Investigating the sex-specific effects of socialization on voluntary ethanol self-administration in rats using an eVape system

Christopher D. Walker[1], Hannah G. Sexton[1,2], and Mary-Louise Risher[1,2,3,†]

[1]Department of Biomedical Sciences, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, USA

[2]Hershel 'Woody' Williams Veteran Affairs Medical Center, Huntington WV, USA

[3]Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC, USA

[†]**Addresses for correspondence:**

Mary-Louise Risher, 1700 Third Ave (BBSC 336L), Huntington, WV 25755

Tel: 304 6963894 Fax: 304 6967207 Email: risherm@marshall.edu

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ABSTRACT

Introduction: Peer interactions are a crucial part of social and personal development, particularly during adolescence. Adolescence is characterized as a transitional developmental period between childhood and adulthood that is often associated with increased freedom, self-exploration, and new experiences that are often peer-influenced. Due to this newfound independence, there is a higher

prevalence of alcohol consumption, which is in part due to the heightened social facilitating and rewarding effects of alcohol. Previous work shows that males and females that consume excessive alcohol during adolescence are at an increased risk of developing an alcohol use disorder (AUD) later in life. However, the contributions of social interaction and sexual dimorphism in alcohol consumption, two driving factors influencing AUD risk, are not fully understood. Currently, there are several rat models used to study the characteristics of alcohol use and the emergence of AUD. However, many require the addition of a sweetener to coerce them into consuming liquid ethanol, which has been proven to confound results in adolescent rats. Here we use a novel self-administration ethanol eVape system to investigate the sexual dimorphic nature of socially facilitated ethanol consumption without the use of sweeteners.

Methods: Adolescent and adult male and female Sprague-Dawley rats underwent a novel voluntary chronic intermittent self-administration ethanol vapor paradigm. Nosepoke initiated self-administration eVape chambers (La Jolla Alcohol Research, Inc.) administered 20mg/L of vaporized ethanol or air (control) into the chamber in response to each nose poke. Beginning postnatal day (PND)30 or PND70 animals were placed in vapor chambers for 4 hours every other day for a total of 40 sessions. All animals underwent 10 sessions with their cagemate (social access) followed by 10 sessions in isolation (isolated access), a 10 day forced abstinence period, 10 sessions in isolation (isolated access), and 10 sessions with their cagemate (social access).

Results: These data reveal that while female rats consumed more alcohol than age-matched males, male rats increase ethanol preference regardless of age. In addition, all rats regardless of sex or age consumed more ethanol during the first social access session than during the subsequent isolated access sessions. Interestingly, there was an increase in ethanol consumption in adult

females during the second social access session compared to the previous isolated access session that was not observed in males.

Conclusion: These data demonstrate that female and male rats, regardless of age are vulnerable to socially facilitated ethanol consumption. This is consistent with human data that show increased levels of alcohol consumption among adolescents and young adults is associated with high levels of alcohol use within their social group (Sudhinaraset, Wigglesworth, & Takeuchi, 2016). However, only male rats demonstrate escalation across sessions. This may indicate that male rats are more vulnerable to escalated drinking and the emergence of ethanol dependence compared to females regardless of peer interaction. These data demonstrate that the self-administration ethanol eVape system is an effective alternative to other methods of voluntary ethanol administration for investigating factors that contribute to alcohol use and escalation.

INTRODUCTION

Adolescence is a transitional developmental period between childhood and adulthood that is often characterized as a time of increased independence and increased opportunity to participate in novel experiences. Social interactions during this time are critical for cognitive development leading to a sense of 'self' or the development of one's own identity (Burnett & Blakemore, 2009; Sebastian, Burnett, & Blakemore, 2008). Through peer reinforcement, positive and/or negative, adolescents adopt behaviors that reflect those of their social groups. For example, adolescents are more prone to participating in risky behavior, such as consuming alcohol, when interacting with their peers than they are when alone. Peer reinforcement can be direct, via peer pressure or the offer to supply alcohol or indirect, in which the adolescent models the behavior they perceive to fit the social norms of their chosen social group (Borsari & Carey, 2003; Jackson, Sher, & Park, 2005).

Parallels are beginning to emerge between human findings and rat models of binge ethanol (EtOH) exposure, making them an appreciable model for conducting alcohol studies (Crews et al., 2019; Spear & Swartzwelder, 2014). Adolescent rats are similar to adolescent humans as they are less sensitive than adults to the motor impairing, aversive, and sedative effects of acute EtOH and more sensitive to the social facilitating and rewarding effects of EtOH (Anderson, Varlinskaya, & Spear, 2010; Ramirez & Spear, 2010; Silveri & Spear, 1998; Varlinskaya & Spear, 2002). Using an adolescent binge drinking model in rats, our lab and others have demonstrated that there are behavioral, cognitive, and electrophysiological changes that persist into adulthood (Spear & Swartzwelder, 2014). This model has yielded substantial insight, however it is limited due to the involuntary nature of EtOH consumption. Involuntary EtOH administration has been necessary because rats have an aversion to the taste of EtOH, however some studies have suggested that experimenter-administrated EtOH may impact the motivation to voluntarily consume EtOH later (Gilpin, Karanikas, & Richardson, 2012; Walker & Ehlers, 2009). In addition, the use of sweetened EtOH can confound the assessment of later EtOH consumption (Broadwater, Varlinskaya, & Spear, 2013). As a consequence, involuntary approaches prevent the field from investigating how patterns of EtOH consumption change across adolescence and into adulthood in a rat model. These limitations also prevent investigation into how socialization influences early onset EtOH consumption and potential escalation later in life.

In an effort to develop a voluntary EtOH delivery system, de Guglielmo (2017) developed a vapor chamber model that would allow rodents to voluntary self-administer EtOH vapor. The apparatus provides for the voluntary self-administration of EtOH or clean air after the animal triggers one of

two nosepokes. This system results in alcohol dependence without the use of artificial sweeteners, food reinforcement, water restriction, or forced alcohol administration that can result in potential experimental confounds.

This novel voluntary self-administration vapor model allows us to observe how social interactions affect adolescent and adult drinking behaviors in male and female rats. Male and female adolescent and adult rats underwent 40 sessions of EtOH access with and without their cagemate to determine the impact of peer interaction on EtOH consumption. These data demonstrate that male rats increase EtOH preference across time regardless of the age at which exposure began. This escalation was not observed in female rats, however adolescent and adult females consumed more EtOH than age-matched males. All rats, regardless of age and sex consumed more EtOH during the initial peer interaction sessions versus alone. There was an appreciable increase in EtOH consumption in the female's final peer interaction sessions that was not observed in males. Together, these data provide insight into the sexually dimorphic nature of voluntary social EtOH consumption in a rat model without the use of sucrose fading or restriction paradigms.

METHODS

Male and female PND 24 and PND 64 Sprague-Dawley rats (Hilltop, USA) were double-housed and maintained in a temperature- and humidity-controlled room with *ad libitum* access to food and water (n=8 per group). Animals were handled daily and allowed to acclimatize for 5 days on a reverse 12-hr/12-hr light:dark cycle (lights off at 6:00 am). All procedures were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory

Animal Care and the National Research Council's Guide for Care and Use of Laboratory Animals and approved by the Marshall University IACUC.

Vapor Chamber Apparatus. The apparatus consists of a 36.83 cm x 25.4 cm x 22.86 cm airtight chamber equipped with two nose pokes and two cue lights located immediately above each nose poke (de Guglielmo, Kallupi, Cole, & George, 2017). One nose poke is active and one is inactive. The active nose poke when initiated releases ethanol vapor (20 mg/L for 2 mins) into the chamber. The inactive nose poke results in no response. The chamber is ventilated with 15 L/min of air at all times. Nosepoke activity was monitored using infrared beam breaks.

Chronic intermittent ethanol vapor self-administration. Beginning PND30 and PND70 animals were habituated to the procedure room for 30 minutes, weighed, and placed in the vapor chamber for 4 hours every other day. The active nose poke that initiated EtOH vapor release into the chamber was initially randomized but remained the same throughout the remainder of the sessions. All animals received 10 sessions with their cage mate (voluntary social access, SA) followed by 10 sessions in isolation (voluntary isolated access, IA). All animals then underwent 10 days forced abstinence in which they remained in their homecage. Following forced abstinence, animals underwent another 10 sessions of isolated access (IA) followed by another 10 sessions of cage mate access (SA), i.e., a reversal of the earlier access paradigm (Figure 1).

Elevated Plus Maze. To determine changes in anxiety, 24 hr after session 20 animals were tested in the elevated plus maze as previously described in (Wilson, Vazdarjanova, & Terry, 2013). The apparatus consisted of a plus shaped maze made of opaque plexiglas, two open arms and two

closed arms of the same size (50 cm x 10 cm) but with side walls (40 cm). The apparatus was elevated 50.8 cm above the floor. Lighting was set at 35-40 lux, lumen/m^2. The rats were habituated to the room for 30 minutes and then placed one at a time in the central area of the maze facing an open arm. Rats were allowed to explore the apparatus for 10 min and then returned to their homecage. Between animals the apparatus was cleaned with 5% vinegar solution. Activity was recorded using an overhead camera. Total number of entries into open versus closed arms and time spent in open versus closed arms was quantified using Anymaze software (Stoelting, IL, USA).

Withdrawal Score. EtOH behavioral withdrawal signs were assessed 24-hr after the 10th, 20th, 30th, and 40th session (Macey, Schulteis, Heinrichs, & Koob, 1996). Abnormal gait, tail rigidity, ventromedial limb retraction, irritability to touch (vocalization), and body tremors were assigned a score of 0-2 based on severity. 0 = no sign, 1 = moderate sign, 2 = severe sign. Signs were summed and used as a quantitative measure of severity of withdrawal.

Statistical Analysis

Comparisons between session, age, and sex were made using repeated measures Analysis of Variance. Post hoc analysis was conducted using Tukey's multiple comparison test. All analyses were conducted using GraphPad Prism 8 (GraphPad Software, San Diego, California, USA). Statistical significance was assessed using an alpha level of 0.05. All data are presented in figures as the mean +/− S.E.M.

RESULTS

<u>% EtOH Preference (Figure 2)</u>: Using a non-escalating paradigm to mimic intermittent social drinking these data revealed a significant effect of sex ($F_{3, 28} = 5.5$, p=0.0042) and session ($F_{39, 1092} = 5.128$, p<0.0001) across all 40 sessions. When analyzed based on session block (first SA, first IA, second IA block, and second SA) there was a significant effect of session block ($F_{3, 84} = 20.84$, p<0.0001) and sex ($F_{3, 28} = 5.5$, p=0.0042). Post hoc analysis revealed that in males exposed to EtOH beginning PND30 there was a significant increase in EtOH preference (as indicated by an increase in active vs. inactive nosepokes) from the first SA block to the second IA block (p=0.0025) and the second SA block (p<0.0001). There was also a significant increase from the first IA block to the second SA block (p=0.0039). There was no significant effect of session block in the female rats exposed to EtOH beginning PND30. In males exposed to EtOH beginning PND70 there was a significant increase in EtOH preference from the first SA block to the second IA block (p=0.0002) and second SA block (p=0.0003). There was no significant effect of session block in the female rats exposed to EtOH beginning PND70. These data indicate that there is an increased preference for EtOH across the 40 sessions in male rats regardless of when exposure to EtOH begins that is not observed in females.

<u>Minutes of EtOH Vapor (Figure 3):</u> 3 way ANOVA (sex x age x session) revealed a significant effect of sex ($F_{1, 1120} = 5.844$, p=0.0158), session ($F_{39, 1120} =20.973$, p<0.0001), and age ($F_{1, 1120} = 435.3$, p<0.0001) across all 40 sessions. While there was not a significant interaction between session x sex ($F_{39, 1120} =1.305$, p=0.1014), there was a significant interaction between session x age ($F_{39, 1120} =1.790$, p=0.0022) with a trend towards a sex x age interaction ($F_{1, 1120} =3.189$, p=0.0744).

To further assess the impact of SA versus IA sessions we conducted within group 3 way ANOVA (sex x age x session block). There was an overall effect of sex ($F_{1, 3} = 76.69$, $p<0.0001$), session block ($F_{3, 3} = 36.41$, $p<0.0001$), but no effect of age (p=0.3485) or interaction between measures (p>0.05). Post hoc analysis revealed that female PND30 rats consumed more EtOH in their first SA session block when compared to subsequent IA session blocks (first IA block, p<0.0001, second IA block, p<0.0001) as well as the second SA block, p=0.0009. Demonstrating a significant decrease in EtOH consumption when female PND30 rats transitioned to the first and second IA session blocks. There was no significant difference between the first and second IA session blocks (p=0.9995) despite the intermediate forced abstinence period. While rats consumed significantly less in the second SA block than the first SA block, they consumed more in the second SA block than in the IA block (p<0.0018 (first IA block), p=0.0027 (second IA block)) demonstrating an overall increase in EtOH consumption during SA blocks versus IA blocks.

Female PND70 rats consumed more EtOH in their first SA session block when compared to the subsequent IA session blocks (p<0.0001 (first IA block), and p=0.003 (second IA block)). There was a significant increase in EtOH consumption when female rats transitioned back to the second SA session when compared to the second IA sessions (p=0.0007). There was no significant difference between the first and second IA session blocks (p=0.4911) despite the intermediate forced abstinence period. A significant increase in EtOH consumption was also observed when comparing the first IA block to the second SA block (p<0.0001) demonstrating an overall increase in EtOH consumption during SA blocks when compared to IA blocks.

Male rats beginning consumption at PND30 demonstrated a session block effect. Male rats consumed more EtOH in their first SA session block when compared to subsequent session blocks ($p<0.0001$ (first IA block), $p<0.0001$ (second IA block), $p<0.001$ (second SA block). There was no significant difference between the first and second IA session blocks ($p=0.9985$) despite the forced abstinence period.

Males beginning consumption at PND70 demonstrated a session block effect. Post hoc analysis within group comparison revealed that the male rats consumed more EtOH in their first SA session block when compared to subsequent session blocks ($p<0.0001$ (first IA block), $p<0.0001$ (second IA block), $p=0.0144$ (second SA block). There was no significant increase in EtOH consumption when transitioning back to the second SA block when compared to the second IA block ($p=0.3038$). There was no significant difference between the first and second IA session blocks ($p>0.9999$) despite the forced abstinence period.

<u>Timeout Nose Pokes (Figure 4)</u>: To ensure that all animals learned the tasks equally well and to determine whether impulsive nose poking contributes to differences in EtOH consumption we assessed total timeout nose pokes, theorizing that the number of timeout nose pokes should decline as the animals learned the task. 3 way ANOVA (sex x age x session) revealed a significant sex ($F_{1,29} = 26.27$, $p<0.0001$), age ($F_{39,1092} = 23.78$, $p<0.001$), and session effect ($F_{1,28} = 11.21$, $p=0.0023$). Post hoc analysis revealed that these effects were mainly driven by differences in timeout nose pokes during early sessions when animals were learning the task. Secondary analysis revealed that female PND30 rats nose poked during timeout significantly more than male and female PND70 rats during sessions 1-4 (Session 1 $p<0.0001$, $p<0.0001$, session 2 $p<0.0001$, $p<0.0001$, session 3

$p=0.0001$, $p<0.0001$, and session 4 $p<0.0001$, $p<0.0001$, respectively). By session 5 there was no significant difference between groups based on age or sex when compared across identical sessions ($p>0.05$). As with female PND30 rats, male PND30 rats nosed poked significantly more than PND70 male and female rats during session 1 ($p<0.0001$, $p<0.0001$) and session 2 ($p<0.0001$, $p<0.0001$). By session 3 there was no significant difference between groups based on age or sex when compared across identical sessions ($p>0.05$) suggesting that impulsive nose poking is not an underlying factor influencing long-term differences in EtOH consumption.

Elevated Plus Maze (Figure 5): To determine whether differences in anxiety may be an underlying factor influencing EtOH consumption we conducted the EPM 24 hours after session 20. Statistical analysis revealed no significant differences in time spent in open ($F_{3, 28}=2.878$, $p=0.0537$) and closed arms ($F_{3, 28}=1.981$, $p=0.1397$) or in distance moved in closed ($F_{3,28}=1.268$, $p=0.3043$) and open ($F_{3,28}=2.032$, $p=0.1321$) arms.

Weight (Figure 6): There was a significant effect of sex ($F_{1,39}=5251$, $p<0.0001$), age ($F_{1,39}=16098$, $p<0.0001$), and session ($F_{39,39}=215.4$, $p<0.0001$) and a significant interaction between session x sex ($F_{39,39}=43.16$, $p<0.0001$), session x age ($F_{39,39}=28.07$, $p<0.0001$), sex x age ($F_{1,39}=204.8$, $p<0.0001$), and session x sex x age ($F_{39,39}=6.907$, $p<0.0001$). Female and male PND30 rats began sessions weighing the same ($p>0.9999$) but less than female and male PND70 rats ($p<0.001$). PND30 male rats continued to grow at a quicker rate than PND70 male rats reaching equivalent weights by session 40 ($p=0.8556$), whereas female PND30 rat weights remained lower than the female PND70 counterparts ($p<0.0001$).

Somatic Symptoms of Withdrawal (Figure 7): 24 hours after the 10th, 20th, 30th, and 40th session rats were scored for withdrawal. All rats showed minimal to moderate signs of withdrawal, however there was no significant effect of sex, age, session, or interaction on overall withdrawal score ($p>0.05$). When broken down based on separate withdrawal measures there was an overall effect of sex on ventromedial limb retraction (VLR) ($F_{1,3}=7.658$, $p=0.0066$) however post hoc analysis revealed no individual differences ($p>0.05$). There was a significant main effect of age on vocalization (VOC) ($F_{1,3}=47.91$, $p<0.0001$) and tail rigidity (TR) ($F_{1,3}=12.1$, $p=0.0007$) and a main effect of session ($F_{1,3}=5.317$, $p=0.0018$) and age ($F_{1,3}=4.131$, $p=0.0445$) when body tremors (BT) were assessed but post hoc analysis revealed no individual differences ($p>0.05$).

DISCUSSION

Over the course of this study male and female adolescent (PND30) and adult (PND70) Sprague-Dawley rats were place in self-administration vapor chambers for 4 hours over 40 sessions. To begin, male and female adolescent and adult rats were placed with their cagemate in the vapor chambers and allowed access to vaporized EtOH or air via a nosepoke for 10 sessions. The animals were then socially isolated in the vapor chambers for an additional 10 sessions, followed by a brief abstinence period. The animals were once again socially isolated in the vapor chambers for 10 sessions before undergoing social access in the vapor chambers for a final 10 sessions. The animals were subjected to elevated plus maze 24 hours after session 20 to determine if there were differences in anxiety that may contribute to alcohol consumption. Animals were observed 24 hours after the 10th, 20th, 30th, and 40th sessions to be scored for withdrawal.

Our first goal was to determine if sex and age differences in EtOH preference previously observed in other models was consistent with the voluntary EtOH vapor self-administration model used here. We observed an increase in preference for EtOH in males regardless of the age at which EtOH exposure began. This same escalation in EtOH preference was not observed in either female age group. This is consistent with previous work in which EtOH escalation was observed in the two bottle choice model in adolescent and adult male rats when compared to age matched females (Li et al., 2019; Varlinskaya & Spear, 2009). Female rats at PND30 and PND70 consumed more EtOH than male age-matched controls across all session blocks. This is consistent with previous studies that have demonstrated using the 2 bottle choice that females consume more EtOH than males in continuous or intermittent EtOH exposure experiments (Li et al., 2019; Priddy et al., 2017) .

Next, we wanted to test if social interaction would facilitate changes in alcohol consumption. Our model allowed us to observe how social interaction (SA) and isolation (IA) contributes to differences in EtOH consumption. This paradigm also allowed us to observe whether abstinence would escalate EtOH consumption. All rats regardless of age and sex consumed more EtOH during the first SA session block than subsequent IA session blocks. While not exceeding or reaching the same level of consumption that was observed in the first SA block, there was a significant increase during the second SA block when compared to the first and second IA block in both female groups. While there is limited data on how SA influences EtOH consumption, previous work has demonstrated that prior EtOH exposure does facilitate subsequent adolescent social interaction in a rat model (Varlinskaya & Spear, 2002, 2009).

Due to the lower amount of EtOH consumed in IA sessions compared to the second SA session, we can correlate increased female EtOH consumption in adulthood and social behavior. This pattern was not observed in either males and could be attributed to males aging out of the social facilitating effects of EtOH before females or due to the males heightened sensitivity to ethanol-induced sedation (Varlinskaya, Doremus-Fitzwater, & Spear, 2010; Varlinskaya, Truxell, & Spear, 2014).

CONCLUSION

This novel voluntary self-administration vapor model allows us to observe how social interactions affect adolescent and adult drinking behaviors in male and female rats. Female rats consumed more EtOH than age-matched males, however male rats regardless of age escalated EtOH consumption over time. Adolescent and adult rats regardless of sex or age consumed more EtOH during peer interactions, this was particularly apparent in females. Together, these data provide insight into the sexually dimorphic nature of voluntary EtOH consumption and the impact of social interaction during EtOH access in a rat model without the use of sucrose fading or restriction paradigms.

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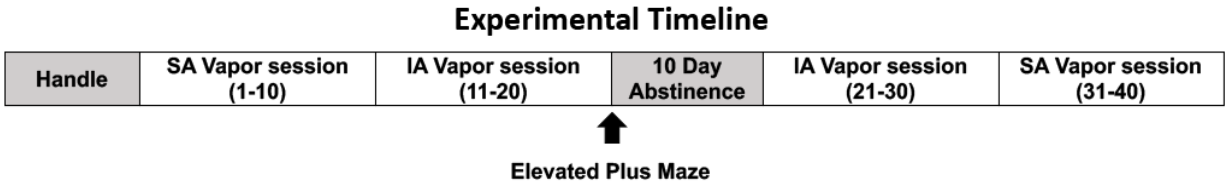

Figure 1. Experimental timeline for voluntary self-administration EtOH vapor chambers. Voluntary social access (SA); voluntary isolated access (IA).

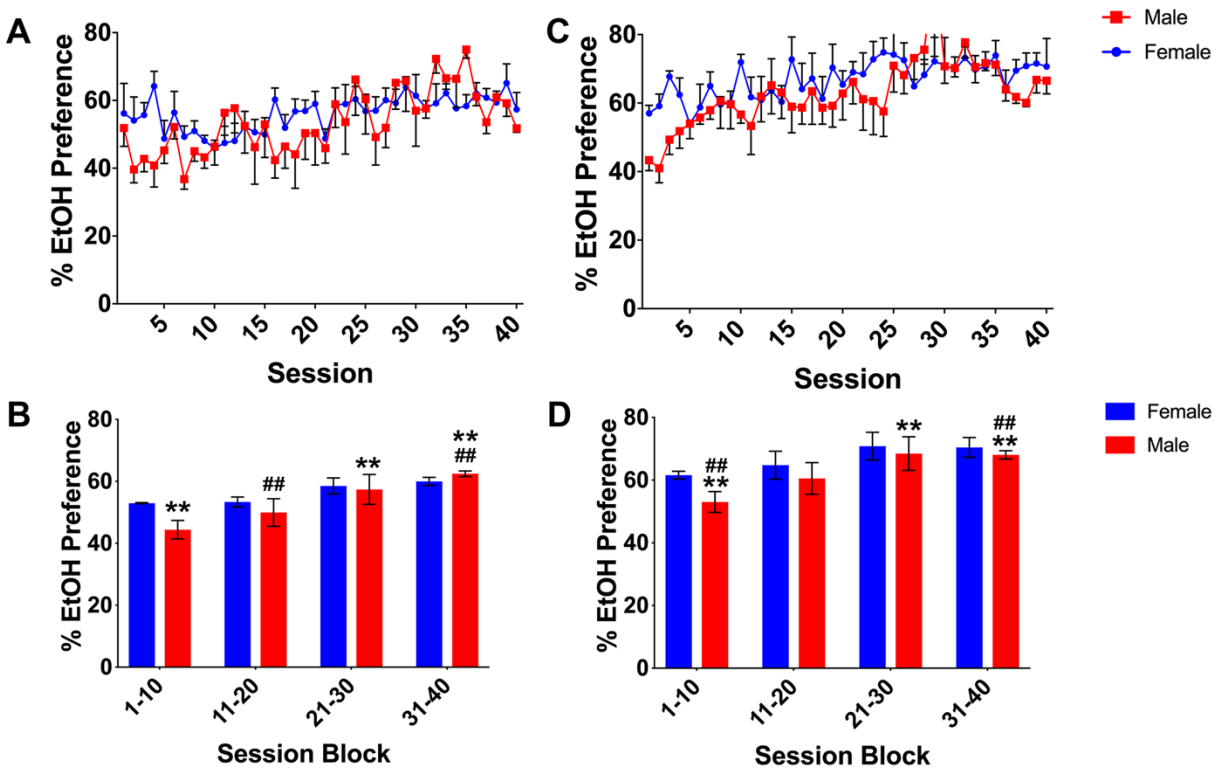

Figure 2. EtOH Preference. %EtOH preference over 40 sessions of voluntary self-administration of EtOH vapor (A) PND30 female and male rats, (B) summary of EtOH preference per session block in PND30 female and male rats. **** $p < 0.01$ when comparing PND30 male session block 1-10 to session blocks 21-30 and 31-40. ## $p < 0.01$ when comparing PND30 male session blocks 11-20 and 31-40. %EtOH preference over 40 sessions of voluntary self-administration of EtOH vapor (C) PND70 female and male rats, (D) summary of EtOH preference per session blocks in PND70 female and male rats. ** p <0.01 when comparing PND70 male session blocks 1-10, 21-30, and 31-40. ## p<0.01 when comparing PND70 male session blocks 1-10 to 31-40. There was a significant overall effect of sex ($p = 0.0042$) and session ($p < 0.0001$) across all 40 sessions that is not indicated in figure.

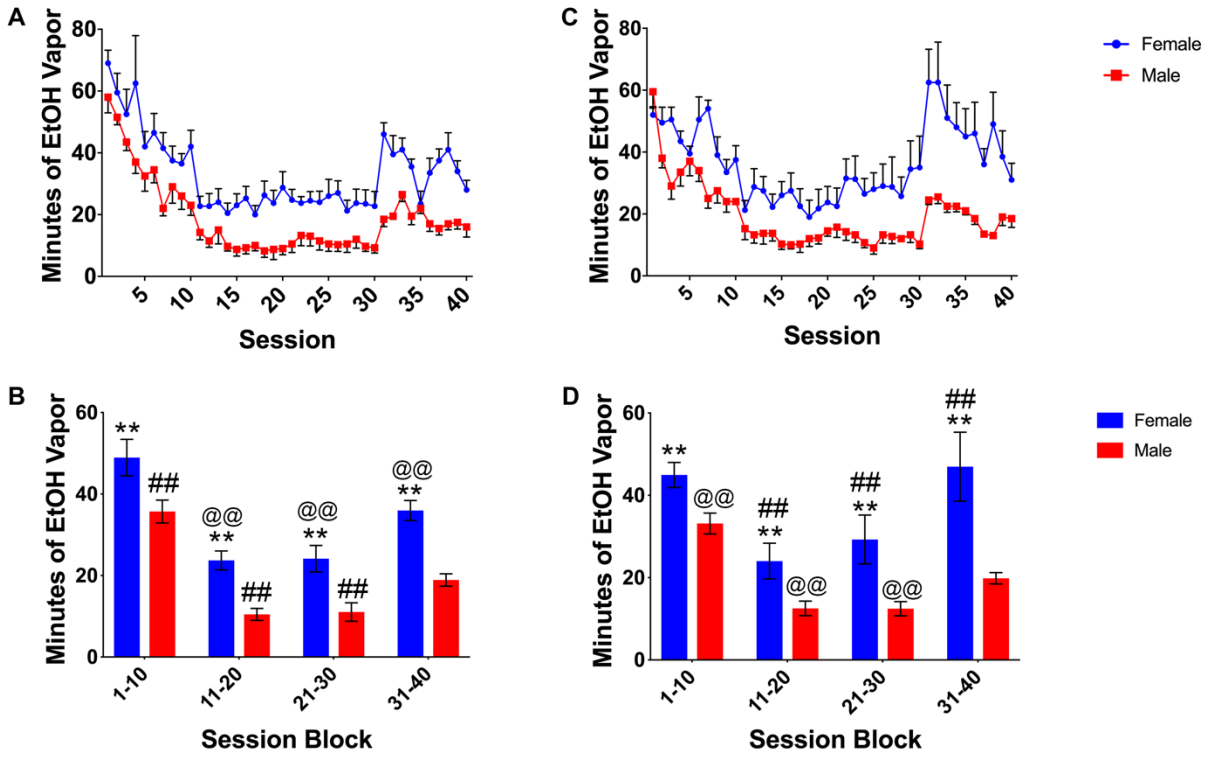

Figure 3. Minutes of EtOH Vapor. Minutes of EtOH over 40 sessions of voluntary self-administration (A) PND30 female and male rats, (B) summary of minutes of EtOH per session block in PND30 female and male rats. **** $p < 0.01$ when comparing PND30 female session block 1-10 to the subsequent session blocks 11-20, 21-30, and 31-40. @@ $p<0.001$ when comparing PND30 female session block 31-40 to the 11-20 and 21-30. ## $p < 0.01$ when comparing PND30 male session blocks 1-10, 11-20, and 21-30. Minutes of EtOH over 40 sessions of voluntary self-administration (C) PND70 female and male rats, (D) summary of minutes of EtOH per session block in PND70 female and male rats. ** $p <0.01$ when comparing PND70 female session block 1-10 to 11-20 and 21-30. ## $p < 0.01$ when comparing PND70 female session blocks 31-40 to 11-20 and 21-30. **@@** $p < 0.01$ when comparing PND70 male session blocks 1-10, 11-20, and 21-30. There was a significant overall effect of sex ($p = 0.0158$), session ($p < 0.0001$), and age ($p <0.0001$) across all 40 sessions that is not indicated in figure.

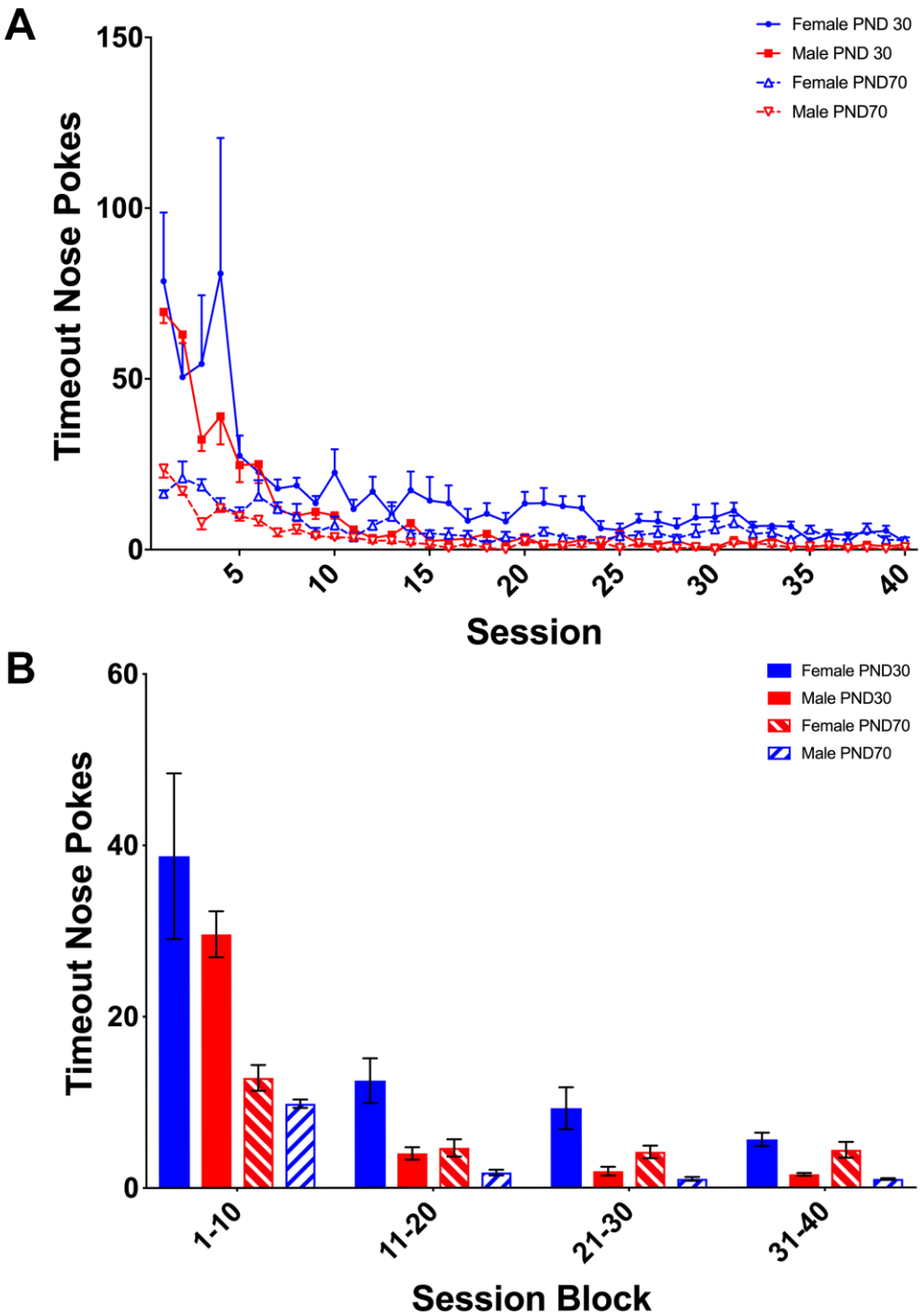

Figure 4. Timeout Nose Pokes. Total time out nose pokes across all 40 sessions (A) PND30 and PND70 female and male rats. (B) Summary of total nose pokes per session block in PND30 and PND70 female and male rats.

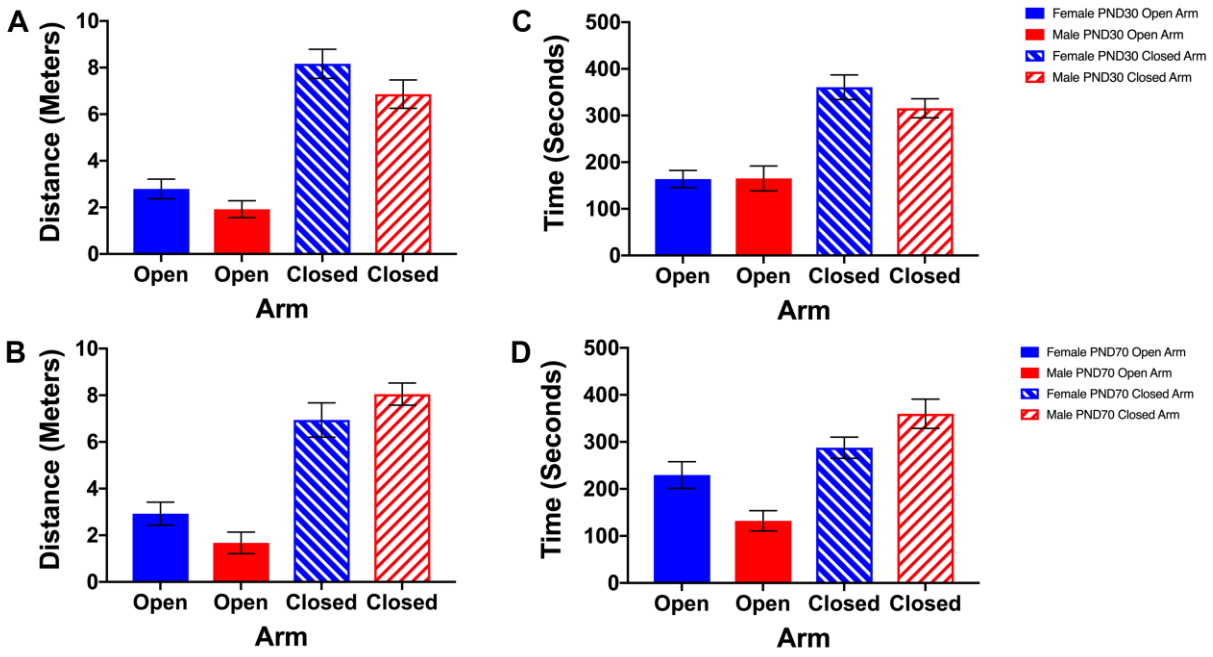

Figure 5. Elevated Plus Maze (EPM). Distance and time spent in open and closed arms of the elevated plus maze. (A) Distance traveled in open and closed arms by PND30 female and male rats. (B) Distance traveled in open and closed arms by PND70 female and male rats. (C) Time spent in open and closed arms by PND30 female and male rats. (D) Time spent in open and closed arms by PND70 female and male rats.

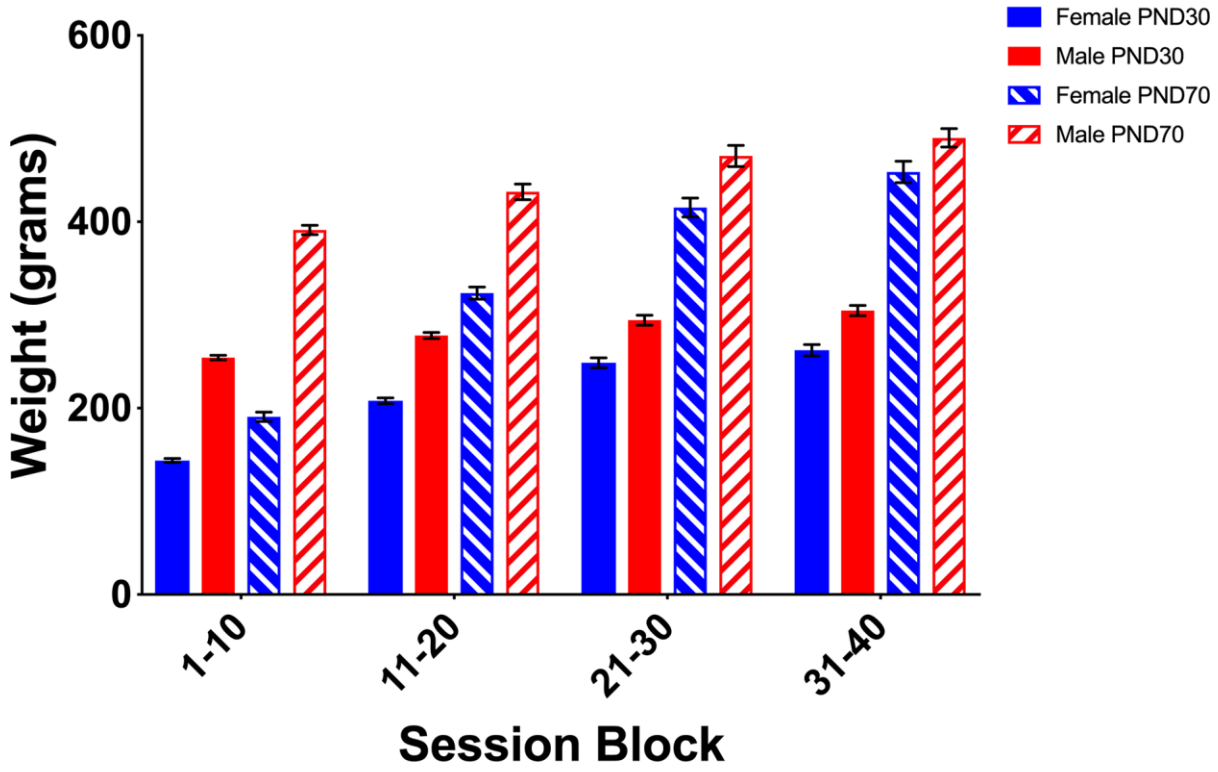

Figure 6. Animal Weights. Summary of PND30 and PND70 female and male rat weights across session blocks.

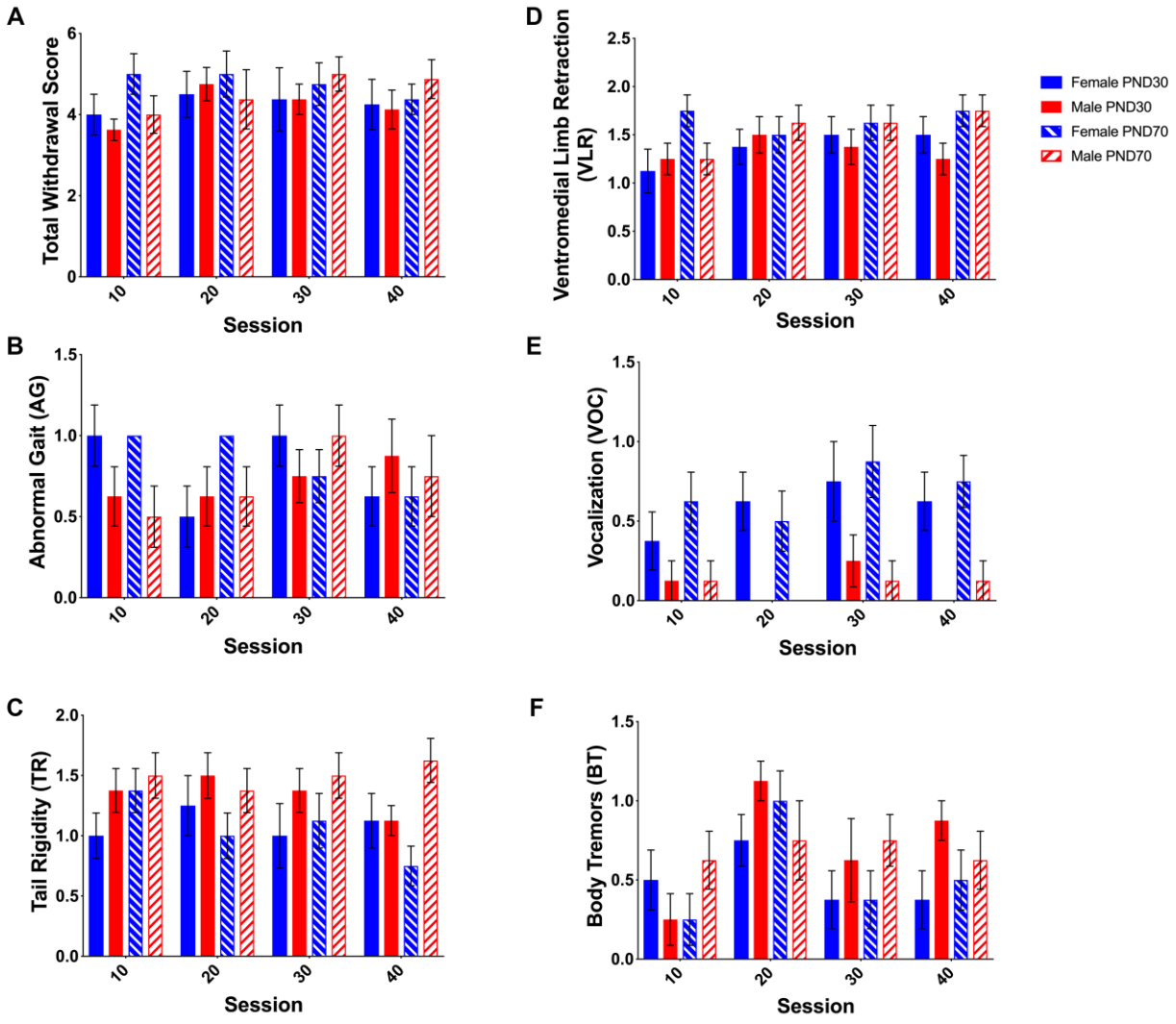

Figure 7. Withdrawal Signs. (A)Total Withdrawal Score, (B) Abnormal Gait (AG), (C)Tail Rigidity (TR), (D) Ventromedial Limb Retraction (VLR), (E) Vocalization (VOC), and (F) Body Tremors (BT) assessment of PND30 and PND70 female and male rats 24 hours after the 10th, 20th, 30th, and 40th voluntary self-administration EtOH vapor sessions.