension tool (Hayes, 2017) in SPSS. Finally, bivariate correlations were conducted separately in men and women to assess associations between the individual PSQI subscales and AUDIT scores. We also include a qq plot (**Supplementary Figure 1**) justifying our choice of linear regression. No violations for linear regression were found, including non-normality of standardized residuals, form of relations, or heteroscedasticity.

RESULTS

Sample Characteristics

A total of 111 participants (n = 45 male and n = 66 female) took part in this study. Sample characteristics are presented in **Table 1**. Men reported more drinking days in the past month compared to women (p = 0.04). No significant sex differences were observed on any other drinking or demographic measures (ps > 0.05). Both women and men had mean AUDIT scores well above the threshold for hazardous drinking, mean PSQI scores indicating poor sleep, and mean ISI scores that met criteria for moderately severe clinical insomnia. The racial makeup of the sample was White (n = 93), American Indian or Alaska Native (n = 3), Asian (n = 1), Black or African-American (n = 5), and Mixed Race (n = 9). The ethnic makeup of the sample was Non-Hispanic/Latino (n = 102), Hispanic/Latino (n = 7), and unreported (n = 2). Educational history was obtained from only a partial subset of the sample (n = 61, M = 15.43 years, SD = 1.89).

Sex Differences in the Relationship Between Sleep Quality and Alcohol-Related Problems

Results of the regression analysis testing sex differences in the association between sleep quality and alcohol problems are presented in **Table 2**. Of the three covariates included in the model, only the PSS was significantly related to AUDIT scores

TABLE 1 | Mean age, drinking habits, depression, sleep quality, and stress in men and women.

	Women (n = 66)	Men (n = 45)	
	M (SD)	M (SD)	Contrasts
Age	24.8 (4.7)	26.8 (6.2)	ns
AUDIT	17.3 (7.2)	18.0 (7.0)	ns
Drinking (past 30 days)			
# of drinking days	15.0 (7.6)	18.0 (7.7)	Sig*
# of binge episodes	10.5 (5.8)	11.1 (6.8)	ns
CESD-R	16.9 (5.6)	14.9 (6.2)	ns
ISI	19.6 (3.1)	18.9 (2.8)	ns
PSQI	14.7 (2.5)	13.7 (2.8)	ns
PSS	24.6 (6.0)	22.6 (7.1)	ns

Group contrasts tested by independent samples t-tests; Sig indicates p < 0.05.*

TABLE 2 | Hierarchical regression analysis of sex differences in the relationship between sleep quality and alcohol-related problems.

AUDIT scores			
	R^2	B (se)	Beta
Step 1	0.06		
Age		0.03 (0.12)	0.02
PSS		**0.32 (0.15)**	**0.29***
CESD-R		−0.09 (0.17)	−0.07
Step 2	**0.11***		
Age		−0.05 (0.13)	−0.04
PSS		**0.32 (0.15)**	**0.30***
CESD-R		−0.15 (0.17)	−0.13
Sex		1.74 (1.38)	0.12
PSQI		**0.63 (0.26)**	**0.24***
Step 3	**0.15***		
Age		−0.01 (0.13)	−0.01
PSS		**0.34 (0.15)**	**0.31***
CESD-R		−0.14 (0.16)	−0.12
Sex		1.57 (1.36)	0.11
PSQI		**1.09 (0.34)**	**0.41****
Sex × PSQI		**−1.05 (0.50)**	**−0.26***

*$p < 0.05$, **$p \leq 0.01$. Bold values indicate $p < 0.05$.*

and this effect was seen in all three steps of the model. This effect suggested that individuals with more stress had higher AUDIT scores. PSQI scores were also positively related to AUDIT scores in the second and third steps of the model, such that individuals with poorer sleep quality reported greater alcohol-related problems. Moreover, the Sex × PSQI interaction term was significant ($p = 0.04$), indicating that the association between PSQI and AUDIT scores differed by sex. Using the PROCESS extension tool (Hayes, 2017) in SPSS, we probed this interaction by testing the significance of the simple slopes for both women and men. Women showed a significant, positive relationship between PSQI and AUDIT scores, B = 1.09, $t = 3.20$, $p < 0.01$. By contrast, PSQI scores were not significantly related to AUDIT scores in men, $B = 0.04$, $t = 0.10$, $p = 0.92$. **Figure 1** shows the bivariate relationship between PSQI and AUDIT scores for women and men separately.

Bivariate Correlations Between Pittsburgh Sleep Quality Index Subscales and Alcohol Use Disorders Identification Test Scores in Men and Women

Bivariate correlations between each of the PSQI subscales and AUDIT scores were conducted separately in men and women. Results of these correlations can be found in **Table 3**. Two significant associations were seen in women. Sleep Duration, $r = 0.40$, $p = 0.001$, and Sleep Disturbances, $r = 0.28$, $p = 0.02$, were significantly positively associated with AUDIT scores such that less total sleep time and more sleep disturbances were associated with greater AUDIT scores. By contrast, there were no significant associations between any of the subscales and AUDIT scores in men.

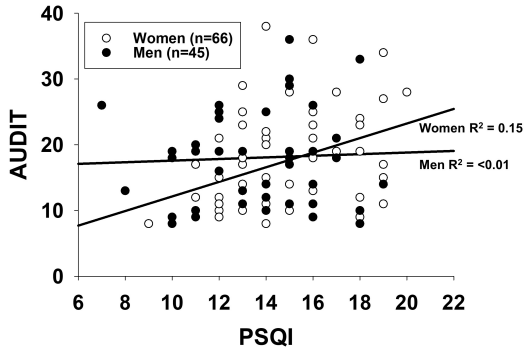

FIGURE 1 | Bivariate correlations of Pittsburgh Sleep Quality Index (PSQI) and Alcohol Use Disorders Identification Test (AUDIT) scores in men and women. PSQI and AUDIT scores were significantly positively correlated in women ($p < 0.01$), but not in men ($p = 0.75$).

DISCUSSION

This study examined sex differences in associations between sleep quality and alcohol use in a sample of heavy drinkers with insomnia. Findings showed that poor sleep quality was significantly associated with more alcohol-related problems in women, but not in men. This effect was found even after controlling for several potential confounding variables such as age, global stress, and symptoms of depression. Based on bivariate correlational analyses, the PSQI Sleep Duration and Sleep Disturbances subscales may be driving the observed relationship. In line with previous research (Rice and Van Arsdale, 2010; Tavolacci et al., 2013; Ruisoto et al., 2020), we also found that stress was associated with alcohol-related problems. These findings build on initial studies which have suggested a stronger relationship between sleep and alcohol use in women compared to men (Haario et al., 2013; Ogeil et al., 2015), and help clarify some of the mixed findings from previous reports.

This is the first study, to our knowledge, to test sex differences in the association between poor sleep and alcohol-related problems in a sample of heavy drinkers with insomnia. Prior reports have come primarily from community samples with

TABLE 3 | Pearson correlations (r) between individual Pittsburgh Sleep Quality Index (PSQI) subscales and Alcohol Use Disorders Identification Test (AUDIT) scores in men and women.

	Women ($n = 66$)	Men ($n = 45$)
	AUDIT	AUDIT
Subjective sleep quality	0.23	0.27
Sleep latency	0.01	−0.07
Sleep duration	**0.40****	−0.11
Habitual sleep efficiency	0.15	0.27
Sleep disturbances	**0.28***	0.20
Use of sleep medication	0.12	−0.13
Daytime drowsiness	0.10	0.01

*$p < 0.05$, **$p \leq 0.01$. Bold values indicate $p < 0.05$.*

relatively low rates of problematic drinking, and these studies have typically shown a stronger association between poor sleep and alcohol-related problems in men. The one exception is the study conducted by Ogeil et al. (2015) that tested sex differences in sleep-alcohol associations among social drinkers (i.e., mean AUDIT scores = 8/11 for women/men). This study found that among the riskiest drinkers, sleep and alcohol were more strongly linked in women than men. Our results take this a step further and show that among heavy drinkers at very high risk (i.e., mean AUDIT scores = 17/18 for women/men), the association is especially pronounced in women and non-existent in men. Taken together, these findings suggest that poor sleep is especially likely to accompany heavy drinking in women.

The sex difference in the association between poor sleep and alcohol-related problems observed here lays the groundwork for future studies investigating sex differences in the directionality of this relationship. One hypothesis is that disrupted sleep is more likely to lead to increased alcohol consumption in women than in men. While there is clear evidence from prospective studies to suggest that insomnia and sleep disturbances can play a causal role in the onset of alcohol use and alcohol-related problems (Wong et al., 2004, 2015; Roberts et al., 2008; Hasler et al., 2014, 2016), little is known about sex differences in this relationship. Additionally, a significant number of drinkers report using alcohol to self-medicate insomnia (Johnson et al., 1998; Brower et al., 2001), in which case poorer sleep would lead to greater alcohol use. Although an initial report suggested such self-medication may be more common in men (Johnson et al., 1998), it is possible that this trend may be changing with increasing alcohol use among women. In sum, future studies are needed to determine whether the current findings suggest that poor sleep in women is playing a causal role in increased alcohol consumption.

Alternatively, another hypothesis suggests that greater alcohol use leads to poorer sleep for women than for men (i.e., women may be more sensitive than men to the acute impairing effects of alcohol on sleep). Indeed, one laboratory study (Arnedt et al., 2011) suggests that this is the case. In this study, young adult social drinkers consumed an oral dose of alcohol (target breath alcohol concentration = 100 mg %) 1 h prior to bedtime. Sleep was monitored throughout the night using polysomnography, and results showed that alcohol impaired objective sleep quality indicators to a greater extent in women than in men. Specifically, alcohol decreased total sleep time and sleep efficiency, and increased number of awakenings and minutes awake after sleep onset, in women, but not in men. These findings suggest that our observed association between poor sleep and alcohol use in women may be because heavy drinking causes more pronounced sleep impairment in women.

This study has some limitations. First, there were more women than men in our sample. The observed effect size in men for the relationship between sleep and drinking was quite low, suggesting that the sex difference was not due to greater power in women than in men. However, it will be important to replicate these findings with equal numbers of each sex. Additionally, study measures consisted entirely of self-report measures. Future studies would benefit from inclusion of more objective measures, including actigraphy watches and polysomnography to assess sleep and wrist biosensors to assess alcohol consumption.

In sum, our findings offer important contributions to the literature. Our primary finding, that poor sleep is linked to greater alcohol problems in heavy drinking females (but not males) with insomnia, is particularly novel. These findings further support the hypothesis that sleep quality and alcohol use are linked in women who drink heavily. Future studies are needed to determine the degree to which poor sleep is a sex-specific cause or consequence (or both) of heavy drinking in women. These studies may benefit in focusing specifically on the role of sleep duration and sleep disruptions, since our results point to those two factors as being particularly associated with alcohol problems in women but not in men. This line of research has the potential to contribute important information for the development of sex-specific prevention and treatment efforts for female heavy drinkers, particularly women who suffer from both alcohol-related problems and insomnia.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Kentucky Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JW and MM designed the study. JV collected the data, conducted the statistical analyses, and wrote the first draft of the manuscript. All authors revised the manuscript critically for intellectual content and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.875168/full#supplementary-material

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Ovarian Steroids Mediate Sex Differences in Alcohol Reward After Brain Injury in Mice

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Intoxication is a leading risk factor for injury, and TBI increases the risk for later alcohol misuse, especially when the injury is sustained in childhood. Previously, we modeled this pattern in mice, wherein females injured at postnatal day 21 drank significantly more than uninjured females, while we did not see this effect in males. However, the biological underpinnings of this sex difference have remained elusive. In this study, we utilize this preclinical model and traditional endocrine manipulations to assess the effect of perinatal sex steroids on post-injury ethanol response. We found that perinatal androgen administration and adult ovariectomy prevented the development of conditioned place preference to ethanol in females, while there was not an effect of gonadectomy either developmental time point on the severity of axonal degeneration. Finally, although TBI increased the number of microglia in males, there was no corresponding effect of gonadectomy, which suggests that males exhibit prolonged neuroinflammation after brain injury irrespective of circulating sex steroids. Taken together, our results indicate a potential role for ovarian sex steroids in the development of greater alcohol preference after a juvenile TBI in female mice.

Keywords: traumatic brain injury, alcohol, sex steroids, organizational effects, androgens, ovaries

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INTRODUCTION

The rate of traumatic brain injury (TBI) is notably increasing, with the latest report suggesting that 2.8 million individuals experience a TBI in the United States annually compared to 1.7 million according in a 2011 study (Coronado et al., 2011; Taylor et al., 2017). Intoxication is a leading risk factor for traumatic brain injury (TBI), and alcohol use disorder (AUD) is the most commonly diagnosed psychiatric disorder among TBI patients (Whelan-Goodinson et al., 2009). Moreover, the relationship between TBI and alcohol is bidirectional. Recent studies have suggested that TBI itself might increase the risk of future alcohol misuse (Adams et al., 2012; Corrigan et al., 2014; Wu et al., 2016). By some estimates more than half of substance abuse patients seeking treatment have a history of TBI (Sacks et al., 2009). Therefore, drinking increases the risk of TBI and TBI increases drinking, leading to a destructive cycle. Importantly, little is known about how injury itself increases the risk of developing AUD.

This phenomenon is most prominent in those who experienced a TBI in childhood (Corrigan et al., 2013). For many reasons, it is vital to study this population, as children comprise a large subset of those effected by TBI, and TBI is a leading cause of death and disability among this age

group (Langlois et al., 2006; Coronado et al., 2011). Additionally, the TBI population is more likely to experience anxiety, unemployment, and substance use disorders in general (Adams et al., 2012; Karver et al., 2012; Ilie et al., 2015).

Our lab previously reported sex differences in alcohol-related behaviors in adult mice that experienced a mild TBI early in life. Female mice injured at 21 days-old drank significantly more than uninjured females; however, this effect was not observed in males (Weil et al., 2016b). Moreover, injured female mice developed a conditioned place preference to ethanol, whereas males and uninjured females did not. Importantly, these findings resemble epidemiological data which indicate women who experience a TBI in adolescence are much more likely to misuse alcohol than women injured at any age, while this age-related distinction is not observed for men (Corrigan et al., 2020). Although there are sex differences noted in the epidemiological and preclinical literature surrounding TBI, most studies of TBI have focused exclusively on males, and this has left the consequences of injury among women largely understudied. This neglect is especially problematic as there are some indications that women fare worse after injury, including needing surgical intervention more often than men and exhibiting greater durations of posttraumatic amnesia and hospitalization (Farace and Alves, 2000; Oliverio et al., 2020). Given the mounting evidence that TBI can also increase the risk of AUD, this further highlights the need to address this gap in the literature. Of note, although women are less likely than men to receive a diagnosis of AUD, this disparity appears to be decreasing (Keyes et al., 2008), and women face numerous risks as they tend to progress through the stages of addiction at a faster rate, experience greater impact to their health, and are less likely to receive proper treatment (Foster et al., 2014; Mccrady et al., 2020).

The biological mediators of this sex difference have remained unknown. Noting that these studies have revealed sex differences in alcohol-related behaviors, many of which do not emerge until puberty, we postulate that sex hormones are involved in this process. Within the neuroendocrine literature, sex steroid functions are categorized into organizational and activational roles. Organizational effects generally occur early in development and establish the capacity of the nervous system to promote male-typic or female-typic reproductive behaviors later in life. Perinatal production of androgens by the testes (and subsequent aromatization to estrogens within the nervous system) results in the masculinization and de-feminization of both the external genitalia and the nervous system. In contrast, it is the absence of androgens in females which feminizes and de-masculinizes the CNS. Activational effects occur later in life when sex steroids modulate sex-typical reproductive behaviors. Alterations, in the organizational aspect of sex steroids affects the activational component of sex steroids. For instance, the introduction of androgens perinatally in females will prevent ovarian cyclicity (Gorski, 1973). Here, we hypothesized that sex differences in alcohol seeking behavior after TBI are driven in part by organizational and/or activational effects of sex steroids. To test this, we manipulated sex steroids through perinatal androgen exposure in females and gonadectomies in males and performed a mild closed-head injury early in life. In adulthood, we assessed activational effects of sex steroids on alcohol-related behavior via gonadectomy. We report that while injury severity was not altered by these sex steroid manipulations, perinatal androgen exposure and adult gonadectomy in females prevented the development of a conditioned place preference following injury. Taken together, our data indicate that sex steroids play a role in moderating alcohol-related behavior following a juvenile TBI.

MATERIALS AND METHODS

Animals

Swiss-Webster mice were purchased from Charles River (Wilmington, MA) and bred at Ohio State University (OSU). Pups were weaned at postnatal day (PND) 21 and housed in standard mouse cages under a 14:10 light-dark cycle. Mice were provided with access to food and water *ad libitum*, and all experiments were performed in accordance with approval from the OSU Institutional Animal Care and Use Committee.

Hormone Manipulations

To measure organizational effects of sex steroids, neonatal pups underwent hormone manipulations. Female mice were injected subcutaneously with testosterone, 100mg (per pup) crystalline testosterone (Sigma Aldrich) dissolved in 100 μL olive oil (Thermo Scientific) or oil alone on PND 4 (Klein et al., 2002). Male mice were gonadectomized (GDX) under cryoanesthesia at PND 4 or underwent a sham operation (Anderson et al., 2005). Briefly, the skin was disinfected with alternating swabs of betadine and 70% ethanol, a vertical midline incision was made, and the testes were removed with forceps. The incision was sutured, and pups were returned to their home cage on a heating pad. The sham procedure included an incision but no manipulation of the gonads. To measure activational effects of sex steroids, mice underwent GDX or control procedures at PND 60 as previously reported (Aubrecht et al., 2015) producing the following groups: females (oil + control "control", oil + GDX "GDX", T + GDX), males (control, neonatal GDX, adult GDX). Note that to control for age differences at the time of surgery, all male mice underwent two surgical procedures (at PND 4 and PND 60), undergoing either two control surgeries, or one control and one GDX. All mice were randomly assigned to each group.

Traumatic Brain Injury

A mild closed head injury (or sham procedure) was performed on mice at PND 21. Mice were anesthetized with isoflurane at 3% for induction and 1.5% during the operation. The injury was performed using an Impact One device (Leica Biosystems, Richmond, IL) while the head was stabilized in the stereotaxic frame. Bupivacaine (1mg/kg) was injected SC at the incision site, the skull was exposed, and the device impactor was placed on its surface at –1 AP and 1 ML relative to Bregma. The 2mm diameter tip of the impactor was then depressed 1mm into the skull at 3m/sec with a 30msec dwell time. The process was the same for sham mice, excluding the depression of the plunger. Mice were then returned to their homecage, and additional endocrine manipulations were performed at PND 60 as discussed above. The following groups were generated: Females (control/sham

n = 9, control/TBI n = 9, GDX/sham n = 8, GDX/TBI n = 8, T + GDX/sham n = 9, T + GDX/TBI n = 10), Males (control/sham n = 7, control/TBI n = 7, neonatal GDX/sham n = 7, neonatal GDX/TBI n = 7, adult GDX/sham n = 8, adult GDX/TBI n = 8). See **Figure 1A** for the timeline of hormonal manipulations, TBI (or sham surgery), and behavioral analyses.

Conditioned Place Preference

The ethanol CPP protocol (adapted from O'Neill et al., 2013; Cunningham, 2014) beginning PND 80 as follows. A plastic container held two chambers of different tactile (thick and thin grid) and visual patterns (checkered and lined) that were separated by a barrier with a small passageway. On the first day, mice were injected intraperitoneally with saline (10mL/kg) and allowed to cross freely between the chambers for 5 min to allow the mice to habituate and determine baseline side bias. Treatment/cue pairings were counterbalanced and randomly assigned. For days 2, 4, 6, and 8, mice were injected with a 20% ethanol solution (2 g/kg), returned to their home cages for 5 min, then confined to an assigned side of the container for 5 min. On alternating days (3, 5, 7, and 9), mice were injected with saline and confined to the opposite-patterned chamber for 5 min. Finally, on the 10th day, mice were injected with saline and allowed to travel freely between the chambers. Conditioned place preference was determined by assessing the amount of time spent exploring the chamber associated with ethanol on day 10 relative to day 1.

Tissue Processing

Tissue was collected after transcardial perfusion with 4% paraformaldehyde after mice were overdosed with sodium pentobarbital. The forebrain was sectioned coronally into 40 μm slices. Immunohistochemistry was performed for the microglia-specific protein Iba1 using an antibody purchased from Wako (1:1000 anti-Iba1, rabbit) as previously reported (Karelina et al., 2017). Briefly, tissue was washed with 0.1M phosphate buffered saline, quenched with hydrogen peroxide, and incubated overnight with anti-Iba1 antibody. The next day tissue was incubated with a goat anti-rabbit biotinylated secondary antibody (1:500) and visualized using the ABC-DAB method. To assess axonal degeneration, a silver stain was performed with the FD NeuroSilver^{TM} Kit II from FD Neurotechnologies following the manufacturer's instructions (Columbia, MD).

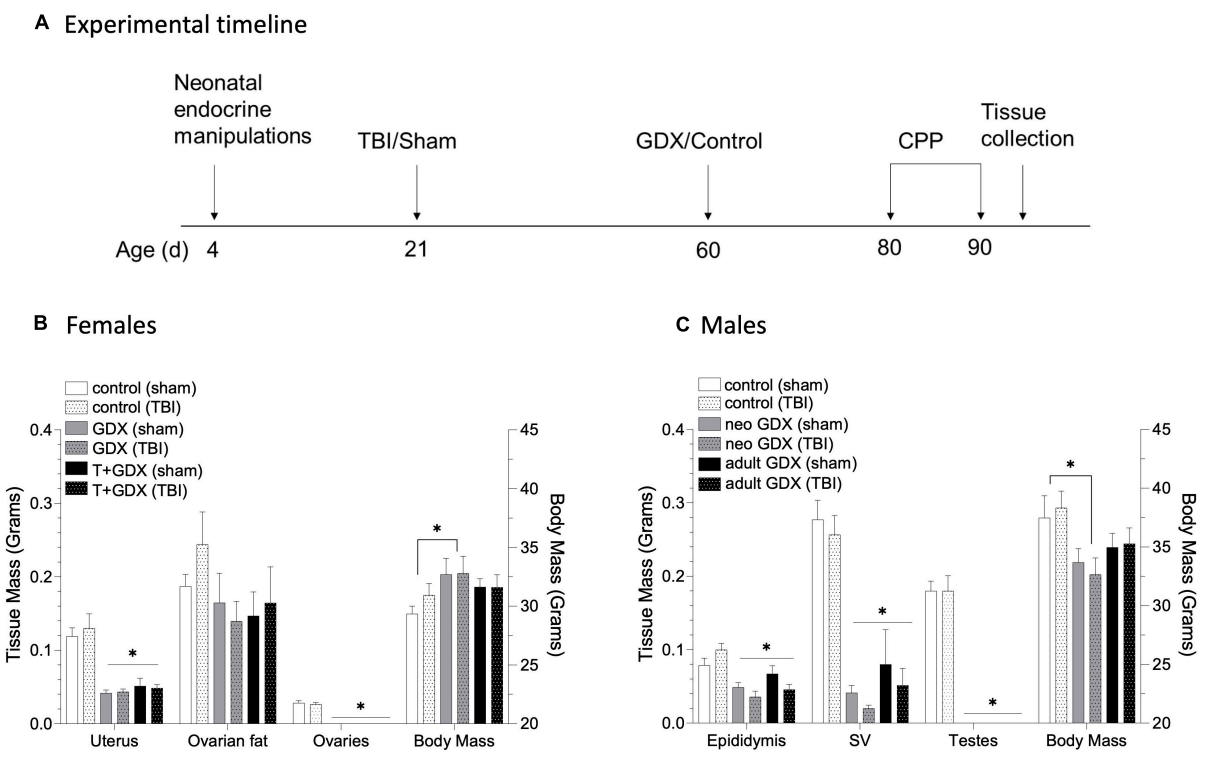

FIGURE 1 | Timeline of experimental procedures and tissue responses to endocrine manipulation. **(A)** Male and female mice underwent neonatal endocrine manipulations at PND 4 (T or oil for females, GDX or control surgery for males). TBI or sham surgery was conducted at PND 21. Additional endocrine manipulations were conducted at PND 60 (females underwent GDX or control surgery, males that underwent early life control surgery had either GDX or control surgery in adulthood, and males that underwent early life GDX had a control surgery). Females (control/sham n = 9, control/TBI n = 9, GDX/sham n = 8, GDX/TBI n = 8, T + GDX/sham n = 9, T + GDX/TBI n = 10), Males (control/sham n = 7, control/TBI n = 7, neonatal GDX/sham n = 7, neonatal GDX/TBI n = 7, adult GDX/sham n = 8, adult GDX/TBI n = 8). All mice were tested for conditioned place preference beginning PND 80. **(B)** Uterine and ovarian tissue masses and body mass in female mice and **(C)** epididymal, seminal vesicle and testicular tissue masses and body mass in male mice. An asterisk (*) indicates statistically significant difference from the relevant control group within each sex, ANOVA $p < 0.05$. Data are presented as mean ± SEM.

Microscopy

Photomicrographs of Iba1 staining were obtained at a 20X magnification (Nikon E800 microscope) and cell counts were conducted using FIJI (Schindelin et al., 2012). Cell counts were obtained from defined regions of interest (ROI) (prefrontal cortex, ROI = 0.04 mm^2; amygdala, ROI = 0.04 mm^2; nucleus accumbens, ROI = 0.072 mm^2), averaged across the hemispheres, and reported as cells per mm^2. Axonal degeneration was observed based on silver staining in the white matter tracts of the forebrain. The silver score was determined as previously reported qualitatively using a point-value system: such that 0 = little to no axonal degeneration, 1 = sparse silver staining limited to the corpus callosum, 2 = moderate silver staining in the corpus callosum and other white matter tracts, and 3 = very dense silver staining throughout multiple white matter tracts (Karelina et al., 2021). All analyses were performed blind to experimental conditions.

Statistical Analysis

Statistical analysis was performed using SPSS Version 26 (IBM Corp., Armonk, NY, United States). CPP responses, silver staining, and microglial immunohistochemistry were assessed separately for males and females via a two-way ANOVA (injury x endocrine manipulation). Reproductive tissue and body masses were assessed via a two-way ANOVA (injury x endocrine manipulation). Significant overall ANOVA results were followed up by a one-way ANOVA (factor = injury) between specified groups for CPP, or a Tukey HSD *post hoc* test for reproductive tissue and body mass. Effect sizes are reported as partial eta squared (η_p^2). Results are considered significant when $p \leq 0.05$.

RESULTS

Perinatal Testosterone Administration and Gonadectomies Reduce the Masses of Steroid-Sensitive Tissue

To determine the role of circulating sex steroids on alcohol-related behavior after brain injury, we manipulated gonadal steroid availability at critical developmental periods (perinatally and at adolescence). The effectiveness of our endocrine manipulations was verified by assessing reproductive tissue masses at necropsy (see **Figure 1** for time line). Endocrine manipulations altered tissue masses, but injury did not ($p > 0.5$ for all sham vs. TBI comparisons). Uterine mass was reduced in GDX and T + GDX females compared to control mice ($F_{2,51} = 34.74$, $P < 0.00001$, $\eta_p^2 = 0.591$), ovarian fat pad mass was also reduced but not significantly ($F_{2,50} = 20.154$, $P = 0.14$, $\eta_p^2 = 0.127$). Body mass was also altered by endocrine manipulations ($F_{2,52} = 3.141$, $P = 0.05$, $\eta_p^2 = 0.114$) such that GDX females were heavier than controls. For males, epididymides ($F_{2,\ 43} = 13.874$, $P < 0.00001$, $\eta_p^2 = 0.41$) and seminal vesicle masses ($F_{2,42} = 48.985$, $p < 0.00001$, $\eta_p^2 = 0.715$) were reduced in NEO GDX and Adult GDX groups. Body mass was altered by endocrine manipulations ($F_{2,41} = 6.064$, $p = 0.005$, $\eta_p^2 = 0.228$) such that NEO GDX male mice were lighter than intact mice.

Sex Steroid Manipulation in Females Prevents Development of Conditioned Place Preference Following Injury

An overall ANOVA including all of the sex steroid manipulations revealed no significant sex differences in CPP responses to ethanol (all $p > 0.05$), however this is not surprising given the sex-specific variability in both CPP after TBI, and the substantial difference in surgical procedures between male and female neonates (T/oil injections in females vs. GDX/Sham surgery in males). To confirm that we have replicated our previously reported findings (Weil et al., 2016b), we conducted an ANOVA to compare CPP responses among control (no sex steroid manipulations) male and female mice, and reveal that control female mice have significantly greater CPP responses to ethanol after TBI compared to intact males ($F_{1,28} = 6.022$, $p = 0.022$, $\eta_p^2 = 0.201$). CPP behavior in males and females varied in response to gonadal manipulations and TBI. Among females there was no overall effect of injury ($F_{1,39} = 0.335$, $p > 0.05$, $\eta_p^2 = 0.009$, **Figure 2A**) or of endocrine manipulations ($F_{2,39} = 0.574$, $p > 0.05$, $\eta_p^2 = 0.029$). However, there was a significant interaction ($F_{2,39} = 4.19$, $p = 0.022$, $\eta_p^2 = 0.177$) such that injury increased CPP responses among control (gonadal unmanipulated) females ($F_{1,13} = 5.756$, $p = 0.034$, $\eta_p^2 = 0.324$) but had no effect in any other group ($P > 0.05$ in all cases). Injured females that were treated with T perinatally and then gonadectomized exhibited significantly lower CPP responses than did intact females ($F_{1,15} = 6.414$, $p = 0.025$, $\eta_p^2 = 0.330$). In contrast, males exhibited no effects of injury ($F_{1,36} = 0.338$, $p > 0.05$, $\eta_p^2 = 0.009$, **Figure 2B**), endocrine manipulation ($F_{2,36} = 1.371$, $p > 0.05$, $\eta_p^2 = 0.071$), or interaction between the two variables ($F_{2,36} = 1.976$, $p > 0.05$, $\eta_p^2 = 0.099$).

Sex Steroid Manipulation Does Not Affect Axonal Degeneration or Microglial Cell Count

Silver staining was used as indicator of axonal degeneration such that a higher silver score indicated greater axonal degeneration. Injured females ($F_{1,43} = 33.16$, $p < 0.0001$, $\eta_p^2 = 0.435$) and males ($F_{1,35} = 20.55$, $p < 0.0001$, $\eta_p^2 = 0.37$) exhibited greater evidence of axonal degeneration, as assessed by silver staining than did sham-injured mice (**Figure 3**). But there was no overall significant sex difference or significant effect of endocrine manipulations among either sex ($p > 0.05$ in all cases) or interactions between the variables. We next examined microglial cell counts in regions of the mesolimbic pathway and prefrontal cortex (PFC), given the known relationship between activity in the amygdala, nucleus accumbens, and PFC and alcohol use disorder (Abernathy et al., 2010; Roberto et al., 2012; Seif et al., 2013; Grodin et al., 2018). Interestingly, microglial cell numbers in key forebrain structures was increased by TBI in injured males but not females (**Figure 4**). In the amygdala, nucleus accumbens, and PFC of females there were no injury or endocrine manipulation effects, nor were there interactions

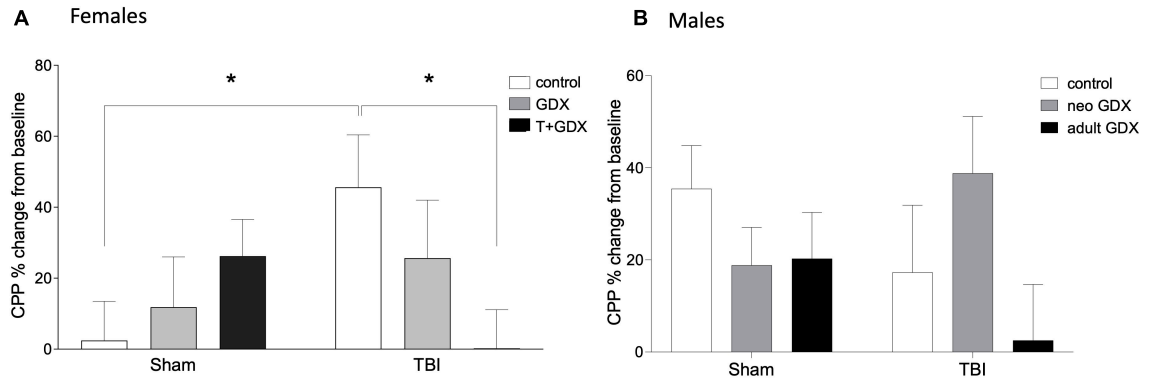

FIGURE 2 | Conditioned place preference to ethanol. Percent change of conditioned place preference to alcohol from baseline in **(A)** female and **(B)** male mice. An asterisk (*) indicates statistically significant difference from control/TBI females, ANOVA $p < 0.05$. Data are presented as mean ± SEM.

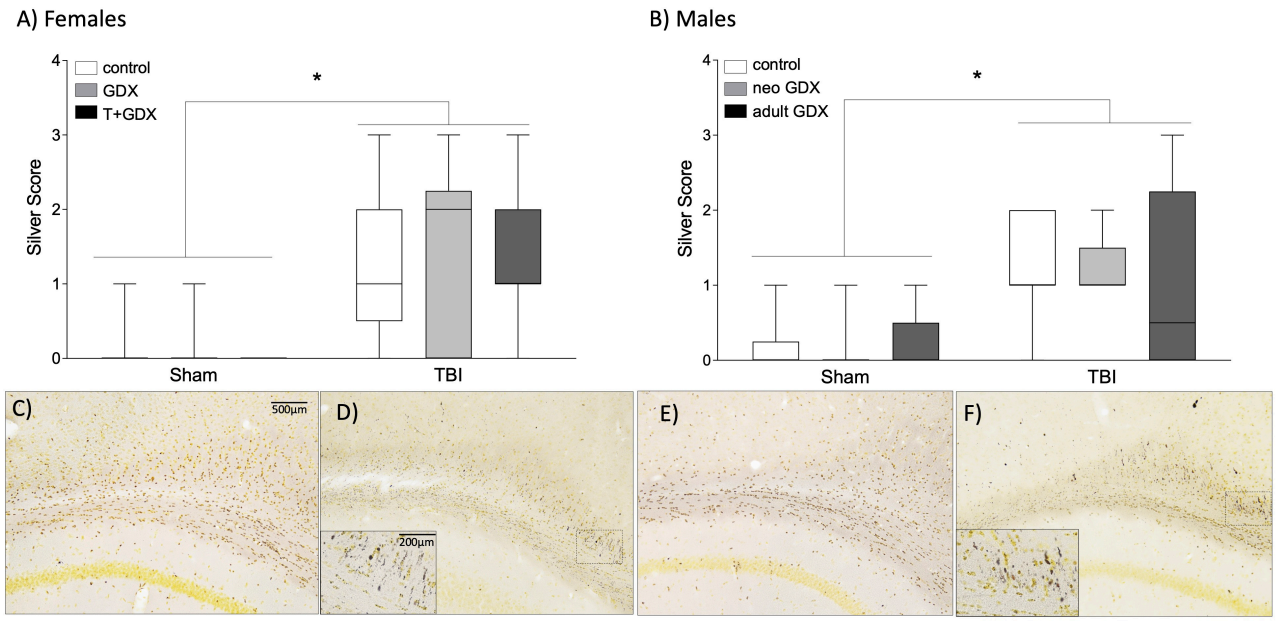

FIGURE 3 | Axon degeneration. Axonal damage scores based on silver staining in **(A)** female and **(B)** male mice. An asterisk (*) indicates statistically significant difference from sham injured mice, ANOVA $p < 0.05$. Boxplots show representative range (minimum and maximum), first quartile, median, and third quartile data for each group. Representative photomicrographs are shown for **(C)** female sham, **(D)** female TBI, **(E)** male sham, and **(F)** male TBI. Scale bar = 500 μm, inset scale bar = 200 μm.

between the variables ($P > 0.05$ in all cases). In contrast, males, regardless of endocrine manipulation have increased microglial cell counts throughout the forebrain. For instance, in the amygdala ($F_{1,21} = 5.404$, $p = 0.35$, $\eta_p^2 = 0.265$) and prefrontal cortex ($F_{1,21} = 5.101$, $p = 0.039$, $\eta_p^2 = 0.254$) injured males exhibited significantly greater microglial cell numbers than did sham-injured mice. The nucleus accumbens did not exhibit this effect ($F_{1,21} = 1.752$, $p = 0.205$, $\eta_p^2 = 0.105$). A direct comparison of microglial cell numbers in TBI mice between the sexes confirmed a sex difference such that males exhibited significantly greater numbers of microglia in the PFC ($F_{1,29} = 8.036$, $p = 0.009$, $\eta_p^2 = 0.229$), and nucleus accumbens ($F_{1,35} = 6.162$, $p = 0.018$, $\eta_p^2 = 0.157$).

DISCUSSION

Given the noted sex difference in alcohol-related behaviors after TBI in both clinical and basic research, we aimed to gain a deeper understanding of the role of circulating sex steroids as a potential mediator of this phenomenon. Our results represent the first known attempt to delineate the role of sex steroid hormones in drug seeking behavior after TBI. Here we demonstrate that while TBI-induced axonal degradation is not affected by sex steroid manipulations, perinatal testosterone administration and adult gonadectomy in females prevents the development of a conditioned place preference to ethanol. Additionally, although there was an effect of injury on microglial

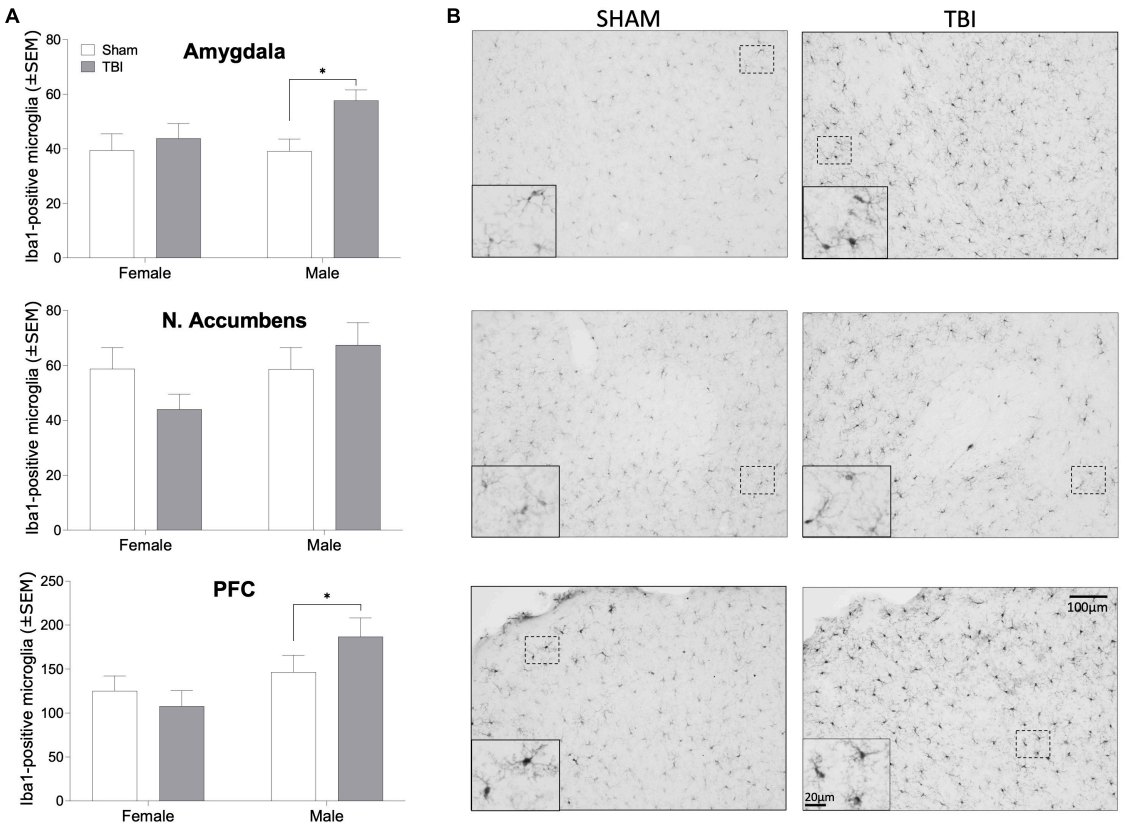

FIGURE 4 | Iba1-positive microglia. **(A)** Bar graphs depicting mean (± SEM) microglial cell counts per mm^2 in brain regions important for alcohol reward in male and female mice. **(B)** representative photomicrographs of Iba1 immunohistochemistry in male mice. An asterisk (*) indicates significant main effect of injury, ANOVA $p < 0.05$. Scale bar = 100 μm, inset scale bar = 20 μm.

cell count in males, there was no effect of testosterone or gonadectomy at either developmental timepoint on microglia for either males or females. These results represent the first known evidence towards both organizational and activational effects of sex steroids as potential regulators of alcohol-related behavior after TBI in females.

Masculinization of Females by Sex Steroid Manipulations Eliminates Ethanol Conditioned Place Preference

We hypothesized that sex steroids were the mediators of this sex difference for two primary reasons. First, both neonatal testosterone treatment and adult gonadectomy in female rodents have been shown to affect their response to ethanol. For instance, ovariectomized females drink significantly less than other females (Becker et al., 1985) and estradiol administration increases drinking in ovariectomized mice (Ford et al., 2002). Furthermore, defeminization of female rats through neonatal sex steroid exposure reduces alcohol consumption (Almeida et al., 1998). Secondly, the epidemiological data suggest that women are most at risk of exhibiting later alcohol misuse when a TBI is incurred during puberty (Corrigan et al., 2020).

Sexual differentiation of brain and behavior results from the contribution of appropriately timed steroid hormone exposure (or the lack thereof) and differential gene expression associated with sex chromosome complement (Breedlove and Hampson, 2002). In the current, study we treated female mice with androgens during the early postnatal period. The exposure to androgens, which are aromatized into estrogens in the brain, permanently prevents the development of ovarian cyclicity and other female-typic behavioral patterns. We utilized the CPP paradigm as it specifically assesses the hedonic effects of alcohol that we have previously demonstrated were altered by TBI (Weil et al., 2016b). The current study replicates previous findings: intact female mice injured early in life developed a conditioned place preference to ethanol, while the uninjured females did not (Weil et al., 2016b). Treatment with testosterone neonatally *and* adult gonadectomy abolished the enhanced CPP response to alcohol after TBI. Thus, both female typical sex steroid exposure perinatally and in adulthood are necessary for the TBI-induced potentiation of alcohol reward. In contrast, neither sex steroid manipulations (perinatal or in adulthood), nor TBI was sufficient to enhance alcohol CPP in male mice. Thus, the enhanced CPP response among brain injured females appears to require normal ovarian cyclicity and cannot be replicated by neonatal castration of genetic males.

Role of Traumatic Brain Injury Pathophysiology as a Regulator of Alcohol-Seeking Behavior

A separate possibility underlying the disparity in alcohol response between males and females is a difference in injury severity. For instance, some data from clinical populations suggest that women fare worse than men after TBI and exhibit greater delays in recovery (Farace and Alves, 2000). However, given that axonal degeneration is not significantly different between unmanipulated males and females after a TBI, we assert that this is not the cause of the sex difference represented in our model. Moreover, axonal degeneration was affected by injury but not significantly altered by neonatal testosterone treatment or gonadectomy. Since the axon injury scores do not mirror the alcohol-related behavior, these behavioral responses appear to occur independently of injury severity.

We also considered that the variations in alcohol response could be due to differential neuroinflammation, as there is an established link between neuroinflammation and substance abuse (Kohno et al., 2019). Moreover, we previously showed that treatment with an inhibitor of microglial activation reduced post-TBI drinking in male mice (Karelina et al., 2018). There are both sex differences in neuroimmune physiology and effects of steroid hormones on immune function (Villa et al., 2016). We had predicted that greater microglial activity in brain regions related to reward processing might correlate with CPP responses among injured mice. Traumatic brain injuries and other early life perturbations can produce a persistent state of increased reactivity to immune challenges (Fenn et al., 2014). Most drugs of abuse, including alcohol can drive inflammatory signaling (Alfonso-Loeches et al., 2010; Lacagnina et al., 2017). There is a substantial literature linking early life stress and adversity with both alterations in microglial physiology *and* increased susceptibility to substance abuse in both preclinical and clinical studies (Enoch, 2011; Crews et al., 2017; Pascual et al., 2017; Johnson and Kaffman, 2018). Taken together, we had reasoned that TBI would prime microglial immune responses such that the administration of alcohol would produce exaggerated inflammatory responses. Sex differences in this relationship could therefore mediate the sex differences in CPP responses to alcohol among injured mice gonadectomy, such that microglial count was greater in the PFC and amygdala of males that underwent a TBI. This is in line with existing evidence that the neuroinflammatory response is greater in males after a TBI (Villapol et al., 2017). One limitation of these experiments is that the length of time between injury and tissue collection (2 + months); thus, testosterone treatment or gonadectomy promote acute changes in neuroinflammation that may resolve over time. Additionally, examining microglial cell numbers only provides a relatively limited amount of information as to the reactivity, gene expression, and secreted factors. This finding suggests that although injury promotes a neuroinflammatory response in males, this occurs independently of sex hormones. Furthermore, this finding reduces the possibility that neuroinflammation is directly responsible for the increase in alcohol consumption after a TBI. neuroinflammatory response in males, this occurs independently of sex hormones. Furthermore, this finding reduces the possibility that neuroinflammation is directly responsible for the increase in alcohol consumption after a TBI.

These data have important implications for case management of individuals with a history of childhood traumatic brain injury. Substance abuse prevention and treatment have the potential to produce significantly better outcomes in this patient population because (1) substance abuse impairs rehabilitation and produces poorer overall outcomes in those with a history of brain injuries and (2) alcohol intoxication is a major risk factor for subsequent TBIs which can have devastating permanent consequences (Weil et al., 2016a, 2019; Corrigan et al., 2020). Moreover, there is also epidemiological evidence that sex differences exist in the risk of developing substance abuse disorders after brain injury. Humans differ from rodents in that males tend to consume more alcohol whereas the opposite is true among laboratory rodents (Barker et al., 2010). Thus, from a public policy standpoint it will be beneficial to invest in strategies that reduce the risk of developing substance abuse disorders for individuals of both sexes that have experienced TBI. However, these data should highlight the importance of considering the role of sex and sex steroids as potential contributors to substance-abuse related outcomes after brain injuries.

CONCLUSION

Overall, these findings provide substantial evidence that sex hormones are involved in the increase in alcohol-related behaviors after a juvenile TBI in female mice. The ultimate reasons for sex differences in the response to alcohol remain unspecified but at a proximate level, ovarian steroids appear to be necessary both perinatally and in adulthood to produce the sex difference in alcohol responses. While this is an important first step to identifying a role for circulating sex steroids as mediators of drug seeking behavior after TBI, there are equally important follow up questions that still need to be addressed to understand the mechanisms by which sex steroids may mediate these behaviors. Key among these is the question of how alcohol use prior to injury affects post-TBI alcohol use, with a particular focus on sex differences. Moreover, future studies are needed to assess the roles of important variables that were controlled in this study, including impact severity and location.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ohio State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

ZW and KK contributed to conception and design of the study. JF, RV-C, BW, and RO performed the experiments. RO wrote the first draft of the manuscript. ZW and KK analyzed the data and contributed to the writing and design of the figures. All authors contributed to manuscript revision, read, and approved the submitted version.

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Sex differences in cognitive flexibility are driven by the estrous cycle and stress-dependent

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Stress is associated with psychiatric disorders such as post-traumatic stress disorder, major depressive disorder, anxiety disorders, and panic disorders. Women are more likely to be diagnosed with these stress-related psychiatric disorders than men. A key phenotype in stress-related psychiatric disorders is impairment in cognitive flexibility, which is the ability to develop new strategies to respond to different patterns in the environment. Because gonadal hormones can contribute to sex differences in response to stress, it is important to consider where females are in their cycle when exposed to stress and cognitive flexibility testing. Moreover, identifying neural correlates involved in cognitive flexibility could not only build our understanding of the biological mechanisms behind this crucial skill but also leads to more targeted treatments for psychiatric disorders. Although previous studies have separately examined sex differences in cognitive flexibility, stress effects on cognitive flexibility, and the effect of gonadal hormones on cognitive flexibility, many of the findings were inconsistent, and the role of the estrous cycle in stress-induced impacts on cognitive flexibility is still unknown. This study explored potential sex differences in cognitive flexibility using an operant strategy shifting-paradigm after either control conditions or restraint stress in freely cycling female and male rats (with estrous cycle tracking in the female rats). In addition, we examined potential neural correlates for any sex differences observed. In short, we found that stress impaired certain aspects of cognitive flexibility and that there were sex differences in cognitive flexibility that were driven by the estrous cycle. Specifically, stress increased latency to first press and trials to criterion in particular tasks. The female rats demonstrated more omissions and perseverative errors than the male rats; the sex differences were mostly driven by proestrus female rats. Interestingly, the number of orexinergic neurons was higher in proestrus female rats than in the male rats under control conditions. Moreover, orexin neural count was positively correlated with number of perseverative errors made in cognitive flexibility testing. In sum, there are sex differences in cognitive flexibility that are driven by the estrous cycle and are stress-dependent, and orexin neurons may underlie some of the sex differences observed.

KEYWORDS

stress, cognitive flexibility, sex differences, orexin, estrogen

Introduction

Stress is associated with a variety of psychiatric disorders such as post-traumatic stress disorder (PTSD), major depressive disorder (MDD), anxiety disorders, and panic disorders (Carr et al., 2013; Bangasser and Valentino, 2014). Interestingly, women are more likely to be diagnosed and often have different symptoms and severity of stress-related psychiatric disorders than men (Nestler et al., 2002; Keane et al., 2006; Bangasser and Valentino, 2014; Swaab and Bao, 2020). Therefore, investigating sex differences and underlying biological mechanisms is crucial to the diagnosis and treatment of these disorders.

A key phenotype in stress-related psychiatric disorders is cognitive impairment (Ben-Zion et al., 2018; Boisseau and Garnaat, 2018; Doss et al., 2021). Importantly, stress is related to cognitive deficits such as impaired cognitive flexibility (Powell and Ragozzino, 2017). Cognitive flexibility is the ability to change or switch between mental sets and develop new strategies to adapt and respond to different patterns in the environment (Powell and Ragozzino, 2017; Irwin et al., 2019). Thus, this is an important skill for daily functioning. Understanding more about how stress affects the brain and cognition is vital in treating stress-related psychiatric disorders.

Although our ultimate goal is to understand how stress affects humans, studying stress in animal models allows for control of more variables. Importantly, there are shared physiological and behavioral responses to stress in both humans and rodents, which makes studying the stress response in rodents translatable to humans (Schöner et al., 2017). Among all stress protocols in rodents, restraint stress is one of the simplest and most common approaches (Campos et al., 2013; Taslimi et al., 2019). This study examines how acute restraint stress affects cognitive flexibility performance in male and female Sprague Dawley rats in order to better understand the biological underpinnings of any sex differences observed.

Previous research has assessed cognitive flexibility using attentional set-shifting paradigms in both humans and animals (Brown and Tait, 2016). The paradigms are focused on flexibility in shifting between thoughts and actions and involve problem-solving and exploration (Ionescu, 2012; Marko and Riečanský, 2018). In humans, the Wisconsin Card Sorting Test (WCST) is a common test for cognitive flexibility, while in rodents, the Attentional Set Shifting Paradigm is the most common (Brown and Tait, 2016; Miles et al., 2021). In both procedures, intradimensional (ID) and extradimensional (ED) cues are used, and the subject must learn to shift both within and between dimensions to complete the test (Brown and Tait, 2016).

In this study, an automated operant strategy-shifting paradigm is used in which the dimensions are lever positions and light cues; rodents must undergo initial discrimination between the levers, followed by reversal of the levers and, lastly, an extradimensional shift to light cues (Floresco et al., 2008; Hurtubise and Howland, 2016; Grafe et al., 2017a; Gargiulo et al., 2020). Reversal learning involves a change in response strategy in the same stimulus dimension (e.g., lever position), actively suppressing a previously learned response strategy while acquiring a new competing strategy. The extradimensional shift (sometimes referred to as "strategy shifting" or "set shifting") also requires changing a response strategy and suppressing a previously learned rule, but it is across stimulus dimensions (e.g., from lever position to a light cue) (Brady and Floresco, 2015). Both of these tasks require high-level cognitive processes that are necessary for behavioral flexibility and are mediated by unique subregions in the frontal cortex (Brown and Tait, 2016). Most previous research studies on adults indicate that the extradimensional shift is more difficult than reversal learning (Buss, 1956; Harrow and Friedman, 1958). Importantly, this operant strategy-shifting paradigm also allows for assessment of different kinds of errors, including perseverative errors, which are persistent responses made by a subject on the basis of a previous rule, and these error types are common in stress-related psychiatric disorders, demonstrating cognitive rigidity and an inability to adapt to change (Uddo et al., 1993; Vasterling et al., 1998; Van Laethem et al., 2016; Miles et al., 2021). Moreover, this behavioral paradigm allows for quantification of omissions, which are failures to respond to a cue and appear to be more common in patients with PTSD, indicating slower cortical processing and attentional deficits (Korgaonkar et al., 2021). Thus, this paradigm is translationally relevant.

Thus far, there have been limited studies exploring sex differences in cognitive flexibility, with the majority of research indicating that women require more trials for reversal learning compared to men, but that there were no sex differences in ED shifts (LaClair et al., 2019; Hilz et al., 2022). Moreover, studies exploring the effects of stress on cognitive flexibility tend to only include male subjects and are equivocal in their findings. In short, studies have demonstrated that the effects of stress on cognitive flexibility vary depending on the length of the stress (with chronic stress showing greater impairments in cognitive flexibility than acute stress), stages of the task (some stress affects ID tasks while others affect ED tasks), and the time after stress (with both long-term or short-term effects) (Thai et al., 2013; Hurtubise and Howland, 2016; Sullivan et al., 2019). Sex differences have been observed in the effects of stress on cognitive flexibility in some studies, although the findings have also been inconsistent between animal and human research. For example, rodent research indicates that stress impairs cognitive flexibility more strongly in females, and human research indicates that men are more susceptible to cognitive flexibility deficits after stress (Shields et al., 2016; Goldfarb et al., 2017; Grafe et al., 2017a). Research studies measuring other aspects of cognition (including spatial memory) indicate that chronic restraint stress may enhance

performance in women compared with men, although sex differences may be task-dependent and are heavily influenced by the phase of the light cycle (Bowman et al., 2009; Huynh et al., 2011; Peay et al., 2020). These changes in cognition after restraint stress stem from sex-specific changes in the brain (Conrad et al., 2017). The inconsistency between findings from different studies indicate that more research is needed to investigate sex differences in the effects of stress on cognitive flexibility and to explore the underlying mechanisms for a more comprehensive understanding.

Although previous research has explored the effect of repeated restraint stress on cognitive flexibility, the experimental paradigm included the effect of restraint stress on learning (e.g., 5 consecutive days of repeated restraint was administered, followed by 3 days of training for the cognitive flexibility task; finally, the cognitive flexibility test was administered) (Grafe et al., 2017a). Conversely, in this study, the training for cognitive flexibility was conducted for 3 days, and on the 4th day, acute restraint was administered immediately prior to the cognitive flexibility testing. Thus, in this study, we are examining how stress affects cognitive flexibility performance and eliminating the effect that stress may have on learning the task.

Because gonadal hormones can contribute to sex differences in response to stress (Becker et al., 2005; Oyola and Handa, 2017; Heck and Handa, 2019), it is important to consider where women are in their cycle when exposed to stress and cognitive flexibility testing. In general, estrogen has been shown to promote stress response, while progesterone inhibits the stress response (Becker et al., 2005; Oyola and Handa, 2017; Heck and Handa, 2019). In rodents, the reproductive cycle is called the estrous cycle, which happens for 4 to 5 days (Becker et al., 2005) and includes the diestrus, proestrus, and estrus phases (Becker et al., 2005; Oyola and Handa, 2017). The diestrus in rodents is equivalent to the follicular phase in humans when estrogen gradually increases. In the proestrus phase, both estrogen and progesterone levels increase (progesterone starts to increase later than estrogen) and peak before ovulation. The estrous phase is when ovulation occurs and estrogen and progesterone begin to decline (Becker et al., 2005; Oyola and Handa, 2017). Previous studies have separated these phases into low and high gonadal hormone phases (i.e., diestrus/estrus vs. proestrus) (Goldman et al., 2009). Importantly, when estrogen is lowest during the diestrus phase, female rodents are observed to secrete stress hormones in a similar manner to male rodents (Heck and Handa, 2019). In contrast, stress hormones are higher in proestrus than in diestrus (Oyola and Handa, 2017; Heck and Handa, 2019).

There have been inconsistent findings on the impacts of gonadal hormones on cognitive flexibility. For example, one rodent study has found that 17β-estradiol treatment in females leads to worse set-shifting performance compared to both males and ovariectomized control females (Hilz et al., 2022). However, another rodent study has shown that in ovariectomized female rats, 17β-estradiol treatment results in poorer learning in simple discrimination but improved learning in extradimensional set-shifting (Lipatova et al., 2016). Furthermore, in male rats, high testosterone has been reported to impair both extradimensional set-shifting and reversal learning (Wallin and Wood, 2015; Tomm et al., 2022). Thus, more research is necessary to better understand how changes in gonadal hormones may impact cognitive flexibility. Additionally, studies that consider how stress affects cognitive flexibility while considering gonadal hormone status are nonexistent, which is the reason for this study.

Identifying neural correlates involved in cognitive flexibility could not only build our understanding of biological mechanisms behind this crucial skill but also leads to more targeted treatments for psychiatric disorders associated with impairments of cognitive flexibility. Many brain regions are involved in cognitive flexibility. Here, we focus on two regions: the prefrontal cortex and the lateral hypothalamus, which have been shown to play a role in stress-induced changes in cognitive flexibility (McAlonan and Brown, 2003; Placek et al., 2013; Arnsten, 2015; Lipatova et al., 2016; Grafe et al., 2017a; Durairaja and Fendt, 2021). Importantly, previous research has indicated that the orbital prefrontal cortex (OFC) is important for reversal learning (McAlonan and Brown, 2003), whereas the medial prefrontal cortex (mPFC) is required for extradimensional set-shifting (Placek et al., 2013; Lipatova et al., 2016; Durairaja and Fendt, 2021). Moreover, orexin neuropeptides produced in the lateral hypothalamus have been shown to impair reversal learning both in control and stressed animals (Grafe et al., 2017a; Durairaja and Fendt, 2021). Hormonal fluctuations associated with the estrous cycle can alter both prefrontal and orexin activities (Porkka-Heiskanen et al., 2004; Duclot and Kabbaj, 2015). Thus, understanding the effects of stress on these brain regions and sex differences in the effects (especially sex differences mediated by the estrous cycle) may help us develop sex-specific treatments for stress-related disorders.

This study explored potential sex differences in cognitive flexibility using an operant strategy-shifting paradigm (Floresco et al., 2008; Grafe et al., 2017a; Gargiulo et al., 2020) after either control conditions or acute restraint stress in freely cycling female and male rats (with estrous cycle tracking in the female rats). In addition, we examined both the PFC and orexin neurons in the lateral hypothalamus as potential neural correlates for any sex differences observed. In short, we found that there are sex differences in cognitive flexibility that are driven by the estrous cycle and are stress-dependent, and that orexin neurons in the lateral hypothalamus may underlie some of the sex differences observed. As cognitive flexibility is affected in many stress-related psychiatric

disorders, better understanding how stress and the estrous cycle affect this phenotype at the neurobiological level is important in individualized diagnosis and treatment of these disorders.

Methods

Subjects and overview of procedures

Sprague-Dawley rats ($n = 24$ male and $n = 40$ female; Envigo, Indianapolis, IN; run in 4 cohorts) were same-sex pair-housed and accommodated in a 12-h light/dark cycle with lights on and off at 7am and 7pm, respectively. The rats were acclimated to the animal facility for at least 2 weeks after arrival. Food and water were available *ad libitum*. When the rats were at least 65 days of age, daily measurements of tail temperature (using infrared technology, IR Rodent Thermometer; BIOSEB, Vitrolles, France), body weight, and vaginal lavage (in the female rats only) were conducted until the conclusion of the experiment (see Figure 1A for experimental timeline). Thus, the male and female rats were handled for a comparable amount of time, which has been reported to affect behavioral performance (Bohacek and Daniel, 2007). However, we did not perform anal swabs on males as a control for the vaginal swab procedure in females, as some previous studies have (Talboom et al., 2014). Tail temperature was not significantly different between the male and female rats over the course of the study; therefore, data are not shown. Five days before operant strategy-shifting training, the animals were singly housed and underwent food restriction. During food restriction, the rats were restricted to 80% of free-feeding weight as a guideline; water was still available *ad libitum* (Gargiulo et al., 2020). Daily body weights were collected to ensure that the rats were gaining weight according to Sprague Dawley growth data (Envigo, Indianapolis, IN) and did not lose any weight during food restriction for the operant strategy shifting paradigm; the data are not shown. The entire operant strategy-shifting paradigm lasted for 4 days, including 3 days of training and 1 day of testing. On test day, the rats were randomly divided into two conditions: control ($n = 12$ males and $n = 20$ females) and 30-min restraint stress ($n = 12$ males and $n = 20$ females). Restraint video recordings for 2 of the stressed females were not fully captured (beginning minute of restraint was missing), so they could not be analyzed. Thus, $n = 18$ for the female restraint stress data rather than $n = 20$. Thirty minutes after the operant strategy-shifting task ended, the rats were sacrificed, and their brains were collected for slicing and staining procedures. Three male rats did not learn the operant strategy-shifting task, thus, total $n = 21$ for male behavioral data rather than $n = 24$. The first cohort of rats could not be analyzed for c-fos as the PFC slices were not in good-enough condition for quantification; thus, the n for c-fos data is 16 for the male and 32 for the female rats. All the procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Bryn Mawr College.

Procedures

Lavage

When the female rates were at least 65 days of age, vaginal lavage was performed every morning to track the estrous cycle until the end of the experiment, as previously described (Gargiulo et al., 2020). Briefly, a glass pipette with warm water inside was pressed and released several times on the surface of the vaginal orifices of the female rats. The sample was then transferred to a specimen slide with acrylic paint circles and was observed under a Leica microscope (DM4 B; Leica Camera, Wetzlar, Germany) at 5× magnification. The estrous cycle phases from the vaginal cytology samples were determined as previously described (Becker et al., 2005). In short, samples that displayed predominantly leukocytes (and some larger round cells without nuclei) were categorized as diestrus. Samples that had primarily nucleated epithelial cells were categorized as proestrus. Samples that principally included cornified cells were categorized as estrus. Representative images of vaginal lavage samples categorized into each phase of the estrous cycle are included in Supplementary Figure 1.

Restraint stress

The 12 male and 20 female rats randomly assigned to the stress condition were exposed to a single acute restraint stress just prior to the operant strategy-shifting test. Briefly, the rats were placed in a Broome-style transparent restraint tube for 30 min. Previous research has indicated that corticosterone in the plasma increases significantly by 15 min of restraint and peaks at 30 min of restraint; thus, this duration of restraint is sufficient to induce a reliable stress response (Jaferi et al., 2003). A camera recorded the time it took the experimenter to restrain a rat as well as the struggle behavior displayed by the rodent for the first 10 min of restraint. Time to restrain was defined as the time it took the experimenter to secure the rodent in the restrainer (in seconds). Struggle behavior was quantified as cumulative duration of movement (in seconds); this was hand-coded by an experimenter blind to other experimental conditions (e.g., sex or estrous phase). Total stress behavior was quantified as the summation of time to restrain and struggle behavior. The detailed steps of the restraint stress procedure are as previously described (Grafe et al., 2017a; Gargiulo et al., 2020). After the stress exposure, the rats were immediately transferred into the operant chamber to perform the operant strategy-shifting test.

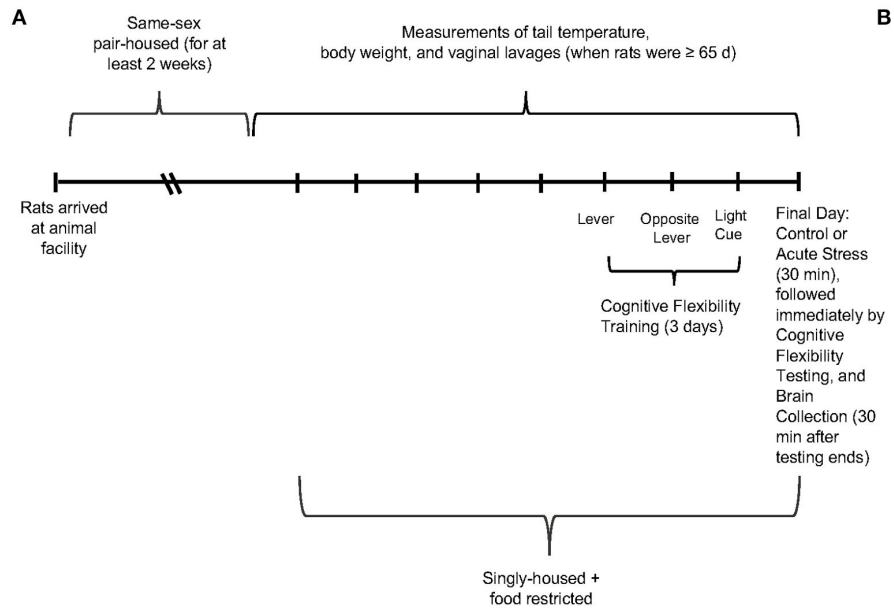

FIGURE 1
(A) Experimental paradigm depicting the timeline for collection of tail temperature/body weight/vaginal lavage measures, operant strategy shifting training/testing, restraint stress, and brain collection. **(B)** Schematic illustrating the operant set shifting paradigm on test day.

Operant strategy-shifting paradigm

The operant strategy-shifting paradigm spans 4 days. For both training and tests, each rat was placed in the operant chamber with a house light, two retractable levers with two stimulus lights above them, and a food pellet dispenser for reinforcement for these tasks (MED-PC, St. Albans, VT, United States). The training was completed during the first 3 days (each rat first learned to press the right lever on day 1, left lever on day 2, and respond to light cues above the levers on day 3). For each day of training, 50 correct trials had to be completed before the training ended. There were no light cues on the first 2 days of the training. For day 3 of the training, stimulus lights appeared above both levers for 5 s, signaling that the rat should make a response; the correct lever (with food pellet reward) was chosen randomly for each trial. If the rat did not press a lever when the lights were on, the house light and stimulus lights were turned off for 5 s, and no food pellet was delivered; this lack of response is defined as an omission. If the rat pressed a lever when the stimulus lights were on and the side was randomly assigned to be the correct side, the house light and stimulus lights stayed on for 3 s, while a food pellet was delivered, and then all the lights were turned off for 7 s before the next trial started; this is defined as a correct response. If the rat pressed a lever when the lights were on and the side was randomly assigned to be the incorrect side, the house light and stimulus lights were turned off for 10 s, and no food pellet was delivered; this is defined as an error. These conditions were matched during testing. Side bias for a particular lever was calculated after day 3 of training, and the rats were started on their least preferred side on test day.

On the 4th day (test day), the rats underwent the three tasks consecutively, where a light cue would appear above one lever for each trial (see Figure 1B for schematic). Briefly, the test day began with side discrimination, where the rats learned to press the lever on one side of the operant chamber to be rewarded regardless of where the light cue was illuminated, followed by side reversal, also called reversal learning, where the rats learned to press the lever on the other side of the operant chamber to be rewarded regardless of where the light cue was illuminated and, finally, light discrimination, also called extradimensional set-shifting or set-shifting, where the rats learned to press the lever under the illuminated light cue to be rewarded (Floresco et al., 2008; Hurtubise and Howland, 2016; Grafe et al., 2017a; Gargiulo et al., 2020). Each rat had to press the correct lever 8 times consecutively to complete each task. The data for the number of correct responses, errors (including types of errors), omissions, and latency to first press were collected with the computer software that operated the chamber. Error types were characterized by logistic regression to determine if the rats perseverated on a previous rule (perseverative error) or failed to acquire or maintain a new rule (regressive error) as described previously (Snyder et al., 2014). Briefly, every trial attempted was categorized as correct or incorrect and regressed by trial number. A logistic curve of best fit was generated, and the trial

number after which the value of the curve became greater than or equal to chance performance value of 50% was noted. Errors that occurred before this trial were categorized as perseverative, and errors that occurred after were categorized as regressive. More details of the procedures for the training and testing phases have been previously described (Gargiulo et al., 2020).

Immunohistochemistry

All the rats were sacrificed by rapid decapitation 30 min after they finished the light discrimination task (the final task for test day). Their brains were extracted and submerged in 4% paraformaldehyde for 3 days and then transferred to and stored in 30% sucrose. Each brain was sectioned into 40-μm slices using a freezing Leica microtome (SM2000R; Leica Microsystems, Wetzlar, Germany) and stored on 12-well plates with cryoprotectant in each well. The first series of tissues (Bregma 3.7 to −0.26 mm) was stained for *c-fos* expression in the prefrontal cortex, and the third series of tissues (Bregma, 2.3to −4.3 mm) was stained for cells that express orexin in the lateral hypothalamus.

C-fos in the PFC

The detailed immunochemical staining procedures for *c-fos* in the PFC and OFC took 2 days as previously described (Gargiulo et al., 2020). In short, we incubated slices in a mouse anti-c-fos primary antibody solution (1:500; ab208942, Abcam, Cambridge, United Kingdom) with 3% normal donkey serum (NDS) and phosphate-buffered saline with Triton-X (PBS/Tx) overnight at room temperature. On the 2nd day, we incubated the slices in the biotin-SP-conjugated donkey anti-mouse secondary antibody (1:250; 715-065-150; Jackson ImmunoResearch, West Grove, United States) with 3% NDS and PBS/Tx for 2 h at room temperature, followed by the avidin biotin peroxidase complex, and 3,3'-diaminobenzidine (DAB) solution. *C-fos* expression was quantified in the prelimbic cortex (PRL), infralimbic cortex (IL), and orbitofrontal cortex (OFC). The bregma levels of the images were from 2.2 to 3.7 mm for the IL and 3.2 to 4.7 mm for the PRL and the OFC. For each rat, an average of 8 pictures per brain area was analyzed. The *c-fos* expression for each image was counted with a macro using ImageJ, with a rolling radius of 40, a Gaussian Blur sigma of 5 and 0.75, a particle size of.02-10, and a particle circularity of.5-1. The *c-fos* counts for each bregma level were determined by averaging the counts across all the images (both left and right hemispheres) within that bregma level. The final *c-fos* expression for each PFC subregion in each rat was determined by averaging counts across all the bregma levels analyzed for that region.

Orexin in the lateral hypothalamus

The immunofluorescence procedure for staining orexin in the lateral hypothalamus took 2 days as previously described (Grafe et al., 2017a; Gargiulo et al., 2021). Briefly, on the 1st day, slices were incubated in a rabbit anti-orexin-A primary antibody solution (1:500, ab255294; Abcam, Cambridge, United Kingdom) with 3% NDS and PBS/Tx overnight at room temperature. On the 2nd day, we incubated the tissue in donkey an anti-rabbit Alexa Fluor 488 secondary antibody (1:500, a-21206; Thermo Fisher Scientific, Waltham, United States) with 3% NDS and PBS/Tx for 1 h at room temperature. Orexin-expressing cells in the lateral hypothalamus were hand-counted using the Multi-point Tool in ImageJ. The bregma levels of the images were from −1.3 to −4.52mm. An average of 22 images was analyzed for each rat. The orexin cell counts within each bregma level were determined by averaging the counts across both the left and right hemispheres. The final number of cells expressing orexin was determined by averaging counts across all the bregma levels analyzed in each rat.

Statistical analysis

All the data are presented as mean ± standard error of the mean. The Graphpad prism software (version 9.3.1) was employed for statistical analysis. Outliers were identified as 2 standard deviations above or below the mean. Independent sample *t*-tests (Student's *t*-test for groups with homogeneity of variances and Welch's *t*-test for groups without homogeneity of variances) were conducted to examine the relationship between sex and task performance for each task during the training phase. Task performance during training was assessed with Trials to criterion, Errors, Time to criterion, and Latency to first press for each task (plus Omission for learning to respond to light cue). A one-way ANOVA followed by the Tukey's *post-hoc* tests was conducted to analyze the relationship between sex (male vs. female rats) and stress (control vs. stress) on behaviors during restraint (measured by Time to Restrain, Struggle Behavior, and Total Stress Behavior). Two-way ANOVAs followed by Tukey's *post-hoc* tests were conducted to examine the relationship between sex (male vs. female rats) and stress (control vs. stress) for each task during the testing phase. To analyze gonadal hormone status, we separated the female rats into low vs. high gonadal hormone groups (i.e., diestrus/estrus vs. proestrus), as previously described (Goldman et al., 2009). Raw data examining each estrous cycle phase separately can be found in Supplementary Figures 2–5. Two-way ANOVAs followed by Tukey's *post-hoc* tests were conducted to examine the relationship between gonadal hormonal status (male vs. diestrus/estrus female vs. proestrus female rats) and stress (control vs. stress) for each task during the testing phase. In both cases, the same measurements were collected as during the training phase, plus Omissions for Side Discrimination and Side Reversal, as well as Perseverative Errors and Regressive Errors for Side Reversal and Light Discrimination. Two-way ANOVAs followed by Tukey's *post-hoc* tests were also carried out to examine the relationship between sex (male vs. female

rats) and stress (control vs. stress) on *c-fos* expression in the IL, PrL, and OFC subregions of the PFC and on orexin-expressing cells in the lateral hypothalamus. Additionally, two-way ANOVAs followed by Tukey's *post-hoc* tests were performed to examine the relationship between gonadal hormonal status (male vs. diestrus/estrus female vs. proestrus female rats) and stress (control vs. stress) on orexin-expressing cells in the lateral hypothalamus. Lastly, correlations were performed to determine the relationship between *c-fos* expression or orexin expression and task performance during the cognitive flexibility testing phase. The level of significance for all the analyses was set at $p < 0.05$.

Results

Sex differences in the training phase of the operant strategy-shifting paradigm

On the 1st day of training for the operant lever pressing task, the female rats made fewer errors than the male rats (learning to press the right lever, male rats = 12 ± 1.5 errors vs. female rats = 8.3 ± 1.1 errors, $t_{(59)} = 2.031$, $p = 0.047$; Figure 2A). However, the female rats made more errors than the male ones on day 2 of the training (learning to press the left lever, male rats = 40.5 ± 4.3 errors vs. female rats = 53.7 ± 0.0 errors, $t_{(58)} = 2.009$, $p = 0.044$; Figure 2B). In addition, the female rats exhibited a shorter latency to first press than the male rats on day 2 of the training (male rats = 278.6 ± 48.3 s vs. female rats = 98.9 ± 19.4 s, t (31) = 3.451, $p < 0.001$; Figure 2C). However, there were no sex differences in trials to criterion and time to criterion on the 1st and 2nd days of the training (data not shown). On the 3rd day of the training (learning to respond to the light cue), the female rats again showed a significantly shorter latency to first press compared to the male rats (female rats = 88.5 ± 12.7 vs. male rats = 231.5 ± 33.2 s, t (30) = 4.023, $p < 0.001$; Figure 2D), while trials to criterion, number of errors, time to criterion, and number of omissions did not show significant differences between the female and male rats (data not shown). Overall, our results suggest that during the training phase of the cognitive flexibility test, the female rats made fewer errors when learning to press the lever differentiated by its location on day 1 of the training but made more errors than the male rats on day 2 of the training on the opposite lever. Moreover, the female rats demonstrated a shorter latency to first press than the male rats on the 2nd and 3rd days of the training.

Restraint stress behaviors did not differ between the male and female rats

There were no significant differences in time to restrain, struggle time, and total stress behavior between the male and female rats (Figure 3). The results suggest that the male and female rats exhibited similar stress behaviors when exposed to acute restraint stress.

Acute stress effects and sex differences in cognitive flexibility

We first examined the relationship between stress (control vs. stress) and sex (male vs. female) in each task in the cognitive flexibility test (Figure 4). Stress had a main effect on the latency to first press for the first task of the test [side discrimination, $F_{(1,53)} = 6.952$, $p = 0.011$, $\eta^2 = 0.115$; Figure 4A, for graph depicting only the main effect of stress, refer to Supplementary Figure 6A] and on trials to criterion for the 3rd task of the test [light discrimination, $F_{(1,52)} = 4.286$, $p = 0.043$, $\eta^2 = 0.075$; Figure 4C; for graph depicting only the main effect of stress, refer to Supplementary Figure 6C]. Specifically, stress increased the latency to first press in both sexes in the side discrimination task. Moreover, stress increased the trials to criterion in the light discrimination task.

Sex exerted main effects on the number of omissions in the 2nd task [side reversal, $F_{(1,53)} = 6.9$, $p = 0.011$, $\eta^2 = 0.11$; Figure 4B; for graph depicting only the main effect of sex, refer to Supplementary Figure 6B] and the number of perseverative errors in the 3rd task [light discrimination, $F_{(1,48)} = 8.627$, $p = 0.005$, $\eta^2 = 0.148$; Figure 4D; for graph depicting only the main effect of sex, refer to Supplementary Figure 6D]. In short, the female rats demonstrated a higher number of omissions and perseverative errors than the male rats in these tasks. There were no significant sex differences or effects of stress for the other measurements of task (data not shown). In sum, the results suggest that stress led to some impairment in task performance by evoking longer latency to first press for the side discrimination task and greater trials to criterion for the light discrimination task (Figures 4A,C). In addition, the data indicate that the female rats showed worse cognitive flexibility performance than the male rats, demonstrated by a higher number of omissions in the side reversal task and more perseverative errors in the light discrimination task (Figures 4B,D).

The estrous cycle and its interaction with stress to affect cognitive flexibility

To determine the if particular estrous cycle phases drove the sex differences, two-way ANOVAs were conducted to examine the relationship between gonadal hormone status (male vs. diestrus/estrus female vs. proestrus female rats) and stress (control vs. stress) on measurements of performance with each of the three tasks (Figures 5–7). In the side discrimination task

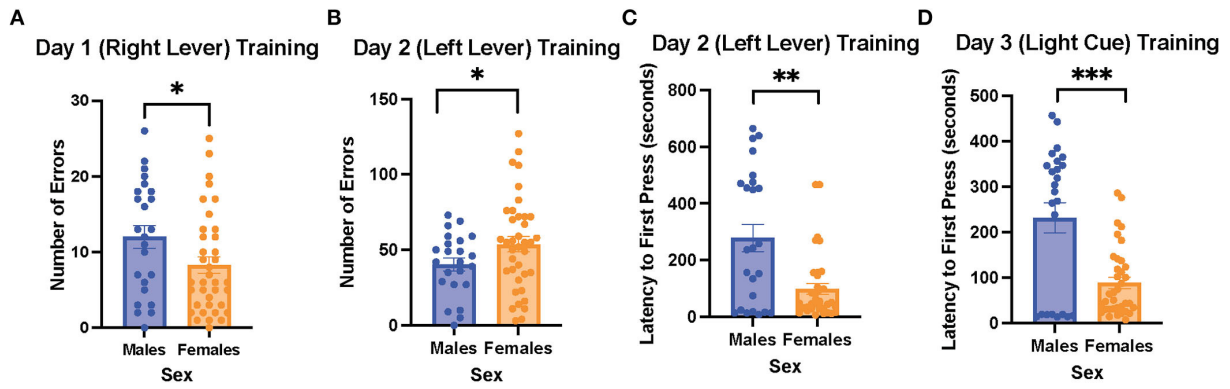

FIGURE 2
Female rats made fewer errors than male rats when learning to press the right lever on the 1st day of training, but they made more errors than the male rats when learning to press the left lever on the 2nd day of the training; the female rats also showed a shorter latency to first press than the male rats when learning to press the left lever and respond to the light cue. **(A)** Number of errors on the 1st day of the training (learning to press the right lever) in the males and female rats. **(B)** Number of errors on the 2nd day of the training (learning to press the left lever) in the male and female rats. **(C)** Latency to first press on the 2nd day of the training (learning to press the left lever) in the male and female rats. **(D)** Latency to first press on the 3rd day of the training (learning to respond to the light cue) in the male and female rats. Independent samples *t*-tests were conducted to compare the measurements between the mean of males ($n = 24$) and the mean of females ($n = 40$). Error bars are plotted as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and***$p < 0.001$.

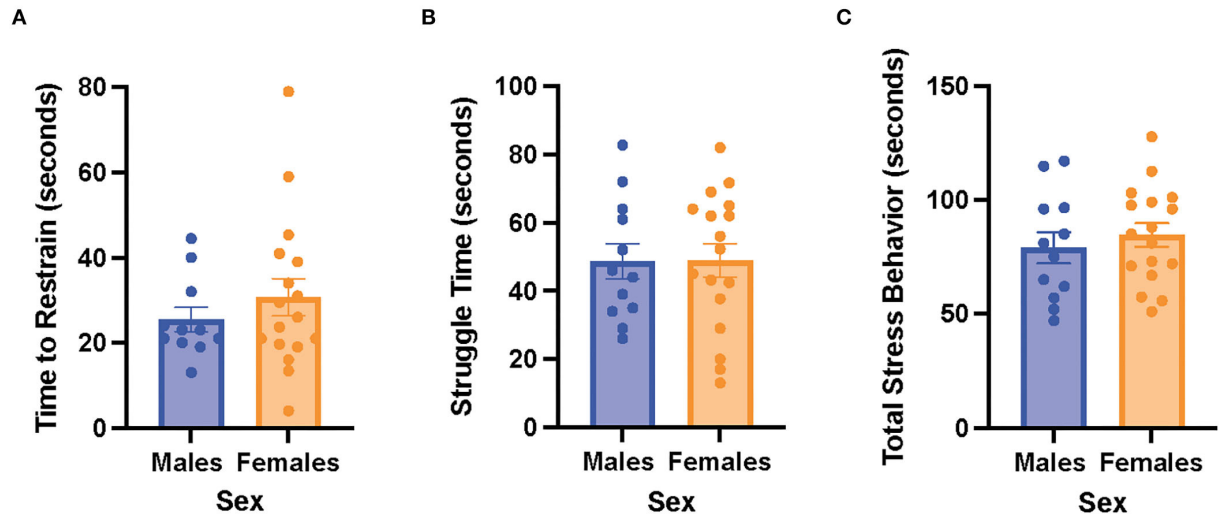

FIGURE 3
Male and female rats did not differ in restraint stress behaviors. **(A)** Time to restrain in the male and female rats. **(B)** Struggle time in the male and female rats. **(C)** Total stress behavior in the male and female rats. Independent samples *t*-tests were conducted to compare the measurements between the mean of males ($n = 12$) and the mean of females ($n = 18$). Error bars are plotted as mean ± SEM.

(the first task during the testing phase, Figure 5), there were no significant main effects of gonadal hormone status or stress on trials to criterion (Figure 5A), number of errors (Figure 5B), or time to criterion (data not shown). However, there was a main effect of gonadal hormone status on the number of omissions [$F_{(2,53)} = 3.41$, p = 0.041; $\eta^2 = 0.113$; Figure 5C; for graph depicting only the main effect of gonadal hormone status, refer to Supplementary Figure 7A]. Although the female rats appear to have higher omissions than the male rats in general, the *post-hoc* tests did not indicate that any particular group was significantly different from another. In contrast, there was a main effect of stress on latency to first press [$F_{(1,55)} = 4.644$, $p = 0.035$, $\eta^2 = 0.038$; Figure 5D; for graph depicting only the main effect of stress, refer to Supplementary Figure 7B]. Specifically, stress increased the latency to first press, and this appeared to be driven by the male and diestrus/estrus female rats in the stress condition. In sum, the data suggest that the female rats made more omissions than the male rats in the side discrimination

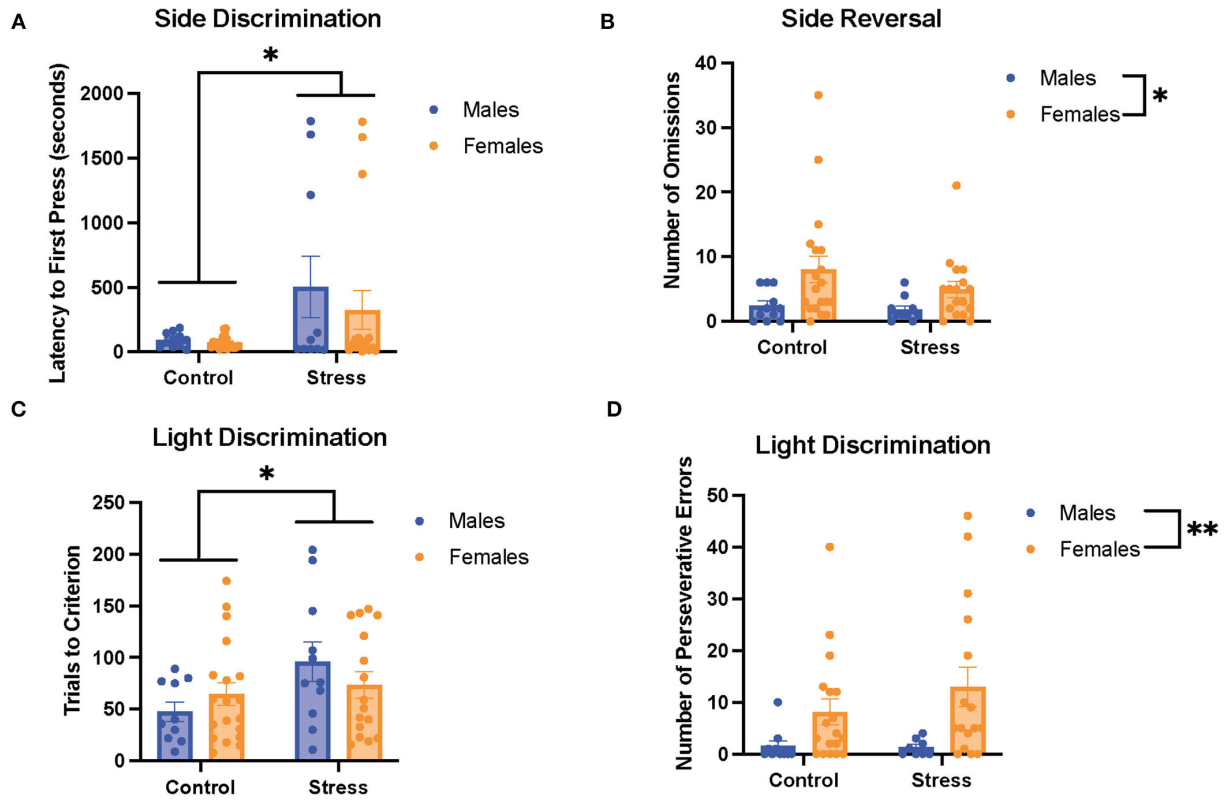

FIGURE 4
Acute stress and sex impacted performance in the side discrimination, side reversal, and light discrimination tasks. **(A)** Stress increased latency to first press in the side discrimination task in both sexes. **(B)** Female rats exhibited a higher number of omissions than male rats in the side reversal task regardless of stress condition. **(C)** Acute stress increased trials to criterion in the light discrimination task (main effect of stress). **(D)** Female rats demonstrated a higher number of perseverative errors in the light discrimination task compared with male rats. Two-way ANOVAs followed by the Tukey *post-hoc* tests were conducted to examine the relationship between stress and sex, and the interaction between the two variables on the measurements of task performance for the male rats ($n = 21$, control = 11, and stress = 10) and female ($n = 40$: control = 20, and stress = 20) rats. Error bars are plotted as mean ± SEM. *$p < 0.05$ and ** $p < 0.01$.

task, although no particular estrous cycle phase appeared to drive this difference. Moreover, stress led to longer latency to first press in the side discrimination task (Figure 5).

In the side reversal task (the second task during the testing phase; Figure 6), there were no main effects of gonadal hormone status, stress, or interactions between the two variables on trials to criterion (Figure 6A), time to criterion (data not shown), number of errors (data not shown), number of perseverative errors (Figure 6B), and number of regressive errors (data not shown). Interestingly, there were main effects of gonadal hormone status and stress, and an interaction between the two variables on the number of omissions [gonadal hormone status, $F_{(2,54)} = 4.34$, $p = 0.018$, $\eta^2 = 0.114$; stress, $F_{(1,54)} = 6.193$, $p = 0.011$, $\eta^2 = 0.081$; gonadal hormone status × stress, $F_{(3,49)} = 2.81$, $p = 0.049$, $\eta^2 = 0.128$; Figure 6C; for separate graphs depicting the main effects of gonadal hormone status or stress, refer to Supplementary Figures 7C,D, respectively]. The *post-hoc tests* demonstrated significantly more omissions by the control proestrus female rats than the control or stressed male rats, as well as the control or stressed diestrus/estrus female and stressed proestrus female rats ($p = 0.002$,.001,.007,.010, and.013, respectively). Moreover, there was an interaction between gonadal hormone status and stress on latency to first press [$F_{(2,54)} = 3.553$, $p = 0.035$, $\eta^2 = 0.108$, Figure 6D]. Specifically, the control proestrus female rats exhibited higher latency to first press than the control male or control diestrus/estrus female rats. In sum, female rats in the proestrus phase without exposure to stress exhibited the highest number of omissions and highest latency to first press in the side reversal task.

In the light discrimination task (third task during the testing phase; Figure 7), there were no main effects of gonadal hormone status, stress, or interactions between the two variables on trials to criterion (Figure 7A), number of errors (data not shown), time to criterion (data not shown), number of regressive errors (data not shown), or latency to first press (Figure 7D). The two-way ANOVAs revealed a main effect of gonadal hormone

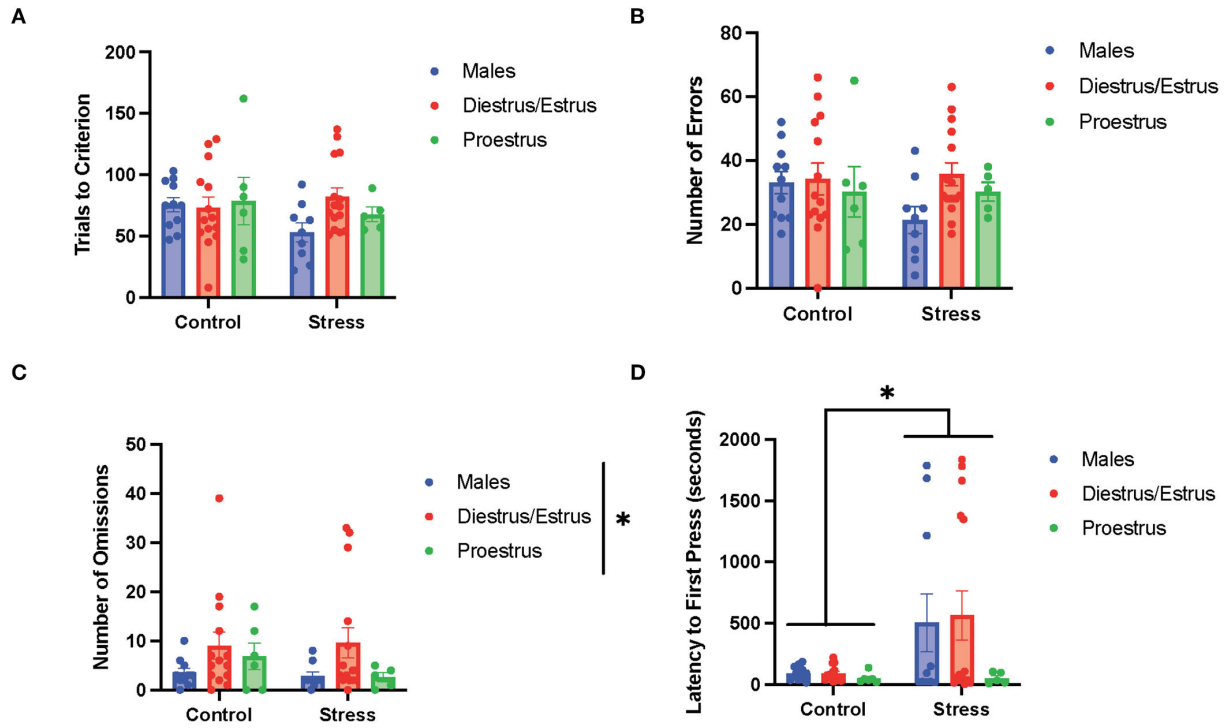

FIGURE 5
Gonadal hormone status and acute stress impacted performance in the side discrimination task. **(A)** There were no effects of gonadal hormone status or acute stress on trials to criterion. **(B)** There were no effects of gonadal hormone status or acute stress on number of errors. **(C)** There was a main effect of gonadal hormone status on the number of omissions. Although the female rats appear to have higher omissions than the male rats in general, the *post-hoc* tests did not indicate that any particular group was significantly different from another. **(D)** Stress increased the latency to first press for the male and diestrus/estrus female rats. Two-way ANOVAs followed by Tukey *post-hoc* tests were conducted to examine the relationship between stress and gonadal hormone status, and the interaction between the two variables on the measurements of side discrimination task performance for the male ($n = 21$, control = 11, stress = 10) and female ($n = 40$: control diestrus/estrus = 14, control proestrus = 6, stress diestrus/estrus = 14, and stress proestrus = 6) rats. Error bars are plotted as mean ± SEM. *$p < 0.05$.

status on perseverative errors [$F_{(2,51)} = 4.338$, $p = 0.018$, $\eta^2 = 0.141$; Figure 7B; for graph depicting only the main effect of gonadal hormone status, refer to Supplementary Figure 7E]. Although the female rats appear to have higher perseverative errors than the male rats in general, the *post-hoc* tests did not indicate that any particular group was significantly different from another. Another two-way ANOVA revealed a main effect of gonadal hormone status, as well as an interaction between gonadal hormone status and stress on the number of omissions in the light discrimination task [gonadal hormone status, $F_{(2,52)} = 4.277$, $p = 0.019$, $\eta^2 = 0.125$; gonadal hormone status × stress, $F_{(2,52)} = 3.79$, $p = 0.029$, $\eta^2 = 0.107$; Figure 7C; for graph depicting the main effect of gonadal hormone status, refer to Supplementary Figure 7F]. Moreover, the *post-hoc* tests revealed that the control proestrus female rats had a higher number of omissions than the control male and control diestrus/estrus female rats ($p = 0.008$ and $= 0.0138$, respectively; Figure 7C). In summary, the results suggest that the female rats committed more perseverative errors than the male rats, although no particular estrous cycle phase drives this difference (Figure 7B). Additionally, control female rats in the proestrus phase made the highest number of omissions (Figure 7C), but this effect diminished with stress exposure.

The role of the PFC in sex differences and acute stress effects on cognitive flexibility

A two-way ANOVA was conducted to examine the relationship between sex (male vs. female) and stress (control vs. stress) on *c-fos* expression in the PFC (more specifically in the IL, PrL, and OFC subregions). There were no main effects of sex, stress, or an interaction between sex and stress on *c-fos* expression in the 3quantified subregions of the prefrontal cortex (Figure 8). There were also no significant main effects of gonadal hormone status, stress, or an interaction between the two variables in the three subregions of the PFC (data not shown). However, there were negative correlations between *c-fos* expression in the mPFC (IL and PRL) and omissions in the side reversal and light discrimination tasks, respectively [data not shown; $r_{(37)} = -0.34$, $p = 0.04$; $r_{(38)} = -0.35$, $p = 0.03$]. Specifically, more activation in the mPFC was associated

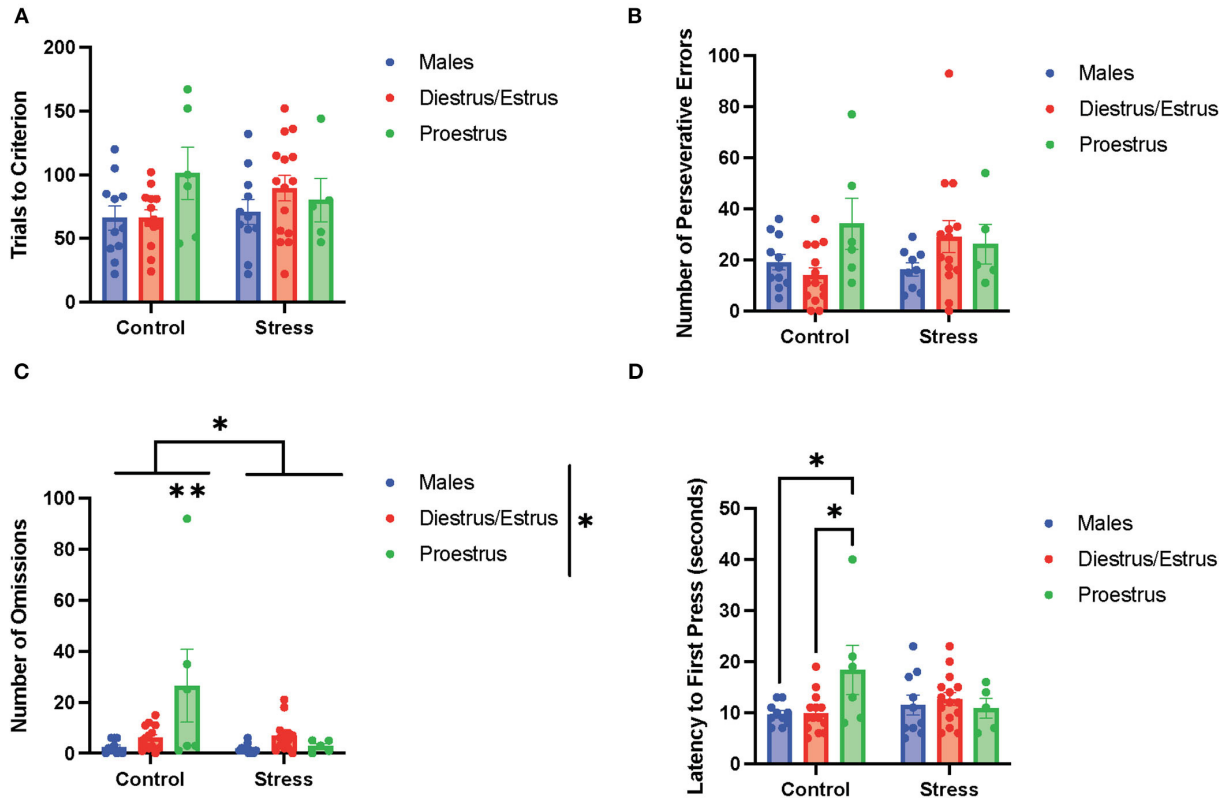

FIGURE 6
Gonadal hormone status and acute stress impacted performance in the side reversal task. **(A)** Gonadal hormone status and acute stress did not affect the trials to criterion. **(B)** Gonadal hormone status and acute stress did not affect the number of perseverative errors. **(C)** There was a main effect of stress and gonadal hormone status, and an interaction between the two variables on the number of omissions; this was driven by the most omissions by the control proestrus female rats. **(D)** There was an interaction between gonadal hormone status and stress on latency to first press. Specifically, the control proestrus female rats exhibited a higher latency to first press than the control male or control diestrus/estrus female rats. Two-way ANOVAs followed by Tukey *post-hoc* tests were conducted to examine the relationship between stress and gonadal hormone status, and the interaction between the two variables on the measurements of side reversal task performance for the males ($n = 21$: control = 11, and stress = 10) and female ($n = 40$: control diestrus/estrus = 14, control proestrus = 6, stress diestrus/estrus = 14, and stress proestrus = 6) rats. Error bars are plotted as mean ± SEM. *$p < 0.05$ and **$p < 0.01$.

with fewer omissions in those tasks. In sum, the activation in the different subregions of the PFC does not appear to differ between sexes or change significantly with acute stress. However, the activity in the mPFC is correlated with behavioral performance, such that more mPFC activation is associated with fewer omissions.

The role of orexins in sex differences and acute stress effects on cognitive flexibility

A two-way ANOVA was conducted to examine the relationship between sex (male vs. female) and stress (control vs. stress) on the number of orexin-expressing cells in the lateral hypothalamus. There was a main effect of sex and an interaction between sex and stress on the number of detected orexin-expressing cells [sex, $F_{(1,55)} = 10.44$, $p = 0.002$, $\eta^2 = 0.1498$; sex × stress, $F_{(1,55)} = 4.281$, $p = 0.043$, $\eta^2 = 0.061$; Figure 9A, for graph depicting only the main effect of sex, refer to Supplementary Figure 8A]. The *post-hoc* test revealed that the female rats in both control and stress conditions had more orexin-expressing cells than the male rats in the control condition ($p = 0.003$ and 0.03 respectively; Figure 9A). In addition, a two-way ANOVA was conducted to examine the relationship between gonadal hormone status (male vs. diestrus/estrus female vs. proestrus female rats) vs. stress (control vs. stress) on the number of orexin-expressing cells in the lateral hypothalamus. Th results revealed a main effect of gonadal hormone status and an interaction between gonadal hormone status and stress on the number of orexin-expressing cells [gonadal hormone status, $F_{(2,54)} = 6.812$, $p = 0.002$, $\eta^2 = 0.181$; gonadal hormone status × sex $F_{(2,54)} = 3.431$, $\eta^2 = 0.091$; Figure 9B; for graph depicting only the main effect of gonadal hormone status, refer to Supplementary Figure 8B].

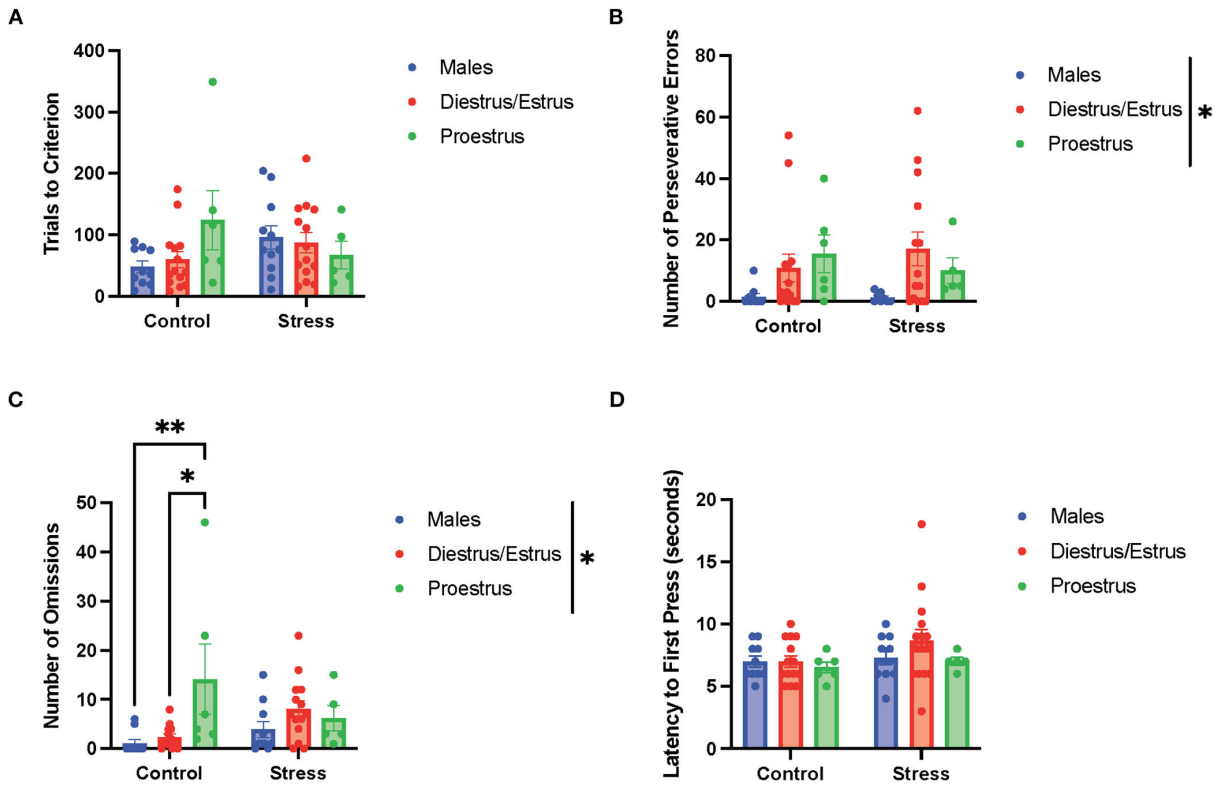

FIGURE 7
Gonadal hormone status and acute stress impacted performance in the light discrimination task. **(A)** Gonadal hormone status and acute stress did not affect trials to criterion. **(B)** There was a main effect of gonadal hormone status on the number of perseverative errors. Although the female rats appear to have higher perseverative errors than the male rats in general, the *post-hoc* tests did not indicate that any particular group was significantly different from another. **(C)** There was a main effect of gonadal hormone status and an interaction between gonadal hormone status and stress on the number of omissions in the light discrimination task. Moreover, the *post-hoc* tests revealed that the control proestrus female rats had a higher number of omissions than the control male and control diestrus/estrus female rats. **(D)** Gonadal hormone status and acute stress did not affect the latency to first press. Two-way ANOVAs followed by Tukey *post-hoc* tests were conducted to examine the relationship between stress and gonadal hormone status, and the interaction between the two variables on the measurements of light discrimination task performance for the male ($n = 21$: control = 11, and stress = 10) and female ($n = 40$, control diestrus/estrus = 14, control proestrus = 6, stress diestrus/estrus = 14, and stress proestrus = 6). Error bars are plotted as mean ± SEM. *$p < 0.05$ and **$p < 0.01$.

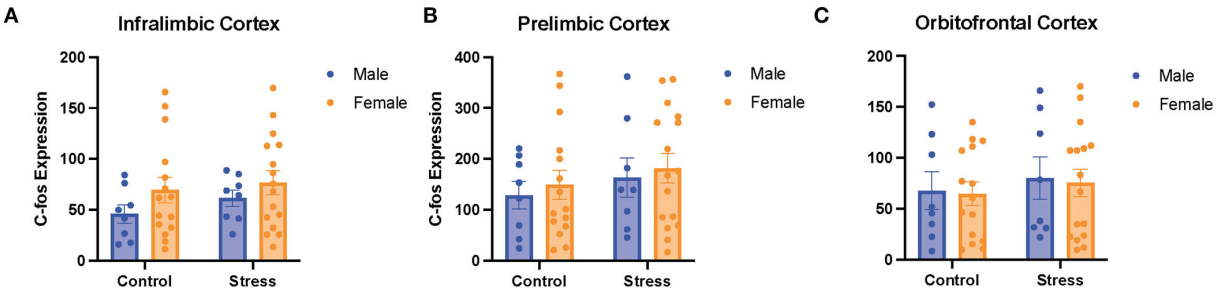

FIGURE 8
There was no effect of stress or sex on *c-fos* expression in different subregions of the PFC. *C-fos* expression in the **(A)** infralimbic cortex, **(B)** prelimbic cortex, and **(C)** orbitofrontal cortex for the male and female rats in the control or stress conditions. Two-way ANOVAs followed by Tukey *post-hoc* tests were conducted to examine the relationship between stress, sex, and *c-fos* expression for the male ($n = 16$: control = 8, and stress = 8) and female ($n = 32$, control = 16, and stress = 16) rats. Error bars are plotted as mean ± SEM.

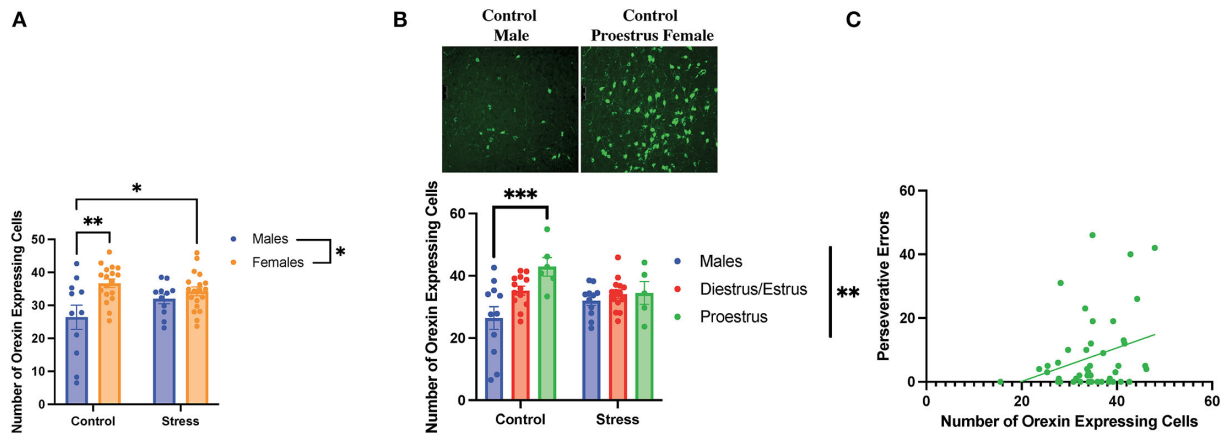

FIGURE 9
Sex, gonadal hormone status, and stress affected the number of orexin-expressing cells. **(A)** There was a main effect of sex and an interaction between sex and stress on the number of orexin-expressing cells in the lateral hypothalamus. Specifically, the female rats in both the control and stress conditions had a higher number of orexin-expressing cells than the control male rats. **(B)** There was a main effect of gonadal hormone status and an interaction between gonadal hormone status and stress on the number of orexin-expressing cells. Interestingly, the proestrus female rats in the control group had more orexin-expressing cells than the male rats in the control group (representative image above of the number of orexin-expressing cells in the control male rats vs. the control proestrus female rats at Bregma level −3.3 mm). However, stress brought the male and female rats to comparable levels. **(C)** Number of orexin-expressing cells is positively correlated with perseverative errors in the light discrimination (LD) task. Two-way ANOVAs followed by Tukey *post-hoc* tests were conducted to examine the relationship between stress and sex or gonadal hormone status, and the interaction between the two variables on the number of orexin-expressing cells for the male rats. A correlation was performed to determine the relationship between the number of orexin-expressing cells and perseverative errors in the light discrimination task for the male ($n = 24$, control = 12, and stress = 12) and female ($n = 40$: control = 20, stress = 20; control diestrus/estrus = 14, control proestrus = 6, stress diestrus/estrus = 14, and stress proestrus = 6) rats. Error bars are plotted as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

The *post-hoc* tests revealed that the control proestrus female rats had more detectable orexin-expressing cells than the control male rats ($p = 0.001$). The data demonstrate that there is a sex difference in the number of orexin-expressing cells in the control condition; namely, that the female rats express more detectable orexin cells than the male rats, and that this is driven by the proestrus female rats. In addition, the data indicate that stress brings the male rats to a similar level of orexin-expressing cells as the female rats in the restraint stress condition. Interestingly, the number of orexin-expressing cells was positively correlated with perseverative errors made in the light discrimination task [$r_{(36)} = 0.4$, $p = 0.015$; Figure 9C]; thus, orexin neurons may be important in set-shifting performance. In sum, when not exposed to stress, the female rats in proestrus exhibit more orexin-expressing cells than the male rats; stress equalizes the number of detectable orexinergic neurons in the male and female rats. Importantly, orexin neurons may play a role in errors made during extradimensional shifts during tests of cognitive flexibility.

Discussion

Stress is associated with psychiatric disorders such as post-traumatic stress disorder (PTSD) and major depressive disorder (MDD) (Carr et al., 2013; Bangasser and Valentino, 2014), which are characterized in part by cognitive inflexibility (Powell and Ragozzino, 2017; Ben-Zion et al., 2018; Boisseau and Garnaat, 2018; Doss et al., 2021) and are more common in women (Nestler et al., 2002; Keane et al., 2006; Bangasser and Valentino, 2014; Swaab and Bao, 2020). It is important to consider where women are in their cycle when exposed to stress and cognitive flexibility testing, as gonadal hormones can contribute to sex differences in response to stress (Becker et al., 2005; Oyola and Handa, 2017; Heck and Handa, 2019). Although previous studies have separately examined sex differences in cognitive flexibility (LaClair et al., 2019; Hilz et al., 2022), stress effects on cognitive flexibility (Thai et al., 2013; Hurtubise and Howland, 2016; Shields et al., 2016; Goldfarb et al., 2017; Grafe et al., 2017a; Sullivan et al., 2019), and the effect of gonadal hormones on cognitive flexibility (Wallin and Wood, 2015; Lipatova et al., 2016; Hilz et al., 2022), many of the findings were inconsistent, and the role of the estrous cycle in stress-induced impacts on cognitive flexibility is still unknown. This study explored sex differences in cognitive flexibility using an operant strategy-shifting paradigm after either control conditions or restraint stress in freely cycling female and male rats (with estrous cycle tracking in the female rats). In addition, we examined potential neural correlates for any changes in behavior observed not only to build our understanding of biological mechanisms behind cognitive flexibility but also to lead to more targeted treatments

for psychiatric disorders associated with impairments of cognitive flexibility.

Sex differences in the training phase of the operant strategy shifting paradigm

While the female rats initially performed better than the male rats during the 1st day of the training for the operant strategy-shifting paradigm, the female rats perseverated on the incorrect lever on the 2nd day of the training, making more errors than the male rats. Our results are consistent with previous findings in primates that females exhibit worse reversal learning (LaClair et al., 2019). In addition, we did not observe sex differences on day 3 of the training (light cue, a new dimension), which is consistent with previous findings in primates that females and males require comparable trials to criterion for extradimensional shift learning (LaClair et al., 2019). The better learning observed in the female rats on the first day might be because they are more sensitive to the food reward following dietary restriction, leading to better initial stimulus response learning with the food reward as response. Indeed, previous studies have shown that food reward may be stronger in female rats than in male rats (Sinclair et al., 2017). Interestingly, we also found that the female rats pressed the lever more quickly than the male ones on the 2nd and 3rd days of the training. Considering that shorter latency to first press has been interpreted as motivation for food reward in previous studies (Workman et al., 2013), our results suggest that the female rats might be more motivated to obtain the food reward during the training, which can also potentially be explained by the greater perception of food reward by female rats (Sinclair et al., 2017).

Acute stress effects on cognitive flexibility

We found that acute restraint stress impaired task performance on test day, demonstrated by longer latency to first press (side discrimination task) and more trials to criterion (light discrimination task). The longer latency to first press may suggest that acute stress reduces the motivation for food reward, consistent with results from a previous study exposing rodents to repeated stress (Sullivan et al., 2019). However, our finding that acute stress led to impaired performance on the light discrimination task is not consistent with a previous study examining acute stress in male rats (Thai et al., 2013). Importantly, the operant strategy-shifting paradigm used in this previous study was different from the current study; each task was completed on different days, with either control or stress groups prior to set shifting and reversal (Thai et al., 2013). Moreover, the stress impairments during the light discrimination task in our study were consistent with studies that exposed animals to repeated stress or chronic unpredictable stress (Hurtubise and Howland, 2016). Thus, it appears that acute stress is sufficient to impair adaptive response strategies in a new stimulus dimension, which requires high-level cognitive processes (Brady and Floresco, 2015; Brown and Tait, 2016). Lastly, we did not observe any improvements in reversal learning following 30-min acute restraint stress in the male rats as previously reported, but again, a different operant strategy-shifting paradigm was used (Thai et al., 2013). Interestingly, we did observe that acute stress reduced the number of omissions for the proestrus female rats. This suggests that acute stress improves adaptive response strategies for simpler tasks in stimulus dimensions (Buss, 1956; Harrow and Friedman, 1958).

Sex differences in cognitive flexibility

We found that the female rats had worse performances than the male rats in both the side reversal and light discrimination tasks on test day, demonstrated by more omissions and perseverative errors, respectively. The impaired side reversal performance in the female rats on test day is parallel with the poor performance by the female rats during the 2nd day of the training (where they had to press the opposite lever from the 1st day of training). However, the impaired performance in the side reversal task on test day is mostly due to omissions, which are failures to respond to a cue, indicating slower cortical processing and attentional deficits (Korgaonkar et al., 2021). Thus, it appears that the female rats demonstrate slower cortical processing than the male rats when trying to adapt to new rules in a stimulus dimension (Brady and Floresco, 2015). In contrast, in the light discrimination task, when a new dimension was involved, the female rats made more perseverative errors than the male rats. This indicates that the female rats might be less likely to inhibit strategies involving previously related dimension in order to explore new strategies. These results indicate that female rats may be more subject to perseveration than male rats, which is defined as the inability to abandon an established strategy for a new strategy despite the fact that the old strategy is no longer useful (Landry and Mitchell, 2021). These errors demonstrate cognitive rigidity and inability to adapt to change (Uddo et al., 1993; Vasterling et al., 1998; Van Laethem et al., 2016; Miles et al., 2021). This sex difference in cognitive flexibility can be explained by the previous observation that female rats form habitual behaviors more quickly or that they are more committed to habitual behaviors than male rats (LaClair et al., 2019). However, it has also been proposed that this sex difference in habitual behaviors is dependent on estrogen levels (LaClair et al., 2019). Therefore, gonadal hormone changes during the

estrous cycle likely influence female cognitive flexibility, as discussed below.

The estrous cycle and its interaction with stress to affect cognitive flexibility

Our results demonstrate that the control proestrus female rats drove most of the sex differences that we observed in cognitive flexibility. Specifically, the proestrus female rats exhibited worse performance in the side reversal and light discrimination tasks than all the other groups primarily because of more omissions and perseverative errors. We will first discuss the reversal performance of the proestrus female rats, followed by their set shifting performance and, lastly, we will consider the effects that stress had on the proestrus female rats in these tasks.

We expected a poor reversal performance by the proestrus female rats, as estradiol levels are highest during proestrus compared to the other phases of the estrous cycle (Becker et al., 2005; Heck and Handa, 2019), and estradiol treatment causes impairments in reversal learning in marmosets (Lacreuse et al., 2014). However, it is important to consider that progesterone levels are also high in the proestrus phase (Heck and Handa, 2019). Other studies have indicated that performance in reversal learning is best when estradiol levels are high and progesterone levels are low (Kromrey et al., 2015). However, there is no consensus on whether progesterone shows positive or detrimental effects on cognition (Barros et al., 2015). In short, the increased omissions during reversal in the proestrus female rats indicate that high levels of estrogen or progesterone can slow cortical processing while adapting to new rules in a stimulus dimension (Korgaonkar et al., 2021).

We also expected a poor performance in the light discrimination task by the control proestrus female rats, as a previous study demonstrated impaired set-shifting performance in ovariectomized female rats with 17β-estradiol treatment (Hilz et al., 2022). It is important to note that our results contradict two other studies, which are carried out on female rhesus monkeys and female rats, respectively, and showed that estradiol treatment improves set shifting (Voytko et al., 2009; Lipatova et al., 2016). However, as the first study was performed on menopausal monkeys and the rodent study used a different paradigm to assess extradimensional shift, it is possible the research model or paradigm used could explain the conflicting results. It is equally important to consider how progesterone levels during the proestrus phase may affect set shifting. Other studies found that administration of progestin during development increases omissions and perseverative errors in cognitive tasks in adulthood (Willing and Wagner, 2016; Fahrenkopf et al., 2021). The observed omissions and perseverative errors made by the proestrus female rats during the light discrimination task indicate both slower cortical processing and cognitive rigidity (Uddo et al., 1993; Vasterling et al., 1998; Van Laethem et al., 2016; Miles et al., 2021). More research studies will have to be conducted to determine if estrogen, progesterone, or both, contribute to these impairments and the mechanism by which this occurs.

We expected that stress would further exacerbate the cognitive flexibility impairments in the proestrus female rats, as high estrogen and progesterone levels are associated with higher HPA response (Oyola and Handa, 2017; Heck and Handa, 2019). On the contrary, our results indicate that stress abolished the impairments in cognitive flexibility in the proestrus female rats by reducing omissions. This phenomenon might be due to the higher vigilance, cue sensitivity, and enhanced associative learning induced by acute stress (Stelly et al., 2020). Since omissions in cognitive flexibility tasks can reflect attention and vigilance (Vasterling et al., 1998), the decrease in omissions of proestrus female rats exposed to acute stress can demonstrate a higher level of vigilance or sensitivity to cues. In sum, these results imply that acute stress can exert a positive effect on cognitive flexibility in female rats with high estrogen and progesterone levels (proestrus female rats), potentially by increasing vigilance specifically for tasks involving higher level of difficulty or susceptibility to perseveration.

The role of the PFC in sex differences and acute stress effects on cognitive flexibility

We found that there were no main effects of sex, estrous phase, stress, or interactions between these variables on *c-fos* expression in the 3 quantified subregions of the prefrontal cortex (IL, PrL, and OFC). As previous literature indicates that the PFC is important for cognitive flexibility tasks (McAlonan and Brown, 2003; Placek et al., 2013; Lipatova et al., 2016), we expected to find that the control proestrus female rats would show changes in their PFC activation to reflect impairments in their behavioral performance. However, as previously mentioned, we did not find group differences. Interestingly, we did find negative correlations between *c-fos* expression in the mPFC and omissions in the side reversal and light discrimination tasks. Thus, it appears that activity in the mPFC is correlated with behavioral performance, such that more mPFC activation is associated with fewer omissions. As previously mentioned, omissions in attention tasks appear to be more common in patients with PTSD, indicating slower cortical processing (Vasterling et al., 1998). Thus, it makes sense that more PFC activity is associated with fewer omissions in our task.

Our findings that stress did not significantly alter *c-fos* expression in the PFC is not consistent with previous findings that stress can impair functions of PFC (Arnsten, 2009).

However, many of the previous studies examined the effects of chronic, rather than acute, stress. Given that acute stress affected cognitive flexibility performance, our results suggest that acute stress may exert some effects on cognitive flexibility without changing PFC neural activation.

Orexin neurons play a role in cognitive flexibility

We found that the control female rats had a higher number of detectable orexin-expressing cells than the control male rats. However, acute stress brought both sexes to a similar level of orexin- expressing cells. A previous study has found that acute restraint stress can induce increases in activities of orexin neurons (Grafe et al., 2017b; Grafe and Bhatnagar, 2018), which may explain the higher number of detectable orexin-expressing cells in response to stress observed in our study. In addition, there may be a ceiling effect of orexin production (limited by quantities or rates of production), such that stress cannot increase the number of detectable orexin cells in female rats beyond their already high control levels.

We also found that the control proestrus female rats showed higher orexin expression than the control male rats, which is consistent with previous findings (Porkka-Heiskanen et al., 2004). Interestingly, the control proestrus female rats also showed worse performance in the side reversal and light discrimination tasks than the control male rats. This may suggest that high orexin expression can interfere with reversal learning and extradimensional shifting. In support of this, we found that the number of orexin-expressing cells was positively correlated with the number of perseverative errors in the light discrimination task. In sum, this suggests that high levels of orexin expression may underlie perseveration.

Limitations

One limitation of our study is that it lacked direct measurements of levels of gonadal hormones such as estrogen and progesterone. In our study, the effects of estrogen and progesterone were extrapolated from levels of the sex steroids associated with each estrous phase based on previous studies (Becker et al., 2005). However, more direct measurements of estrogen levels would be beneficial for more straightforward investigations of the effects of estrogen on task performances. In addition, as testosterone has been also shown to impact performance in cognitive flexibility tasks (Wallin and Wood, 2015; Tomm et al., 2022), and testosterone levels can vary by individuals (Viau, 2002), measurements or experimental designs with testosterone could add more insights into the understanding of sex differences in cognitive flexibility (Shansky, 2019).

Another limitation of our study was the inability to measure the perception of stress in rats, which is a limitation of animal research in general. Differences in stress perception could possibly explain the opposite effects of acute stress on cognitive flexibility in different estrous phases. In rats, cortisol levels can potentially reflect stress perception (Goldfarb et al., 2017; Gabrys et al., 2019). However, the cutoff level of cortisol corresponding to the perception of controllability and uncontrollability can be difficult to determine. In addition, since sympathetic response has also been shown to affect cognitive flexibility (Lapiz and Morilak, 2006; Tait et al., 2007; Hurtubise and Howland, 2016), measurements of sympathetic responses such as noradrenaline level would be important for understanding the effects of acute stress on cognitive flexibility.

Finally, we did not counterbalance the order of cues (i.e., lever location vs. light cue) in our attentional set shifting paradigm. Some research using the pot digging version of the attentional set shifting paradigm suggests that counterbalancing cues is important. Specifically, researchers have found that there are some differences in the simple discrimination stage depending on which cue is introduced first (i.e., higher number of trials to criterion if the odor is the relevant cue), which may then affect subsequent performance (Heisler et al., 2015). Although our operant lever pressing version of the task does not include odor or media cues, it may still be important to counterbalance the lever location cue vs. the light cue in future studies. Importantly, in the lever location dimension, we did calculate side bias during the training and accounted for any observed bias on test day.

Conclusions and future directions

Our findings provide an insight into how the estrous cycle impacts cognitive flexibility and, moreover, how stress and the estrous cycle interact to affect cognitive flexibility performance in rats. Future studies should investigate whether the positive effects of stress on female rats in the proestrus phase are only limited to acute stress or if chronic stress would exert similar effects. Moreover, future studies should determine how the gonadal hormone milieu affects learning this cognitive flexibility task (during training). In addition, more research is needed to better understand how orexin activity in the lateral hypothalamus underlies cognitive flexibility performance. Indeed, understanding the neural circuits of cognitive flexibility would provide more clarity as to how sex differences in behavior are mediated. Previous research has indicated the importance of dopaminergic input on cognitive flexibility (Floresco et al., 2008; Haluk and Floresco, 2009; Klanker et al., 2013; Radke et al., 2019; Tomm et al., 2022); as orexin neurons project to the VTA (Peyron et al., 1998), understanding how these neural substrates interact could provide more clarity on sex differences in cognitive flexibility after stress. Furthermore, future research should more directly determine the role of gonadal hormones

in these circuits, which would lead to a more comprehensive understanding of sex differences in cognitive flexibility. Lastly, future studies should incorporate objective measures of the stress response, including cortisol and noradrenaline levels, to examine the relationship between gonadal hormones, stress response and perception, and cognitive flexibility performance.

Our study contributes to the understanding of sex differences and impacts of stress on cognitive flexibility, as well as the role of the estrous cycle in cognitive flexibility. Since impairments of cognitive flexibility are related to the stress-related psychiatric disorders, which are more prevalent in women than men, unveiling the sex differences and effects of hormone status on cognitive flexibility is crucial to the improvement of diagnosis and treatment of these stress-related psychiatric disorders. Therefore, the findings from our study could potentially inform the development of individualized treatment for psychiatric disorders associated with impaired cognitive flexibility.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Bryn Mawr Institutional Animal Care and Use Committee.

Author contributions

AG and LG contributed to the conception and design of the study. AG, JH, IR, AH, XL, KS, and LG performed the experiments, collected the data for the manuscript, and performed the data analysis. JH wrote the first draft of the manuscript. JH, AG, and LG wrote sections of the manuscript. All authors contributed to revision of the manuscript, read, and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.958301/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Representative vaginal lavage pictures categorized into each stage of the estrous cycle. **(A)** Samples that displayed predominantly leukocytes (and some larger round cells without nuclei) were categorized as diestrus. **(B)** Samples that had primarily nucleated epithelial cells were categorized as proestrus. **(C)**Samples that principally included cornified cells were categorized as estrus.

SUPPLEMENTARY FIGURE 2
Gonadal hormone status and acute stress impacted performance in the side discrimination task. **(A)** There was a main effect of gonadal hormone status on the trials to criterion, with the diestrus female rats showing the highest trials to criterion regardless of stress. **(B)** There was a main effect of gonadal hormone status on the number of errors, with the diestrus female rats showing the highest number of errors regardless of stress condition. **(C)** There was no effect of stress or gonadal hormone status on the number of omissions. **(D)** Stress increased the latency to first press for the male and diestrus female rats. Two-way ANOVAs followed by Tukey *post-hoc* tests were conducted to examine the relationship between stress and gonadal hormone status, and the interaction between the two variables, on the measurements of side reversal task performance for the male ($n = 21$: control = 11, and stress = 10) and female ($n = 40$, control diestrus = 6, control proestrus = 6, control estrus = 8, stress diestrus = 8, stress proestrus = 6, and stress estrus = 6) rats. Error bars are plotted as mean ± SEM. $^{*}p < 0.05$.

SUPPLEMENTARY FIGURE 3
Gonadal hormone status and acute stress impacted performance in the side reversal task. **(A)** Gonadal hormone status and acute stress did not affect the trials to criterion. **(B)** Gonadal hormone status and acute stress did not affect the number of perseverative errors. **(C)** There was a main effect of stress and gonadal hormone status, and an interaction between the two variables, on the number of omissions; this was driven by the most omissions of the control proestrus female rats. **(D)** Gonadal hormone status and acute stress did not affect the latency to first press. Two-way ANOVAs followed by Tukey *post-hoc* tests were conducted to examine the relationship between stress and gonadal hormone status, and the interaction between the two variables, on the measurements of side reversal task performance for the male ($n = 22$: control = 11, and stress = 11) and female ($n = 40$, control diestrus = 6, control proestrus = 6, control estrus = 8, stress diestrus = 8, stress proestrus = 6, and stress estrus = 6). Error bars are plotted as mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$.

SUPPLEMENTARY FIGURE 4
Gonadal hormone status and acute stress impacted performance in the light discrimination task. **(A)** Gonadal hormone status and acute stress did not affect trials to criterion. **(B)** There was a main effect of gonadal hormone status on the number of perseverative errors; stressed female rats in the estrus phase made the highest number of errors. **(C)** There

was a main effect of gonadal hormone status on the number of omissions; the control proestrus female rats had the highest number of omissions. **(D)** Stress increased the latency to first press. Two-way ANOVAs followed by Tukey *post-hoc* tests were conducted to examine the relationship between stress and gonadal hormone status, and the interaction between the two variables, on the measurements of light discrimination task performance for male ($n = 22$: control = 11, and stress = 11) and female ($n = 40$, control diestrus = 6; control proestrus = 6; control estrus = 8, stress diestrus = 8, stress proestrus = 6, and stress estrus = 6). Error bars are plotted as mean ± SEM. $^{*}p < 0.05$.

SUPPLEMENTARY FIGURE 5
Sex, gonadal hormone status, and stress affected the number of orexin-expressing cells. **(A)** There was a main effect of sex, and an interaction between sex and stress, on the number of orexin-expressing cells in the lateral hypothalamus. Specifically, the female rats in both the control and stress conditions had a higher number of orexin-expressing cells than the control male rats. **(B)** There was a main effect of gonadal hormone status, and an interaction between gonadal hormone status and stress, on the number of orexin-expressing cells; the proestrus female rats in the control group had more orexin-expressing cells than the male rats in the control group (representative image above of the number of orexin-expressing cells in the control male rats vs. the control proestrus female rats at Bregma level−3.3 mm). However, stress brought the male and female rats to comparable levels. **(C)** Number of orexin-expressing cells is positively correlated with perseverative errors in the light discrimination (LD) task. Two-way ANOVAs followed by Tukey *post-hoc* tests were conducted to examine the relationship between stress and sex or gonadal hormone status, and the interaction between the two variables, on the number of orexin-expressing cells for the males ($n = 24$, control = 12 and stress = 12) and female ($n = 40$: control = 20; stress = 20; control diestrus = 6; control proestrus = 6; control estrus = 8; stress diestrus = 8; stress proestrus = 6, and stress estrus = 6). A correlation was performed to determine the relationship between the number of orexin-expressing cells and perseverative errors in the light discrimination task. Error bars are plotted as mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$.

SUPPLEMENTARY FIGURE 6
Main effects of stress or sex displayed in graph format for original Figure 4 data. Acute stress and sex impacted performance in the side discrimination, side reversal, and light discrimination tasks. **(A)** Stress increased latency to first press in the side discrimination task. **(B)** Female rats exhibited a higher number of omissions than male rats in the side reversal task. **(C)** Acute stress increased trials to criterion in the light discrimination task. **(D)** Female rats demonstrated a higher number of perseverative errors in the light discrimination task than the male rats ($n = 31$ control, 30 stress; $n = 21$ male and 40 female rats).

SUPPLEMENTARY FIGURE 7
Main effects of stress or gonadal hormone status displayed in graph format for original Figures 5–7 data. **(A)** Gonadal hormone status had a main effect on the number of omissions in the side discrimination task. **(B)** Stress increased the latency to first press in the side discrimination task. **(C)** Gonadal hormone status had a main effect on the number of omissions in the side reversal task. **(D)** Control animals demonstrated more omissions than stressed animals in the side reversal task. **(E)** Gonadal hormone status had a main effect on the number of perseverative errors in the light discrimination task. **(F)** Gonadal hormone status had a main effect on the number of omissions in the light discrimination task. ($n = 31$ control, 30 stress; $n = 21$ male, 28 diestrus/estrus female, and 12 proestrus female rats).

SUPPLEMENTARY FIGURE 8
Main effects of stress, sex, or gonadal hormone status displayed in graph format for original Figure 9 data. **(A)** Female rats have a higher number of orexin-expressing cells than male rats. **(B)** Gonadal hormone status has a main effect on the number of orexin-expressing cells in the lateral hypothalamus. ($n = 24$ male and 40 female rats; $n = 24$ male, 28 diestrus/estrus female, and 12 proestrus female rats).

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Sex-dependent effects of multiple acute concurrent stresses on memory: a role for hippocampal estrogens

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Memory disruption commonly follows chronic stress, whereas acute stressors are generally benign. However, acute traumas such as mass shootings or natural disasters—lasting minutes to hours and consisting of simultaneous physical, social, and emotional stresses—are increasingly recognized as significant risk factors for memory problems and PTSD. Our prior work has revealed that these complex stresses (concurrent multiple acute stresses: MAS) disrupt hippocampus-dependent memory in male rodents. In females, the impacts of MAS are estrous cycle-dependent: MAS impairs memory during early proestrus (high estrogens phase), whereas the memory of female mice stressed during estrus (low estrogens phase) is protected. Female memory impairments limited to high estrogens phases suggest that higher levels of estrogens are necessary for MAS to disrupt memory, supported by evidence that males have higher hippocampal estradiol than estrous females. To test the role of estrogens in stress-induced memory deficits, we blocked estrogen production using aromatase inhibitors. A week of blockade protected male and female mice from MAS-induced memory disturbances, suggesting that high levels of estrogens are required for stress-provoked memory impairments in both males and females. To directly quantify 17β-estradiol in murine hippocampus we employed both ELISA and mass spectrometry and identified significant confounders in both procedures. Taken together, the cross-cycle and aromatase studies in males and females support the role for high hippocampal estrogens in mediating the effect of complex acute stress on memory. Future studies focus on the receptors involved, the longevity of these effects, and their relation to PTSD-like behaviors in experimental models.

KEYWORDS

stress, memory, estrogen, aromatase, hippocampus, PTSD, ELISA, mass spectrometry

Introduction

Studies on the effects of stress on memory have largely centered on chronic stress, which is well established to disrupt hippocampal memory (Kleen et al., 2006; Peay et al., 2020). Whereas acute stress generally enhances memory (Uysal et al., 2012; Brivio et al., 2020), acute traumatic events composed of simultaneous physical, emotional, and social stresses are increasingly recognized to provoke stress-related mental illnesses, including PTSD, and associated spatial memory impairments (Lawyer et al., 2006; Cherry et al., 2010, 2011; Millan et al., 2012; Tempesta et al., 2012; Lowe and Galea, 2017; Lowe et al., 2017; Musazzi et al., 2017; Novotney, 2018; Bell et al., 2019; Harrison, 2019). Additionally, sex differences in stress-related disorders are pronounced, with women generally having higher rates or more severe symptoms (Christiansen and Hansen, 2015; Olff, 2017).

We have previously discovered that exposure to simultaneous short stresses [multiple concurrent acute stresses (MAS)], impairs spatial memory and disrupts thin dendritic spines in hippocampal CA1 in male rodents (Chen et al., 2008, 2010, 2016; Maras et al., 2014). Interestingly, in female mice, the impacts of MAS are estrous cycle dependent. Surprisingly, MAS impair memory when mice are stressed during early proestrus, when levels of estrogens are high. Notably, spatial memory was protected from MAS during estrus, characterized by lower estrogens (Hokenson et al., 2021).

Proestrus-selective stress-induced memory disruptions were unexpected. While estrogens can modulate stress hormone release (Viau and Meaney, 1991; Lund et al., 2006; Heck and Handa, 2019), estrogens are neuroprotective from stress (Wei et al., 2014; Luine, 2016; Azcoitia et al., 2019). Others reported that higher estrogen levels associate with deleterious effects of stress (Shors et al., 1998; Gupta et al., 2001; Rubinow et al., 2004; Shansky et al., 2004, 2009). These disparate findings suggest a nuanced role of estrogens, where their interactions with stress and memory are likely influenced by dose, origin, interaction with other hormones, stressor type, and the brain regions and networks involved (Holmes et al., 2002; McLaughlin et al., 2008; Barha et al., 2010; Babb et al., 2014; Korol and Pisani, 2015; Graham and Daher, 2016; Graham and Scott, 2018; Cohen et al., 2020; Duong et al., 2020).

Given the profound effects of MAS on hippocampus (Chen et al., 2008, 2010, 2013, 2016; Maras et al., 2014; Hokenson et al., 2021), the important role of estradiol in learning and memory for males and females (Frick et al., 2015, 2018; Luine et al., 2018; Chen et al., 2021) and because hippocampal estradiol levels are reported to be higher in male and proestrus female compared to estrous female rats (Hojo et al., 2004; Kato et al., 2013; Hojo and Kawato, 2018), we hypothesized that high levels of hippocampal estradiol are required for, and perhaps mediate, MAS-provoked memory impairments in both male and female mice. Here we tested this notion by blocking aromatase, an enzyme required for the production of estrogens, for 1 week leading up to MAS. This blockade prevented MAS-induced spatial memory deficits in male and female mice, supporting a deleterious role of estrogens in MAS-induced memory impairments.

We sought to quantify circulating and hippocampal estradiol levels to confirm reported levels by sex and cycle (Hojo et al., 2009; Kato et al., 2013; Hojo and Kawato, 2018) and to assess the efficacy of aromatase inhibition. We identified apparent reductions in the serum and hippocampal estradiol-immunoreactivity in mice treated with aromatase inhibitors, but analyses by mass spectrometry indicate that the compound measured using ELISA might not be estradiol.

Materials and methods

Animals

C57BL/6J 2–5-month-old virgin male and female mice were purchased from Jackson Laboratories or bred in-house. Two to five same-sex mice were group housed in individually ventilated cages (Envigo 7092-7097 Teklad corncob bedding, iso-BLOX^{TM} nesting) and had *ad libitum* access to water and food (Envigo Teklad 2020x global soy protein-free extruded). The vivarium was maintained between 22 and 24°C on a 12-h light/dark cycle (lights on 6:30 a.m.). Stress and behavior tests occurred during the light phase.

Multiple concurrent acute stresses (MAS)

Male and female mice were assigned to a home-cage ("unstressed") control or a multiple concurrent acute stresses (MAS) group (**Figure 1A**). Two to nine mice were individually restrained in a ventilated 50 ml plastic tube and jostled on a laboratory shaker in a room bathed with loud (90 dB) rap music and bright lights for 2 h (Hokenson et al., 2020). Behavioral tests were conducted 2 h post cessation of MAS, when plasma corticosterone and total object exploration time is equivalent between stressed and control animals (Maras et al., 2014; Chen et al., 2016; Hokenson et al., 2021).

Estrous cycle monitoring

Estrous cycle phases were monitored daily *via* vaginal cytology. Cells were stained with the Shandon Kwik-Diff Kit (Thermo Fisher 9990700) and cycle phases were classified based on relative cell type composition (Byers et al., 2012; McLean et al., 2012; Hokenson et al., 2021). To account for the potential effects of daily smearing on behavior, male mice were "faux" smeared with a cotton swab (Sava and Markus, 2005). Smears

were taken during the morning alongside the administration of formestane/vehicle, 1 h prior to MAS.

Pharmacology

The conversion of androgens to estrogens (**Figure 2A**) was blocked with subcutaneous administration of the steroidal, aromatase inactivator formestane (4-Hydroxyandrost-4-ene-3,17-dione, Sigma-Aldrich F2552; Yue et al., 1995; Nißlein and Freudenstein, 2007) at 25 mg/kg/day (or corn oil vehicle) each morning for 8 days, with the final dose given 1 h before MAS. Treatments were assigned randomly but per cage to avoid cross-contamination. Formestane cross-reacted with our estradiol ELISA (a concentration analogous to max expected levels was interpreted as having >200 pg/ml estradiol), thus for estradiol quantification in serum and hippocampus we employed mice treated intraperitoneally with the non-steroidal inhibitor letrozole [4,4'-(1H-1,2,4-triazol-1-ylmethylene) *bis*-benzonitrile, 2 mg/kg/day (Kafali et al., 2004)] or vehicle (1% DMSO in saline). Only formestane was used for behavioral studies. Corn oil and 1% DMSO in saline vehicle treated mice were pooled for analyses of time in diestrus and uterine index.

Object location memory (OLM)

For OLM (Vogel-Ciernia and Wood, 2014; **Figure 1A**), mice (n = 7–9/group) were handled (2 min/day, 6 days) then habituated to an empty apparatus (10 min/day for ≥5 days) leading up to MAS. Mice were trained (2 h after MAS) for 10 min with two identical objects. In the 5-min testing session 24 h later, one object was displaced while the other remained in the same location (counter-balanced). Object investigation was scored using BORIS version 7 (Friard and Gamba, 2016) by two independent observers unaware of the experimental conditions and was defined as the mouse's nose pointed ≤1 cm toward the object. Performance is expressed as the ratio of time spent exploring the object in the novel vs. the familiar location (a ratio of 1.0 indicates no preference). Two mice were excluded for under exploration (<5 s during testing), one mouse was excluded due to object bias during training (a ratio >2.0), one mouse was excluded for incorrect cycle phase, and two mice were excluded for being statistical outliers.

Tissue collection

Uterus dissection

Uteri, whose weights fluctuate with cycle and estrogen manipulation (Yue et al., 1995; Zysow et al., 1997; Zhou et al., 2010; Xiao et al., 2018), were weighed (wet weight) and normalized to body weight by computing a "uterine index" {[uterine weight (g)/body weight (g)] × 100; n = 7–14/group; Hokenson et al., 2021}.

Serum and fresh-frozen hippocampus

Mice were euthanized by rapid decapitation. Trunk blood was collected (within 1–2 min), clotted for 30 min (RT), centrifuged (1,100 *g*, 15 min), then the clear supernatant (serum) was collected and stored at −20°C. For hippocampi, brains were immediately removed from the skull. Hippocampus was dissected on ice (2 min), flash frozen on dry ice, weighed, then stored at −80°C.

Tissue extraction

To remove interfering substances and enhance estradiol signal, serum and hippocampi were extracted. 100 µl thawed serum was extracted twice (5:1 ratio diethyl ether: serum). After 30 min, samples were frozen in a methanol/dry ice bath and the organic (unfrozen) phase was transferred to a new glass tube, dried, then stored until analysis (Krentzel et al., 2020; Proaño et al., 2020). Hippocampi (20 mg) were processed using liquid-liquid and solid-phase extraction (Chao et al., 2011; Tuscher et al., 2016). Fresh frozen hippocampus was homogenized in 250 µl ice-cold 0.1 M phosphate buffer (PB) *via* pestle (Zymo H1001). Ether extractions (repeated three times) were performed by adding 375 µl diethyl ether, vortexing (30 s), centrifuging (10,000 *g*, 10 min, 4°C), and incubating in a methanol/dry ice bath. The organic phase (unfrozen) was transferred to a glass culture tube and dried (50°C water bath). 100% methanol: dichloromethane (1:1) was dripped into the tubes and evaporated under an airstream. For solid-phase extractions, solvents were eluted through C18 columns (Empore™ Extraction Cartridge C18-SD 3 ml, Supelco 66872-U) with positive pressure (adapter Supelco 57020-U). Columns were first conditioned [250 µl 100% methanol, then 250 µl double-distilled water (×2)]. Samples were eluted (resuspended in 250 µl of 0.1 M PB), washed (×2 250 µl double-distilled water), and two organic elutions (250 µl 100% methanol) were collected. Organic layers were evaporated under airstream/50°C water bath. Methanol: dichloromethane was again dripped into tubes and evaporated under an airstream. Dried samples were stored at −20°C. An estradiol control was run during each extraction round to calculate recovery (99% ± 14%).

Estradiol enzyme-linked immunosorbent assay (Estradiol ELISA)

Estradiol-immunoreactivity (estradiol-IR) was quantified by the Calbiotech Mouse/Rat Estradiol ELISA Kit (ES180S-100,

Supplementary Table S1). Samples (duplicates) were compared to a standard curve generated by the provided standards. Dried extracted serum (n = 2–9/group) or hippocampus (n = 3–13/group) were resuspended in 1% BSA in 1× PBS [(Silva et al., 2013) and correspondence with the manufacturer], a buffer found to have little interference with the assay. Some serum (n = 3–8/group) was dispensed directly onto the plate without extraction as per the manufacturer's instructions. Absorbances were read (450 nm) with a microplate reader (BioTek® Synergy HTX). Concentrations are expressed as estradiol-IR (picograms) relative to grams hippocampus wet weight or serum volume after correction for recovery.

Mass spectrometry

Dried extracted hippocampi (n = 2–7/group) were resuspended in isopropanol. Thermo Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled with Vanquish UHPLC system was used. LC-MS system was controlled by Xcalibur software (Thermo). Metabolite separation was conducted by Xbridge BEH amide column (2.1 × 150 mm, 2.5 μm particle size, 130 A° pore size; Waters, Milford, MA). LC gradient was generated using LC solvents (solvent A) 20 mM ammonium acetate, 20 mM ammonium hydroxide in 95:5 acetonitrile: water, pH 9.45; (Solvent B) acetonitrile. The chromatography gradient of solvent A and solvent B was run at a flow rate of 150 μl/min: 0 min, 90% B; 2 min, 90% B; 3 min, 75% B; 4.5 min, 0% B; 6 min, 0% B; 7 min, 90% B; 9 min, 90% B; 10 min, 90% B. Autosampler temperature was set to 4°C and column temperature to 25°C. MS analysis was performed with a full-scan mode for measurement of samples (m/z range 260–280, negative ion mode). Tissue sample extracts were compared to standards: 17β-estradiol (Cayman 10006315), 17α-estradiol (Cayman 20776), and 17β-estradiol-d_2 (Cayman 9002846) dissolved in isopropanol. To obtain MS/MS spectra for estradiol and hippocampus peak, a Targeted Selected Ion Monitoring (Targeted SIM) mode coupled with a data-dependent MS/MS (dd-MS2) scan was used. SIM scans were acquired based on the inclusion of the parent ion (271.1704 m/z) with a normalized collision energy (NCE) of 80. MS/MS spectra were then collected at a resolution of 70,000 (271.1704 m/z) with an automatic gain control (AGC) target value of 1 × 106 and maximum fill times of 100 ms. Hippocampi were first spiked with 17β-estradiol-d_2 prior to extraction and raw ions of measured compound were adjusted to correct for recovery (69% ± 4%) and then normalized to hippocampus weight.

Statistical analyses

Analyses employed GraphPad Prism v9.3.1 (Windows). Behavioral data were analyzed with 3-way ANOVA, with factors of sex, drug, and MAS. Time in diestrus was analyzed with 2-way repeated measures ANOVA with drug and time as factors. Ordinary one-way ANOVA was used to analyze estradiol concentrations. Brown-Forsythe ANOVA tests were used when population standard deviations differed. If an interaction was statistically significant (α = 0.05), *post-hoc* tests with Sidak's multiple comparisons (or Dunnett's T3 for Brown-Forsythe ANOVA) were performed. For estradiol quantification, planned comparisons to compare across sex and cycle or to examine the effects of the drug within sex were employed. The correlation between estradiol-IR and uterine index was computed with Pearson's correlation. Outliers were excluded by ROUT when applicable. Data are presented as means ± SEM.

Results

Aromatase inhibition with formestane protects spatial memory from MAS in male and female mice

To test the potential role of estrogens in the effects of MAS on object location memory (OLM), aromatase was inhibited in male and female mice for 1 week leading up to stress (Figure 1A). Male mice exposed to MAS or female mice experiencing MAS during early proestrus had poor spatial memory when compared to controls (Figure 1B) as previously described (Chen et al., 2016; Hokenson et al., 2021). Treatment with the aromatase inhibitor formestane protected memory in both sexes (Figure 1B). Three-way ANOVA showed an effect of drug ($F_{(1,54)}$ = 6.28, p = 0.015) and an interaction of drug × MAS ($F_{(1,54)}$ = 17.0, p < 0.001). There were no effects of sex ($F_{(1,54)}$ = 0.21, p = 0.649) or MAS ($F_{(1,54)}$ = 2.98, p = 0.090), nor interactions between drug × sex ($F_{(1,54)}$ = 0.80, p = 0.376), sex × MAS ($F_{(1,54)}$ = 0.17, p = 0.683), or drug × sex × MAS ($F_{(1,54)}$ = 0.69, p = 0.411). *Post-hoc* testing indicated a difference in performance between vehicle MAS males and formestane MAS males ($t_{(54)}$ = 3.51, p = 0.004) and a difference between vehicle MAS (proestrus) females and formestane MAS females ($t_{(54)}$ = 3.35, p = 0.006). There were no differences between vehicle control males and formestane control males ($t_{(54)}$ = 0.05, p > 0.999) or vehicle control (proestrus) females and formestane control females ($t_{(54)}$ = 1.59, p = 0.393, Supplementary Table S2).

Notably, differences in OLM were not attributed to differences in object exploration or bias during training. During training, the ratio of time spent exploring the object to be moved over the familiar object did not differ across groups (Figure 1C, Table 1). There was an effect of sex on total object investigation time during both the training and testing sessions (Figures 1D,E, Table 1), however, there were no effects of drug or stress on exploration time (Table 1).

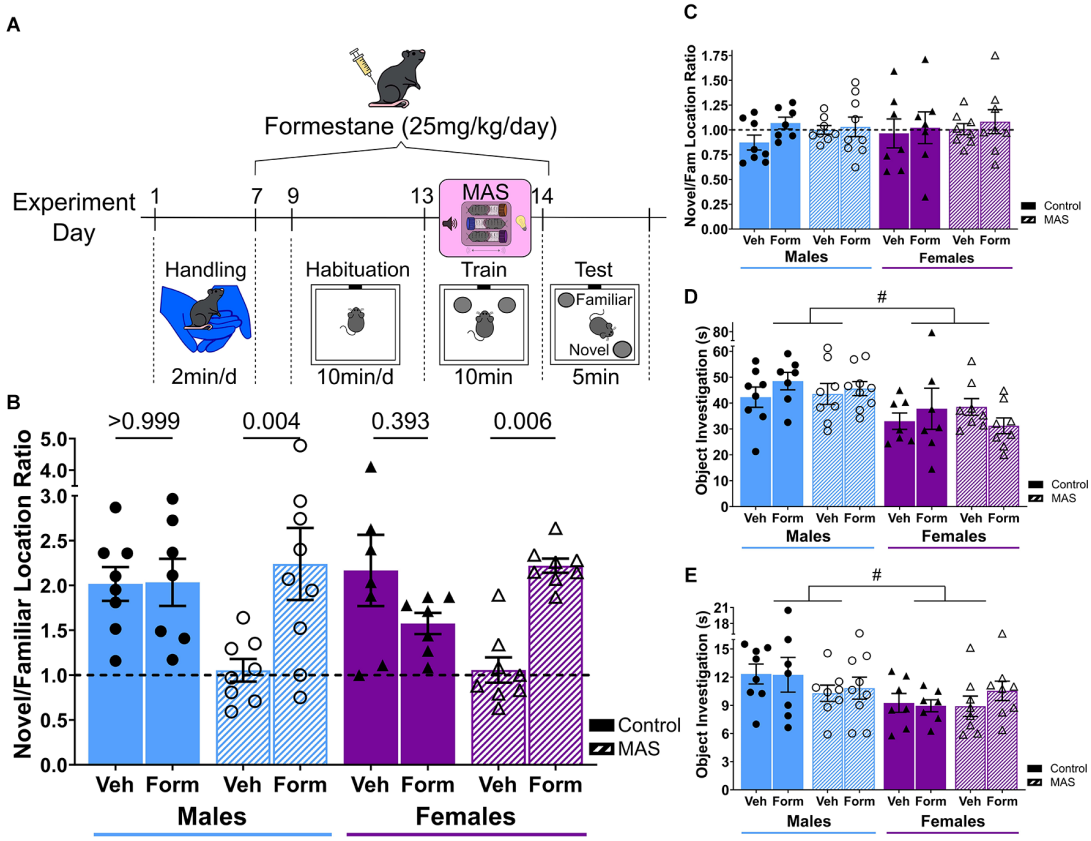

FIGURE 1
Aromatase inhibition prevents MAS-induced object location memory deficits in male and female mice. **(A)** Mice were handled, then given daily, subcutaneous administrations of the aromatase inhibitor formestane for 1 week leading up to MAS, concurrently with habituation to the object location memory (OLM) apparatus. Two hours after MAS, mice were trained with two identical objects for 10 min then tested 24 h later. **(B)** During the 5 min OLM test, vehicle treated male and proestrus female mice exposed to MAS had poor spatial memory compared to controls, however, male and female mice treated with formestane prior to MAS preferentially explored the moved object (n = 7–9/group). **(C)** During OLM training, mice did not display any object bias regardless of group assignment. **(D)** During OLM training, time spent investigating the objects did not vary with drug or stress, but males had longer total investigation compared to females. **(E)** Likewise, during OLM testing, time spent investigating the objects did not vary with drug or stress, but males had longer total investigation compared to females. $^{\#}$Main effect ($p < 0.05$). Post test p-values are provided above the corresponding comparisons. Individual points represent individual mice. Data are presented as mean ± SEM.

Together these results replicate our prior findings that MAS impair spatial memory in male and early proestrus female mice. Blocking the production of estrogens with an aromatase inhibitor leading up to MAS protects OLM in males and females, suggesting that high levels of estrogens are required for MAS to disrupt spatial memory.

Aromatase inhibition disrupts cycling and decreases systemic estradiol levels

One week of aromatase inhibition with inhibitors formestane or letrozole disrupted estrous cycling in female mice, pausing cycles in the diestrus phase (low estrogens; McLean et al., 2012). The percent of the observation period the mouse spent in diestrus was increased during aromatase inhibition compared to vehicle (**Figure 2B**). Two-way repeated measures ANOVA identified an interaction of drug × duration of diestrus ($F_{(2,53)}$ = 9.25, $p < 0.001$), effect of drug ($F_{(2,53)}$ = 28.48, $p < 0.001$) and effect of duration ($F_{(1,53)}$ = 24.18, $p < 0.001$), but no effect of subject ($F_{(53,53)}$ = 0.77, p = 0.826). *Post hoc* results reveal a difference of diestrus period length during treatment between vehicle and formestane ($t_{(106)}$ = 7.47, $p < 0.001$) and vehicle and letrozole ($t_{(106)}$ = 5.61, $p < 0.001$), but no difference between formestane and letrozole ($t_{(106)}$ = 0.62, p = 0.901). There were no differences in diestrus length between groups before treatment (**Supplementary Table S3**).

Aromatase inhibition decreased uterine weights compared to proestrus uteri (**Figure 2C**). There was a significant difference in uterine *indices* ($F^{*}_{(3,25)}$ = 31, $p < 0.001$, Brown-Forsythe ANOVA). *Post hoc* results reveal differences in uterine indices between proestrus and estrus ($t_{(9.7)}$ = 5.0, p = 0.003), proestrus and formestane ($t_{(13)}$ = 4.7, p = 0.003), proestrus

TABLE 1 Three-way ANOVA results of object bias during training and objection investigation times during training and testing.

Object bias during training session

Factor	F	DFn, DFd	*P* value
Drug	1.59	1,54	0.212
Sex	0.13	1,54	0.719
MAS	0.46	1,54	0.501
Drug × Sex	0.11	1,54	0.745
Drug × MAS	0.27	1,54	0.608
Sex × MAS	0.003	1,54	0.957
Drug × Sex × MAS	0.42	1,54	0.520

Object investigation time during training session

Factor	F	DFn, DFd	*P* value
Drug	0.25	1,54	0.620
Sex	11.6	1,54	0.001
MAS	0.05	1,54	0.816
Drug × Sex	0.87	1,54	0.355
Drug × MAS	1.97	1,54	0.166
Sex × MAS	0.003	1,54	0.959
Drug × Sex × MAS	0.47	1,54	0.494

Object investigation time during testing session

Factor	F	DFn, DFd	*P* value
Drug	0.32	1,54	0.577
Sex	6.30	1,54	0.015
MAS	0.49	1,54	0.486
Drug × Sex	0.08	1,54	0.785
Drug × MAS	0.64	1,54	0.426
Sex × MAS	2.16	1,54	0.148
Drug × Sex × MAS	0.17	1,54	0.684

and letrozole ($t_{(7.1)}$ = 11, $p < 0.001$), estrus and letrozole ($t_{(17)}$ = 9.2, $p < 0.001$), and formestane and letrozole ($t_{(16)}$ = 6.5, $p < 0.001$). Notably, there was no difference in uterine indices between estrus and formestane mice ($t_{(23)}$ = 0.22, $p > 0.999$, Supplementary Table S4).

Serum estradiol was quantified using ELISA. Because formestane, with a steroid-like structure, was recognized by the anti-estradiol antiserum, we quantified the hormone levels only in samples from letrozole treated mice. There were differences in serum estradiol-IR ($F_{(4,21)}$ = 5.82, p = 0.003, ordinary one-way ANOVA, Figure 2D) between male vehicle and male letrozole ($t_{(21)}$ = 3.16, p = 0.028), proestrus vehicle and estrus vehicle ($t_{(21)}$ = 3.18, p = 0.027), and a near difference between proestrus vehicle and female letrozole ($t_{(21)}$ = 2.63, p = 0.090, additional *post hoc* comparisons described in Supplementary Table S5). However, there was no difference between male and proestrus estradiol ($t_{(21)}$ = 0.22, $p > 0.999$). In female mice, there was a positive correlation between serum estradiol-IR and uterine index across proestrus, estrus, and letrozole treatment (Pearson R^2 = 0.218, p = 0.014, Figure 2E). Of note, nearly all apparent estradiol concentrations were below the lower detection limit of the assay (3 pg/ml).

To improve the assay, we extracted serum prior to ELISA. Extraction increased average concentrations and estradiol levels differed across groups ($F^*_{(4,8.05)}$ = 8.05, p = 0.007, Figure 2F; Brown-Forsythe ANOVA). Though these *post hoc* comparisons did not reach the threshold for significance, letrozole tended to decrease estradiol-IR, proestrus levels tended to be higher than estrus, and male levels were below female (Supplementary Table S5). Together, these findings indicate that aromatase inhibition, which protects male and female mice from MAS, reduces systemic estradiol.

Systemic aromatase inhibition decreases ELISA-measured hippocampal estradiol-immunoreactivity, yet the compound is not recognized as estradiol on mass spectrometry

To test if **hippocampal** estradiol, reportedly high in males and proestrus females (Kato et al., 2013; Hojo and Kawato, 2018; but see Caruso et al., 2013; Marbouti et al., 2020) enables MAS-induced memory problems, we quantified hippocampal estradiol across sex, cycle, and aromatase inhibition using ELISA. Letrozole reduced apparent hippocampal estradiol-IR in both sexes, however, levels were surprisingly lower in proestrus females compared to estrous females and males ($F_{(4,34)}$ = 13.84, $p < 0.001$, Figure 3A, ordinary one-way ANOVA). *Post-hoc* tests revealed differences in hippocampal estradiol-IR between male vehicle and male letrozole ($t_{(34)}$ = 5.05, $p < 0.001$), male vehicle and proestrus vehicle ($t_{(34)}$ = 3.09, p = 0.024), proestrus vehicle and estrus vehicle ($t_{(34)}$ = 2.84, p = 0.045), proestrus vehicle and female letrozole ($t_{(34)}$ = 3.28, p = 0.014), and estrus vehicle and female letrozole ($t_{(34)}$ = 5.23, $p < 0.001$), but no difference between male vehicle and estrus vehicle ($t_{(34)}$ = 0.13, $p > 0.999$, Supplementary Table S6).

To validate hippocampal estradiol levels, we analyzed similarly processed tissue by mass spectrometry and detected peaks with the expected molecular weight of 17β-estradiol (271.17 g/mol). Quantifying measured ions/g of hippocampus, concentrations in female proestrus were again lower compared to male and estrous females (one-way ANOVA: $F_{(2,10)}$ = 11.40, p = 0.003, Figure 3B). *Post-hoc* tests revealed a difference in concentration between male and proestrus ($t_{(10)}$ = 3.00, p = 0.040) and proestrus and estrus ($t_{(10)}$ = 4.42, p = 0.004), but no difference between male and estrus ($t_{(10)}$ = 0.42, p = 0.968, Supplementary Table S6).

However, further analyses indicated that the peak identified in hippocampus was not estradiol. Elution times differed: 2.24 min for the compound, 2.47 min for 17β-estradiol standard (Figure 3C). This unknown peak was not identified in extracted water or estradiol samples. Given the identical molecular weight, we hypothesized that the compound might be the 17β-

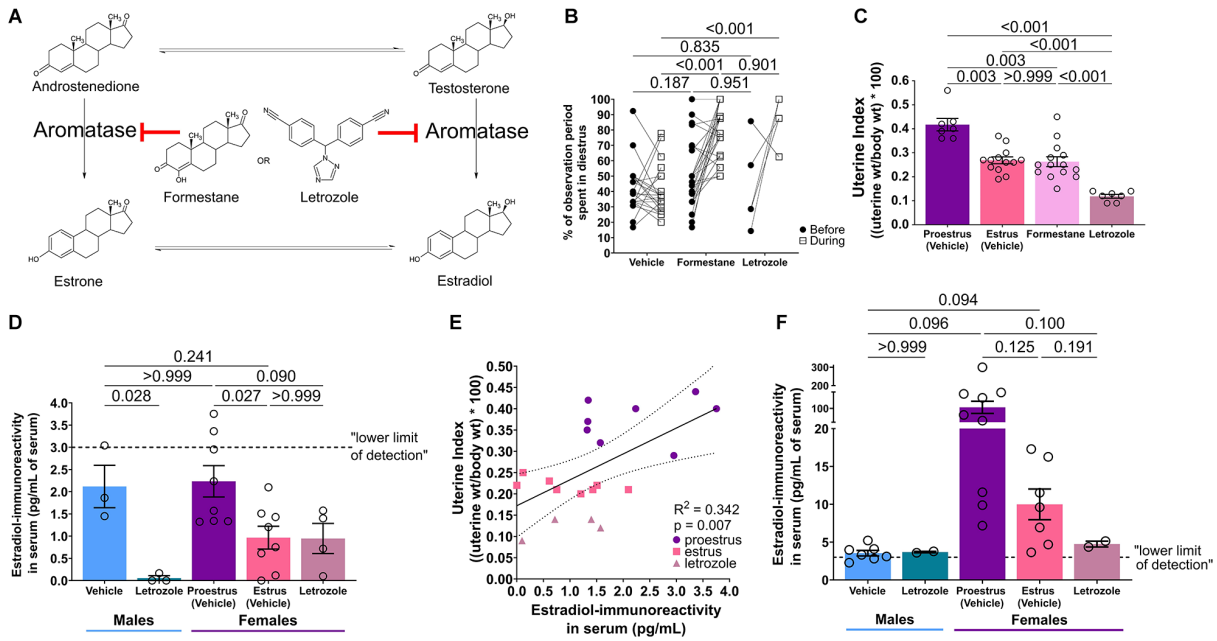

FIGURE 2
Aromatase inhibition disrupts estrous cycling and decreases systemic estradiol levels. **(A)** Aromatase inhibitors, formestane and letrozole, block the conversion of androgens to estrogens. **(B)** During treatment, aromatase inhibitors increase the percentage of time the female mouse spends in the diestrus phase of the cycle (n = 7–24/group). **(C)** Uterine indices are decreased in estrus compared to proestrus mice. Treatment with aromatase inhibitors likewise decreases uterine indices (n = 7–14/group). **(D)** Estradiol-immunoreactivity was quantified in serum directly measured by ELISA (unextracted). Letrozole treatment tended to decrease estradiol levels and proestrus levels were elevated compared to estrus. However, male vehicle serum estradiol levels were high, similar to proestrus female levels. Most values are below the lower limit of detection of the assay (n = 3–8/group). **(E)** There is a positive correlation between estradiol-immunoreactivity in serum measured by ELISA and uterine index in female mice (n = 20). **(F)** Estradiol-immunoreactivity was additionally quantified in serum that was extracted prior to ELISA. Again, letrozole treatment tended to decrease estradiol levels and proestrus levels were elevated, though with even larger variability, compared to estrus. With extraction, male serum estradiol levels were below female (n = 2–9/group). Post test p-values are provided above the corresponding comparisons. Individual points represent individual mice and matched points represent a mouse at different time points. Data are presented as mean ± SEM.

estradiol isomer: 17α-estradiol (Toran-Allerand et al., 2005). While it was difficult to distinguish enantiomer peaks without a chiral column or derivatization, the hippocampal peak still eluted earlier, suggesting that the compound detected here, and presumably through ELISA, was neither 17β-estradiol nor 17α-estradiol. We additionally examined MS/MS profiles of the estradiol standard vs. hippocampus (**Figures 3D,E**). Based on the disparate fragmentation patterns of estradiol standard and hippocampus, we conclude that these are not the same compound.

Therefore, we conclude that available commercial methodologies identify a compound in hippocampus that is not estradiol. Intriguingly, aromatase inhibition reduces its levels, yet its identity remains to be established.

Discussion

Here we confirm that multiple acute concurrent stresses (MAS) disrupt hippocampus-dependent memory in male mice, and in females stressed during proestrus (**Figure 1B**; Chen et al., 2016; Hokenson et al., 2021). High levels of estrogens are required in both sexes, as reducing estrogens by inhibiting aromatase prevents MAS from disrupting memory (**Figure 1B**). These findings are surprising for both sexes. First, they support a deleterious role of high levels of estrogens in females. Second, they suggest a role for hippocampal estrogens in males. Whereas hippocampal estradiol is reportedly high (Hojo et al., 2009; Kato et al., 2013; Hojo and Kawato, 2018) there has been little work on its putative role. Here we suggest the novel notion that hippocampal estrogens in males may act to repress stressful memories.

We previously established that MAS-induced memory disruption and dendritic spine loss in males require convergent activation of corticotropin releasing hormone receptor 1 (CRHR1) and glucocorticoid receptor (GR; Chen et al., 2008, 2016). As high levels of estrogens are required for memory disruption in males and females (**Figure 1B**), these stress-induced disruptions may rely on the synergistic activation of estrogen receptors with CRHR1 and GR. Concurrent activation of these receptors, which converge on RhoA-pCofilin signaling (Chen et al., 2008, 2013; Kramár et al., 2009a,b, 2013),

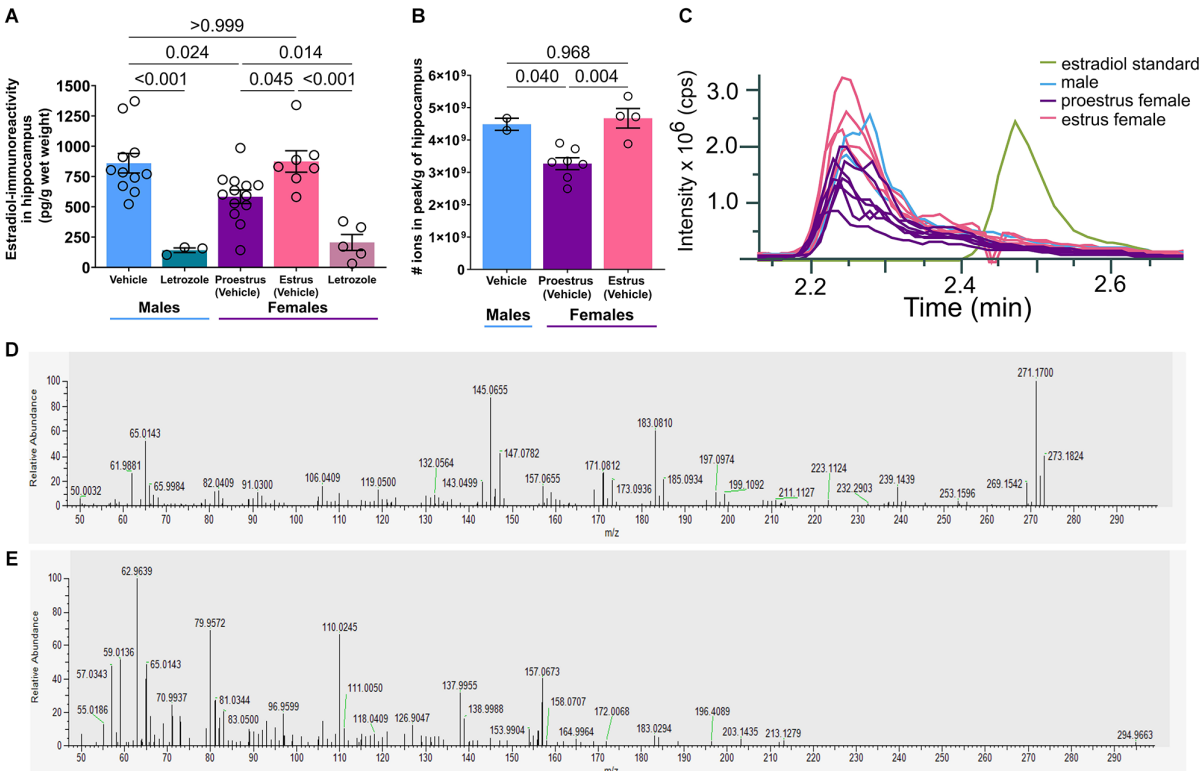

FIGURE 3
Systemic aromatase inhibition decreases hippocampal, ELISA-measured estradiol-immunoreactivity but estradiol was not detected by mass spectrometry. **(A)** Estradiol-immunoreactivity in the hippocampus (pg/g of hippocampus wet weight) as measured by estradiol ELISA. Letrozole reduces hippocampal estrogen. Estradiol in proestrus female hippocampus is lower than estradiol in male or estrous female hippocampus (n = 3–13/group). **(B)** The number of ions in the measured peaks (m/z 271.17) normalized to g of the hippocampus. Again, concentration in proestrus female hippocampus is lower than in male or estrous female hippocampus (n = 2–7/group). **(C)** The m/z 271.17 peak in hippocampus samples elute at 2.24 min while a 17β-Estradiol standard elutes at 2.47 min, indicating that these peaks are not 17β-Estradiol. **(D)** MS/MS spectrum of the [M-H]- ion (m/z 271.1704) for a 1:1 mixture of deuterated-labeled and unlabeled estradiol standards. **(E)** MS/MS spectrum of the [M-H]- ion (m/z 271.1704) for an unknown compound identified in a male hippocampus. Post test p-values are provided above the corresponding comparisons. Individual points represent individual mice. Data are presented as mean ± SEM.

may destabilize dendritic spines. Alternatively, estrogens may influence the levels or activity of CRH or glucocorticoids. Indeed, a potential role for estradiol in augmenting CRH expression has been identified (Lalmansingh and Uht, 2008; Qi et al., 2020).

Here we tested the potential role of high estrogens in MAS-induced memory deficits using the aromatase blocker formestane. While estradiol-IR reductions with letrozole were generally quite large (**Figures 2D–F**, **3A**), formestane-induced reductions may be more modest. Indeed, formestane exerts less aromatase inhibition (84%–93%) than letrozole (>98%; Lønning, 2003), and likewise less potently reduces plasma estrogens (Jones et al., 1992; Geisler et al., 2002). Nevertheless, the pronounced effects of formestane on cycling and uterine weights (**Figures 2B,C**) and reported reduction in circulating estradiol with similar doses (Yue et al., 1995; Nißlein and Freudenstein, 2007) suggest that estrogens were significantly reduced. While estradiol, especially originating from the hippocampus, is critical for memory in female and male mice (Martin et al., 2003; Tuscher et al., 2016; Marbouti et al., 2020) control formestane treated females had only a mild reduction in preference for the moved object compared to control vehicle treated females (**Figure 1B**), suggesting that reduction of estrogens was incomplete. Alternatively, it is possible that week-long aromatase inhibition could increase testosterone or have direct androgenic properties (Séralini and Moslemi, 2001). While this may propose a protective role of androgens, our current work using estrogen receptor blockers suggest estrogen receptor activation plays a direct role in MAS-induced memory impairment.

ELISA is widely used in animal and clinical research due to its relatively low cost, convenience, high throughput, and safety compared to radioactive assays (Sakamoto et al., 2018). However, several issues were found here. Estradiol ELISAs have difficulties quantifying low concentrations, such as in males, ovariectomized, or aromatase-inhibited animals (Hsing et al., 2007; Huhtaniemi et al., 2012; Schumacher et al., 2015; Niravath et al., 2017). We have used the Calbiotech ELISA to distinguish proestrus and estrous female unextracted

serum (Hokenson et al., 2021). This was accomplished, though nearly all values were below the lower limit of detection (**Figure 2D**). These low values are not unusual given low mice serum levels (Nilsson et al., 2015; Handelsman et al., 2020). Surprisingly, male serum levels were unexpectedly high, similar to proestrus females (**Figure 2D**). Purification may increase estradiol-IR by removing interfering substances (Chao et al., 2011; Tuscher et al., 2016; Boyaci et al., 2020; Krentzel et al., 2020; Proaño et al., 2020). Indeed, extracting serum prior to ELISA enhanced estradiol-IR and distinguished male and female values (**Figure 2F**) but were higher than expected of mouse serum (Nilsson et al., 2015; but see Marbouti et al., 2020, analyzed by ELISA). Artificially high estradiol-IR could be due to the tendency for ELISA to overestimate levels (McNamara et al., 2010). Indeed, the Calbiotech plate may not distinguish between intact vs. ovariectomized mice (Haisenleder et al., 2011).

Regarding hippocampal estradiol, we expected lower levels in estrus females vs. proestrus females and males (Kato et al., 2013). Instead, ELISA found the lowest estradiol-IR in proestrus females (**Figure 3A**). Therefore, we turned to mass spectrometry and given the high levels of apparent estradiol-IR by ELISA (**Figure 3A**), anticipated that estradiol levels would be quantifiable without derivatization. Indeed, we found peaks of the expected molecular weight in our samples and, similar to ELISA, a reduction in proestrus female levels (**Figure 3B**). However, the peaks in the hippocampus were not estradiol based on different retention times and MS/MS profiles (**Figures 3C–E**). Only hippocampi were analyzed through mass spectrometry, so it is unknown whether the same unknown peak is found in serum, or if ELISA and mass spectrometry detect the same compound. These factors lead us to conclude that ELISA-measured estradiol values should be considered with extreme caution (Schumacher et al., 2015).

We probably failed to detect estradiol with our current mass spectrometry approach because estradiol quantities were too low or extraction procedures insufficient. While large quantities of tissue estradiol have been quantified without derivatization (McNamara et al., 2010), other groups have successfully detected small quantities through estradiol-specific derivatization (Kato et al., 2013; Jalabert et al., 2022) or signal-enhancing additives (Lozan et al., 2017).

In conclusion, aromatase inhibition protects male and female mice from the memory impairing effects of MAS, suggesting that high levels of estrogens are required for MAS to disrupt memory. Future studies will probe the specific estrogen receptors involved. Additionally, studies are clearly warranted to definitively measure estradiol in murine hippocampus.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by University of California—Irvine's Institutional Animal Care and Use Committee (IACUC).

Author contributions

RH and TZB designed the research. RH, YA, AS, and SJ conducted the research. RH, AS, YA, SJ, CJ, and TZB analyzed the data. RH and TZB wrote and edited the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.984494/full#supplementary-material.

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Work hard, play hard: how sexually differentiated microglia work to shape social play and reproductive behavior

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Microglia are brain-resident immune cells that play a critical role in synaptic pruning and circuit fine-tuning during development. In the adult brain, microglia actively survey their local environment and mobilize inflammatory responses to signs of damage or infection. Sex differences in microglial gene expression and function across the lifespan have been identified, which play a key role in shaping brain function and behavior. The levels of sex hormones such as androgens, estrogens, and progesterone vary in an age-dependent and sex-dependent manner. Microglia respond both directly and indirectly to changes in hormone levels, altering transcriptional gene expression, morphology, and function. Of particular interest is the microglial function in brain regions that are highly sexually differentiated in development such as the amygdala as well as the pre-optic and ventromedial hypothalamic regions. With a focus on hormone-sensitive developmental windows, this review compares male and female microglia in the embryonic, developing, and adult brain with a particular interest in the influence of sex hormones on microglial wiring of social, reproductive, and disordered behavior circuits in the brain.

KEYWORDS

microglia, sex, neurodevelopment, social behavior, sex hormones

Introduction

Microglia are brain-resident immune cells that compose 10%–15% of total brain cells (Kettenmann et al., 2011). *In vivo* imaging studies show homeostatic microglia as highly dynamic cells that survey the local brain environment (Davalos et al., 2005) and rapidly mobilize in response to injury or disease (Gomez-Nicola and Perry, 2015). Under inflammatory conditions, microglia become ameboid with retracted processes and a larger cell body (Nimmerjahn et al., 2005), which facilitates phagocytosis of cellular debris and the release of inflammatory cytokines and chemokines (Streit, 2000).

In addition to their immune-responsive activity, microglia carry out important age-specific functions (Thion and Garel, 2017). Microglia have been implicated as critical regulators of neuronal cell number, synapse formation, axon targeting, and interneuron lamination (Thion and Garel, 2017). These diverse functions are supported by recent advances in transcriptomic

analysis which have revealed different microglial states that vary across developmental age, sex, and region (Hammond et al., 2019) of the healthy brain. One of the most rapidly expanding areas in microglial research is that of sex differences. This previously overlooked biological variable is emerging as a critical factor mediating differential microglial functions between the sexes in both health and disease.

Drawing on human and rodent studies, this review explores how sex influences the diversity of microglial roles throughout the lifetime with an emphasis on the role of sex hormones and brain environment in differentially influencing microglia function. During critical developmental windows, when the brain is highly pliable, microglia work to sculpt neuronal connectivity, often in a sex-specific manner. The work of microglia in regions of the brain that are highly differentiated between males and females directly influences sex-specific play behavior, social interactions, and reproductive behaviors (**Figure 1**). Disruption of microglial development can impair circuits and behaviors in a sex-specific manner, which may play a role in sex-biased prevalence of neurodevelopmental and neuropsychiatric disorders.

Microglial origins

During embryonic development, microglia are uniquely derived from erythromyeloid progenitors (Ginhoux et al., 2010). In mice, progenitors develop in the yolk sac and migrate through the vasculature to colonize the developing brain by E9.5, before the formation of the blood-brain-barrier (Alliot et al., 1999; Ginhoux et al., 2010; Kierdorf et al., 2013). In the absence of a functional circulatory system in NXC1 knockout mice, microglia fail to populate the brain (Ginhoux et al., 2010), highlighting the importance of the vasculature in embryonic microglial migration. Embryonic microglia also regulate axon development and interneuron positioning in the developing cortical layers (Squarzoni et al., 2014), however, to date the majority of these studies have not examined sex as a variable.

In humans, ameboid microglia are seen entering the human forebrain through the meninges, choroid plexus, and the ventricular zone between 4.5 and 5.5 gestational weeks (Verney et al., 2010). Once the progenitors enter the developing brain, they respond to a unique set of CNS-derived factors (Navascués et al., 2000; Gosselin et al., 2014, 2017) that drive a period of

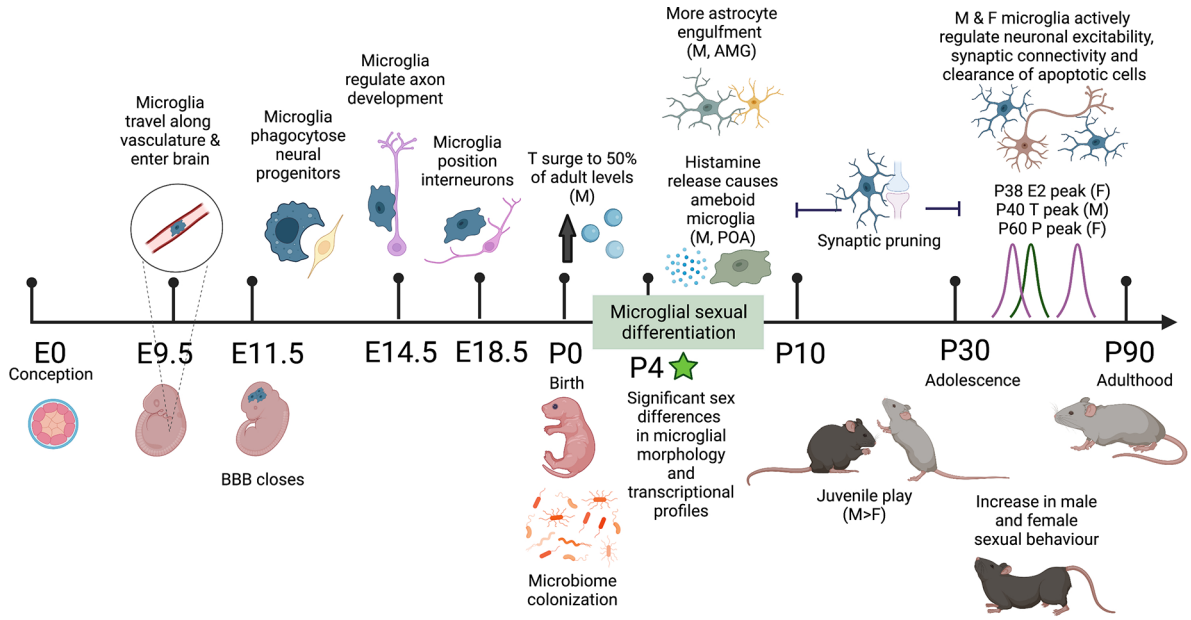

FIGURE 1
Schematic timeline of the sex differences observed in mouse microglial development and adult behavior. Microglia colonize the brain along the vasculature at E9.5 and fully colonize the brain by E11.5 upon closure of the blood-brain barrier (BBB). In embryonic development, microglia perform a range of organizational functions in the healthy brain. Male and female microglial transcriptomes are comparable throughout embryogenesis but significantly diverge after birth, correlating with male testosterone (T) surge and microbiome colonization in both males and females. By P4 significant sex differences in microglial morphology are observed including more ameboid microglia and more microglial-astrocyte engulfment in males compared to females. Between P10 and P30 both male and female microglia undergo synaptic pruning. During this adolescent period, males display more juvenile play behavior. In early adulthood, there are observed increases in male and female sexual behavior along with peak levels of sex hormones: T in males and estradiol (E) and progesterone (P) in females. In the healthy adult mouse brain, microglia self-proliferate for the rest of life, continuing to sense and respond to their local environment and regulate neuronal excitability, synaptic connectivity, and clearance of apoptotic cells. Differential programming of microglia early in life alters their responses to immune challenges throughout life and may play a role in sex-biased prevalence rates in neurodevelopmental and neuropsychiatric disease. Created with BioRender.com.

intense proliferation (Swinnen et al., 2013) and differentiation. Similar profiles are observed in rodents and non-human primates, suggesting evolutionary conservation across mammals (Dalmau et al., 2003; Billiards et al., 2006; Monier et al., 2007; Cunningham et al., 2013). Once the blood-brain barrier closes, microglia perpetually self-renew within the brain parenchyma, without replacement from peripheral sources, for the lifetime of the organism (Ajami et al., 2007).

Microglia in the developing postnatal brain

In early postnatal development, microglia are critical for neural circuit formation through synaptic pruning. In classic studies of the retinogeniculate circuit, Stevens and colleagues demonstrated that microglia phagocytose synaptic inputs from the retina during the first postnatal week. In this system, less-active synapses are tagged with complement proteins and subsequently targeted by microglia for engulfment (Schafer et al., 2012). Neural activity guided microglial pruning appears critical for proper circuit development in other brain regions as well, including the visual cortex (Tremblay et al., 2010), hippocampus (Paolicelli et al., 2011), striatum (Kopec et al., 2018), and frontal cortex (Mallya et al., 2019; Schalbetter et al., 2022). However, the open critical window in which pruning occurs appears to vary by brain region and is sensitive to individual experience. Microglial pruning can also be neuronal type specific, with a subset of $GABA_B$ receptor expressing microglia selectively pruning GABAergic interneurons during cortical development (Favuzzi et al., 2021). Interestingly, microglia can also induce the formation of post-synaptic filopodia, potentially playing a role in synapse *formation* (Weinhard et al., 2018). Increasingly, studies are recognizing the importance of examining microglial pruning in the context of both sexes, often revealing sex-specific differences in critical windows and neuronal targets (Kopec et al., 2018; Rosin et al., 2021; Bolton et al., 2022).

Microglia in the adult brain

In the healthy adult brain, microglia adopt a ramified morphology consisting of a small cell body and highly branched protrusions (Swinnen et al., 2013). In the absence of damage or infection, microglia actively survey their local brain environment. Each microglial cell appears to have a home territory that it monitors for damage or infection (Kettenmann et al., 2011). Microglia make regular transient contacts with neuronal synapses, astrocytes, and other brain cells (Nimmerjahn et al., 2005; Wake et al., 2009). These transient contacts likely contribute to healthy microglial regulation of neuronal excitability, synaptic activity, connectivity, neurogenesis, and clearance of apoptotic cells. For example, microglia can regulate hippocampal long-term potentiation (Costello et al., 2011; Rogers et al., 2011), a measure of synaptic plasticity and long-term memory. Microglia are also critical regulators of adult neurogenesis through targeted phagocytosis of apoptotic cells in the subgranular zone (Sierra et al., 2010). Microglia also clear naturally dying cells in the cerebellum (Ayata et al., 2018). Microglia can dampen neuronal activity by converting neuronal activity induced extracellular ATP to adenosine. The resulting increase in adenosine suppresses neuronal activity (Badimon et al., 2020) and failure in this system results in aberrant neuronal activity and seizures.

Sex hormones and sexual differentiation of the brain

There is a growing appreciation for the role of the immune system, specifically microglia, in the sexual differentiation of the brain (McCarthy, 2020). Male and female microglia differ across development: there are notable sex differences in the number, function, and transcriptome of microglia in various brain regions and in disease (Hanamsagar et al., 2017; Guneykaya et al., 2018; Rahimian et al., 2019; Delage and Cornil, 2020; Han et al., 2021). These sex differences arise and contribute to the sexual differentiation of the brain beginning in embryogenesis.

The embryonic brain begins as bipotential, capable of adopting female or male typical phenotypes. In mammals, sex is largely determined by the presence of the Y chromosome gene Sry (Sex-determining region of the Y) that encodes for testis-determining factor protein (Tdf). In the presence of Sry, testes develop and secrete gonadal steroid hormones which masculinize the brain. In the absence of Sry, ovaries and associated female phenotypes develop (Lenz et al., 2012). In humans, sexual differentiation of the brain occurs between 8 and 24 gestational weeks and is further organized during periods of puberty when gonadal sex hormones (such as androgens, estrogens, and progesterone) surge (Bakker, 2019).

The bipotential of the early embryo is also reflected in the microglial transcriptome which is largely similar between males and females at E18 but significantly diverges during postnatal development (Hanamsagar et al., 2017; Thion et al., 2018). The transcriptional divergence between male and female microglia corresponds with the early postnatal hormone surge in males and with microbiome colonization upon birth (Thion et al., 2018) in both sexes. An absence of the gut microbiome during development more dramatically alters transcription and epigenetic regulation in male microglia compared to female. Altogether, this supports a model recently put forth by Bordt et al. (2020) and VanRyzin et al. (2020) in which both intrinsic regulation by sex chromosomes and extrinsic influences from sex

hormones and the environment combine to shape sex-specific aspects of microglial development.

Sex hormones in early life

In male rodents, there is a surge in testicular androgen production beginning in the last few days of gestation that lasts until shortly after birth (Lenz and McCarthy, 2015). In primates, androgen surges occur from the end of the first trimester into the second trimester and again at birth (Lenz and McCarthy, 2015). During this window, aromatase catalyzes the conversion of testosterone into estradiol or dihydrotestosterone (Lephart, 1996). Estradiol (E2) acts as the primary driver of masculinization of the embryonic rodent brain (McCarthy et al., 2018). Interestingly, sex differences in microglia morphology and number manifest after the perinatal testicular androgen surge (Schwarz et al., 2012). These differences occur in multiple brain regions, including the hypothalamic medial preoptic area (POA; Lenz et al., 2013), amygdala (VanRyzin et al., 2019), and hippocampus (Nelson et al., 2017). By postnatal day (P) 4, sex has a significant effect on microglial morphology, specifically, within the parietal cortex, hippocampus, and amygdala where males show more ameboid microglia compared to females (Schwarz et al., 2012).

In addition to interacting with microglia directly, sex hormones can also influence the local brain microenvironment and indirectly impact microglia. For example, in male rats, aromatization of testosterone to estradiol in the POA causes nearby mast cells to release histamine, resulting in more ameboid microglia (Lenz et al., 2018). However, these sex differences naturally reverse by P30, with female rats showing more ameboid morphology than males in adulthood (Schwarz et al., 2012). In adult mice, females also show more ameboid microglial morphologies at baseline, resulting in LPS-induced shifts from highly branched to ameboid only in males (Hanamsagar et al., 2017).

Sex hormones during adolescence and adulthood

Microglia may also be critical for the active feminization of the brain and behavior. Although estradiol is responsible for masculinization of the brain early in life, prepubertal secretion of estradiol is required for sexual receptivity in females (Bakker et al., 2003). Microglia contain E2 receptors (Sierra et al., 2008) and thus are responsive to estradiol changes in the brain.

Microglia E2 receptors may differentially regulate inflammatory responses between the sexes. Estradiol has been shown to enhance microglial reactivity to immune challenges during puberty in the ventromedial nucleus (VMN) of the hypothalamus (Velez-Perez et al., 2020), a key brain region for female sexual behaviors (McCarthy, 2010).

Ovariectomized mice also show impaired induction of several immune genes in response to lipopolysaccharide (LPS) or live viral infection (Soucy et al., 2005). Conversely, in adult rats and mice systemic delivery of estrogen blocks LPS-induced changes in microglia morphology across several brain regions. The estrogen-mediated suppression of LPS response is absent in female ERα-null mice but intact in ERβ-nulls. ERα-null mice also developed aberrant microglial function with age in both males and females, suggesting important roles for ERα in microglial regulation in both sexes (Vegeto et al., 2003). Similar immune repressive effects of estrogens have been observed in rat microglial cultures (Vegeto et al., 2001). Together these studies support a complex role for ERα in both suppressing and augmenting microglial immune activity in the context of inflammation. Future work addressing the limitations of this work should employ genome wide transcriptomic approaches in combination with microglia specific and inducible models of ERα deletion to help clarify how estrogen modulates microglial activity across development and disease states.

Transcriptomic profiling of the adult whole brain microglia revealed differences in gene expression, with male microglia having a higher expression of genes related to inflammation and female microglia having a higher expression of genes associated with morphogenesis and cytoskeletal organization (Villa et al., 2018). Sex differences in gene expression were also found in hippocampal microglia (Hanamsagar et al., 2017; Villa et al., 2018). These sex differences in adulthood could be driven by sex hormone singling in the brain, sex chromosomes and/or sex specific programming during development. The expression of several target genes in female hippocampal microglia did not vary across the estrous cycle (Hanamsagar et al., 2017). Transcriptional profiles also appear resistant to sex-specific changes in the local brain environment by adulthood, as female mouse microglia transplanted into the male brain maintain a female-oriented gene expression profile (Villa et al., 2018). However, masculinization of female pups during *development* (postnatal week 1) resulted in microglial expression of several target genes that more closely resembled males than females (Villa et al., 2018), supporting the hypothesis that the effect of estrogens during early development may induce permanent sex-specific microglial transcriptional programs that are not sensitive to manipulations in the adult. Together, these studies support a critical developmental window in which both intrinsic and extrinsic factors can influence microglial development.

Microglia and behavior

It is widely accepted that early life experiences have lasting impacts on health and behavior later in adulthood. Early life changes in gonadal hormone levels have a long-lasting influence

on sex-specific adolescent and adult rodent behaviors such as social behavior, aggression, alloparenting rough- and -tumble play, conspecific sniffing, and sexual behavior (Sisk and Zehr, 2005; Bale et al., 2010). Microglia respond to gonadal hormones through surface receptors and through sensing changes in the local brain environment. Interestingly, microglia have also been shown to mediate social (Kopec et al., 2018), cognitive (Cornell et al., 2022), and mood disorder (Branchi et al., 2014; Nelson and Lenz, 2017) behaviors in rodents. Since microglia are responsible for refining neural circuitry in the developing brain (Stevens et al., 2007), they may serve as a causal link in sex hormone-regulated behaviors.

Social behavior

A key study by VanRyzin et al. (2019) demonstrated that the P0 testosterone surge in male rats is responsible for changes in microglia that sculpt sexually differentiated social circuits in the rodent brain. They observed that male rats possessed more phagocytic microglia in the amygdala between P0 and P4 that were specifically engulfing newly proliferating astrocytes. Lower numbers of astrocytes in the medial amygdala correlated with increased juvenile play bouts in males compared to females, suggesting long-term impacts on the developing amygdala circuits mediating play behavior. In support of this hypothesis, treating females with testosterone at P0 led to increased phagocytic microglia in the amygdala and social play behaviors comparable to their male counterparts in adolescence. Furthermore, inhibiting complement dependent phagocytosis in the male amygdala between P0 and P2 increased the number of newborn cells at P4 and in adolescence decreased the levels of male social play to that of females (VanRyzin et al., 2019). Together this suggests a causal link between sex-specific microglial functions in amygdala development and sex-specific differences in play later in life.

Microglial synaptic pruning also shapes social behaviors. Transient depletion of frontal cortex microglia in adolescence resulted in disruptions in adult behaviors including an impaired ability to discriminate between a novel and familiar mouse (Schalbetter et al., 2022). These deficits were not specific to social behaviors as depleted mice also showed memory impairments. The behavioral deficits were linked to altered synaptic engulfment and subsequent reduced neuronal dendritic branching and synapse density as adults. The same depletion experiments conducted in adults did not produce deficits, defining a critical window for microglial pruning of synaptic circuits in the frontal cortex (Schalbetter et al., 2022). While this study was conducted only in males, Kopec et al. (2018) identified sex-specific microglial pruning windows underlying social behaviors. They demonstrated that microglial phagocytosis of dopamine receptors in the nucleus accumbens during key adolescent time windows shaped male rat social play behavior. In females, dopamine receptor elimination during adolescence was not mediated by microglial engulfment. However, blocking complement mediated phagocytosis did impact female social play, suggesting an alternative mechanism for microglial regulation of play in females. Together, these studies provide compelling evidence for sex-specific microglial mediated impacts on neural circuits underlying sex-differences in adolescent social behaviors. These findings may be highly relevant for neurodevelopmental disorders such as autism spectrum disorder (ASD) that are characterized by both microglial abnormalities and deficits in social interactions.

Sexual behavior

The net result of embryonic sexual differentiation is to set up the brain architecture for subsequent production of female or male typical social and reproductive behaviors after puberty (Arambula and McCarthy, 2020). The POA serves as an example region in which microglia play a direct role in controlling sexual differentiation and associated male sexual behavior. During early postnatal life, aromatization of testosterone into estradiol increases the production of prostaglandin E2 (PGE_2) in the POA (Lenz et al., 2013). Microglia produce PGE_2 and express PGE_2 receptors (Minghetti et al., 1997), making them prime candidates for mediating POA masculinization. Studies treating female rats with estradiol or PGE_2 before birth "masculinized" this area of the brain, increasing ameboid microglial numbers to those of males. Additionally, treating females with estradiol in early postnatal life, caused male-typical mating behaviors. Intriguingly, inhibition of microglia with co-treatment of minocycline prevented male copulatory behavior in the females, demonstrating a clear role for microglia in mediating the masculinization effects of PGE_2 in the POA and the associated male sexual behaviors, in adulthood (Lenz et al., 2013).

Brain and behavior disorders

One of the most consistent findings in post-mortem human brains across brain disorders is the altered expression of genes critical for neuronal and immune function (Voineagu et al., 2011; Gupta et al., 2014; Parikshak et al., 2016; Gandal et al., 2018; Vogel Ciernia et al., 2020). Transient pharmacological depletion of embryonic mouse microglia during gestation results in long-term, sex-specific impacts on behavior, including hyper-activity in juvenile females and anxiolytic behavior in adult females (Rosin et al., 2018). Similar depletion of rat microglia during the postnatal testosterone surge (P0–4) results in sex-specific alterations in nest seeking and ultrasonic vocalizations in pups, memory and anxiety-like behavior impairments in juveniles, and impairments in adult male

sex-behaviors (VanRyzin et al., 2016). Together these studies and others like them, suggest that the removal of microglia during key developmental windows can have long-term consequences on the developing neuronal circuits and result in sex-specific impacts on behavior.

A growing body of literature suggests that a wide variety of environmental exposures are associated with an increased risk of neurodevelopmental disorders, including infection during pregnancy (Hallmayer et al., 2011; Kalkbrenner et al., 2014; Fang et al., 2015; Lee et al., 2015). Maternal immune activation (MIA) mouse models lead to long-lived changes in microglial phagocytosis activity (Fernández de Cossío et al., 2017), interneuron function (Thion et al., 2019), axon targeting (Squarzoni et al., 2014), transcription (Mattei et al., 2017; Vogel Ciernia et al., 2018), and morphology (O'Loughlin et al., 2017). For example, offspring from dams induced with allergic airway inflammation during pregnancy showed impaired social approach and increased repetitive behaviors, similar to both social and repetitive behaviors observed in ASD (Schwartzer et al., 2015). Microglia isolated from juvenile female offspring from this model showed altered transcription and epigenetic regulation indicative of enhanced microglial sensitivity to the brain environment (Vogel Ciernia et al., 2018).

Other maternal perturbations can also disrupt microglial development including exposures to drugs of abuse, air pollution, and maternal stress. For example, prenatal opioid exposure reduced adolescent microglial pruning of dopamine receptors in the nucleus accumbens specifically in male rats. This pruning deficit was associated with impaired opioid extinction in adult males but not females, suggesting impacts of early life drug exposure on microglial pruning may alter drug taking behaviors in adulthood (Smith et al., 2022).

Similar sex-specific vulnerabilities were identified in male mice exposed to diesel exhaust particles during gestation. Exposed males showed altered microglial morphology, increased microglia-neuron interactions, and increased inflammatory cytokine production. The male-specific alterations in microglial responses were consistent with male-specific behavioral and metabolic deficits (Bolton et al., 2012, 2013, 2014), suggesting a sex-specific causal link between microglial dysfunction, altered brain development, and behavioral abnormalities in males.

Early life stress can also reprogram microglia in a sex-specific fashion. Offspring that experienced embryonic cold stress showed sex-specific alterations in the transcriptional profiles of embryonic hypothalamic microglia. Male microglia had more significantly upregulated genes than female microglia, rendering them more sensitive to maternal cold stress. Gestational cold stress impaired adult social preference in both sexes but was rescued by gestational microglial depletion only in males, suggesting that the adult social deficits are driven by non-microglial mechanisms in females (Rosin et al., 2021). Recent work from Block et al. (2022) combined prenatal air pollution exposure with postnatal maternal stress. They identified male-specific impairments in social behaviors that were linked to altered microglial pruning of developing thalamocortical synapses in the anterior cingulate cortex specifically in males. Inhibiting microglial phagocytosis within the anterior cingulate cortex during the critical developmental pruning window mimicked the pollution plus stress male phenotype, supporting the role of male-specific alterations in microglial function and behavioral impairments related to neurodevelopmental disorders (Block et al., 2022).

Conclusions

The significance of understanding sex differences in the brain is becoming increasingly important with the growing prevalence of sex biased neurodevelopmental and neuropsychiatric disorders. Future therapeutic strategies should be guided by research into differential organization of the male and female brain, with a particular focus on microglia and sex hormones. With the recent advances in *in vivo* imaging and transcriptomics, the field is poised to understand how sex impacts microglial sculpting of brain circuits and behavior. The work highlighted here emphasizes the need to include both sexes in studies of microglia and provides the key rationale for sex-specific approaches to therapeutic development for neuroinflammation.

Author contributions

All authors listed have made equal direct intellectual contribution to the work and approved it for publication.

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Conflict of interest

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

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Sex-specific behavioral outcomes of early-life adversity and emerging microglia-dependent mechanisms

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Early-life adversity (ELA) is known to alter brain circuit maturation as well as increase vulnerability to cognitive and emotional disorders. However, the importance of examining sex as a biological variable when researching the effects of ELA has not been considered until recently. This perspective discusses the sex-specific behavioral outcomes of ELA in both humans and animal models, then proposes microglia-mediated mechanisms as a potential underlying cause. Recent work in rodent models suggests that ELA provokes cognitive deficits, anhedonia, and alcohol abuse primarily in males, whereas females exhibit greater risk-taking and opioid addiction-related behaviors. In addition, emerging evidence identifies microglia as a key target of ELA. For example, we have recently shown that ELA inhibits microglial synapse engulfment and process dynamics in male mice, leading to an increase in excitatory synapse number onto corticotrophin-releasing hormone (CRH)-expressing neurons in the paraventricular nucleus of the hypothalamus (PVN) and aberrant stress responses later in life. However, ELA-induced synaptic rewiring of neural circuits differs in females during development, resulting in divergent behavioral outcomes. Thus, examining the role of microglia in the sex-specific mechanisms underlying ELA-induced neuropsychiatric disorders is an important topic for future research.

KEYWORDS

early-life adversity, sex differences, cognitive deficits, depression, substance abuse, microglia, synaptic pruning, CRH neurons

Introduction

Early-life adversity (ELA) is now well-known to alter the development of multiple brain circuits and increase long-term risk for cognitive and emotional disorders, including depression and substance abuse (Gershon et al., 2013; Davis et al., 2017; Luby et al., 2017; Dahmen et al., 2018; Birnie and Baram, 2022; Hakamata et al., 2022). Decades of human research have established ELA, often defined as low socioeconomic status, poor family functioning, sexual or physical abuse, and/or an absence of parental nurturing, as

a strong predictor of poor mental and physical health later in life (Springer et al., 2007; Vegt et al., 2009; Lovallo et al., 2013; Targum and Nemeroff, 2019). Rodent models of ELA have also been developed to provoke the cognitive and emotional dysfunction associated with mental disorders, so as to better elucidate their biological underpinnings (Baram et al., 2012; Molet et al., 2014; Rincón-Cortés and Sullivan, 2016).

Early on, studies of ELA identified aberrant reactivity of the hypothalamic–pituitary–adrenal (HPA) axis as a major outcome (Levine et al., 1991; Gilles et al., 1996; Nicolson, 2004; Tarullo and Gunnar, 2006). More recent research has allowed us to gain a better understanding of the mechanisms underlying abnormal stress reactivity, as well as other neural circuits involved. Several animal studies have found that ELA alters the development of brain regions involved in reward and stress, such as the nucleus accumbens, amygdala, and hypothalamus (Brenhouse and Andersen, 2011; Bolton et al., 2018a; Nieves et al., 2020; Wendel et al., 2021; Haikonen et al., 2022; Levis et al., 2022). In addition, recent work indicates that microglia, the resident immune cells of the brain, may play an important role in sculpting these developmental changes due to the critical role they play in synaptic pruning via engulfment of synaptic elements (Delpech et al., 2016; Hoeijmakers et al., 2017; Sellgren et al., 2017; Bolton et al., 2022). For example, we recently demonstrated that reduced synaptic engulfment by microglia during ELA leads to increased excitatory synapse number on corticotropin-releasing hormone (CRH)-expressing neurons of the hypothalamus and aberrant stress reactivity in adulthood (Bolton et al., 2022). However, few studies have investigated sex as a biological variable when examining the effects of ELA, even though sex differences are known to exist both in ELA's later outcomes (Bath, 2020) and microglial development (Schwarz et al., 2012; Hanamsagar et al., 2017). In this perspective, we will highlight the sex-specific behavioral outcomes of ELA identified thus far and discuss emerging evidence for microglia-dependent mechanisms in an effort to catalyze this exciting area of research.

Sex-specific behavioral outcomes of early-life adversity in humans

Numerous studies have shown that ELA is associated with depression, substance abuse, and cognitive deficits in humans (reviewed in Dube et al., 2003; Short and Baram, 2019; Lemoult et al., 2020). For example, a study of institutionalized children found marked delays in cognitive development and decreased IQ, with the degree of impairment correlated with the duration of institutionalization (Nelson et al., 2007). Further studies have also shown that ELA may have sex-specific effects on cognition. In an assessment of ELA-induced cognitive dysfunction, an association between a history of childhood trauma and worse working and spatial memory in a standardized neuropsychological test battery was found in males only (Aas et al., 2011). In addition, ELA has been associated with a smaller hippocampal volume in men, which is a risk factor for cognitive deficits and other neuropsychiatric disorders. For example, Lawson et al. (2017) found that low socioeconomic status was associated with reduced hippocampal volumes in males but not females. Similarly, Samplin et al. (2013) revealed that childhood emotional abuse was associated with decreased hippocampal volume in males only. However, a history of emotional abuse was correlated with increased levels of depressive symptoms in both males and females (Samplin et al., 2013). These results indicate that while females might be more resilient to ELA-induced cognitive dysfunction and associated structural changes, they are not necessarily more resistant to its effects on emotional function.

ELA is a strong predictor of developing depressive disorders later in life (Mandelli et al., 2015). One of the most widely reported findings of individuals struggling with depression is hyperactivity of the HPA axis. Since ELA has also been found to increase the activity of the HPA axis, Heim et al. (2008) sought to determine if ELA was associated with an increased cortisol response to a CRH challenge in men with and without major depressive disorder (MDD). The study found that men with a history of childhood trauma showed an increase in ACTH and cortisol in response to a CRH injection as compared to controls. In addition, men with MDD that were exposed to childhood trauma exhibited increased responsiveness in comparison with controls and depressed men without childhood trauma (Heim et al., 2008). These results indicate that ELA leads to HPA axis hyperactivity in men, although women were not included in the study. Interestingly, other studies assessing ELA-induced mental illness found that women who were exposed to ELA were more likely to experience depression than men (Colman et al., 2013), suggesting that stress may manifest itself differently in men.

One way the long-term effects of ELA may manifest differently is through an increased susceptibility to drug and alcohol abuse, which has been observed in several studies on the sex-specific impact of ELA in humans. For example, Evans et al. (2017) found that the risk for substance abuse of a variety of different drugs was generally higher in men than in women exposed to childhood adversity. However, as the number of childhood adversity experiences increased, women's risk for substance abuse disorders exceeded that of men (Evans et al., 2017). Furthermore, multiple studies examining the impact of ELA on alcohol abuse found that men had a higher risk of abusing alcohol than women (Strine et al., 2012; Colman et al., 2013). The higher rates of drug and alcohol abuse in men and the higher rate of depression diagnoses in women exposed to ELA suggest that while women may be more likely to seek treatment for psychiatric symptoms, men may be more likely to turn to drugs and alcohol as a coping strategy.

Sex-specific behavioral outcomes in animal models of early-life adversity

In order to study the biological mechanisms of ELA, it is generally modeled in animals by manipulating either the quantity or quality of maternal care (Bolton et al., 2017). For rodents, two commonly applied models of ELA are maternal separation (MS) and limited bedding and nesting (LBN). Maternal separation is characterized by the daily separation of pups from the dam, ranging from one to eight hours a day over several days or even weeks (reviewed in Tractenberg et al., 2016). These repeated separations result in intermittent stress and an overall reduction in the quantity of maternal care (Pryce et al., 2005). The LBN model is applied continuously during an early sensitive period [typically postnatal days (P)2–9] by limiting the number of nesting materials provided and adding a mesh platform to the dam's cage, which prevents the dam from constructing a typical, full nest. In this paradigm, the impoverished environment leads to fragmented and unpredictable maternal care, as measured by more frequent exits from the nest and shorter bouts of licking and grooming (Molet et al., 2016a; Walker et al., 2017). Although the majority of ELA models utilize rodents, non-human primate models have also been developed, including childhood maltreatment as a model of ELA for the rhesus macaque. In this paradigm, infants are placed with known abusive females who have been previously recorded engaging in infant abuse (McCormack et al., 2006). These animal models have all been shown to result in impairments to cognitive and emotional development, along with alterations in stress reactivity of the HPA axis (reviewed in Bolton et al., 2017; van Bodegom et al., 2017).

The LBN model has been widely used to investigate the impact of ELA on cognitive function, although historically, the sex-specific impact of ELA on cognitive development has not been well-defined. Recent studies have shown that ELA may have sex-selective effects on hippocampus-dependent memory deficits. For example, multiple studies have found that LBN provokes deficits in hippocampus-dependent spatial memory in a stress-free novel object location task in adult male (>P60) rodents (Molet et al., 2016b; Davis et al., 2017; Bolton et al., 2020; Xu et al., 2022). This cognitive dysfunction was also found in LBN male mice in a more stressful water-maze task (Naninck et al., 2015). The MS model elicited similar results, in which MS male mice showed impairments during spatial (Stoneham et al., 2021) and non-spatial memory tasks (Banqueri et al., 2017), as compared to controls. In contrast, in females, ELA did not result in hippocampus-dependent spatial memory deficits, as measured by the water maze (Naninck et al., 2015) and novel object location task (Loi et al., 2017). Interestingly, ELA did elicit memory impairments in the novel object location task transiently during adolescence (Bath et al., 2017). However, this deficiency did not carry into early adulthood, indicating that ELA impairment of spatial memory is more severe and long-lasting in males than females (Bath et al., 2017).

ELA has also been shown to impact emotional function, such as depressive-like behavior and reward-related behavior, in a sex-dependent manner in animal models. For example, we have found that ELA induces anhedonia, as measured by reduced sucrose preference and social play, in male rats (Bolton et al., 2018a). Similarly, recent work reported a decrease in cocaine- and opioid-seeking behavior in male ELA rats (Bolton et al., 2018b; Levis et al., 2022), suggesting that ELA induces anhedonia for both natural and drug rewards. In contrast, ELA in female rats provokes increased addictive-like behavior with opioids and no anhedonia (Levis et al., 2021). Interestingly, Okhuarobo et al. (2020) found that ELA escalated alcohol intake in male, but not female, mice, suggesting that the emotional dysfunction due to ELA may result in different types of "self-medicating" in males vs. females, in agreement with the human studies.

Because depression is linked with alterations of the HPA axis, it is not surprising that previous work has shown that ELA has a significant impact on HPA axis reactivity. For example, LBN rearing elevated plasma corticosterone by the end of the experience (P9), and this increase persisted into adulthood (Rice et al., 2008). Furthermore, these changes are accompanied by enhanced glutamatergic innervation and activity of CRH-expressing neurons in the PVN (Gunn et al., 2013; Bolton et al., 2022). Daviu et al. (2020) recently utilized the looming-shadow threat task as a behavioral read-out of PVN-CRH+ neuron activity. They discovered that an escape response to the simulated threat (i.e., looming shadow) is preceded by an increase in the activity of PVN-CRH+ neurons, and inhibiting their activity decreases the escape response (Daviu et al., 2020). When this behavioral test was applied in the context of ELA, it was revealed that adult LBN male mice escaped significantly more than control mice, in agreement with their increased PVN-CRH+ neuronal activity (Short et al., 2021; **Figure 1A**). In contrast, we have recently shown that LBN rearing from P2-P10 does not significantly alter the defensive behavioral response of adult female mice in the looming shadow task (**Figure 1A**), suggesting a sex-specific impact of ELA on the behavioral threat response in adulthood.

Some studies using MS as a model for ELA have shown increased anxiety-like behavior in MS mice, particularly in males (Daniels et al., 2004; Malcon et al., 2020; Tsotsokou et al., 2021), although this is inconsistent (Fabricius et al., 2008; Bondar et al., 2018; Wang D. et al., 2020). On the other hand, numerous studies applying the LBN model have found that exposure to ELA does not affect anxiety-like behavior in the open field or elevated-plus maze test in male rats and mice (Naninck et al., 2015; Molet et al., 2016a; Bolton et al., 2018a, 2020; **Figure 1B**). Interestingly, we have recently found that female mice reared in LBN cages display increased risk-taking behavior in the elevated-plus maze test, spending more time in the open arms than controls (**Figure 1B**). Similarly, Viola and colleagues found that ELA in female mice

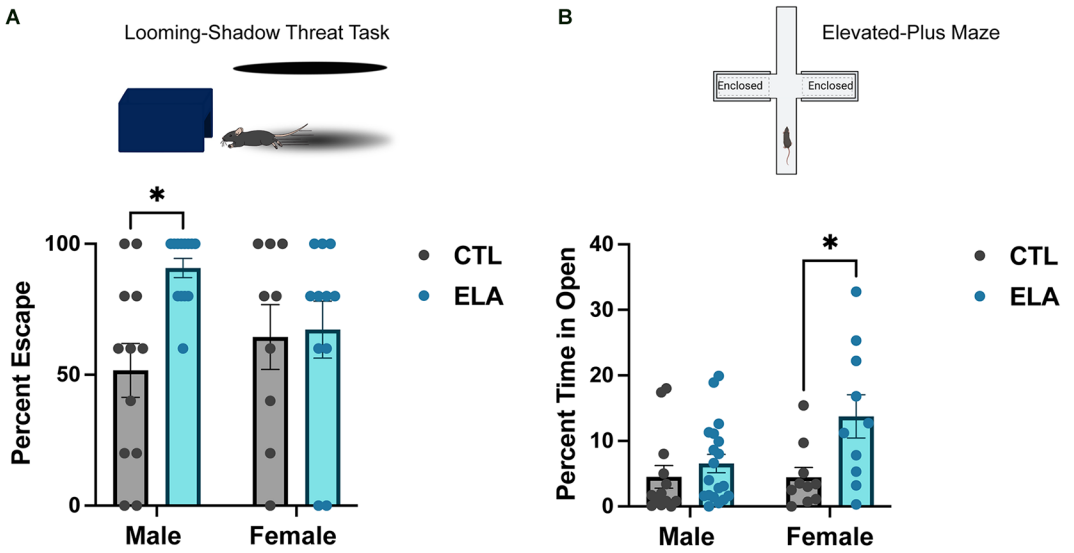

FIGURE 1
Sex-specific behavioral outcomes of early-life adversity. **(A)** ELA provokes an increased escape response to a looming-shadow threat in male, but not female, adult mice (trend for ELA x Sex interaction; $F_{(1,41)} = 3.71$, $p = 0.06$; *post-hoc* test, $p < 0.05$). **(B)** On the other hand, ELA increases risk-taking behavior, as measured by an increased percent time spent in the open arms in the elevated-plus maze, in female, but not male, adult mice (trend for ELA x Sex interaction; $F_{(1,48)} = 3.31$, $p = 0.08$; *post-hoc* test, $p < 0.05$). Figure created with BioRender.com. Data are mean ± SEM; $p \leq 0.1$ criteria used for interaction terms to trigger subdivision for lower-order tests; $^*p < 0.05$ *post-hoc* test.

decreased HPA axis reactivity, impaired risk assessment, and increased risking-taking in a task used to assess risk-taking under reward-seeking conditions (Viola et al., 2019). Together, these data suggest that ELA does not robustly impact anxiety-like behavior in males, but may even induce the opposite in females, by decreasing anxiety-like behavior and increasing risk-taking behavior.

Emerging microglia-dependent mechanisms

Microglia play an important role in regulating brain development, via pruning synapses and shaping neuronal function (reviewed in Delpech et al., 2015; Wu et al., 2015; Ngozi and Bolton, 2022). Notably, significant perturbations of microglial function during development due to injury, infection, or even stress can result in aberrant microglial synaptic pruning, thus causing the "rewiring" of brain circuits and ultimately behavioral changes. For example, deleting *Cx3cr1* in mice temporarily reduces microglial numbers during development, leading to deficient synaptic pruning, impaired functional brain connectivity, and deficits in social behavior (Zhan et al., 2014). As a result of findings such as these, the impact of environmental manipulations, such as ELA, on microglial function has become an important topic of research. Various human studies have identified inflammation as one potential mechanism by which ELA leads to poor behavioral outcomes later in adulthood (McQuaid et al., 2019; Pinto Pereira et al., 2019; O'Connor et al., 2020). However, stress can also inhibit and impair microglia. For example, Hoeijmakers et al. (2017) reported that LBN rearing altered microglial morphology and decreased Iba1+ staining coverage in the hippocampus of male mice at P9. Similarly, microglia in the hippocampus of P28 MS male mice exhibit increased expression of genes related to phagocytic activity and decreased expression of inflammatory genes relative to controls, indicating that ELA perturbs the function of microglia in the hippocampus (Delpech et al., 2016). In addition to the hippocampus, other studies have found that ELA disrupts microglial morphology and function in the prefrontal cortex (PFC) of male rodents during development (Reshetnikov et al., 2020; Wang R. et al., 2020), although females were not included in any of these experiments.

ELA may have differential effects on microglial function in males and females due to known sex differences in microglial maturation. In an analysis of microglia colonization in the developing brain, Schwarz et al. (2012) found that there are more microglia, and microglia of more "activated" (or less mature) morphologies, in certain regions, such as the hippocampus and amygdala, in males than in females early in postnatal life. Intriguingly, this balance shifts to the opposite pattern by puberty, such that adolescent and adult females have more "activated" microglia in the same brain regions (Schwarz et al., 2012). In the context of ELA, work by Bachiller et al. (2022) discovered that the extent of Iba1+ staining coverage is increased in the hippocampus of MS male, but not female, mice at P15.

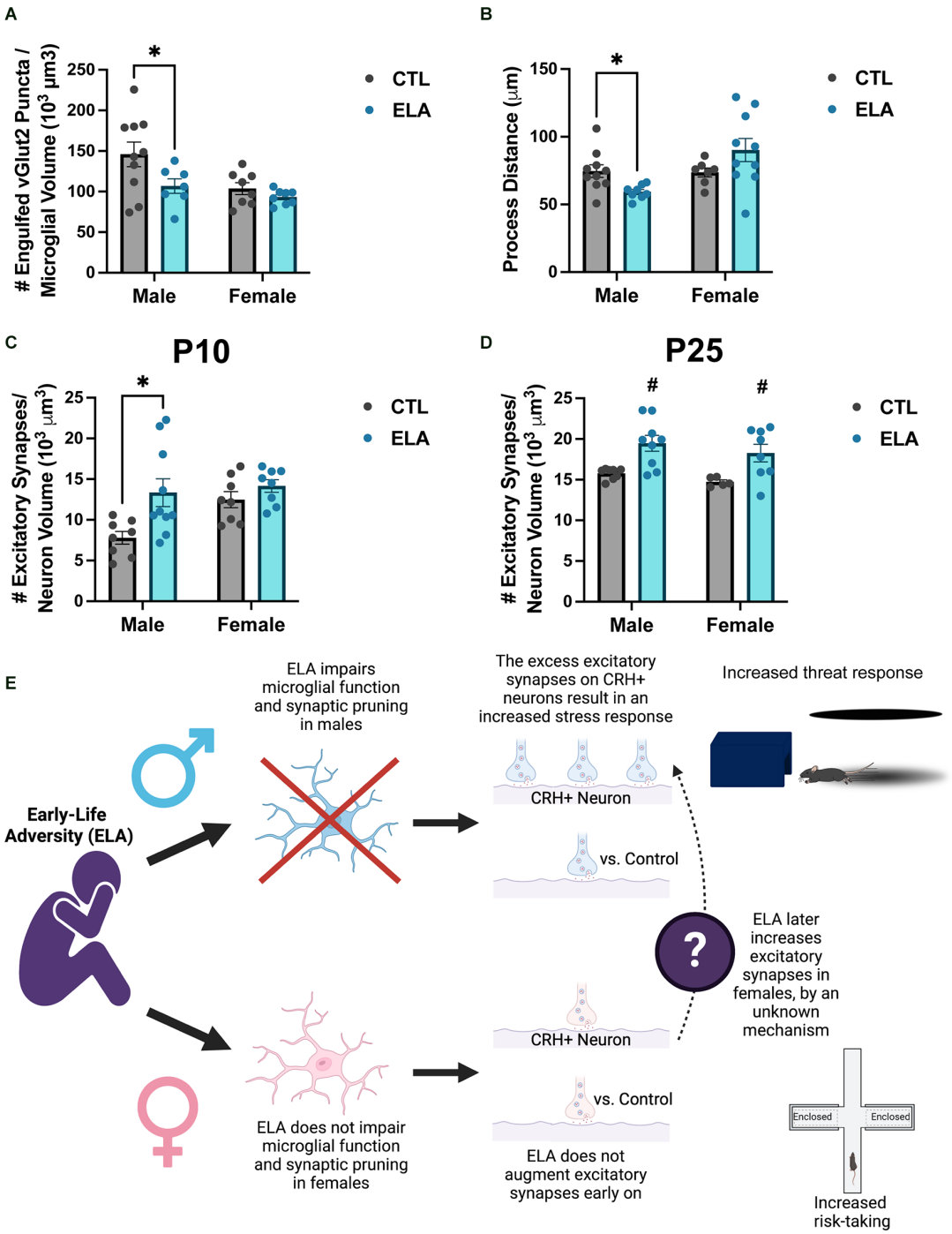

FIGURE 2
ELA alters microglial function and synaptic pruning in a sex-specific manner, leading to sex differences in the ELA-induced augmentation of excitatory synapse number on PVN-CRH+ neurons across development. **(A)** ELA diminishes the number of excitatory synaptic puncta engulfed by microglia at P8 in male, but not female, mice (trend for ELA x Sex interaction; $F_{(1,29)} = 1.74$, $p = 0.1$; *post-hoc* test, $p < 0.05$). **(B)** ELA inhibits the microglial process dynamics, as measured by total distance moved by microglial processes, at P8 in males, but not in females, even tending to do the opposite (significant ELA x Sex interaction; $F_{(1,29)} = 7.38$, $p = 0.01$; *post-hoc* test, $p < 0.05$). **(C)** ELA augments the number of excitatory synapses on PVN-CRH+ neurons at P10 in male, but not female, mice (trend for ELA x Sex interaction; $F_{(1,30)} = 2.45$, $p = 0.1$; *post-hoc* test, $p < 0.05$). **(D)** By P25, there is a significant increase in the number of excitatory synapses on PVN-CRH+ neurons in both male and female ELA mice (significant main effect of ELA; $F_{(1,26)} = 17.5$, $p = 0.0003$). **(E)** Conceptual figure displaying the proposed microglia-dependent mechanisms by which ELA results in sex-specific behavioral outcomes. Figure created with BioRender.com. Data are mean ± SEM; $p \leq 0.1$ criteria used for interaction terms to trigger subdivision for lower-order tests; $^{*}p < 0.05$ *post-hoc* test; $^{\#}p < 0.05$ main effect of ELA; adapted from Bolton et al. (2022).

However, the same study found an increase in the percentage of microglia with an "activated" morphology in the PFC of MS females in comparison to male counterparts (Bachiller et al., 2022). Thus, it is possible that experiencing adversity during a developmental period in which microglia show sex differences in number and morphology in certain brain regions could impact males and females differently, thereby resulting in sex-specific and region-specific ELA-induced changes in synaptic pruning and ultimately divergent behavioral outcomes.

Recent evidence shows that the ELA-induced disruption of microglial function described above may in fact cause the developmental circuit changes observed following ELA. We have previously shown that the ELA-induced anhedonia observed in male rats is accompanied by changes in the connectivity of reward- and stress-related circuits in the brain (Bolton et al., 2018a). Given the importance of microglia in shaping circuits, we then focused on the impact of ELA on microglia surrounding CRH-expressing neurons in the paraventricular nucleus (PVN) of the hypothalamus (Bolton et al., 2022). We found that ELA inhibited microglial function in P8 male CX3CR1-GFP+/- mice, both in terms of synapse engulfment (**Figure 2A**) and process dynamics (**Figure 2B**), leading to an increase in excitatory synapse number on PVN-CRH+ neurons at P10 and P25 (**Figures 2C,D**). Importantly, selective chemogenetic "reactivation" of microglia during ELA in males prevented the development of the synapse excess on PVN-CRH+ neurons and the aberrant stress response in adulthood (Bolton et al., 2022). However, female microglia were not significantly impaired by ELA at P8 (**Figures 2A,B**), and the developmental trajectory of the ELA-induced synaptic rewiring was different from males, although by P25 females also had increased numbers of excitatory synapses on their PVN-CRH+ neurons (**Figure 2B**). Together, these findings indicate that microglia play an important role in the sex-specific mechanisms underlying the ELA-induced behavioral outcomes described above (proposed mechanism illustrated in **Figure 2E**).

Discussion

In this perspective, we present evidence that there are sex-specific behavioral outcomes of ELA in both humans and animal models, as well as microglia-dependent mechanisms that differ by sex. While techniques such as two-photon imaging are driving important discoveries in animal research, further advancements need to be made in technologies such as positron emission tomography (PET) to enable high-resolution functional imaging of microglia in humans. Importantly, many earlier studies excluded female subjects for simplification, due to the misconception that females presented more variability (Shansky, 2019). However, in recent years, researchers have begun to examine sex differences in ELA-induced cognitive deficits, affective disorders, and microglial dysfunction, thus making important contributions to our understanding of the outcomes of ELA and the underlying mechanisms. Moving forward, it is critical that we all include sex as a biological variable in our studies in order to fully capture the complexity of the enduring effects of ELA, as well as the promise for preventative interventions and treatments in all individuals.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committees of the University of California—Irvine and Georgia State University.

Author contributions

MG and JB wrote and edited the article, performed the experiments and analyzed the data. JB created the figures for the data included in this Perspective article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Effects of sex and retention interval on the retrieval and extinction of auditory fear conditioning

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Fear memory retrieval is relevant to psychiatric disorders such as post-traumatic stress disorder (PTSD). One of the hallmark symptoms of PTSD is the repeated retrieval and re-experiencing of the initial fear memory even long after the traumatic event has occurred. Women are nearly twice as likely to develop PTSD following a trauma than men, thus sex differences in the retrieval of fear memories is highly relevant for understanding the development and maintenance of PTSD. In the current study, we aimed to examine sex differences in the retrieval and extinction of either recent or remote fear memories. To do so, we conditioned male and female rats either 1 day (recent) or 28 days (remote) prior to testing retrieval and extinction. While there was no effect of sex or retention interval on initial retrieval, we found that remotely conditioned females exhibited higher rates of freezing than remotely conditioned males in later retrieval/extinction sessions, suggesting a sex difference in the retrieval and/or extinction of remote, but not recent, fear memories. Overall, these results are the first to demonstrate a sex difference in the extinction of remote fear memory, and this may contribute to the differential expression of fear-related disorders like PTSD in men and women.

KEYWORDS

fear conditioning, remote memory, sex differences, freezing, extinction, retrieval

Introduction

The ability to form and later retrieve fear memories is highly adaptive. Fear memories promote survival by guiding behavioral responses to avoid potential threats in the future. However, fear memory processes can also contribute to the ontology and maintenance of psychiatric disorders. For example, one of the hallmark diagnostic

criteria for post-traumatic stress disorder (PTSD) is chronic re-experiencing of the memory of the traumatic event, even long after the event occurred (National Institute of Mental Health, 2022). The lifetime prevalence of PTSD in the United States is nearly 7% (Kessler et al., 2005); however, women represent a significantly higher proportion of cases, with some reports suggesting that women are two- to three-times more likely to be diagnosed with the disorder than men following a traumatic event (Kessler et al., 2005; Koenen and Widom, 2009). Thus, in order to understand and effectively treat PTSD, it is critical to understand the behavioral and neurobiological differences between males and females with respect to fear memories.

Fear memories are often studied in rodents using contextual fear conditioning. In this procedure, rats or mice are placed in the conditioning apparatus and receive mild foot-shock(s). Re-exposure to the conditioning apparatus, or context, elicits conditioned fear responses (e.g., Fanselow, 1980). Using this procedure, several studies have examined how contextual fear learning and memory may differ between males and females. For example, an early study found that male rats froze more than females when re-exposed to the original conditioning context (Maren et al., 1994). While this finding has been replicated (Pryce et al., 1999; Chang et al., 2009; Poulos et al., 2015; Colon et al., 2018; Russo and Parsons, 2021) there are also contradicting reports in the literature (e.g., Dachtler et al., 2011; Fenton et al., 2014).

In addition to potential differences in the expression of contextual fear, there is some evidence that generalization of contextual fear to a novel context is influenced by sex. For instance, Keiser et al. (2017) found greater degrees of generalization in female mice compared to males, and Asok et al. (2019) reported more generalization in female mice tested 3 weeks after initial conditioning, specifically when testing occurred first in the novel context. Further, using step-through avoidance conditioning, Lynch et al. (2013) demonstrated that males and females have equivalent context discrimination when tested early after conditioning. However, when the retention interval increased to either 5 or 7 days, female rats showed more generalization (greater responding in the second context) than males. Nevertheless, despite the apparent converging evidence of greater generalization in females than males in most studies, there is contradicting evidence that males show stronger generalization of contextual fear to a second context, raising the possibility that some sex differences may be parameter-specific (Colon et al., 2018).

In addition to contextual cues, fear responses can also be elicited by discrete cues that were present in the environment during the aversive event. For example, in Pavlovian fear conditioning, discrete cues (e.g., tones, lights) gain the ability to elicit fear responses through pairings with mild-foot shock. Although there are some exceptions, a general pattern in the literature is that that males and females exhibit relatively similar conditioned fear to discrete cues during retrieval tests [Maren et al., 1994; Voulo and Parsons, 2017; Colon et al., 2018; but see Graham et al. (2009) and Gresack et al. (2009)]. However, the majority of these studies have tested retrieval shortly after conditioning (e.g., within 24 h). Thus, less is known about sex differences in cued fear retrieval when the interval between acquisition and testing is much longer. The use of longer retention intervals may be particularly relevant for studying fear memories in PTSD, as PTSD diagnoses require the presence of memory-related symptoms for at least 1 month (American Psychiatric Association, 2013); additionally, these patients tend to have chronic, recurring symptoms, including persistent, disruptive memories (National Institute of Mental Health, 2022).

The purpose of the present study was to compare male and female rats in the retrieval of cued fear conditioning acquired either recently or remotely. We chose to examine differences between recent and remote memories because it is broadly acknowledged that as memories age, their neurobiological correlates undergo significant reorganization (e.g., systems consolidation; Frankland and Bontempi, 2005). However, whether and/or how these processes differ between females and males, and whether these differences present behaviorally, is yet unknown. One prior study has examined retrieval of cued fear in male and female rats at retention intervals of either 1 or 14 days (Colon et al., 2018), and reported no difference between sexes, although these authors suggested a ceiling effect may have impacted their ability to detect potential effects. The present study therefore extends this prior work in at least two ways. First, to complement the study by Colon et al. (2018), which tested remote memory at a 14-day retention interval, we compared retrieval in groups with retention intervals of either 1- or 28-days. The use of the longer retention interval (28 days) allowed us to assess if sex differences emerge at later time points. Second, all rats received multiple sessions of tone retrieval. The purpose of this was to gradually extinguish fear to the tone, allowing us to assess the impact of sex on fear retrieval/extinction across a broad range of the response scale.

Materials and methods

Subjects

The subjects were 63 (31 male, 32 female) experimentally naïve Long-Evans rats (Envigo Laboratories, Indianapolis, IN, USA) 75–90 days old upon arrival. Rats were allowed 1 week to acclimate to the vivarium while housed in pairs. On the first day of behavioral procedures rats were then individually housed with plastic tunnels for enrichment in 12 × 7.5 × 7.5 in plastic caging for the remainder of the experiment. Rats were assigned to one of four groups: Male Remote (n = 15), Male Recent (n = 16), Female Remote (n = 16), and Female Recent (n = 16) Food and water were available ad libitum

(LabDiet 5P00 Prolab RMH 3000, LabDiet, St. Louis, MO, USA) in a climate-controlled colony room on a 12:12 light-dark cycle. Throughout the experiment, rats were monitored and cared for in compliance with the Association for the Assessment and Accreditation of Laboratory Animal Care guidelines and the University of Vermont Institutional Animal Care and Use Committee.

Behavioral apparatus

Behavioral procedures occurred in 16 conditioning chambers (Med Associates, Inc., St. Albans, VT, ENV-007; 24 cm W × 30.5 cm L × 29 cm H), which were modified to create 4 sets of distinct "contexts." All chambers had the following common features. Each chamber was housed in a sound-attenuating cabinet (Med Associates, ENV-017M; 66 cm W × 56 cm L × 56 cm H) outfitted with an exhaust fan to provide airflow and background noise (68 dB). All 16 chambers were outfitted with a food cup, recessed in the center of the front wall, a retracted lever (Med Associates, ENV-112CM), located on the right of the front wall, and an inactive nose-poke aperture (2 cm in diameter) located 3 cm above the food cup. All chambers also had a panel light (Med Associates, ENV-221M) on the right front wall (16 cm above the grid floor), a house light (Med Associates, ENV-215M) centered on the back wall 24 cm above the grid floor, and a speaker (Med Associates, ENV-224AM) located 20 cm above and to the right of the food cup. Only the house light was illuminated throughout the experiment. The speaker was used to deliver a 2000 Hz tone for 10 s (the conditioned stimulus, CS), and the grid floor was used to deliver a 1.0-mA, 1.0-s shock (the unconditioned stimulus, US). Security cameras were mounted to the wall outside each sound-attenuating cabinet, and an 8-cm hole in the chamber wall allowed for video recording from the wall opposite the door.

Sets of four chambers were modified to create four different contexts. For the first distinct context ("Bedding" context), the ceiling and side walls were clear acrylic plastic, the front and back walls were brushed aluminum, and the grid floor was stainless-steel rods (5 mm in diameter) spaced 1.5 cm apart (center-to-center). In addition, approximately 6 oz of woodchip bedding was placed in the tray below the grid floor. For the second set of boxes ("Anise" context), the ceiling and door were covered with laminated black and white checkerboard paper with 3.5 cm black and white squares, and three panels on the back wall were covered in black electrical tape to provide a distinct visual feature. The grid floor was staggered, such that every other bar was on a different plane offset by 0.5 cm, and the tray below the grid floor was painted black. Approximately 5 mL of 10% Anise extract (McCormick, Baltimore, MD, USA) was placed in a plastic dish on the floor directly outside the chamber (inside the cabinet) to the right of the chamber door at the beginning of every session to serve as a distinct olfactory cue.

For the third set of boxes ("Vicks" context), the ceiling and door were covered with wallpaper made from laminated gray construction paper. There was an additional panel light (which remained off) and retracted lever on the left side of the front wall. The floor consisted of alternating stainless-steel rods with different diameters (0.48 and 1.27 cm), spaced 1.6 cm apart from center to center, and the tray beneath the floor was painted gray. Prior to each session approximately 0.5 mL of Vicks VapoRub ointment (Vicks, Cincinnati, OH, USA) was placed in the plastic dish outside the door to chamber. For the fourth set of boxes ("Coconut" context), the ceiling and door were covered with rows of blue dots (3 cm in diameter) that were spaced approximately 1.75 cm apart. There was also an additional panel light (off) and retracted lever on the left side of the front wall. In these chambers the floor consisted of stainless steel rods (5 mm in diameter) arranged such that there was a slight arch in the floor between front and back wall: the highest rod at the center was approximately 1 cm higher than the two rods at either end of the grid floor. A small dish of 10% coconut extract (McCormick, Baltimore, MD, USA) was also placed on the floor to the right of the chamber door.

In the current experiment, each group of rats experienced two contexts, counterbalanced as Context A and B. Half of the rats experienced the Bedding and Anise boxes (counterbalanced as Context A and B) and the other half of the rats experienced the Vicks and Coconut boxes (counterbalanced as Context A and B). Assignment to a particular pair of contexts was counterbalanced across sex and retention interval. Thus, half of the rats in each of the four behavioral conditions (i.e., Male Remote, Male Recent, Female Remote, Female Recent) were trained in the Bedding/Anise pair, and the other half in the Vicks/Coconut pair.

Behavioral procedures

All behavioral procedures were conducted between 8:00 am and 2:00 pm, and the timing of procedures was kept consistent for each group.

Conditioning

All rats received a single day of auditory fear conditioning in Context A. Each session consisted of 3 presentations of the CS, a 10-s tone, which terminated with the onset of the US, a 1-mA, 1-s shock. The first trial began 3 min after rats were placed in the chambers. The time between shock and the next CS presentation was 64 s. Subjects remained in the chambers for 90 s after the last trial before being returned to their home-cages. Half of the rats remained in their home cages for a 28-day retention interval. The other half received a 24-h retention interval. As shown in **Figure 1**, we staggered the start of the experiment

(conditioning), so that all rats received the subsequent phases of the experiment on the same day and were therefore the same age at the time of testing.

Context tests

Following either a 1- or 28-day retention interval (see **Figure 1**), all rats were then given a test session in Context A and Context B on the same day, separated by approximately 3 h. During each session, rats were returned to the apparatus for a 4.5-min period in which no tones or shocks were presented and freezing to the context was monitored. The order of testing in A and B was counterbalanced within each group of rats such that half of the rats were tested in Context A first and Context B second, and the other half had the reverse order.

Context B re-exposure

Over the course of the next 2 days, rats were exposed to Context B alone for four 20-min sessions (see **Figure 1**). There were two sessions per day, separated by approximately 3 h. During these sessions, no tones or shocks were presented. The purpose of these sessions was to reduce any generalized fear to the context alone prior to testing tone retrieval.

Tone retrieval and extinction

Tone retrieval was tested in Context B. Each session consisted of 30 presentations of the tone with no shocks presented (64 s ITI). The first trial began 3 min after rats were placed in the chamber, and rats were removed from the chamber following the last CS presentation. For two consecutive days (see **Figure 1**), there were three sessions per day, separated by approximately 1.5 h. Thus, there were a total of six sessions of tone retrieval/extinction.

Estrous cycle monitoring

In order to monitor the estrous cycle, vaginal smears were collected from all female rats (both Recent and Remote groups) for 4 days prior and 4 days after conditioning for the Remote group, and again starting 4 days prior to conditioning for the Recent group, continuing through the end of the experiment. Smears were collected by inserting a cotton swab dampened with distilled water less than a centimeter into the vaginal canal (to avoid inducing pseudopregnancy) and rolling the tip against the vaginal wall. Samples were then transferred to dry glass slides. All samples were taken each day between 11:45 am and 1:30 pm. Following the end of the experiment, all slides were stained using 0.1% Crystal Violet stain (e.g., McLean et al., 2012), cover-slipped, and evaluated under a light microscope at 10X objective. Number, proportion, and type of cells (nucleated epithelial cells, cornified epithelial cells, leukocytes, and neutrophils) were used to determine if rats fell within one of four stages: proestrus, estrus, metestrus, or diestrus (Cora et al., 2015; Hilz et al., 2019). All samples were evaluated by two trained observers.

Behavioral observations and data analysis

Freezing was the main dependent measure, defined as total motor immobility except for breathing (Blanchard and Blanchard, 1969; Fanselow, 1980). For the conditioning session, freezing is reported during the 64-s period before the first trial (baseline freezing) and during each of the 10-s tones (CS freezing). In addition, during the conditioning session, we measured activity bursts to the 1-s foot shocks and a control period of 1-s prior to the first CS as a measure of shock reactivity. Shock reactivity was assessed so as to account for any group differences in the experience of shock that could potentially influence differences in learning as measured in later tests (e.g., Wiltgen et al., 2001). Rat position data was collected from every frame of the video data by a trained observer (who clicked on the target point on each randomly presented video frame), smoothed using a three frame rolling average to reduce jitter, and summarized by adding the distances (in normalized pixels) between these points over all pairs of frames during each period. During the context test sessions as well as the re-exposure sessions in Contexts A and B, freezing is reported for the first 4.5 min of each session. During the tone retrieval/extinction sessions, freezing is reported for the 64-s period prior to the first tone (pre-CS period), as well as during tone presentations.

Automated scoring of freezing was conducted using the following method: video streams were acquired in near-infrared (720P resolution, 29.97 frames per second) by Anpviz IPCameras (model IPC-B850W) mounted in each chamber. Streams were delivered over a dedicated ethernet network and captured by a computer running ffmpeg. Recordings were subsequently scored by first computing the absolute difference in pixel intensity at every pixel on each pair of subsequent frames. A per-frame activity measure was produced by averaging this difference over all pixels. Inspection of the distribution of (log10-transformed) activity scores revealed a clear bimodal distribution of activity, with the mode of the lowest scores reflecting video noise and mode of the higher scores reflecting rat movement. These distributions varied almost solely by chamber/camera. Presumptive freezing was therefore defined as occurring, on a per-chamber basis, when the activity score fell below the value visually marking the beginning of the rat-movement related portion of the distribution. Activity scores were then averaged in 1 s bins, and only 1 s bins that fell below the threshold were defined to represent freezing [approximating procedures used by the Fanselow laboratory, e.g., Fanselow et al. (2019)]. Algorithmically scored freezing correlated well with freezing scored by trained human observers, with all R-values exceeding 0.80. Tone retrieval/extinction videos were further screened to exclude immobility due to sleeping, boredom, or fatigue from the freezing measure. A trained observer watched video from every CS presentation for each rat, and trials

where the animal was clearly sleeping, in a sleep-related posture, or otherwise displaying no fear-related behavior were manually marked as non-freezing. Classification of a small subset of more ambiguous trials was confirmed by a second observer, yielding the final adjusted scores. Freezing data were statistically analyzed using between subjects analysis of variance (ANOVA) and repeated measures ANOVA where appropriate.

In addition to freezing behavior, this is some evidence that female rates engage in other, escape-like responses (e.g., "darting," flight; Gruene et al., 2015; Greiner et al., 2019) more than males. Because higher performance of these alternate responses can potentially result in the misattribution of lower freezing in females as lower fear (Gruene et al., 2015), we examined the presence of darting during the six tone retrieval/extinction sessions in males and females. The same videos used to score freezing were used to score instances of darting during all of the 10-s tones in all sessions. Darting was defined and scored as "rapid, forward movement across the chamber that resembled an escape-like response" (Gruene et al., 2015).

Results

Estrous cycle

While the estrous cycle was monitored throughout the duration of the experiment, adequate analyses based on phase were not possible given that sample sizes for individual phases and/or low vs. high hormone phases were too underpowered (e.g., during tone retrieval/extinction sessions 1–3, only 5 out of the 31 females were in the proestrous phase) to reliably detect a statistical influence of estrous phase.

Conditioning

Freezing

Mean percent freezing during conditioning is presented in **Figure 2A**. Freezing during the baseline period was analyzed with a 2 (sex: male vs. female) × 2 (retention interval: 1 vs. 28 days) ANOVA (for means and standard errors, see **Table 1**). Unexpectedly, there was a significant main effect of retention interval [$F_{(1,59)} = 7.20$, $p = 0.009$, $\eta_p^2 = 0.109$], with remotely conditioned rats exhibiting slightly lower percent freezing ($M = 0.65$, $SD = 1.25$) than recently conditioned rats ($M = 2.64$, $SD = 3.97$) during the 64-s baseline period. Although significant, the difference between means was remarkably small (∼2%). No other significant differences were observed between groups.

Freezing during the CS presentations in the conditioning session was analyzed with a 2 (sex: male vs. female) × 2 (retention interval: 1 vs. 28 days) × 3 (CS presentation) repeated-measures ANOVA. This revealed a significant effect of CS presentation [$F_{(2,118)} = 185.56$, $p < 0.001$, $\eta_p^2 = 0.759$]. Additionally, there was a significant main effect of retention interval [$F_{(1,59)} = 4.77$, $p = 0.033$, $\eta_p^2 = 0.075$] as well as significant CS presentation × retention interval interaction [$F_{(2,118)} = 6.42$, $p = 0.002$, $\eta_p^2 = 0.098$]. Follow-up comparisons to further analyze this interaction revealed no differences between groups during the first CS presentation [$F_{(1,59)} = 1.55$, $p = 0.218$]. While the Recent group froze significantly less than the Remote group during the second CS presentation [$F_{(1,59)} = 11.77$, $p = 0.001$, $\eta_p^2 = 0.166$], the difference was fleeting, as levels of freezing were equivalent by the third and final CS presentation [$F_{(1,59)} = 0.33$, $p = 0.566$]. There was no main effect of sex ($p = 0.426$) nor sex × retention interval interaction ($p = 0.092$). Neither the CS × sex ($p = 0.59$), nor the CS × sex × retention interval interaction were significant ($p = 0.116$).

Shock activity

Burst activity during the pre-CS period and during the shocks can be seen in **Figure 2B**. Total movement during the pre-CS period was analyzed with a 2 (sex) × 2 (retention interval) ANOVA. As expected, movement during the pre-CS period did not differ by sex or by retention interval (p's > 0.05). Average movement during the shocks was analyzed in a 2 (sex) × 2 (retention interval) × 3 (shock periods) ANOVA. While neither the main effect of sex nor the effect of retention interval was significant [$F_{(1,59)} = 0.49$, $p = 0.486$; $F_{(1,59)} = 0.39$, $p = 0.534$, respectively], there was a significant sex × retention interval interaction [$F_{(1,59)} = 4.64$, $p = 0.035$, $\eta_p^2 = 0.073$]; however, pairwise comparisons revealed that group differences were only marginal {Male Remote < Female Remote [$F_{(1,59)} = 4.01$, $p = 0.050$]; Male Remote < Male Recent group [$F_{(1,59)} = 3.80$, $p = 0.056$]}.

Context tests

Freezing

Overall mean freezing in Contexts A and B during the context tests is presented in **Figure 3A**. Surprisingly, freezing was not significantly higher in context A than B, regardless of experimental group: a repeated-measures ANOVA revealed a lack of significant effects of context [$F_{(1,59)} = 3.33$, $p = 0.073$], retention interval [$F_{(1,59)} = 2.43$, $p = 0.124$] or sex [$F_{(1,59)} = <0.001$, $p = 0.987$].

To further probe contextual fear retrieval, we also examined freezing across 4, 1-min bins during the context tests with a 4 (bin: 1–4) × 2 (context: A vs. B) × sex × retention interval repeated-measures ANOVA. There was a significant main effect of bin [$F_{(3,177)} = 20.82$, $p < 0.001$ $\eta_p^2 = 0.261$] and bin × retention interval interaction [$F_{(3,177)} = 5.03$, $p = 0.002$,

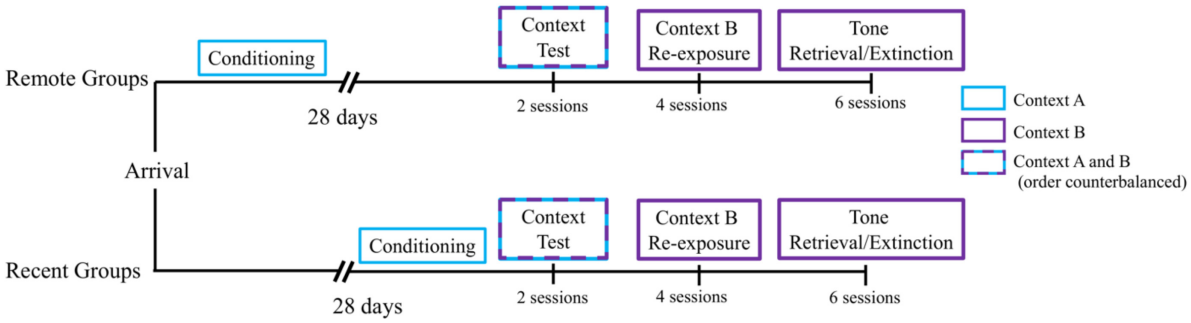

FIGURE 1
Experimental timeline. All groups received a single session of auditory fear conditioning in Context A, followed by a 28-day retention interval (Remote Groups) or a 1-day retention interval (Recent Groups) before being tested for context fear retrieval in Contexts A and B (order counterbalanced). On the subsequent 2 days, all groups received 2 daily sessions of Context B Re-exposure, for a total of 4 sessions. Next, all groups received 3 daily sessions of tone retrieval/extinction in Context B for 2 days, for a total of 6 sessions.

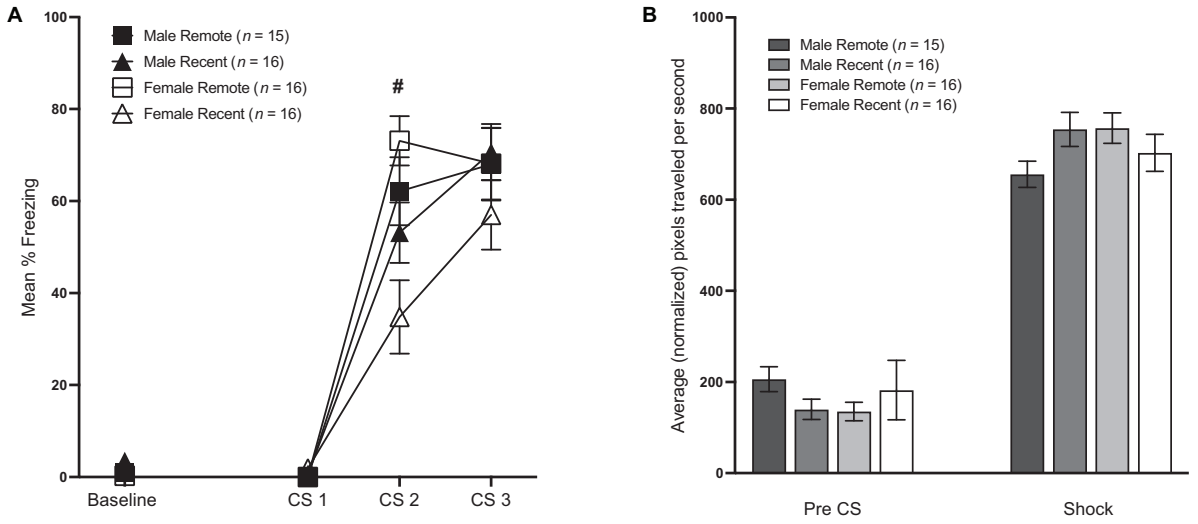

FIGURE 2
(A) Mean percent freezing (±SEM) during conditioning. Baseline represents the 64-s baseline period preceding the first tone. CS 1–3 = the 10-s tones that preceded shock. There was a small but significant difference in freezing during the baseline period, such that Remote groups froze less than Recent groups. During conditioning, Recent groups froze significantly less than Remote groups during CS 2 (# indicates a significant difference between Recent and Remote, $p < 0.05$), but there were no differences in freezing during CS 1 or CS 3 (see Section "Results" for details). **(B)** Shock reactivity during conditioning. Average movement in the 1-s before the first shock [Pre-CS (±SEM)] and average movement across the three 1-s shocks (±SEM).

TABLE 1 Mean percent freezing (±SEM) during baseline periods for each group during conditioning and tone extinction/retrieval sessions.

	Conditioning	Tone extinction/retrieval					
Group		**1**	**2**	**3**	**4**	**5**	**6**
Male remote	1.03 (0.39)	3.28 (1.09)	15.23 (3.66)	32.72 (7.27)	44.00 (6.92)	32.92 (9.03)	29.03 (6.38)
Male recent	3.46 (1.26)	4.10 (1.00)	27.87 (8.10)	31.49 (7.22)	57.54 (8.96)	25.54 (6.49)	29.03 (7.07)
Female remote	0.29 (0.21)	10.76 (4.19)	22.91 (5.23)	45.74 (8.36)	32.00 (5.73)	30.67 (6.38)	37.74 (6.87)
Female recent	1.83 (0.56)	8.41 (3.48)	25.05 (5.96)	28.00 (5.28)	33.23 (6.68)	31.08 (5.73)	31.49 (5.09)

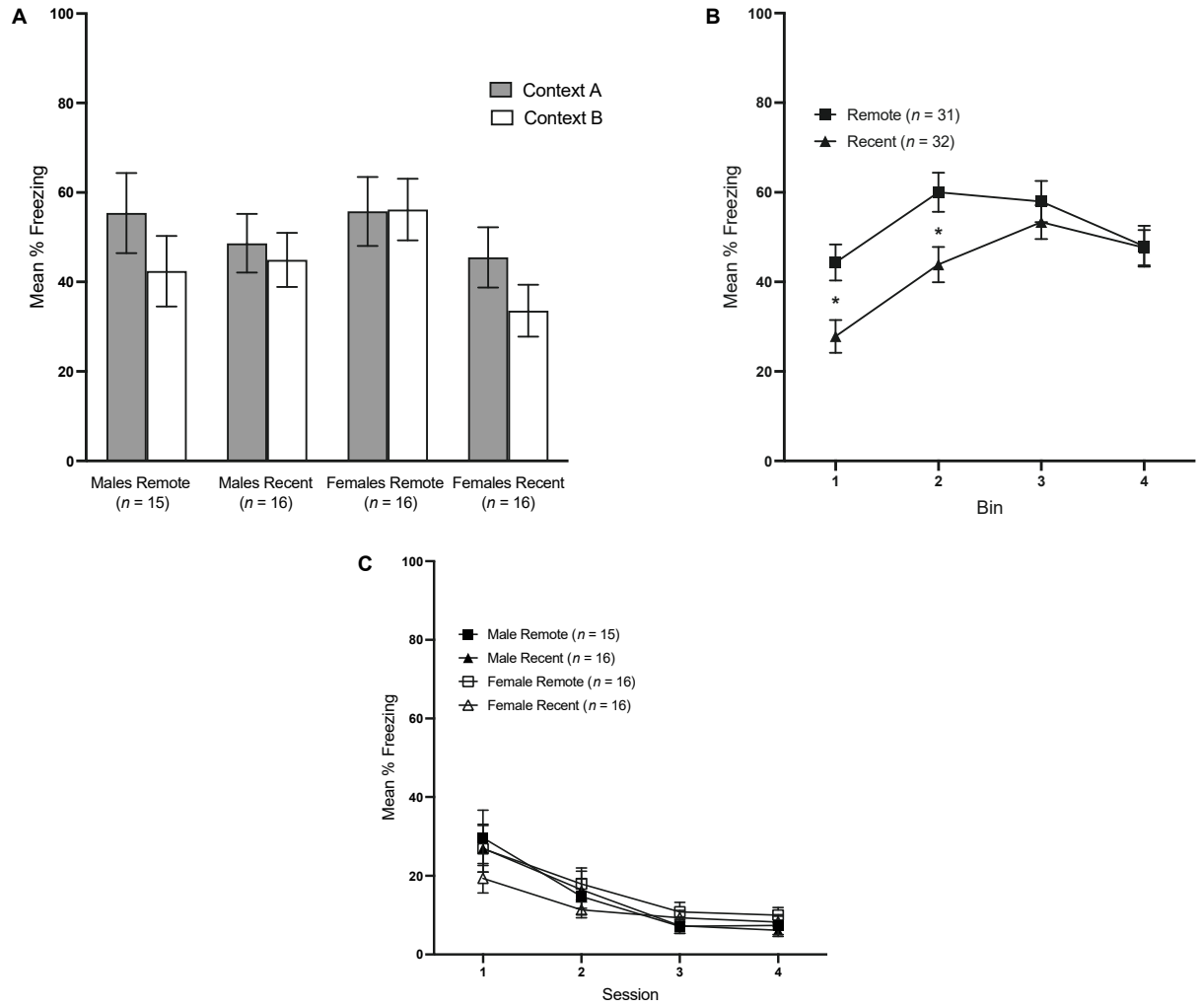

FIGURE 3
(A) Mean percent freezing (±SEM) in Context A vs. Context B for all groups during the context retrieval test. **(B)** Mean percent freezing (±SEM) during the context tests across 4 1-min bins, collapsed across context and sex. **(C)** Mean percent freezing (±SEM) during sessions 1–4 of Context B Re-exposure. *Indicates significant with $p < 0.05$.

$\eta_p^2 = 0.079$]. No other main effects or interactions reached significance. Freezing in the Recent and Remote groups across bins is presented in Figure 3B, collapsed across sex and context, since neither were significant. Pairwise comparisons revealed a significant difference between Recent and Remote groups at bin 1 [$F_{(1,59)} = 7.062$, $p = 0.010$, $\eta_p^2 = 0.107$] and bin 2 [$F_{(1,59)} = 5.44$, $p = 0.023$, $\eta_p^2 = 0.084$]. In both instances, Remote groups froze significantly more than Recent groups, indicating that, in the first half of the context tests, Remote groups demonstrated higher levels of freezing than Recent groups.

Context B re-exposure

Mean percent freezing across the four sessions of Context B re-exposure is shown in Figure 3C. A 4 (session) × 2 (sex) × 2 (retention interval) repeated-measures ANOVA revealed a significant effect of session, $F_{(3,177)} = 31.0$, $p < 0.001$, $\eta_p^2 = 0.344$, and no other significant factors, indicating that extinction of any generalized fear to Context B proceeded equivalently in all four groups.

Tone retrieval/extinction

Mean percent freezing across sessions of tone retrieval/extinction can be seen in Figure 4A (Remote Groups) and Figure 4B (Recent Groups). Baseline levels of freezing were compared for each session in a 2 (sex) × 2 (retention interval) ANOVA. Freezing during the tone within each session was analyzed in separate 6 (5-trial blocks) × 2 (sex) × 2 (retention interval) repeated-measures ANOVAS.

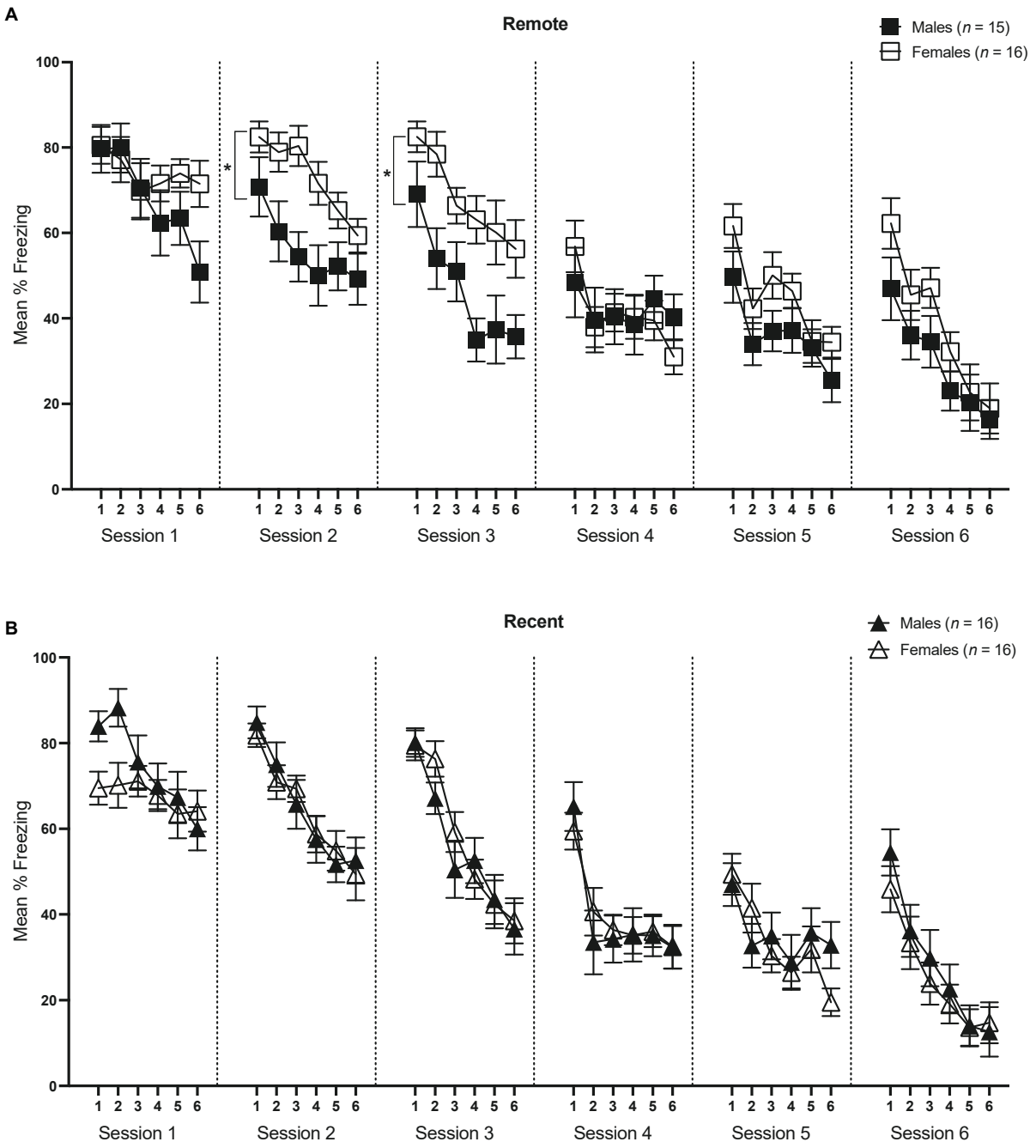

FIGURE 4
Mean percent freezing (± SEM) during the tone retrieval/extinction tests in the Remote [upper panel **(A)**] and Recent [lower panel **(B)**] groups across blocks of 5 trials during each session. *Indicates significant with $p < 0.05$.

For session 1, ANOVA revealed a significant effect of block [$F_{(5,295)} = 14.46$, $p < 0.001$, $\eta_p^2 = 0.197$] and significant block × sex interaction [$F_{(5,292)} = 5.30$, $p < 0.001$, $\eta_p^2 = 0.082$]. Importantly, there was no significant difference in the first 5-trial block between males and females [$F_{(1,59)} = 2.45$, $p = 0.124$], suggesting that initial tone-shock retrieval did not differ between the sexes. However, sexes did vary slightly at other points in the session—males appeared to freeze more in block 2, although this was marginally significant ($p = 0.050$), and females froze more in block 6 ($p = 0.032$). Of particular interest was the change in freezing rates across the session in both sexes. To address this, an ANOVA assessing freezing rates over blocks was conducted separately for males and females. These analyses indicated that while

males showed a significant decline in freezing across the session [$F_{(5,145)}$ = 22.01, p < 0.001, η_p^2 = 0.432], there was no significant change in levels of freezing in females [$F_{(5,150)}$ = 1.11, p = 0.358]. Furthermore, there were no main effects of sex [$F_{(1,59)}$ < 0.001, p = 0.985], retention interval [$F_{(1,59)}$ < 0.001, p = 0.996], or sex × retention interval interaction [$F_{(1,59)}$ = 2.28, p = 0.136]. Finally, freezing during the baseline period did not significantly differ between males and females or Recent and Remote groups (p's > 0.05). Overall, results from session 1 indicate that initial tone retrieval was not affected by sex or retention interval, but that as the session progressed, males began to exhibit initial extinction (reduction in freezing) of auditory fear, while females maintained continued high levels of freezing to the tone.

For session 2, the effects of block and sex were significant [$F_{(5,295)}$ = 34.09, p < 0.001, η_p^2 = 0.366; $F_{(1,59)}$ = 4.47, p = 0.039, η_p^2 = 0.07, respectively]. While the main effect of retention interval was not significant [$F_{(1,59)}$ = 0.003, p = 0.957], there was a significant sex × retention interval interaction [$F_{(1,59)}$ = 4.88, p = 0.031, η_p^2 = 0.076]. Follow-up pairwise comparisons revealed that females froze significantly more than males within the Remote condition [$F_{(1,59)}$ = 9.20, p = 0.004, η_p^2 = 0.135], whereas there was no difference in degree of freezing between males and females within the Recent condition (p = 0.946). There were no significant differences in baseline freezing levels (p's > 0.05). Thus, while freezing significantly declined across blocks in all groups, freezing was comparable between males and females in the Recent condition, but differed significantly between sexes in the remote condition, with females maintaining a higher level of freezing than males across the session.

The sex difference in the Remote condition that was observed in session 2 continued in session 3. There was a significant main effect of block [$F_{(5,295)}$ = 41.99, p < 0.001, η_p^2 = 0.416] as well as sex [$F_{(1,59)}$ = 8.48, p = 0.005, η_p^2 = 0.126]. The main effect of retention interval was not significant [$F_{(1,59)}$ = 0.09, p = 0.766], though there was a significant sex × retention interval interaction [$F_{(1,59)}$ = 5.44, p = 0.023, η_p^2 = 0.084]. As in session 2, in session 3 the Female Remote group froze significantly more than the Male Remote group [$F_{(1,59)}$ = 13.53, p = 0.001, η_p^2 = 0.186], and again, this effect was not seen in the recently conditioned groups [$F_{(1,59)}$ = 0.17, p = 0.681]. There were no significant differences in baseline freezing levels (p's > 0.05).

In session 4, there was a significant main effect of block [$F_{(5,295)}$ = 15.79, p < 0.001, η_p^2 = 0.211], suggesting that freezing declined across the session, though a lack of any other significant effects indicated that all groups reduced freezing comparably. Baseline responding was significantly higher in males than females [$F_{(1,59)}$ = 6.02, p = 0.017, η_p^2 = 0.093], though this difference did not appear in freezing during the tones.

Similarly, no significant differences were observed between groups in either session 5 or session 6. The only significant effect in each session was that of block [Session 5: $F_{(5,295)}$ = 16.48, p < 0.001, η_p^2 = 0.218; Session 6: $F_{(5,295)}$ = 30.08, p < 0.001, η_p^2 = 0.392]. There were no significant differences in baseline freezing in either session between groups (p's > 0.05). Together, these results suggest that freezing reliably declined across later sessions of extinction, though after session 3 the effects of sex and retention interval were no longer present.

In order to examine the progression of extinction across sessions between groups, we also examined mean percent freezing during tone presentations for each session, averaged over all 30 CS presentations [see **Figure 5A** (Remote groups) and **Figure 5B** (Recent groups)]. Average freezing during each session was analyzed with a 6 (Session) × 2 (sex) × 2 (retention interval) repeated-measures ANOVA.

There was a significant main effect of session [$F_{(5,295)}$ = 178.77, p < 0.001, η_p^2 = 0.059] as well as a significant sex × session interaction [$F_{(5,395)}$ = 3.67, p = 0.003], Furthermore, the sex × retention interval × session interaction approached significance [$F_{(5,295)}$ = 2.12, p = 0.063], which is consistent with the individual within-session analyses conducted previously: in session 2, the sex × retention interval interaction was significant [$F_{(1,59)}$ = 4.88, p = 0.031, η_p^2 = 0.076], and the same was true in session 3 [$F_{(1,59)}$ = 5.44, p = 0.023, η_p^2 = 0.084]. As previously described, the higher overall freezing observed in females is driven by the sex difference in the Remote condition, specifically.

There is some evidence that, in addition to freezing, rats exhibit a darting response to conditioned fear cues, and that female rats do so at higher rates than males. In order to assess darting, we manually scored the presence of rapid, escape-like movements across the conditioning chamber during the 10 s tone for all rats across all tone presentations during extinction/retrieval sessions. We observed a single instance of darting, exhibited by one Female Recent rat during session 1; no other instances were observed. Thus, there was no discernable difference between males and females in this measure in the current study.

General discussion

Understanding sex differences in fear conditioning is critically important for appropriate treatment of many psychopathologies that involve dysregulated fear learning, such as PTSD. The purpose of this study was to compare retrieval of auditory fear conditioning between male and female rats that were tested at either a recent (1 day) or remote (28 days) time-point. Our results are relevant to both initial tone retrieval and subsequent extinction. We observed no group differences in retrieval of auditory fear during the early portion of session 1 [see also Colon et al. (2018)]. In contrast, during sessions 2 and 3, we observed a marked sex difference in freezing during the tone, with females freezing more than males—but only in the

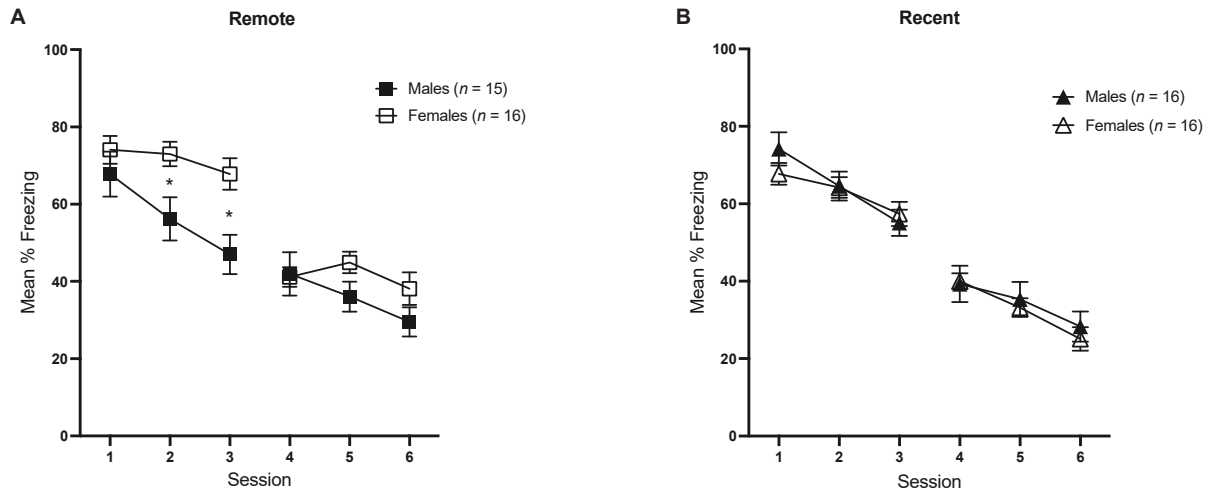

FIGURE 5
Overall mean percent freezing (±SEM) during sessions of tone retrieval/extinction in the Remote [**(A)** left] and Recent [**(B)** right] groups. *Indicates significant with $p < 0.05$.

Remote groups; no sex difference was observed in the Recent Groups.

During initial conditioning we observed group differences that were not anticipated: there was a small but significant difference in baseline freezing between Recent and Remote groups (Remote groups showed lower freezing at baseline), while Recent groups exhibited significantly lower freezing during the second tone than Remote groups. It should be noted that, while all testing for context and tone retrieval/extinction occurred when rats were the same age, conditioning occurred when Recent groups were a few weeks older than Remote groups. The effects of age on fear conditioning are most frequently attributed to conditioning that occurs in adolescence vs. adulthood (e.g., Toledo-Rodriguez and Sandi, 2007), and both the Recent and Remote groups were fully adult when conditioning took place, therefore the potential influence of age is not clear. In addition, conditioning occurred on different days for these groups, and there may have been environmental factors (e.g., noise or activity in the vivarium on the days around conditioning) that contributed to differences in freezing. We note that the difference observed between the Recent and Remote groups during the conditioning session was only present on trial 2, and that by trial 3 there were no differences in overall freezing levels. As noted, during sessions 2 and 3 of tone retrieval/extinction, we observed a sex difference in the Remote condition, and not the Recent condition. Because there were no sex differences observed during initial conditioning, it seems unlikely that the recent vs. remote difference observed during conditioning fully explains the results observed during later tone testing.

Our analysis of the first session of tone retrieval/extinction showed that initial retrieval did not differ based on sex or retention interval (see **Figures 4**, **5**). A study by Colon et al. (2018) tested tone retrieval in males vs. females either 1 or 14 days after auditory conditioning and found similar results: retrieval did not differ between sexes at either retention interval. Our findings are complementary and extend this research to suggest that sex differences do not emerge even at later time-points after conditioning (here, 28 days). Colon et al. (2018) acknowledged that freezing may have been at a ceiling in their study, making sex differences difficult to detect. This was not the case in the present study, as both sexes were at approximately 75% mean freezing at the onset of testing.

The primary finding from our study was that, despite initial retrieval being relatively equivalent between sexes, a dissociation emerged between recent and remote memories that was sensitive to sex: remotely conditioned females showed higher levels of freezing in sessions 2 and 3 relative to their male counterparts, while there was no difference between recently conditioned males and females throughout the entirety of tone extinction/retrieval. One possibility is that the excitatory tone-shock association was more strongly encoded/consolidated in females in the Remote group, resulting in stronger resistance to extinction in those rats. Indeed, there are several lines of research indicating sex differences in the neurobiological processes involved in the consolidation of fear memory (e.g., Devulapalli et al., 2021; Farrell et al., 2021; Florido et al., 2021; Crestani et al., 2022), largely focusing on protein signaling within the amygdala. Alternatively, it may be that the original fear memory was encoded similarly, but extinction proceeded differentially between the sexes. The results in the Female Remote group are consistent with prior studies that have shown that females exhibit slower extinction following cued fear

conditioning compared to males [e.g., Baran et al., 2009; Fenton et al., 2016; Greiner et al., 2019; but see Voulo and Parsons (2017)]. However, these studies used relatively short retention intervals (e.g., 1 day) and still observed this sex difference, whereas we did not see any differences in extinction between recently conditioned male and female groups [see also Gruene et al. (2015), Voulo and Parsons (2017), and Binette et al. (2022)]. Overall, our results are the first to a show sex-specific effect in extinction of remote fear memory.

One of the limitations of our study was that we did not see differential retrieval in the fear-conditioning context (A) vs. the neutral context (B). This was an unexpected result of this study: our laboratory has previously observed differential responding across contexts with other behavioral paradigms using very similar arrangements of contextual cues (e.g., Tavakkoli et al., 2020). Nevertheless, it is interesting to note two factors of the context test that we did observe. Firstly, we found that Remote groups showed higher freezing in the first half of the test than the Recent Groups, which is consistent with previous reports of stronger fear following longer retention intervals (e.g., Poulos et al., 2016). Greater freezing for the Remote groups could reflect incubation of fear, or it could reflect weaker conditioning in the Recent groups. Secondly, we did not detect any sex differences in context retrieval, which is consistent with some of the literature regarding context fear [but see, e.g., Maren et al. (1994), Poulos et al. (2015), and Russo and Parsons (2021)]. For example, Dachtler et al. (2011) observed similar freezing between male and female wild-type mice when they returned to the conditioning context after a 24-h retention period. Additionally, Kosten et al. (2006) observed equivalent freezing between male and female rats during a context test that occurred 24-h after tone-fear conditioning. Thus, it is not unprecedented that male and females show equal contextual fear.

Previous studies have indicated that female rats have a higher propensity than males to exhibit darting, an escape-like response to fear cues (Gruene et al., 2015; Colom-Lapetina et al., 2019; Mitchell et al., 2022). However, in the current study, this behavior was not present; only one instance of darting was observed throughout tone retrieval/extinction [see also Colon et al. (2018)]. It is possible that specific conditioning parameters are necessary to observe this behavior: darting behavior increases with more CS-US pairings during conditioning, initially emerging between 5 and 7 CS-US pairs (Gruene et al., 2015; Mitchell et al., 2022) and commonly when milder foot-shock US's are used (less than 1 mA; Mitchell et al., 2022). These differ from the parameters used in this study (3 CS-US pairings, 1 mA shock). Alternatively, unlike freezing, darting is not a conditioned behavior but rather occurs as a result of non-associative processes [see Trott et al. (2022)].

While our study was not adequately powered to investigate the role of gonadal hormones in the sex difference we observed [see also Voulo and Parsons (2017)], previous work has indicated that gonadal hormone state, such as phase of estrous or menstrual cycle, can influence multiple aspects of fear learning and memory [for reviews, see Dalla and Shors (2009), Maeng and Milad (2015), Ramikie and Ressler (2018), and Velasco et al. (2019)]. While low vs. high hormone state has previously been shown to have no effect on the acquisition of freezing to discrete cues (Milad et al., 2009; Carvalho et al., 2021), the literature is mixed as to how hormone state interacts with contextual fear learning; some reports indicate facilitated freezing to the context when estrogen and progesterone are high (e.g., Jasnow et al., 2006) where others show the opposite effect (e.g., Gupta et al., 2001) and still others show a lack of any effects of hormone state (e.g., Chang et al., 2009).

Relevant to our study were findings from a study by Milad et al. (2009), in which authors analyzed sex differences and the effect of relative hormone state (low vs. high) during extinction learning and extinction retention (performance in an extinction test 24 h after the initial test). These authors reported that sex differences only emerged when hormone state was taken into consideration. Importantly, they found that hormone state primarily influenced extinction recall rather than initial extinction learning: females in a high hormone state during the initial extinction learning showed lower freezing the following day (thus, better extinction recall) than those in the low hormone state, and hormone state on the extinction recall day did not appear to matter. Given these findings, it is important to note that hormone state may play a role in the sex difference in the extinction of remote fear observed in the current study, but due to the uneven distribution of females in different phases throughout the experiment, we were unable to detect any effect statistically.

In summary, the present experiment extends prior work investigating sex differences in recently vs. remotely acquired memory (Colon et al., 2018). Our results regarding initial retrieval were complementary to those previously reported: we saw no meaningful differences between males and females regardless of the age of the fear memory during the initial trials of tone retrieval. However, we did observe a sex difference following with the longer retention interval during extinction, with the Female Remote group retaining higher levels of freezing (and thus showing greater resistance to extinction) than the Male Remote group. There are a number of possible mechanisms for this difference that have yet to be empirically examined, including sex differences in fear memory consolidation as well as the role of hormone state. Overall, the present study suggests a dissociation between recent and remote memories which is at least in part sensitive to sex, and this may provide a basis for further research into sex-specific mechanisms of fear-related disorders.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by University of Vermont Institutional Animal Care and Use Committee.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Conflict of interest

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

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Age- and sex-specific effects of maternal separation on the acoustic startle reflex in rats: early baseline enhancement in females and blunted response to ambiguous threat

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Early life adversity (ELA) increases the incidence of later-life anxiety disorders. Dysregulated threat processing, including responsivity to ambiguous threats, is an indicator of anxiety disorders and can be influenced by childhood experiences. The acoustic startle response is a defensive reflex displayed by mammals when exposed to sudden intense stimuli reflecting individual variations in vigilance. These measures can be altered by previous experience and experimental modifications, including the introduction of unconditioned aversive stimuli. Rats emit ultrasonic vocalizations (USVs) in the 22 KHz range in negative contexts. As such, 22 KHz USVs are an ethologically relevant social cue of environmental threat shown to induce anxiety-like behavior in recipient rats. Because the timing of symptom manifestation after early life adversity can differ between sexes, the current study sought to identify the age- and sex-specific effects of daily maternal separation (MS) on responsivity to ambiguous threat in rats. In Experiment 1, rat pups underwent MS or control rearing from postnatal day (P) 2–20, then underwent behavioral testing beginning on P24, 34, or 54 to determine whether MS modified the baseline startle response or the modulation of startle by 22 KHz USVs. In Experiment 2, rats were tested in a light-enhanced startle paradigm at P54 after MS or control rearing to determine whether MS influenced light-enhanced startle. Results show an enhancement of the baseline startle magnitude by MS in females at P34. At P54, MS reduced the modulation of the startle response by 22 KHz USVs and prevented light-enhanced startle, indicating an MS-induced deficit in defensive responsivity when exposed to potential threat.

KEYWORDS

early life adversity (ELA), development, sex, rat, acoustic startle, ultrasonic vocalization (USV)

Introduction

Exposure to adversity early in life often leads to detrimental affective phenotypes that involve heightened responsivity to potential threats (Edmiston and Blackford, 2013; Silvers et al., 2016). Because early life adversity (ELA)-related disorders often first emerge in adolescence (Kessler et al., 2001; Andersen and Teicher, 2008; Davey et al., 2008), intervening variables found in clinical studies make the role that ELA plays in these diseases difficult to interpret. Moreover, the challenging nature of developmental studies has mired the ability to uncover the mechanisms underpinning discernible sex differences in ELA-attributable vulnerability. In fact, ELA yields an earlier age of onset for anxiety and depression, with more robust effects in girls than in boys (Marcus et al., 2005; Rudolph and Flynn, 2007; Schuch et al., 2014; Weeks et al., 2014).

The acoustic startle reflex is an evolutionarily conserved defensive behavioral response to sudden and intense stimuli (Koch and Schnitzler, 1997). In rodents, the reflex is demonstrated by a contraction of the head and neck muscles, measurable by the latency to onset, latency to peak response, magnitude, and duration. Exaggerated responses on these outcome measures are indicative of increased arousal and hypervigilance. ELA has been associated with either enhanced or blunted startle responses depending on whether outcomes were measured in a neutral context or with emotionally-valenced stimuli (Jovanovic et al., 2009; Klauke et al., 2012; Quevedo et al., 2015). Heightened startle at baseline, a measure of physiological arousal associated with trait anxiety (Beck and Catuzzi, 2013), is found in adults with a history of childhood trauma (Jovanovic et al., 2009; Klauke et al., 2012). We know of no studies to date that directly investigated sex differences in acoustic startle following ELA, however, there are mixed reports of sex differences in post-traumatic stress disorder-related acoustic startle (Beck and Catuzzi, 2013), likely due to wide varieties in the type of trauma exposure.

Affective picture viewing studies in humans have demonstrated that the startle reflex is modulated by emotional valence, such that, relative to neutral pictures, startle is potentiated when viewing unpleasant pictures that depict a possible threat and attenuated when viewing pleasant pictures (Lang et al., 1990). Potentiated startle in the presence of uncertain threat is a core feature of several internalizing psychopathologies (Gorka et al., 2017), and is a reliable measure of corticolimbic-driven threat sensitivity in both rodents and humans (Davis et al., 2010; Thome et al., 2018). In humans, viewing or anticipating images of fearful faces, in particular, has been shown to induce potentiation of the startle reflex, whereas neutral and happy faces do not facilitate the same response (Anokhin and Golosheykin, 2010). Compared to other affective pictures, facial expressions produce a greater autonomic response and more pronounced activation of brain regions involved in processing emotionally-valenced stimuli, like the amygdala (Hariri et al., 2002). This response to fearful faces is positively correlated with the subjective experience of anxiety, indicating that the degree to which startle is enhanced reflects changing emotional states. However, adults who experienced ELA display a diminished startle response during anticipation of such aversive stimuli (Stout et al., 2021). Suppressed potentiation of startle can result from coordinated efforts of peripheral and central inhibitory mechanisms that control threat responsivity and develop differently after ELA, namely pro-inflammatory immune signaling (Beck and Catuzzi, 2013; Brenhouse, 2022), Hypothalamic-pituitary-adrenal (HPA)-derived glucocorticoid signaling (Lee et al., 1994), and corticolimbic regulation between the amygdala and anterior cingulate (Pissiota et al., 2003).

After experiencing ELA, the onset of psychiatric disturbance is protracted (Kessler et al., 2001; Andersen and Teicher, 2008; Davey et al., 2008), and symptom onset may differ between males and females. This is particularly relevant during the adolescent transition, which is characterized by increased reactivity, risk-taking, and emotional sensitivity (Spear, 2000). It is therefore important to track the development of symptomatology to identify sex-specific vulnerabilities and windows for intervention. In humans without ELA exposure, there is evidence that startle potentiation by aversive stimuli emerges during adolescence, but this trajectory could be offset by early childhood neglect (Quevedo et al., 2015). Few studies in humans have directly compared individuals on the basis of sex, but differing patterns of results have been found between studies examining gender-homogenous populations (Medina et al., 2001; Costanzo et al., 2016). This demonstrates the need to investigate sex-specific effects of ELA on potentiated startle, and further, to directly compare males and females.

Traditional paradigms in animals to assess potential threat responsivity have limitations (Molendijk and de Kloet, 2015; Demin et al., 2019), due to the lack of translatability or relevance to human experience. Importantly, childhood maltreatment-evoked anxiety in humans largely involves sensitivity to social threats (Sandre et al., 2018), as measured in fearful-face paradigms (Dannlowski et al., 2012). Here, we assessed ELA-associated changes in responsiveness to a socially relevant, emotionally-valenced stimulus in rats (Demaestri et al., 2019). Specifically, responsivity to recordings of threatening ultrasonic vocalizations (USV) emitted by a conspecific was used to determine sex-specific changes to threat sensitivity in ELA-exposed rats. USVs in the 22 KHz range are typically emitted by rats as early as juvenility when in anxiety- or fear-provoking situations, while 55 KHz range USVs are typically emitted in appetitive situations (Burgdorf et al., 2008; Wöhr et al., 2016). We have previously shown that 22 Hz USV presentation can elicit anxiety-like

and defensive behaviors while activating neurons in regions underlying threat detection and responsiveness, such as the basolateral amygdala (BLA) and bed nucleus of the stria terminalis (BNST; Demaestri et al., 2019). Similarly, others have found enhanced startle magnitude in response to 22 KHz USV playback, but these experiments were only carried out in male rats (Inagaki and Ushida, 2017). Moreover, the effects of ELA on the startle response during exposure to 22 KHz USVs have not been investigated in other ages. A widely used assessment of startle modulation in anxiogenic contexts is the light-enhanced startle test (de Jongh et al., 2002). Sustained exposure to a bright light, a non-social aversive stimulus, enhances the startle response (Davis et al., 1997; de Jongh et al., 2002), but ELA was found to prevent light-enhanced startle in adult female, but not male, rats (de Jongh et al., 2005). Because the current study sought to determine the effects of ELA on startle in response to 22 KHz USVs, we also conducted a light-enhanced startle experiment in order to obviate potential unknown sex-specific responses to USV stimuli.

Maternal separation (MS) is an ELA paradigm in which pups are separated from their dam and littermates for several hours per day from postnatal day (P) 2–20. MS has been shown to disrupt the mother-infant relationship and is an experimental model for the study of childhood neglect (Lehmann and Feldon, 2000; Walker and McCormick, 2009; Brenhouse and Bath, 2019). MS has been described as an environment of deprivation and, to a lesser extent, threat (McLaughlin et al., 2014), and we have recently reported that MS confers an unpredictable environment as well (manuscript under review). Adversity within the dimensions of threat and unpredictability has particularly been associated with anxiety-like behaviors due to functional and developmental impairments of connectivity between regions such as the BLA, BNST, and prefrontal cortex (PFC; Tottenham et al., 2010; Hein and Monk, 2017; Spadoni et al., 2022). With a developmental study in males and females, we have observed that MS yields hyperinnervation from the basolateral amygdala to the prefrontal cortex, with females, in particular, displaying this hyperinnervation earlier in development (as early as P25), along with disrupted maturation of BLA-medial PFC functional connectivity (Honeycutt et al., 2020). Therefore, we hypothesized that if MS disrupts the development of threat responsive circuits that regulate acoustic startle, with females impacted earlier than males, then MS exposure will enhance baseline threat sensitivity earlier in females. We further hypothesized that if the circuitry controlling affective regulation of startle is disrupted following MS, MS will blunt a typical potentiation of startle in the presence of 22 KHz USV playback or light, with an exploratory investigation of when during development effects on USV potentiation would emerge.

Methods

Animals

All experiments were performed in accordance with the 1996 Guide for the Care and Use of Laboratory Animals (NIH) with approval from the Institutional Animal Care and Use Committee at Northeastern University. Animals were housed under standard laboratory conditions in polycarbonate wire-top caged with pine shave bedding, a plexiglass tube for enrichment, and food and water available *ad libitum*. The facility was kept on a 12-h light/dark cycle (light period between 07:00 and 19:00) with regulated temperature (22–23°C) and humidity (37%–53%). Throughout all procedures, animals were left undisturbed except for weekly cage cleanings and to take pup weights on postnatal day (P) 9 and P20.

Maternal separation

Male and female Sprague-Dawley rats originally obtained from Charles River Laboratories (Wilmington, MA) were mated in-house. One male and one female were caged together until pregnancy was confirmed by checking for the presence of sperm in a vaginal swab each morning for a maximum of 4 days. All females were primiparous, and pregnant dams were housed singly upon confirmation of pregnancy. Parturition was checked daily, and the day of birth was denoted as P0. Each litter was randomly assigned to maternal separation (MS) or control (Con) treatment conditions. On P1, litters were culled to 10 pups with five males and five females when possible. Pup sex was determined by anogenital distance on P1.

MS litters underwent separations daily from P2 to P20. From P2 to P10, each pup was placed in an individual plastic cup containing pine shavings from the home cage to maintain a familiar odor. Cups were placed in a circulating water bath kept at 37°C for 3.5 h (09:30–13:0 h). From P11 to P20, when pups are able to adequately thermoregulate, each pup was placed in an individual mouse cage containing clean bedding mixed with a small handful of home cage bedding for 4 h (09:30–13:30). MS dams remained in the home cage in a separate room for the duration of the separation.

Subjects were identified by toe clips, which were performed on P5. Con litters were left undisturbed except for normal husbandry procedures, including toe clips on P5 and weights on P9 and P20. We have previously reported (Grassi-Oliveira et al., 2016), and consistently observe, that MS animals gain less weight during the MS paradigm than Con animals but that MS and Con animals are equal in weight at P25, P35, and P55. To control for the experimenter interaction received

by MS pups, Con pups were also briefly handled by an experimenter on P12 and P15 for 3 min each. On P21, all rats were weaned and pair-housed in standard caging with sex- and condition-matched cage mates. No more than two male and two females per litter were used in each experimental group.

Acoustic startle test

Experiments 1 and 2 used the acoustic startle hardware and software package from Med Associates (Med Associates product number: MED-ASR-PRO1). The startle cabinets were equipped with sound attenuating foam on all walls and doors. Each cabinet held a grid rod animal holder on a startle platform containing the load cell. The load cell and load cell amplifier were used to convert force on the platform to a voltage representing the startle response. Speakers for delivering white noise background and startle noise bursts were positioned 1" behind the animal holder. The grids rods on the back of the animal holder provide ventilation and do not interfere with sounds. Four different chambers were counterbalanced between sexes and rearing groups.

Experiment 1a: effects of MS on development of the acoustic startle response

Experimental design is illustrated in **Figure 1**. Con and MS rats were tested beginning on P24 (10 Con males, 11 MS males, 10 Con females, 10 MS females), P34 (11 Con males, nine MS males, 10 Con females, 10 MS females), or P54 (eight Con males, 11 MS males, 10 Con females, 10 MS females) in a 2-day paradigm. Different animals were used for each age, so no animal was tested more than once. Because the rats' weights differ between ages and sexes, the startle boxes were calibrated to accommodate the weight of each rat being tested. The purpose of Day 1 was to establish a baseline startle response for each animal. On Day 1, rats were transported to the testing room (∼50 lux) and left to acclimate for 10 min. After acclimation, each rat was placed into the animal holder in the unlit startle cabinet. The experiment began with 5 min of white background noise to acclimate the animal to the startle cabinet. Then, 100 stimulus tones were presented at 30-s intervals. Each stimulus was a 50 ms white noise tone of 95 dB, 100 dB, 105 dB, or 110 dB presented in random order with a 3 ms rise/fall time. The background noise level was set to 70 dB throughout the experiment. At the end of 100 trials, rats were removed from the boxes and returned to their home cages. Boxes were cleaned with 40% EtOH between runs.

Experiment 1b: effects of MS on 22 KHz USV-modulated acoustic startle

The purpose of Day 2 was to determine the rats' response to startle stimulus tones after being exposed to a 22 KHz USV, a social cue signifying potential threat. Twenty-four hours after the Day 1 test, rats were transported to the testing room and left to acclimate for 10 min. Similar to Day 1, rats were placed in the animal holders, and the experiment began with a 5-min acclimation period. Then, rats were presented with a 5-min 22 KHz USV recording previously obtained from an adult male rat exposed to cat urine. The USV was recorded using a condenser ultrasonic microphone (Avisoft-Bioacoustics CM16/CMPA; frequency range 2–200 KHz), and played back using a D/A converter/power amplifier and ultrasonic speaker (Avisoft-Bioacoustics USG Player 416H, Vifa speakers) situated inside the startle cabinet 1" behind the animal holder. After the USV playback, rats were presented with 30 stimulus tones at 30-s intervals. Stimulus tones were 50 ms in duration, 95 dB, 100 dB, 105 dB, or 110 dB in volume, and presented in random order with a 3 ms rise/fall time. At the end of Day 2, rats were removed from the boxes and returned to their home cages.

Experiment 2: effects of MS on light-enhanced acoustic startle

After observing the effects of USV in the first cohort of P55 animals, we aimed to test responses to a different anxiogenic context. Con and MS rats (11 Con males, 10 MS males, 10 Con females, nine MS females) were tested beginning on P54 in a 2-day light-enhanced startle paradigm. Startle boxes were calibrated to accommodate the weight of each rat. On Day 1, rats were transported to the testing room and allowed 10 min of acclimation to the room. After the acclimation period, each rat was placed in the calibrated animal holder in the startle cabinet. The experiment began with a 5-min acclimation followed by 30 stimulus tones presented at 30-s intervals. Each tone was a 50 ms 105 dB white noise burst with a 3 ms rise/fall time. Because the baseline data from Experiment 1 separated by stimulus intensity showed that the 95 dB stimulus did not reliably generate a strong startle response (data not shown), and to avoid ceiling effects at 110 dB, Experiment 2 presented stimulus tones only at 105 dB. The background noise level was set to 70 dB throughout the experiment. At the end of the 30 trials, rats were removed from the boxes and returned to their home cages. Boxes were cleaned with 40% EtOH between runs.

Day 2 of the experiment aimed to determine the rats' response to stimulus tones while exposed to light, a naturally aversive stimulus. Notably, the illumination was chosen for moderate intensity in order to provide a diffusely, rather than

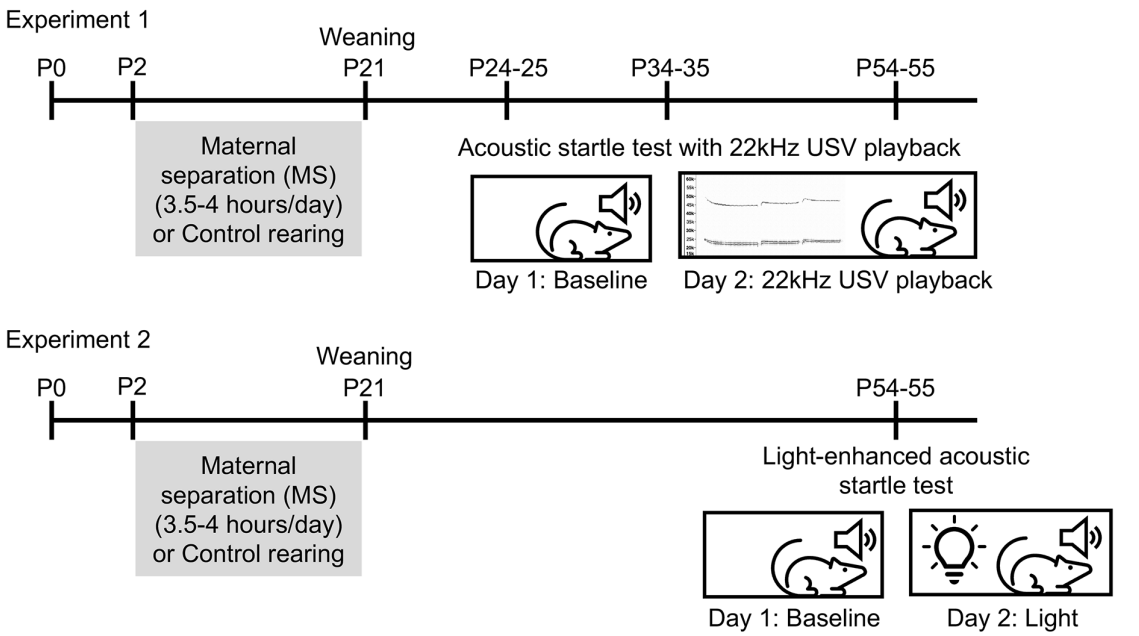

FIGURE 1
Experimental timelines. In Experiment 1, rats underwent maternal separation (MS) or control rearing from P2 to 20 until weaning on P21. Separate cohorts of rats underwent the acoustic startle test with 22 KHz USV playback on either P24–25, P34–35, or P54–55. In Experiment 2, rats underwent MS from P2 to 20 and were tested in light-enhanced startle on P54–55.

acutely, threatening environment. Twenty-four hours after the Day 1 test, rats were transported to the experimentation room and allowed to acclimate for 10 min. Rats were then placed in the calibrated animal holder in the startle cabinet. A wireless LED puck light located 4 inches behind the animal holder inside each startle cabinet was turned on before the start of the experiment (∼70 lux). Rats underwent the same experimental protocol as Day 1 (5-min acclimation to background white noise followed by 30 105 dB stimulus tones). At the end of Day 2, rats were returned to their home cages.

TABLE 1 Descriptions of outcome measures from the acoustic startle test.

Dependent variable	Definition
Latency to onset (ms)	Time between white noise burst onset and response onset
Latency to peak (ms)	Time between white noise burst onset and response peak
Peak value (arbitrary units)	Greatest startle amplitude in the response window
Duration (ms)	Time between response onset and end of response

Statistical analysis

Outcome measures represent the average across all trials on Day 1 or Day 2 for each subject. For baseline measures, we report the latency to onset (ms), latency to peak (ms), peak startle value (arbitrary units), and duration (ms), described in Table 1. Total and average startle magnitudes during the startle period were also analyzed, but data are not shown because results were redundant to the results of the peak value. The response to USV or light presentation was represented by the subject's Day 2 value as a percentage of the Day 1 value for each outcome measure. Statistical tests were performed in jamovi software (The jamovi project, Version 1.6). To determine the effects of MS and sex on the baseline startle response and the percent change from baseline after USV or light presentation, two-way ANOVAs were performed for each outcome measure. Effects were considered significant when $p < 0.05$. When there was a significant interaction between rearing condition and sex, Tukey's *post hoc* comparisons were performed to determine group differences between males and females.

Results

Experiment 1a: effects of MS on the baseline startle response on P24, 34, and 54

All statistics for experiment 1a can be found in Supplementary Table 1. In P24 animals at baseline, there were no effects of sex, rearing, or sex by rearing

interaction on the latency to onset, peak value, or duration (Figures 2A,C,D). Females had a shorter latency to peak than males ($F_{(1,37)} = 6.1045$, $p = 0.018$, $\eta_p^2 = 0.142$), but there were no effects of rearing or sex by rearing interaction on latency to peak (Figure 2B).

At P34, there were no effects of sex, rearing, or sex by rearing interaction on latency to onset, latency to peak, or duration (Figures 2E,F,H). The magnitude of the startle response was greater in MS animals, demonstrated by increased peak value ($F_{(1,36)} = 12.476$, $p = 0.001$, $\eta_p^2 = 0.257$), and there was a sex by rearing interaction ($F_{(1,36)} = 6.163$, $p = 0.018$, $\eta_p^2 = 0.146$; Figure 2G). *Post hoc* comparisons revealed significant increases in peak startle magnitude in MS females compared to Con females ($t = -4.264$, $p < 0.001$, $d = -1.907$), and no difference in males ($t = -0.74$, $p = 0.88$, $d = -0.333$).

A sex difference was observed at P54, with males startling at a greater magnitude and for longer than females (peak value: $F_{(1,35)} = 9.394$, $p = 0.004$, $\eta_p^2 = 0.212$: duration: $F_{(1,35)} = 23.1727$, $p < 0.017$, $\eta_p^2 = 0.398$; Figures 2K,L). There were no main effects of rearing condition on any startle outcome measure. However, there was a sex by rearing interaction on latency to onset ($F_{(1,35)} = 5.734$, $p = 0.022$, $\eta_p^2 = 0.141$; Figure 2I) and latency to peak ($F_{(1,35)} = 5.304$, $p < 0.027$, $\eta_p^2 = 0.132$; Figure 2J). *Post hoc* comparisons revealed that Con males had a shorter latency to onset than Con females ($t = -2.883$, $p = 0.032$, $d = -1.367$), but MS males and females did not differ ($t = 0.405$, $p < 0.977$, $d = 0.177$). No group-wise differences were found in the latency to peak.

Experiment 1b: effects of MS on 22 KHz USV-modulated startle

All statistics for experiment 1b can be found in Supplementary Table 2. At P25, there was a main effect of sex on the change in the latency to onset, with males decreasing their latency to onset more than females ($F_{(1,37)} = 4.66$, $p = 0.037$, $\eta_p^2 = 0.112$; Figure 3A), but there was no effect of rearing or sex by rearing interaction. There was a main effect of rearing on the change in latency to peak, with MS animals displaying a decrease in latency to peak compared to Con animals ($F_{(1,37)} = 7.01$, $p = 0.012$, $\eta_p^2 = 0.163$; Figure 3B). There was no effect of sex, rearing, or sex by rearing interactions in the change in peak value or duration after USV presentation (Figures 3C,D). At P35, the change in startle response from baseline after USV presentation was not affected by sex or rearing on any outcome measure (Figures 3E–H). At P55, MS reduced the change in startle magnitude from baseline after USV presentation, blunting the enhancement of startle by USV presentation (%Δ peak value: $F_{(1,35)} = 4.127$, $p = 0.050$, $\eta_p^2 = 0.132$; Figure 3K). Additionally, females displayed reduced change from baseline compared to males (%Δ peak value: $F_{(1,35)} = 7.469$, $p = 0.010$, $\eta_p^2 = 0.176$). There was no sex by rearing interaction in peak value change. There were no rearing, sex, or rearing by sex interactions in the change in latency to onset, latency to peak, or duration at P55 (Figures 3I,J,L).

Experiment 2: effects of MS on P55 light-enhanced startle

All statistics for Experiment 2 can be found in Supplementary Tables 3, 4. At baseline, there were no effects of sex, rearing, or sex by rearing interactions on the latency to onset, latency to peak, or peak value (Figures 4A–C). However, the duration of startle in females was lower than in males (main effect of sex: $F_{(1,36)} = 51.801$, $p < 0.001$, $\eta_p^2 = 0.590$), and duration was also shorter in MS animals compared to Con (main effect of rearing: $F_{(1,36)} = 5.025$, $p = 0.031$, $\eta_p^2 = 0.122$; Figure 4D). In the light-enhanced experiment, there were no sex- or rearing-dependent differences in the change from baseline on any startle measure (Figures 4E–H). In order to confirm replication of previously reported light-enhanced startle using brighter illumination, we performed a repeated measures ANOVA (full results in Supplementary Table 5) with between-subjects factors of sex, and rearing, and peak values from the no-light test (baseline) and the light test as repeated measures to determine whether there was an effect of light exposure. Results show a main effect of light exposure ($F_{(1,36)} = 6.096$, $p = 0.018$, $\eta_p^2 = 0.145$) and a light by rearing interaction ($F_{(1,36)} = 4.943$, $p = 0.033$, $\eta_p^2 = 0.121$). In Con animals only, there was a difference between the no-light test and the lighted test (Tukey's *post hoc*, Con/no light vs. Con/light: $t = -3.405$, $p = 0.009$).

Discussion

The purpose of this study was to establish sex-dependent effects of MS on the acoustic startle response over development and to characterize the effects of two distinct aversive sensory stimuli, 22 KHz USVs, and light exposure. As hypothesized, MS affected females earlier in development than males, enhancing the baseline response in young adolescent females. In older adolescents, MS changed the modulation of startle by 22 KHz USV playback and light exposure, preventing potentiation.

In Experiment 1a, we investigated age-dependent and sex-dependent effects of MS on the baseline startle response. In P24 animals, MS did not impact the baseline startle response, but females exhibited a shorter latency to peak than in males regardless of rearing condition, which aligns with another study that showed shorter latencies in P25 female rats (Boctor and Ferguson, 2009). Latency is typically negatively correlated with startle amplitude, increasing amplitude with shorter latencies (Pilz and Schnitzler, 1996), but we did not observe a sex difference in peak startle amplitude at this age. After 22 KHz

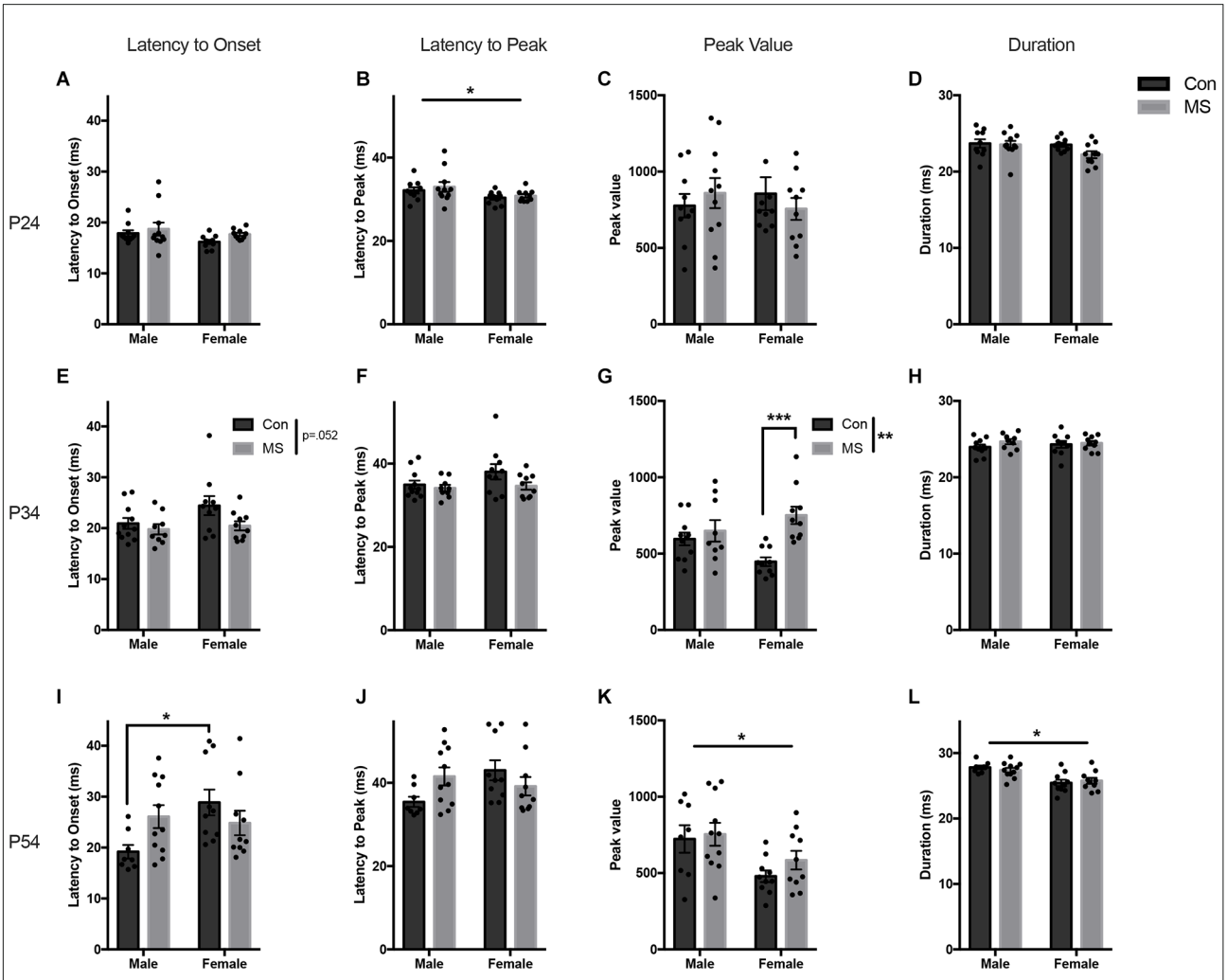

FIGURE 2
Results from Experiment 1a, the effects of maternal separation (MS) on the baseline startle response. Graphs depict the latency to onset (ms), latency to peak (ms), peak value (arbitrary units), and duration (ms) on P24 **(A–D)**, P34 **(E–H)**, and P54 **(I–L)**. Control-reared groups are depicted by dark gray bars and MS-reared groups are depicted by light gray bars. Figures represent the mean group values ±SEM. Individual subjects are depicted by data points. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

USV playback on P25, latency to peak was shortened compared to baseline in Con animals, and MS further exaggerated this change from baseline. Shorter latency to peak generally reflects increased alertness and is associated with other behaviors congruent with hypervigilance (Lebow et al., 2012). Interestingly, at this age, the MS-related change in responsivity became apparent only after exposure to ambiguous threat. Latency to peak includes the time from the onset of the acoustic pulse to the peak of the motor reaction. Therefore, the measure includes both the neural delay encompassing the delay to startle, and the mechanical delay between the onset and peak of the startle. Latency to startle onset decreases with age and continues to mature through the third postnatal week of life. In weanling rats, louder stimuli begin to elicit shorter latencies to respond, demonstrating maturation of the ventral cochlear nucleus (Sheets et al., 1988). As startle delay is dependent on the three-synaptic startle reflex arc between the cochlear nucleus, caudal pontine reticular nucleus (PnC), and motor nuclei (Koch, 1999), and startle duration (time from startle onset to peak) relies on motor and neuromuscular activity (Sipos et al., 2001), it is possible that early sex differences in the baseline response may reflect sex differences in the maturation of the brainstem circuitry, downstream motor pathways, or musculature.

The effects of MS on the baseline startle response emerged in early adolescents on P34, with females displaying elevated peak value if they had undergone MS. Heightened acoustic startle at baseline is reflective of hypervigilance and increased arousal. This enhanced responsiveness is phenotypic of patients with panic disorder, PTSD, and obsessive-compulsive disorder

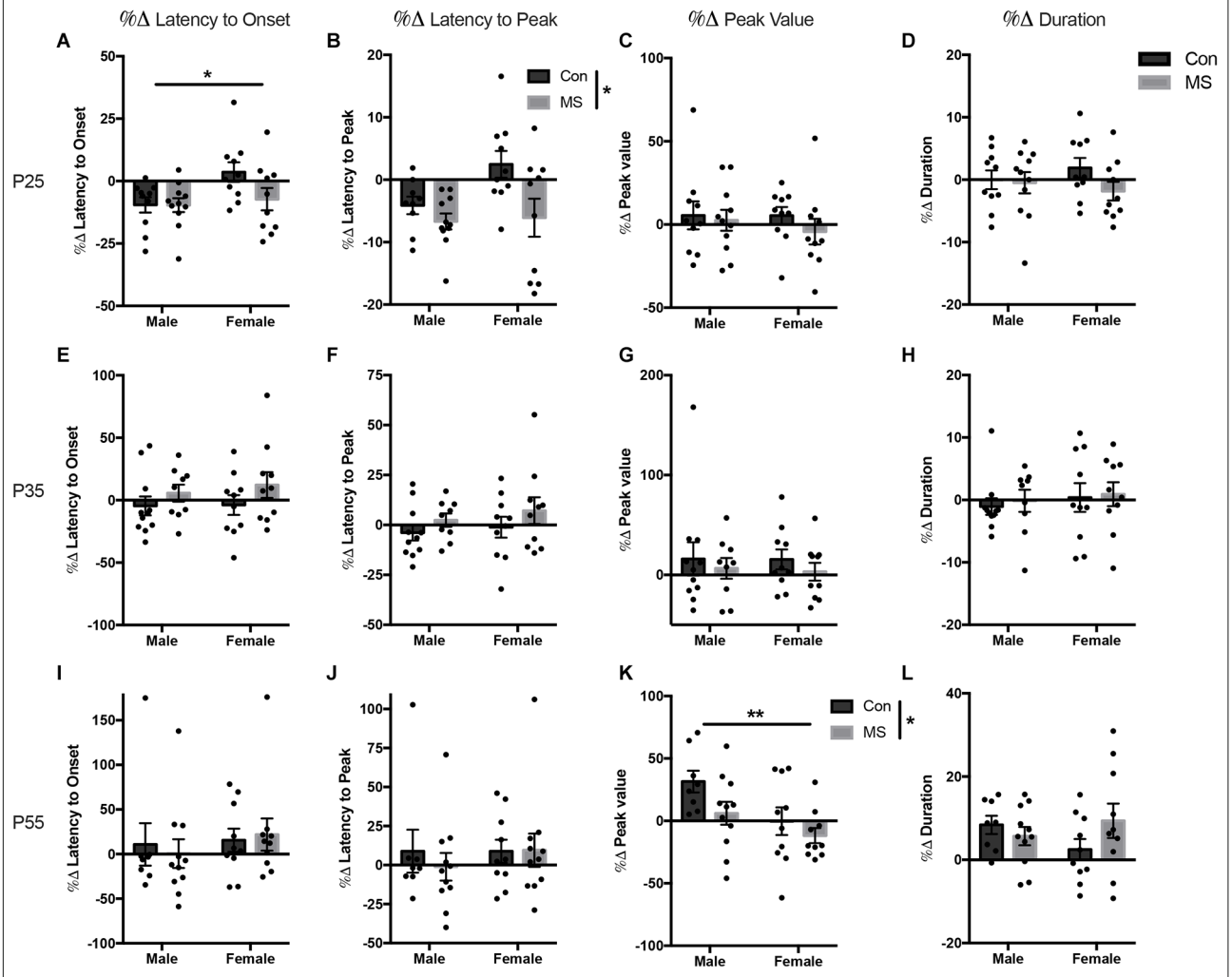

FIGURE 3
Results from Experiment 1b, the effects of maternal separation (MS) on the change in startle response from baseline after exposure to 22 KHz USVs. Graphs depict the change in each measure as a percentage of the value from the baseline test. Percent change from baseline in latency to onset, latency to peak, peak value, and duration on P24 **(A–D)**, P34 **(E–H)**, and P54 **(I–L)**. Control-reared groups are depicted by dark gray bars and MS-reared groups are depicted by light gray bars. Figures represent the mean group values ±SEM. Individual subjects are depicted by data points. *$p < 0.05$, **$p < 0.01$.

(Grillon et al., 1994; Morgan et al., 1995; Kumari et al., 2001). As such, the baseline acoustic startle response is considered an operationalization of hypervigilance observable in a range of anxiety disorders (Seiglie et al., 2019) and is a sensitive measure of individual differences in reactivity. Human research has found that adolescents at high-risk for anxiety disorders due to parental history of such conditions showed gender-specific changes in baseline and fear-potentiated startle (Grillon et al., 1997, 1998). Girls, but not boys, in the high-risk group had a heightened startle response at baseline compared to low-risk girls, but no differences in their state of anxiety were found at the time of testing (Grillon et al., 1998). Therefore, increased acoustic startle at baseline may be an early indicator of premorbid vulnerability to anxiety disorders, and the gender difference may reflect unique developmental susceptibilities to generational anxiety. In rats, the sex-specific effect of MS on the startle response in adolescents has not been extensively studied. Previous studies have found adolescent females to be more susceptible to anxiety-like behavior after MS than male rats using other assays measuring defensiveness and hypervigilance (Jin et al., 2018; Tschetter et al., 2021). Similar effects have been seen in depressive-like behavior, specifically an earlier onset of depressive-like behavior after early life adversity in female adolescents compared to males (Goodwill et al., 2019). Notably, since female rats' pubertal maturation occurs earlier than males (Grassi-Oliveira et al., 2016) and we did not assess pubertal status in this study, there may have been more females than in males who had initiated puberty by P34. Therefore

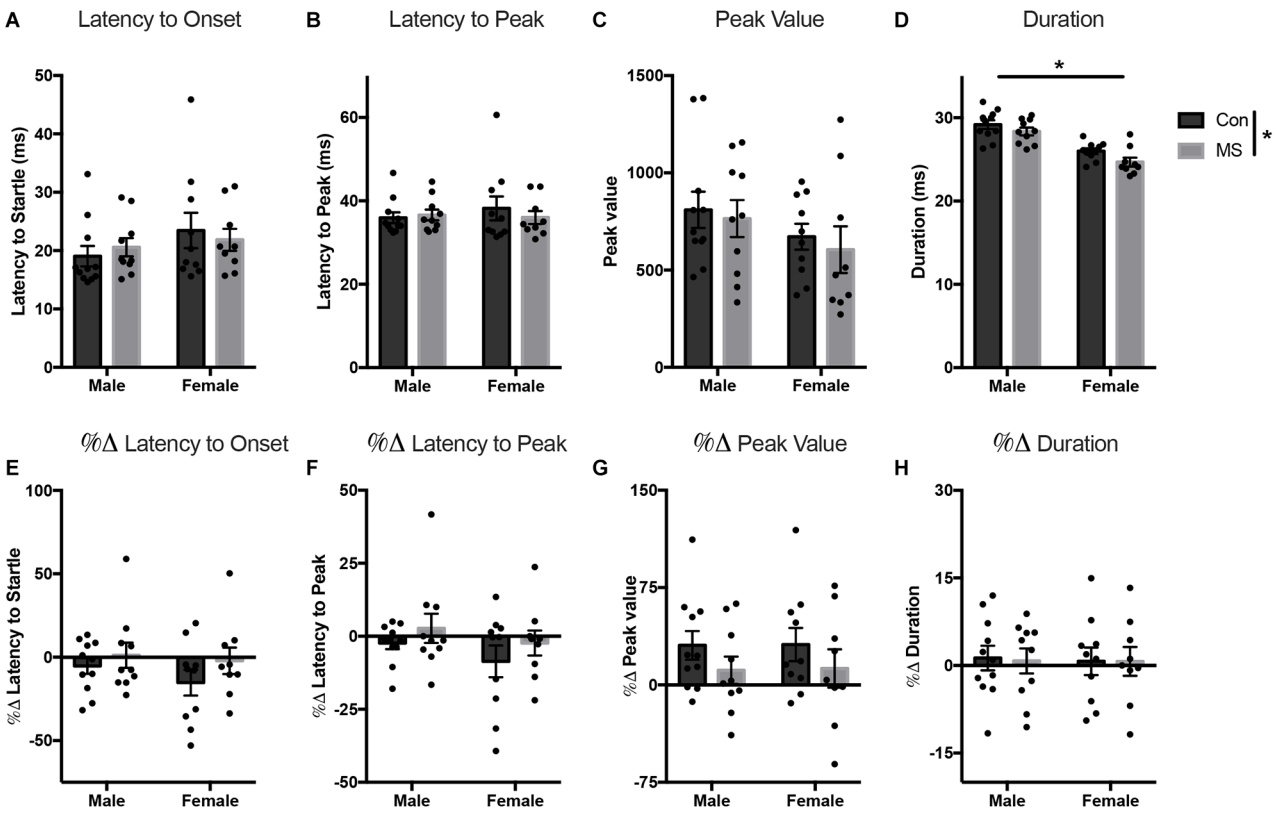

FIGURE 4
Results from Experiment 2. Effects of MS on baseline and light-enhanced startle on P54–55. Graphs depict the latency to onset (ms) **(A)**, latency to peak (ms) **(B)**, peak value (arbitrary units) **(C)**, and duration (ms) **(D)** at baseline and the percent change from baseline in latency to onset **(E)**, latency to peak **(F)**, peak value **(G)**, and duration **(H)** during exposure to light. Control-reared groups are depicted by dark gray bars and MS-reared groups are depicted by light gray bars. Figures represent the mean group values ±SEM. Individual subjects are depicted by data points. *$p < 0.05$.

pubertal hormones may be a factor in the observed sex-specific effects of MS.

MS-induced startle enhancement in young adolescent females may be driven by sex-specific changes in underlying circuits sensitive to MS. The baseline startle response is mediated by Corticotropin-releasing factor (CRF) receptors in the brain, and CRF-enhanced startle is dependent on the BNST (Lee and Davis, 1997). MS has been shown to exert changes in CRF mRNA expression throughout the brain, including the central amygdala (CeA), BNST, medial PFC, locus coeruleus, and paraventricular nucleus of the hypothalamus (Plotsky and Meaney, 1993; Plotsky et al., 2005), all regions involved in the defensive response (LeDoux and Pine, 2016). Although adolescent-specific alterations are less extensively studied, one study found increased CRF mRNA in the PVN of adolescent female rats after MS, but no changes in the BNST or CeA, and no changes in males (Tschetter et al., 2021). Female adolescents exposed to MS demonstrate earlier manifestation of excitatory/inhibitory imbalances in the PFC, driven by reduced expression of GABAergic markers and hyperinnervation from the amygdala (Honeycutt et al., 2020; Ohta et al., 2020). Previous work from our lab has also identified accelerated maturation of PFC innervation from the BLA in females, and females sustained this hyperinnervation through later adolescence (Honeycutt et al., 2020). These may play a role in the female-specific enhancement of acoustic startle after MS.

In P54 animals, there was no effect of MS on baseline startle, but males had a greater startle magnitude and a longer response than females. It is typical for male rodents to have a larger startle response than females, likely due to their higher body weight, muscle mass, and motor strength (Lehmann et al., 1999; Plappert et al., 2005). In response to the 22 KHz USV, MS decreased the degree to which the peak value was changed from baseline, and females had a smaller change in response than males. Con males demonstrated startle enhancement after USV playback, while females did not clearly show a potentiation or inhibition. Overall, enhancement of the startle response to 22 KHz USVs was attenuated by MS.

Aversive USVs have been used to elicit anxiogenic responses in both male and female adults (Demaestri et al., 2019; Wöhr et al., 2020). Twenty-two KHz calls are emitted during social

aggression, fear learning with foot shock, cocaine and opiate withdrawal, and predator exposure (Brudzynski, 2005). As a signal of potential danger in the environment, they can elicit either behavioral inhibition or hyperlocomotion in the recipient depending on the context of the alarm call and the strain of the recipient (Beckett et al., 1997). Previous work has found increased anxiety-like behavior in the elevated zero maze and reduced locomotion when exposed to 22 KHz USVs (Demaestri et al., 2019), while others showed that alarm calls caused more escape behavior in the open field (Beckett et al., 1997; Neophytou et al., 2000). In these studies, behavioral inhibition and fleeing were both accompanied by increased c-fos activation in the basolateral amygdala. Activation of other subregions of the amygdala, thalamus, hypothalamus, and periaqueductal gray was concomitant with fleeing behavior after USV exposure (Beckett et al., 1997; Wöhr et al., 2020). Efferent pathways from these regions project to the PnC of the primary acoustic startle reflex circuit, which can enhance or inhibit the startle reflex through direct connections to motor nuclei (Davis et al., 1997; Koch, 1999; Walker et al., 2003). Startle inhibition is modulated by midbrain nuclei, including the substantia nigra reticulata, pedunculopontine tegmentum, and laterodorsal tegmental area, which receive inputs from the prefrontal cortex, amygdala, and BNST (Davis, 2006). The attenuation of the USV-modulated startle seen in the present study after MS may be caused by changes in the developmental programming of forebrain regions, directly and indirectly, affecting the startle circuit. The central amygdala has been implicated as a major modulator of classically conditioned fear-potentiated startle, while the BNST and BLA have both been shown to regulate light-enhanced startle (Beck and Catuzzi, 2013). It is not currently known whether the BLA-PFC pathway, which we have previously shown to be developmentally and sex-specifically affected by MS, underlies the attenuation of the USV-modulated startle, but these circuits will be important targets for future testing.

Importantly, nearly all of the USV playback research has been performed on male recipient rats with male rat stimulus vocalizations, despite the evidence that male and female rats respond differently to alarm calls, and their own vocalizations have different qualities and tendencies of emission (Boulanger-Bertolus and Mouly, 2021; Lenell et al., 2021). One study found reduced locomotion in male and female rats exposed to alarm calls, but females retained this behavioral inhibition even after the stimulus ended (Wöhr et al., 2020). In our paradigm, the USV playback occurs just before the startle stimulus trials began so the observed startle would not be in direct response to the USV itself. If females remain more behaviorally inhibited after USV exposure, this could explain the failure to potentiate in females.

In Experiment 2, Con animals were potentiated by exposure to light, but MS animals failed to enhance startle in this aversive context. A previous study similarly found that MS blunted light-enhanced startle, but this finding was exclusive to females (de Jongh et al., 2005). This differs from the present results, which do not demonstrate a sex-specific effect. Differences in the experimental paradigms, such as stimuli amplitude and time between testing sessions, could account for disparate findings. In the present study, startle stimuli were all 105 dB, as opposed to the 95–105 dB stimuli used in the previous study. Furthermore, the previous study used Wistar rats and an MS procedure from P2 to P14, while here we used Sprague Dawley rats and MS lasted from P2 to P20. Different strains respond to acoustic startle differently at baseline, in demonstration of prepulse inhibition, and in response to drug exposure (Buuse, 2004). Here, Experiment 1 provided the basis for using 105 dB stimuli to produce sufficient and consistent startle amplitudes, but strain differences could be attributable to distinct hearing capabilities and responses to specific stimuli, which should be considered when designing and comparing experiments.

Blunted startle potentiation in response to aversive stimuli is consistent with recent human studies showing that early childhood adversity caused reduced startle responses in negative contexts. One study found that men who experienced childhood trauma had weaker startle responses compared to men with no history of trauma specifically during anticipation of negative stimuli (Stout et al., 2021). In another study, women who suffered from PTSD due to childhood abuse demonstrated reduced startle in the presence of a danger cue, and this association was strongest in individuals with more severe clinical symptoms representing avoidance (Lis et al., 2020).

Conclusion

The present study found intriguing sex differences in the effects of MS on the acoustic startle response depending on age and type of aversive stimulus exposure. Females were affected by MS earlier than males, demonstrating a more exaggerated defensive response at baseline in early adolescence. Interestingly, 22 KHz USV-enhancement was only found in control males later in adolescence, although MS decreased the change in startle response from baseline after exposure to USVs regardless of sex. This differed from the light-enhanced experiment, in which Con animals displayed potentiation by light regardless of sex, but MS animals failed to potentiate in the presence of light. This demonstrates that males and females may respond differently to specific aversive stimuli, and different sensory modalities should be further explored. Overall, the present experiments demonstrate sex- and age-specific deficits in defensive responsiveness after MS, specifically by preventing ethologically relevant adaptive responses to potential threat.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Northeastern University IACUC.

Author contributions

LG, AP, and HB contributed to writing and editing of the manuscript. LG and HB designed the studies. LG and AP collected data for the studies. HB contributed funding for the study and supervised all stages of data collection, analysis, and interpretation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.1023513/full#supplementary-material.

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Ranking the contribution of behavioral measures comprising oxycodone self-administration to reinstatement of drug-seeking in male and female rats

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Introduction: Rates of relapse to drug use during abstinence are among the highest for opioid use disorder (OUD). In preclinical studies, reinstatement to drug-seeking has been extensively studied as a model of relapse—but the work has been primarily in males. We asked whether biological sex contributes to behaviors comprising self-administration of the prescription opioid oxycodone in rats, and we calculated the relative contribution of these behavioral measures to reinstatement in male and female rats.

Materials and methods: Rats were trained to self-administer oxycodone (8 days, training phase), after which we examined oxycodone self-administration behaviors for an additional 14 days under three conditions in male and female rats: short access (ShA, 1 h/d), long access (LgA, 6 h/d), and saline self-administration. All rats were then tested for cue-induced reinstatement of drug-seeking after a 14-d forced abstinence period. We quantified the # of infusions, front-loading of drug intake, non-reinforced lever pressing, inter-infusion intervals, escalation of intake, and reinstatement responding on the active lever.

Results: Both male and female rats in LgA and ShA conditions escalated oxycodone intake to a similar extent. However, males had higher levels of non-reinforced responding than females under LgA conditions, and females had greater levels of reinstatement responding than males. We then correlated each addiction-related measure listed above with reinstatement responding in males and females and ranked their respective relative contributions. Although the majority of behavioral measures associated with oxycodone self-administration did not show sex differences on their own, when analyzed

together using partial least squares regression, their relative contributions to reinstatement were sex-dependent. Front-loading behavior was calculated to have the highest relative contribution to reinstatement in both sexes, with long and short inter-infusion intervals having the second greatest contribution in females and males, respectively.

Discussion: Our results demonstrate sex differences in some oxycodone self-administration measures. More importantly, we demonstrate that a sex-dependent constellation of self-administration behaviors can predict the magnitude of reinstatement, which holds great promise for relapse prevention in people.

KEYWORDS

escalation, front-loading, female, vulnerability, opioid, relapse, sex difference

Introduction

Recent attention has focused on the dramatic increase in extensive abuse of prescription opioid drugs and heroin by women, which can lead to opioid use disorder (OUD) and even death through overdose. The 2020 "National Survey on Drug Use and Health: Women" reviewed sex/gender differences in prescription opioid medications and heroin use in the USA from 2015 to 2020 and found that the number of women using heroin is increasing at a faster rate than for men, even though non-medical prescription opioid drug misuse is declining for both sexes (SAMHSA, 2020). A consistent finding has been that the likelihood of substance abuse does not differ between men and women when controlling for access (McHugh et al., 2018). This suggests that vulnerability to initiate drug use is not a major source of sex differences in the addiction cycle. Rather, sex differences in high resolution patterns of opioid use and how these contribute to drug craving and reinstatement are more likely to provide information useful for treatment.

OUD is characterized by chronic cycles of compulsive drug-taking, a loss of control in limiting intake, withdrawal-induced negative affective states, craving, and relapse (Koob, 2020). Clinical evidence points to the negative emotional states of withdrawal combined with high rates of relapse as the major barrier to successful treatment of OUD (Hunt et al., 1971; O'Brien, 2005; Chartoff and Connery, 2014). Considerable research over the past 2–3 decades has identified experimental parameters, neural circuits, and a vast array of molecular substrates underlying both withdrawal-induced aversive states and relapse-like behavior (Chartoff et al., 2006; Russell et al., 2016; Hearing, 2019; Koob, 2020). What remains unknown is how behavioral patterns that manifest during escalation of opioid self-administration directly contribute to the vulnerability and magnitude of reinstatement of drug-seeking after periods of abstinence. Compounding this gap in knowledge is that even less is known about basic behavioral responses to opioid self-administration in females, let alone their ability to predict reinstatement (Becker and Chartoff, 2019). The primary goals of this study were to (1) quantify and compare addiction-relevant behavioral measures observed during oxycodone self-administration in male and female rats to determine if there are sex differences in any individual behavioral measures, and (2) rank the relative contributions of all behavioral measures to post-abstinence reinstatement-responding in male and female rats. Importantly, the question of how each behavioral measures contributes to reinstatement in males and females does not require there to be sex differences in those measures *per se*. Each goal is significant for different reasons. First, the more granular data we can obtain about self-administration behaviors in males and females, the more we can learn about underlying neurobiological mechanisms. Second, the ability to weigh the relative contributions of behaviors displayed during drug self-administration to reinstatement—in males and females separately—suggests this approach may facilitate the ability to accurately predict risk of relapse in men and women with OUD.

To determine how measures and patterns of oxycodone self-administration might be used to predict relapse-like behavior, we used a rat model of OUD that incorporates key features of OUD in people, including escalation of drug intake when availability of drug is extended (i.e., long-access, LgA)(Ahmed et al., 2000; Belin-Rauscent et al., 2016; Towers et al., 2019), followed by 14 days of forced abstinence and a subsequent reinstatement test in which rats underwent a standard self-administration session with the same cues and contexts previously paired with oxycodone, but presses on the active lever did not deliver drug (Zhou et al., 2009; Venniro et al., 2016). We focused on self-administration behavioral measures relevant to OUD in people for analysis of their predictive ability: oxycodone infusions, escalation of intake, front-loading, and

inter-infusion intervals. The concept of using addiction-like behaviors to generate individualized measures that can predict vulnerability to reinstatement or relapse to SUD itself is not new (Kruzich et al., 1999; Belin et al., 2009; O'Neal et al., 2020; Panlilio et al., 2020), but has not, to our knowledge, been examined in both males and females or within the context of abused prescription painkillers like oxycodone.

Materials and methods

Animals

Adult female (N = 52) and male (N = 51) Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) were used. Body weights upon arrival were 200–225 g (female) and 250–275 g (male). Upon arrival, rats were group housed (4 rats/cage) and were acclimated for 1 week in a 12-h light-/dark-cycle (7:00 am/7:00 pm lights on/off) with food and water available *ad libitum*. Following surgeries, all rats remained singly housed. All experiments were conducted during the light phase. All guidelines recommended by the Institutional Animal Care and Use Committee of McLean Hospital and by the National Institutes of Health guidelines for the care and use of laboratory animals were followed.

The data analyzed for this study is a combination of data generated by 3 different investigators in the lab for their individual projects. All 3 experiments were run in the same operant chambers and used the same IVSA paradigm shown in **Figure 1**: an 8-d training phase (1-h/d), a 14-d escalation phase (either 1 or 6 h/d), a 14-d forced abstinence phase, and a cue-induced reinstatement test. The first experiment included 26 female and 26 male rats. Approximately 6 weeks prior to catheterization surgery for oxycodone self-administration, intracranial self-stimulation (ICSS) stimulating electrodes were surgically implanted in the medial forebrain bundle at the level of the lateral hypothalamus, as in Carlezon and Chartoff (2007) and Ebner et al. (2010). The IVSA paradigm used in rats from Experiment 1 was identical to the other groups and is described below in the Methods. See **Supplementary methods** for details of the ICSS portion of Experiment 1. The second experiment included 21 female (saline, N = 10; oxycodone, N = 13) and 22 male (saline, N = 9; oxycodone, N = 11) rats that underwent the IVSA paradigm described below. The third experiment included 3 male and 5 female rats that underwent the IVSA paradigm described below. For simplicity, data from the rats of Experiments 2 and 3 have been combined.

An important question is whether the presence of the ICSS electrode and/or ICSS behavior altered self-administration behavior such that combining data from these experimental cohorts is not feasible. As such, examples of IVSA data from Experiment 1 (IVSA + ICSS) and Experiment 2 (IVSA alone) are compared in **Supplementary Figure 1**. We show no significant differences between experimental groups for self-administration infusions, front-loading behavior, and reinstatement responding.

Intravenous catheter implantation

All rats were implanted with chronic indwelling silastic intravenous (i.v.) jugular vein catheters (0.51 mm internal diameter; SAI Infusion Technologies, IL, USA) as described (Mavrikaki et al., 2017, 2020). Briefly, rats were anesthetized with a ketamine/xylazine mixture (ketamine, 80 mg/kg; xylazine 8 mg/kg, IP), and catheters were implanted into the right jugular vein, secured to the vein with non-absorbable sutures, and the tubing passed subcutaneously to exit dorsally from the rat's back. After surgery, rats were injected once with ketoprofen (5 mg/kg, SC) to reduce pain. For maintenance, catheters were flushed daily with 0.2 ml of heparinized saline (30 USP units/ml) and once a week with 0.2 ml gentamicin (10 mg/ml). Catheter patency was tested at least once per week by i.v. infusion of 150–200-μl methohexital sodium (2 mg in 200 μl for the males and 1.5 mg in 150 μl for the females, i.v.) and assessing loss of righting reflex. If the latency to loss of righting was greater than 10 s, the catheter was not considered patent, and the rat was not tested.

Intravenous self-administration

Intravenous self-administration (IVSA) was performed during the light phase in operant conditioning chambers (11.625" L 9.78" W 10.75" H; Med Associates, St. Albans, VT, USA) with grid floors and contained in sound- and light-attenuating outer chambers (ENV-007CT; Med Associates). A syringe pump located outside each chamber was connected to a swivel within each chamber to permit IV drug infusions. After 1-week recovery from catheter implantation surgery, rats were trained to self-administer oxycodone (provided by the NIDA Drug Supply Program) at 60 μg/kg per infusion on a fixed ratio 1 (FR1) schedule of reinforcement. For training, 1-h sessions [i.e., short access (ShA)] were conducted once per day (M-F) for 8 d, as in Schlosburg et al. (2013) and Mavrikaki et al. (2019). Rats then entered the escalation phase, comprising 14 d of oxycodone self-administration (M-F) under either ShA (1 h/day) or long access (LgA; 6 h/day) conditions. A subset of rats underwent this IVSA paradigm with saline instead of oxycodone (N = 10 females, 9 males). Each self-administration trial began with the chamber house light turning on and the extension of two lever manipulanda signaling drug availability. A press on the active lever resulted in illumination of a cue light over the active lever and the house light turning off. Coincident with presentation of cues, a syringe pump delivered 100 μl of

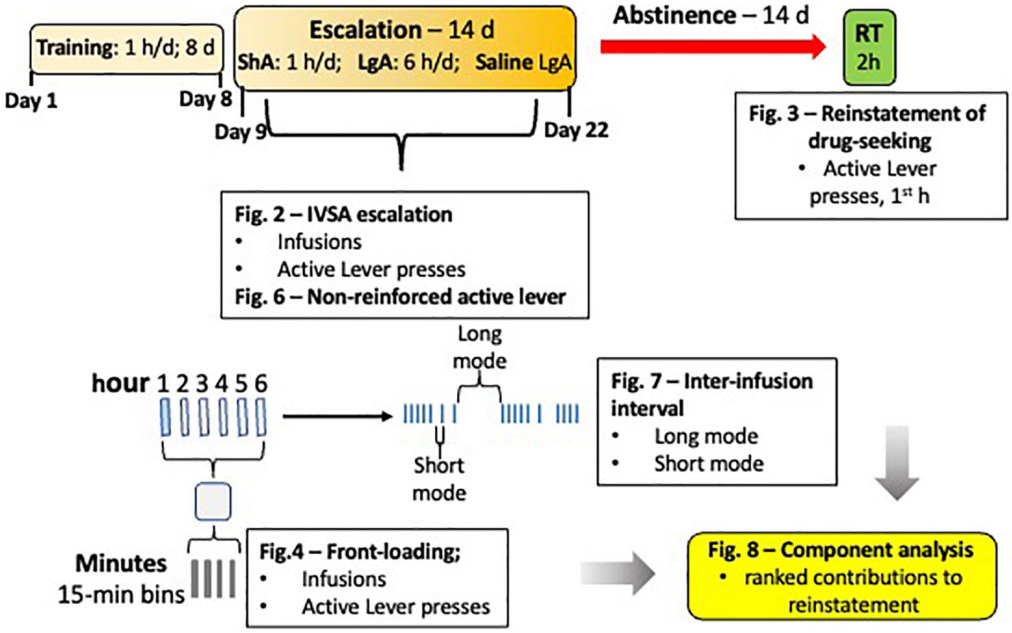

FIGURE 1
Experimental Schematic. The IVSA paradigm is depicted with the orange boxes, forced abstinence with the red arrow, and the reinstatement test with the green box. A blow up of one example day is shown below the overall schematic, with an example IVSA session represented as long access (LgA), with 6 bars representing 6 h [First blue bar indicates a short access (ShA) session)]. Below that, 1 h of one session is represented by 4 smaller bars representing 15-min bins. To the right of the blow up of a representative session is an example of infusion patterns, with blue ticks representing infusions. Text boxes represent the behavioral measures analyzed for sex differences and used in regression analyses to determine contribution to reinstatement responding and ability to predict magnitude of reinstatement.

oxycodone (60 μg/kg per infusion) lasting 4 s. Following the 4-s infusion was a 6-s timeout period during which any press on the active lever had no consequence. Following the full 10-s cycle, the cue light turned off and the house light turned on to again signal drug availability. Presses on the inactive lever were recorded but had no consequences. We recorded and analyzed total infusions, active lever presses (those that led to a drug infusion and those that occurred during a timeout), and inactive lever presses.

Cue-induced reinstatement test

After the last IVSA day, rats were returned to their home cages in the vivarium for 14-d of forced abstinence; a design based on studies using heroin (Zhou et al., 2009; Fanous et al., 2012). After the abstinence period, rats were placed in the same operant chambers in which they had previously self-administered oxycodone. A 2-h reinstatement test was conducted, with conditions identical to those during self-administration except a press on the active lever did not result in drug (oxycodone or saline) infusion. Both Zhou et al. (2009) and Fanous et al. (2012) showed that cue-induced reinstatement responding on the active lever is significantly higher after 14 days compared to 1 day of forced abstinence, consistent with time-dependent increases in drug-seeking; i.e., incubation of craving (Shalev et al., 2001). In our study, we only conduct one cue-induced reinstatement test (after 14-d abstinence), which means we are not directly measuring the incubation phenomenon. As such, we compare reinstatement responding in males and females among the three treatment groups (LgA, ShA, and saline self-administration).

Statistical analyses

Behavioral data was collected using automated procedures (MedPC-IV, MedAssociates) and processed using custom written scripts in R (R Core Team, 2019). Further preparation of data and statistical analysis was done with R and GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). For treatment by sex by time comparisons, 3-way ANOVAs with repeated measures on time were used. In the event of significant interactions or main effects, data were collapsed into the relevant two factors, and 2-way ANOVAs were performed. For sex by time comparisons, 2-way ANOVAs with repeated measures on time were used. In the event of significant interactions or main effects, Sphericity was not assumed, so the Geisser-Greenhouse correction was used. To understand the difference between various time-points, multiple comparison tests were performed.

One goal of these studies was to identify key behaviors measured during the escalation phase of oxycodone self-administration that correlate with reinstatement-responding after prolonged abstinence from self-administration. Various measures of drug self-administration behavior have been related to addiction severity (O'Neal et al., 2020). We tested for correlations between reinstatement responding and 6 measures of oxycodone self-administration behavior. The six behavioral measures were all taken from the escalation phase of oxycodone self-administration (days 9–22), and are as follows *(1) infusion score,* the average total number of infusions; *(2) front-loading,* the average number of infusions during the first 15 mins of the self-administration day; *(3) Active (no reward),* the average number of non-reinforced active lever presses normalized to the number of infusions; *(4) escalation score,* the difference in oxycodone infusions between day 9 and 22); *(5 and 6) inter-infusion interval (long and short),* the average of the mode of time-difference (s) between two successive bursts of oxycodone infusions. Inter-infusion intervals showed a bimodal distribution, suggesting there were two predominant time-differences (long and short). First univariate (Pearson's) correlation analysis was performed to determine if each of these behavioral measures were significantly associated with active lever presses during the reinstatement test. Additionally, Pearson's correlation between these measures was performed to determine whether the measures themselves were correlated with each other, also known as collinearity. To understand how measures of addiction-like behavior (that are correlated with each other) differentially affect reinstatement of drug-seeking in females and males, multivariate (partial least squares) regression analysis was used (Kuhn and Johnson, 2018) and variable importance in projection was measured. Variable importance in projection is a measure calculated using the weighted sums of the absolute regression coefficients (Kuhn, 2008). See **Figure 1** for an Experimental Schematic.

Results

Male and female rats escalate oxycodone intake under both long access and short access conditions

Currently, there is relatively little information comparing oxycodone self-administration behaviors between male and female rats. Here we report data on several behavioral measures associated with oxycodone self-administration during an escalation phase in which male and female rats have either long access (LgA) or short access (ShA) to drug, or in which male and female rats self-administer saline. It has typically been found that laboratory animals will escalate drug intake when allowed LgA, but not ShA, to drug (Ahmed and Koob, 1998; Ahmed et al., 2000). Escalation of heroin intake has been reported in male and female mice (Towers et al., 2019), suggesting similar effects will be observed with oxycodone. In our study, rats underwent an 8-d training phase, in which rats could self-administer oxycodone (60 mg/kg/infusion) for 1 h/day (1 h/d, M-F), followed by a 14-d escalation phase in which rats could self-administer oxycodone under ShA (1-h/d) or LgA (6-h/d) conditions (M-F). A separate group of rats self-administered saline under LgA conditions. The number of infusions and active lever presses increased over the course of the escalation phase (days 9–22) under both LgA and ShA conditions. Main effects of Day were found for Infusions (LgA: $F_{(4.53,240.1)} = 10.20$, $p < 0.0001$, **Figure 2A**; ShA: $F_{(4.50,115.6)} = 5.81$, $p < 0.0001$, **Figure 2B**) and Active Lever presses (LgA: $F_{(2.79,148.0)} = 8.96$, $p < 0.0001$, **Figure 2D**; ShA: $F_{(3.88,101)} = 2.618$, $p = 0.041$, **Figure 2E**). Rats that self-administered saline did not show an increase in responding throughout the escalation phase (**Figures 2C,F**). No Sex × Day interactions or main effects of Sex were observed under either LgA or ShA conditions. To control for possible non-specific motor effects, which could indicate a lack or reduction of learning the drug-lever pairing—we found no significant changes in inactive lever presses over time or by sex (**Supplementary Figure 2**).

Despite the lack of sex effects in oxycodone self-administration over the course of the escalation phase, the number of infusions and active lever presses was visually higher in males than females. To determine if there was a sex difference in overall drug intake or lever pressing, we compared total infusions and active lever presses in males and females during the escalation phase (days 9–22). Unpaired *t*-tests for all each (**Figures 2G,H**) showed no significant sex effects. In addition, we calculated the difference in oxycodone infusions (**Figure 2I**) and active lever presses (**Figure 2J**) between escalation days 9 and 22 as a measure of escalation and compared this escalation score between males and females. Unpaired *t*-tests for each treatment condition revealed no significant sex differences.

To specifically determine if rats escalated their drug intake, we compared the number of infusions and active lever presses in males and females on day 22 to those on day 9 for each treatment condition (LgA, ShA, saline). Two-way ANOVA (Sex × Day) with repeated measures on Day showed no Sex × Day interactions or main effects of Sex under any condition. However, there were main effects of Day for infusions and active lever presses under all conditions. Bonferroni's multiple comparison tests showed that both males and females had significantly higher oxycodone infusions and active lever presses on escalation day 22 (ESC d22) compared to ESC d9 in LgA and ShA rats (**Supplementary Figure 3**). Main effect of Day for Infusions [LgA: $F_{(1,54)} = 29.72$, $p < 0.0001$, **(A)**; ShA: $F_{(1,26)} = 20.43$, $p = 0.0001$, **(B)**; Sal: $F_{(1,17)} = 6.12$, $p = 0.024$, **(C)**] and main effect of Day for Active lever presses [LgA: $F_{(1,49)} = 23.56$, $p < 0.0001$, **(D)**; ShA: $F_{(1,26)} = 17.64$, $p = 0.0003$, **(E)**; Sal: $F_{(1,17)} = 9.56$, $p = 0.007$, **(F)**].

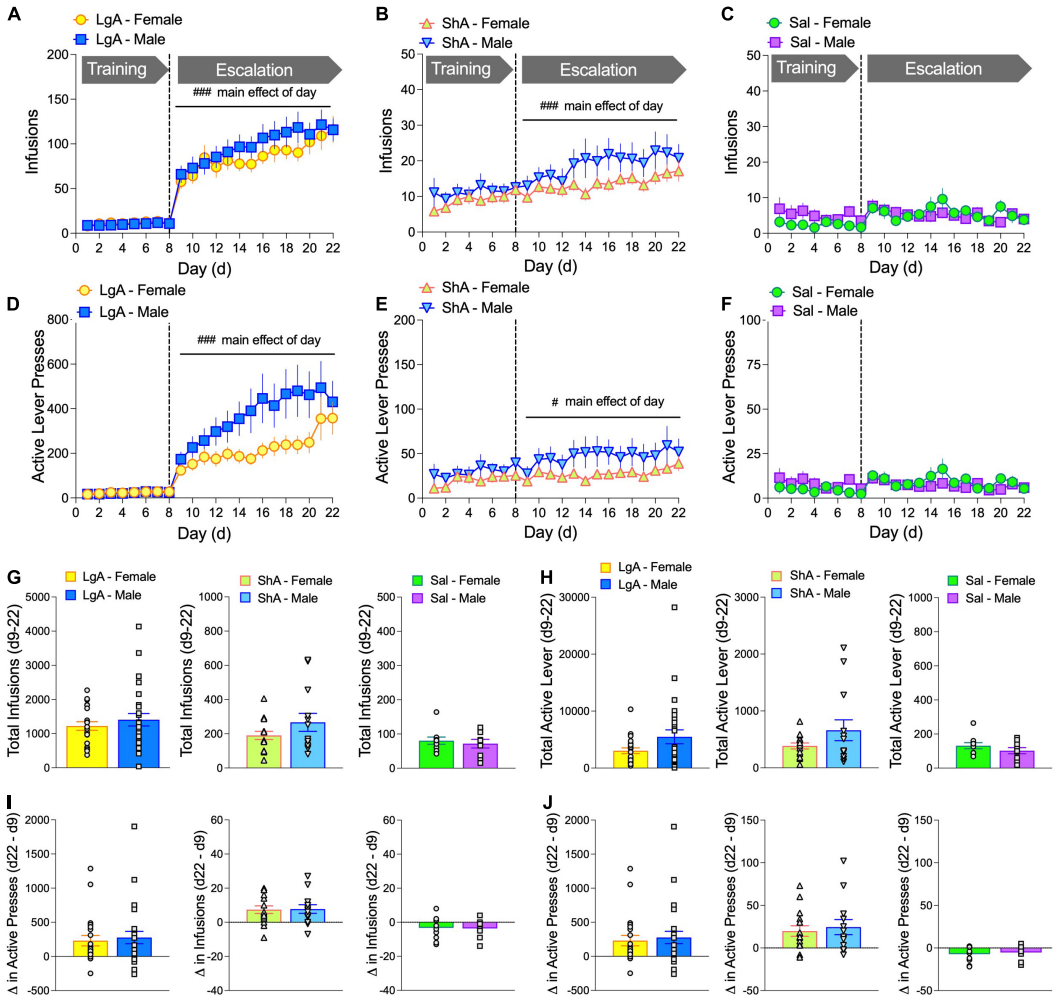

FIGURE 2
Female and male rats escalate oxycodone self-administration under both long access (LgA, 6 h) and short access (ShA, 1 h) conditions. Oxycodone self-administration behavior is shown as # of infusions/day **(A–C)** and # of active lever presses/day **(D–F)** for male and female rats across the 22 days of the self-administration paradigm. Groups included rats that self-administered oxycodone under LgA conditions **(A,D)** or under ShA conditions **(B,E)** during the escalation phase (days 9–22) and rats that self-administered saline **(C,F)**. Under both LgA and ShA, but not saline, conditions, there was a main effect of Day. There were no sex differences in total infusions **(G)** or active lever presses **(H)** during the escalation phase (d9–22). There were no sex differences in the change in number of infusions **(I)** or active lever presses **(J)** from escalation day 9 to escalation day 22, although the net change for LgA and ShA, but not saline, rats was >0, indicating escalation of intake (see **Supplementary Figure 2**). $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, main effect of Day. *N*: LgA (22 females, 26 males); ShA (15 females, 13 males); Sal (10 females, 9 males).

Reinstatement responding is higher in female compared to male rats after 14-d of forced abstinence from oxycodone self-administration

A major goal of these studies was to determine if behavioral measures observed during oxycodone self-administration—relevant to DSM-5 criteria for OUD (American Psychiatric Association, 2013)—can be ranked in importance to their contribution to reinstatement of drug-seeking after a period of abstinence. As such, our experimental design included a 14-d forced abstinence period after the last self-administration day (day 22) followed by a cue-induced reinstatement test. This design was intended to approximate a common experience in people with substance use disorder in which periods of heavy drug use are followed by periods of abstinence (often forced, e.g., incarceration) followed again by reinstatement to drug-taking (Reiner et al., 2018).

Importantly, there is a large body of literature showing that craving for drug can "incubate" (increase), resulting in different levels of reinstatement in a time- and drug-dependent manner (Reiner et al., 2018). In our study, all rats experienced 14 days of abstinence, which did not allow measurement of the incubation of craving. As such, we compared the magnitude of reinstatement responding between males and

females among the three treatment conditions (LgA, ShA, Sal). Active lever pressing during the reinstatement test was higher in females compared to males across treatment groups (**Figure 3**), which depended on a main effect of Sex ($F_{(1,85)}$ = 4.57, p = 0.035). Reinstatement responding was highest in rats that had self-administered oxycodone under LgA conditions, which depended on a main effect of Treatment ($F_{(2,85)}$ = 17.06, $p < 0.0001$).

Female and male rats exhibit front-loading self-administration behavior

It is well established that drug self-administration typically starts with a "front-loading phase," in which an animal makes several lever presses in rapid succession to quickly bring blood/brain drug levels to a concentration that is reinforcing. This is followed by a "maintenance phase" in which drug self-administration occurs at more regular intervals thought to maintain sufficient drug levels to sustain the desired hedonic set point (Wise and Bozarth, 1987; Gerber and Wise, 1989). However, repeated opioid use usually leads to tolerance and dependence (Wise and Koob, 2014); conditions in which progressively increasing intake is required to front-load and maintain drug effectiveness (Ahmed and Koob, 1998, 2005).

To determine if male and female rats exhibit front-loading behavior during the escalation phase of self-administration and if front-loading changes from the beginning to the end of escalation (d9 vs d22), we analyzed the first hour of self-administration data in 15-min bins on escalation day 9 (ESC d9) and ESC d22 (**Figure 4**). We found that oxycodone (or saline) infusions and active lever presses were greatest in the first 15 min of the self-administration sessions on both ESC d9 and ESC d22 under every treatment condition (LgA, ShA, and Sal). This robust evidence for front-loading depended on either Day × Time interactions (**Figures 4A,D,E**) or main effects of Time (**Figures 4B,C,F**) in the 3-way ANOVAs (Sex × Day × Time) conducted for each treatment condition. An example of a Day × Time interaction is found in active lever pressing in LgA rats (**Figure 4D**): ($F_{(2.17,99.68)}$ = 3.83, p = 0.022), and an example of a main effect of time is found in infusions in Sal rats (**Figure 4C**): ($F_{(3.0,51.0)}$ = 20.46, $p < 0.0001$). The fact that rats self-administering saline had higher infusions (**Figure 4C**) and active lever presses (**Figure 4F**) in the first 15 min of the sessions suggests that a portion of front-loading behavior can be attributed to locomotor stimulation from placing the rats in the operant chambers each day. The 3-way ANOVAs for this front-loading data also revealed that oxycodone, but not saline, self-administration behavior was higher across the first hour of ESC d22 compared to ESC d9 in LgA and ShA rats, consistent with development of tolerance. There were no effects of sex on front-loading behavior.

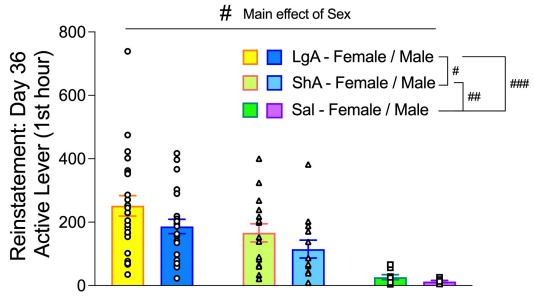

FIGURE 3
Reinstatement responding after 14 days of forced abstinence: effects of sex and prior self-administration condition. Data are shown as the average number (±SEM) of active lever presses during the first hour of the Reinstatement Test in male and female rats that had self-administered oxycodone in the escalation phase under LgA or ShA conditions or self-administered saline (Sal). Across treatment groups, females had higher levels of reinstatement responding than males (main effect of Sex). Further, treatment group dictated the level of reinstatement responding, with LgA > ShA > Sal (main effect of Treatment). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, main effects of sex and treatment, as indicated by brackets. *N*: LgA (19 females, 20 males); ShA (15 females, 13 males); Sal (8 females, 6 males).

Temporal features of long access oxycodone self-administration sessions at the beginning and end of the escalation phase

Male and female rats do not differ in the number of oxycodone infusions or active lever presses during LgA sessions of the escalation phase (d9–22; **Figures 2A,D**). However, it is not known whether self-administration behavior fluctuates within a 6-h session or between early (d9) and late (d22) 6-h sessions in male and female rats. As such, we quantified oxycodone infusions (**Figure 5A**) and active lever presses (**Figure 5B**) for each hour of the 6-h IVSA sessions in LgA rats on escalation days 9 (ESC d9) and ESC d22. We found that rats exhibited front-loading behavior at the hourly level, as the number of infusions was highest in the first hour of the 6-h session on both ESC d9 and ESC d22. This depended on a main effect of Hour in the 3-way ANOVA (Sex × Day × Hour) for infusions: ($F_{(5.0,230)}$ = 3.62, p = 0.004). Front-loading was not observed with active lever presses, and no significant effects of sex were found in any condition. Finally, hourly infusions and active lever pressing were higher across the 6-h sessions on ESC d9 compared to ESC d22, which depended on main effects of Day in the 3-way ANOVAs for infusions ($F_{(0.76,34.94)}$ = 29.29, $p < 0.001$) and active lever presses ($F_{(0.45,20.61)}$ = 17.88, p = 0.003).

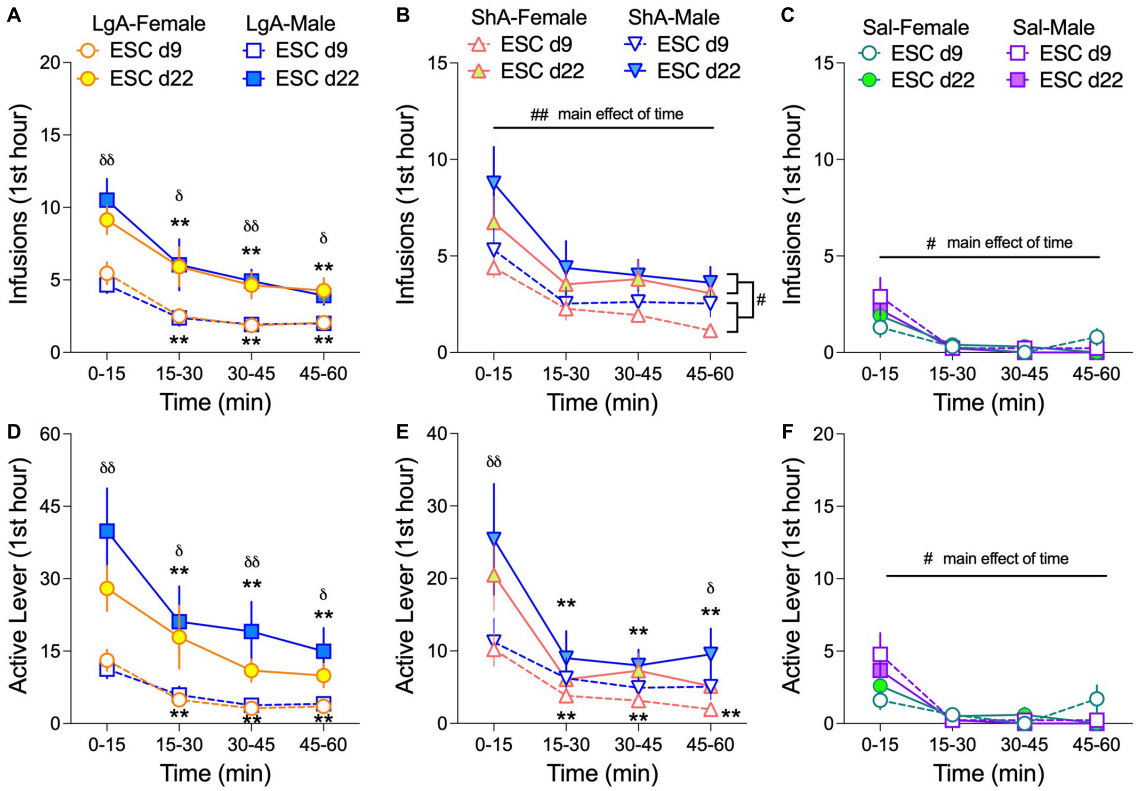

FIGURE 4
Female and male rats front-load oxycodone intake. Data are shown as the average number (±SEM) of infusions **(A–C)** and active lever presses **(D–F)** in 15-min bins from the first hour of escalation day 9 (ESC d9) and day 22 (ESC d22) in male and female rats. For each treatment condition: LgA **(A,D)**, ShA **(B,E)**, and Sal **(C,F)**, 3-way ANOVAs (Sex × ESC day × Time) were calculated. For oxycodone self-administration under LgA and ShA conditions, rats self-administered more oxycodone in the first 15-min bin compared to the last 15-min bin (i.e., front-loading), and overall oxycodone intake was greater on ESC d22 compared to ESC d9. This was determined through main effects or interactions of ESC day and Time. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, main effect of Time; $^{**}p < 0.01$, Dunnett's multiple comparisons to the 0–15 min bin; $^{\delta}p < 0.05$, $^{\delta\delta}p < 0.01$ Bonferroni's multiple comparisons of ESC d9 to ESC d22 per time bin. N: LgA (19 females, 20 males); ShA (15 females, 13 males); Sal (8 females, 6 males).

Males have higher levels of non-reinforced active lever presses compared to females during the escalation phase of long access oxycodone self-administration

Each active lever press that leads to drug delivery triggers a 4-s infusion (100 μl) followed by a 6-s time out period. Any active lever presses that occur during the total 10 s after an infusion-triggering active lever press are not reinforced with drug delivery. To quantify continued lever pressing during these non-reinforced periods, we summed the normalized non-reinforced lever presses for each day of IVSA using the formula [(active press—infusion)/infusion]. The more non-reinforced lever presses per session, the higher the value of this output. A value of 1 means there were at least twice as many active lever presses as infusions, whereas a value between 0 and 1 means the number of active lever presses was less than twice the number of infusions. We observed that both male and female rats self-administering oxycodone in 1-h sessions—during the training or escalation phases—had a normalized, non-reinforced active lever press value of ∼1 (Figures 6A,B). This suggests that rats typically press the active lever about twice as often as they receive oxycodone infusions. In rats self-administering saline; however, we observed a normalized, non-reinforced active lever press value of ∼0.5 (Figure 6C). Taken together, these observations are consistent with a higher motivation to seek and acquire reinforcing stimuli (e.g., oxycodone) but not non-reinforcing stimuli (e.g., saline).

During the escalation phase under LgA, but not ShA or saline, conditions, a sex difference emerged such that normalized, non-reinforced active lever presses were higher in males compared to females (Figure 6A). This depended on a main effect of Sex ($F_{(1.0,46)} = 4.15$, $p = 0.047$). A main effect of Day was also found ($F_{(4.44,204.2)} = 4.83$, $p = 0.0006$), but pairwise comparisons revealed no significant differences among escalation days. The sex difference in normalized, non-reinforced active lever presses was also observed when these data

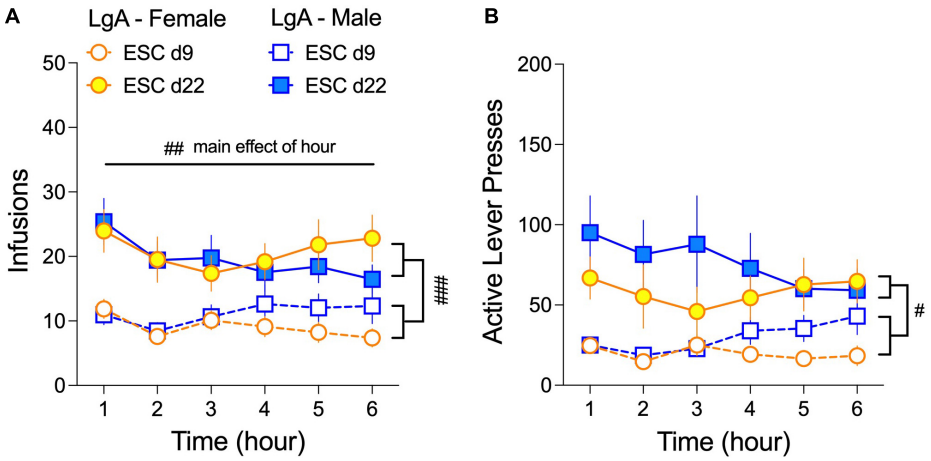

FIGURE 5
Female and male rats self-administer oxycodone throughout the 6-h LgA sessions during the escalation phase. The average number (±SEM) of oxycodone infusions **(A)** and active lever presses **(B)** for each of the 6 h of escalation days 9 (ESC d9) and 22 (ESC d22) are plotted for males and females. Infusions and active lever presses are higher for both sexes on ESC d22 compared to ESC d9 (main effect of Day), although there is an overall, yet modest, decrease in responding across the 6-h (main effect of Hour). No sex differences were observed. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, main effects of day and hour, as indicated. N: LgA (19 females, 20 males); ShA (15 females, 13 males); Sal (8 females, 6 males).

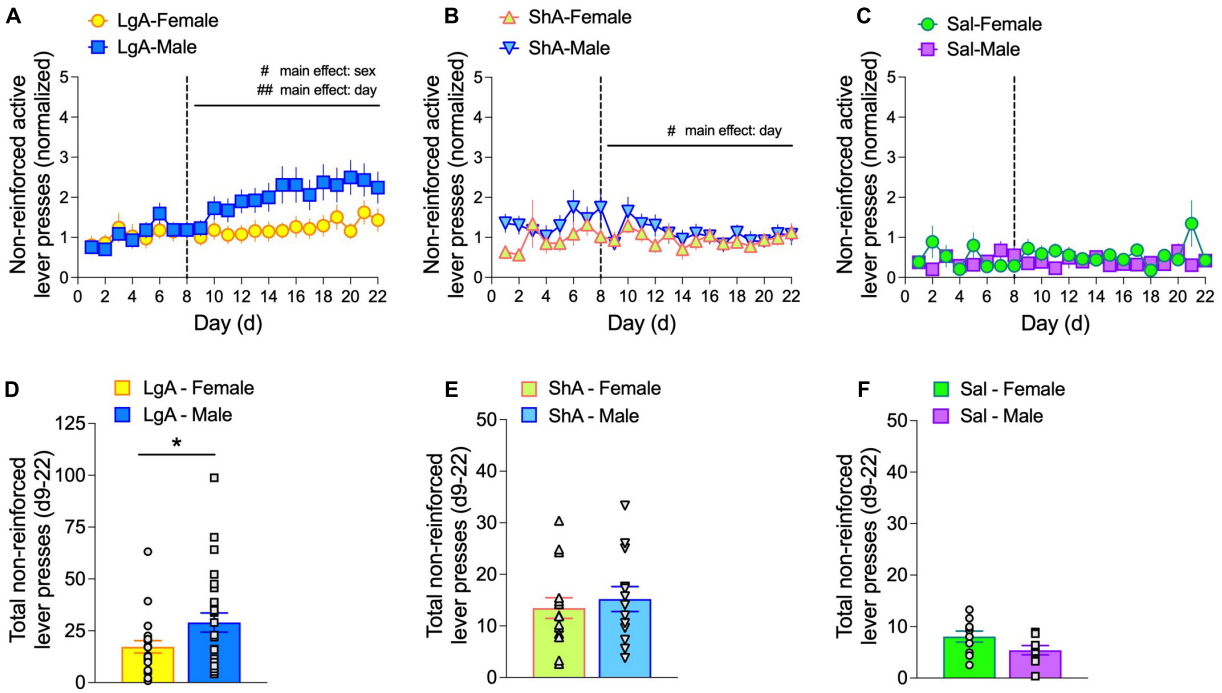

FIGURE 6
Males exhibit higher levels of non-reinforced active lever pressing than females under LgA self-administration conditions. Non-reinforced active lever pressing is expressed as: [(non-reinforced active lever presses—infusions)/infusions]. Data are shown as non-reinforced active lever pressing per self-administration day (d1–22) for LgA **(A)**, ShA **(B)**, and Sal **(C)** escalation phase treatment groups and as the total of non-reinforced active lever pressing during the escalation phase (d9–22) for LgA **(D)**, ShA **(E)**, and Sal **(F)** escalation phase treatment groups. Under LgA conditions, non-reinforced active lever pressing depended on main effects of Sex and Day **(A)**, with males showing significantly higher responses than females **(D)**; unpaired *t*-test). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, main effects of sex and day, as indicated; $^{*}p < 0.05$ unpaired *t*-test. *N:* LgA (22 females, 26 males); ShA (15 females, 13 males); Sal (10 females, 9 males).

were summed over the escalation phase in LgA rats (Figure 6D), but not ShA or Sal rats (Figures 6E,F, respectively).

The time (interval) between bursts of oxycodone infusions decreases as escalation of intake increases in male and female long access rats

Development of a burst-like pattern of drug self-administration has been associated with addiction-like behavior in cocaine self-administration studies (Belin et al., 2009). Historically, burst-like patterns are defined by a minimum number of drug infusions over a specified time-window. However, this approach does not account for the variable pattern of infusions during a burst nor the dynamic nature of burst-like patterns over time. To address this, we used inter-infusion intervals (s) as a proxy for drug self-administration patterns. An evenly spaced drug self-administration pattern would have a normal/gaussian distribution of inter-infusion intervals. A burst-like drug self-administration pattern would have a bimodal distribution (Figures 7A–C). Based on empirical observation of oxycodone infusion histograms from self-administration sessions (e.g., Figure 7A), the larger mode (long-mode) typically indicates time elapsed between two burst-like bouts of oxycodone self-administration, whereas the smaller mode (short-mode) typically indicates time elapsed between two successive infusions within a burst-like bout. Both LgA and ShA rats undergoing oxycodone self-administration showed this bimodal distribution of inter-infusion intervals. In male and female LgA rats, the long-mode intervals decreased as drug escalation increased from days 9 to 22 (Figure 7D). This was supported by a main effect of Day ($F_{(1.0,43)}$ = 16.88, p = 0.002) and Bonferroni's multiple comparison tests. In contrast, the short-mode intervals remained unchanged (Figure 7F). Together, these findings suggest that the nature of the bursts remains the same, but their frequency increases.

Sex differences in the relative contributions of oxycodone self-administration behaviors to reinstatement responding

An important goal of this study was to determine how the various measures of addiction-like behavior reported here contribute to reinstatement responding, with the relative contributions of behaviors ranked for females and males separately. The behaviors we ranked for this analysis are: (1) Infusions during the escalation phase, (2) Front-loading of drug intake in the first 15 min of each self-administration session during the escalation phase, (3) Non-reinforced lever presses during the escalation phase, (4) Short, and (5) Long inter-infusion intervals during the escalation phase, and (6) Escalation of drug intake (day 22 infusions minus day 9 infusions for each rat). To accomplish this, univariate (Pearson's) correlation analysis was performed separately on male and female data to determine the extent to which each behavioral measure was associated with active lever presses during the reinstatement test (Figures 8A,B; gray highlighted plots). For females, we found significant correlations between reinstatement responding and Infusions, Front-loading, and Long inter-infusion intervals (Figure 8A; R scores listed in each matrix box). For males, we found significant correlations between reinstatement responding and Infusions, Front-loading, Non-reinforced active lever presses, and Short inter-infusion intervals (Figure 8B).

Since many of these behavioral measures were also significantly correlated with each other (Figures 8A,B; unshaded plots), this indicated collinearity of the data. Therefore, to rank the importance of each measure to reinstatement responding in males and in females, we used partial least squares regression, a form of multi-variable linear regression analysis (diagrammed in Figure 8D). The various collinear measures were weighted and combined to generate a single component ("Component," Figure 8D), which was optimally correlated with post-abstinence reinstatement responding (Figure 8D orange plot, females; Figure 8D blue plot, males). The relative contribution of each measure to active lever presses during the post-abstinence reinstatement test was reported in a sex-specific manner (Figure 8E). Front-loading (average number of infusions during the first 15 mins of each self-administration day during the escalation phase) was most strongly associated with reinstatement responding for both sexes. The behavioral measures with the second highest relative contribution to reinstatement were long-mode inter-infusion intervals for females and short-mode inter-infusion intervals males. The number of oxycodone infusions was ranked third in relative contribution to reinstatement for both females and males. For females, only those three behavioral measures (front-loading, long inter-infusion intervals, and infusion number) were significantly correlated with reinstatement responding, so the relative contributions of other behaviors (Figure 8E, behaviors listed below horizontal dashed line) were deemed insignificant. In contrast, non-reinforced active lever presses also contributed significantly to reinstatement responding in males. Interestingly, escalation itself did not show a significant correlation with reinstatement in either sex (Figures 8A,B; first row, last column).

Discussion

In this study we found that the majority of addiction-like behavioral components that comprise escalation of oxycodone

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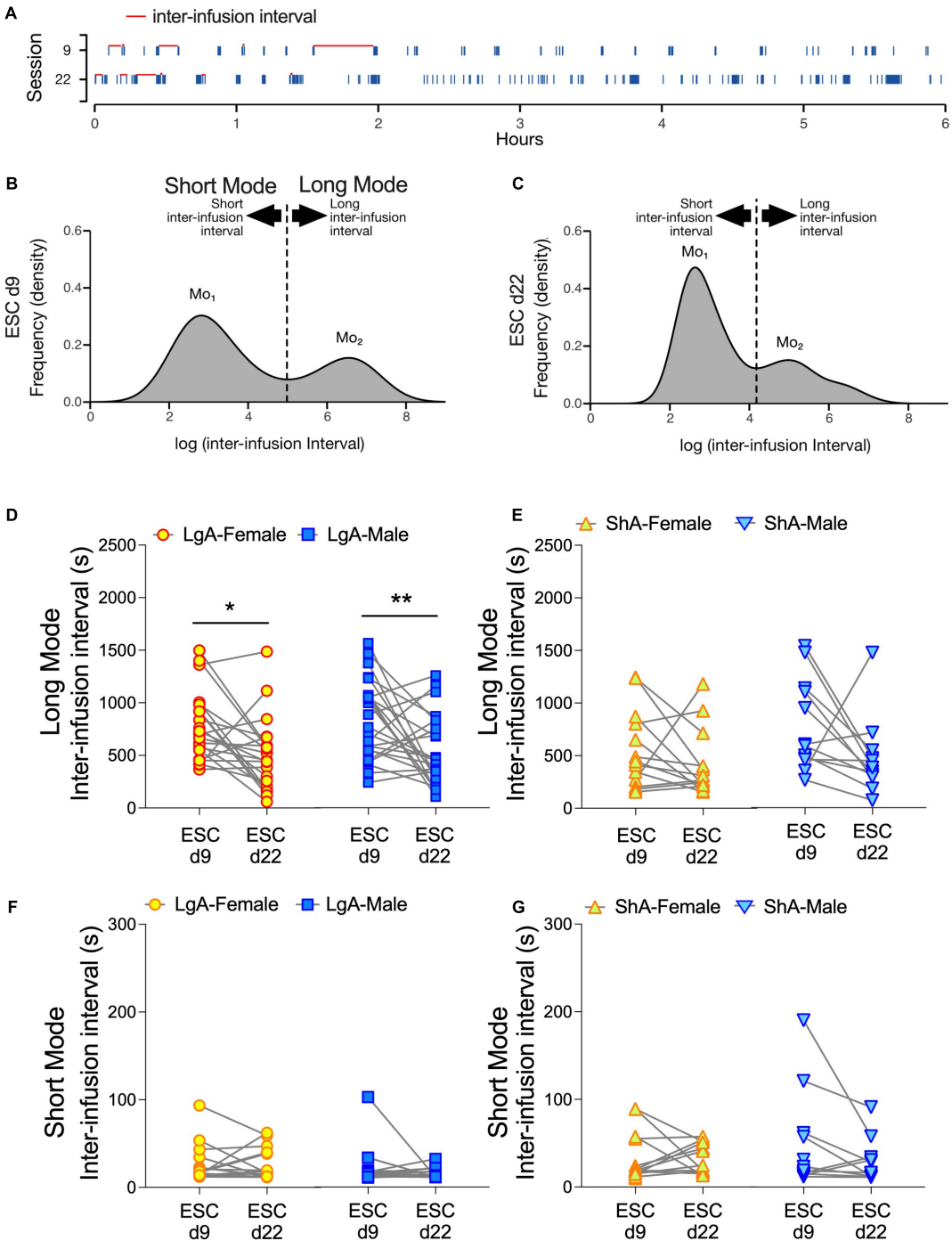

FIGURE 7
Latency between bursts of oxycodone infusions decreases in females and males under LgA, but not ShA, self-administration conditions. Schematics illustrating methods to calculate interval latencies between oxycodone infusions are generated from representative data and shown in **(A–C)**. In panel **(A)**, raster plots show oxycodone infusions (blue ticks) from the escalation days 9 and 22 from a LgA rat. All inter-infusion intervals (s) are quantified example intervals demarcated with red lines **(A)**. Frequency distributions of log-transformed inter-infusion intervals across the 6-h self-administration days reveal bimodal distributions that consist of a population of long (long mode), and a population of short (short mode), intervals **(B,C)**. Mo_1 represents the inter-infusion interval in the short mode with the highest frequency of occurrence, and Mo_2 represents the inter-infusion interval in the long mode with the highest frequency of occurrence. Data in panels **(D–G)** are dot and line plots representing average inter-infusion intervals for the long mode **(D,E)** and short mode **(F,G)** on escalation days 9 (ESC d9) and 22 (ESC d22) for each rat under LgA **(D,F)** and ShA **(E,G)** conditions. 2-way ANOVAs (Sex × ESC day) show a main effect of ESC day and a significant decrease in Long Mode inter-infusion intervals in both males and females under LgA conditions on ESC d22 compared to ESC d9 **(D)**. $^*p < 0.05$, $^{**}p < 0.01$, Bonferroni's multiple comparisons of ESC d9 to ESC d22 per sex. LgA (22 females, 26 males); ShA (15 females, 13 males).

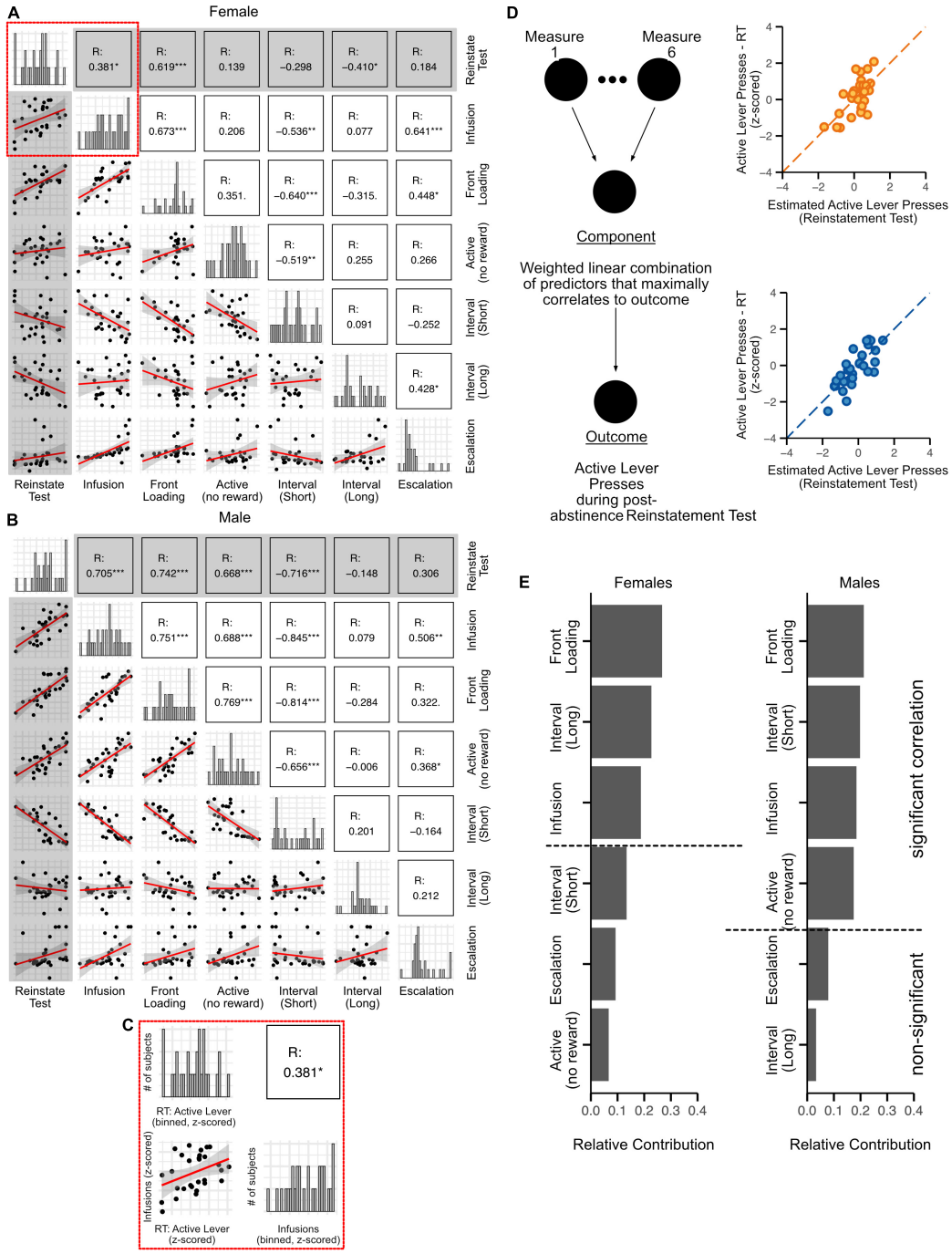

FIGURE 8
Relative contribution of behavioral measures associated with oxycodone self-administration to post-abstinence reinstatement responding. The 6 behavioral measures quantified in the previous figures [Infusions, front-loading, non-reinforced active lever pressing, Inter-infusion intervals (short and long modes), Escalation] were segregated by sex, *z*-scored, and correlated with reinstatement responding (Pearson's). For both females **(A)** and males **(B)**, histograms across the diagonal represent *z*-scored distributions of each measure. The inset **(C)** provides enlarged visualization of each component of the matrix. In the histograms, the X-axis represents the *z*-scored measures, while the Y-axis represents the number of rats. The correlation plot in panel **(C)** shows the relationship between *z*-scored active lever presses in the reinstatement test (RT: active lever) and *z*-scored oxycodone infusions (infusion). The correlation matrix (below the diagonal of histograms) represents the cross-correlation between all the measures, while the matrix on top shows the correlation coefficients (R). Significant correlations are indicated with asterisks. Gray highlighted squares show comparisons of reinstatement test responding with each behavioral measure: correlation plots are in the gray-shaded column and correlation coefficients (R) are in the gray-shaded row. **(D)** Schematic showing the partial least square regression model used to determine the role of each measure to reinstatement responding. The dot plots in panel **(D)** (orange circles, females and blue circles, males) were generated with the partial least square regression model and demonstrate a tight fit between observed reinstatement responses vs predicted ones. Bar plots were generated indicating the relative contribution of each behavioral measure to reinstatement test responding in female and male rats **(E)**. N: 30 females, 29 males.

self-administration and reinstatement of drug-seeking after a 2-week forced abstinence period are similar in male and female Sprague Dawley rats. However, our approach of analyzing more granular components of the process from acquisition of self-administration behavior to patterns of self-administration during the escalation phase and reinstatement showed that the number of non-reinforced active lever presses (those that occur between infusions) was significantly higher in males than females self-administering oxycodone under LgA conditions. In addition, reinstatement responding on the active lever previously paired with oxycodone was significantly higher in females. The second goal of this study was to compute the relative contribution of these granular components of self-administration behavior to the magnitude of reinstatement-responding. We found that many of the same behavioral components contributed the most to reinstatement responding in both males and females, but the rank order of their contribution was sex-dependent. For example, inter-infusion intervals during the escalation phase of self-administration was the second highest contributor for both sexes, except for females the intervals referred to those between bursts of oxycodone infusions and for males the intervals referred to those between individual infusions. We do not think it appropriate to try to directly relate either the behavioral components themselves or the sex differences within to the human condition of OUD or gender differences in OUD. However, we suggest that our findings highlight that broad behaviors (self-administration) and human conditions (OUD) are composed of numerous factors, each of which may reveal sex-dependent mechanistic processes and that it is possible to generate algorithms based on active self-administration (drug-taking) data that can help predict the magnitude of reinstatement to drug-seeking.

In both humans and animal models of addiction-like behavior, it is accepted that the transition from initial drug use to substance use disorder (SUD) involves numerous steps at the behavioral and neurobiological level (Self and Nestler, 1995; Robinson and Berridge, 2001; Wise and Koob, 2014). In this study, we used the extended drug access rodent model of addiction, which is thought to mimic many of these steps (Ahmed and Koob, 1998) because it leads to escalation of drug intake—an essential feature of SUD. Within this construct, we focused our analyses on 6 behavioral measures comprising oxycodone self-administration during the escalation phase (see schematic, **Figure 1**) that are related to the progression to addiction-like behavior. These included the total number of infusions, front-loading of drug intake, non-reinforced active lever presses, escalation of intake, inter-infusion, and inter-burst intervals. Since relapse to drug-taking is a primary barrier to recovery from SUD, and perhaps OUD in particular (Hunt et al., 1971; O'Brien, 2005), we used reinstatement of oxycodone-seeking after forced abstinence to complete our animal model of addiction-like behavior (Shalev et al., 2002; Venniro et al., 2016). Our experimental design was sensitive to possible sex differences in each of these behavioral components and to reinstatement responding. The fact that sex differences were only observed in non-reinforced active lever presses during oxycodone self-administration and reinstatement responding after abstinence suggests that opioid self-administration as a whole is similar in males and females, but individual steps contributing to reinstatement can be sex-dependent. Although this study was not intended to probe neurobiological mechanisms underlying the behavioral components measured here, it is likely that molecular, hormonal, and pharmacokinetic mechanisms all play a role in male and female behavioral processes contributing to addiction-like behavior. Of note, we have previously shown no sex differences in plasma or brain levels of oxycodone after i.v. infusion (Mavrikaki et al., 2017). It is important to recognize that males and females can show similar outward behaviors that are driven by different underlying mechanisms (Becker and Chartoff, 2019).

In our study, the majority of self-administration behavioral components were similar in males and females. There were no significant sex differences in infusions, active or inactive lever responses during the acquisition or escalation phases (either LgA or ShA conditions). These results are consistent with several recent studies specifically examining male and female rat opioid self-administration behavior under extended access conditions, forced abstinence periods, and reinstatement tests (Venniro et al., 2017, 2019; Fredriksson et al., 2020; Bossert et al., 2022). Even the more granular measures such as front-loading of oxycodone, maintenance of self-administration behavior over the 6-h sessions, and inter-infusions intervals were similar between males and females. In contrast, males, but not females, showed an increase in the number of non-reinforced active lever presses over the course of the escalation phase (days 9–22). These are lever presses that occurred during the ∼10-s period of a drug infusion cycle in which drug was not available. Studies using intermittent access self-administration paradigms typically refer to these non-reinforced active lever presses as "drug-seeking" (O'Neal et al., 2020; Bakhti-Suroosh et al., 2021). However, caution must be used here, as the periods in which drug was not available were much shorter (10 s) than in intermittent access protocols. It is just as likely the increase in non-reinforced active lever pressing reflects oxycodone-induced psychomotor stimulant action or a type of impulsive motor behavior.

Our finding that cue-induced reinstatement is higher in both LgA and ShA females than males after a 14-d forced abstinence period contrasts with the recent studies mentioned above (Venniro et al., 2017, 2019; Fredriksson et al., 2020; Bossert et al., 2022). In those studies, no sex differences in reinstatement were observed. The experimental designs were not identical to ours, as they typically included a reinstatement test after 1 day of abstinence as well as after a prolonged forced abstinence. However, a critical difference is that those studies were conducted during the dark phase of the light cycle

(reverse light cycle) whereas ours was conducted during the light phase. It is well known that there are sex differences in the circadian timing system that play important roles in determining responses to both endogenous and exogenous factors (Bailey and Silver, 2014), and it has been shown that time of day/circadian rhythms affect the reinforcing properties of opioids (Webb et al., 2015). We do not claim that circadian rhythms are specifically responsible for our finding that reinstatement responding is higher in females than males, but the "hidden variable" (Butler-Struben et al., 2022) of time of day is likely to be a major factor in the conflicting results emerging in sex difference research. Another factor that likely contributes to conflicting results in studies of sex differences is that estrous cycle can impact drug-seeking behavior (Carroll and Anker, 2010; Becker and Koob, 2016)—although relatively few studies have investigated the effect of estrous cycle on opioid-induced behavior. Indeed, we did not track estrous cycle stages in our studies, as most behavioral endpoints spanned days or weeks, making it practically impossible to tag an estrous cycle stage with a particular behavior.

Our second goal was to determine if the behavioral measures comprising self-administration could be ranked in a sex-dependent manner according to their relative importance to the magnitude of reinstatement-responding. A number of studies report measuring relapse vulnerability and addiction severity by the magnitude of reinstatement responding (Deroche-Gamonet et al., 2004; Belin et al., 2009). For example, it has been proposed that the amount and maintenance of responding for the drug-paired lever during the reinstatement test could predict reinstatement to drug-seeking (Kruzich et al., 1999). But those very behaviors constitute reinstatement and seem not be helpful in a clinical setting. A more useful biomarker would be measurable before relapse occurred, not in retrospect. We first demonstrated that a subset of behavioral measures correlated significantly with reinstatement in males (i.e., infusions, front-loading, non-reinforced active lever presses, and short inter-infusion intervals) and a slightly different subset was found in females (i.e., infusions, front-loading, long inter-infusion intervals). Partial least squares regression analysis then allowed us to rank the relative contribution of each behavior to reinstatement. We found that front-loading behavior, in which rats self-administer more drug at the beginning of each session than at the end, had the highest relative contribution to reinstatement in both sexes. After front-loading, the behavioral measures that ranked the highest for their contribution to reinstatement were sex-dependent. The second ranked contributor was long inter-infusion intervals for females and short inter-infusion intervals for males, followed by number of infusions for both sexes and finally non-reinforced active lever presses for males but not females. The important conclusion from these data is not that we have identified a magic set of factors that "cause" addiction, as the analysis was limited to the measures we input into the analyses. Rather, these findings are significant because they demonstrate that a battery of concrete behavioral measures reflecting active drug-taking behavior can be compared to a human-specific correlation matrix similar to the one we generated in **Figure 2** to predict the magnitude, and ultimately the risk, of relapse. Our data also highlight that the predictive behavioral measures and/or their rank order may be sex-dependent, which is consistent with findings that men and women with OUD report different reasons for continued opioid abuse (McHugh et al., 2018).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by McLean Hospital Institutional Animal Care and Use Committee.

Author contributions

EC conceived the experimental questions and the required analysis methods, wrote the manuscript, and secured funding. SG, YA-C, and JB conceived of the experiments that generated the data used in this study. SG, YA-C, JB, GD, MN, NC, TL, and EK performed experiments. EC, SG, YA-C, and GD analyzed data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.1035350/full#supplementary-material

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Social play experience in juvenile rats is indispensable for appropriate socio-sexual behavior in adulthood in males but not females

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Social play is a dynamic and rewarding behavior abundantly expressed by most mammals during the juvenile period. While its exact function is debated, various rodent studies on the effects of juvenile social isolation suggest that participating in play is essential to appropriate behavior and reproductive success in adulthood. However, the vast majority of these studies were conducted in one sex only, a critical concern given the fact that there are known sex differences in play's expression: across nearly all species that play, males play more frequently and intensely than females, and there are qualitative sex differences in play patterns. Further limiting our understanding of the importance of play is the use of total isolation to prevent interactions with other juveniles. Here, we employed a novel cage design to specifically prevent play in rats while allowing for other forms of social interaction. We find that play deprivation during the juvenile period results in enduring sex-specific effects on later-life behavior, primarily in males. Males prevented from playing as juveniles exhibited decreased sexual behavior, hypersociability, and increased aggressiveness in adulthood, with no effects on these measures in females. Importantly, play deprivation had no effect on anxiety-like behavior, object memory, sex preference, or social recognition in either sex, showing the specificity of the identified impairments, though there were overall sex differences in many of these measures. Additionally, acute play deprivation impaired performance on a test of prosocial behavior in both sexes, indicating a difference in the motivation and/or ability to acquire this empathy-driven task. Together, these findings provide novel insight into the importance and function of juvenile social play and how this differs in males and females.

KEYWORDS

behavior, social play, play, social isolation, social behavior, sex differences

Introduction

Broadly seen in most mammalian species from rodents to humans, social play (also known as rough-and-tumble play or play-fighting) has fascinated neuroscientists and evolutionary biologists alike for decades. Though animals spend upwards of 20% of their time participating in play during adolescence (Pellis and Pellis, 2009), this well-conserved behavior serves no obvious purpose, yet appears fundamental for appropriate development. Various studies have found that juvenile social isolation in rats leads to impairments in social behavior (Hol et al., 1999; Van den Berg et al., 1999; Von Frijtag et al., 2002), cognition (Einon et al., 1978; Baarendse et al., 2013; Yusufishaq and Rosenkranz, 2013), and sexual behavior (Gerall et al., 1967; Cooke et al., 2000), increases anxiety- and depression-like behavior (Parker and Morinan, 1986; Wright et al., 1991; Arakawa, 2003; Leussis and Andersen, 2008; Lukkes et al., 2009; Cuesta et al., 2020), and impacts susceptibility to addiction-related behaviors (Whitaker et al., 2013; Baarendse et al., 2014).

While these studies provide insight on the impacts of juvenile social isolation writ large, there are three important caveats that preclude the ability to apply them to assess the importance of social play specifically. First, isolation prevents *all* social interaction—both play and non-play—so it is unclear in most of these studies whether and to what extent any identified impairments are due to the lack of play vs. the lack of general social interaction, as well as the additional stress that total isolation induces (Begni et al., 2020). Second, in many of these studies, animals were isolated through adulthood, when testing was conducted; as such, it is also unclear whether and to what extent there may be an effect of acute isolation on the observed phenotypes. Finally, and most notably, the majority of these studies were conducted in only one sex—typically, male subjects—thereby preventing the ability to assess whether there are sex differences in the identified impairments.

This final caveat is of critical importance given there are robust sex differences in social play that are seemingly as well-conserved as play itself. Across nearly all species that play, from rodents to humans, male juveniles play more frequently and intensely than females (see VanRyzin et al., 2020a for review). Additionally, there are known sex differences in the qualitative characteristics of social play interactions (Pellis and Pellis, 1990, 1997). Studies investigating the neural underpinnings of this behavioral sex difference have identified various nodes within the larger circuitry of social behavior, including the medial amygdala (VanRyzin et al., 2019) and lateral septum (Bredewold et al., 2014), which exert sex-specific influences on play following sexual differentiation early in life as part of typical brain development. Indeed, deficits in play are core symptoms of neurodevelopmental disorders like autism spectrum disorder, attention-deficit/hyperactivity disorder, and early onset schizophrenia (Alessandri, 1992; Jones et al., 1994; Møller and Husby, 2000; Jordan, 2003; Helgeland and Torgersen, 2005), many of which also exhibit robust sex differences in diagnosis and symptomology (Aleman et al., 2003; Ramtekkar et al., 2010; Arnett et al., 2015; Halladay et al., 2015; Giordano et al., 2021; Prosperi et al., 2021). The robust and conserved nature of this sex difference, then, speaks to its importance and begs the question: does play serve a different purpose in males compared to females?

Here, we investigate the impact of juvenile play experience on later-life behaviors and how this may differ as a consequence of sex. Juvenile rats of both sexes were deprived of play *via* one of two different methods or were housed under normal conditions, i.e., controls. For the first play deprivation method, we created perforated Plexiglass cage dividers ("play barriers") that could be placed into standard home cages to physically separate the two juvenile rats inside (**Figure 1A**). Improving upon the previous methodology, this manipulation prevents play while still allowing for other forms of social interaction in the visual, auditory, olfactory, and tactile domain. Interaction with a conspecific across a similar physical barrier has been shown to be socially rewarding (Peartree et al., 2012) and to reduce anxiety-like behavior as compared to full isolation, an additional benefit (Klapper-Goldstein et al., 2020). For the second play deprivation method, we eliminated all social interactions (play and non-play) by socially isolating animals *via* single-housing, as done by others. Animals were placed in these or control housing conditions as juveniles, then re-housed in standard group housing around puberty, after which we assessed the impact on various adult behaviors. Supporting our hypothesis, we found that social play experience impacts later-life endpoints in a sex-specific manner. Play deprivation preferentially impacted behaviors within the socio-sexual domain, decreasing sexual behavior, increasing sociability, and increasing aggressiveness in adulthood in males but not females, providing valuable insight into sex differences in the function of this fundamental adolescent behavior.

Materials and methods

Animals and housing conditions

Adult Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were maintained on a 12:12 h reverse light-dark cycle with food and water available *ad libitum*. Animals were mated in our facility and allowed to deliver normally under standard laboratory conditions, with the day of birth designated as postnatal day 0 (P0). Both male and female pups were used, with condition groups and sexes balanced across multiple litters. Rats were weaned on P21 in same-sex, sibling pairs and housed in polycarbonate cages (20 × 40 × 20 cm) with corncob bedding. For the short duration barrier (SDB) experiments, rats either remained in control housing conditions

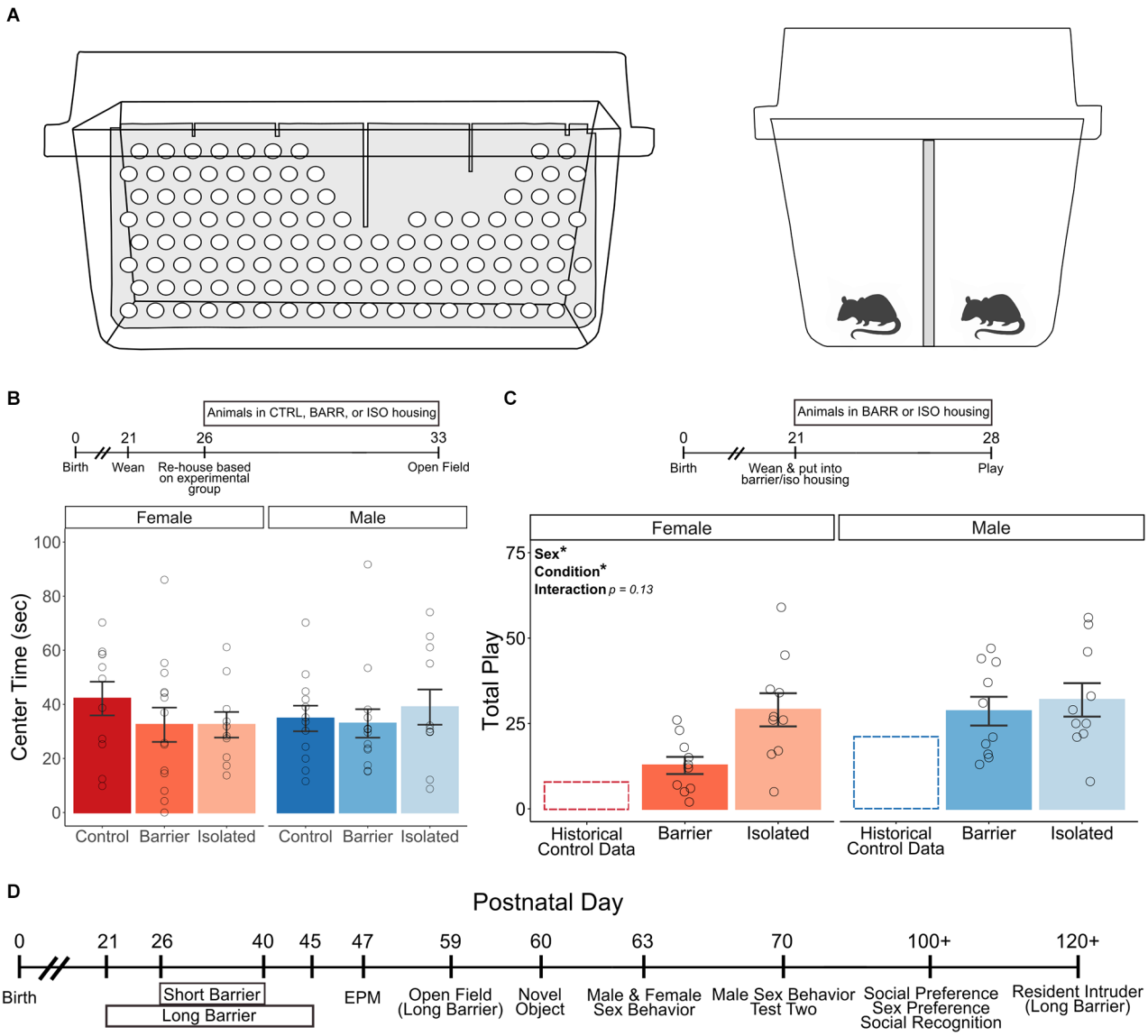

FIGURE 1
Experimental approach and assessment of acute effects of barrier separation/isolation. Cartoons depicting the perforated Plexiglass cage divider (in light gray) used to prevent play in BARR animals **(A)**, viewed from the side (left panel, no animals shown) and straight on (right panel). Acute play deprivation has no effect on anxiety-like behavior as evidenced by center time in an open field test **(B)**, with timeline of experiment shown above graph); additionally, the ability and motivation to engage in play is maintained (**C**, showing the intact play in BARR and ISO animals of both sexes compared to historical control data, with timeline of experiment shown above graph). A timeline of experimental procedures for the remainder of the study is shown in **(D)**. All behavioral tests shown on the timeline were conducted in both the short and long duration barrier studies unless otherwise noted. Bars indicate group means ± SEM, and open circles represent data from individual rats. *$p < 0.05$, $n = 10–14$ per group.

or were subjected to one of two play deprivation groups from P26–40: barrier separation (BARR) or full social isolation (ISO). On P26, a thin, transparent Plexiglass cage divider (Total Plastics, Baltimore, MD, USA; approximately 45 × 21 × 0.5 cm) containing 98 evenly spaced 1.5 cm diameter holes were inserted into the middle of the home cage of BARR rats. This Plexiglass barrier created two identical compartments within the home cage and served to separate the pair of animals and therefore prevent them from physically engaging in social play behavior, while still allowing for visual, olfactory, auditory, and tactile communication between the two. In contrast, on P26, ISO rats were placed alone in a new standard cage and subjected to full social isolation. Control rats continued to be housed in pairs for the full extent of this time period. Animals remained in these conditions until P40, when BARR and ISO animals were re-housed in the same same-sex, sibling pairs as before, under

standard housing conditions. For the long duration barrier (LDB) experiments, the same experimental procedures were applied, except animals were placed in the appropriate housing conditions for a longer period: from P21 (upon weaning) through P45. A total of 196 rats were used across all experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee's regulations at the University of Maryland School of Medicine.

Behavioral testing

All behavioral testing was performed during the dark phase of the light-dark cycle under red light illumination. Unless stated otherwise, all behavioral tests were performed and scored offline by an experimenter blind to condition (at all times) and sex (when appropriate).

Social play (P28)

In a separate cohort of animals used for initial experiments, subjects were individually placed with a sex- and age-matched, control-housed stimulus animal into an enclosure (49 × 37 cm, 24 cm high) with TEK-Fresh cellulose bedding (Envigo, Indianapolis, IN, USA). Only BARR and ISO animals were used in this experiment, as the goal was to assess whether the motivation and/or ability to play was maintained in animals despite the altered housing conditions. Animals were allowed to acclimate to the arena for 2 min, then video recorded for 10 min. Videos were manually scored offline to quantify the number of pounces, pins, and boxing behaviors, summed together as the "total play" exhibited in the test. Further detail on the scoring parameters for each of the three assessed play behaviors can be found in VanRyzin et al. (2020b).

Open field (P33 or P59)

Rats were individually placed in an open polycarbonate arena (78 × 78 cm, 40 cm high) underlaid with a grid delineating perimeter and center regions and video-recorded for 10 min. Videos were manually scored offline for the number of gridline crossings and time spent in the center region of the arena.

Elevated plus maze (P47)

Rats were individually placed in the center of a black polycarbonate plus maze consisting of two open (102.5 × 12 cm) and two closed (102.5 × 12 cm, 45.5 cm high) opposing arms, elevated 72 cm from the ground. Rats were allowed to explore the maze for 5 min while automatically recorded using a video camera and ANY-maze video tracking software (Stoelting, Wood Dale, IL, USA) to determine the percentage of the test duration spent in the open arms of the maze and the total distance traveled.

Novel object recognition (P60)

Rats were individually placed in the same open polycarbonate arena used for the open field test for 5 min and allowed to investigate a pair of identical objects placed on opposite ends of the arena. Following this initial exposure, rats were placed back in their home cages. One hour later, rats were returned to the arena, where they were exposed to the now familiar object and a novel object, again on opposite ends of the arena. Videos were recorded during both sessions and manually scored offline using a virtual stopwatch for the time spent investigating each object on the second test, from which the discrimination ratio [(time with novel object − time with familiar object)/(time with novel object + time with familiar object)] was calculated. The position of objects within the arena and the order of object exposure was counterbalanced across groups.

Female sex behavior (P63)

For the SDB study, intact female rats were hormonally primed ahead of the sexual behavior test by receiving subcutaneous injections of 10 μg of estradiol benzoate (Millipore Sigma, St. Louis, MO, USA) in 0.1 ml sesame oil (Millipore Sigma) 1 and 2 days before testing (P61 and P62, respectively), and 500 μg of progesterone (Millipore Sigma) in 0.1 ml sesame oil 6 h before testing on P63. For the LDB study, female subjects were allowed to naturally cycle without any hormonal priming. Vaginal smears were taken daily to determine the estrus cycle stage, and females were tested on the day of proestrus.

For the sex behavior test itself, females were placed in an enclosure (49 × 37 cm, 24 cm high) with TEK-Fresh cellulose bedding (Envigo, Indianapolis, IN, USA) with an adult male stimulus rat for 10 min. Videos were manually scored for the number of lordoses in response to a mount by the stimulus male and the number of proceptive behaviors (number of hops, darts, and solicitations) as previously described (VanRyzin et al., 2016).

Male sex behavior (P63 and P70)

Intact male rats were tested for expression of copulatory behaviors in response to a hormonally primed female stimulus rat. Two tests of male sex behavior took place: one on P63, and another 1 week later on P70. For both tests, stimulus females were hormonally primed as described above, with subcutaneous injections of estradiol benzoate 1 and 2 days before and progesterone 6 h before testing. For the sex behavior tests, males were placed in an enclosure as described above with a primed adult female stimulus rat for 20 min. Videos were manually

scored offline for the number of mounts, intromissions, and ejaculations as previously described (VanRyzin et al., 2016). The refractory period, or the resting period following an ejaculation before the male rat began exhibiting mounts and intromissions again, was also recorded and used to determine the active time (full time of the 20 min test − length of the refractory period). From this, each animal's mount rate (the total number of mounts, intromissions, and ejaculations divided by the active time and multiplied by 60 to get the mounts per minute) was calculated for each test, as well as the percentage of animals in each group that ejaculated on one or both tests.

Social preference (P100+)

For the social preference test, a two-chambered open-topped polycarbonate apparatus (100 × 50 cm, 35 cm high) was used. In the corner of one chamber ("social chamber"), a novel same-sex adult (P60+) stimulus rat was placed under a small (20 × 20 × 20 cm) clear polycarbonate box ("stimulus box") containing small 1.25 cm holes to allow the test rat to see, hear, smell, and have some tactile interactions with the stimulus rat. In the corner of the other chamber ("empty chamber"), an identical clear polycarbonate box was placed without a stimulus rat. Test rats were individually placed in the neutral zone of this apparatus and allowed to freely explore for 5 min while video recorded and automatically tracked using ANY-maze software. The time spent in each chamber and the time spent nearby (within 5 cm, deemed the "interaction zone") each stimulus box was recorded, and the percentage of time near the social box (time spent in the interaction zone of the social chamber/time spent in the interaction zone of the social chamber + time spent in the interaction zone of the empty chamber) was calculated, as well as the ratio of time spent near the social chamber to time spent near the empty chamber. The position of the social chamber and the empty chamber within the arena was counterbalanced across groups.

Sex preference (P100+)

Using the same apparatus and procedure as described above for the social preference test, the sex preference test assessed the amount of time a test rat spent interacting with a same-sex vs. an opposite-sex stimulus animal. In this test, one chamber of the apparatus contained a novel adult (P60+) male rat under the stimulus box, while the other chamber contained a novel adult female rat under the stimulus box. As before, test rats were individually placed in the neutral zone of the apparatus and allowed to freely explore for 5 min while video recorded and automatically tracked using ANY-maze. From this, the percentage of time spent closely interacting with the opposite-sex stimulus animal (time spent in the interaction zone of the opposite-sex chamber/time spent in the interaction zone of the opposite-sex chamber + time spent in the interaction zone of the same-sex chamber) was calculated, as well as the ratio of time spent near the opposite-sex chamber to time spent near the same-sex chamber. The position of the chamber containing the male stimulus animal and the chamber containing the female stimulus animal within the arena was counterbalanced across groups.

Social recognition (P100+)

To allow for habituation and increase social motivation, rats were singly housed in a test cage identical to their home cage for 2 h before the start of the test. After 2 h, a novel same-sex juvenile (P24–30) stimulus rat was placed into the test cage with the test rat for 5 min ("Train" trial) and their interactions were video-recorded. After 5 min, the stimulus rat was removed, and the test rat remained in the test cage for a retention interval of 30 min, after which the same stimulus rat (now familiar) was placed back in the test cage. Interactions between the test rat and the stimulus rat were again recorded for 5 min ("Test" trial). Videos were manually scored offline for the time spent by the test rat investigating the stimulus rat in both tests, and the ratio of the time spent interacting with the stimulus rat on the Test trial (familiar) compared to the Train trial (novel) was calculated. Separately, a control experiment was independently conducted which followed the same procedure; however, a novel stimulus rat was placed in the cage with the test rat in both the Train trial and the Test trial, to control for any effects of a second interaction trial in general, unrelated to recognition of the stimulus animal or the lack thereof.

Resident intruder assay (P120+)

We assessed aggressive behavior in the resident-intruder assay in adult males using a procedure modified from Koolhaas et al. (2013). Males were isolated in their home cages for 48 h prior to the start of the test. On the test day, a novel, smaller (weighing >150 g less than the test animal) stimulus male was placed into the cage with the test male. Their interactions were recorded for 15 min, after which the stimulus male was removed from the test animal's home cage. After a 30 min inter-trial interval, the test was repeated with a novel stimulus male. Videos were manually scored offline for the following behaviors: keep downs, in which the test animal pins down the stimulus animal by placing its front paws on the chest of the stimulus; lateral threats, in which the test animal pushes its flank towards the stimulus; upright postures, in which both animals stand up on their hind legs and grasp at each other's front legs; mounting behavior; and overall clinch attacks. The

total number of aggressive behaviors displayed in each test was calculated.

Empathy/prosocial helping behavior

We assessed empathy behavior using a paradigm modified from Kight et al. (2021). Rats of both sexes were weaned on P21 into CTRL or BARR housing conditions, as described above. On P26, animals were individually placed in an enclosure (49 × 37 cm, 24 cm high) with TEK-Fresh cellulose bedding (Envigo, Indianapolis, IN, USA) containing an empty and open 14 × 8 × 9 cm clear polycarbonate confinement box and allowed to explore for 10 min to habituate to the box and arena. This confinement box contained a hinged door that is blocked by a lever when closed, requiring the animal outside the confinement box to push the lever open in order for the animal inside the confinement box to freely escape. On P27, one animal from a sex- and condition-matched cagemate pair was placed inside the confinement box. A funnel filled with ice was positioned over the confinement box, dripping cool water inside and thus motivating the animal to seek release. Video recording and a count-up timer began when the other member of the cagemate pair (the test subject) was placed in the arena. If at any time the test subject released their cagemate from the confinement box, the recording was stopped and the time of release was recorded. Animals were allowed to interact for 10 s, then swiftly returned to CTRL or BARR housing conditions. If 15 min elapsed without release, the lever blocking the door was loosened slightly by the experimenter (turned 90 degrees such that there was no gap in the door but that the lever no longer blocked the exit) to facilitate learning. If an additional 5 min passed (20 min total testing time), the recording was stopped and the door opened further to allow for free exit. In this case, the time of release was denoted as 20 min (the maximum). This procedure was repeated once daily until P38, with each member of the cagemate pair serving the same role each day (i.e., there was no switching as to which member of the pair was the test subject vs. the confined subject).

Statistical analysis

Specific values for test statistics, p values, and effect sizes are listed in **Table 1** and referenced in the text, when appropriate. Statistical analysis was performed using RStudio (RStudio Team, 2021; version 1.4.1106) and GraphPad Prism (GraphPad Software, San Diego, CA, USA; version 7.04). All data were initially analyzed with a Shapiro-Wilk normality test to determine if data were normally distributed. Data which were normally distributed were then analyzed with a two-way ANOVA with factors for sex and juvenile housing condition (CTRL, BARR, or ISO) unless otherwise stated. If a significant main effect of housing condition or a significant interaction was detected, *post hoc* analysis was conducted using a Tukey's honestly significant difference test to determine which groups differed. If a significant main effect of condition was detected in cases in which a one-way ANOVA was conducted (i.e., tests in which sex was not a factor, such as male sex behavior), *post hoc* analysis was conducted using Fisher's least significant difference procedure, as there were three groups (Meier, 2006). In some cases (Novel Object Recognition, Social Preference, and Sex Preference tests), one-sample t-tests were used to determine whether group means differed from chance; additionally, paired t-tests were used to compare the Train trial to the Test trial for the Social Recognition test. Data which were not normally distributed were analyzed using Kruskal-Wallis tests or Wilcoxon rank sum tests when appropriate, as described in **Table 1**. Analyses were considered significant when $p < 0.05$.

Factor analysis

We chose *a priori* to conduct factor analysis on the Short Duration Barrier and Long Duration Barrier studies separately, to determine whether the identified factors would be similar or different across the two experiments. Only subjects that had complete datasets were included in the factor analysis. Data were normalized by log transformation, then initially analyzed for adequacy for factor analysis using the Kaiser-Meyer-Olkin factor adequacy test and Bartlett's test of sphericity for the correlation matrix of the dataset. In both the SDB and LDB studies, female data was deemed insufficiently correlated and therefore inadequate for factor analysis based on these indices (Kaiser-Meyer-Olkin MSA below 0.5 for both studies; Bartlett's sphericity $p > 0.05$). For this reason, we conducted a factor analysis on only the male data, a choice supported by the fact that the vast majority of our observed effects of play deprivation were seen in males but not females. Using male data alone, datasets from both the SDB and LDB study indicated appropriateness for factor analysis, with an overall Kaiser-Meyer-Olkin MSA above 0.5 (SDB: MSA = 0.53; LDB: MSA = 0.53) and a significant p-value for Bartlett's test (SDB: $p = 0.001$; LDB: $p < 0.001$). This resulted in an overall n of 23 for both studies (SDB: $n = 6$ for CTRL; $n = 8$ for BARR; $n = 9$ for ISO. LDB: $n = 8$ for CTRL; $n = 7$ for BARR; $n = 8$ for ISO).

Factor analysis was then conducted using varimax rotation with a factor-loading cutoff of 0.5. The resulting factors were retained if their eigenvalue was greater than 1 (based on the Kaiser criterion), which generated a three-factor solution for both the SDB and LDB datasets. Behavioral variables which had low communality (below 0.3) and did not load onto any of the three factors above the factor loading cutoff of 0.3 were removed, and analysis was repeated using the parameters described above, again identifying a three-factor solution for both datasets. We then calculated individual factor scores for each subject and analyzed these scores for an effect of condition independently by factor using a one-way ANOVA, as described further above.

TABLE 1 Summary of statistical parameters.

Test	Data structure	Type of test	Description of analysis	Test value	*p*-value	Effect size
Open Field—Acute Deprivation						
Center Time	Non-normal	Wilcoxon rank-sum test	Effect of sex	$W = 657$	0.919	$r = 0.0126$
	Non-normal	Kruskal-Wallis test: males	Effect of condition	$\chi^2_{(2)} = 0.893$	0.64	$\eta^2_{(H)} = -0.033$
	Non-normal	Kruskal-Wallis test: females	Effect of condition	$\chi^2_{(2)} = 1.9$	0.387	$\eta^2_{(H)} = -0.003$
Play—Acute Deprivation						
Total Play	**Normal distribution**	**Two-way ANOVA**	**Main effect: sex**	$\mathbf{F_{(1,36)} = 4.929}$	**0.036**	$\eta^2_{(H)} = -0.101$
	Normal distribution	**Two-way ANOVA**	**Main effect: condition**	$\mathbf{F_{(1,36)} = 5.358}$	**0.026**	$\eta^2_{(H)} = -0.11$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(1,36)} = 2.357$	0.134	$\eta^2_{(H)} = 0.048$
Elevated Plus Maze—SDB						
Time in Open Arms (%)	Normal distribution	Two-way ANOVA	Main effect: sex	$F_{(1,70)} = 2.835$	0.097	$\eta^2 = 0.038$
	Normal distribution	Two-way ANOVA	Main effect: condition	$F_{(2,70)} = 1.330$	0.271	$\eta^2 = 0.037$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(2,70)} = 0.107$	0.899	$\eta^2 = 0.003$
Distance Traveled	**Normal distribution**	**Two-way ANOVA**	**Main effect: sex**	$\mathbf{F_{(1,70)} = 4.704}$	**0.034**	$\boldsymbol{\eta^2 = 0.065}$
	Normal distribution	Two-way ANOVA	Main effect: condition	$F_{(2,70)} = 0.183$	0.833	$\eta^2 = 0.005$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(2,70)} = 0.912$	0.406	$\eta^2 = 0.025$
Elevated Plus Maze—LDB						
Time in Open Arms (%)	**Normal distribution**	**Two-way ANOVA**	**Main effect: sex**	$\mathbf{F_{(1,62)} = 7.654}$	**0.007**	$\boldsymbol{\eta^2 = 0.106}$
	Normal distribution	Two-way ANOVA	Main effect: condition	$F_{(2,62)} = 3.038$	0.055	$\eta^2 = 0.089$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(2,62)} = 0.149$	0.862	$\eta^2 = 0.005$
Distance Traveled	**Normal distribution**	**Two-way ANOVA**	**Main effect: sex**	$\mathbf{F_{(1,62)} = 8.595}$	**0.005**	$\boldsymbol{\eta^2 = 0.113}$
	Normal distribution	**Two-way ANOVA**	**Main effect: condition**	$\mathbf{F_{(2,62)} = 10.326}$	**<0.001**	$\boldsymbol{\eta^2 = 0.250}$
		Two-way ANOVA: Tukey post-hoc	***CTRL vs. BARR***	***n/a***	**<0.001**	$\boldsymbol{d = 1.655}$
		Two-way ANOVA: Tukey post-hoc	*CTRL vs. ISO*	*n/a*	0.544	$d = 0.298$
		Two-way ANOVA: Tukey post-hoc	***BARR vs. ISO***	***n/a***	**0.003**	$\boldsymbol{d = 0.937}$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(2,62)} = 0.239$	0.788	$\eta^2 = 0.008$

(*Continued*)

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TABLE 1 (Continued)

Test	Data structure	Type of test	Description of analysis	Test value	*p*-value	Effect size
Open Field Test—LDB						
Center Time	**Non-normal**	**Wilcoxon rank-sum test**	**Effect of sex**	$W = 1{,}080.5$	<0.001	$r = 0.428$
	Non-normal	Kruskal-Wallis test: males	Effect of condition	$\chi^2_{(2)} = 0.024$	0.988	$d = -0.055$
	Non-normal	Kruskal-Wallis test: females	Effect of condition	$\chi^2_{(2)} = 0.234$	0.89	$d = -0.052$
Line Crossings	**Normal distribution**	**Two-way ANOVA**	**Main effect: sex**	$F_{(1,70)} = 19.673$	<0.001	$\eta 2 = 0.221$
	Normal distribution	**Two-way ANOVA**	**Main effect: condition**	$F_{(2,70)} = 4.649$	0.013	$\eta^2 = 0.117$
		Two-way ANOVA: Tukey post-hoc	***CTRL vs. BARR***	***n/a***	0.01	$d = 0.709$
		Two-way ANOVA: Tukey post-hoc	*CTRL vs. ISO*	*n/a*	0.117	$d = 0.509$
		Two-way ANOVA: Tukey post-hoc	*BARR vs. ISO*	*n/a*	0.512	$d = 0.311$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(2,70)} = 0.777$	0.464	$\eta^2 = 0.022$
Novel Object—SDB						
Discrimination Ratio	**Normal distribution**	**One sample *t*-test**	**Male CTRL vs. 0**	$t_{(11)} = 3.369$	0.006	$d = 0.972$
	Normal distribution	**One sample *t*-test**	**Male BARR vs. 0**	$t_{(13)} = 4.537$	<0.001	$d = 1.213$
	Normal distribution	**One sample *t*-test**	**Male ISO vs. 0**	$t_{(12)} = 3.437$	0.005	$d = 0.953$
	Normal distribution	**One sample *t*-test**	**Female CTRL vs. 0**	$t_{(13)} = 2.822$	0.014	$d = 0.754$
	Normal distribution	**One sample *t*-test**	**Female BARR vs. 0**	$t_{(13)} = 3.322$	0.006	$d = 0.888$
	Normal distribution	**One sample *t*-test**	**Female ISO vs. 0**	$t_{(10)} = 3.932$	0.003	$d = 1.186$
	Normal distribution	Two-way ANOVA	Main effect: sex	$F_{(1,72)} = 0.421$	0.519	$\eta^2 = 0.008$
	Normal distribution	Two-way ANOVA	Main effect: condition	$F_{(2,72)} = 1.334$	0.27	$\eta^2 = 0.036$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(2,72)} = 0.634$	0.533	$\eta^2 = 0.017$
Novel Object—LDB						
Discrimination Ratio	**Normal distribution**	**One sample *t*-test**	**Male CTRL vs. 0**	$t_{(10)} = 2.631$	0.025	$d = 0.793$
	Normal distribution	**One sample *t*-test**	**Male BARR vs. 0**	$t_{(8)} = 3.806$	0.005	$d = 1.269$
	Normal distribution	**One sample *t*-test**	**Male ISO vs. 0**	$t_{(14)} = 3.943$	0.001	$d = 1.018$
	Normal distribution	**One sample *t*-test**	**Female CTRL vs. 0**	$t_{(10)} = 4.947$	<0.001	$d = 1.492$
	Normal distribution	**One sample *t*-test**	**Female BARR vs. 0**	$t_{(11)} = 3.541$	0.005	$d = 1.022$
	Normal distribution	**One sample *t*-test**	**Female ISO vs. 0**	$t_{(12)} = 0.001$	0.003	$d = 1.165$

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TABLE 1 (Continued)

Test	Data structure	Type of test	Description of analysis	Test value	*p*-value	Effect size
	Normal distribution	Two-way ANOVA	Main effect: sex	$F_{(1,65)} = 0.756$	0.388	$\eta^2 = 0.013$
	Normal distribution	Two-way ANOVA	Main effect: condition	$F_{(2,65)} = 0.416$	0.661	$\eta^2 = 0.013$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(2,65)} = 0.723$	0.489	$\eta^2 = 0.022$
Female Sex Behavior—SDB						
Lordosis Quotient	Non-normal	Kruskal-Wallis test	Effect of condition	$\chi^2_{(2)} = 3.257$	0.196	$\eta^2_{(H)} = 0.034$
Proceptive Behaviors	Normal distribution	One-way ANOVA	Effect of condition	$F_{(2,37)} = 0.137$	0.873	$\eta^2 = 0.007$
Female Sex Behavior—LDB						
Lordosis Quotient	Non-normal	Kruskal-Wallis test	Effect of condition	$\chi^2_{(2)} = 3.52$	0.172	$\eta^2_{(H)} = 0.056$
Proceptive Behaviors	Non-normal	Kruskal-Wallis test	Effect of condition	$\chi^2_{(2)} = 1.61$	0.447	$\eta^2_{(H)} = -0.014$
Male Sex Behavior—SDB						
Average Number of Mounts	Normal distribution	One-way ANOVA	Effect of condition	$F_{(2,35)} = 2.786$	0.075	0.216
Average Number of Intromissions	Normal distribution	One-way ANOVA	Effect of condition	$F_{(2,35)} = 2.165$	0.13	0.137
Average Mount Rate	**Normal distribution**	**One-way ANOVA**	**Effect of condition**	$\mathbf{F_{(2,35)} = 4.818}$	**0.014**	**0.11**
		Kruskal-Wallis: Fisher's LSD post-hoc	***CTRL vs. BARR***	***n/a***	**0.03**	$\mathbf{d = 0.618}$
		Kruskal-Wallis: Fisher's LSD post-hoc	***CTRL vs. ISO***	***n/a***	**0.005**	$\mathbf{d = 0.981}$
		Kruskal-Wallis: Fisher's LSD post-hoc	*BARR vs. ISO*	n/a	0.407	$d = 0.221$
Percent that Ejaculated	Non-normal	Chi-square goodness of fit test	% ejaculated vs. 90% - CTRL	$\chi^2 = 0.818$	0.366	$\varphi = 0.273$
		Chi-square goodness of fit test	% ejaculated vs. 90% - BARR	$\chi^2 = 0.127$	0.722	$\varphi = 0.095$
		Chi-square goodness of fit test	% ejaculated vs. 90% - ISO	$\chi^2 = 0.419$	0.518	$\varphi = 0.18$
Male Sex Behavior—LDB						
Average Number of Mounts	Normal distribution	**One-way ANOVA**	**Effect of condition**	$\mathbf{F_{(2,33)} = 3.949}$	**0.029**	$\mathbf{\eta^2 = 0.193}$
		One-way ANOVA: Fisher's LSD post-hoc	***CTRL vs. BARR***	***n/a***	**0.03**	$\mathbf{d = 0.654}$
		One-way ANOVA: Fisher's LSD post-hoc	***CTRL vs. ISO***	***n/a***	**0.014**	$\mathbf{d = 0.626}$
		One-way ANOVA: Fisher's LSD post-hoc	*BARR vs. ISO*	*n/a*	0.767	$d = 0.06$
Average Number of Intromissions	Non-normal	**Kruskal-Wallis test**	**Effect of condition**	$\mathbf{\chi^2_{(2)} = 8.304}$	**0.016**	$\eta^2_{(H)} = 0.191$

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TABLE 1 (Continued)

Test	Data structure	Type of test	Description of analysis	Test value	*p*-value	Effect size
		Kruskal-Wallis: Fisher's LSD post-hoc	***CTRL vs. BARR***	***n/a***	**0.026**	***d* = 0.796**
		Kruskal-Wallis: Fisher's LSD post-hoc	***CTRL vs. ISO***	***n/a***	**0.012**	***d* = 0.906**
		Kruskal-Wallis: Fisher's LSD post-hoc	*BARR vs. ISO*	n/a	0.763	$d = 0.182$
Average Mount Rate	Non-normal	Kruskal-Wallis test	Effect of condition	$\chi^2_{(2)} = 3.779$	0.151	$\eta^2_{(H)} = 0.054$
Percent that Ejaculated	Non-normal	Chi-square goodness of fit test	% ejaculated vs. 90% - CTRL	$\chi^2 = 0.056$	0.814	$\varphi = 0.084$
		Chi-square goodness of fit test	**% ejaculated vs. 90% - BARR**	$\chi^2 = 10$	**0.002**	**φ = 1**
		Chi-square goodness of fit test	**% ejaculated vs. 90% - ISO**	$\chi^2 = 11.701$	**<0.001**	**φ = 0.949**
Social Preference—SDB						
Percent of Time Near Social Box	**Normal distribution**	**One sample *t*-test**	**Male CTRL vs. 50%**	$t_{(11)} = 8.363$	**<0.001**	***d* = 2.414**
	Normal distribution	**One sample *t*-test**	**Male BARR vs. 50%**	$t_{(13)} = 19.047$	**<0.001**	***d* = 5.091**
	Normal distribution	**One sample *t*-test**	**Male ISO vs. 50%**	$t_{(12)} = 6.702$	**<0.001**	***d* = 1.859**
	Normal distribution	**One sample *t*-test**	**Female CTRL vs. 50%**	$t_{(13)} = 10.423$	**<0.001**	***d* = 2.786**
	Normal distribution	**One sample *t*-test**	**Female BARR vs. 50%**	$t_{(13)} = 7.674$	**<0.001**	***d* = 2.051**
	Normal distribution	**One sample *t*-test**	**Female ISO vs. 50%**	$t_{(11)} = 6.631$	**<0.001**	***d* = 1.914**
	Normal distribution	**Two-way ANOVA**	**Main effect: sex**	$F_{(1,73)} = 18.582$	**<0.001**	$\eta^2 = 0.142$
	Normal distribution	**Two-way ANOVA**	**Main effect: condition**	$F_{(2,73)} = 8.168$	**<0.001**	$\eta^2 = 0.126$
	Normal distribution	**Two-way ANOVA**	**Interaction: sex × condition**	$F_{(2,73)} = 10.672$	**<0.001**	$\eta^2 = 0.165$
		Two-way ANOVA: Tukey post-hoc	*Male CTRL vs. Female CTRL*	n/a	0.778	$d = 0.579$
		Two-way ANOVA: Tukey post-hoc	*Female BARR vs. Female CTRL*	n/a	1	$d = 0.006$
		Two-way ANOVA: Tukey post-hoc	***Male BARR vs. Female CTRL***	**n/a**	**<0.001**	***d* = 2.939**
		Two-way ANOVA: Tukey post-hoc	*Female ISO vs. Female CTRL*	n/a	0.99	$d = 0.248$
		Two-way ANOVA: Tukey post-hoc	*Male ISO vs. Female CTRL*	n/a	0.999	$d = 0.147$
		Two-way ANOVA: Tukey post-hoc	*Female BARR vs. Male CTRL*	n/a	0.786	$d = 0.501$

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TABLE 1 (Continued)

Test	Data structure	Type of test	Description of analysis	Test value	*p*-value	Effect size
		Two-way ANOVA: Tukey post-hoc	***Male BARR vs. Male CTRL***	**n/a**	**<0.001**	***d* = 1.911**
		Two-way ANOVA: Tukey post-hoc	*Female ISO vs. Male CTRL*	n/a	0.984	$d = 0.247$
		Two-way ANOVA: Tukey post-hoc	*Male ISO vs. Male CTRL*	n/a	0.936	$d = 0.247$
		Two-way ANOVA: Tukey post-hoc	***Male BARR vs. Female BARR***	**n/a**	**<0.001**	***d* = 2.515**
		Two-way ANOVA: Tukey post-hoc	*Female ISO vs. Female BARR*	n/a	0.991	$d = 0.215$
		Two-way ANOVA: Tukey post-hoc	*Male ISO vs. Female BARR*	n/a	0.999	$d = 0.126$
		Two-way ANOVA: Tukey post-hoc	***Female ISO vs. Male BARR***	**n/a**	**<0.001**	***d* = 2.056**
		Two-way ANOVA: Tukey post-hoc	***Male ISO vs. Male BARR***	**n/a**	**<0.001**	***d* = 2.156**
		Two-way ANOVA: Tukey post-hoc	*Male ISO vs. Female ISO*	n/a	1	$d = 0.082$
Ratio Time Near Social Box	**Non-normal**	**Wilcoxon rank-sum test**	**Effect of sex**	$W = 449$	**0.001**	$r = 0.365$
	Non-normal	**Kruskal-Wallis test: males**	**Effect of condition**	$\chi^2_{(2)} = 19.374$	**<0.001**	$\eta^2_{(H)} = 0.483$
		Kruskal-Wallis: Wilcoxon post-hoc	***Male CTRL vs. BARR***	$W = 15$	**<0.001**	$r = 0.696$
		Kruskal-Wallis: Wilcoxon post-hoc	*Male CTRL vs. ISO*	$W = 98$	0.295	$r = 0.218$
		Kruskal-Wallis: Wilcoxon post-hoc	***Male BARR vs. ISO***	$W = 170$	**<0.001**	$r = 0.738$
	Non-normal	Kruskal-Wallis test: females	Effect of condition	$\chi^2_{(2)} = 0.557$	0.757	$\eta^2_{(H)} = -0.039$
Social Preference—LDB						
Percent of Time Near Social Box	**Normal distribution**	**One sample *t*-test**	**Male CTRL vs. 50%**	$t_{(11)} = 6.639$	**<0.001**	$d = 1.917$
	Normal distribution	**One sample *t*-test**	**Male BARR vs. 50%**	$t_{(11)} = 6.794$	**<0.001**	**d = 1.961**
	Normal distribution	**One sample *t*-test**	**Male ISO vs. 50%**	$t_{(10)} = 3.837$	**0.003**	$d = 1.157$
	Normal distribution	**One sample *t*-test**	**Female CTRL vs. 50%**	$t_{(11)} = 5.178$	**<0.001**	**d = 1.495**
	Normal distribution	**One sample *t*-test**	**Female BARR vs. 50%**	$t_{(11)} = 5.547$	**<0.001**	**d = 1.601**
	Normal distribution	**One sample *t*-test**	**Female ISO vs. 50%**	$t_{(12)} = 2.520$	**0.027**	$d = 0.699$
	Normal distribution	**Two-way ANOVA**	**Main effect: sex**	$F_{(1,66)} = 9.471$	**0.003**	$\eta^2 = 0.121$

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TABLE 1 (Continued)

Test	Data structure	Type of test	Description of analysis	Test value	*p*-value	Effect size
	Normal distribution	Two-way ANOVA	Main effect: condition	$F_{(2,66)} = 1.192$	0.31	$\eta^2 = 0.03$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(2,66)} = 0.239$	0.788	$\eta^2 = 0.006$
Ratio Time Near Social Box	**Non-normal**	**Wilcoxon rank-sum test**	**Effect of sex**	$\mathbf{W = 354}$	**<0.001**	$\mathbf{r = 0.390}$
	Non-normal	Kruskal-Wallis test: males	Effect of condition	$\chi^2_{(2)} = 0.134$	0.935	$\eta^2_{(H)} = -0.058$
	Non-normal	Kruskal-Wallis test: females	Effect of condition	$\chi^2_{(2)} = 2.51$	0.285	$\eta^2_{(H)} = 0.015$
Sex Preference—SDB						
Percent Time Near Opposite-Sex Box	**Normal distribution**	**One sample *t*-test**	**Male CTRL vs. 50%**	$\mathbf{t_{(11)} = 3.953}$	**0.002**	$\mathbf{d = 1.141}$
	Normal distribution	**One sample *t*-test**	**Male BARR vs. 50%**	$\mathbf{t_{(13)} = 4.009}$	**0.001**	$\mathbf{d = 1.071}$
	Normal distribution	**One sample *t*-test**	**Male ISO vs. 50%**	$\mathbf{t_{(12)} = 2.423}$	**0.032**	$\mathbf{d = 0.672}$
	Normal distribution	**One sample *t*-test**	**Female CTRL vs. 50%**	$\mathbf{t_{(13)} = 2.355}$	**0.035**	$\mathbf{d = 0.629}$
	Normal distribution	**One sample *t*-test**	**Female BARR vs. 50%**	$\mathbf{t_{(13)} = 3.007}$	**0.01**	$\mathbf{d = 0.804}$
	Normal distribution	**One sample *t*-test**	**Female ISO vs. 50%**	$\mathbf{t_{(11)} = 3.558}$	**0.004**	$\mathbf{d = 1.027}$
	Normal distribution	Two-way ANOVA	Main effect: sex	$F_{(1,73)} = 0.037$	0.849	$\eta^2 = 0.001$
	Normal distribution	Two-way ANOVA	Main effect: condition	$F_{(2,73)} = 0.559$	0.574	$\eta^2 = 0.015$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(2,73)} = 0.996$	0.374	$\eta^2 = 0.026$
Ratio Time Near Opposite-Sex Box	Non-normal	Wilcoxon rank-sum test	Effect of sex	W =795	0.887	$r = 0.017$
	Non-normal	Kruskal-Wallis test: males	Effect of condition	$\chi^2_{(2)} = 3.334$	0.189	$\eta^2_{(H)} = 0.037$
	Non-normal	Kruskal-Wallis test: females	Effect of condition	$\chi^2_{(2)} = 0.017$	0.992	$\eta^2_{(H)} = -0.054$
Sex Preference—LDB						
Percent Time Near Opposite-Sex Box	**Normal distribution**	**One sample *t*-test**	**Male CTRL vs. 50%**	$\mathbf{t_{(11)} = 3.313}$	**0.007**	$\mathbf{d = 0.956}$
	Normal distribution	One sample *t*-test	Male BARR vs. 50%	$t_{(11)} = 1.966$	0.075	$d = 0.567$
	Normal distribution	**One sample *t*-test**	**Male ISO vs. 50%**	$\mathbf{t_{(11)} = 2.339}$	**0.04**	$\mathbf{d = 0.675}$
	Normal distribution	**One sample *t*-test**	**Female CTRL vs. 50%**	$\mathbf{t_{(11)} = 4.881}$	**<0.001**	$\mathbf{d = 1.409}$
	Normal distribution	One sample *t*-test	Female BARR vs. 50%	$t_{(11)} = 1.557$	0.148	$d = 0.449$
	Normal distribution	**One sample *t*-test**	**Female ISO vs. 50%**	$\mathbf{t_{(12)} = 4.034}$	**0.002**	$\mathbf{d = 1.119}$
	Normal distribution	Two-way ANOVA	Main effect: sex	$F_{(1,67)} = 0.741$	0.392	$\eta^2 = 0.011$
	Normal distribution	Two-way ANOVA	Main effect: condition	$F_{(2,67)} = 0.811$	0.449	$\eta^2 = 0.023$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(2,67)} = 1.167$	0.317	$\eta^2 = 0.033$
Ratio Time Near Opposite-Sex	Non-normal	Wilcoxon rank-sum test	Effect of sex	$W = 595$	0.437	$r = 0.092$
	Non-normal	Kruskal-Wallis test: males	Effect of condition	$\chi^2_{(2)} = 1.5$	0.472	$\eta^2_{(H)} = -0.015$
	Non-normal	Kruskal-Wallis test: females	Effect of condition	$\chi^2_{(2)} = 5.059$	0.08	$\eta^2_{(H)} = 0.09$

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TABLE 1 (Continued)

Test	Data structure	Type of test	Description of analysis	Test value	*p*-value	Effect size
Social Recognition—SDB						
Interaction Time	**Normal distribution**	**Paired *t*-test**	**Male CTRL-Train vs. Test**	**$t_{(12)} = 2.336$**	**0.038**	**$d = 0.592$**
	Normal distribution	Paired *t*-test	Male BARR-Train vs. Test	$t_{(11)} = 1.892$	0.085	$d = 0.518$
	Normal distribution	**Paired *t*-test**	**Male ISO-Train vs. Test**	**$t_{(12)} = 2.491$**	**0.028**	**$d = 0.746$**
	Normal distribution	**Paired *t*-test**	**Female CTRL-Train vs. Test**	**$t_{(9)} = 4.316$**	**0.002**	**$d = 1.276$**
	Normal distribution	**Paired *t*-test**	**Female BARR-Train vs. Test**	**$t_{(8)} = 2.692$**	**0.027**	**$d = 1.055$**
	Normal distribution	**Paired *t*-test**	**Female ISO-Train vs. Test**	**$t_{(8)} = 4.791$**	**0.001**	**$d = 1.455$**
Ratio of Investigation Duration	**Non-normal**	**Wilcoxon rank-sum test**	**Effect of sex**	**$W = 253$**	**<0.001**	$r = 0.446$
	Non-normal	Kruskal-Wallis test: males	Effect of condition	$\chi^2_{(2)} = 0.03$	0.985	$\eta^2_{(H)} = -0.056$
	Non-normal	Kruskal-Wallis test: females	Effect of condition	$\chi^2_{(2)} = 0.22$	0.896	$\eta^2_{(H)} = -0.072$
Social Recognition-LDB						
Interaction Time	**Normal distribution**	**Paired *t*-test**	**Male CTRL-Train vs. Test**	**$t_{(11)} = 2.653$**	**0.022**	**$d = 0.713$**
	Normal distribution	**Paired *t*-test**	**Male BARR-Train vs. Test**	**$t_{(11)} = 2.425$**	**0.033**	**$d = 0.707$**
	Normal distribution	**Paired *t*-test**	**Male ISO-Train vs. Test**	**$t_{(11)} = 3.019$**	**0.012**	**$d = 0.629$**
	Normal distribution	**Paired *t*-test**	**Female CTRL-Train vs. Test**	**$t_{(11)} = 6.737$**	**<0.001**	**$d = 1.861$**
	Normal distribution	**Paired *t*-test**	**Female BARR-Train vs. Test**	**$t_{(11)} = 10.557$**	**<0.001**	**$d = 3.492$**
	Normal distribution	**Paired *t*-test**	**Female ISO-Train vs. Test**	**$t_{(12)} = 5.839$**	**<0.001**	**$d = 1.477$**
Ratio of Investigation Duration	**Non-normal**	**Wilcoxon rank-sum test**	**Effect of sex**	**$W = 110$**	**<0.001**	**$r = 0.718$**
	Non-normal	Kruskal-Wallis test: males	Effect of condition	$\chi^2_{(2)} = 0.126$	0.939	$\eta^2_{(H)} = -0.057$
	Non-normal	Kruskal-Wallis test: females	Effect of condition	$\chi^2_{(2)} = 3.552$	0.169	$\eta^2_{(H)} = 0.046$
Resident Intruder Assay-LDB						
Average Number of Keep Downs	Normal distribution	One-way ANOVA	Effect of condition	$F_{(2,27)} = 1.773$	0.189	$\eta^2 = 0.116$
Average Number of Lateral Threats	Non-normal	Kruskal-Wallis test	Effect of condition	$\chi^2_{(2)} = 4.08$	0.13	$\eta^2_{(H)} = 0.077$
Average Number of Upright Postures	Non-normal	Kruskal-Wallis test	Effect of condition	$\chi^2_{(2)} = 4.856$	0.088	$\eta^2_{(H)} = 0.106$
Average Number of Clinch Attacks	Non-normal	Kruskal-Wallis test	Effect of condition	$\chi^2_{(2)} = 0.799$	0.671	$\eta^2_{(H)} = -0.045$
Average Number of Aggressive Behaviors (Total)	**Normal distribution**	**One-way ANOVA**	**Effect of condition**	**$F_{(2,27)} = 4.815$**	**0.016**	**$\eta^2 = 0.263$**
		One-way ANOVA: Fisher's LSD post-hoc	*CTRL vs. BARR*	*n/a*	0.056	$d = 0.863$

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TABLE 1 (Continued)

Test	Data structure	Type of test	Description of analysis	Test value	*p*-value	Effect size
		One-way ANOVA: Fisher's LSD post-hoc	***CTRL vs. ISO***	***n/a***	**0.005**	**$d = 1.481$**
		One-way ANOVA: Fisher's LSD post-hoc	*BARR vs. ISO*	*n/a*	0.3	$d = 0.456$
Empathy/Prosocial Helping Behavior						
Average Mean Latency to Release Cagemate—Days 1–6	**Normal distribution**	**Two-way ANOVA**	**Main effect: sex**	**$F_{(1,15)} = 4.757$**	**0.046**	**$\eta^2 = 0.217$**
	Normal distribution	**Two-way ANOVA**	**Main effect: condition**	**$F_{(1,15)} = 6.049$**	**0.027**	**$\eta^2 = 0.287$**
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(1,15)} = 0.157$	0.697	$\eta^2 = 0.01$
Average Mean Latency to Release Cagemate—Days 7–12	Normal distribution	Two-way ANOVA	Main effect: sex	$F_{(1,15)} = 4.028$	0.063	$\eta^2 = 0.217$
	Normal distribution	Two-way ANOVA	Main effect: condition	$F_{(1,15)} = 0.344$	0.566	$\eta^2 = 0.022$
	Normal distribution	Two-way ANOVA	Interaction: sex × condition	$F_{(1,15)} = 0.99$	0.336	$\eta^2 = 0.062$
Factor Analysis–SDB						
Factor Scores—Anxiety	Normal distribution	One-way ANOVA	Effect of condition	$F_{(2,20)} = 0.439$	0.651	$\eta^2 = 0.042$
Factor Scores—Salience	**Normal distribution**	**One-way ANOVA**	**Effect of condition**	**$F_{(2,20)} = 4.029$**	**0.034**	**$\eta^2 = 0.287$**
		One-way ANOVA: Fisher's LSD post-hoc	***CTRL vs. BARR***	***n/a***	**0.0285**	**$d = 1.521$**
		One-way ANOVA: Fisher's LSD post-hoc	***CTRL vs. ISO***	***n/a***	**0.015**	**$d = 1.33$**
		One-way ANOVA: Fisher's LSD post-hoc	*BARR vs. ISO*	*n/a*	0.797	$d = 0.119$
Factor Scores—Sociosexual Behavior	**Normal distribution**	**One-way ANOVA**	**Effect of condition**	**$F_{(2,20)} = 3.674$**	**0.044**	**$\eta^2 = 0.269$**
		One-way ANOVA: Fisher's LSD post-hoc	***CTRL vs. BARR***	***n/a***	**0.019**	**$d = 1.265$**
		One-way ANOVA: Fisher's LSD post-hoc	*CTRL vs. ISO*	*n/a*	0.447	$d = 0.55$
		One-way ANOVA: Fisher's LSD post-hoc	*BARR vs. ISO*	*n/a*	0.059	$d = 0.877$

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TABLE 1 (Continued)

Test	Data structure	Type of test	Description of analysis	Test value	*p*-value	Effect size
Factor Analysis-LDB						
Factor Scores—Anxiety I	Normal distribution	One-way ANOVA	Effect of condition	$F_{(2,20)} = 0.537$	0.593	$\eta^2 = 0.051$
Factor Scores—Anxiety II	Normal distribution	One-way ANOVA	Effect of condition	$F_{(2,20)} = 3.306$	0.058	$\eta^2 = 0.248$
Factor Scores—Sociosexual Behavior	**Normal distribution**	**One-way ANOVA**	**Effect of condition**	$\boldsymbol{F_{(2,20)} = 4.196}$	**0.03**	$\boldsymbol{\eta^2 = 0.296}$
		One-way ANOVA: Fisher's LSD post-hoc	***CTRL vs. BARR***	***n/a***	**0.009**	$\boldsymbol{d = 1.706}$
		One-way ANOVA: Fisher's LSD post-hoc	*CTRL vs. ISO*	*n/a*	0.167	$d = 0.746$
		One-way ANOVA: Fisher's LSD post-hoc	*BARR vs. ISO*	*n/a*	0.147	$d = 0.683$

Results

To ensure our manipulation did not induce overt behavioral pathology on its own, we began by assessing the acute effects of play deprivation. During the juvenile age (at either P26 or P21 for the open field and playfulness tests, respectively), same-sex sibling pairs of rats of both sexes were placed into control (CTRL) or barrier (BARR) cages or were single-housed in isolation cages (ISO). One week later, we assessed anxiety-like behavior *via* the open field test. In a separate cohort of BARR and ISO animals, we also assessed playfulness. We found no effect of sex or housing condition on center time in the open field (**Figure 1B**). As seen in previous studies of juvenile isolation (Panksepp and Beatty, 1980; Ikemoto and Panksepp, 1992), we found that the ability and motivation to participate in play was not only intact in BARR and ISO animals but was increased relative to our historical data for control-housed animals (**Figure 1C**; Argue and McCarthy, 2015). Interestingly, when we assessed the BARR and ISO play data, we detected a significant main effect of both sex ($p = 0.036$) and treatment ($p = 0.026$), with males of both housing conditions and ISO animals of both sexes playing significantly more than females and BARR animals, respectively. However, this effect of the condition seems to be driven more strongly in females than in males. Although the sex × condition interaction did not reach statistical significance ($p = 0.13$), the mean difference in play levels between female BARR and ISO animals (12.7 vs. 29.0 play events) is much larger than that of male BARR and ISO animals (28.6 vs. 31.9 play events). This suggests that the increased social interaction allowed by the BARR housing condition is sufficient to reduce play motivation in females but not males, an unexpected finding speaking to the differential role that play may serve in females compared to males. Overall, these initial studies indicate that the modified housing used to prevent play does not in itself increase anxiety-like behavior or impair the ability to play if given the opportunity and provides intriguing evidence in support of the idea that play may serve different purposes across the sexes.

For adult behavioral experiments, rats were weaned on P21 into same-sex sibling pairs. In the short duration barrier experiments (SDB), rats were then placed into barrier or isolation cages starting on P26 or remained in control housing conditions (**Figure 1D**). Animals remained in these conditions until P40, a 2-week period encompassing the peak of the window in which social play is maximally expressed (Panksepp, 1981), after which they were returned to standard group housing for the remainder of the experiments. To determine whether there were any sex differences in resiliency to play deprivation, we repeated our behavioral assays in the long duration barrier experiments (LDB). In this study, animals were placed in control, barrier, or isolation conditions for as long as could be tolerated based on size: starting at weaning (P21) and lasting until P45, an age when rats of both sexes have typically entered puberty, at which

point playfulness dramatically decreases (Meaney and Stewart, 1981; Panksepp, 1981). Given the relatively short period of time in which playfulness is seen in rats, the additional 10 days of play deprivation in the LDB study (an additional 5 days before and 5 days after the SDB deprivation period) represents an appreciable expansion in play deprivation time as compared to the SDB study, while maintaining specificity to the time period in which play is the predominant social behavior.

Anxiety-like behavior

To determine whether juvenile play deprivation affected anxiety-like behavior later in life, we conducted two tests of anxiety-like behavior. First, we assessed performance on the elevated plus maze at P47, one week after BARR and ISO animals were returned to standard housing conditions in the SDB study and two days after the return to standard housing in the LDB study. Second, we assessed performance on the open field test at P59. This latter open field test was only conducted in the LDB study, as the SDB animals were exposed to the open field in our initial studies (**Figure 1C**) and as such it was no longer novel. In both tests, we assessed measures of both anxiety-like behavior and hyperactivity.

Elevated plus maze

In both the SDB and LDB studies, we found no effect of housing condition on time in the open arms (**Figures 2A,C**). However, replicating other studies (Johnston and File, 1991; Knight et al., 2021), we observed a small but significant sex difference in the LDB animals with a trending effect in the SDB animals ($p = 0.007$ and 0.097, respectively) whereby males spent significantly less time in the open arms of the maze. Mirroring these findings, we observed a significant main effect of sex in the distance traveled in the maze in both studies (SDB: $p = 0.034$; LDB: $p = 0.005$; **Figures 2B,D**), with males exploring the maze significantly less than females. In the LDB animals, we also observed a main effect of condition ($p < 0.001$) on distance traveled. *Post hoc* analysis indicated that BARR animals of both sexes were significantly more active in the maze than both CTRL ($p < 0.001$) and ISO ($p = 0.003$) animals.

Open field test

Similar to performance on the elevated plus maze, we found that males across the three conditions exhibited more anxiety-like behavior, as they spent significantly less time in the center of the open field arena than females ($p < 0.001$; **Figure 2E**). Additionally, we observed an increase in hyperactivity in animals deprived of play (**Figure 2F**). *Post hoc* analysis following a significant main effect of both sex ($p < 0.001$) and condition ($p = 0.013$) on the number of gridline crossings indicated that CTRL animals of both sexes were significantly less locomotive than BARR animals ($p = 0.01$), with a trending effect when compared to ISO animals as well ($p = 0.117$). As before, females across conditions were also more locomotive on this test than males.

Novel object recognition

The performance of animals in both the SDB and LDB studies on this task suggested intact object memory within our retention interval (1 h), as evidenced by a discrimination ratio (DR) significantly different from chance (DR = 0; **Figures 3A,B**) for all six groups. However, there was no effect of sex or housing condition on the discrimination ratio, indicating no difference in object memory.

Sex behavior

Female sex behavior

As described further in the materials and methods section, females in the SDB study were hormonally primed and assessed for copulatory behavior with a sexually experienced adult male rat. We observed no effect of juvenile housing condition on female sexual behavior, as there were no differences in receptivity (lordosis quotient, **Figure 4A**) or proceptivity (number of proceptive behaviors, **Figure 4B**) across groups. To ensure the artificial hormonal priming was not obscuring a potential deficit, we allowed for natural cycling in the LDB study, conducting the behavioral assay when female subjects were in proestrus and thus sexually receptive. Even in naturally cycling conditions, we again observed no effect of play deprivation on either sexual receptivity or proceptivity (**Figures 4C,D**).

Male sex behavior

In contrast to females, there was a significant impairment in sexual behavior in adult males prevented from playing as juveniles. To account for potential changes in performance due to experience, male sex behavior testing was conducted twice, one week apart on P63 and P70, and values were averaged by subject. We observed either significant or trending effects on the average number of mounts (SDB: $p = 0.075$; LDB: $p = 0.029$; **Figures 5A,E**) and the average number of intromissions (SDB: $p = 0.13$; LDB: $p = 0.016$; **Figures 5B,F**) in both the SDB and LDB animals, with BARR and ISO males exhibiting an often stepwise decrease in the numbers of both sexual behaviors compared to CTRL males. This resulted in a significant reduction in the average mount rate (the total number of mounts, intromissions,

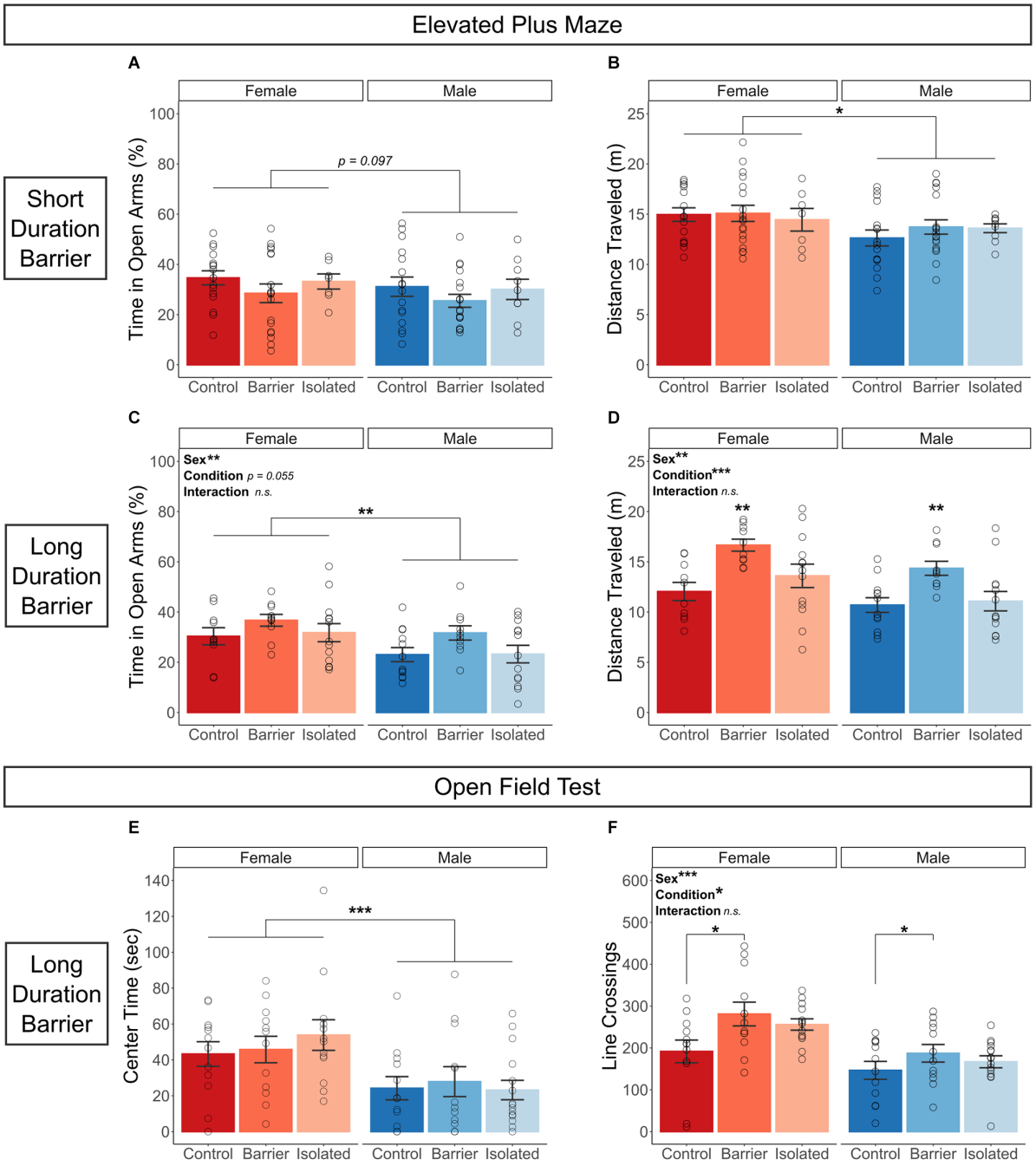

FIGURE 2
Juvenile play deprivation induces later-life hyperactivity but has no effect on anxiety-like behavior; however, males exhibit more anxiety like behavior than females. Percentage of time spent in the open arms **(A)** and distance traveled **(B)** in an elevated plus maze on P47 in the short duration barrier study, with the same measures shown for the long duration barrier study **(C,D)**. In **(D)**, **indicates BARR animals traveled significantly farther than both CTRL and ISO animals, with $p < 0.01$. An open field test was also conducted at P59 in the long duration barrier experiment, with center time (in seconds) and the number of gridline crossings shown in **(E,F)**, respectively. In **(F)**, *indicates CTRL animals exhibited significantly fewer line crossings than BARR animals with $p < 0.05$. Bars indicate group means ± SEM, and open circles represent data from individual rats. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 10–15$ per group.

and ejaculations divided by the active time) in the SDB animals ($p = 0.014$; Figure 5C) which was also trending in the LDB animals ($p = 0.151$; Figure 5G). *Post hoc* analysis of data from the SDB animals revealed both BARR ($p = 0.03$) and ISO ($p = 0.005$) males had a significantly lower mount rate than their CTRL counterparts.

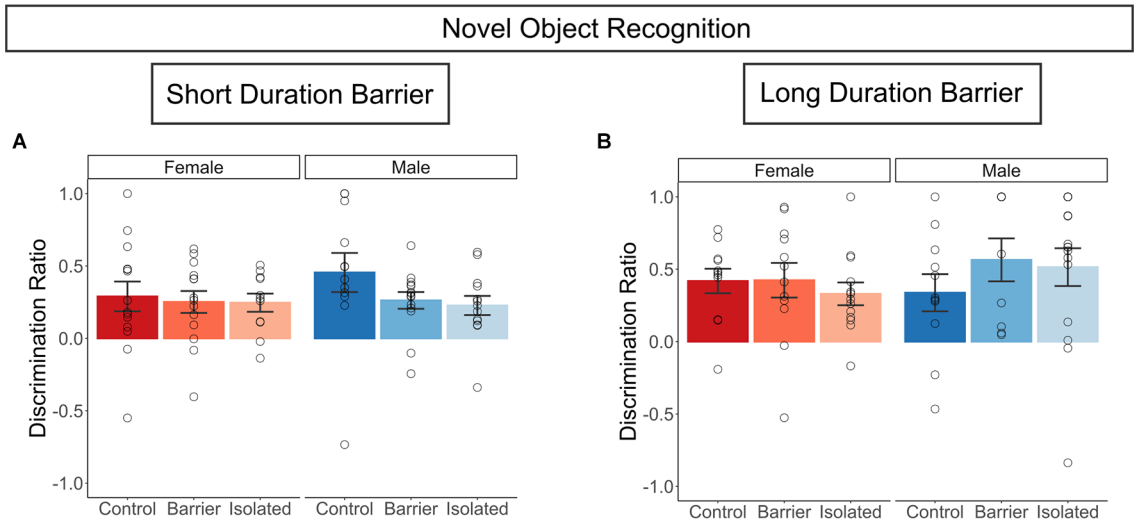

FIGURE 3
Juvenile play deprivation has no effect on novel object memory in adulthood. Discrimination ratio (**A,B**, calculated as the time spent investigating the novel object minus that of the familiar object, divided by the total time spent investigating either object), in the short and long duration barrier studies, respectively, at P60. Bars indicate group means ± SEM, and open circles represent data from individual rats. *n* = 9–15 per group.

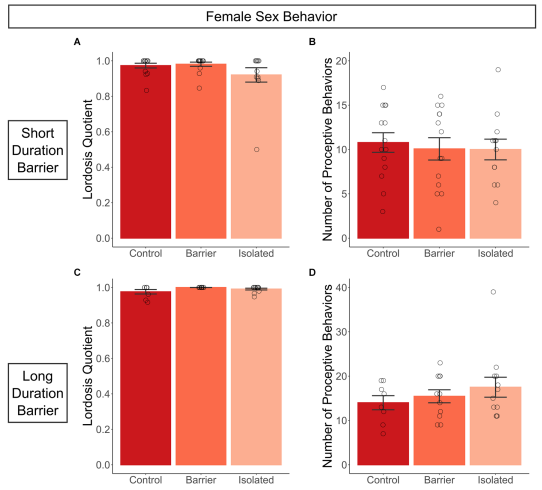

FIGURE 4
Juvenile play deprivation has no effect on copulatory behavior in adult females. Lordosis quotient **(A)**, the number of lordoses exhibited by the female divided by the total number of mounts by the stimulus male, and the number of proceptive behaviors **(B)**, the total number of hops, darts, and solicitations, exhibited in a 10-min test with a sexually experienced adult male stimulus rat on P63 for the short duration barrier study, with the same measures shown for the long duration barrier study in **(C,D)**. Bars indicate group means ± SEM, and open circles represent data from individual rats. *n* = 7–14 per group.

We also assessed the proportion of animals that ejaculated across the two tests of sexual behavior. It is well known in the field of rodent sexual behavior that around 10% of all sexually mature male rats will be "duds" (otherwise known as "sexually sluggish"), meaning that they do not ejaculate in the course of a 20–30 min assay with a sexually receptive female (Dewsbury, 1972; Pattij et al., 2005; Portillo et al., 2006). To determine if play deprivation affected the incidence of "duds", we calculated the proportion of animals in each group that ejaculated in one or both of the 20-min sex behavior tests, compared to the proportion that did not ejaculate in either test. We then conducted individual chi-square goodness of fit tests comparing the distribution of each group (Male CTRL, BARR, and ISO) to the expected distribution (90% ejaculators, 10% non-ejaculators/"duds"). In the SDB animals, the distribution of ejaculators vs. non-ejaculators did not significantly differ from the expected distribution across any of the three groups (**Figure 5D**). In the LDB animals, CTRL males also did not show a significant difference (**Figure 5H**). However, the distribution of ejaculators vs. non-ejaculators in BARR ($p = 0.002$) and ISO ($p < 0.001$) males significantly differed from the expected distribution. In both groups, only ∼60% of animals ejaculated in one or both tests, while ∼40% were "duds". Together, these studies indicate that juvenile play deprivation impairs both the number/rate of sexual behaviors (SDB and LDB) and the proportion of animals which successfully ejaculate (LDB) in adult males, a notable finding given the paramount importance sexual behavior plays in reproductive fitness.

Social preference

In both the SDB and LDB studies, all six groups demonstrated significant social preference compared to chance (50%), exhibiting a much higher percentage of time interacting closely with the social stimulus box compared to the empty

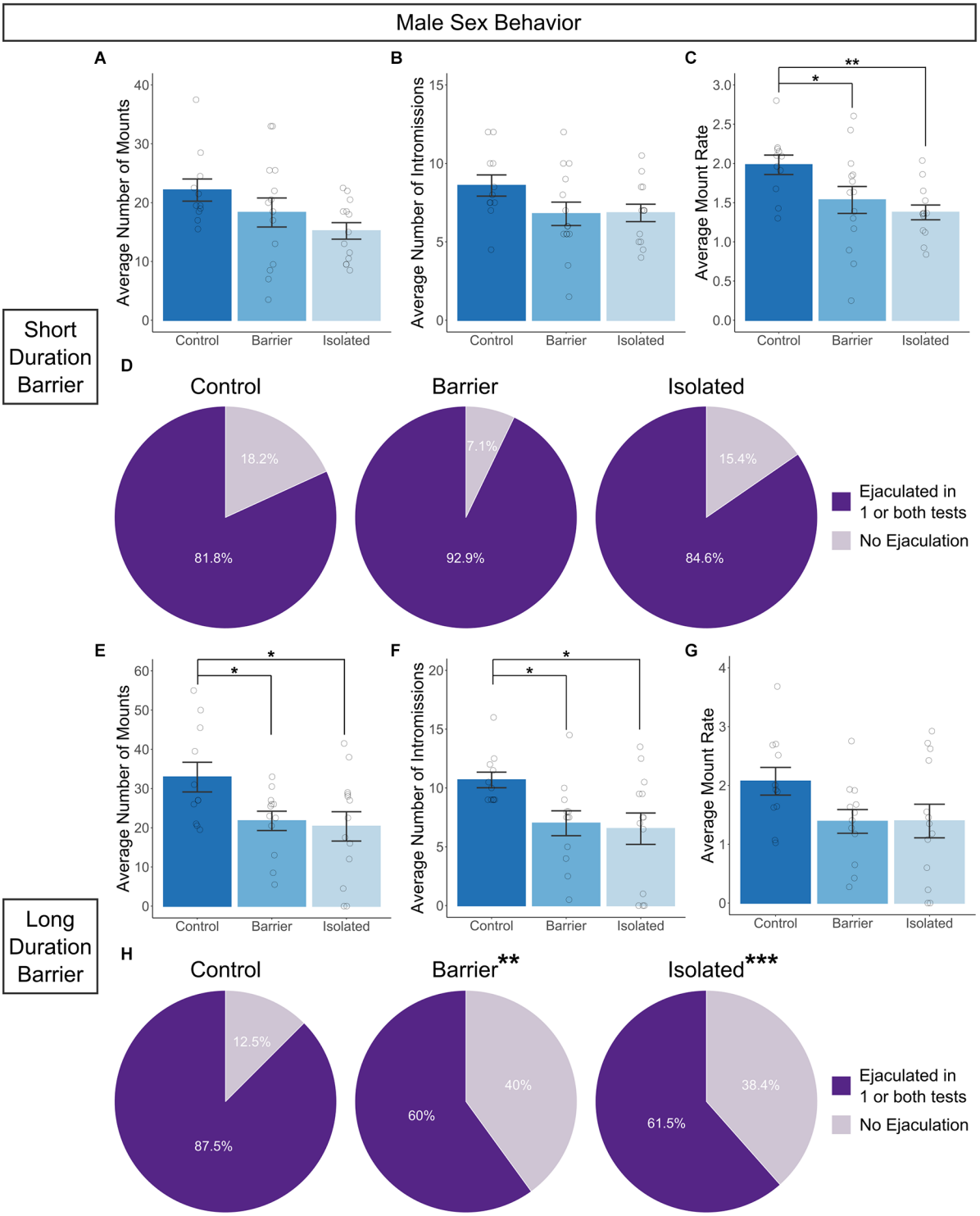

FIGURE 5
Juvenile play deprivation induces deficits in adult male copulatory behavior. Quantification of the average number of mounts **(A)**, intromissions **(B)**, and the mount rate **(C)**, (the total number of all mounts, intromissions, and ejaculations divided by the active time, multiplied by 60 to indicate the number of mounts per minute) across two 10-min tests with a hormonally primed adult female stimulus rat on P63 and P70 in the short duration barrier experiment. The same measures are shown in **(E–G)** for the long duration barrier experiment. Panels **(D,H)** indicate the proportion of animals in each group which ejaculated on one or both tests. Bars indicate group means ± SEM, and open circles represent data from individual rats. *$p < 0.05$, **$p < 0.01$, $n = 8$–14 per group. In **(H)**, ** and ***indicate that the proportion of animals which ejaculated is significantly different (**$p < 0.01$, ***$p < 0.001$) from 90%, the amount expected for control animals based on historical data.

stimulus box. In the SDB study, we additionally observed a significant sex × condition interaction on the percent of time spent near the social stimulus box ($p < 0.001$; **Figure 6A**; ratio data and representative traces shown in **Figure 6B**). *Post hoc* analysis indicated that male BARR animals had a significantly higher social preference compared to all other groups, including male CTRL ($p < 0.001$) and male ISO ($p < 0.001$) animals. This appeared to be a true difference in sociability not affected by differences in overall exploration time, as there was no effect of condition on the time spent near either stimulus box (**Supplementary Figure 1A**). However, while a small but significant ($p = 0.003$) sex difference in the percentage of time near the social box was detected in the LDB study (**Figure 6C**; ratio data shown in **Figure 6D**), with males showing increased social preference relative to females, there was no effect of juvenile housing condition.

Sex preference

In both the SDB and LDB studies, all six groups demonstrated significant opposite-sex preference as compared to chance (50%), with the exception of the two BARR groups in the LDB study, in which the one-sample *t*-test vs. chance was trending (Male BARR: $p = 0.075$; Female BARR: $p = 0.148$). However, no significant differences were detected as a result of condition on the percent of time spent near the opposite-sex stimulus box (**Figures 7A,C**), indicating sex preference in adulthood is unaffected by juvenile play deprivation. While there were no sex differences on this measure in either the SDB or LDB animals, a main effect of sex was detected when considering the data as a ratio of the time spent near the opposite sex compared to the same-sex stimulus box in the LDB animals (**Figure 7D**) but not the SDB animals (**Figure 7B**), with males exhibiting stronger opposite-sex preference than females.

Social recognition

In the social recognition assay, subjects were assessed for the amount of time spent interacting with a novel stimulus animal ("Train" trial) compared to the amount of time spent interacting with that same (now familiar) animal on a second trial ("Test" trial) after a 30 min inter-trial interval (Lemaire, 2003). In both the SDB and LDB studies, all six groups demonstrated social recognition (**Figures 8A,C**), as their interaction time on the Test trial was significantly decreased compared to that of the Train trial, with the exception of the Male BARR group in the SDB study, which was trending ($p = 0.085$). This decreased interaction time appeared to represent true social recognition, as opposed to an artifactual decrease as a result of being assessed in a second consecutive test, as there was no difference in interaction time between the Train and Test trials for any of the six SDB or LDB groups in a control experiment in which subjects received a novel stimulus animal in both trials (**Supplementary Figure 2**). Interestingly, we detected a strong sex difference in the ratio of investigation duration, with males exhibiting ratios closer to 1, indicating less intact social recognition (SDB: $p < 0.001$; LDB: $p < 0.001$; **Figures 8B,D**); this followed a sex difference we observed in the overall interaction times, whereby males spent much more time interacting with the stimulus animal across both trials than females did. However, there was no effect of juvenile housing condition, indicating play deprivation has no effect on later-life social recognition.

Resident intruder assay

In the LDB study, we conducted two tests of the resident intruder assay using male subjects, separated by a 30 min inter-trial interval with a novel intruder rat each time. We scored various aggressive behaviors as described in Koolhaas et al. (2013) and averaged values by subject. We detected trending effects of condition on the average number of keep downs ($p = 0.189$; **Figure 9A**), lateral threats ($p = 0.13$; **Figure 9B**), and upright postures ($p = 0.088$; **Figure 9C**), but no difference in the number of clinch attacks (**Figure 9D**). Summing these behaviors together, we calculated the average number of total aggressive behaviors exhibited by subjects across the two tests (**Figure 9E**) and observed a significant main effect of condition on this measure ($p = 0.016$). *Post hoc* analysis indicated CTRL males exhibited significantly fewer total aggressive behaviors than ISO males ($p = 0.005$), with a trending effect when compared to BARR males ($p = 0.056$), demonstrating that juvenile play deprivation significantly increased aggressiveness in adulthood.

Empathy/prosocial helping behavior

In a separate experiment from the SDB and LDB studies, we assessed empathy using a test of prosocial helping behavior in animals acutely deprived of play starting a week before and continuing through the 12 days of testing. Rats of both sexes were housed in CTRL or BARR conditions starting on P21 and remained in these conditions until and throughout testing, which began on P27 and lasted until P38 (**Figure 10A**). In this assay, test subjects are evaluated for whether and how quickly they release their sex- and condition-matched sibling cagemate from a confinement box over the 12 days of repeated daily testing (**Figure 10B**). Animals deprived of play exhibited significantly longer latencies to free their cagemates on the first six days of the task compared to controls ($p = 0.027$; **Figure 10C**). The performance of play-deprived animals improved to control levels by the second half of testing, as there was no effect of condition on this measure on days 7–12 of the assay (**Figure 10D**). There

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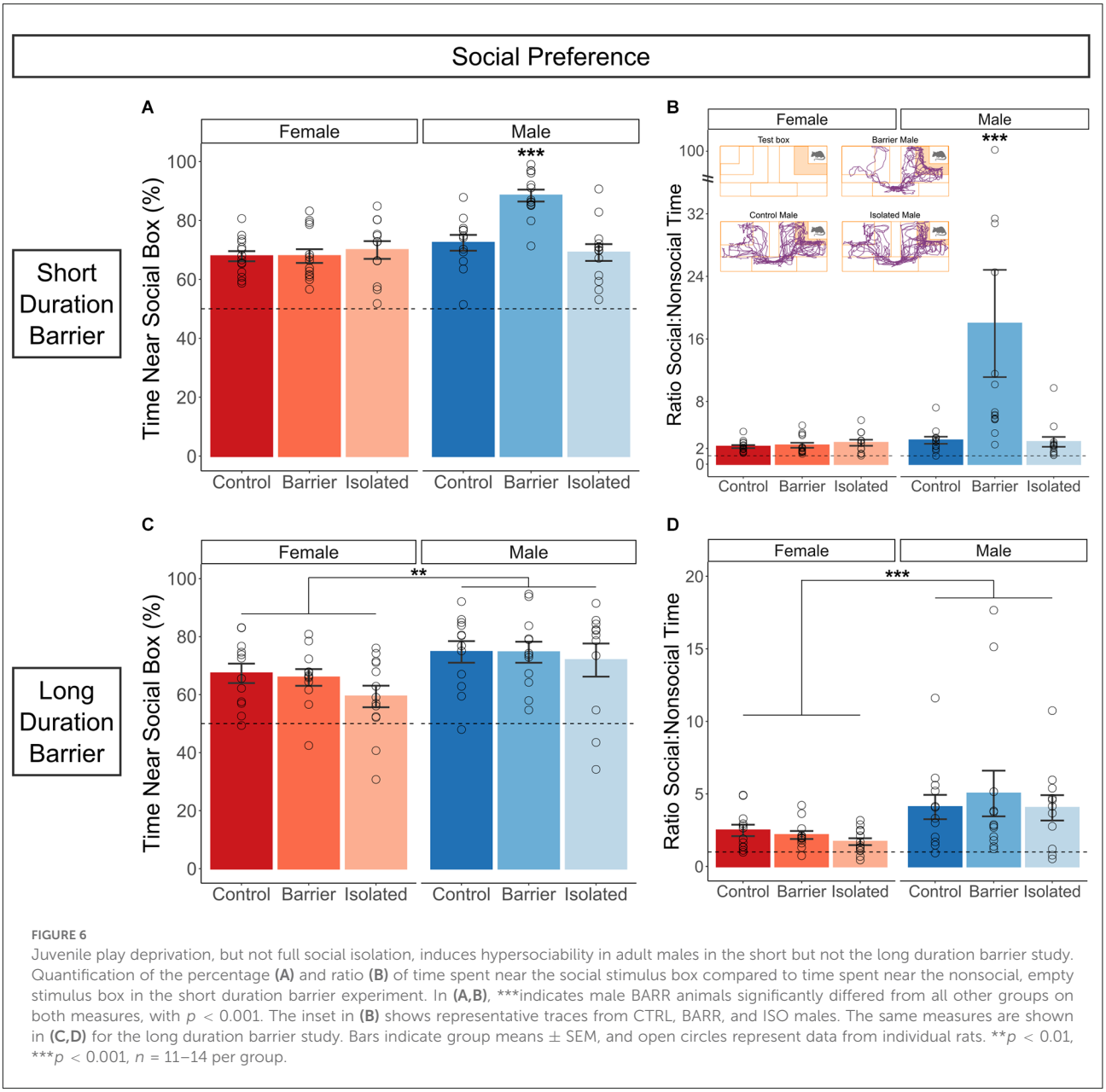

FIGURE 6
Juvenile play deprivation, but not full social isolation, induces hypersociability in adult males in the short but not the long duration barrier study. Quantification of the percentage **(A)** and ratio **(B)** of time spent near the social stimulus box compared to time spent near the nonsocial, empty stimulus box in the short duration barrier experiment. In **(A,B)**, ***indicates male BARR animals significantly differed from all other groups on both measures, with $p < 0.001$. The inset in **(B)** shows representative traces from CTRL, BARR, and ISO males. The same measures are shown in **(C,D)** for the long duration barrier study. Bars indicate group means ± SEM, and open circles represent data from individual rats. **$p < 0.01$, ***$p < 0.001$, $n = 11–14$ per group.

was also a sex difference in this task in both stages of testing, with males on average exhibiting significantly longer latencies to release their cagemates in the early stage of testing (Days 1–6; $p = 0.046$) with a trending effect in the late stage of testing (Days 7–12; $p = 0.063$).

Factor analysis

Upon completing our analyses, we wondered if any larger patterns or categories of later-life behaviors were specifically altered by play deprivation in the juvenile period. To investigate this, we performed factor analysis, a method for assessing observed variables and their variances to determine whether there are latent constructs ("factors") that explain the patterns of phenotypes seen in a particular dataset. We conducted this analysis on the SDB and LDB results separately because we were interested in whether the analysis would pull out factors that were similar or different across the two experiments. As is standard for this method, we conducted this analysis on various outcomes from the battery of behavioral experiments, focusing on those most strongly correlated and thus appropriate for inclusion in factor analysis (see "Materials and methods" Section). Consequently, we conducted these analyses on only the male data, as the female data in both the SDB and LDB studies was deemed insufficiently correlated and therefore

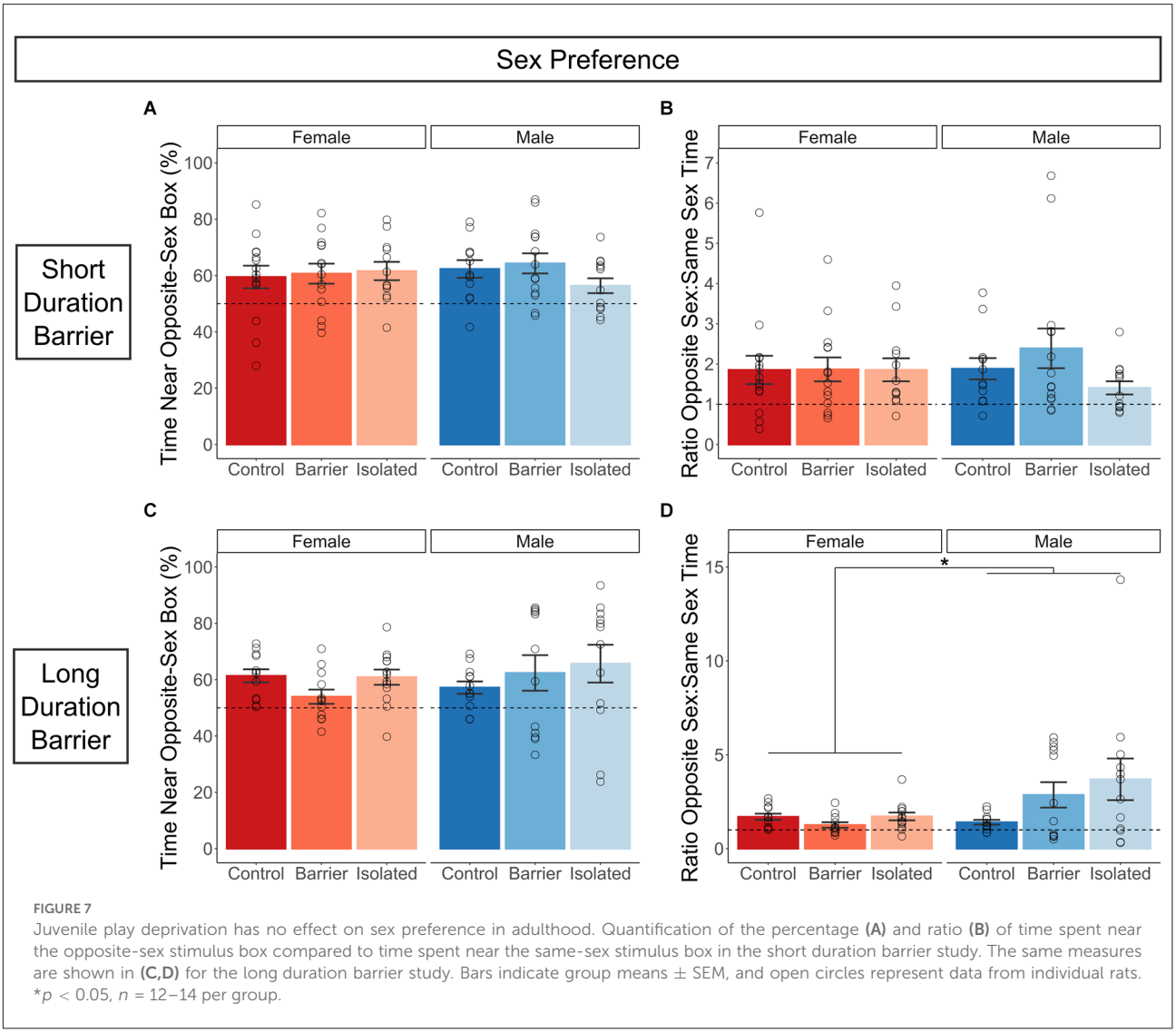

FIGURE 7
Juvenile play deprivation has no effect on sex preference in adulthood. Quantification of the percentage **(A)** and ratio **(B)** of time spent near the opposite-sex stimulus box compared to time spent near the same-sex stimulus box in the short duration barrier study. The same measures are shown in **(C,D)** for the long duration barrier study. Bars indicate group means ± SEM, and open circles represent data from individual rats. *$p < 0.05$, $n = 12–14$ per group.

inappropriate for factor analysis based on the Kaiser-Meyer-Olkin Measure of Sampling Adequacy (MSA; below 0.5 for both studies) and Bartlett's sphericity test (p-value above 0.05 for both studies).

In the SDB study, six behavioral measures were deemed appropriate for inclusion in our analysis. We identified three factors to be sufficient to explain the data, accounting for 66.5% of the total variance (Figure 11A). The first factor (31.3% of total variance) contained positive loadings for distance traveled and percent of the time in the open arms of the elevated plus maze, with negative loading for social recognition. As these enriched behaviors generally reflected measures of a latent anxiety-like phenotype, we renamed it accordingly ("Anxiety"). The second factor (19.3% of total variance) contained positive loadings for discrimination ratio on the novel object recognition task and sex behavior mount rate, both phenotypes that require recognition of the salience of a given stimulus; thus, we renamed the factor as such ("Salience"). Finally, the third factor (15.9% of total variance) contained positive loadings for sex behavior mount rate and social preference, two tests within the socio-sexual domain (hence titled "Socio-sexual behavior").

In the LDB study, nine behavioral measures were deemed appropriate for inclusion. From this analysis, we again identified three factors to be sufficient to explain the data, accounting for 58% of the total variance (Figure 11C). The first two factors appeared to reflect a latent anxiety-like phenotype, separating based on the specific anxiety-like behavior test in question. Factor one (23.6% of total variance) contained strong positive loadings for center time and line crossings in the open field test, with negative loading for novel object discrimination ratio, while factor two (17.5% of total variance) contained strong positive loadings for distance traveled and open arm time

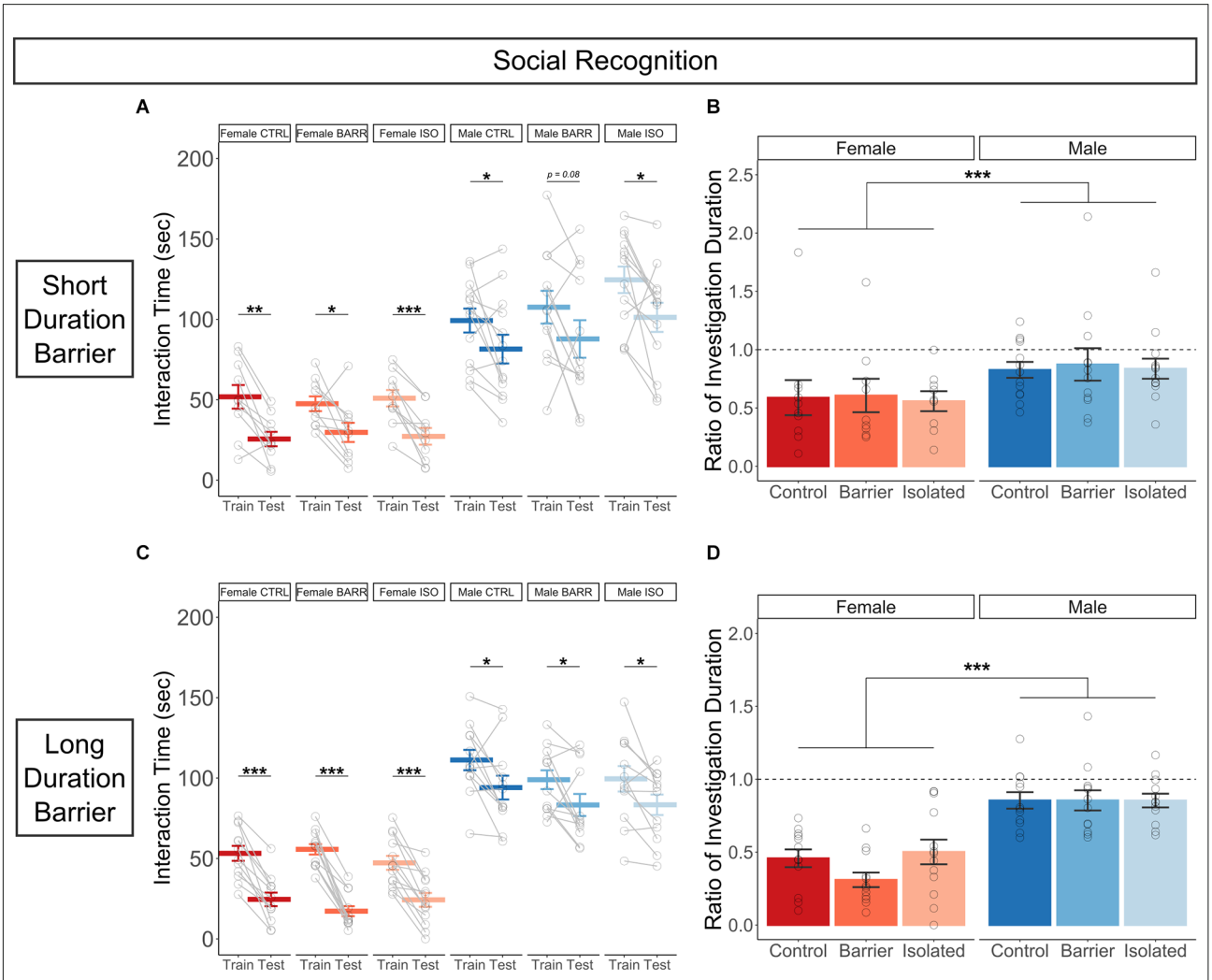

FIGURE 8
While juvenile play deprivation has no effect, there are sex differences in social recognition in adulthood. Quantification of the amount of time spent interacting with a stimulus rat on both the Train (first trial in which the stimulus rat is novel) and the Test (second trial following a 30 min inter-trial interval in which the stimulus rat is now familiar) trials **(A)** and the ratio of interaction time on the Test trial compared to that of the Train trial **(B)** for the short duration barrier study. The same measures are shown in **(C,D)** for the long duration barrier study. Bars indicate group means ± SEM, and open circles represent data from individual rats. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 9–13$ per group.

on the elevated plus maze. As such, we renamed the factors as "Anxiety I" and "Anxiety II", respectively. Similar to the SDB study, the third factor (16.9% of total variance; hence titled "Socio-sexual behavior") contained loadings for various socio-sexual behaviors: positive loadings for sex preference and social preference and negative loading for sex behavior mount rate.

To determine whether the expression of these factors differed across our three groups, we calculated factor scores for each subject (SDB: **Figure 11B**; LDB: **Figure 11D**). No significant differences were observed for the Anxiety-related factors in either the SDB or the LDB study. However, in the SDB animals, there was a significant effect of condition on the Salience factor ($p = 0.034$). *Post hoc* analysis detected a significant difference between CTRL animals and those deprived of play on this factor (BARR: $p = 0.029$; ISO: $p = 0.015$), with CTRL males exhibiting higher positive scores on this Salience factor than both play-deprived groups. Most notably, we also detected a significant main effect of condition on the Socio-sexual Behavior factor in both the SDB ($p = 0.044$) and LDB ($p = 0.03$) animals. *Post hoc* analyses indicated BARR males had a significantly higher positive score for this factor than CTRL males in both studies (SDB: $p = 0.019$; LDB: $p = 0.008$), and there were trending effects when compared to ISO males in both studies as well (SDB: $p = 0.059$; LDB: $p = 0.147$). Together, these analyses suggest that socio-sexual behavior is a distinct category of later-life behavior specifically sensitive to play deprivation in juvenile males.

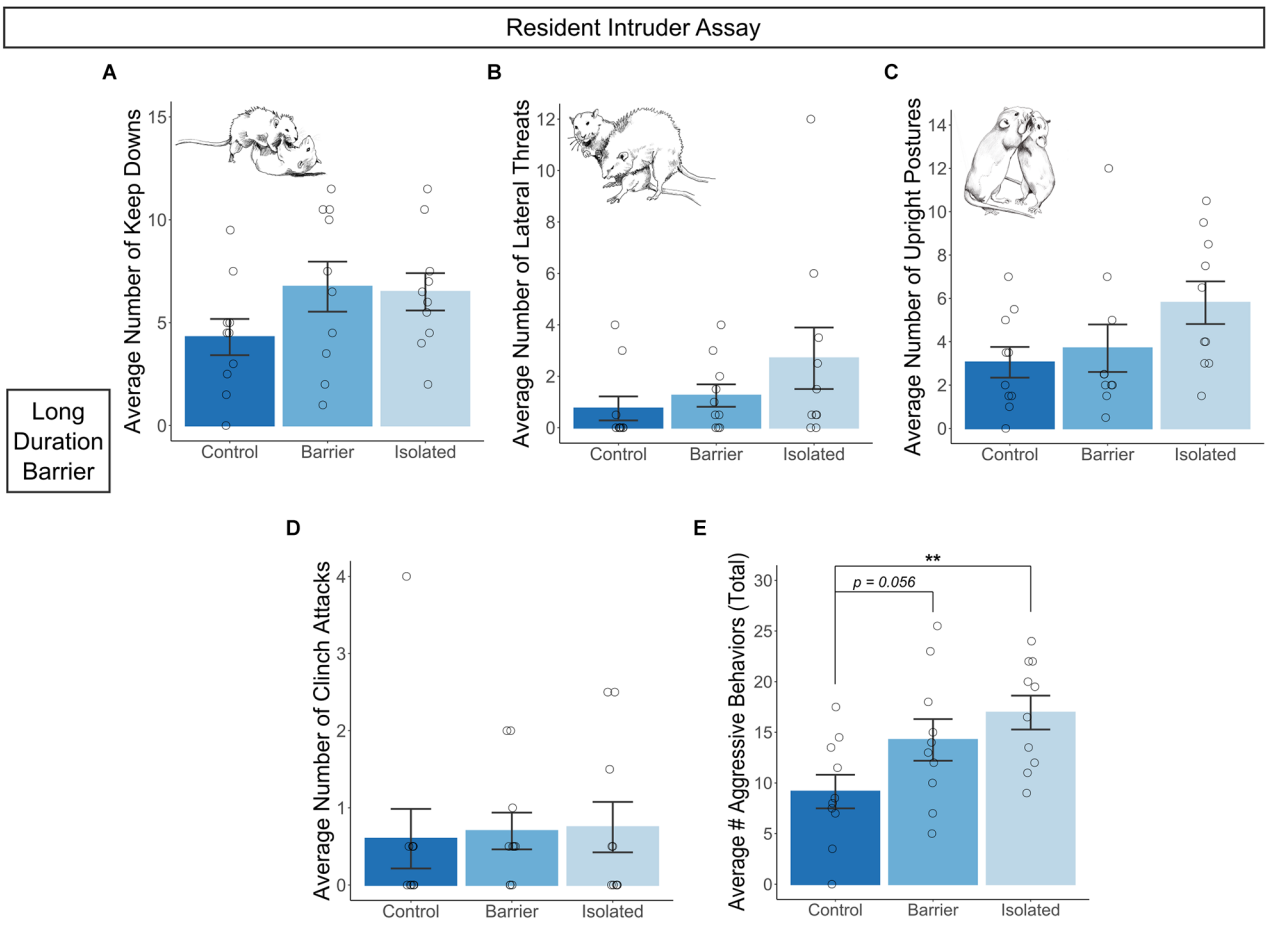

FIGURE 9
Juvenile play deprivation increases adult aggressive behavior in males. Quantification of the average number of keep downs **(A)**, lateral threats **(B)**, upright postures **(C)**, and clinch attacks **(D)** exhibited by each resident male subject towards a novel intruding stimulus male in two 15-min tests in the long duration barrier experiment. Cartoon insets in **(A–C)** depict the assessed behavior in question. The average number of total aggressive behaviors is shown in **(E)**. Bars indicate group means ± SEM, and open circles represent data from individual rats. $^{**}p < 0.01$, $n = 10$ per group.

Discussion

Here, we report persistent alterations in the expression of various adult behaviors following juvenile play deprivation in rats. We demonstrate that social play experience is critical for the development of appropriate socio-sexual behavior in males, as males prevented from playing as juveniles exhibited altered sexual behavior, sociability, and aggressive behavior. These effects are complex and pervasive, seen weeks to months after all animals were returned to group housing, and impact reproductively critical behaviors. In females, however, play is more dispensable. While rats of both sexes were hyperactive shortly after play deprivation and displayed impairments in a test of empathy motivation when acutely prevented from playing, we observed no effects in females on any other test in the diverse battery we conducted.

Given the importance for reproductive fitness, the most striking of our findings is the lasting effect of play deprivation on male sexual behavior. Males prevented from playing for as little as 2 weeks (in the SDB study) showed a significant reduction in mount rate across two tests of sexual behavior. This was seen in both BARR and ISO males, indicating that the lack of play, not the lack of social interaction writ large, was the critical driver of this impairment. Moreover, this impairment did not seem to represent a simple delay in the learning process, but rather a persistent reduction in the rate of copulatory behavior; while we present the data here as an average, the deficit in BARR and ISO males was evident across both successive tests. Furthermore, males prevented from playing for as little as 3.5 weeks (in the LDB study) also exhibited deficits in ejaculation. Around 40% of BARR and ISO males did not ejaculate in either test of copulatory behavior, a significant deviation from what was seen in CTRL males (12.5%) and from the expected distribution

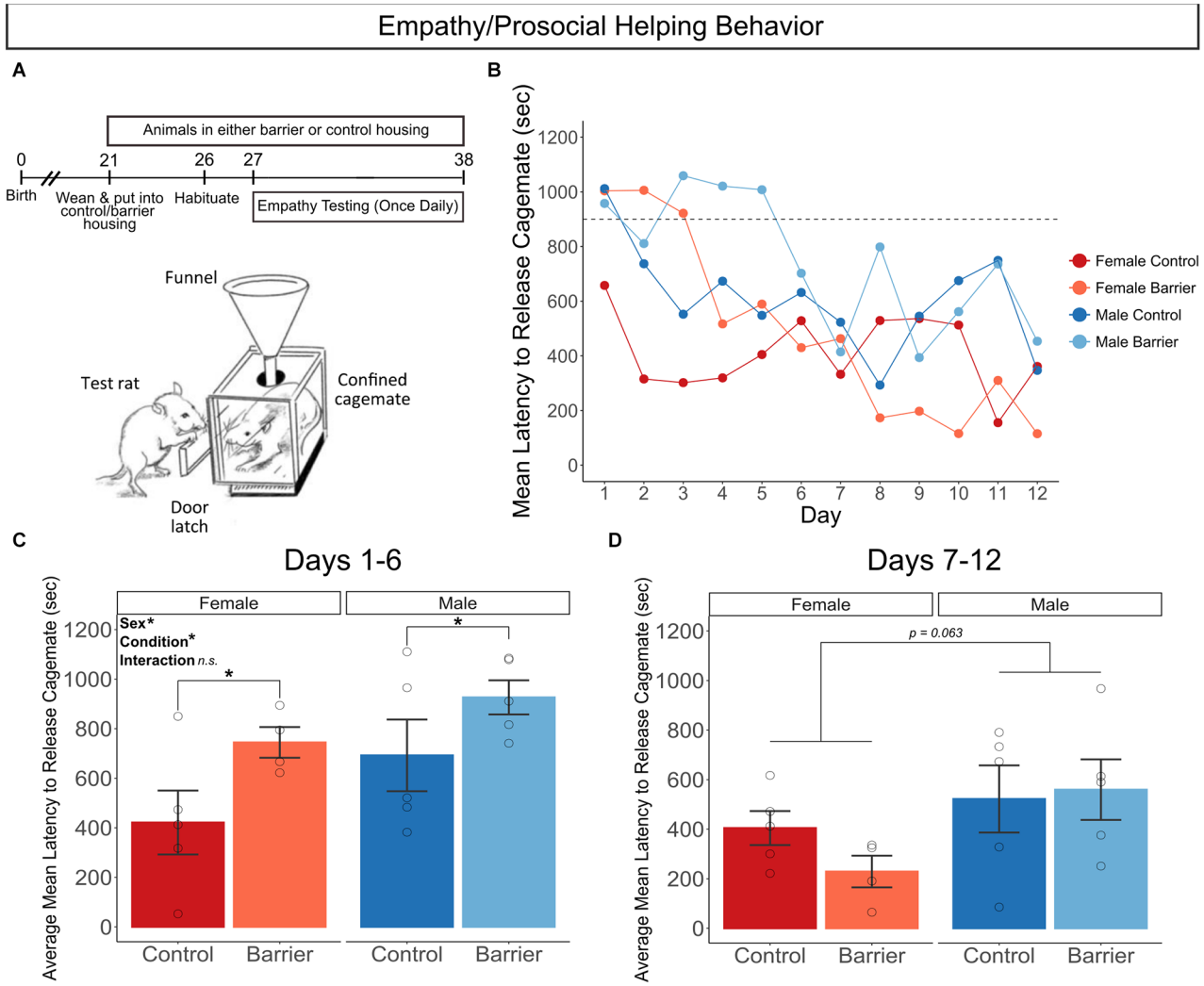

FIGURE 10
Juvenile play deprivation impairs performance on a test of empathy/prosocial helping behavior. **(A)** Timeline of experiment and cartoon of the prosocial behavior test apparatus, showing the test rat opening the door latch to release its cagemate confined in the box. Quantification of the mean latency (in seconds) to release cagemate across the 12 days of testing **(B)**, with dashed line at 900 s indicating when the door latch was loosened by the experimenter if the test animal had not already released the confined cagemate. Quantification of the average mean latency to release cagemate across the first 6 **(C)** and the last 6 **(D)** days of the test procedure. Bars indicate group means ± SEM, and open circles represent data from individual rats. *$p < 0.05$, $n = 4–5$ per group.

of non-ejaculators (~10%) based on decades of research on male rat sexual behavior (Dewsbury, 1972; Pattij et al., 2005; Portillo et al., 2006). Remarkably, these impairments were close to the magnitude seen in other studies in which male copulatory behavior is altered by more substantial pharmacological or genetic manipulations, despite the fact that our manipulation merely prevented juveniles from playing for a short window of time. For example, the decrease in the number of mounts we observed was of similar magnitude to that seen in males treated with a cyclooxygenase-2 inhibitor for 2 weeks during the perinatal sensitive period (Amateau and McCarthy, 2004), and the reduction in the percentage of animals which successfully ejaculated we observed was similar to that seen in males in which both the androgen receptor and estrogen receptor alpha were genetically deleted (Trouillet et al., 2022).

We also found that play deprivation induces hypersociability in adulthood. In the SDB study, BARR males exhibited social preference that was significantly higher than that seen in all other groups. This hypersocial phenotype was seen across multiple SDB cohorts, and the effect size was substantial (Cohen's *d* ranging between 1.9 and 2.9 for all pairwise comparisons between Male BARR and the other groups for percent of time near the social box; **Figure 6A**). However, there was no effect on social preference in the LDB animals. This differential

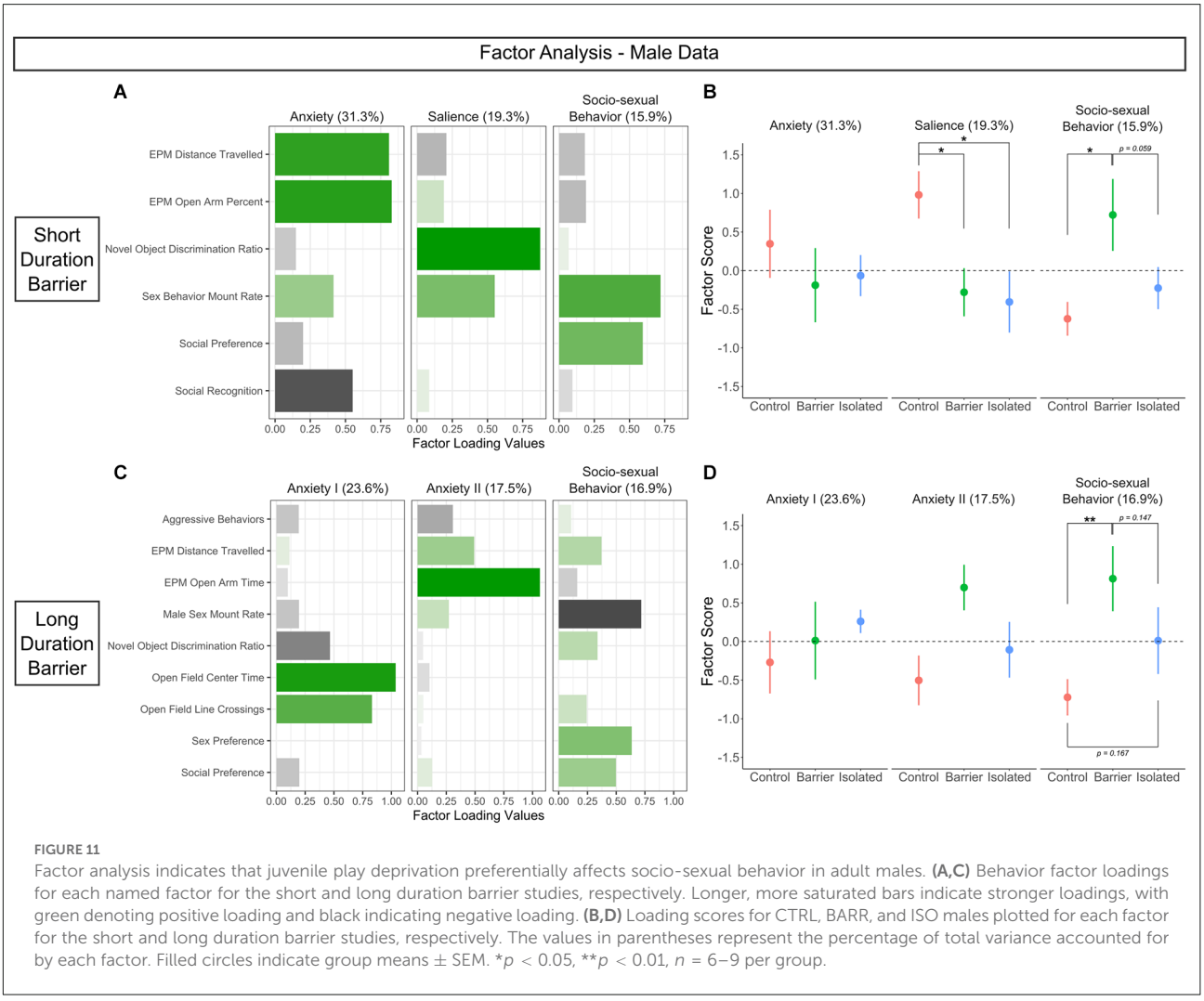

FIGURE 11
Factor analysis indicates that juvenile play deprivation preferentially affects socio-sexual behavior in adult males. **(A,C)** Behavior factor loadings for each named factor for the short and long duration barrier studies, respectively. Longer, more saturated bars indicate stronger loadings, with green denoting positive loading and black indicating negative loading. **(B,D)** Loading scores for CTRL, BARR, and ISO males plotted for each factor for the short and long duration barrier studies, respectively. The values in parentheses represent the percentage of total variance accounted for by each factor. Filled circles indicate group means ± SEM. $*p < 0.05$, $**p < 0.01$, $n = 6–9$ per group.

effect in the SDB compared to the LDB study may be due to an as-yet-unidentified sensitive period for social development around the time of weaning. Work from Hol et al. (1999) demonstrated that social isolation from P22–28, but not P29–35, decreases sociability in adulthood in male rats. Isolation across the full window, from P22–35, also decreased sociability. This finding was mirrored by a study in females which found that isolation from P19–70 also decreases sociability (Hermes et al., 2011). In contrast, isolation starting later in adolescence, from P28–70, was shown to produce hypersociability in both sexes, although this study was conducted in mice (Rivera-Irizarry et al., 2020). Looking at the intersection of these studies, it is possible that two distinct sensitive periods exist, perhaps governed by the maturation of distinct sets of social circuitry: one window from ∼P19–28, in which the lack of play experience causes hyposociability, and another starting at ∼P35, in which the lack of play experience causes hypersociability. In this view, it is possible we observed no effect on sociability in the LDB study as the play deprivation spanned across those windows (from P21–45), canceling out any effects. It is also possible that the additional stress caused by full isolation may alter the direction and magnitude of these effects, given ours were seen only in BARR, but not ISO, males. Future studies should more closely investigate the possibility of these distinct sensitive periods and whether they may differ based on sex, given the hypersocial phenotype we observed was seen only in males.

One of many theories regarding the function of social play is the "play-as-practice" theory, which argues that the individual behaviors constituting social play (e.g., pounces, pins, and boxing behaviors in rats) resemble adult agonistic behaviors for a reason: because play serves as practice for later-life aggressive encounters (Fagen, 1981; Smith, 1997). This theory has received criticism for various reasons, primarily that there are several key differences in the microstructure of juvenile play-fighting and adult aggressive behaviors, thus hindering play's applicability as a means of physical "practice" (Pellis and Pellis, 1987). We here provide further evidence

against the play-as-practice theory, as we demonstrate that juvenile play deprivation increases aggressive behavior in adult males, not decreases as a straightforward interpretation of the theory would predict. Nonetheless, a more complex interpretation of the theory may be better justified. While it seems evident that play does not serve as simple practice for the motor skills needed for later-life agonistic behavior, perhaps what is gained from play is experience in its reciprocal nature. This reciprocity—necessitating monitoring one's own actions and those of a partner, recognizing social signals indicating the partner's responses and intentions, and adjusting one's actions appropriately to maintain the back-and-forth of the play bout—is a fundamental feature of social play (Pellis and Pellis, 2017), so play may serve to provide experience in these social skills, allowing for the maturation of brain circuitry to the same effect. Animals deprived of play may lack this experience, thereby hindering their ability to appropriately recognize and/or respond to social signals to de-escalate an agonistic encounter, resulting in increased aggression as we observed. Indeed, recent work in *Drosophila* identified a transcriptional regulator believed to govern this same effect, suppressing aggression in a social experience-dependent manner (Ishii et al., 2022), and long-term post-weaning social isolation has been shown to induce qualitative differences in the attack patterns of male rats (Toth et al., 2011).

We also conducted a test of prosocial motivation in animals acutely deprived of play starting a week before and continuing throughout the 12 days of testing. BARR animals of both sexes performed worse on this task, taking significantly longer to free their distressed cagemate from a confinement box on the first 6 days of testing. This impairment was resolved by the latter half of testing, with the performance of play-deprived animals improving to that seen in controls on days 7–12 of the paradigm. This finding may serve as another example of the concept that play aids in developing the ability to appropriately sense and respond to social signals. In this view, BARR animals may perform worse on the empathy test due to a deficit in their perception of and/or responsiveness to the social signals generated by their distressed cagemate. This impairment may have improved over time due to the increased social experience afforded as part of the test (while animals were allowed only 10 s to interact with their cagemate following release from the confinement box, this social experience may have accumulated over time), or because animals had sufficient experience with the paradigm by the latter half of testing such that it allowed BARR animals to "catch up" to controls. We also observed a sex difference in this assay: males generally performed worse than females, as they exhibited a significantly higher latency to release their cagemate on average in the first half of testing. This too may speak to the heightened importance of play experience in males compared to females, given even at baseline males are less successful on this socially-driven task.

Importantly, we saw no effects of play deprivation on any other assessed behavior, with the exception of a small but significant effect on locomotion shortly after animals were returned to standard group housing. Our findings in which we observed play deprivation had no effect—on anxiety-like behavior, object memory, sex preference, and social recognition in both sexes and on sex behavior and social preference in females—are just as informative as our findings in which we did observe an effect, in that they show what play experience does *not* seem to be important for. The effects of play deprivation (and therefore, the importance of play itself) seem to preferentially center around socio-sexual behaviors in adulthood, a concept further supported by our factor analyses, which in an independent and unbiased manner identified factors in both the SDB and LDB studies that represent socio-sexual behavior and significantly differed in control and play-deprived males.

This specificity to socio-sexual behavior makes sense, given play is itself a social behavior and involves the activity of many regions in the social circuitry, such as the medial amygdala, lateral septum, and hypothalamus (summarized in Siviy, 2016 and VanRyzin et al., 2020a). These regions have distinct roles in socio-sexual behavior in adulthood as well, often driven by distinct, separable cell type-specific and projection-specific mechanisms (Unger et al., 2015; Wong et al., 2016; Ishii et al., 2017; Kohl et al., 2018). The maturation of these cell subtypes and/or projections, then, may be modulated in an experience-dependent (and, potentially, sex-dependent) manner based on playfulness. Like the importance of visual experience for the development of ocular dominance in V1 neurons (Wiesel and Hubel, 1963) or somatosensory experience for dendritic spine dynamics in the barrel cortex (Lendvai et al., 2000), play experience may be fundamental to the developmental firing and wiring of individual cells and projections that establishes appropriate adult circuit organization. However, the fact that the impairments we observed following play deprivation were generally specific to males begs the question: in this view, what, if anything, governs social circuit maturation in females? Does social experience (play or non-play) have any role? In our initial studies, we found that the additional non-play social experience provided by the BARR vs. the ISO housing condition is sufficient to decrease play motivation in females but not males (**Figure 1C**), suggesting that play itself is the critical feature driving the motivation to play in males. Perhaps in females, the significance of play is more so the social experience in general, and less so the experience of play itself. Even then, it appears little social experience during the juvenile window is necessary for appropriate social development in females, given we observed few impairments following even full social isolation. Future studies should investigate whether the window for experience-dependent social circuit organization is shifted in females compared to males, or if, perhaps, female social circuit maturation is not experience-dependent at all.

While our BARR manipulation improves upon previous methodology in that it prevents play while allowing for other forms of social interaction, there are nonetheless limitations. BARR animals are also deprived of other tactile non-play social interactions such as social grooming, and there may be other variables such as the difference in physical space allowed by the BARR vs. CTRL and ISO conditions that may affect development. Nevertheless, the convergence of evidence from our study and others using different manipulations (e.g., reducing play by rearing juveniles with an adult or a peer from a less playful strain) points to play experience being the critical driver of the impairments observed (Schneider et al., 2016; Burleson et al., 2016; Pellis et al., 2017; Stark and Pellis, 2020, 2021). Additionally, while females appeared largely resilient to play deprivation across the myriad of behavioral assays we conducted, this may not be true for all traits, or there may be latent differences in our assessed traits not detectable by the paradigms we used to evaluate them. Regardless, this female resilience is notable, supporting our hypothesis that the purpose of play differs across the sexes, at least in the rat.

Embedded within our studies is also a deep assessment of sex differences and similarities across a wide variety of behavioral tests in rats. When possible (i.e., in assays that are not only applicable to animals of one sex), males and females were included in all experiments, all of which were adequately powered to detect sex differences if they were present. We statistically tested sex as a variable, calling a finding a "sex difference" if and only if we detected a significant main effect of sex or a significant interaction. This was as opposed to independently assessing males and females and noting a sex difference if we observed an effect in one sex but not the other, a common misconception of the appropriate way to assess sex differences (Garcia-Sifuentes and Maney, 2021). As the research community works to better address sex as a biological variable (SABV), especially following the implementation of the United States National Institutes of Health's 2016 policy requiring consideration of SABV in all funded research, we hope this work is useful to behavioral neuroscientists interested in the differences and similarities in male and female rats across various behavioral domains.

Finally, our results are especially relevant in the wake of the COVID-19 pandemic. We began these studies shortly before shutdowns began in early 2020, impacting access to play for millions of children worldwide, and as such our experiments became even more relevant. While our studies were conducted in rats, social play is remarkably similar across the many mammalian species in which it is expressed and has strong face validity in humans. Our results would argue that boys may be more vulnerable to a reduction in access to play than girls. This potential sex difference in the influence of the pandemic on child health and brain development is something that should be monitored as we collectively move forward.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, University of Maryland School of Medicine.

Author contributions

AM and MM designed research and wrote the manuscript. AM, JV, and RF performed research. AM and JV analyzed data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.1076765/full#supplementary-material.

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Developmental exposure to corn grown on Lake Erie dredged material: a preliminary analysis

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While corn is considered to be a healthy food option, common agricultural practices, such as the application of soil amendments, might be introducing contaminants of concern (COC) into corn plants. The use of dredged material, which contain contaminants such as heavy metals, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), as a soil amendment is increasing. Contaminants from these amendments can accumulate in corn kernels harvested from plants grown on these sediments and potentially biomagnify in organisms that consume them. The extent to which secondary exposure to such contaminants in corn affect the mammalian central nervous system has been virtually unexplored. In this preliminary study, we examine the effects of exposure to corn grown in dredge amended soil or a commercially available feed corn on behavior and hippocampal volume in male and female rats. Perinatal exposure to dredge-amended corn altered behavior in the open-field and object recognition tasks in adulthood. Additionally, dredge-amended corn led to a reduction in hippocampal volume in male but not female adult rats. These results suggest the need for future studies examining how dredge-amended crops and/or commercially available feed corn may be exposing animals to COC that can alter neurodevelopment in a sex-specific manner. This future work will provide insight into the potential long-term consequences of soil amendment practices on the brain and behavior.

KEYWORDS

soil amendment, neurotoxicity, neurodevelopment, open field, object recognition

1. Introduction

The United States is the greatest producer and consumer of corn in the world (Gloy, 2017; McConnell, 2021). Additionally, corn is a major source of carbohydrates for humans and livestock and recommended as part of a balanced diet (satisfying vegetable and fiber requirements) by governmental and non-governmental authorities (U.S. Department of Health and Human Services, and U.S. Department of Agriculture, 2015; U.S. Department of Agriculture, and U.S. Department of Health and Human Services, 2020; McConnell, 2021); however, corn may contain contaminants of concern (COC), naturally occurring

compounds that are typically regulated due to potentially toxic effects, acquired from the soil on which it is grown. This may be especially true for corn grown on soils amended with dredged sediments that are increasingly sourced from industrial areas. For example, sediments dredged from Lake Erie shipping lanes have been proposed as a soil amendment in Ohio due to their ability to mitigate nutrient depletion as it is rich in nutrients, organic matter and has high water retention (Bhairappanavar et al., 2018). Dredged sediments have also been shown to contain COC including heavy metals, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, pharmaceuticals and other emerging COC, chemicals or toxicants that may have an impact on human health but are not yet regulated (Hull and Associates Inc, 2018). These contaminants persist and accumulate within the environment and can potentially biomagnify in animals who consume plants grown on these sediments (Darmody et al., 2004; Ebbs et al., 2006). The COC within dredged sediments poses particular concern for the developing brain since early developmental periods are characterized by heightened susceptibility to toxicants. Because dredged material in Ohio will likely be applied to fields growing corn for animal consumption, commercially available feed corn was used as a reference comparison to maintain biological relevance. While this feed corn may not be entirely devoid of COC, future studies will assess the effects of corn from a variety of sources in comparison to a group with no corn exposure.

Gestational and perinatal exposure to many COC found in dredged material may affect neurodevelopmental processes such as neurogenesis, gliogenesis, synaptogenesis, apoptosis, and migration, and can alter a variety of behaviors (Bushnell et al., 2002; Naveau et al., 2014; Hossain et al., 2016; Rao Barkur and Bairy, 2016). Numerous studies suggest that the developing perinatal brain is more susceptible to the effects of COC, as these toxicants can cross the placenta and interfere with the developmental processes mentioned above (Knudsen, 2004). One region that is particularly sensitive to environmental toxins is the hippocampus (Stoltenburgdidinger, 1994; Reddy et al., 2007; Pierozan et al., 2020). Exposure to COC during gestation and lactation can impair the growth of this region and can cause long-term changes in hippocampal-dependent behaviors involving learning and memory, sometimes in a sex-specific manner (Curtis et al., 2011; Karlsson et al., 2015). Because corn grown on dredged material is likely to contain numerous bioaccumulated COC that alter neurodevelopment and behavior in isolation, the current preliminary study examines the effects of pre- and postnatal exposure to this corn and/or commercial feed corn on the neural, physical, and behavioral development of male and female Long-Evans rats.

2. Methods

2.1. Subjects

Adult male and female Long Evans rats (approximately 3–4 months of age) were purchased from Envigo (Indianapolis, IN, USA) to be used as breeders. The offspring of these breeders were used as experimental subjects. All animals were kept on a 12:12 h light/dark cycle with lights off at 20:00 and were housed in polycarbonate cages with corn cob bedding and glass water bottles. Animals were weaned on postnatal day (P) 25 and were housed in pairs or triplets with same-sex littermates. Throughout the study, animals had *ad libitum* access to food and water. The diet utilized was the Teklad Rodent Diet 8604 (Envigo), which is primarily composed of soybeans, wheat and corn. Experimental corn exposure (described below) was a supplement to this *ad libitum* diet. All experimental procedures were approved by Bowling Green State University's (BGSU) Institutional Animal Care and Use Committee (IACUC, protocol: 1485417) prior to the study.

2.2. Growth of dredge-amended corn and COC profiles

Corn (*Zea mays*) was grown on dredged sediments at the Great Lakes Dredged Material Center for Innovation (GLDMCI) located in Toledo, Ohio (41.6700354oN–83.5029711oW). Sediments were originally dredged from Toledo Harbor and the Maumee River in 2016–17 and allowed to dewater between 2017 and 18 before being planted with corn (variety: W2903DP; Wellman Seeds Inc., Delphos, OH, USA) during the 2019 growing season (Rúa et al., 2023). Existing vegetation was sowed into the dredged material prior to sowing rows of corn. All plots were sprayed with a commonly used herbicide mixture containing glycophosate (1.5 qt/acre, 53.8%) and ammonium sulphate (1.5 lb/acre, 48%) in 20 gallons of water per acre (Rúa et al., 2023). This herbicide mixture is standard practice and is not unique to corn growth with dredge amendments. Ears of corn were hand harvested at the end of the growing season and then dried in a drying oven at 55°C for at least 48 h and then stored with desiccant until use. Analysis of COC profiles of commercial feed and dredge-amended corn is ongoing as part of a larger collaboration.

2.3. Corn dosing

Animals were randomly assigned to receive corn grown on dredged material or commercially available feed corn grown without the use of soil amendments (Tractor Supply Inc). Whole corn kernels were presented as a dietary supplement (without food restriction) at a dose of 6 g/kg. One cohort of subjects received this exposure in adulthood. These animals were given either type of corn for 20 consecutive days from P120—P139 (5 males and 4 females in each corn group: Adult Exposure Group). This preliminary study was included to investigate the potential for acute toxic effects in adults prior to administering this corn to pregnant dams. As these data are preliminary and since no sex differences were anticipated, this smaller cohort of animals was utilized for the adult exposure groups. In a second cohort (12 males and 11 females in each corn group: Developmental Exposure Group), corn exposure took place during gestation and lactation. Dams were given either corn type from embryonic day 0 throughout gestation and lactation until the day of weaning. All animals rapidly consumed all the corn

within several minutes. The offspring of these exposed dams were used to assess behavior and neuroanatomy in adulthood. Eleven females and 12 males were developmentally exposed to commercial feed corn, while 11 females and 12 males were exposed to dredge-amended corn. There was no effect of corn exposure on litter size, pup sex ratio or body weight (data not shown).

2.4. Behavior testing

Behavioral tests were conducted in 9ft. × 9ft. temperature-controlled room within the vivarium. All animals, regardless of testing day, were acclimated in their home cages to the testing room for at least 5 min, but no longer than 15 min, before being tested. All arenas were cleaned with 30% ethanol and allowed to dry between subjects. All tests were scored by experimenters blind to corn exposure during behavior testing.

Subjects exposed to the corn as adults were behaviorally tested in the Elevated Plus Maze (EPM) 1 day after their last dose of corn. The EPM is composed of two perpendicular arms, one arm being walled or enclosed and the other being open and is used to measure anxiety-like behavior (Lister, 1987). For a period of 5 min, experimenters manually assessed the amount of time spent in the open arms, the latency to enter an open arm, the number of entries into open arms and the number of total arm entries. Animals in the developmental study were weaned on P25 and began behavior testing on P70 with the EPM. EPM behavior was administered first to assess baseline anxiety-like behavior prior to further behavior testing and maze/apparatus exposure. The following day, animals were tested in the Open Field task (OF). The OF arena was a 70 cm × 70 cm plexiglass box with walls 40 cm high and a 15 cm × 15 cm "center area." Animals were allowed to explore the box freely for a 5 min period while the experimenter recorded the latency to enter the center, the amount of entries into the center and the amount of time in the center. Center activity in the OF has also been associated with anxiety-like behavior (Zohner, 1968; Tartar et al., 2009).

The next day, animals were tested in the Novel Object Recognition task (NOR), which examines exploratory behavior toward novel objects, and recognition memory (Ennaceur and Delacour, 1988; Ennaceur and Meliani, 1992; Lueptow, 2017). This test consisted of two trials. In the first, the animal is placed in the OF test box with two identical objects equidistant from two corners of the box. Animals were allowed to freely explore the objects for a period of 5 min, and the experimenter recorded interaction time with each object. After a 2 h inter-trial interval, there is a second trial where one of the identical objects is replaced with a novel object, and the amount of interaction time with each object is recorded. More time with the novel object is considered to indicate greater recognition memory, as rodents typically choose to interact with novel elements in their environment. Objects used in the NOR test were of similar size and weight, used previously with no inherent preference indicated, and cleaned between subjects. These three behavioral tests were included in this preliminary experiment as they are standard tests used to assess exploratory behavior, anxiety and recognition memory in laboratory subjects. Future work will employ a more comprehensive battery of behavior tests to assess other facets of cognition.

2.5. Histology—assessment hippocampal volume

Following behavior testing, a subset of subjects ($n = 4$ males and 3 females per feeding group; total $n = 14$) were anesthetized with a lethal dose of sodium pentobarbital and perfused with 4% paraformaldehyde. After brain extraction, tissue was stored in 4% paraformaldehyde for 48 h and then moved to a 30% sucrose solution for 72 h, after which it was sliced. Fixed tissue was cut into 40-micron with a freezing microtome and sections every 4th section containing the hippocampus was mounted on glass slides. Sections were stained with methylene blue-azure II as previously described (Markham et al., 2006; Willing and Juraska, 2015). Volumetric measurements were gathered using MicroBrightField Biosciences Stereoinvestigator software. Using a 2.5X objective, the hippocampus (including all layers/subregions), beginning at −1.80 mm to −5.60 from bregma, was traced on each section for each subject (approximately 25 sections per subject). Hippocampal areas were summed together to reach a combined area per subject, then multiplied by 40 microns, a thickness constant, resulting in an estimate of hippocampal volume. A subset of behavioral subjects was chosen to investigate the possibility of neuroanatomical changes between commercial corn and dredge corn groups.

2.6. Statistical analyses

For both Adult Exposure and Developmental Exposure experiments, behavioral measures of the EPM, OF, and NOR were compared using two-way ANOVA (Corn type × Sex) followed by Tukey HSD *post-hoc* tests. For the NOR test, an additional analysis for total object exploration was conducted using Students *t*-tests (conducted separately for males and females). Hippocampal volume was analyzed using a two-way ANOVA (Corn type × Sex). All statistical tests were conducted using SPSS Version 27.0.0.0 and *p*-values were determined to be significant when $p < 0.05$.

3. Results

3.1. Adult exposure

There was no main effect of corn treatment on any measure in the EPM [closed entries, $F(1,14) = 0.00705$, $p = 0.934$; open entries, $F(1,14) = 1.35$, $p = 0.264$; end visits, $F(1,14) = 0.422$, $p = 0.562$; open arm time, $F(1,14) = 2.08$, $p = 0.171$; open latency, $F(1,14) = 1.16$, $p = 0.300$; data not shown). There was a main effect of sex on open arm time [open arm time, $F(1,14) = 4.96$, $p = 0.043$; data not shown] but not for any other measure of the EPM. This effect was driven by dredge-amended corn females that exhibited increased time spent in the open arms. There were no significant differences in body weight [$F(1,14) = 0.00944$, $p = 0.924$] or

brain weight [$F(1,14) = 0.0214$, $p = 0.886$] observed between corn treatments.

3.2. Developmental exposure

3.2.1. Behavior

There were no significant effects of corn type on performance in the EPM [closed entries, $F(1,42) = 1.518$, $p = 0.23$, open entries, $F(1,42) = 0.863$, $p = 0.36$, end visits, $F(1,42) = 0.701$, $p = 0.41$, open arm time, $F(1,42) = 0.627$, $p = 0.43$, open latency, $F(1,42) = 0.141$, $p = 0.71$; data not shown]. In the OF test, there was no difference between groups for time spent in the center of the arena [$F(1,42) = 1.824$, $p = 0.18$], but there was a significant difference in the number of center entries by corn treatment [$F(1,42) = 5.672$, $p = 0.02$; Figure 1B]. The commercial feed corn group exhibited more center entries than the dredge-amended group; this effect was most prominent in dredge-amended females, who had the lowest average of center entries. There was also a significant difference in center latency between males and females [$F(1,42) = 6.873$, $p = 0.012$; Figure 1A] with females exhibiting increased latencies. A non-significant trend was also found between corn treatment and center latency [$F(1,42) = 3.933$, $p = 0.054$, Figure 1A], with subjects exposed to dredge-amended corn having a longer latency. There was not a significant interaction between sex and corn treatment for center latency [$F(1,42) = 0.595$, $p = 0.45$].

In the NOR test there was a significant effect when examining interaction time with the familiar object compared to the novel object between corn treatments. Subjects in the commercial feed corn treatment spent significantly more time with the familiar object than subjects in the dredge amended corn treatment [$F(1,42) = 6.354$, $p = 0.016$; Figure 2A]. However, an analysis of total object exploration time (combining interaction time from trials 1 and 2) revealed that compared to subjects fed commercial feed corn, exposure to dredge-amended corn reduced total object interaction (novel and old objects, combined) in males ($t = 2.375$, $p = 0.027$; Figure 2B), but not females ($t = 0.122$, $p = 0.904$). There was no effect of corn type on the time (Figure 2C) or the percentage of time interacting with the novel object (F1,42 = 0.761, $p = 0.388$; Figure 2D; Percent Novel = Amount of Time with Novel / Total Object Interaction Time).

3.2.2. Hippocampal volume

There was not a significant main effect of corn type on hippocampal volume [$F(1,10) = 3.325$, $p = 0.098$]. There was a significant effect of sex [$F(1,10) = 22.84$, $p < 0.001$; Figure 3] with males having a greater hippocampal volume than females. Additionally, there was a significant interaction between corn treatment and sex [$F(1,10) = 23.29$, $p < 0.001$]. A *post-hoc* test revealed that developmental exposure to dredge-amended corn reduced hippocampal volume in adult male, but not female, rats compared to rats fed commercial feed corn ($p_{tukey} = 0.002$; Figure 3B).

4. Discussion

The purpose of the present study was to begin to examine potential long-term effects of corn grown on dredged sediments on mammalian behavior and neurodevelopment. Though the present study is preliminary, these results suggest that a dietary supplement of dredge-amended corn during an early critical period of brain maturation can affect both behavior and neuroanatomy compared to diets supplemented with commercially available feed corn. While adult exposure to dredge-amended corn in adulthood did not affect body weight, brain weight or behavior in the EPM, exposure during early development did have several significant effects. Developmental exposure to dredge-amended corn led to fewer entries into the center area of the OF, a slightly increased latency to enter the center, and a sex-specific decrease in time spent interacting with objects in the NOR. Additionally, developmental exposure to dredge-amended corn led to a sex-specific decrease in adult hippocampal volume.

Subjects from the developmental exposure study reflect a population that is more susceptible to COC influence. Toxicant exposure during critical periods of development can permanently alter brain development and behavior. Gestational and lactational exposure to corn grown on dredged sediment and commercially available feed corn allows for the examination of potentially indirect exposure to COC. The EPM and OF tests were utilized in the developmental exposure study to measure anxiety-like behavior. The OF test revealed that exposure to dredge-amended corn led to an increase in the number of center entries compared to commercial feed corn. While there were no significant findings from the EPM test, decreased center entries in an OF test does suggest a modest effect on anxiety-like or exploratory behavior induced by dredge-amended corn. Aside from anxiety, dredge-amended corn could also be affecting motivation and/or exploratory behavior. Interestingly, females of the dredge-amended corn group had the lowest average of center entries of all groups, suggesting that potential effects of dredge on anxiety-like behavior may reflect a heightened susceptibility in females. Results from the NOR test revealed that the commercial feed corn group had more interaction time with familiar old object in the second trial of the test. Though no effects were seen in the percentage of time spent with the novel object, this finding could suggest that commercial feed corn subjects had decreased recall of the old object, or that they experienced increased averse-to-novelty behaviors. However, this effect might have been driven in part by the fact that males exposed to dredge-amended corn spent less time overall interacting with objects in trials 1 and 2.

Developmental exposure to dredge-amended corn also reduced adult hippocampal volume, but only in male subjects. Previous studies have reported sex-specific effects following early environmental toxicant exposure. As complete contaminant profiles of dredge and commercial corn have not yet been determined; this preliminary study relies on previous work suggesting broad toxicant mixtures in dredge material (Hull and Associates Inc, 2018). These toxicant mixtures have been shown to induce sex-specific effects on the brain and behavior. For example, researchers dosed pregnant mice with either a single endocrine disrupting chemical (EDC) or a mixture. Results indicate that male progeny exposed to the mixture displayed a sex-specific behavior reversal in exploration time during the novel object recognition task (Sobolewski et al., 2014). Although an exposure to a single EDC or the mixture resulted in behavioral differences, this sex-specific reversal may suggest mixtures of EDC could impact male and female brains disproportionately. A similar study found that,

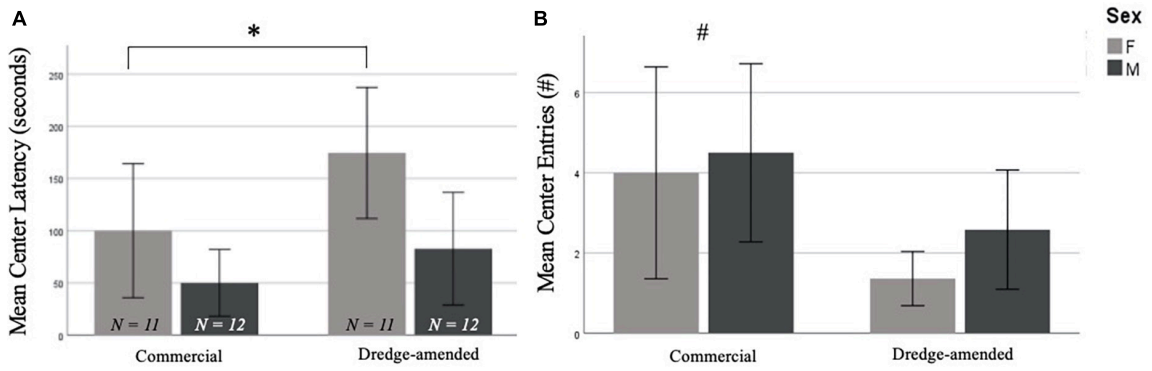

FIGURE 1
Early exposure to dredge-amended corn affects adult Open Field Test (Total $N = 46$; $N = 12$ males and 11 females per corn group). Mean center latency in seconds **(A)** and mean number of center entries **(B)** in the Open Field Test with standard error bars. Females in both commercial and dredge-amended groups exhibited increased center latencies compared to males in either group (sex-specific difference indicated by *). A non-significant trend showed increased latencies in the dredge-amended group. A corn treatment difference (indicated by #) was observed for the number of center entries. Dredge-amended groups had significantly fewer center entries compared to the commercial feed corn group.

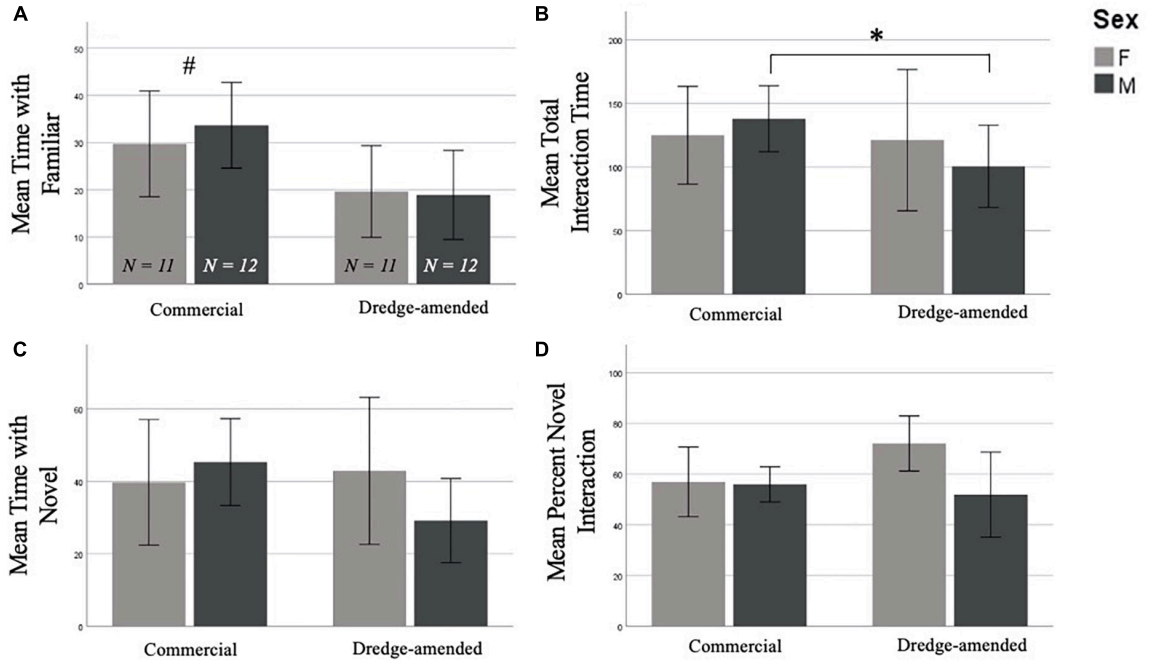

FIGURE 2
Early exposure to dredge-amended corn affects adult Novel Object Recognition Test (Total $N = 46$; $N = 12$ males and 11 females per corn group). Mean time spent with the familiar object **(A)**, mean combined trial 1 and trial 2 object interaction time **(B)**, mean time spent with the novel object **(C)**, and mean percent novel interaction **(D)**, percent novel interaction (Amount of Time with Novel/Total Object Interaction Time) of the Novel Object Recognition Test with standard error bars. There was an effect of corn treatment (indicated by #) on interaction with the familiar object; additionally, and dredge-amended males spent significantly less time interacting with objects compared to commercial feed corn males (indicated by *). Time spent with the novel object and percent novel measurements did not result in significant differences between corn treatment or sex.

following prenatal exposure to an EDC mixture delayed puberty in males but not in females (Gore et al., 2022). Since studying toxicant mixtures is often more clinically relevant given the mechanism of human exposure, most research in neurotoxicology focuses on assessing a single toxin in order to better determine a mechanism of action (Cory-Slechta, 2005).

Previous work suggests that the hippocampus is a target for a variety of toxicants. Broadly, prenatal exposure to environmental toxins has been shown to impair memory and cause cell loss in the hippocampus (Karlsson et al., 2011, 2015). Heavy metal exposure decreases recognition memory and dendritic spine density which may lead to increased cell death (López et al., 2009; Pulido et al., 2019). Early exposure to the PAH BaP impairs memory and reduces CA1 dendrites (Das et al., 2019). Prenatal BPA exposure decreases CA1 spine synapses, although the same effect was not seen when BPA was administered during the juvenile period suggesting an age-dependent effect (Elsworth et al., 2013). Chronic mercury exposure induces TNFα protein expression in the hippocampus,

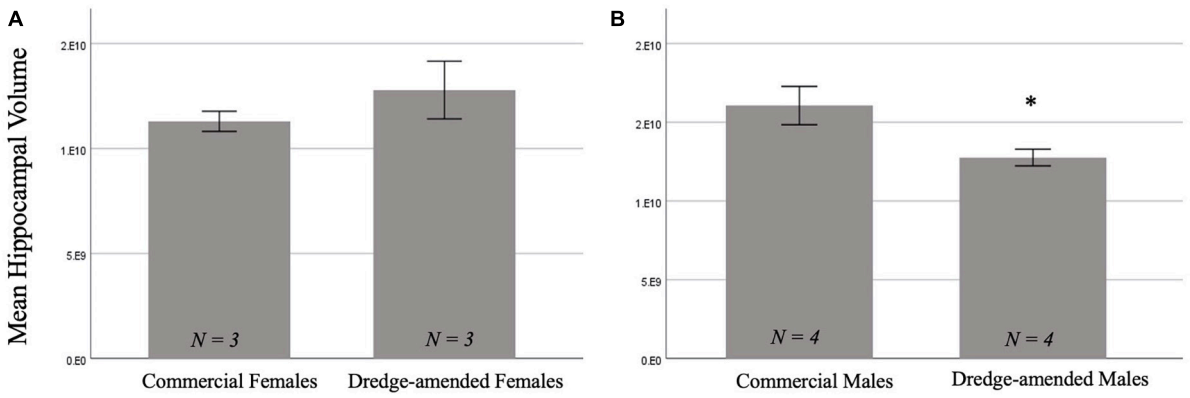

FIGURE 3
Early exposure to dredge-amended corn affects adult hippocampal volume. Mean hippocampal volume measurements for commercial and dredge-amended females [(**A**); $N = 6$] and males [(**B**); $N = 8$] with standard error bars. Dredge-amended males had a decrease (indicated by *) in hippocampal volume compared to their commercial counterparts. There was no significant effect of corn treatment in females.

which could lead to apoptotic events, a result only observed in male subjects (Curtis et al., 2011). Prenatal toxicant exposure *via* dredge-amended corn consumption could induce increased cell death that could manifest in a reduction in hippocampal volume.

These sex-specific effects, including those found in the present study, may be due to a neuroprotective mechanism facilitated by estrogen (Yao et al., 2011). Hippocampal cells are notably affected by estrogen across the lifespan. For example, CA1 dendritic spine and synapse density show a positive correlational relationship with estradiol administration in ovariectomized rats, estrogen promotes synaptic plasticity, and estrogen promotes neurite outgrowth *in vitro* cultures (*Reviewed in* Brann et al., 2007). There are conflicting findings in the research investigating estrogen as a neuroprotective mechanism. Although the importance of gonadal steroid hormones cannot be overlooked, there is another mechanism that may influence sex-specific brain and behavioral differences. Research suggests that thyroid hormone could also contribute to sex-specific differences. There are sex-specific peaks in thyroid hormone and thyroid hormone has been shown to contribute to cell neurogenesis during development, cell migration, and adult neurogenesis (Bernal, 2007; Remaud et al., 2014; reviewed in Baksi and Pradhan, 2021). Prenatal PCB exposure may interfere with thyroid hormone signaling in progeny (Gauger et al., 2004), which could lead to sex-specific differences in neural development and behavior later in life. While broad mixtures of toxicants may affect a number of neuroendocrine and developmental facets, with this preliminary study we are hesitant to suggest a mechanism driving the behavioral and neuroanatomical affects documented here. Future work will incorporate the full toxicant profile of dredge-amended and commercial feed corn into the proposed mechanism by which corn supplements affect neural development.

This study's preliminary nature demonstrates the need for more research into implications of using dredged sediment as a soil amendment. Future studies will employ multiple doses of corn supplementation to determine a potential dose-response curve including corn that has been approved for human consumption, as commercially available feed corn may also contain COC. Additionally, a comparison will be made to animals without corn supplementation. A thorough battery of behavioral tests that assess multiple forms of cognition, including spatial learning/memory will be utilized. Future studies should also seek to determine the mechanisms by which dredge-amended and commercial feed corn alter the brain at the cellular level, assessing regional cell number and markers for apoptosis. This work should be pursued, as COC can magnify as they move up the food chain. If deviations from typical behavioral and neural development are being observed in a primary consumer, those same effects could be magnified in higher trophic levels due to increased exposure *via* bioaccumulation. Determining the potential effects of such soil amendments could have important clinical implications for animals and humans.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by the Bowling Green State University Institutional Animal Care and Use Committee (IACUC).

Author contributions

JW, MR, and LS: conceptualization and methodology. KF, MC, and VR: investigation. KF and JW: data curation and writing. JW and LS: funding acquisition. All authors contributed to the article and approved the submitted version.

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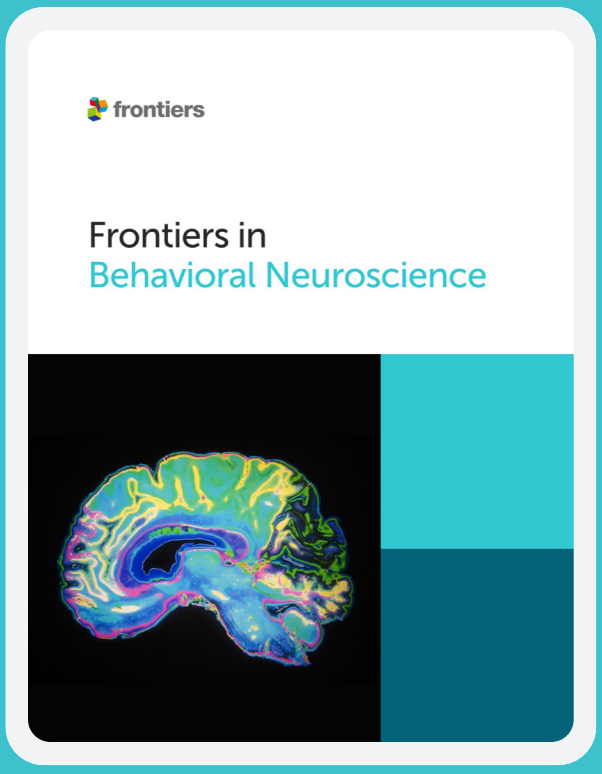

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