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Sexually dimorphic effects of a modified adolescent social isolation paradigm on behavioral risk factors of alcohol use disorder in Long Evans Rats

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Abstract

Early life stress (ELS) is a major risk factor for alcohol use disorder (AUD) and comorbid neuropsychiatric conditions. We previously demonstrated that an adolescent social isolation (aSI) model of ELS significantly increased behavioral risk factors for these disorders (e.g. anxiety-like behaviors, alcohol drinking) in male, but not female rats. Since many neurodevelopmental milestones are accelerated in females, we investigated whether an earlier/shorter isolation window (PND 21–38) would yield comparable phenotypes in both sexes. In two experiments, Long Evans rats were socially isolated (SI) or group-housed (GH) on postnatal day (PND) 21 and locomotion was assessed in the open field test (OFT; PND 30). Experiment 1 also assessed behavior on the elevated plus-maze (EPM) (PND 32). In Experiment 2, all rats were single housed on PND 38 to assess home cage alcohol drinking. Experiment 1 revealed that SI females had increased locomotor activity in the OFT but did not differ from GH subjects on the EPM. The OFT results were replicated in both sexes in Experiment 2 and both male and female SI rats had significantly greater ethanol consumption during an eight day continuous access paradigm. In contrast, during subsequent intermittent two-bottle choice drinking, only SI females displayed greater ethanol intake and preference and increased consumption of a quinine-adulterated alcohol solution. These findings demonstrate that early life social isolation can promote AUD vulnerability-related phenotypes in female rats but that there are profound sex differences in the vulnerability window to this early life stressor. Uncovering the neural mechanisms responsible for these sexually dimorphic differences in sensitivity to ELS may shed light on the biological substrates associated with vulnerability to AUD and comorbid disorders of negative emotion in men and women.

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Declaration of Competing Interest

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

Keywords

Early life stress; Social isolation; Sex differences; Alcohol use disorder; Disorders of negative emotion

1. Introduction

Exposure to stress during childhood and adolescence is a major risk factor for alcohol use disorder (AUD) [20,22,31,51,52] and other neuropsychiatric conditions that are highly comorbid with AUD, such as post-traumatic stress disorder (PTSD) [13,16,25,43,52], depression [39, 55,68], and anxiety [39]. In the human population, early life stress (ELS) involves exposure to acute and/or chronic stressors, including physical or sexual abuse, neglect, violence, witnessing violence and/or death, war, terrorism, and natural disasters, among other experiences [16,43,66]. Many researchers have hypothesized that the experience of stress during critical windows of development results in neurological, epigenetic, and endocrine changes that may lead to the development of psychiatric conditions in later life [9,43,48,55,66]. Therefore, it is of critical importance to understand the biological responses to ELS in order to improve prevention and treatment of AUD and comorbid disorders.

In addition to ELS, biological sex has been a known risk factor for AUD and related comorbid conditions for decades. Historically, men have been more likely to be diagnosed with AUD and are more likely to die from alcohol-related harm than females [72]; however, rates of alcohol use between males and females have been narrowing [71]. Data from the National Survey on Drug Use and Health [54] suggests that adolescent females and males (ages 12–17) now have comparable rates in past-year drinking (20.6 % females, 15.0 % males) and past-year binge drinking (4.7 % females, 3.0 % males). Recent evidence has also shown higher binge, heavy, and high-risk drinking, as well as increased AUD prevalence, in adult (aged 18+) women compared to men [26,28]. Recent data have also revealed that males and females had similar relative percent increases in alcohol-related deaths when comparing the first year of the COVID-19 pandemic (2020) and the year prior (2019) [72]. Of note, there was also a 35 % increase in deaths with a cause of alcohol-related mental and behavioral disorders between 2019 and 2020 [72], highlighting the relationship between AUD and comorbid conditions. Indeed, there exist striking sex differences in the prevalence of many of the disorders of negative emotion which frequently co-occur with AUD. For example, women are about twice as likely as men to meet the diagnostic criteria for PTSD according to the DSM-5 classification, even after controlling for differences in trauma type [50,15,40]. Similarly, women are twice as likely to be diagnosed with depression compared with men, an effect that is initially observed during puberty and remains throughout adulthood [61]. The lifetime prevalence of generalized anxiety disorder is also higher in women (6.6 %) compared to men (3.6 %) [2]. Of note, the onset of anxiety and depressive disorders peaks during adolescence, leading early adulthood and childhood and adolescence to be considered the “core risk phase” for the development of anxiety and depressive disorders [6,61].

Furthermore, developmental differences have been observed between males and females during childhood and adolescence. One longitudinal MRI study that examined developmental trajectories of healthy children and adolescents found that both cortical and subcortical gray matter trajectories show peak sizes 1 to 2 years earlier in females than males and these peaks correspond to the average age of puberty [44]. Other studies have observed sexually dimorphic developmental trajectories of white matter tracts [62], pubertal timing and tempo [46], as well as in EEG measures [27], where age–related changes were observed earlier in girls than boys. Despite evidence of sexually dimorphic developmental trajectories in humans across structural, functional and physiological domains, the manner in which biological sex interacts with early life stress to potentially promote later neuropsychiatric conditions remains largely unclear (for review see [32]). Taken together, these observations support the importance of taking developmental trajectory into account when examining sex differences in vulnerability windows to early life stress.

Given the heterogeneity of stress in the human experience, rodent models are particularly useful to examine neurobiological, physiological, and behavioral responses to stress due to the ability to precisely control the nature and duration of the stressor. To study the neurobiological underpinnings that might explain why ELS increases the risk of developing AUD and disorders of negative emotion, our lab has employed a rat adolescent social isolation (aSI) paradigm to model early life stress [10,12,14]. Our standard protocol involves procuring male Long Evans rats on postnatal day (PND) 21 and housing them in groups of 4 in large plexiglass cages for one week to habituate to the facility. Then, on PND 28, rats are randomly divided into two housing conditions until PND 70. Socially isolated (SI) rats are single housed in smaller cages and group housed (GH) animals remain in groups of four in the larger cages. Extensive work from our lab and others has shown that, relative to GH rats, adult SI animals exhibit numerous behavioral phenotypes linked with vulnerability to AUD and comorbid conditions, including locomotor hyperactivity in a novel environment [14,65], increases in anxiety-like behaviors [47,65,75], and deficits in fear extinction learning [65]. Moreover, SI rats also display increases in appetitive (i.e., seeking) and consummatory (i.e., intake) measures of operant ethanol self-administration [47] and enduring escalations in home cage ethanol consumption and preference [14,47,65]. This model has also been shown to promote maladaptive neurobiological changes in several brain regions associated with addiction, anxiety, and depression (see [45] for comprehensive review). For example, when examined in adulthood, SI subjects exhibit increases in the intrinsic excitability of pyramidal cells in the basolateral amygdala [59], synaptic hyperexcitability and disrupted plasticity in the ventral hippocampus [1], and alterations in dopamine terminal functioning [36,37,74,75] and kappa opioid receptor modulation of dopamine release [38] in the ventral striatum. Surprisingly, when tested in female rats, this ELS paradigm failed to promote many of these phenotypes [11]. The lack of reproducibility using this model in female rats is particularly concerning given the repeated clinical observations suggesting that ELS is a major risk factor for AUD and comorbid conditions in both sexes.

One possible reason that the aSI paradigm did not engender AUD-related phenotypes in female rats may be that there are sex differences in the vulnerability window to various forms of ELS. Indeed, it is well known that many important neurodevelopment milestones occur earlier in females than males. In addition to reaching puberty earlier than male

rats [63], several brain structures such as the prefrontal cortex, amygdala, striatum, and hippocampus mature earlier in females, as well [29,69]. Additional studies suggest that the gamma-aminobutyric (GABA) and dopamine neurotransmitter systems also develop earlier in female rodents [41,56]. There are also reports of sex-dependent changes in neural development as a result of ELS. For example, the number of parvalbumin-containing interneurons in the PFC was significantly reduced among rats with a history of ELS compared to controls; however, this finding emerged earlier in life among females compared to males [33]. Honeycutt et al. [34] reported that both male and female rats had accelerated maturation of BLA-PFC innervation in response to ELS and that such innervation was significantly correlated with anxiety-like behavior. Once again, this neurobiological adaptation occurred earlier in females compared to males.

Given the accelerated pace of female neurodevelopment, the current study aimed to examine whether a modified (earlier and shorter) aSI protocol could yield behavioral and drinking phenotypes in female rats. Specifically, in Experiment 1 we employed the social isolation protocol between PND 21–38 with female Long Evans rats and assessed their locomotor response to a novel environment and behavior on the elevated plus-maze, two measures that repeatedly yielded specific phenotypes when using the aSI model in male Long Evans rats. In Experiment 2, we characterized this early aSI paradigm in male and female Long Evans rats by replicating the locomotor response data from Experiment 1 and also assessed home cage drinking behaviors.

2. Materials and methods

2.1. Subjects

Experiment 1 used 56 adolescent female Long Evans rats to assess the effects of social isolation on behavior during the open field test and elevated-plus maze. Rats were aged 21 days, weighing approximately 35 g (± 0.88 g), upon arrival (Envigo Laboratories, Indianapolis, IN). Experiment 2 used an additional 32 adolescent male (n = 16) and female (n = 16) Long Evans rats to replicate the open field test behavior and assess the effects of social isolation on home cage ethanol drinking (Fig. 2). Rats were aged 21 days upon arrival (Envigo Laboratories, Indianapolis, IN). Males weighed approximately 33 g (± 0.91 g) while females weighed approximately 35 g (± 1.12 g) upon arrival. On the day of arrival, animals were randomly selected to be GH (4/cage) or SI. GH animals were housed in large plexiglass cages (33.0 × 58.7 cm; Nalgene, Rochester, NY), while SI rats were housed in standard cages (20.3 × 26.7 cm; Allentown Inc., Allentown, NJ). GH and SI rats were housed in the same room and lived under the same conditions other than SI rats being physically restricted from any physical contact and/or social interaction with their peers. All rats in Experiment 1 were maintained on a reverse 12 h light/dark cycle while all rats in Experiment 2 were maintained a standard 12 h light/dark cycle, due to facility limitations. Food and water were available ad libitum for the duration of all studies. All animal care procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Wake Forest Institutional Animal Care and Use Committee.

2.2. Measures of behavioral risk factor phenotypes

2.2.1. Open field test—On PND 30, locomotor response to a novel environmental was assessed using the open field test (OFT; model-RXYZCM, Digiscan animal activity monitors; Omnitech, Columbus, OH). Subjects were placed in the back left corner of acrylic plastic chambers (42 × 42 × 30 cm) equipped with a 7.5 W frosted incandescent light and infrared photodetectors located 2.5 cm above the floor in arrays of 8 photobeams per wall. Total distance traveled was assessed for 30 min in 5 min time bins. Behavior was assessed during the dark cycle for Experiment 1 and the light cycle for Experiment 2.

2.2.2. Elevated plus-maze—On PND 32, unconditioned anxiety-like behavior was assessed using the elevated plus-maze among rats from Experiment 1 (EPM; Med Associates, St. Albans, VT). Rats were placed in the center junction and behavior was assessed for 5 min (300 s). Measures of anxiety-like behavior included time spent on the open arms and the number of open entries, while closed arm entries was used as a proxy of general locomotion. Behavior was assessed during the animal's dark cycle.

2.3. Home cage ethanol drinking procedure

On PND 38, all animals in Experiment 2 were single housed in standard cages and acclimated to two water bottles on their home cage. Starting on PND 39, rats were given concurrent access to ethanol and water for 23 h for 8 consecutive days. Ethanol concentration increased every other day (3 %, 5 %, 7 %, and 10 % (v/v)). Beginning on PND 49, an intermittent access two-bottle choice home cage drinking procedure was used for the remainder of the study [64,70]. Rats were given 24 h access to ethanol and water on Monday, Wednesday, and Friday and two bottles of water on the remaining days. Rats completed the intermittent access procedure for 15 weeks. For two weeks each, rats were given access to 10 %, 15 %, 20 %, and then 30 % ethanol (v/v). Following one week of forced abstinence, rats were given access to 30 % and 20 % ethanol for one respective week per concentration and then 10 % ethanol for two weeks. To assess how quinine would affect ethanol consumption, 100 μM of quinine was added to the 10 % ethanol bottle on Monday and Wednesday of the 14th week. Unadulterated 10 % ethanol was available on Friday of the 14th week and Monday of the 15th week. Finally, 25 μM of quinine was added to the 10 % ethanol on Wednesday and Friday of the 15th week. Both bottles were weighed following 30 min and 24 h of ethanol access. Total fluid consumption, preference ratios (ethanol consumed / (water + ethanol consumed)), and g/kg (1000 / [body weight] * ([intake] / 5 * 0.0397 * [ethanol concentration])) were calculated at the 30 min and 24 h time points. The placement of the bottles was switched each session to avoid side preference.

2.4. Statistical analyses

In Experiment 1, a repeated measures two-way ANOVA was used to analyze total distance traveled by Time (6 levels: 5 min time bins from 0 to 30 min) and Housing (2 levels: SI, GH). Welch's *t*-tests were used to analyze differences between SI and GH females regarding cumulative total distance traveled on the OFT and differences in open arm time, open arm entries, and closed arm entries on the EPM. In Experiment 2, a repeated measures three-way ANOVA was used to analyze total distance traveled using within-subject factors of Time

(6 levels) and between-subjects factors of Housing (2 levels) and Sex (2 levels: males, females). Similarly, repeated measures three-way ANOVAs were used to analyze drinking data. For the eight sessions of continuous access, Sex (levels = males, females) and Housing (levels = SI, GH) served as between-subjects factors while Concentration (levels = 3, 5, 7, 10 (%)) served as a within-subject factor. For the remaining 12 weeks of intermittent access, Week (levels = each week of access) was used as the within-subjects factor. The Greenhouse-Geisser correction was applied when the assumption of sphericity was violated. To further probe these relationships, simple main effect and interaction analyses were performed and followed up with pairwise comparisons using Bonferroni adjustments. Data were analyzed using GraphPad Prism version 9.0.0 or R version 4.3.1. Descriptive statistics are reported as mean ± SEM.

3. Results

3.1. Experiment 1

3.1.1. Open field test—A repeated-measures two-way ANOVA revealed statistically significant differences between SI and GH females (Housing: $F_{(1, 54)} = 10.62$, $p = .002$; Time: $F_{(2.5, 137.2)} = 132.4$, $p < .0001$; Housing x Time interaction: $F_{(5, 270)} = 2.59$, $p = .026$) (Fig. 1A). While distance traveled decreased between both groups as time elapsed, the reduction in locomotor activity was stronger in GH females, especially between minutes 10 and 25. When averaging cumulative distance traveled throughout the entire 30 min session, Welch's two sample *t*-test revealed that SI rats traveled significantly more than GH rats ($t_{(50.3)} = 4.99$, $p < .0001$, 95 % CI [698.0, 1637.5]; Fig. 1A inset). SI rats traveled approximately 1.5x as much as GH rats, with SI rats traveling an average of 3296 ± 186 cm. and GH only traveling an average of 2128 ± 748 cm. in 30 min.

3.1.2. Elevated plus-maze—Welch's two sample *t*-tests revealed no differences in open arm time ($t_{(38.6)} = -1.68$, $p = .101$, 95 % CI [−27.73, 2.56]), number of open arm entries ($t_{(42.3)} = -1.71$, $p = .095$, 95 % CI [−2.18, 0.18]), nor number of closed arm entries ($t_{(53.8)} = 0.77$, $p = .45$, 95 % CI [−0.75, 1.67]) between SI and GH rats (Fig. 1B–D).

3.2. Experiment 2

3.2.1. Open field test—A three-way repeated measures ANOVA revealed statistically significant main effects of Time ($F_{(3.5, 97.8)} = 105.96$, $p < .0001$, $\eta^2 = 0.70$), Housing ($F_{(1, 28)} = 39.24$, $p < .0001$, $\eta^2 = 0.34$), and Sex ($F_{(1, 28)} = 12.62$, $p = .001$, $\eta^2 = 0.14$) (Fig. 3). There was a significant Housing x Time interaction ($F_{(3.5, 97.8)} = 4.95$, $p = .002$, $\eta^2 = 0.10$). All other interactions were not statistically significant (ps > 0.05). Simple two-way ANOVAs were assessed at both levels of sex. We report statistically significant main effects of housing condition when assessed among females ($F_{(1, 14)} = 17.6$, $p < .001$, $\eta^2 = 0.33$) and males ($F_{(1, 14)} = 21.8$, $p < .001$, $\eta^2 = 0.35$) (Fig. 3 insets), as well as significant main effects of Time (ps < 0.0001). To probe differences at each 5 min time bin per sex, simple main effects were assessed when data were grouped by sex and time bin with a Bonferroni adjustment applied. SI females traveled significantly more than GH females between minutes 5 and 30 while SI males traveled significantly more than GH males between minutes 5 and 20 ($p_{sadj} < 0.05$).

3.2.2. Home cage drinking

Continuous Access.: To first assess the effects that social isolation had on two-bottle choice ethanol drinking, rats had continuous (23 h) access to ethanol and water at increasing concentrations. When assessing ethanol intake (g/kg), a 3-way repeated measures ANOVA revealed that Housing served as a significant main effect ($F_{(1, 28)} = 28.98$, $p < .0001$, $\eta^2 = 0.32$) while there were no significant main effects of Sex ($F_{(1, 28)} = 0.53$, $p = .47$) nor Concentration ($F_{(1.8, 50.4)} = 2.20$, $p = .13$). These data reveal that regardless of sex, SI rats drank significantly more ethanol over the 8 days of continuous access than those reared in group housing. Simple pairwise comparisons, grouped at the level of each sex, revealed that SI females consumed significantly more ethanol than GH females at 5 % ($p_{adj} = 0.013$) and 7 % ($p_{adj} = 0.005$) while a significant difference between SI and GH males was only observed at 7 % ($p_{adj} = 0.006$) (Fig. 4A).

During continuous access, a difference between SI and GH animals also emerged when assessing preference for ethanol. Overall, SI rats had greater preference for ethanol than GH rats (Housing: $F_{(1, 28)} = 26.64$, $p < .0001$, $\eta^2 = 0.31$; Sex: $F_{(1, 28)} = 0.34$, $p = .57$; Housing x Concentration: $F_{(2.1, 57.4)} = 4.71$, $p = .012$, $\eta^2 = 0.08$). Among females and males, preference decreased as the concentration of ethanol increased during continuous access (Concentration: $F_{(2.1, 57.4)} = 54.72$, $p < .0001$, $\eta^2 = 0.51$). At 3 % ethanol, SI and GH females had comparable preferences for ethanol at 71.0 % (± 5.9 %) and 53.7 % (± 7.7 %), respectively ($p_{adj} = 0.17$). Preference for ethanol decreased more dramatically among GH females when the concentration was increased to 5 %, reducing preference to 26.9 % (± 5.3 %) for GH females while SI females still had a preference of 63.6 % (± 6.7 %) ($p_{adj} = 0.01$). There remained a significant difference between SI and GH preference at 7 % ($p_{adj} = 0.006$) but this disparity disappeared when the concentration was increased to 10 % ($p_{adj} = 0.30$). Among males, there was no significant difference between SI and GH preference at 3 %, with mean preferences being 62.4 % (± 6.1 %) for SI and 44.6 % (± 7.6 %) for GH ($p_{adj} = 0.16$). Preference for both groups decreased as ethanol concentration increased. Like females, the reduction in preference was more apparent among GH animals, and pairwise multiple comparisons test between SI and GH males revealed a significant difference in preference at 7 %, with a mean preference of 42.5 % (± 5.8 %) for SI males and 13.1 % (± 2.9 %) for GH males ($p_{adj} = 0.006$). Preference was lowest for both groups at 10 %, with no detectable group differences ($p_{adj} = 0.21$).

It's possible that the difference in ethanol preference was influenced by differences in water intake. SI animals consumed significantly less water compared to GH animals during the eight days of continuous ethanol access (Housing: $F_{(1, 28)} = 20.06$, $p = .0001$, $\eta^2 = 0.29$; Concentration: $F_{(2.1, 57.6)} = 71.03$, $p < .0001$, $\eta^2 = 0.53$; Sex: $F_{(1, 28)} = 4.16$, $p = .051$; Housing x Concentration: $F_{(2.1, 57.6)} = 3.49$, $p = .04$). To this note, multiple comparisons tests revealed significant differences at similar concentrations that preference differences were observed. For both females and males, these differences were observed at 5 % (females $p_{adj} = 0.012$; males $p_{adj} = 0.045$) and 7 % (females $p_{adj} = 0.008$; males $p$adj = 0.008).

Intermittent Access.: Following the eight days of continuous access, ethanol was available on an intermittent schedule (Mon-Wed-Fri) at increasing concentrations for 8 weeks, was

removed for one week (forced abstinence), and then was available on an intermittent schedule at decreasing concentrations for an additional 4 weeks. When assessing daily ethanol intake (g/kg) using a repeated measures three-way ANOVA, a Sex x Housing interaction was observed ($F_{(1, 28)} = 8.62$, $p < .01$, $\eta^2 = 0.11$) so simple two-way interaction and main effect analyses were conducted grouped at each level of sex. Among females, we observed a significant main effect of Housing ($F_{(1, 14)} = 21.70$, $p < .001$, $\eta^2 = 0.34$) and Week ($F_{(3.3, 46.5)} = 4.63$, $p = .005$, $\eta^2 = 0.18$), but no significant Housing x Week interaction ($F_{(3.3, 46.5)} = 0.79$, $p = .52$) (Fig. 4B). Unlike females, we did not observe any significant main effects nor a significant Housing x Week interaction among males (Housing: $F_{(1, 14)} = 0.69$, $p = .42$; Week: $F_{(2.8, 38.8)} = 1.50$, $p = .23$; Housing x Week: $F_{(2.8, 38.8)} = 0.93$, $p = .43$). While the difference between SI and GH females was apparent throughout the duration of intermittent access, g/kg between SI and GH females particularly diverged during week 7, the week we introduced the highest ethanol concentration tested (30 %) ($p_{adj} = 0.02$). Following the one week of forced abstinence, SI females maintained significantly higher g/kg compared to GH females for four weeks ($ps_{adj} \leq 0.01$).

The intermittent drinking regimen promotes binge-like ethanol intake at the onset of each drinking session, with subjects consuming ~25 % of their daily intake during the first 30–120 min [57,64,65]. To that end, we also assessed ethanol drinking during the first 30 min. of each session. As observed with daily intake, a repeated measures three-way ANOVA revealed a significant Sex x Housing interaction ($F_{(1, 28)} = 8.62$, $p < .01$, $\eta^2 = 0.11$). Simple main effect analyses revealed that social isolation only resulted in increased drinking compared to GH controls in females (Housing: $F_{(1, 14)} = 21.90$, $p < .001$, $\eta^2 = 0.41$; Week: $F_{(3.8, 52.9)} = 6.75$, $p < .001$, $\eta^2 = 0.21$; Housing x Week: $F_{(3.8, 52.9)} = 0.92$, $p = .46$), but not males (Housing: $F_{(1, 14)} = 1.37$, $p = .26$; Week: $F_{(4.1, 58.0)} = 4.13$, $p = .005$, $\eta^2 = 0.18$; Housing x Week: $F_{(4.1, 58.0)} = 1.84$, $p = .13$) (Fig. 4D).

Upon examination of 24 h preference, we observed a significant main effect of Housing ($F_{(1, 18)} = 8.25$, $p < .01$, $\eta^2 = 0.10$) and Week ($F_{(3.0, 83.4)} = 14.33$, $p < .0001$, $\eta^2 = 0.24$), but not Sex ($F_{(1, 28)} = 1.43$, $p = .24$), suggesting that SI rats had significantly greater preference for ethanol regardless of sex. We also observed a significant Sex x Week interaction ($F_{(3.0, 83.4)} = 3.61$, $p < .05$, $\eta^2 = 0.08$), indicating sex differences in preference across the intermittent access paradigm. Simple main effect analyses further revealed that Housing served as a significant main effect for females ($F_{(1, 14)} = 13.50$, $p < .01$, $\eta^2 = 0.22$), but not males ($F_{(1, 14)} = 0.76$, $p = .40$), while Week served as a significant main effect for both females ($F_{(2.9, 40.3)} = 8.91$, $p < .001$, $\eta^2 = 0.31$) and males ($F_{(2.3, 32.4)} = 9.02$, $p < .001$, $\eta^2 = 0.27$). Taken together, these results suggest that SI females (26.35 % ± 2.50 %) had significantly greater preference for ethanol across the weeks of intermittent access compared to GH females (14.55 % ± 2.19 %) while SI (25.62 % ± 2.74 %) and GH (21.79 % ± 1.89 %) males had comparable preference.

Differences in preference were even more striking when examined at 30 min. Across the 12 weeks of drinking, SI females had an average preference of 63.35 % (± 2.62 %) when assessed after the first 30 min of access, while GH females only had a preference of 46.75 % (±3.09 %). Both SI (71.95 % ± 2.17 %) and GH (71.57 % ± 2.59 %) males had greater preference during the first 30 min than females. Indeed, we report significant main effects

of Sex ($F_{(1, 28)}$ = 19.12, p < .001, η^2 = 0.15), Housing ($F_{(1, 28)}$ = 4.92, p < .05, η^2 = 0.04), and Week ($F_{(4.2, 117.9)}$ = 17.24, p < .0001, η^2 = 0.32), as well as significant interactions between Sex x Housing ($F_{(1, 28)}$ = 4.49, p < .05, η^2 = 0.04) and Sex x Week ($F_{(4.2, 117.9)}$ = 1.58, p < .01, η^2 = 0.10). Similar to the 24 h data, simple main effect analyses revealed that Housing served as a significant main effect for females ($F_{(1, 14)}$ = 9.43, p < .01, η^2 = 0.14), but not males ($F_{(1, 14)}$ = 0.005, p = .95), while Week served as a significant main effect for females ($F_{(4.2, 58.5)}$ = 15.20, p < .0001, η^2 = 0.45) and males ($F_{(3.1, 43.8)}$ = 5.67, p = .002, η^2 = 0.23). Unlike the continuous access data, the differences in preference could not be attributable to differences in water intake as repeated measures three-way ANOVAs did not reveal a significant main effect of Housing nor a significant interaction between Housing x Sex when assessing 24 h or 30 min water consumption (ps > 0.05).

Quinine-adulteration.: 100 μm quinine was added to the 10 % ethanol bottles on the first two sessions of the 14th week of intermittent access. A 2-way ANOVA revealed a significant Sex x Housing interaction ($F_{(1, 28)}$ = 5.60, p = .025, η^2 = 0.17) but no significant main effects. This concentration of quinine suppressed ethanol consumption among all groups such that pairwise comparisons revealed no differences in ethanol intake (Fig. 4C). When assessing 30 min consumption, there was a significant Housing x Sex interaction ($F_{(1, 28)}$ = 12.29, p = .002, η^2 = 0.31) and no significant main effects. Post-hoc comparisons revealed no differences between SI and GH females (p_{adj} = 0.11) but did reveal that SI males drank significantly less adulterated ethanol than GH males (p_{adj} = 0.01) (Fig. 4E). Following two sessions of unadulterated 10 % ethanol consumption, a lower concentration of quinine (25 μm) was added to the ethanol solution. When assessing how 25 μm of quinine affected ethanol consumption, we report a significant Sex x Housing interaction ($F_{(1, 28)}$ = 14.94, p < .001, η^2 = 0.35) (Fig. 4C). Pairwise comparisons revealed that SI females drank significantly more adulterated ethanol than GH females (p_{adj} = 0.002) while SI males drank significantly less adulterated ethanol than GH males (p_{adj} = 0.03). These same patterns were observed at 30 min (Sex x Housing: $F_{(1, 28)}$ = 14.42, p < .001, η^2 = 0.34) (Fig. 4E).

4. Discussion

The current studies revealed that isolating young female rats during an earlier developmental window than historically used in males yielded a behavioral phenotype consistent with heightened vulnerability to AUD. Specifically, we report that both SI male and female rats, isolated from PND 21 – 38, had increased general locomotor activity in a novel environment while only isolated females showed increased home cage ethanol drinking in an intermittent paradigm compared to GH controls. Additionally, SI females demonstrated aversion-resistant drinking when challenged with quinine-adulterated alcohol while SI males did not. These findings provide initial evidence that SI can result in behavioral phenotypes that model vulnerability to stress-related disorders and AUD in both male and female rats but that the vulnerability window to this early life stressor may be sexually dimorphic.

In both experiments conducted in this study, SI males and females had increased locomotor activity in a novel environment compared to GH controls. This finding adds to the consistent reports that SI rats are significantly more active in the open field test compared to GH subjects [11,14,65], and, to our knowledge, is the first report of this finding in female

rats. Heightened novelty responding is considered a preclinical predictor of increased stimulant [7,18,56] and ethanol [53] intake. Data from Experiment 2 suggest that we can also observe this phenotype in male SI animals earlier than we've historically assessed (~ PND 30 vs. PND 56 [65]). Due to facility limitations, Experiments 1 and 2 had to be conducted under opposite light cycles (standard vs. reverse); however, it is of interest that these differences did not influence the OFT results, highlighting the generalizability of this finding. Interestingly, while both male and female SI animals demonstrated increased locomotor activity, only SI females developed an enduring escalation of ethanol intake and preference later in life under this adapted aSI paradigm, decoupling the positive relationship between increased locomotor activity and later drinking phenotypes.

In Experiment 1 we also report that SI females spent less time and made fewer entries into the open arm of the EPM compared to GH females; however, this result was not statistically significant. Previous work from our lab has also failed to detect differences in behavior on the EPM among SI and GH females when using a later and longer isolation window (PND 28 – 70) [11]. The EPM is commonly considered a preclinical model of anxiety-like trait behaviors because treating rats with anxiolytic or anxiogenic drugs resulted in increased and decreased exploration of the open arms in dose-dependent manners, respectively [30]. However, it is noteworthy that this seminal work was originally validated in male rats only. Indeed, subsequent studies have highlighted that the EPM may be less effective in capturing "anxiety-like" behavior in female rats [24]. Likewise, previous work from our lab has shown anxiogenic effects of chronic intermittent ethanol vapor (CIE) in male rats [23] on these open-arm parameters in the EPM while no observable differences were detected in CIE-treated females [5]. Together, these findings add to a growing body of literature suggesting that the EPM may be better suited to assess trait anxiety-related behaviors in male, rather than female, rats. Additionally, the absence of a significant anxiety-like phenotype in female SI rats may suggest that other factors, such as incentive salience, may have contributed to the greater ethanol drinking in these subjects.

We also found that this earlier SI regimen resulted in enduring increases in ethanol intake and preference in female rats during both continuous and intermittent access. These effects have been observed and replicated in male rats but have never been observed in females using our original aSI paradigm [12,14,65]. One major difference between this SI paradigm and the regimen we have previously used is that SI animals are immediately assigned to a housing condition upon arrival, rather than habituating to the vivarium for one week. Therefore, we cannot rule out that the current findings are a result of compounded stressors (e.g., stress of shipping *and* adolescent SI rearing). However, McElroy et al. [49] recently reported that among rats who were bred in-house and then assigned to SI or GH conditions at PND 21, female SI rats had significantly greater daily ethanol intake compared to GH females, a finding that was not observed between the male cohorts. While the subjects in the McElroy et al. (2023) study endured the housing conditions longer than the subjects in the current study (PND 80 vs. 38), the fact that females specifically demonstrated greater ethanol drinking across both independent studies leads us to believe that SI rearing is driving the observed results rather than the compounded stress of shipping. Additionally, home cage ethanol access began on postnatal day 80 in McElroy et al. (2023), a time period closer to the time in which our previous aSI paradigm would begin home cage drinking (PND 100)

but weeks after drinking was initiated in the current study (PND 39). While it is possible that the observed differences between our current and former findings are mediated by sex differences in vulnerability to the onset of ethanol access, the concordance of our findings with another preclinical early life stress paradigm that initiated social isolation on PND 21 [49] leads us to believe that the time at which SI begins is a critical independent variable. Nevertheless, future studies are needed to directly compare parametric differences between males and females (e.g., age of ethanol access, length of isolation) in preclinical models of vulnerability to early life stress.

Drinking despite negative consequences is a core diagnostic symptom of AUD that is often modeled in rodents by adulterating alcohol with quinine to assess aversion-resistant drinking [17,67]. To that end, we also investigated the effect of SI on the consumption of 10% ethanol + 25 or 100 uM quinine. We observed that SI females consumed more ethanol when challenged with the moderate (25 μM), but not high (100 μM), concentration of quinine. In marked contrast, GH male rats actually had greater ethanol intake compared to SI males at the high (100 μM) quinine challenge during the first 30 min of drinking sessions, and slightly greater ethanol intake at the moderate (25 μM) challenge when assessed at 30 min and 24 h.

Notably, while this SI regimen led to an initial increase in continuous home cage ethanol intake in males, this effect did not persist during the intermittent phase of the drinking study and male SI rats did not exhibit increases in aversion-resistant ethanol intake. Collectively, these findings reveal that SI can result in long-lasting increases in ethanol drinking in male and female rats but that there appear to be profound differences in the developmental period during which both sexes are sensitive to this early life stressor. Interestingly, other preclinical models of ELS, such as maternal separation and limited bedding and nesting, also report sex-specific consequent drinking phenotypes, such that males, but not females, with a history of ELS have greater ethanol intake in adulthood compared to controls (see [60] for full review).

These findings raise the important question of what might account for the sex differences in vulnerability to ELS in various rodent models. While additional studies will be needed to address this question, we hypothesize that one reason why we, and others, have not observed differences in females with and without a history of ELS may be because these paradigms were first developed and optimized using male rodents and then generalized to females. Given that many developmental milestones occur earlier in females, it is possible that these experimental paradigms have not been accurately capturing “early life” for female rodents.

For example, it is well known that female rats reach sexual maturity earlier than male rats and therefore enter puberty, a critical developmental phase within the adolescent period, sooner [63]. Indeed, others have argued against the use of postnatal day as the only definition of adolescence, due to observations that males and females do not necessarily develop on comparable timelines [58,63]. In addition to differences in the timing of puberty, many other developmental processes occur on sex-specific time courses, with key developmental milestones often occurring sooner in female rodents. For example, key corticolimbic brain regions, such as the prefrontal cortex, amygdala, and striatum, mature

later in adolescence in males compared to females, with males reaching overall greater peak volumes in these regions by adulthood [29]. Likewise, hippocampal microglia and synapses develop earlier in females compared to males [69]. In addition to anatomical sex differences in development, there are also physiological processes that develop along sex-dependent time courses. For example, GABAergic neurotransmission functions as a primarily excitatory system in rats until at least the second postnatal week in male rats and then switches to serve as a hyperpolarizing, inhibitory neurotransmitter as glutamate becomes the driving excitatory neurotransmitter system, a process known as “the GABA switch” [8]. More recent evidence has revealed that this switch happens 7–10 days earlier in female rats [58]. Moreover, sex differences in the GABAergic system persist through adolescence. Studies report differences in GABAergic interneuron expression in the hippocampus and prefrontal cortex throughout rodent adolescence [19, 73]. Findings from Wu et al. [73] suggest that these developmental sex differences may be mediated by gonadal hormones, such as estradiol.

The GABA system is particularly compelling as it has been reported that ELS results in disruptions to distinct subpopulations of GABAergic interneurons [21]. For example, ELS has been show to decrease the expression of parvalbumin GABAergic interneurons in the rat hippocampus and basolateral amygdala [21], brain regions known to be involved in stress as well as alcohol-related behaviors [1,3,4,5]. These regions may also be contribute to the effects of chronic stress and alcohol on negative emotion-like behaviors (i.e., hyperkatifeia). Therefore, future studies should seek to examine effects of stress and alcohol exposure in these particular regions throughout early life and adolescence, with a particular focus on known male and female developmental events.

Numerous studies have also revealed that adolescent (PND 28 – 70) social isolation has profound effects on the dopaminergic system in male rats [35,36,37]. For example, it has been shown that males with a history of SI have decreased baseline dopamine (DA) levels in the basolateral amygdala and increased DA transporter levels as well as a heightened DA response to an acute ethanol challenge in the basolateral amygdala and nucleus accumbens [12]. Additionally, one study has reported that at baseline, female Wistar rats socially isolated from PND 21 – 35 have comparable levels of D1 and D2 DA receptors in the dorsal striatum, as well as comparable DA transporter levels in the dorsal striatum when compared to GH controls; however, when assessed 24 h after receiving a single dose of diethylpropion (an amphetamine-like drug), SI females had significantly lower D2 levels and significantly higher DA transporter levels [42]. Taken together, these studies highlight how adolescent social isolation results in long-term changes in the DA system in both sexes. We have posited that SI-induced changes to the DA system may contribute to SI rats’ vulnerability to alcohol use, a proposal that has also been generalized to the vulnerability to other drugs of abuse [42]. Thus, it is possible that sex differences in the development of the DA system may also help to explain why we have only observed SI to result in increased drinking in males and females under separate methodologies (PND 21–70 in males vs. PND 21–38 in females). For example, D1 receptors in the nucleus accumbens peak, and then are subsequently downregulated, earlier in females (~PND 20–30) compared to males (~PND 30–38) [41]. Furthermore, it is possible that we did not observe drinking differences between SI and GH males because in the current isolation procedure, their dopamine

receptors in the nucleus accumbens have not yet peaked. However in our original isolation window these receptors are beginning to peak during the first week of separation (beginning PND 28). Comparably, females in the current aSI paradigm who were isolated at PND 21 were perhaps separated right before their sex-specific peak in expression. Therefore, future studies may seek to investigate the effects of social isolation stress using this modified paradigm to examine resulting changes in the dopaminergic system in female rodents.

5. Conclusions

The results of the present study demonstrate for the first time that aSI can lead to enduring increases in ethanol intake in female rats. When considered in the context of historical social isolation studies with male rats, these results support the notion that there are sex differences in the vulnerability window to this ELS model. Under this modified aSI paradigm, where rats were isolated between PND 21 – 38, females showed a long lasting increase in ethanol intake and preference. However, males only transiently increased alcohol consumption. Notably, this is the opposite of what was observed in females when an aSI model was used that encapsulated male adolescence and early adulthood (isolated PND 28–70). We hypothesize that these sex differences in the vulnerability window to aSI may arise due to developmental events that are accelerated in females compared to males, including the dopaminergic and GABAergic systems that are known to be sensitive to stress and alcohol exposure. Future studies will be needed to test the causal role of these developmental processes in the sexually dimorphic effects of aSI and other models of ELS on AUD-related behavior. Ultimately, it may be possible to identify the biological substrates associated with vulnerability to AUD and comorbid disorders of negative emotions by identifying the neural mechanisms responsible for these sexually dimorphic differences in ELS sensitivity. These findings may also inform on personalized treatment approaches for alcohol use disorder and comorbid disorders of negative emotion.

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Data availability

Data will be made available on request.

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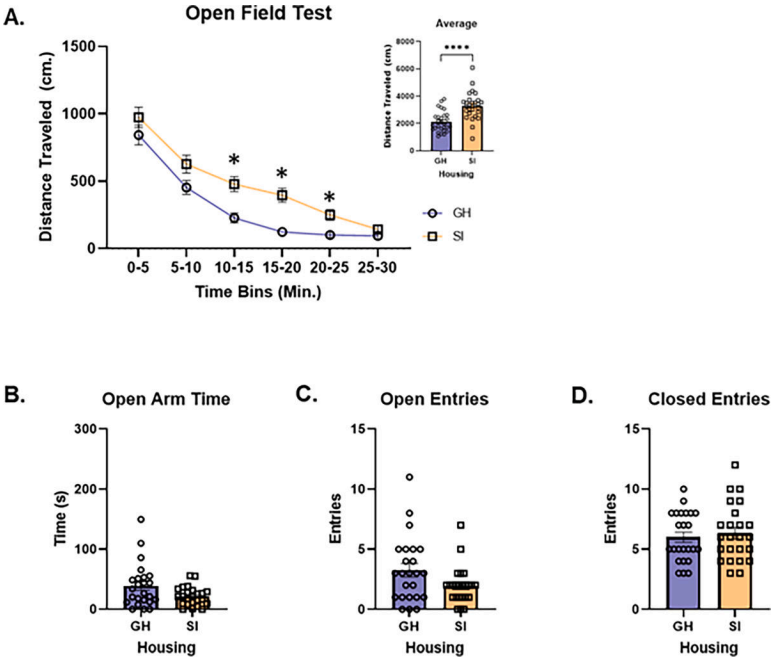

Fig. 1.

Behavioral data from Experiment 1. (A) Distance traveled in the open field test by five minute time bins between socially isolated (SI) vs. group-housed (GH) females. The inset represents the cumulative distance traveled throughout the entire 30 min session by housing group. Asterisks on the line graph represent adjusted *p*-values from Sidak's multiple comparisons test. Asterisks on bar graphs represent *p*-values from Welch's *t*-test. **** $p <$ .0001, * $p < .05$. (B) Time spent on the open arm, (C) the number of open entries, and (D) the number of closed entries on the elevated plus-maze between SI and GH females. Data are presented as mean ± SEM.

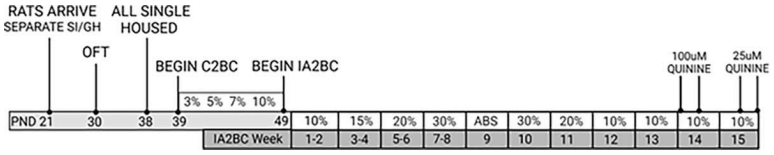

Fig. 2.

Experiment 2 timeline. Rats arrived to the facility on postnatal day (PND 21) and were immediately assigned into an adolescent housing condition. Rats remained in their respective housing conditions until PND 38, at which time all animals were single housed to assess home cage ethanol drinking using the ethanol concentrations indicated by the percentage (%). During weeks 14–15 rats were given concurrent access to adulterated quinine and water on 2 out of 3 drinking sessions per week, indicated with a vertical hash mark. Abbreviations: PND= postnatal day. SI = social isolation. GH = group-housed. OFT = open field test. C2BC = continuous access two-bottle choice. IA2BC = intermittent access two-bottle choice. ABS= forced abstinence. Created with Biorender.com.

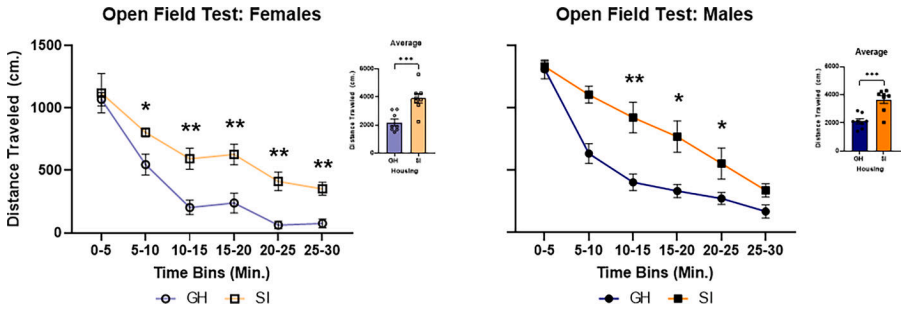

Fig. 3.

Open field test data from Experiment 2. Distance traveled in the open field test by five minute time bins, insets represent cumulative distance traveled throughout the entire 30 min session. Circles represent socially isolated rats, squares represent group-housed rats. Open symbols represent female rats, closed symbols represent male rats. Data are presented as mean ± SEM. Asterisks on line graphs represent adjusted p-values from Bonferroni-adjusted pairwise comparisons. Asterisks on bar graphs represent p-values of the simple main effect of Housing for each level of Sex when assessing total distance traveled. *** $p < .001$, * $p < .05$.

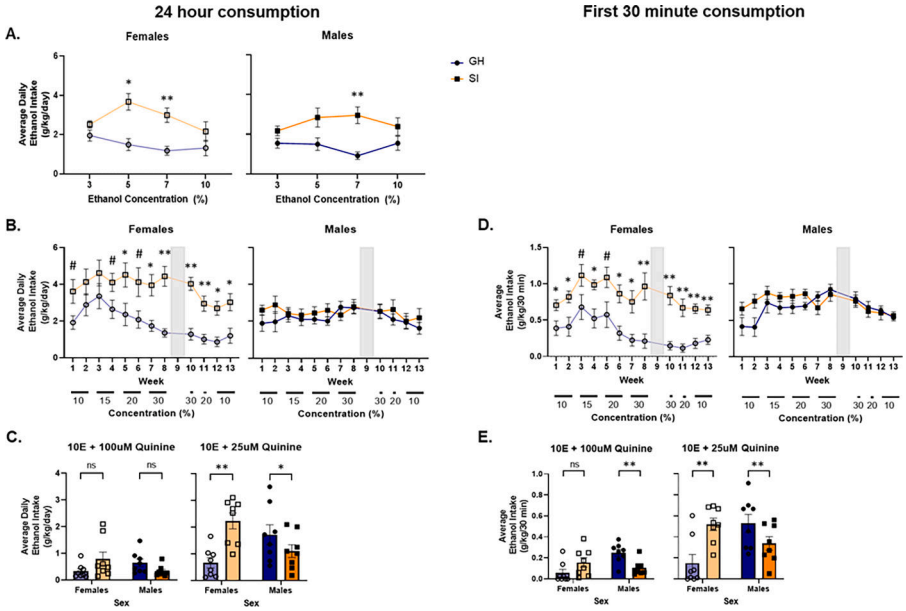

Fig. 4.

Two-bottle choice consumption (g/kg) in male and female socially isolated vs. group-housed rats. (A) Continuous and (B) intermittent access daily consumption at varying ethanol concentrations was evaluated before (C) quinine-adulterated consumption was assessed. 30 min consumption was also recorded when assessing intake of (D) unadulterated and (E) quinine-adulterated ethanol. Circles represent socially isolated rats, squares represent group-housed rats. Open symbols represent female rats, closed symbols represent male rats. The gray horizontal box in panels B and D represents one week of forced abstinence. Data are presented as mean ± SEM. Asterisks represent a *p*-value from Bonferroni-adjusted pairwise comparisons. ** $p < .01$, * $p < .05$, # $p \leq .10$.