DOES BINGE DRINKING INDUCE PMDD-LIKE DYSFUNCTION FOR FEMALE C57BL/6J MICE? IMPLICATIONS FOR SEX DIFFERENCES IN ADDICTION VULNERABILITY

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This work is dedicated to my sister, Tasha, my brothers Malik and Ako and to the memory of my brother Aaron.

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ABSTRACT

Melón, Laverne C Ph.D., Purdue University, May 2014. Does Binge Drinking Induce PMDD-like Dysfunction for Female C57Bl/6J mice? Implications for Sex Differences in Addiction Vulnerability. Major Professor: Stephen L. Boehm.

It has traditionally been posited that women show a telescoped development of alcohol use disorders (Kuhn, 2011). In particular, a number of clinical studies support striking sex differences in the progression from initial use of alcohol to dependence on the compound; with women showing a faster progression through landmark events associated with the development of alcohol addiction (Randall et al., 1999). However, recent studies have challenged this tenet (Keyes et al., 2010). The work presented herein was designed to determine whether females are indeed more vulnerable to the development of behavioral maladaptations following binge drinking and whether sex differences in GABAA receptor regulation might underlie this vulnerability. Using a mouse model of binge drinking this dissertation established that, compared to males, females escalate their binge drinking at a faster rate and maintain altered responsivity to the locomotor effects of alcohol after extended abstinence from binge drinking. Female mice also displayed significant increases in ethanol preference and intake in a continuous, two-bottle choice protocol following a shorter history of binge drinking than males. The final goal was to determine if binge drinking results in unique patterns of anxiety- or depressive-like symptoms in males and females and whether these behaviors would be associated with the dimorphic regulation of GABAA receptor subunits across the prefrontal cortex and hippocampus. Male binge drinkers displayed anxiety-like behavior during early withdrawal that dissipated after 2 weeks of abstinence. There were no significant changes in the expression of or 2 GABAA receptor subunit mRNA at this time point in the regions analyzed. Females also

showed temporary anxiety-like behavior during early withdrawal from binge drinking. Additionally, females displayed significant depressive-like behavior after 2 weeks of abstinence from binge drinking. In particular, diestrus-phase females displayed significantly greater immobility in the forced-swim test after ethanol exposure and no longer maintained the reduced swim-time behavior associated with this phase of the cycle at baseline (when compared to the estrus-phase). qPCR analysis of hippocampal tissues from diestrus females supported a significant reduction in expression of 2 GABAA subunit mRNA after binge drinking. This effect was not noted for RNA isolated from hippocampal tissues taken during the estrus phase of bingers. These final data suggest possible interaction of estrous-cycle and binge drinking history that may result in the unique expression of deficits following binge drinking for females. Taken together, this work supports sex and estrous dependent effects of binge drinking on behavior and gene regulation.

1. CHAPTER 1: SEX AND AGE DIFFERENCES IN HEAVY BINGE DRINKING AND ITS EFFECTS ON ALCOHOL RESPONSIVITY FOLLOWING ABSTINENCE

1.1 Introduction

Adolescence is a major stepping-stone in mammalian development. It is a period characterized by substantial changes in brain structure, systems and connectivity, and includes reorganization of neurochemical networks, and increases in synaptic pruning and myelination (Bava and Tapert, 2010; Giedd, 2004; Spear and Brake, 1983; Tamnes et al., 2011). The dramatic brain changes that occur at this time period may leave the central nervous system especially vulnerable to adulteration by drugs and alcohol. Consequently, the high rate of binge alcohol consumption in this age group elicits concern (Johnston et al., 2008). Alcohol use during this time period may not only perturb the neuroenvironment, but may also stunt maturation and increase susceptibility to the development of dependence and abuse (Crews et al., 2007; Witt, 2010). Indeed, there is a strong relationship between age of first drink and rate of alcohol dependence (Dawson et al., 2008; Hingson et al., 2006; Pitknen et al., 2005). Our research team has previously shown a positive relationship between binge alcohol consumption during adolescence and higher than average consumption of the drug during adulthood (Moore et al., 2010). Interestingly, we have also shown that both sensitivity to alcohol during adolescence, and the effects of adolescent alcohol exposure on adult receptivity to the drug, may be modulated by genetic background (Melón and Boehm, 2011; Moore et al., 2010). This is not surprising, as a substantial body of literature supports a role for genetic background in the progression from recreational drug use or social drinking to abuse and addiction. Furthermore, though most alcohol consumers initiate use prior to the end of adolescence, only a small

percentage of those go on to develop an alcohol use disorder. However, little is known about how the interaction between genetics and ontogeny alters the effect of adolescent exposure on the risk of developing addiction during adulthood.

Given the ethical limitations of human research, animal models are crucial to our ability to clarify the independent and/or synergistic roles of genetics and ontogeny with respect to the vulnerability to develop alcohol use problems (Zucker et al., 2008). Unfortunately, many animal models of voluntary alcohol consumption yield higher alcohol intake among adolescents than adults (Doremus et al., 2005; García-Burgos et al., 2009; Maldonado et al., 2008; Moore, et al., 2010; Vetter et al., 2007). Although this highlights the face and ecological validity of these animal models in representing alcohol related behaviors seen in human adolescents, it makes it difficult to isolate the importance of age of exposure from the general pathological effects of high alcohol intake. Put another way, in experimental models where adolescent rodents actually consume more alcohol than their adult conspecifics, it is impossible to infer whether the effects seen following this early pre-exposure were due to the age at which the animals were drinking, or to the amount of alcohol to which the animals were exposed. With this in mind, we adapted the recently characterized Drinking in the Dark-Multiple Scheduled Access (DID-MSA) paradigm (Bell et al., 2006; Bell et al., 2011) in order to induce home cage binge drinking in mice. Like the Drinking-in-the-Dark (DID) paradigm (Rhodes et al., 2005; 2007), this procedure is an oral self-administration protocol that takes place in the animals home environment. Although the original DID-MSA protocol has been shown to induce age-dependent binge drinking behavior in rats (Bell et al., 2011), preliminary evidence from our laboratory suggested that this adapted access schedule could produce similar alcohol consumption across adolescent and adult mice.

The goals of the present series of experiments were threefold: 1) to characterize the level of consumption and intoxication achieved using the DID-MSA procedure in adolescent and adult C57Bl/6J (B6) mice; 2) to assess whether age of exposure moderates the development of functional tolerance to intoxication following multiple

binge sessions and 3) to evaluate whether age of exposure affects later sensitivity to alcohol. We hypothesized that this modified DID-MSA protocol would initiate high but comparable levels of intake in B6 adults and adolescents and that later sensitivity to alcohol would be affected by age of exposure in this strain. Given our ultimate interest in exploring the interaction of ontogeny and genetics in moderating the effects of alcohol exposure, we also included the alcohol non-preferring, DBA/2J inbred mouse strain to see whether this type of scheduled drinking procedure could induce any level of relevant alcohol intake in these mice.

1.2 Methods

1.2.1 Subjects

Male and female DBA/2J (D2) and C57BL/6J (B6) adult (PD 60± 3) and adolescent (PD 30± 3) mice were purchased from Jackson Laboratory (N= 251 mice). Animals arrived at the Indiana University-Purdue University Indianapolis School of Science animal facility at PD 21±3 or PD 56± 3. Animals were singly housed in standard shoebox cages and were habituated to the facility for seven days. Mice were maintained across two holding rooms, each kept at 21± 1 degrees Celsius and approximately 50% humidity. An anteroom, where all mice were moved for daily weights, separated the holding rooms. Behavioral testing and blood retrieval also occurred in this anteroom. Food and water were available ad libitum, except during alcohol access periods. All procedures were approved by the Indiana University-Purdue University Indianapolis School of Science Institutional Animal Care and Use Committee and were consistent with the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

1.2.2 Drugs and Drinking Solution

For drinking, 95% Ethanol (Ethanol; Pharmco Products Inc., Brookfield, CT) was diluted with tap water to a 20% v/v solution. For intraperitoneal injections, 95% Ethanol was diluted with 0.9% physiological saline to a 20% v/v solution and administered by varying injection volume for a 1.75 g/kg dose.

1.2.3 Experiment 1: Alcohol pre-exposure using the drinking in the dark-multiple scheduled access (DID-MSA) protocol

The drinking protocol was adapted from Bell and colleagues (Bell et al., 2011). Each day, mice received access to water or a 20% unsweetened ethanol solution during three, 1-hour access periods. Each access period was separated by two hours, during which all mice had ad libitum access to water. Immediately following lights-out, regular water bottles were removed from all cages and replaced with a 10mL plastic Mohr pipette affixed to a ball bearing sipper. This modified drinking tube contained either water or the ethanol solution and volumes were recorded before and after each hourly access period. The regular heavy duty glass water bottles (16 ounces) were placed atop the modified tubes. This helped to reduced leakage by keeping the modified tubes in place. Additionally, two leak cages (one with a modified tube containing water, and one with a modified tube containing the ethanol solution) were maintained on each animal rack, and were read at the end of each access period. An average hourly leak was calculated for each solution (water or 20% ethanol), for the entire experiment. These constants were subtracted from all respective intake values.

1.2.4 Experiment 2: Assessment of intoxication and blood ethanol concentration during DID-MSA

We were interested in evaluating the level of intoxication achieved using this DID-MSA procedure with B6 and D2 mice. Additionally, we wanted to assess the degree

of functional tolerance seen following multiple binges using this DID-MSA procedure. Therefore, mice were assessed for signs of motor incoordination immediately following either the first (1H), second (2H) or third (3H) hour of access to ethanol (or water) on days 7 and 14 of drinking. Mice were pseudorandomly assigned to either group 1H, 2H or 3H. Motor incoordination was measured using the balance beam apparatus. Given the potential confound due to size differences between the adults and adolescents (Broadwater et al., 2011; Doremus et al., 2006; Moore et al., 2011; Linsenbardt, et al., 2010) we used one hardwood balance beam for adults (122cm long X 2cm wide X 4cm tall) and a second hardwood balance beam for adolescents, scaled to 3/4 the size of the adult beam (91.5 cm long X 1.5cm wide X 3 cm tall). Each beam was affixed atop two 48cm tall ring stands. Approximately 2 hours before lights out on days 7 and 14, adolescents and adults were trained on their respective balance beam apparatus. During this training, a mouse was placed onto the starting edge of the balance beam to traverse the length of the beam, to and fro. The eraser end of a pencil was used to nudge mice that paused, or attempted to turn prematurely, along the beam. During the balance beam test, hind foot-slips were counted by the same experimenter that performed the training earlier that morning. Immediately after the mouse traversed the balance beam, a retro-orbital sinus blood sample was collected (25μL).

1.2.5 Experiment 3: Effect of alcohol intake during adolescence on alcohol-induced motor in-coordination and stimulation during adulthood in B6 mice

Only B6 mice were maintained for this portion of the study. Exactly one month following the fourteen days of DID-MSA ethanol access, the same B6 mice from Experiment 1 and 2 were intraperitoneally administered a 1.75 g/kg dose of ethanol (20% v/v). Animals who formerly consumed ethanol as adolescents were PD 73 $\pm$3 and those who consumed ethanol as adults were PD 102$\pm$3. Prior to lights out on this

test day, all mice were trained on the adult sized balance beam. Training proceeded as described earlier. Immediately following the 1.75 g/kg ethanol administration, mice were returned to their home cages. The homecages were placed onto a rack containing homecage activity monitoring systems (Columbus Instruments, Columbus, OH) in order to assess locomotor activity following the 1.75 g/kg ethanol administration. The activity monitor sampled activity in ten separate, one-minute time bins. Immediately following the homecage activity monitoring, mice were made to traverse the length of the balance beam and hind footslips were recorded. At the end of this test, a retro-orbital sinus blood sample was collected (25μL).

1.2.6 Blood Ethanol Concentration Analyses

Blood samples collected following days 7 and 14 of DID-MSA ethanol consumption, and following the 1.75g/kg I.P administration of ethanol, were centrifuged immediately following collection, and plasma supernatant stored at -80°C. Samples were later analyzed for alcohol content using an Analox Ethanol Analyzer (Analox Instruments, Lunenburg, MA) and blood ethanol concentration (BEC) recorded as mg/dL.

1.2.7 Statistical Analyses

DID-MSA ethanol consumption was separately analyzed for B6 and D2 mice using a three-way mixed factor ANOVA, with age (adolescent vs. adult), sex (males vs. females), and day (day 1 through 14; within-subjects variable) as the variables of interest. Pilot data from our laboratory (unpublished results) using a replicate of the high alcohol preferring selected mouse lines (HAP1, Grahame et al., 1999) suggested that this scheduled access procedure increased consumption significantly by the seventh session of drinking. Therefore, an a priori decision was made to assess whether B6 or D2 mice showed a similar escalation of intake by comparing the average daily intake during the first and second weeks of access using a two-way mixed factor ANOVA (age*sex*week). We also analyzed data for B6 separately from D2, as

the B6 mice continued on to Experiment 3, whereas D2 mice were only included in Experiment 1 and 2. Intake on days 7 and 14 (the balance beam test days) and hind footslips were assessed separately using a three-way ANOVA with age, sex, and solution as factors. Homecage locomotor activity and motor intoxication (balance beam hind footslips) following the 1.75g/kg I.P. administration of ethanol were analyzed using a three-way mixed factor ANOVAs, with age of exposure (adolescent vs. adult), sex (males vs. females), and solution consumed (ethanol vs. water) as independent variables. Dunnetts or Tukey post hoc tests were used, as appropriate, to explore significant interactions. Simple linear regressions were used to evaluate the relationship between BEC and hourly ethanol intake. All statistical analyses were carried out using IBM SPSS Statistics, Version 19. Results were considered significant at $p<0.05$.

1.3 Results

1.3.1 Experiment 1: Assessment of intake during the drinking in the dark-multiple scheduled access (DID-MSA) alcohol pre-exposure

The total amount of ethanol consumed across the three 1-hour time bins can be seen for B6 mice in Fig.2 and for D2 mice in Fig. 3. Data were analyzed separately for each genotype. The variables of interest in the initial analyses were age (2), sex (2) and day (14). These data violated the assumption of sphericity; therefore, the Greenhouse-Geisser correction was used when assessing the significance of the F statistic. As such, the degrees of freedom reported reflect this correction. B6 mice showed significant changes in their pattern of drinking across the 14-days of access [Fig.2A; $F(9, 822)= 37.315$, $p<0.0001$]. Planned comparisons support a linear trend ($p<0.0001$) as these mice significantly increased their drinking over time. Ethanol consumption for B6 mice also showed a significant quadratic trend, suggesting that this drinking was sensitive to environmental/procedural changes associated with the behavioral test days. Changes in the pattern of ethanol intake across the 14-days were

also dependent upon sex [$F(9, 822)=4.133$, $p<0.0001$)]. Pairwise comparisons reveal that, when comparing intake on day 1 to successive days of drinking, B6 males do not show any significant increases in drinking until the 10th day of access. In contrast, B6 females begin to show a significant increase in intake by day 5. Daily ethanol drinking for D2 mice also showed a significant effect of day [Fig. 3A; $F(9.8, 853.8)= 5.704$, $p<0.0001$]. There was, however, no significant linear or quadratic trend to the drinking pattern. Instead, this main effect represents general inconsistencies in the pattern of intake across various days. Drinking data for D2 mice also revealed a significant interaction of day and sex, [$F(9.8, 853.8)= 2.4$, $p<0.01$], as the day to day variation in drinking was slightly different across male and female D2 mice.

Drinking data were also analyzed by comparing the average intake from the first week, to that from the second week, using a mixed-3-way ANOVA (age * sex * week). For B6 mice, this analysis revealed a significant effect of week [$F(1,92)=42.5$, $p<0.0001$], as all mice consumed more during their 2nd week of access than their 1st (Fig.2B and C). There was also a significant week* sex interaction [$F(1,92)= 5.4$, $p<0.05$]. Pairwise comparison clarified that a significant sex difference in intake was only supported during the second week of drinking ($p<0.05$). For D2 mice, ethanol intake did not show a significant effect of week [$F(1,87)=0.143$, p=n.s], but there was a significant interaction of sex * week [Figure 3A-B; $F(1,87)=6.05$, $p<0.05$]. This interaction appears to be driven by a marginal decrease in drinking seen for D2 males during the second week of access ($p=0.060$) that resulted in a marginal sex difference in intake during this week ($p=0.056$).

The total amount consumed by control animals, who had access to water in the modified tubes using the DID-MSA protocol, can be seen for B6 mice in Figure 4A and D2 mice in Figure 4B. These data were also analyzed separately for each genotype. Neither B6 nor D2 mice showed any relationship between sex, age or day on the pattern of water consumption.

In addition to g/kg consumed, we assessed total fluid intake to get a measure of general intake behavior. Fluid intakes (mls) were analyzed for B6 mice using

separate mixed 3-way ANOVAs for those with ethanol or water access. For total ethanol consumed, there was a significant interaction of day and sex [$F(9, 832)= 3.38$, $p<0.0001$]. Bonferroni adjusted pairwise comparisons revealed that, generally, both males and females consumed more fluid on the days following their first day of access. However, males reduced their fluid intake back to day 1 levels on day 7 and 8 of access (the day of and following behavioral testing and blood sampling) whereas females consumed more fluid on all subsequent days (p's<0.01). Pairwise comparisons also revealed that the sex difference in fluid intake (with males consuming more fluid than females, p's<0.01) dissipates by the 4th day of access, a consequence of the increase in fluid intake for females. Control mice displayed only a marginal increase in fluid intake across days [$F(6.7, 186.6)=1.89$, $p=0.08$] and did not demonstrate an interaction of day with any other factor. Further, neither sex nor age had a main effect or interaction effect on water intake.

Fluid intakes (mls) were also analyzed by comparing the average intake from the first week of access to that of the second week using mixed 3-way ANOVAs. For ethanol drinkers, there was a significant interaction of week and sex [$F(1,92)=4.19$; $p<0.05$]. Bonferroni adjusted pairwise comparison suggest that males and females both increased their fluid intake across the weeks(p's<0.0001). However, the sex difference in fluid intake that was apparent during the first week of intake (with males consuming more fluid than females; $p<0.05$) dissipates by the second week. Control mice displayed a significant increase in fluid intake across the weeks [$F=9.12$, $p<0.01$], but did not demonstrate an interaction of day with any other factor. Further, neither sex nor age had any effect on water intake

Finally, we assessed whether any of the interactions between sex and ethanol intake behavior noted above may reflect sex bias in weight gain over time. Absolute weight values showed a significant day*age interaction for water drinking mice [$F(13, 364)=6.462$, $p<0.001$]. For ethanol drinkers, there was a significant day*sex*age interaction [$F(13,1196)=3.04$, $p<0.0001$]. This was followed up by separate 2-way ANOVAs (day X sex) for adolescents and adults. Adolescents showed a significant

effect of day [F(13, 585)= 127, p<0.01], but no interaction of this within subjects factor with sex. Adults showed a significant day*sex interaction [F(13,611)=9.09, p<0.0001]. Bonferroni adjusted pairwise comparisons showed that weight remained steady for males until day 12, but increased for females starting by day 6. Weight was also analyzed as a percent change from baseline (day 1 weight, which was taken the morning prior to initiation of the drinking schedule). This analysis also supports an interaction of day*sex for adults [F(13,611)=10.69, p<0.0001], with females significantly increasing their weight by day 5.

1.3.2 Experiment 2: Assessment of Intoxication and Blood ethanol Concentration during DID-MSA

Experiment 2A: Intoxication (Post-drinking balance beam performance)

The degree of intoxication, assessed as hind footslips on a balance beam, achieved each hour was monitored on Day 7 and Day 14. These data are illustrated in Fig. 5. Separate groups of animals were used for each time point and are referred to as 1H (tested following their first hour of ethanol access), 2H (tested following their second hourly session of ethanol access), and 3H (tested following their third hourly session of ethanol access). Water animals were randomly tested following the first, second or third hour of access, but are presented and assessed as one group. Data were analyzed using a 4-factor RM-ANOVA comparing day (7 vs.14; repeated measures variable), sex, age and group (1H, 2H, 3H or water). For B6 mice, alcohol intake in the DIDMSA paradigm resulted in significant intoxication, but the mice failed to demonstrate tolerance to this intoxication across the 14 days of drinking. Specifically, there was no significant effect of day, nor was there a significant interaction between day and any other factor. There was a significant effect of group [F(3,110) = 19.23; p<0.0001]. Dunnetts post hoc comparing each ethanol drinking group (1H, 2H and 3H) to water drinking mice showed that mice with access to ethanol exhibited intoxication after the binge drinking sessions (Fig. 5A; p's<0.01; data shown

collapsed across day). B6 adolescents and adults displayed the same level of intoxication following binge drinking in this paradigm, as there was no main effect of age nor an interaction of this variable with any other factor. Similarly, although adult B6 females consumed greater amounts of ethanol than all other groups, there was no main effect of sex nor an interaction of this variable with age or group. For D2 mice, drinking did not lead to intoxication, as there was no main effect of group (Fig. 5B). Furthermore, performance on the balance beam for these mice did not differ across sex or age. There was a significant effect of Day, as all D2 mice showed a decrease in footslips with subsequent exposures to the balance beam.

Experiment 2B: Blood ethanol concentration

Blood ethanol concentrations achieved each hour were monitored on Day 7 and Day 14 and are detailed in Fig. 6. Ultimately, we wanted to determine whether this DID-MSA protocol could produce equivalent levels of heavy/binge ethanol consumption across adolescents and adults, in order to facilitate our investigations on the interactive effects that genotype and early/adolescent alcohol consumption has on later sensitivity to the drug (without the confound of disparate drinking histories across the age groups). For this, we analyzed the intake and blood ethanol concentration data from day 7 and day 14 using a mixed 4-factor ANOVA with day, sex, age and group (1H, 2H, 3H) as the independent variables. These data for B6 mice are shown in Fig. 6 and are detailed in Table 2. For this high drinking genotype, there was no main effect of day, sex or age. Our analysis did reveal a significant effect of group [$F(2,83) = 3.40$; $p<0.05$], as mice consumed different amounts of alcohol during the 1st, 2nd and 3rd hourly access periods. Tukeys post hoc test confirmed that intakes measured during the 3rd hour of access were significantly greater than those from the 1st hour of access ($p<0.05$), but not the 2nd. BECs achieved by B6 mice were not significantly different across these hourly sessions. Relatedly, we found no evidence supporting an effect of age or sex on the BEC achieved by these mice.

D2 mice maintained their alcohol avoiding phenotype in this drinking protocol, with hourly BECs all below 21 mg/dL (data not shown). This average and its variability were not significantly affected by day of intake, sex, age or hour of access. All groups of B6 mice had an average BEC above 80 mg/dL –the National Institute on Alcohol Abuse and Alcoholism standard for binge drinking– in at least one of the three hourly binge sessions.

As seen in Fig. 6A-F, BECs for B6 mice on both day 7 and day 14 were positively associated with the amount of alcohol consumed in each hourly session. Intakes significantly predicted BEC on days 7 ($R2=0.58$; $p<0.0001$) and 14 ($R2=0.42$; $p<0.0001$). Separate correlation coefficients for each group (by age, sex and hour) are presented in Table 2.

1.3.3 Experiment 3: Effect of adolescent alcohol intake on alcohol-induced motor in-coordination and stimulation during adulthood

Experiment 3A: Balance beam performance following ethanol challenge

The motor-incoordinating and stimulant responses to an ethanol challenge (1.75g/kg; I.P.) following one month of abstinence in B6 mice are illustrated in Fig. 7 and 8, respectively. For motor-incoordination, the number of hind footslips made on the balance beam were analyzed using a three-way ANOVA, with sex, age during binge drinking pre-exposure and solution consumed (during binge pre-exposure) as independent variables. There was a significant main effect of solution [$F(1,126)=3.945$; $p<0.05$], as mice with a history of binge alcohol consumption displayed a dampened ataxic response to this ethanol challenge, when compared to water drinking controls (Fig. 7). This relationship was not altered by sex, or the age at which the binge drinking occurred.

Experiment 3B: Home-cage activity following ethanol challenge

A number of laboratories have demonstrated that B6 mice display a complex, biphasic locomotor response to low dose ethanol (Crabbe et al., 1982; Melón and Boehm, 2011; Tarragón et al., 2012). In particular, these mice often show stimulation 1-5 minutes following ethanol administration and hypolocomotion by 10 minutes post injection. For this reason, we chose to assess the activity data as two separate 5 minute time bins. These data were subject to a three-way ANOVA with sex, age during binge drinking pre-exposure and solution consumed as independent variables. The analysis of the first five minutes following injection revealed a significant interaction of sex and age [$F(1,80) = 13.92$; $p<0.0001$], as females exposed to alcohol or water as adults, all had a greater locomotor response to ethanol than all other groups (Fig. 8A). This effect did not depend on the binge drinking history of the females. Interestingly, females who were exposed to water during DID-MSA as adolescents, showed a locomotor response more similar to that of males than to females exposed to water during DID-MSA as adults. Analysis of the second five minutes following injection revealed a significant three way interaction of sex, age of binge exposure and solution [$F(1,80) = 4.67$; $p<0.05$]. Tukeys post hoc analysis clarified that females who binge drank during adulthood demonstrated significantly higher activity following the ethanol challenge when compared to control females (Figure 8B; $p<0.01$). These females actually had ambulatory counts significantly higher than all other groups (ps <0.01). Post hoc analysis also clarified that control females, during this time bin (6-10 minutes post injection), no longer demonstrated greater activity when compared to other B6 mice.

1.4 Discussion

The present series of experiments have yielded three main demonstrations. First, we established that the DID-MSA procedure yields intoxicating levels of binge-like alcohol consumption in C57BL/6J (B6) and not DBA/2J (D2) mice. Second, we

found that adult B6 females are particularly sensitive to this type of scheduled access, displaying a 120% increase in their intake over the 14 days of drinking. Third, we provide evidence supporting long term changes to ethanol responsivity following binge drinking using this protocol.

1.4.1 Behavioral Intoxication and binge drinking during adolescence

The alcohol intake levels noted during this DID-MSA procedure are comparable to those using other limited access drinking paradigms for all groups except B6 adult females. Similar to other models, we found binge drinking using this procedure to be genotype specific, as D2 mice consumed negligible amounts of alcohol and showed no evidence for intoxication when assessed using the balance beam. In contrast to what has been demonstrated using the DID procedure (Linsenbardt et al., 2011), binge drinking using DID-MSA did not result in the development of functional tolerance across the 14 days of drinking for B6 mice. Although this was surprising, the significant drop in intake noted on the final day of drinking suggests that data from this day should be interpreted with care, as stress from the experimental procedures may have affected the animals behavior on this day. Moreover, given that mice were tested at the end of their hourly binge session, we do not know if differences in the rate of consumption on Day 7 vs Day 14 obfuscate our ability to detect changes in the degree of intoxication measured across the two days. We do attempt to disentangle this potential confound by measuring BECs following the balance beam test and hour of drinking that preceded it, and note statistically comparable BECs achieved following drinking on Day 7 vs Day 14. Still, we contend that there are notable shifts in the correlation between ataxia and BEC on Day 7 vs. Day 14. Specifically, there is a predictable relationship between BEC and ataxia following the first session of access on Day 7 only (data not shown). We are unaware of any published studies showing a significant correlation between BEC and ataxia following drinking. Moreover, those that do report their findings usually see significant ataxia in ethanol drinking

mice but no significant correlation between the degree of ataxia and BEC following drinking (Sharpe et al., 2005). The fact that we do note a significant predictable relationship between these factors and that the correlation wanes following multiple presentations of alcohol (within Day 7 and across Day 7 to 14), suggests some form of tolerance may be developing that we do not tap into with our crude measure. Thus, we must still conclude that we fail to support the development of behavioral tolerance to the intoxicating effects of binge drinking within the 14 days of access to alcohol administered using DID-MSA.

We were also unable to find differences across age or sex in the degree of intoxication noted following each binge session. This finding adds to the currently conflicting body of literature on age differences in sensitivity to the motor impairing effects of ethanol. Studies in rats have generally found adolescents to be less sensitive to ethanol-induced ataxia (Silveri and Spear, 2001; Ramirez and Spear, 2010; Broadwater, Varlinskaya and Spear, 2011). In mice, this relationship has been shown to be dependent upon genotype and sex. Additionally, given their fast metabolic rate, dose significantly moderates the relationship between age and sensitivity to alcohol induced ataxia for mice. B6 adolescents have shown greater sensitivity to ethanol induced ataxia at moderate alcohol doses (1.75 g/kg to 2.5 g/kg; Hefner and Holmes, 2007; Linsenbardt et al., 2009). However, at the 1.5g/kg dose, our lab has been unable to find evidence for significant differences in sensitivity to this response across B6 adults and adolescents. As this dose better approximates the high end of the BEC range achieved during our binge drinking procedure, we believe our collective efforts suggest that this genotype does not show evidence for age-related differences in sensitivity to ethanol induced ataxia at doses relevant to binge intoxication.

1.4.2 Alcohol responsivity following abstinence in adolescent or adult binge drinking

Our efforts herein suggest that binge alcohol consumption perturbs the neurobiological systems that mediate ethanol-induced hyper- and hypo-locomotion, as well as motor incoordination, in a sex- and age- specific manner. For example, B6 mice with binge drinking histories demonstrated dampened sensitivities to the motor incoordinating effect of an ethanol challenge. However, expression of this reduced sensitivity did not depend upon the age of the animal at the time of the binge alcohol exposure. Still, we were able to find evidence of tolerance long after the cessation of binge drinking in both adolescent pre-exposed mice and adult pre-exposed mice. On its own, this is a substantial finding. Though functional tolerance following voluntary consumption has been demonstrated in rats (Gatto et al., 1987; Darbra et al., 2002) and mice (Cronise et al., 2005; Linsenbardt et al., 2011), few have been able to demonstrate long-lasting changes to ethanol induced motor-incoordination as a function of voluntary oral preexposure. Recently, Rimondini and colleagues (2008) demonstrated long-lasting tolerance that persists into protracted abstinence (3 weeks post alcohol cessation) in rats that had 7 weeks of intermittent ethanol vapor. The alcohol exposure paradigm used by those authors is a well established model of dependence, producing persistent increases in voluntary intake and documented neurobiological effects (Roberts et al., 2000; Rimondini et al., 2002), thus we are hesitant to believe that ethanol intakes achieved using this DID-MSA paradigm could approach those necessary to induce comparable persistent changes. It is possible that our demonstration of persistent tolerance may be due to intoxicated practice, which has been shown to prolong demonstration of tolerance in rats up to two weeks post chronic alcohol administration (32 daily doses of 2 or 4 g/kg i.p; Lê et al., 1989). However, it may also be argued that the mice in the present study did not have enough intoxicated exposure to the balance beam (2 times prior to the post-abstinence test; each 1 week apart) to support the development of intoxicated practice, which is shown

following extensive intoxicated experience with the testing apparatus. Future studies should clarify the duration of tolerance following abstinence from voluntary binge-like drinking and determine whether binge consumption using DID-MSA may induce persistent altered preference for alcohol and/or increased consumption of the drug in unlimited/free choice paradigms (i.e. shift too much to fast drinking to too much to often; Leeman et al., 2010).

Regarding the failure to find a specific effect of adolescent binge drinking on the degree of tolerance demonstrated following abstinence, it is possible that the level of alcohol exposure achieved during DID-MSA was high enough to induce adaptation in both adults and adolescents. An alternative explanation is that the neurochemical systems important for the expression of ethanol induced ataxia at this dose range are already developed by PD 30 (when binge drinking was initiated), such that alcohol exposure at this period would result in an adult-like pattern of behavioral adaptation. Indeed, during the binge drinking phase, adolescent mice showed no difference in sensitivity to ethanol induced motor incoordination as compared to adults. Morever, the results add to inconsistent findings from previous works showing that the development of chronic tolerance may be greater during adolescence (Swartzwelder et al., 1998), reduced during adolescence (Matthews et al., 2008) or not different across adolescence and adulthood (Varlinskaya and Spear, 2007). We have previously evaluated tolerance to the ataxic effects of alcohol following injection in mice (Linsenbardt et al., 2009) and found that adolescents developed tolerance with higher (1.75g/kg) but not lower (1.5g/kg) doses. It is therefore possible that in the present studies, our adolescents were consuming alcohol at a level that surpassed the threshold for capturing their reduced ability to develop chronic tolerance.

The initial (first five minutes) locomotor response to an alcohol challenge (Fig. 7A) suggests that females show unique differences in their response to the experimental procedures depending upon their age at the start of the experiment. Among the water-drinking females (drug naive), mice that were initiated into the experiment during adolescence do not show the same heightened locomotor response to ethanol

as females that were initiated as adults. This is a peculiar finding, as these females are all adults at the time of the ethanol challenge injection. However, these naive water drinkers were subjected to unique experiences associated with the experimental design (e.g., limited access to the ball-bearing sipper tubes) and possible stressors, at different developmental stages. Although it was not our intention to model adolescent stress in our experiment, we do concede that the chronic isolation required to administer alcohol and appropriately record intake may be interpreted as a chronic stressor. Additionally, the acute stress experienced following the retro-orbital blood sampling could have worked synergistically with the isolation stress to produce a dampened locomotor response to ethanol noted for females that drank either water or ethanol as adolescents when compared to those that drank as adults. Interestingly, males who started in the experiment as adolescents show a similar locomotor response to males who started as adults, regardless of the solution consumed. Therefore, it is possible that age and sex interacts to modify the effects of early life stress on adult responsivity to an ethanol challenge. Indeed, McCormick and colleagues have demonstrated that adolescent stress results in an augmented expression of locomotor sensitization following repeated exposure to nicotine (McCormick et al., 2004) or amphetamine (McCormick et al., 2005) later in life, and that this occurs only in females. Although the directionality of our effect is opposite that seen by McCormick and colleagues, the fact that we only note a difference for females is similar and adds to the body of evidence supporting sex differences in the effect of adolescent stress on adult responsivity to drugs of abuse

1.4.3 Sex differences in binge drinking and its long-term effects

Among B6 adults, there was a clear effect of sex on the escalation of binge drinking using this paradigm. We believe this DID-MSA model offers an important opportunity to study sex differences in the acquisition of oral alcohol self-administration. For other drugs of abuse, like cocaine and amphetamine, differences across males and

females in the acquisition and maintenance of rewarding compounds has revealed important dimorphic mechanisms underlying the development of addiction (Carroll and Anker, 2010). For alcohol, we have long accepted that female rodents often consume greater amounts of the compound than males, and have made important strides in understanding what underlies this difference. Yet, aside from the heroic efforts of a few investigators, there has been little attention paid to biological sex as an important variable in the acquisition of alcohol consumption (Roth et al., 2004). The data here (Fig. 1) indicates that female B6 mice may acquire heavy alcohol self-administration faster than males, when given limited access to the drug. Of course, a number of factors unrelated to addiction vulnerability may underlie these differences. For example, females may show stronger habituation to the novel, ball-bearing sipper tubes used in this procedure. Given that females do not show dramatic changes in their water consumption using the same procedures, this is unlikely a major factor in the diergic escalation of alcohol self-administration. Still, as alcohol access (in this protocol) initiates at the onset of lights out, females may better adapt their activity patterns to match access to this calorie rich ethanol solution. Indeed, mice have shown evidence for sex differences in their circadian response to zeitgebers (Lee et al., 2004), and food anticipatory activity (FAA) has been demonstrated for drugs of abuse, including limited-access to alcohol (Kosobud et al., 2007). However, a notable sex difference in FAA has not been demonstrated for B6 mice (Feillet et al., 2006). Alternatively, it was possible that males were gaining weight at a faster rate than females, biasing the g/kg calculations. However, as our analyses suggest that females, but not males, are increasing their weight earlier on during the DIDMSA procedure, we can rule out the possibility that the sex-dependent escalation in intake is an artifact of this measure. Specifically, if the males were maintaining a flat level of intake across the two weeks specifically due to sex differences in the slope of their weight change, we would need to see the opposite pattern of weight gain (with males increasing their weight overtime, thus causing their g/kg to not change even if they were indeed escalating their consumption). Lastly, although adolescent females show a significant

increase in their consumption across weeks (Fig.2), they never consume more alcohol than males from either age group. Instead, the increased intake demonstrated by the adolescent females is more a function of their low intakes during the first 5 days of access. Therefore, the escalation noted for adult B6 females may be said to occur following adolescence. Given the important hormonal changes that occur around this time period (i.e. puberty), it is possible that sex differences in the escalation of alcohol consumption for B6 mice reflect an interaction between the effects of alcohol and the activational effects of hormones that increase their synthesis drastically following puberty (i.e. progesterone and its neuroactive metabolites).

In addition to a sex difference in the escalation of intake, we found a marked sex difference in the effect of binge drinking on the locomotor response to ethanol (1.75g/kg; i.p) after one month of abstinence (Fig. 7). There are a number of alternative explanations for the heightened locomotor response noted for adult pre-exposed females. For example, it is possible that the level of alcohol exposure achieved for B6 adult females was enough to cause unique perturbations not seen for the adult pre-exposed males or pre-exposed adolescents. Though, it should be noted that these females did not achieve significantly higher BECs at their level of drinking. Another interpretation concerns a true sex difference in vulnerability to adaptation following binge drinking. Clinical studies suggest that women show a telescoped development of alcohol addiction, progressing through the landmark events associated with the development of alcohol use disorders faster than men (Piazza et al., 1989; Randall et al., 1999). Preclinical studies have also demonstrated sex differences in the development of ethanol dependence (Devaud et al., 1999; 2003; 2006; Kuhn, 2011; Wiren et al., 2006). Preclinical studies also suggest that adult females are more susceptible to the development of psychomotor sensitization following repeated exposure to a variety of compounds including cocaine (Cailhol and Morméde, 1999; Hu and Becker, 2003), nicotine (McCormick et al., 2004) and alcohol (Grahame et al., 2000). Though we did not set out to model the development of psychomotor sensitization to ethanol in the classical sense, there is evidence that alcohol consumption in B6 mice (24 hour,

2-bottle choice) can increase the stimulant effects of an acute alcohol injection (Lessov et al., 2001). Therefore, we may interpret the heightened locomotor response to the ethanol challenge noted for binge drinking females as compared to the naive mice as an example of a between-group sensitized response. This would suggest a sex difference in the development or expression of sensitization following binge drinking in these mice. Further, the sensitization noted following this DIDMSA binge drinking regiment appears less vulnerable to decay that that demonstrated following injection, which degrades by 17 days following the cessation of ethanol treatment (Lessov et al., 1998). It should be noted, however, that the adolescent females did not demonstrate a similar vulnerability following binge exposure. Instead, they showed a reduced response to ethanol, even when compared to control adult-females. However, interpretting the effects of binge drinking during puberty would require future targetted studies, given the possible interaction of ethanol and pubertal development for these females. Vaginal opening for B6 mice occurs around P30 and signals the start of puberty. This period continues until the first signal of estrus and may even be said to progress further into development, when female mice begin having regularly cycled estrous related events. The adolescent females in this experiment were therefore exposed to ethanol across their entire pubertal development. Given the proposed effects that ethanol may have on estrous and the interaction the drug has with many elements necessary to induce reproductive maturation and maintain estrous cycling (i.e. GnRH, GABA, progesterone), it is possible that differences in the post abstinence response to the ethanol challenge dose (where females who drank as adolescents show a different locomotor response to the challenge dose than females who drank only during adulthood), as well as differences in the pattern of binge drinking in general, are induced by ethanols effect on pubertal development. In fact, recent evidence suggests that the switch in the gonadotropin releasing hormone profile to oscillatory activity and surges that define an adult hypothalamic-pituitary-gonadal system, may depend on $GABA_A$ receptors (Han et al., 2002; Herbison and Moenter, 2011) and specifically, the extrasynaptic $GABA_A$ receptors that are particularly sensitive to binge-like con-

centrations of ethanol (17mM; Bhattarai et al., 2011). Future studies should also clarify whether dose, pharmacokinetics, or genuine dimorphic adaptations to alcohol exposure underlie sex differences in the effect of pre-exposure to alcohol on later responsivity to the drug following abstinence. For example, it is generally thought that women reach higher peak BECs than men (Jones and Jones, 1976), though they also demonstrate faster elimination rates (Dubowski, 1976). Given sex differences in body composition (chiefly, reduced total body water for women), and the fact that alcohol is distributed in water-rich tissues, the differences in distribution/peak BEC may be expected. This difference in peak BEC is compounded by sex differences in the gastric metabolism of ethanol.

After alcohol is ingested, a fraction of it is first oxidized by the gastric mucosa. Women have been shown to have less gastric-first pass metabolism of alcohol than men (Baraona et al., 2001), this adds to the effect of higher blood circulation of ethanol and also affects sex differences in the elimination rate of the drug. Whats more, Frezza and colleagues (1990) have shown that sex interacts with prior ethanol experience to affect elimination rate, as first-pass metabolism is completely absent among alcoholic women (they show non-significant differences in peak BEC and in rate of elimination following both orally consumed and intravenously administered ethanol, at 0.3g/kg). Still, given the significant intra and inter-individual variability noted for ethanol pharmacokinetics, this gender difference is supported by some (Jones and Jones 1976, Frezza et al., 1990) but not others (Marshall et al., 1983, Sutcker et al., 1983, Arthur et al., 1984). Differences in the study designs may also account for inconsistencies. For example, although all studies used subjects who had eaten food prior to ethanol administration in order to avoid the effects of fasting on ethanol metabolism, there were considerable differences in factors like the times between meal and ethanol administration and contents of meals (i.e. In one of the studies, all subjects consumed two cups of coffee as part of the meal, which could have altered subjects metabolic rate and could have also altered ethanol clearance rates via its diuretic properties). It has also been suggested that the rate of alcohol elimination is

higher for women. While a number of studies using oral administration have found this to be the case, many have not. Preclinical studies do suggest that testosterone may inhibit the metabolism of ethanol, as male rats show faster elimination rates following castration (Lumeng and Crabb, 1984). Other inconsistencies come from IV administration studies, where males and females often display similar peak BECs. It has been suggested that this is due to sex differences in the ratio of lean body mass per liver mass (Li et al., 2001). Thus, route of administration also affects whether there are measurable/significant sex differences in the pharmacokinetics of ethanol. In terms of our drinking protocol, we need to further probe how pharemacokinetics may be playing a role in the expression of sex differences in the escalation of intake during the binge phase, and in the response to the challenge dose after abstinence.

1.4.4 Tolerance following binge drinking as compared to ethanol challenge

Tolerance is the reduced response to a drug effect. This dampened response may be acquired, in which case it presents as a right-ward shift in the dose-response curve following repeated exposures to a compound. Or, the reduced response may be innate, reflecting individual differences in sensitivity to a compound. Throughout this chapter, I attempted to probe the development of tolerance by looking at whether sensitivity to ethanol-induced ataxia changed following binge drinking. The first attempt looked at the level of ataxia noted immediately following binge drinking, when the mice were voluntarily drinking to a level that induced this measurable level of impairment. The second attempt looked at the level of ataxia noted following an ethanol challenge that consisted of an intraperitoneal administration of 1.75g/kg ethanol. In both attempts, we were interested in probing acquired tolerance after a history of binge drinking.

Acquired tolerance may be mediated by an increased capacity to metabolize the compound following repeated exposures (e.g., repeated exposure resulting in faster

induction of relevant enzymes), by physiological adaptations that occur at the site of action of the drug, by behavioral adaptations that occur from performing the impaired action while under the influence of the drug, or by early induction of the physiological targets of the compound by contextual cues. It is possible that the two different attempts to assess acquired tolerance would reflect different combinations of these elements (pharmacokinetics, pharmacodynamics and learning/behavioral changes). For example, in the situation where the animals are assessed after oral consumption, we may have found that the animals are consuming more ethanol on day 14 as day 7, but show the same level of blood ethanol concentration at the end of drinking and show no change or reduced intoxication or they may drink the same but show reduced BECs on day 14 and reduced ataxia on that day, as compared to day 7. The reduction in intoxication on day 14 could also have been due to changes in the way ethanol interacts with its target tissues at the site of action, thus for example, a reduction in the expression of $GABA_A$ receptor subtypes involved in mediating the effects of ethanol on motor coordination. Lastly, because the animals have experience moving while intoxicated, a reduction in footslips on the balance beam on day 14 could have reflected the behavioral adaptation they developed to compensate for the motor incoordinating effects of ethanol. The data, however, do not support any changes in footslips on the balance beam on day 14, as compared to day 7. Along with the lack of change in BEC across those days, we are unable to support the development of acquired tolerance at any of the three levels. However, it is possible that our behavioral assessment was not sensitive enough to capture the subtle changes in intoxication that may have developed across the days. When the animals are tested after a month of abstinence, they are first given an ethanol challenge that would result in a BEC higher than what was achieved following drinking. This challenge allowed us to compare the post-abstinence challenge response of the animals that had been drinking ethanol to that of the animals for whom this injection would be their first experience with the intoxicating effects of the drug.

Of course, as with any compound, the route of administration can have significant effects on the bioavailability, mainly by altering the rate and extent of absorption. For example intravenous administration of ethanol makes the compound immediately available in the circulatory system-bypassing first-pass metabolism. Therefore, the area under the blood ethanol concentration curve is greater, with a significant increase in peak BEC. Given potential differences in the sensitivity of various receptor systems to ethanol concentration, we can expect different regulatory mechanisms to be activated based on the way ethanol was administered. For example, 4g/kg of ethanol administered intraparetoneally to a mouse would result in a peak BEC ∼400mg/dL or a brain concentration around 88mM brain ethanol concentration. This dose of ethanol could induce significant sedation. On the other hand, a mouse given the opportunity to consume 4g/kg would not be expected to have a brain concentration that exceeds 30mM (∼140mg/dL). This concentration of ethanol would be expected to induce intoxication (I.e. motor in coordination), but not significant sedation. In terms of receptor systems, one would not necessarily expect 30mM ethanol to interact with the same receptor population as 88mM ethanol. For example, recent studies suggest that the population of $GABA_A$ receptors that are located outside of the synapse may be activated by low doses of ethanol, as achieved after the first or second glass of wine (Olsen, 2010). On the other hand, synaptically located $GABA_A$ receptors, that are endogenously opened by the higher concentrations of GABA released by an action potential at the presynaptic neuron, would not necessarily be activated by these low concentrations of ethanol.

Though the peak dose achieved, receptor population initiated, ratio of ethanol to its metabolite acetaldehyde at the site of action (thought to have an opposite effects to ethanol on some behaviors when acting at the VTA to ethanol; see Martí -Prats et al., 2013) may be different after the intraperitoneal vs oral route of administration, a change in the degree of incoordination achieved by this challenge across the ethanol bingers and control mice can still be said to reflect acquired tolerance. What cannot be clarified with the data available is at what level (pharmacokinetic, pharmacody-

namic, behavioral/learned) this tolerance is occurring. For example, chronic ethanol exposure results in the faster elimination of the compound as it usually results in the induction of the secondary pathway of ethanol metabolism via liver microsomes (microsomal ethanol oxidizing system; Leiber and Pirola, 1982). However, alcoholics also demonstrate reduced first-pass metabolism due to a reduction in (or elimination of, as described earlier for female alcoholics) gastric alcohol dehydrogenase activity (Leiber, 1997). It is possible, therefore, that the extensive alcohol consumption access during the binge drinking session increased the clearance rate of ethanol such that the binge-history mice were tested on the balance at a BEC lower than the binge-naive controls.

1.4.5 DID-MSA as a protocol to induce binge drinking in mice

There have been a growing number of drinking protocols with the common goal of inducing high alcohol consumption in animal models of oral alcohol self-administration. Although this redundancy may seem unnecessary to some, these procedures offer opportunities to study unique aspects and consequences of alcohol consumption, a surprisingly complex behavioral phenomenon. The DID-MSA drinking procedure (adapted from Bell et al., 2011) provides a number of advantages over currently used protocols, depending upon the investigators experimental design and variables of interest. Clearly, this drinking protocol results in binge-like consumption and intoxicating levels of alcohol intake in B6 mice (Fig. 5 and 6). However, one may use the simpler and well characterized DID protocol if these are the experimental goals (Rhodes et al., 2005; 2007; Moore et al., 2007). Given the results presented here, the DID-MSA procedure may be useful to investigate the varied consequences specific to adolescent binge drinking (as the procedure equates adolescent binge-drinking with adult male intake levels) and the mechanisms underlying those effects. Additionally, the procedure provides a unique opportunity to study sex differences in the escalation

and/or effects of binge consumption, given the dimorphic response to the protocol by B6 adult males and females.

1.5 Conclusion

To conclude, the experiments presented above support an interaction of sex and age on the effect that binge alcohol intake has on later sensitivity to the drug. These data also support the utility of the DID-MSA paradigm for studying the isolable influence of these two important variables.

2. CHAPTER 2: SEX DIFFERENCE IN THE EFFECT OF BINGE DRINKING ON ALCOHOL INTAKE AND PREFERENCE

2.1 Introduction

Alcohol use disorders develop for a minority of the population of users (Grant et al., 2007). While the total volume of alcohol consumed is an important variable underlying the development of alcohol related problems (Bobak et al., 2004), the pattern of drinking can often be a stronger predictor of risk (Rhem et al., 2001; Murray et al., 2002). For example, in a three-country cross-cultural comparison of alcohol use, epidemiologists found the greatest prevalence of alcohol related problems in the country with the lowest per capita intake of the drug (Bobak et al., 2004). Alcohol use in this country was characterized by high dose in (relatively) infrequent sessions, with a higher proportion of people drinking to intoxication (World Health Organiziation,WHO, 2011).

This pattern of binge drinking is increasing in prevalence across the world (WHO, 2011). The National Institute for Alcoholism and Alcohol Abuse(NIAAA) defines a binge episode as intake that raises the consumers blood ethanol concentration to $\geq$80mg/dL, usually seen following $\geq$4 drinks or $\geq$5 drinks in a two hour drinking session for women and men, respectively (NIAAA, 2004). Although this definition has greatly standardized the literature and research surrounding this pattern of alcohol use (Fillmore et al., 2011), statistics describing alcohol consumption in the United States show that Americans actually intake an average of 8 drinks per session and do so frequently (4 times a month; Center for Disease Control, CDC, 2012). This type of heavy binge drinking has been associated with an increased risk of negative outcomes (Naimi et al., 2003; Wechsler et al., 2005), such as increases in sexual risk taking

(Cook et al., 2005), experiencing sexual violence (Chersich et al., 2007), personal injury (Cherpitel, 1993; Serras et al., 2010) and suicide attempts (Mukumal et al., 2007). In addition to consequences associated with the acute state of intoxication caused by the binge consumption of alcohol, clinical research suggests that chronic binge drinking is an important behavioral substrate in the development of alcohol use disorders (Dawson et al., 2008).

Preclinical studies using rodent models of binge alcohol exposure demonstrate extensive physiological and behavioral changes associated with this pattern of intake (Sparrow et al., 2012; Cox et al., 2013). Though an increase in alcohol preference following voluntary binge drinking has been demonstrated (Strong et al., 2010), until recently this effect appeared restricted to mice who binged during adolescence. In fact, longitudinal studies demonstrating increased risk of vulnerability to alcohol use disorders following binge exposure are fairly limited to investigations in adolescent or young adult populations (McCarty et al., 2004). However, two recent preclinical studies support a later increase in alcohol intake or preference following binge drinking among adults. The first demonstrates the increase after extensive binge drinking-as males and females in the experiment showed handling induced convulsions during wtihdrawal from the 6 week long access protocol (Hwa et al., 2011). The second experiment supports an increase in alcohol preference following a shorter paradigm, but only for male mice (Cox et al., 2013). Therefore, we have little evidence supporting whether moderate to heavy binge alcohol consumption (i.e. drinking that does not induce physical withdrawal symptoms that would require benzodiazapine or similar treatment in a hospital setting) during adulthood can result in increased preference or consumption of the drug when bingeing has ceased. Furthermore, we have no evidence detailing this effect in females (Ward et al., 2009). This particular gap in the research is particularly important, as binge drinking behavior among women and girls has increased dramatically in recent years (Keyes et al., 2008).

In the United States, a recent, now heavily circulated, report from the CDC highlights binge drinking among women and girls as an under-recognized problem

(CDC, Vital Signs, 2012). Specifically, the Center found that almost 14 million women age-18 through 34 binge drink an average of 6 drinks per occasion, 3 times a month (CDC, 2012). In the United Kingdom, binge drinking rates among young women have doubled in the past decade alone, whereas rates among young men have dropped (Smith and Foxcroft, 2009). The prevalence of this risky pattern of alcohol intake among women should be of concern, given clinical reports suggesting that women display a telescoped development of addiction following initiation of drug use.

A long history of cross-sectional and longitudinal studies show that women become addicted to cocaine (Hernandez-Avila et al., 2004), opioids (Hser et al., 1987; but see Hlscher et al., 2009) and heroin (Anglin et al., 1987) following a shorter history of use than men. One of the earliest comparison studies of male and female narcotic addicts found that almost 60

For alcohol, a number of reports have suggested that women transition from recreational use to abuse and dependence more quickly than men (Randall et al., 1999; Hernandez-Avila et al., 2004; Diehl et al., 2007; Schuckit et al., 1998). In particular, these studies showed that although women initiated alcohol use later in life than men (York and Welte, 1994, Chou and Dawson, 1994), they entered treatment facilities at similar ages (Bucholz et al., 1992; Ross et al., 1988). Recent analyses, however, challenge the existence of this telescoping phenomenon (Mann et al., 2005; Keyes et al., 2010). In particular, these researchers fail to find evidence supporting the telescoped development of alcohol use problems for heavy drinking women in the general population, and suggest this phenomenon may be restricted to treatment seeking alcoholics. Furthermore, as gender-driven differences in experiences may have shortened the time between initiation of use and entry into treatment for women (e.g.; drinking during pregnancy may precipitate treatment seeking for younger women), it is possible that telescoping is an artifact of gender-defined social roles and not related to biological sex.

Animal models of alcohol consumption generally support sex differences in overall intake, as well as in performance across various phases of alcohol self-administration.

In both limited access and continuous home cage drinking procedures, female rats and mice have been noted to consume more ethanol in g/kg than male conspecifics whether they be from outbred strains (Lancaster et al., 1996), inbred strains (Li and Lumeng, 1984; Middaugh et al.,1999) or selected lines (Sluyter et al., 2000). However, few of these studies endorse sex differences in the pattern of intake overtime, and thus are unable to support or counter the telescoping phenomenon.

In the first chapter of this dissertation I found that female C57Bl/6J (B6) mice, an inbred strain known for its propensity to binge drink (Rhodes et al., 2007), escalate their binge drinking behavior at a faster rate than males. Additionally, I demonstrated that this sex difference only appears during adulthood and that adult females binge drinking in this paradigm show unique enhanced stimulation following an ethanol challenge after one month of abstinence. These data appear to suggest that adult females from this inbred strain may be particularly vulnerable to the effects of binge alcohol consumption. Therefore, the goals of the present project were to determine whether sex moderates the effect of binge drinking on free choice drinking behavior. Furthermore, I wanted to demonstrate whether binge drinking experience would in fact alter alcohol preference either immediately or following abstinence. Adult male and female B6 mice were given binge exposure to a 20% alcohol solution using the DIDMSA protocol in Phase I. In Phase II, they were shifted to a two bottle choice protocol (24hr access to water or 20%v/v unsweetened ethanol) that was initiated during early or late abstinence. I hypothesized that binge drinking (limited-access during Phase I) would alter free-choice alcohol consumption and preference in a sex specific manner, with females requiring fewer binge exposures to augment their normal free-choice drinking phenotype. Furthermore, as females in our previous study displayed effects of binge drinking after one month of abstinence, I hypothesized that abstinence from binge drinking will not dampen the effects of this pattern of alcohol intake on later preference for the compound for females.

2.2 Methods

2.2.1 Subjects

Male and female C57BL/6J (B6) adult (PD 77± 3) were purchased from Jackson Laboratory (N= 120 mice; 9-11 per group). Animals were singly housed in standard shoebox cages and were habituated to the facility for seven days. Mice were maintained across two holding rooms, each kept at 21±1 degrees Celsius and approximately 50% humidity. Food and water were available ad libitum, except during alcohol access periods. All procedures were approved by the Indiana University-Purdue University Indianapolis School of Science Institutional Animal Care and Use Committee and were consistent with the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

2.2.2 Drinking Solution

For drinking, 95% Ethanol (Ethanol; Pharmco Products Inc., Brookfield, CT) was diluted with tap water to a 20% v/v solution. Animals in the water condition had access to tap water.

2.2.3 PHASE I: Drinking in the dark-multiple scheduled access (DID-MSA) protocol

The drinking protocol was adapted from Bell and colleagues (2011) and is detailed in Chapter 1.

2.2.4 PHASE II: 24-hour two bottle choice preference protocol

Mice were given access to alcohol (20% v/v) and water in modified drinking tubes for 20 days (with ball bearings to prevent leakage). On day 19, drinking was assessed

every two hours in order to predict when, on day 20, bloods should be taken. Therefore, preference ratio and daily intakes are only shown for days 1-18 (when mice were not frequently disturbed). For experiment 1, this preference protocol (PHASE II) was initiated the day following their last binge session(PHASE I). For experiment 2, this preference protocol was separated by the binge protocol by a two-week abstinence period. Retroorbital sinus bloods were taken on the final day of access, 2 hours into the dark cycle (based on assessment of circadian pattern of drinking the day prior to determine peak bout). Leak cages were maintained and an average spill/evaporation value of fluid was subtracted from all raw drinking data. Preference ratio is the amount (mL) of alcohol consumed divided by the total fluid consumed on that day.

2.2.5 Statistical Analyses

DID-MSA ethanol consumption was analyzed using a two-way mixed factor ANOVA, with sex (males vs. females), and day (day 1 through 14; within-subjects variable) as the variables of interest. Based on data in Chapter 1, an a priori decision was made to assess whether mice showed an escalation of ethanol consumption by comparing the average daily ethanol intake during the first and second weeks of access using a two-way mixed factor ANOVA (age*sex*week). Drinking in phase 2 was analyzed using a Repeated Measures ANOVA with day (1-18) and group (3 day binger, 7 day binger, 14 day binger) compared for each sex separately. Average preference ratio was calculated for the 18 days of two bottle choice access and compared using a one way ANOVA where group included 4 levels: water, 3 day bingers, 7 day bingers, 14 day bingers.

2.3 Results

2.3.1 PHASE I: Sex differences in binge ethanol consumption during the drinking in the dark-multiple scheduled access (DID-MSA) alcohol pre-exposure

Daily Ethanol and Water intake

The total amount of ethanol consumed across the three 1-hour time bins can be seen for males and females in Fig. 9A. Data were analyzed for the 14 day group only, so that a complete repeated measures ANOVA could be utilized. The variables of interest in the analyses were sex (2) and day (14). These data violated the assumption of sphericity; therefore, the Greenhouse-Geisser correction was used when assessing the significance of the F statistic. As such, the degrees of freedom reported reflect this correction. All mice showed significant changes in their pattern of drinking across the 14 days of access (p's <0.05). There was a significant interaction of day and sex [$F(8,285)=2.052$, $p<0.05$]. Bonferroni adjusted pairwise comparisons clarify that males only show increased drinking on days 5, 7 and 7 of access (p's<0.001 as compared to day 1). Females show significantly increased drinking that is maintained from days 4 through 14 (p's<0.001 as compared to day 1). The total amount of water consume across the three 1-hour time bins is displayed in Fig. 10. Data were analyzed as described for ethanol, above. These data also violated the assumption of sphericity (Greenhouse-Geisser correction used to assess significance of F statistic). There was a significant effect of day [$F(6,177)=4.368$, $p<0.0001$]. Pairwise comparisons revealed isolated days where mice consumed more water than days prior, but this occurred in no particular pattern (e.g. mice consumed significantly less water on the second day of access when compared to days 5, 7, 9 and 13). There was no significant interaction of sex and day, nor was there a significant main effect of sex.

Average weekly ethanol intake

Ethanol drinking data were also analyzed by comparing the average intake from the first week, to that from the second week, using a mixed-2-way ANOVA (sex * week). This analysis revealed a significant effect of week [$F(1,38)=9.672$, $p<0.01$], as all mice consumed more ethanol during their 2nd week of access than their 1st (Fig.9B). There was also a significant week* sex interaction [$F(1,38)= 3.587$, $p<0.05$]. Pairwise comparison clarified that, unlike males, females significantly increased their ethanol intake in the second week of access, as compared to the first ($p<0.001$). Additionally, we find that, for this experiment, females consumed significantly greater ethanol than males during both the first($p<0.01$) and second ($p<0.0001$) weeks of access.

2.3.2 PHASE II: 24-hour, 2-bottle choice preference drinking

Experiment 1: Sex differences in the effect of binge drinking on ethanol preference and intake in a 24-hour, 2 bottle choice paradigm

Intake

Total daily ethanol consumed in g/kg was analyzed across the 18 days of access in separate repeated measures ANOVAs for males and females (Fig. 11A and 11B).There was a significant main effect of day for both males [$F(10, 583)=8.102$, $p<0.0001$)] and females [$F(9, 492)=4.279$, $p<0.0001$)]. Bonferroni adjusted pairwise comparison suggest that mice had a general increase in intake over time.There was no significant interaction of day with group, however. There was a significant effect of group on the intake values for both males [$F(3, 56) = 8.114$, $p<0.0001$)] and females [$F(3, 56)=13.372$, $p<0.0001$)]. Pairwise comparisons support a significant increase in ethanol intake following 14 days of binge drinking for males ($p<0.0001$) and females ($p<0.0001$). On the other hand, 7 days of binge drinking only increased ethanol intake for females($p<0.001$). Interestingly, a less stringent analysis of the data (using

2-tailed Dunnet's t-tests against baseline intake separately for each sex) suggests that males displayed significantly increased drinking following 7 and 14 days of drinking (p's <0.05) and marginally increased intake following just 3 days of binge drinking (p=0.05).

We also assessed the relationship between the total ethanol consumed during this ad libitum period and the amount of alcohol consumed during the binge paradigm for males and females(Fig. 13A-C). Although 3 days of binge drinking did not significantly alter alcohol intake in the 24-hr phase, the amount of alcohol binged during this time period significantly predicted and amount of 2-bottle ethanol intake for females (R^2= 0.56; p<0.05). This was not the case for females in the 7 day or 14 day drinking groups. Instead, males showed a significant predictive relationship between the amount they consumed during the binge sessions and the amount they consumed when switched to continuous access after 7 (R^2=0.67; p<0.01) and 14 (R^2=0.45; p<0.05) days of drinking.

Finally, we assessed the total fluid intake for these mice(Fig. 12). For males, there was a significant interaction of day and group on total fluid consumed. However, Bonferroni adjusted pairwise comparisons revealed non-systematic differences in the total fluid consumed by the control mice and 3, 7 and 14 day bingers apparent on three days: 5, 11 and 16. On day 5, 3 day bingers were consuming significantly less fluid than 7 day bingers (p¡0.05), on day 11 the 7 day bingers are consuming significantly more fluid than all other groups (ps¡0.05) and on day 16 these same mice are consuming significantly more fluid than water exposed mice (p¡0.05). Females did not show an interaction of day and group.

Preference Ratio

The average preference for ethanol consumed over the 18 days of 24 hour access can be seen in Fig. 14A and 12B for males and females, respectively. Separate one way ANOVAs of ethanol preference ratio were run for each sex with group (water,

3 day bingers, 7 day bingers or 14 day bingers) as the independent variable. There was a significant effect of group for both males [$F(3, 56)=4.862$, $p<0.01$)] and females [$F(3, 56)=10.668$, $p<0.0001$)]. Dunnet's post hoc analysis reveal that males display an increase in ethanol preference following 14 days of binge drinking ($p<0.01$), whereas females show an increase following 7 ($p<0.01$) and 14 ($p<0.0001$) days of binge drinking. T tests were used to determine how each group's binge drinking experience altered the quality of the ethanol choice behavior by comparing their ratio to 0.5 (i.e. no preference). Control males demonstrated an avoidance to this 20% ethanol solution, consuming 42% of their fluid from ethanol [$t(29)=-3.592$, $p<0.001$]. Males in the 3 day [$t(9)=0.463$, p=n.s.] and 7 day [$t(8)=0.293$, p=n.s.] binge history group no longer show this aversion (preference ratio non-significantly different from 0.5). Furthermore, those in the 14 day binge drinking group showed a marginal preference for 20% v/v unsweetened ethanol [$t(10)=2.146$, $p=0.057$]. Control females did not demonstrate an avoidance to this concentration of ethanol, choosing to consume 54% of their daily fluid from the tube containing ethanol. Female mice develop a significant preference for this concentration of ethanol after 7 days [$t(10)=4.476$, $p=0.001$] and 14 days [$t(10)=6.682$, $p=0.0001$] of binge drinking.

Blood Ethanol Concentration

Due to human error, some blood samples were lost. Therefore, data below are on reduced sample sizes (n=6-8 for binge drinking exposure groups and 20-23 for water control groups for each sex). Blood ethanol concentration from the first drinking bout (first two hours of access) on the final day of two-bottle choice is shown in Fig. 15. Males did not demonstrate an effect of exposure group on blood ethanol content: BECs across all exposure groups (3 day bingers, 7 day bingers, 14 day bingers and water controls). For females, there was a significant effect of group on BEC [$F(3,34)=3.3$, $p<0.05$]. Bonferroni adjusted pairwise comparison revealed that

only 7 day bingers achieved a BEC that was significantly higher than water controls during this first drinking bout of the day ($p<0.05$).

2.3.3 Experiment 2: Sex differences in the effect of binge drinking on ethanol preference and intake in a 24-hour, 2 bottle choice paradigm following abstinence

Intake After Abstinence

Ethanol intakes (g/kg) were analyzed across the 18 days of access in separate repeated measures ANOVAs for males and females following abstinence (Fig. 16A and 16B).There was a significant effect of day for males [$F(9, 345)=4.149$, $p<0.0001$)] and females [$F(9, 345)=4.149$, $p<0.0001$)]. Bonferroni adjusted pairwise comparison show no systematic changes in drinking for either sex. Additionally, there was a significant group*day interaction for males [$F(19, 345)=2.113$, $p<0.0001$)] and females [$F(9, 345)=3.275$, $p<0.0001$)]. Pairwise comparisons reveal that, after abstinence, males who binge drank for 14 days do have a tendency to consume more ethanol than controls (e.g., they consumed significantly more than controls on days 11, 12 and 17 of access; p's <0.05).

Preference Ratio After Abstinence

The average preference for ethanol consumed over the 18 days of 24 hour access can be seen in Fig. 17A and 17B for males and females respectively. A one way ANOVA revealed no effect of binge exposure on alcohol preference, as all animals recovered their usual aversion (males) or indifference (females) for this concentration of ethanol after 14 days of abstinence from binge drinking.

2.4 Discussion

The present series of experiments have yielded four important findings. First, we replicate evidence supporting sex differences in the escalation of binge drinking behavior, using the Drinking in the Dark-Multiple Scheduled Access (DIDMSA) paradigm (Melón et al., 2013; Chapter 1). Additionally, these data support recent findings (Cox et al., 2013) that suggest binge drinking may augment later alcohol consumption and preference. We also demonstrate that binge drinking augments ethanol preference and intake of the compound in a sex specific manner. Last, we establish that abstinence can reverse this deleterious effect of binge drinking for both males and females.

2.4.1 Sex difference in the escalation of binge drinking behavior

Animal models of alcohol consumption generally show greater intake of the compound among female rodents. Sex differences in the escalation of alcohol self administration behaviors, however, have rarely been demonstrated. Using the DID-MSA procedure, we have previously shown just this: female mice from the high alcohol accepting C57Bl/6J (B6) inbred strain initiated binge drinking in the paradigm at the same level as males, but escalated their binge drinking behavior across the two weeks of access. In contrast, intake for males remained stable over this time. In the present experiment, B6 females consumed significantly more ethanol than males at the onset of access to alcohol. Although this was unexpected, it highlights the variable nature of this phenotype, even among an inbred strain. We must assume that environmental disparities, either alone or in concert with epigenetic changes across cohorts, resulted in differences in the propensity to binge drinking during the first week. Indeed, females in the present set of experiments drank almost 2 g/kg more ethanol at the start of the study than females in our previous investigation. Interestingly, drinking for males remained stable across experiments.

Still, we find -in agreement with our previous investigation- that females escalate their binge drinking behavior by the second week of this protocol, as binge drinking

levels for males remain stable across the two weeks. There are a number of alternative possibilities underlying this apparent sex difference. First, a recent investigation from our laboratory using male B6 mice in the Drinking in the Dark procedure shows that, with subsequent drinking sessions, mice increase the amount of ethanol they consume in the first few minutes of access along with the rate at which they consume ethanol in these first bouts (Linsenbardt and Boehm, 2013). Given the important role that habituation to the novel ball-bearing sipper tube and flavored fluid may have on the changing rate of daily intake and noted sex differences in habituation to novelty, it is possible that the slope of this relationship (ethanol intake across time in the first 15 minutes of daily binge access) could be steeper for females.

Another alternative possibility underlying the sex difference concerns the important role that circadian rhythms play in drinking patterns in general (Matson and Grahame, 2011), and in DIDMSA in particular. A major difference between this limited access protocol and others we have used in the lab (namely, Drinking in the Dark), is the initiation of alcohol access at the onset of the dark cycle. That the mice consume as much as they do in the DIDMSA protocol is surprising, given the usual circadian pattern of their consummatory behavior (Matson and Grahame, 2011). However, it is possible that, across days, ethanol drinking animals adjust their activity patterns in order to correspond with access to this new calorie rich food. As noted in Chapter 1, however, no sex difference in food aniticipatory activity has been demonstrated for this inbred strain. Still, B6 females do display less precision in their daily onset of activity in constant dark conditions, as compared to B6 males (Iwahaha et al., 2008). Low precision in constant dark has been interpreted as an internal clock that is less rigid and more adaptive to external cues (Subbaraj and Chandrashekaran, 1977). In all, the sex difference in the escalation of intake in this paradigm supports the possibility that female B6 mice may demonstrate a telescoped development of alcohol use problems following binge drinking and this is further probed in the subsequent experiment discussed below.

2.4.2 Binge drinking stimulates later alcohol intake and augments preference

Binge drinking is a dangerous pattern of intake characterized by heavy alcohol exposure, punctuated by periods of abstinence. This cycling or intermittent scheduling of reward access has been shown to be an important behavioral substrate for the development of addiction (Avena, Rada and Hoebel, 2008). Over 4 decades of preclinical investigations of alcohol use support enhanced consumption following various intermittent protocols (Sinclair and Senter, 1968; Wise, 1973; Tomie et al., 2003; Simms et al., 2008). For the most part, this enhanced consumption has been shown following intermittent exposure to high doses of ethanol using vapor procedures(Becker, 1998). This ethanol vapor protocol reliably induces dependence-like behavior (Anton and Becker, 1995). Therefore, exposure to such high doses of ethanol succeeded by periods of withdrawal it thought to induce a kindling of withdrawal symptoms that affects alcohol seeking behaviors (Becker, 1998). This has recently been supported in a new model of intermittent voluntary ethanol consumption that induces severe physical withdrawal in B6 mice (as evidenced, suprisingly, by the expression of handling induced convulsions in this relatively seizure-insensitive inbred strain) to find an associated increase in ethanol preference (Hwa et al., 2011). However, whether binge drinking can alter later preference for ethanol in the early stages of alcohol use (i.e. In pre-dependent or non-dependent populations) has rarely been demonstrated. Of course, protocols that take advantage of pavlovian conditioning (i.e. where the intermittent presentation of alcohol using retractable sippers for example results in greater ethanol consumption than continuous access using fixed-position sippers; Tomie et al, 2006) have shown increased ethanol preference following limited access drinking in the operant chambers (Tomie et al., 2004). Still, these procedures do not necessarily induce binge drinking (although some of the rats do drink to over 80mg/dL; Tomie et al., 2002) and are dependent on an operant procedure that may involve or promote unique reinforcement mechanisms.

Recently, Cox and colleagues (2013) showed that binge drinking in the classic DID protocol (where daily limited access occurs in 4-day cycles separated by two days of no alcohol access) may induce increased preference for ethanol following just 3 cycles of binge drinking (12 days of access, overall). However, it should be noted that control mice in this experiment are consuming surprisingly low levels of ethanol in the 24-hour 2 bottle choice paradigm (>10g/kg at 10%). Strong et al., (2013) used the scheduled-high alcohol consumption procedure (SHAC; mild food restriction followed by fluid restriction and 30minute access to 5% ethanol every 3rd day; reliably induces binge-like drinking, even in heterogenous outbred strains; Finn et al., 2005) to induce binge drinking for adult and adolescent males and females and found that 7 sessions of binge drinking only induced increased preference for ethanol for adolescent females. Our findings support those summarized above (Cox et al., 2013) as adult mice show increased preference for ethanol and increased consumption of the compound when switched from limited (binge drinking; 14 days) to ad libitum access to the compound. These data, taken together, suggest that evidence for dysregulated intake of alcohol may be seen early on following binge drinking (before an animal is dependent).

2.4.3 Sex differences in effect of binge drinking on alcohol preference and intake

In both the first and present chapters, we demonstrated a sex difference in the escalation of binge drinking in the DIDMSA paradigm. With this, we expected that sex would moderate the effect that binge history has on ethanol preference and intake when animals are switched to a continous access procedure. Indeed, females in our study showed significant increases in ethanol intake and preference following just 7 days of binge drinking. However, although not necessarily supported by the original statistical analysis, it is clear (and supported by a less stringent assessment of the data) that males show some alteration in 24-hr ethanol intake following shorter schedules of binge drinking. In fact, this analysis suggests that males may be more

sensitive to the effects of binge drinking on future ethanol intake, as even 3 days of binge drinking marginally altered 24hr-ethanol intakes. Although females show a clear preference for 20% ethanol following binge drinking for 7 and 14 days and males only demonstrate a binge-history associated preference for this concentration of ethanol following 14 days of intake, males show a baseline aversion for this concentration (whereas females show indifference). Thus, it is might be expected that males would require either higher dose or longer experience to alter this aversion than females would need to augment their indifference. Relatedly, the fact that males shift from aversion to indifference after only 3 days of binge drinking might also conflict with the idea that females demonstrate a telescoped development of deregulated alcohol use. It may be that females show greater deregulated use, as female bingers in this experiment consumed an impressive amount of alcohol when switched to the ad libitum, 2-bottle choice protocol.

Following abstinence, neither males nor females maintain enhanced 2-bottle consumption. These data also conflict with prior evidence supporting an alcohol deprivation effect in B6 mice after forced abstinence from alcohol (Tambour, et al., 2008; Melendez, 2006) These data were also surprising, given the findings from Chapter 1. In that chapter, we showed evidence supporting a long lasting (after 1 month of abstinence) effect of binge drinking on response to a challenge dose of ethanol, with all B6 mice showing reduced ataxia to this challenge dose (1.75g/kg) and adult female bingers showing a locomotor response to this dose that was different than adult female water drinking mice). Results from Chapter 2, however, show that the effect of binge drinking on ethanol intake and 2-bottle choice behavior degrades following just two weeks of abstinence. Taken together, these data seem to suggest a disconnect in the expression of preference for ethanol and changes in sensitivity to the drugs motor-related effects noted following binge drinking. It is possible that the change in preference reflects familiarity with the taste/other chemosensory elements of ethanol for the bingers and that this effect. In particular, the preference ratio in these experiments is based upon the animals consumption of ethanol relative to their total fluid

intake; where tap water is the alternatively available fluid. There are a number of features of ethanol, aside from its pharmacological properties, that may make water an inappropriate alternative reinforcer in certain situations, like its caloric value, and taste. However, water is still a primary reinforcer: there are many concentrations of ethanol at which an animal will voluntarily consume more water than ethanol or make more responses on the water-paired lever, whether the animal is or is not water/food deprived (Meisch et al., 1975; Myers and Carey, 1961). However, it may be argued that water is not an appropriate reinforcer for the preference phase because, although all of the mice had experience with water, only the ethanol mice had prior experience with the ethanol made available during this choice phase. In this case, a better alternative reinforcer may have been a novel tastant, as the preference for ethanol vs water in phase 2 may be related to familiarity to the chemosensory properties of ethanol and not necessarily to the pharmacological effects.

Lastly, the differences in the effects of binge drinking on preference and intake early vs late during abstinence may reflect fundamental differences in the "early abstinence" group as compared to the "late abstinence" mice. In the allosteric model of addiction, relapse is thought to be precipitated by a desire to reduce the physiological and psychological disturbance caused by withdrawal from a compound. Put another way, the neuroadaptations that follow intermittent binge/intoxication phases can disrupt homeostasis such that the compound is necessary for the user to feel normal. If such adaptations have occurred during our binge procedure, these animals are given the opportunity to re-initiate drug use every day, at the same time of day. When they are switched to a free choice paradigm, those that get access to ethanol/water the day after their last binge session experience no reduction in their alcohol access schedule (and instead, now have continuous access to the drug). For the mice that go through the two week abstinence, we would expect significant withdrawal (either physical, or psychological; i.e. frustration) to occur from revoking access for this amount of time. One would expect this difference (no abstinence vs abstinence) to result in a larger effect of binge exposure on later preference for the group that experienced

this distress. Indeed, deprivation usually enhances goal-directed behavior and this position is often used to explain the increase in consumption noted after a period of forced abstinence. In our case, the abstinence period actually degraded the effect of binge drinking on free-choice ethanol preference and intake, however. Still, the abstinence and no-abstinence mice represent fundamentally different groups on many levels. Other differences between these groups may be explored in the future to help undertsand abstinence and its relationship to relapse resistence.

2.5 Conclusion

In essense, these experiments suggest that important changes in alcohol preference and regulation of ad libitum intake occur early on in a bingers alcohol use career. Additionally, while we do not specifically support telescoping (i.e. females do not definitively show deregulated intake and preference earlier in their binge drinking history), we do show evidence for important sex differences in the effect of binge drinking.

3. CHAPTER 3: SEX AND ESTROUS SPECIFIC EXPRESSION OF NEGATIVE AFFECT, ANXIETY AND $GABA_A$R SUBUNIT REGULATION DURING ABSTINENCE FROM BINGE DRINKING

3.1 Introduction

The last decade has seen a 29% increase in the frequency of binge drinking episodes in the United States (Brewer and Swahn, 2005). This pattern of consumption has clear and identifiable negative consequences for the binger and for society. For example, binge drinking is associated with an increase in hazardous driving behavior and traffic accidents, with one study citing binge drinkers as being involved in over 14,000 vehicular fatalities in one year alone(Brewer and Swahn, 2005). In addition to the negative consequences associated with acute binge intoxication, frequently occurring binge drinking episodes predicts alcohol-related problems (Wechsler et al., 2000; Dawson, Li and Grant, 2008). Unfortunately, along with the general increase in the prevalence of this drinking pattern cited above, the frequency of individual binge drinking episodes has increased 35% per person, per year, between 1995 and 2001. What's more, this pattern of intake is not restricted to alcohol dependent populations, as half of all binge drinking episodes are reported among moderate users (Naimi et al., 2003). And though clinical investigations of binge drinking usually focus on the adolescent and young adult period (due to the popularity of this pattern of intake during high school and college), this risky pattern of alcohol consumption is not restricted to this age group. Indeed, a recent telephone survey found that 70% of the over 1 billion yearly binge drinking episodes reported in the United states occurred among adults over the age of 26 (Naimi et al., 2003).

Binge drinking is loosely defined as the periodic consumption of intoxicating doses of alcohol. The National Institute on Alcohol Abuse and Alcoholism operationally defines the pharmacological threshold for this level of intake as consumption that increases an individuals blood alcohol content (BAC) to geq80mg/dL. The cycling of such pharmacoactive doses with periods of abstinence/withdrawal is thought to precipitate and even kindle alcohol-use related problems. In particular, recent studies implicate this specific pattern of drinking in the development of negative affect and anxiety during abstinence(Paljärvi et al., 2009; Townshend and Duka, 2005).

In theoretical models of the general development of addiction, the affective disturbances that develop following binge/intoxication are posited to precipitate further drug seeking. It may be in this way that binge drinking is associated with an increased development of alcohol use disorders. Indeed, early work detailing affective responses occurring during and after heavy alcohol consumption by alcoholic men suggested that the euphoria of acute intoxication was often replaced by depressed mood and psychological tensions (Tamerin and Mendelson, 1969). Of course, a significant comorbid expression of anxiety-, mood- and alcohol use disorders has repeatedly been demonstrated in the literature (Schuckit and Hesselbrok, 1994; Milani et al., 2004; Goldstein and Levitt, 2006). Thus, the expression of symptoms related to depressed mood and/or anxiety among patients diagnosed with alcohol use disorders may be interpreted as vulnerability factors that precipitated heavy drinking in the first place, and remain once intoxication has ceased. Recent investigations, however, are beginning to support the opposite causative relationship.

It is becoming clear that certain patterns of alcohol consumption may induce symptoms of negative mood and anxiety among the general population. Indeed, mood disturbance is an important and recognized symptom experienced during hangover and acute alcohol withdrawal (Howland et al., 2010). What is newly supported is the idea that frequently experiencing these acute symptoms (seen following binge drinking) may result in the development of long term emotional dysregulation for the social binger. For example, a general population study of alcohol drinking in men

and women found baseline binge drinking to be the best predictor of the expression of depressive symptoms 5 years later (Paljärvi et al., 2009). This study also supports the frequency of hangovers as an important moderator of binge-drinking induced depressive symptoms. An earlier study of drinking practices and cognitive and mood disturbances among young social drinkers found a similar relationship (Townsend and Duka, 2005). Specifically, social drinkers who were characterized as bingers (calculated based on self-reported measures of intake over the previous 6 months) had a positive mood score that was significantly lower than non-bingers.

The relationship between binge drinking and mood disturbances should be of particular concern in view of the increasing rates of binge drinking among young women and girls.Women are already twice as likely to have had a diagnosis of unipolar depression and anxiety related disorders than men (Kessler et al., 1993; Kendler et al., 1995; Kessler, 2003; Tolin et al., 1996). This gender difference is exaggerated among those with alcohol use disorders. Indeed, a recent report found alcohol dependence to be associated with a mood disorder for women only (Khan et al., 2013). Furthermore, this relationship extends to general mood (and not just clinical co-morbidities) as female alcoholics are reported to be more likely to drink excessively to alleviate negative affective state (Rubonis et al., 1994 recent e Even among non-dependent alcohol drinkers, gender is suggested to moderate the relationship between the expression of affect-related symptoms and binge drinking. For example, Wu and colleagues (2010) demonstrate that the frequency of binge/heavy drinking was significantly associated with anxiety symptoms for adolescent girls but not adolescent boys. A causal relationship between binge drinking and the expression of negative mood and anxiety suggest that binge drinking-induced affect deregulation may become a growing problem, particularly among women and girls. Unfortunately, few preclinical studies have investigated the long term effects of binge alcohol consumption on the expression of depression and anxiety. Furthermore, although a number of clinical studies support the faster development of alcohol use related problems among women as well as the greater prevalence of mood and anxiety related psychopathologies among female al-

coholics, the specific interactive effects of female biological sex (i.e. combined role of gonadal and chromosomal sex) and alcohol exposure are similarly understudied.

In particular, the growing interest in the extrasynaptic-$GABA_A$R (e- $GABA_A$R) subtype as a neuronal target of binge level alcohol alongside this neurosteroid sensitive subtypes established role in the pathogenesis of menstrual/estrous cycle linked disorders suggest that female mice may undergo unique biological and behavioral changes in response to binge alcohol. Animal models of premenstrual dysphoric disorder suggest that an augmentation of neurosteroid-sensitive e-$GABA_A$R receptors may follow the hormonal shifts associated with the estrous cycle or parturition (Maguire et al., 2005; Maguire and Mody, 2008) and that disrupting these shifts may precipitate the negative affect seen in the disorder. Therefore, the goal of the present experiment is to determine whether binge drinking may induce symptoms of anxiety and depression across abstinence (early or protracted). Furthermore, in order to begin to understand the sex specific mechanisms that may underlie the effects of binge drinking, the effects of drinking in females and males are evaluated. These efforts constintute two separate questions. For males (Experiment 1), our goal is to determine plainly whether binge drinking induces anxiety and depressive like behavior during abstinence and whether these changes are associated with $GABA_A$ subunit plasticity in relevant brain regions. We hypothesize that males will fail to display measurable changes in behavior following the two week drinking protocol. For females (Experiment 2), we would like to determine whether binge drinking induce pmdd-like dysfunction and hypothesize that binge drinking will disrupt the normal behavioral changes that occur across the estrous cycle, arresting the females in a state of depressed mood and heightened anxiety (as seen during PMDD). Further, we believe these behavioral effects will be mirrored by disruption of estrous-dependent transcription of extrasynaptic $GABA_A$ subunits during early abstinence.

3.2 Methods

3.2.1 Subjects

Male and female C57BL/6J (B6) adult (PD 77± 3) were purchased from Jackson Laboratory (N= 270 mice). Animals were singly housed in standard shoebox cages and were habituated to the facility for seven days. Mice were maintained across two holding rooms, each kept at 21±1 degrees Celsius and approximately 50% humidity. Food and water were available ad libitum, except during alcohol access periods. All procedures were approved by the Indiana University-Purdue University Indianapolis School of Science Institutional Animal Care and Use Committee and were consistent with the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

3.2.2 Drinking Solution

For drinking, 95% Ethanol (Ethanol; Pharmco Products Inc., Brookfield, CT) was diluted with tap water to a 20% v/v solution. Animals in the water condition had access to tap water.

3.2.3 Drinking in the dark-multiple scheduled access (DID-MSA) protocol

The drinking protocol was adapted from Bell and colleagues (2011) and is detailed in Chapter 1. All male and female mice had exposure to ethanol or water for 14 days using this procedure.

3.2.4 Experiment 1: Effect of binge drinking on behavioral and genetic regulation in C57BL/6J males

After B6 mice had access to alcohol for 14 days following the DIDMSA protocol outlined above, they were tested following 24 hours (early) or 14 days (protracted) of abstinence.

For all behavioral tests, mice were tested during scotophase (at earliest, ZT14). Animals were shuttled in groups of 6-8 in a light-shielded cabinet from their colony room to the testing room for at least 60 minutes of habituation prior to testing.

Porsolts Forced Swim Test (depression)

Mice were placed into a 2000mL glass beaker containing 1300mL of water (23-25C) for 6 minutes. A beaker with a 10cm diameter was used, as this has been shown to decrease the role that group differences in locomotor activity may play as a confounding variable in this test. Immobility is defined as a lack of movement, other than those necessary to keep the head above water. A video camera positioned directly overhead was used to record each session. Immobility usually arises 2 minutes into the FST, therefore only the final 4 minutes of the test was be coded for duration of immobility (seconds). Latency to the first bout of immobility (2 consecutive seconds of floating behavior) was also recorded. Separate groups of mice were used in to measure depression during early (ethanol n=8 , water n=9), and protracted abstinence (ethanol n=11 , water n=9).

Elevated-Plus Maze (anxiety)

A mouse was placed in the center of the maze, with its nose facing the open arms. Behavior was recorded for 5 minutes by a tripod mounted camera for later assessment. Time spent in the open and closed arms of the maze, as well as the center starting square, was assessed. Separate groups of mice were used in to measure depression

during early (ethanol n=8 , water n=8), and protracted abstinence (ethanol n=8, water n=8).

Open Field (anxiety)

The same mice used in the EPM above were used to assess activity and anxiety using the OF. Immediately following EPM testing, mice were placed into a locomotor activity monitoring system, housing a basic, open field. These locomotor monitors are an automated system made of Plexiglass chambers (40 x 40 cm) equipped with 8 pairs of photo-cell beams located 2 cm above the chamber floor (Accuscan Instruments, Columbus, OH). Each individual monitoring system was housed in a sound attenuating chamber (53 x 58 x 43 cm) and furnished with a house light (remained off) and a fan (mounted on the rear wall) for ventilation. The animals remained in these chambers for 15 minutes. Following this test, mice were returned to the homecage and shuttled back to their colony room to await testing in the social approach test 14 days later.

After 14 days of binge drinking, these mice remained in their homecage without alcohol access for 24 hours before their brains were harvested. Mice were moved to a different room for tissue harvesting. All mice were weighed and handled and females received a vaginal smear immediately prior to sacrifice. Brains were harvested within 3 minutes of cervical dislocation and snap frozen in a dry-ice and isopentane slurry. Brains were stored in a 80C freezer for later laser microdissection of the prefrontal cortex(PFC), basolateral amygdala (BLA), hippocampus(HPC) and posterior ventral tegmental area (pVTA).

Laser microdissection

Brains were removed from storage and allowed to thaw to 16C in the cryostat. Tissue was sliced at 10uM and placed on certified RNAse and DNAse free uv-treated polyethelene napthalate slides. Tissue on the slides were stained using a cresyl vi-

olet staining protocol modified to maintain rna integrity. After staining, the slides were immediately placed in a slide box on dry ice until laser micro dissection. For microdissection, the slide was thawed and placed under the viewfinder, using the 10x optical magnification. Leica microdissection system (LMD 6500) was used to excise the tissue which was immediately stored in a pcr tube containing RLT-buffer and beta-mercaptoethanol (Qiagen, Valencia, CA) for downstream isolation of RNA. RNA isolation and cDNA synthesis

Total RNA was isolated using the Qiagen Rneasy mini kit (Qiagen, Valencia, CA). Additional DNAse treatment was incorporated to eliminate DNA contamination. RNA integrity and quantity was determined using the Experian RNA High-sensitivity Analysis kit (Bio-rad). This analysis drastically reduced the number of samples available for the pVTA and BLA. Therefore, downstream steps were only completed on the HPC and PFC tissue. Complimentary DNA was synthesized using iScript cDNA synthesis according to manufacturers protocol.

Although this procedure allowed us to isolate small structures of the mouse brain with confidence that the tissue remained within the neuroanatomical boundaries for said structure, and though we harvested tissue from each slide in under 30minutes, RNA degradation is impossible to halt at room temperature. While some laboratories proceed with isolation of RNA, generation of cDNA and ultimate analysis of expression changes with no assessment of the quality of the RNA isolated (instead stopping at the quantification of their RNA concentration using a spectrophotometer/nanodrop), it is becoming common practice to assess RNA integrity before proceeding with any analysis or generating cDNA. It is here that many samples may be thrown out (mainly due to significant evidence for degradation or DNA contamination of the sample).

qRT-PCR

Reverse transcriptase real time PCR was performed to quantify mRNA levels of $GABA_A$- δ and $GABA_A$- γ in order to assess transcriptional regulation of these GABA(A) receptor subunits across the estrous cycle and following binge drinking. Biorad CFx96 detector and C1000 thermal cycler were used to perform qRT-PCR using SYBR-Green RT-PCR mastermix. GAPDH was used as housekeeping gene. For experimental animals, δ and γ mRNA expression following binge drinking was assessed for each group separately. Raw Ct values were transformed using the comparative Ct method.

3.2.5 Experiment 2: Binge drinking and effect on PMDD-like dysfunction in C57BL/6J females

All procedures described above were used to test the females. In addition, estrous cycle was tracked across the experiment and on test day as described below. Independent groups of mice were used to measure depression during early (Diestrus: ethanol n=6 , water n=8; Estrus: ethanol=8, water n=7), and protracted abstinence (Diestrus: ethanol n=8 , water n=8; Estrus: ethanol=8, water n=8). For anxiety-like behavior, separate groups of mice were used for the early (Diestrus: ethanol n=8 , water n=8; Estrus: ethanol=7, water n=8, and protracted abstinence (Diestrus: ethanol n=8 , water n=8; Estrus: ethanol=8, water n=8) tests.

Estrous Status

Following one weeks habituation to the facility, female mice were administered daily vaginal smears, for 10 days, in order to ensure the use of regularly cycling females in the subsequent experiments. Each mouse was restrained for a maximum of 30seconds in order to introduce 10L of sterile saline into the vaginal opening (Caligioni, 2009). In order to (attempt to) maintain uniformity of experience (to stressors)

across all animals in the studies, males were also restrained daily, for 30seconds. Pictures of the wet smears were taken using a light microscope (Motic, Richmond, British Columbia, Canada) connected to a PC (Hewlett-Packard, Palo Alto, California, USA) and photomicrographs were analyzed by two independent investigators. Animals were considered to be in proestrus if the slide showed a large proportion of nucleated epithelial cells or estrous if the slide showed a large proportion of cornified cells. Diestrus was characterized by the expression of leukocytes and metestrus by a mix of all 3 cell-types (though there is a prevalence of leukocytes).

Vaginal smears were also administered immediately following behavioral testing on final test days. Only data for diestrus and estrus stage females is compared in behavioral tests described below.

3.2.6 Statistical Analyses

For males, we were interested in determining whether binge drinking induced any measurable behavioral and transcriptional changes. Therefore, we used t-tests to compare the effects of binge drinking history on these measures.

For females, we were interested in determining whether estrous cycle-related expression of behaviors were changed by ethanol exposure. Thus, a two-way ANOVA with status (diestrus vs. estrus) and binge history (water vs. ethanol) was used to analyze these data.

3.3 Results

3.3.1 Experiment 1: Effect of binge drinking on behavioral and genetic regulation in C57BL/6J males

Binge drinking

Binge drinking behavior for these males is displayed in Fig. 18. These males did not escalate their binge drinking behavior across the two weeks of the alcohol exposure phase.

Forced Swim Test (Depression)

Behavior in the forced swim test is detailed in Fig. 19 and 20 for early (top panels) and late (bottom) abstinence. An independent t-test was performed to compare differences in the duration of immobility and latency to immobile behavior across the binge exposure groups (ethanol vs water) during early and late abstinence, separately. There was no significant difference of duration of immobility between bingers and water controls during early abstinence, although a trend is apparent during late abstinence, where bingers are immobile for marginally longer than controls (p=0.08). During early withdrawal, males with a history of binge drining took marginally longer to display their first bout of immobility. This trend also dissipates by late abstinence.

Elevated Plus Maze (Anxiety)

There was a significant effect of binge history on the percent of time spent on the open arms of the EPM [t(14)=2.3, p=0.04, Fig. 21]. This effect dissipates after two weeks of abstinence.

Open Field (Anxiety and Activity)

An independent samples t-test showed that males that binged had significantly reduced activity during the early abstinence period [t(18)=3.4, p<0.01, Fig. 22], but this dissipates after extended abstinence. Time spent in the center of the open field, failed to support an increase in anxiety like behavior during early withdrawal. However, during late abstinence, there was a significant effect of binge history on this measure. In particular, males with a history of ethanol consumption spent significantly more time in the center of the maze during protracted abstinence [t(2.2)= 2.2, p<0.05, Fig. 23].

$GABA_A$ δ and γ2 subunit expression following binge drinking

Binge drinking did not alter δ and γ2 mRNA expression for males in the HPC or PFC (Fig. 24).

3.3.2 Experiment 2: Effect of binge drinking on estrous-dependent behavioral and genetic regulation in C57BL/6J females

Binge drinking

Binge drinking behavior for these females is displayed in Fig. 18. Females escalated their binge intake across the two weeks of the binge exposure phase (p¡0.001), as found previously.

Forced Swim Test (Depression)

Behaviors in the forced swim test are detailed in Fig. 25 and 26 for early (top panels) and late (bottom) abstinence. For females, we were interested in the effect that binge drinking has on the usual estrous-dependent expression of immobility in this test, or an interaction between estrous status and binge history. The data were

normally distributed and met the assumption of homogeneity of variances. Therefore, a two-way ANOVA was run.

During early withdrawal, there was no significant effect of solution consumed during the binge preexposure (binge history), nor was there a significant effect of estrous status on either the duration of immobility or the latency to immobility for these mice. Finally, there was no interaction of estrous status and binge history on either of these measures during early abstinence.

After two weeks of abstinence, we find a significant interaction of binge history and estrous status [$F(1,27)=5.48$, $p<0.05$]. Bonferroni adjusted pairwise comparisons clarified two things. First, there was a significant baseline difference in the duration of forced swim across the cycle, as control diestrus females were immobile for significantly less time than estrus females ($p=0.04$). Second, we find that binge alcohol exposure resulted in a significant increase in floating time for females in diestrus, only ($p=0.03$). This increase is also reflected in the ethanol pairwise comparison, where the baseline difference noted for the water drinking mice, no longer exists. For females, there was no effect of estrous on latency to immobility nor was there an interaction of binge history and estrous cycle.

Elevated Plus Maze (Anxiety)

Durng early abstinence, a two-way ANOVA on the effects of binge history and estrous status supported a significant main effect of binge history on the expression of anxiety symptoms [$F(1,27)=4.5$, $p<0.05$, Fig. 27 top]. There was also a marginal ($p=0.056$) effect of estrous on this anxiety-related measure, as estrus females spent

10

Open Field (Anxiety and Activity)

During early abstinence females showed significantly depressed activity during early withdrawal from binge drinking [$F(1,30)=7.7$, $p<0.01$, Fig. 28 top]. However,

there was no significant effect of estrous nor a significant interaction of estrous and binge history. There was also an effect of binge drinking on center time behavior during early abstinence (Fig. 29 top). Specifically, a two-way ANOVA with binge history and estrous revealed a significant effect of binge history [$F(1,30)=7.0$, $p<0.05$], with binge drinking mice displaying less time in the center of the open field. By late abstinence, the activity of binge exposed females in the open field returns to baseline (Fig. 28-29, bottom panels).

$GABA_A$ δ and γ2 subunit expression following binge drinking

Control females (consumed water during phase I) did not show a cycle shift for δ and γ2 expression in the HPC or PFC (Fig. 30 and 31, top panel). Further, binge drinking did not alter expression of δ -$GABA_A$ subunit protein for these females. There was, however, an estrous dependent effect on the expression of binge drinking induced regulation of γ2 subunit in the HPC. Specifically, we found a significant reduction in the expression of γ2 in the HPC following binge drinking in the brains of diestrus females (Fig. 31, bottom panel), as compared to the normal expression of this transcript during this phase [$t(8)=3.7$, $p<0.001$].

3.4 Discussion

This chapter had two goals. The first was to establish whether binge drinking can precipitate negative affect, anxiety and concurrent changes in $GABA_A$ subunit expression during abstinence for C57BL/6J males. The second was to determing whether these behavioral deficits would be noted for females. For the second aim, we needed to account for the normal behavioral and subunit plasticity that occurs across the female estrous cycle, and thus hypothesized that binge drinking would not just cause an overall depressive and or anxiety like effect, but that these changes would reflect dysruption of this cycling of behavior and gene expression. To these ends the combined findings of the experiments presented herein support the expression

of anxiety-like and depressive-like symptoms during abstinence from binge drinking. Additionally, we show some evidence to support an interaction between binge drinking and estrous in the expression of these behaviors. Regarding $GABA_A$ subunit regulation, our hypothesis that binge drinking would disrupt the usual estrous-dependent profile of $GABA_A$ subunit expression in the hippocampus and prefrontal cortex was found untestable, as extraneous (and now confounding) variables associated with the experiment disrupted the basal differences in subunit expression found in experimentally naive mice.

3.4.1 Anxiety and depressive-like behavior during abstinence

The general expression of anxiety like behavior during early abstinence followed by the development of depressive-like behavior during protracted abstinence (as seen for females in this study) is somewhat consistent with previous research. Although our findings generally supports what has been previously demonstrated (Stevenson et al., 2008), we were unable to specifically replicate an effect of drinking on depressive like behavior in males. True, the studies used very different drinking methods, as Stevenson and colleagues demonstrate this affect-dysregulation in B6 males using a 24hr two bottle choice protocol. However, given recent epidemiological data highlighting the important role that binge pattern of consumption has on the development of negative affect (Pljarvi et al., 2008) and the fact that the 24hr preference drinking protocol- which usually results in a lower per session alcohol dose than the procedure used in our experiments -this was surprising. However, Stevenson et al., used a longer access protocol than we did here, leaving the possibility that length of exposure and not just dose is important for the development of depressive-like behavior in males. It is also interesting to note that, although the males in the present experiment exhibited signs of anxiety during early abstinence, expression of this anxiety depended on the behavioral apparatus used to test it; as we saw no change in center time behavior after drinking for any of the animals, but decreased time in the open arms.

In contrast Stevenson et al., showed increased anxiety using center time in the OF as their anxiety index. Furthermore, they found no evidence of withdrawal induced hypolocomotion (as we did). This highlights the possibility that vastly different behavioral mechanisms are vulnerable to adaptation just as a consequence of drinking pattern. The inconsistencies also support the finding that environment interacts with the variables of interest to produce different responses within inbred strains across laboratories (Crabbe, Wahlsten and Dudek, 1999).

3.4.2 Behavioral maladaptations that drive drinking

Although we were not directly comparing the sexes in these studies, the 14 day length of binge pre-exposure was chosen because, as demonstrated in Chapter 2, it significantly augmented preference for both males and females when moved immediately to the two bottle choice protocol but not when two-bottle choice phase was separated by an abstinence period. This design allows us to determine what behavioral maladaptations are phase-locked with the augmented preference, and whether these behaviors decay along with the change in drinking. As such, we find that the anxiety-like behavior associated with early abstinence for both male and female binge drinkers dissipates after two weeks abstinence, much the same as the augmented preference and 2 bottle choice intake. Surprisingly, for females, we found depressive-like behavior expressed during protracted abstinence. This suggests that, even when the problems identifiably associated with binge drinking (i.e. further preference for alcohol), seemingly unrelated issues, like depressed-mood, may persist for some individuals. This should be evaluated further, as it may have important implications for the clinic. One point that needs to be clarified is whether this depressive-like behavior only develops following heavy exposure that approaches dependence.

3.4.3 DID-MSA and alcohol dependence

This drinking protocol was not meant to model dependence. However, whether or not it has induced some level of dependence in these mice is not yet clear and, of course, depends on the operational definition of dependence.

The term dependence generally refers to the expression of physical symptoms following cessation of a psychoactive compound indicative of some level of physiological adaptation. The term was incorporated into the diagnostic manual to refer to compulsive drug taking and to define a feature of substance addiction, as a popular vote believed the word carried less stigma than the word "addiction" (O'Brien, Volkow and Li, 2006). In terms of alcoholism, clinical criteria for diagnosis of dependence suggests that the person endorses 3 of 7 "dependence" criteria (adapted from Office of the Surgeon General, 2007):

1. They drink more than intended or drink for longer than intended.

2. They have had multiple failed attempts to control intake (or a persistent desire to control intake).

3. They spend a significant amount of time using alcohol.

4. They experience negative consequences (physical or psychological) following intake, are aware of this causal relationship between use and their problems, yet continue consumption.

5. They must consume increasing amounts of alcohol to achieve a desired effect (or experience reduction in the effects of the compound at the same dose; i.e. Tolerance)

6. They experience withdrawal symptoms when alcohol consumption has ceased (or they drink to avoid experiencing these symptoms).

Endorsing any 3 of the above 7 criteria distinguishes "alcohol dependence" from alcohol abuse, for which the user would only experience social/ interpersonal/ occupational consequences. Preclinical models of dependence are unable to explore many of the characteristics listed above, as they involve consequences unique to the human experience. Hence, a vast majority of these models focus on physical dependence-

namely indices of tolerance and withdrawal (items 5 and 6 above), as these involve changes that may be measured in lower animals. Furthermore, before the term dependence was incorporated into the DSM IV during the late 80s, alcohol and drug research had already progressed a number of animal models of dependence based upon the pharmacological definition of the term (and not necessarily referring to the 6 criteria listed above). Many of these early models used a liquid diet (where the animal receives up to 40% of its daily caloric requirement in the form of ethanol) and measured the physiological changes that occurred when this liquid diet was removed. As expected, abstinence from 30+ days of such intensive alcohol exposure regimes usually precipitated spontaneous seizures. However, seizure activity were not the only measures used to support dependence in early animal models. In fact, the oft cited Majchrowicz model of physical dependence involves assessing overt signs of seizures, in addition to general hyperreactivity and rigidity. In Waller and colleagues original demonstration that free-choice drinking by the alcohol-preferring P rats induces dependence, the authors demonstrate that the rats consuming alcohol only showed signs of physical disturbance (i.e differences in activity, differences in muscle tone etc; as compared to water drinking controls) following removal of alcohol (during the first 24 hours after cessation of access to alcohol) but not when animals were tested while alcohol access was still available to them. In this way, the authors may conclude that the physical signs seen during the 24hours after ethanol access was terminated reflect an adaptation in the homeostatic set point for the measured behaviors, much the way the allostatic model of addiction suggests that alcoholics are drinking to feel normal.

The behavioral changes noted during abstinence suggests that DID-MSA may be doing more than modeling moderate to heavy binge-drinking. Still, we cannot now say that the level of ethanol exposure seen for the female mice using DID-MSA induces dependence. This can, however, be clarified in future studies where we can either show signs of overt physical symptoms during withdrawal from drinking (such as handling induced convulsions) or show that the animals are performing sub-optimally on some

measure during withdrawal but not when the compound is on board (as per Waller et al., 1982).

3.4.4 Estrous and $GABA_A$R plasticity

In a preliminary study, we found that the relative expression of $GABA_A$R-δ and $\gamma 2$ subunits across the estrous cycle noted for nave animals supports previous work (Maguire et al., 2005; Lovick et al., 2005). However, we could not replicate this in water drinking control mice in the experiments included here. This suggests that the transcription of these particular subunits (-δ, proposed to be an integral part of e-$GABA_A$R and -$\gamma 2$, thought to selectively incorporate in synaptically located $GABA_A$R) across the cycle may be particularly responsive to environmental change. Furthermore, the significant downregulation of $\gamma 2$ subunits in the hippocampus of bingers during diestrus may be thought to support a general interaction of estrous status and binge drinking.

3.5 Conclusion

In essense, these experiments suggest that important changes in alcohol preference and regulation of ad libitum intake occur early on in a bingers alcohol use career. Additionally, while we do not specifically support telescoping (i.e. females do not definitively show deregulated intake and preference earlier in their binge drinking history), we do show evidence for important sex differences in the effect of binge drinking.

4. GENERAL DISCUSSION

The overarching take home message of the data presented herein, supports the growing concern that binge drinking is a dangerous pattern of consumption that may induce long lasting negative consequences. Additionally, across the three chapters we find that this pattern of intake results in sex-specific effects on response to an alcohol challenge, alcohol preference and daily intake, the expression of maladaptive behaviors and gene regulation. Chapter 1 echoes concerns that this pattern of intake is not just dangerous for adolescents and young adults. In fact, both adult and adolescent bingers expressed an attenuated response to the effect of an ethanol challenge on alcohol-induced motor incoordination after extended abstinence. Furthermore, females that binged as adults demonstrated unique differences in the effect of an ethanol challenge on the stimulant response to the compound. Given the diergic escalation of binge consumption in the paradigm introduced and characterized in Chapter 1, the second chapter attempted to tackle the now challenged perspective that females experience atelescoped development of alcohol use disorders. Based on the overwhelming number of preclinical investigations supporting greater vulnerability to the addictive potential of a variety of compounds, like cocaine, nicotine and amphetamines, along with the historic epidemiological data that initiated the telescoping hypothesis, I hypothesized that female B6 mice would alter their usually modest 24hr drinking phenotype and augment their normal preference ratio sooner in their binge drinking histories than males. The results of this chapter do not necessarily support a simple females/males are more vulnerable statement. Instead, as discussed below, each sex expressed a unique relationship between binge history and changes in future alcohol preference and intake whose interpretation -as far as risk is concerned- requires a more nuanced discussion (attempted below). Interestingly, the effects of binge drinking on 24hr intake and preference dissipated after abstinence. This is particularly significant

in light of the results in the final study. Findings in Chapter 3 suggest not only that males and females from this inbred strain express a different pattern of deregulated affect and anxiety following binge drinking, but that these effects (specifically the expression of depressive symptoms during protracted abstinence) do not necessarily coincide with changes that binge drinking may have on alcohol preference and intake in the 24hr 2-bottle choice procedure. Lastly, although it was a guiding hypothesis in the design of these studies, the behavioral and gene expression data only weakly support an influence of binge drinking on the estrous-dependent expression of behavior and $GABA_A$ subunit transcription. In fact, it appears that the stress of the experimental procedures may have had more to do with changing the normal cyclic pattern of behavior and gene regulation than the binge alcohol exposure.

4.1 Binge drinking has transient effects on alcohol use and anxiety-like behavior

Multiple authors suggest that the pattern of alcohol intake, and, in particular, a binge pattern of consumption may be an important predictor of alcohol related problems. Of course, the behavioral endpoints defined as problems in many studies are varied. Certainly, when aspects like cognitive performance or emotional regulation are assessed, binge drinkers usually underperform non-bingers. Unfortunately, many of these studies, even the ones purporting a relationship between binge drinking and alcohol use disorders, are cross sectional in nature and suffer from the usual interpretational shortcomings. From longitudinal studies involving treatment seeking alcoholics it is notable that binge drinking frequency may predict severity of later problems. However, whether binge drinking (or, similarly defined, risky drinking) precipitates later pathological seeking of the compound has only recently begun to be addressed in the general population (Dawson, Li and Grant, 2008). That is not to say binge drinking does not interact with other risk factors to accelerate the development of an alcohol use disorder. Indeed, many of the investigations regarding adult

binge drinkers and their trajectory of alcohol use focus on just this: the interaction of this risky pattern of drinking with another variable, such as a traumatic experience. However, based on our findings, the dearth of clinical studies on this pattern of intake in non-adolescent populations needs to be addressed, especially as its popularity increases.

An interesting and unexpected finding from Chapter 2 was the change in the ethanol preference ratio and 24hr intake noted for males after just 3 days of binge drinking. While easily overlooked, this suggests that B6 males are consuming enough alcohol in this paradigm to precipitate a shift in their normal 24hr alcohol drinking behavior. It is unclear what mechanisms are underlying the shift. It is reasonable to suggest different adaptations would underly a change from aversion (the male baseline ratio for this concentration of ethanol) to indifference than from indifference to preference (as seen for the females). For example, the males may have to overcome a natural aversion to the bitter taste or some other similar sensory quality (burning, smell) to which the females were less sensitive. This may be the adaptation that underlies the shift in choice ratio for the males, as they never express a preference for the 20% v/v ethanol, even after 14 days of binge drinking. Therefore, even with the earlier change in drinking seen for males, females may be thought to be developing a risky pattern of drinking in response to the binge exposure, as 7 and 14 days of binge drinking induces a preference for this concentration of ethanol.

Mice have recently been shown to demonstrate the increase in homecage alcohol drinking found repeatedly with rats following forced deprivation (Melendez, Middaugh and Kalivas, 2006; Tambour, Brown and Crabbe, 2008). Thus, it is surprising that, in Chapter 3, we find the significant effect that binge drinking has on 24hr drinking when switched directly from DIDMSA to that paradigm decays completely when the transfer from binge protocol to 24hr protocol is separated by 2 weeks of abstinence. This recovery of normal drinking behavior after forced abstinence does, however, support findings in humans. A recent study of the effect of forced abstinence associated with Army training on binge drinking rates found that the 27% frequent

heavy episodic drinking rate (binge drinking more than once per week) fell to 9% after basic training (Bray et al., 2010).

In addition to recovery of baseline prefrence and intake after abstinence, findings from Chapter 3 suggest that many of the effects of binge drinking on other behaviors, are transient. Specifically, the increased anxiety and depressed locomotor behaviors seen during early wtihdrawal, dissipate after just two weeks of abstinence. These findings are peculiar, given the long lasting tolerance to the motor incoordinating effects of ethanol noted in Chapter 1.

4.2 Relationship between tolerance, sensitivity and escalated intake

Sensitivity and tolerance can have opposite as well as synergistic effects on ethanol consumption. Sensitivity has generally been thought to be inversely related to the addictive potential of the drug, as sons of alcoholics tend to show less physiological responses to an ethanol challenge (Schuckit, 1994). This reduced physiological response also translates to lower self-reported sensitivity to ethanol (Pollock, 1992). This lower sensitivity to the subjective effects of alcohol would be expected to result in greater consumption (as the subject would need more ethanol to get the same effect of someone with increased sensitivity). Of course, this outcome would depend on the specific effects of ethanol that are being considered. For example, if one is less sensitive to the rewarding effects of ethanol, there may be less motivation to continue consumption. Indeed, light drinkers tend to report lower stimulant-like subjective effects than moderate/heavy drinkers (Holdstock et al., 2000). On the other hand, reduced sensitivity to the sedative effects of ethanol would promote or at least, fail to inhibit, heavy drinking. As such, the differentiator model (Newlin and Thomson, 1990) posits that sensitivity interacts with the biphasic response of alcohol with increased sensitivity to effects on the rising phase of the BEC curve (i.e. Stimulation) promoting alcohol intake, along with lower sensitivity to effects on the descending limb of the curve. Tolerance would be expected to act much the same as sensitiv-

ity to the sedative effects on the descending limb of the BEC curve (which could also be viewed as innate tolerance). That is to say, where the limiting factor in alcohol intake is a behavioral effect during acute intoxication that prevents further intake (i.e. Motor incoordination, sedation), tolerance to this effect(either acquired or innate) may be said to increase an individuals capacity for alcohol consumption. Therefore, one would expect the increase in ethanol g/kg noted for females across the two weeks of DIDMSA to be accompanied by considerable functional tolerance. However, this expectation presumes that motor incoordination (as this is what was measured specifically) directly moderates binge drinking in the DIDMSA model.

As the degree of intoxication does not differ significantly between the groups (specifically, although adult B6 females consumed greater amounts of ethanol than the other groups, there was no main effect of sex nor an interaction of this variable with age or group for motor incoordination), we cannot support this position with our data. A recent report from the Grahame and Boehm labs (Matson et al., 2013) does support a relationship between tolerance to the motor incoordinating effects of ethanol and escalating free choice drinking for the crossed-High alcohol preferring mice. Specifically, this report demonstrates that the escalating free-choice drinking documented for cHAP mice is associated with a reduction in ataxic response to a challenge ethanol dose (1.75g/kg; as compared to water drinker response to this challenge dose). Although B6 mice have demonstrated functional tolerance following binge drinking (Linsenbardt et al., 2011), this behavior was not temporally associated with any shift in drinking (i.e their functional tolerance was not actually associated with any real change in their total ethanol intake or BEC). It may be that, unlike cHAPs, B6 mice are not consuming so much alcohol (in these limited access procedures) that their motor incoordination limits their intake. Instead, it may be that tolerance to the subjective effects (or sensitivity to these effects) of ethanol is what is driving escalated intake for adult females in the DIDMSA procedure. In other protocols, such as chronic intermittent exposure using vapor, escalated voluntary consumption is thought to results from both the significant withdrawal induced by this type of ex-

posure but also tolerance to the subjective effects of ethanol that develops following exposure to such extreme doses. Indeed, Becker and others have used discriminative stimulus effects of ethanol to repeatedly demonstrated significant tolerance to the subjective effects of ethanol for B6 mice that have repeated ethanol exposures following a number of routes of administration (Becker et al., 2006), supporting a low-level response model (Schuckit, 1990) or an inverse relationship between sensitivity to the subjective effects of ethanol and increased drinking (or at least, increased addictive potential of ethanol). Our tolerance data along with the increased locomotor response to ethanol noted for the adult females who consumed the most actually supports a differentiator model (Newline and Thomson, 1990) or an interaction of tolerance, sensitivity and the biphasic effects of ethanol: i.e. tolerance to the aversive effects of ethanol occurring on the descending limb of the BEC curve (i.e. sedation) may be associated with increased capacity to drink or escalating intake, while increases in the sensitivity to the stimulant/positive subjective effects (on the ascending limb of the BEC curve) would similarly promote escalated intake (King et al., 2013). In the case of the data presented above, however, we find a disconnect between escalated intake and the expression of tolerance to the motor incoordinating effects of alcohol.

4.3 Time course of expression of behavioral maladaptations during abstinence from binge drinking

Our data also suggests a surprising disconnect between escalated intake (following binge exposure) and the expression of anxiety and depression during abstinence. In particular, depressive-like behavior emerged after abstinence and its expression was not associated with the expression of increased ethanol preference following binge drinking. Instead, anxiety-like behavior tracked with this measure. This suggests that for the females in this study that demonstrated depressive like behavior during withdrawal, this effect does not drive ethanol preference or daily intake in the two bottle choice paradigm. It is possible that the depressive like behavior is a result of

incubation of the anxiety-like behavior that is precipitated during early withdrawal. Although the anxiety-like behavior degrades, it is possible that the stress associated with it could have precipitated depressive-like behavior in the long-term. Prospective studies of clinical populations support an anxiety-induced depression model, where anxiety is most often a primary condition and it precipitates a secondary, depressive disorder in the long term (Wittchen et al., 2000). Other attempts to probe depressive-like behavior during withdrawal from ethanol do support its expression during early withdrawal. For example, Schulteis and colleagues (1995) found increased ICSS thresholds during the first 6-8hrs following cessation from ethanol exposure that went back down to baseline at 48hrs of withdrawal. It is possible that the differences lie in the dose of ethanol achieved across that study on our data/others (i.e. Stevenson et al., 2008), as these authors used the ethanol vapor procedure and animals were maintained at around a BEC of 200mg/dL. Future studies should explore the relationship between binge-alcohol associated behavioral plasticity and the role that these behavioral changes plays in promoting alcohol drinking and, in particular loss of control drinking.

4.4 Conclusions

Although the model used in our studies does not attempt to approximate the level of drinking required for dependence the data can still add to the growing body of evidence supporting a role for negative affect and anxiety in different disease models of alcohol use disorders. When anxiety disorders and unipolar depression or dysthymia are co-morbidly expressed, it is generally found that the anxiety disorder precedes the development of depression (Witchen, Essau and Krieg, 1991; Kessler et al., 1996; de Graaf et al, 2003). This proposed temporal sequencing of anxiety and depression specifically posits that depression develops as a symptom of the pre-existing anxiety and has been supported even across the daily expression of anxious and depressed mood symptoms (Starr and Davila, 2012). Extending this theory to the develop-

ment of negative affect during protracted withdrawal from drugs of abuse suggests that focusing on pharmacological treatments to block the expression of anxiety during early withdrawal should inhibit the development of depression during protracted withdrawal, and perhaps help to attenuate a depression-induced return to heavy drinking.

4.4.1 Implications for the clinic and the public

One of the important practical findings of the work presented is the demonstration of long term behavioral consequences of binge drinking in adults. Given the history of the 4/5 drink definition of the term as it is now used, most of the clinical work surrounding this pattern of drinking concerns adolescents. For adults, binge drinking is framed as a problem when it interacts with some other condition, like PTSD. But, a number of preclinical studies, including the data presented in this dissertation, suggests that binge drinking, especially the 8/drinks per session, 4 times a month average that is growing in prevalence in the United States, is problematic. Moreover, the fact that the consequences that this pattern of intake had on alcohol consumption/preference and the expression of negative affect were not necessarily temporally linked (i.e. the effect that binge drinking had on baseline preference dissipated after 2 weeks of abstinence, right as the depressive-like symptoms were coming on board) highlights the efforts of Dai Stephens, Theodora Duka and others who have been emphasizing the cognitive and emotional problems that develop from binge drinking that may easily go overlooked (as social drinking is not problem drinking). In essense, these findings support improved monitoring of alcohol consumption across the general population by medical professionals so that alcohol consumers are made aware of the potential long lasting hazards associated with their pattern of use.

APPENDICES

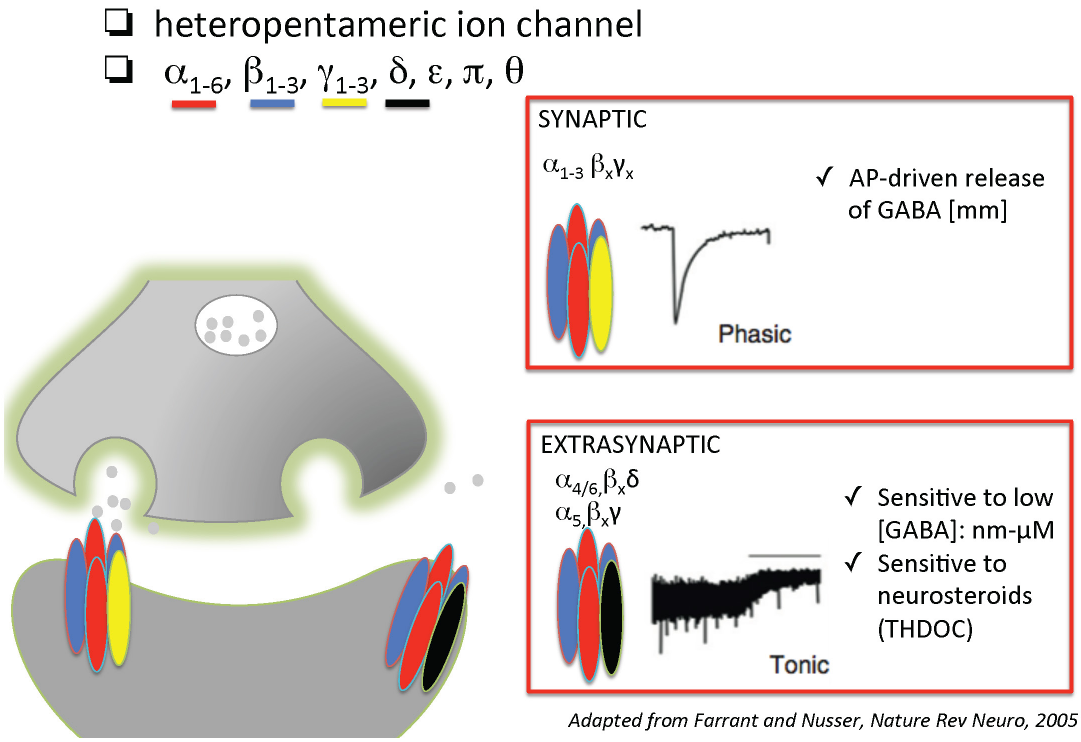

Fig. 1. The ionotropic $GABA_A$ receptor is a heteropentameric structure composed, most often, of two pairs of alternating α and β proteins and a δ or $\gamma 2$ subunit protein. These final subunit proteins are thought to influence translocation of the receptor to the synapse, to mediate phasic inhibition, or to the extrasynaptic space, to mediate tonic inhibition.

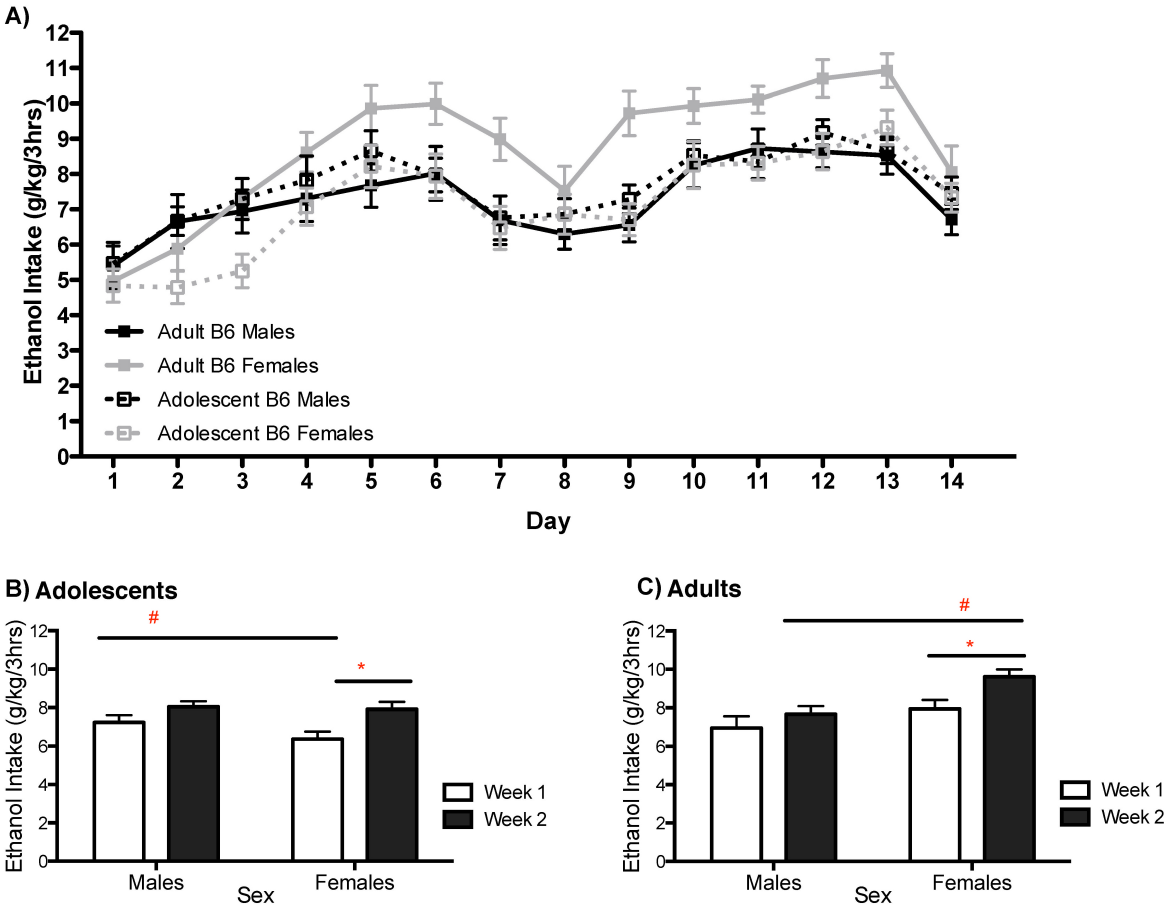

Fig. 2. Females, but not males, increase binge-like ethanol consumption following limited access using DID-MSA. A) Total daily intake across the three hourly binge access periods for adolescent (23-24/sex) and adult (23-25/sex) B6 mice. B) Adolescent females showed an increase in intake across the two weeks (, $p<0.05$), though though they consumed less than adolescent males overall (#, $p<0.05$). C) Adult females consumed more than adult males (#, $p<0.01$) and had greater ethanol intake during the second week of access when compared to the first (, $p<0.05$).

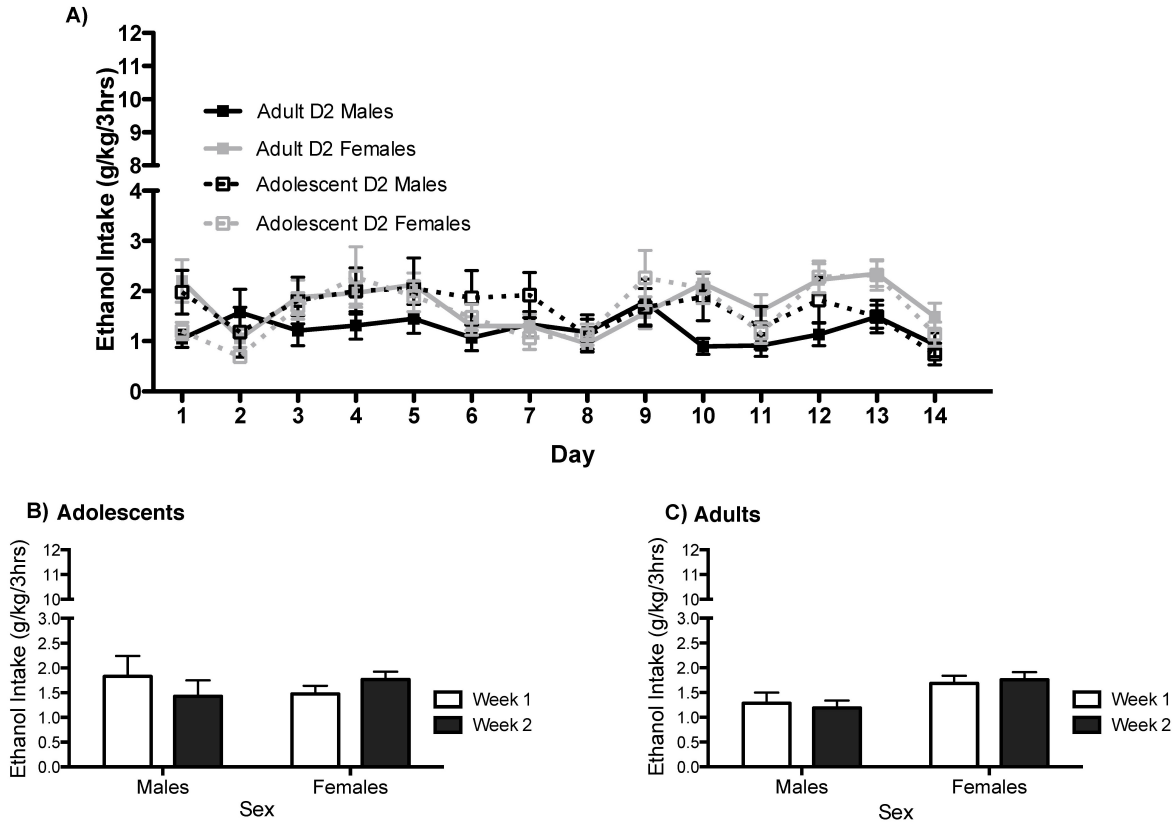

Fig. 3. D2 mice maintain their alcohol avoiding phenotype when given limited access to alcohol using DID-MSA. A) Total daily intake across three hourly binge access periods for D2 adolescents (n=21-23) and adults (23-24). B) D2 adolescent males and females did not alter their intake across the two weeks of access. C) For D2 adults, neither males nor females showed a change in alcohol intake during the second week of access when compared to the first.

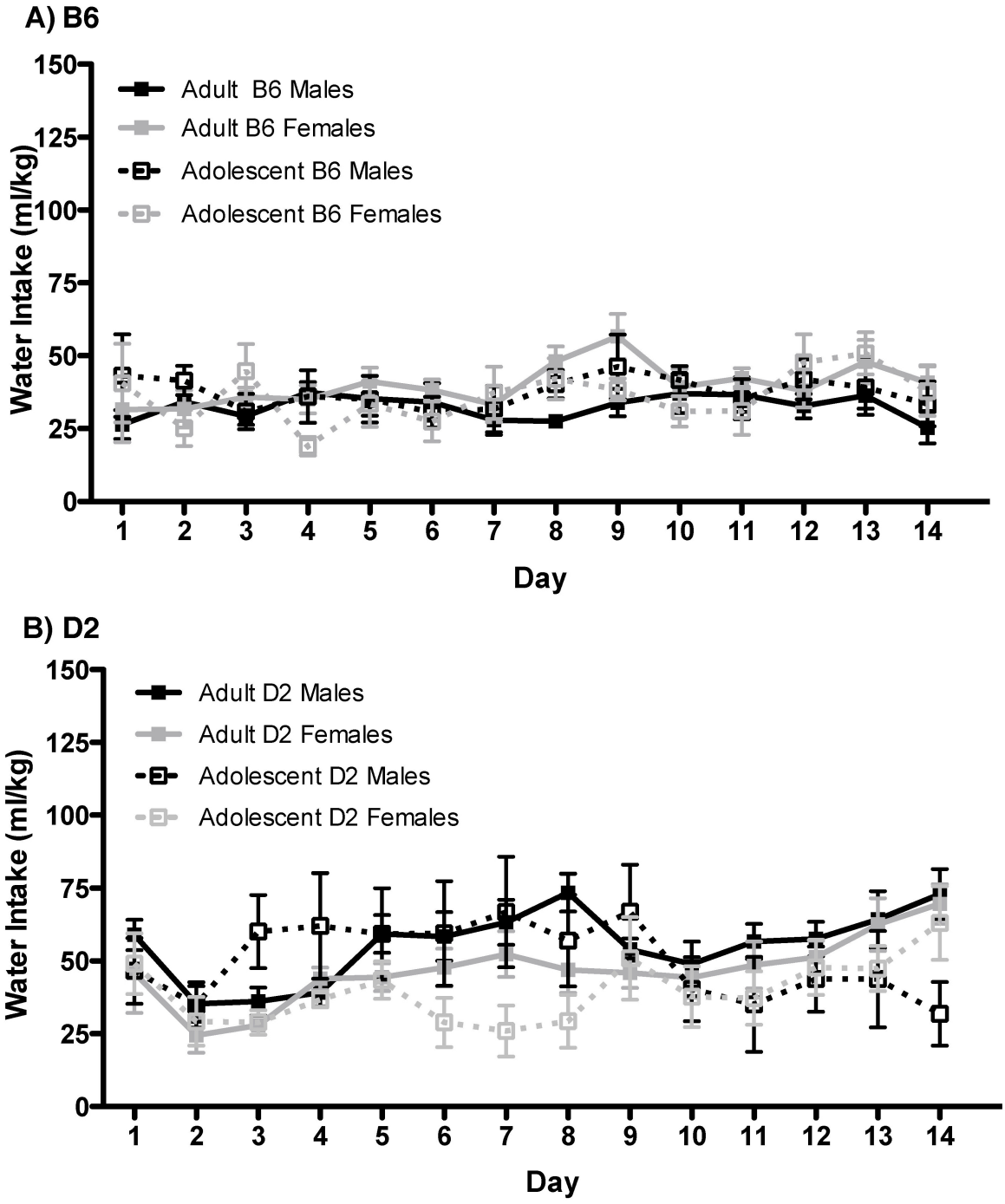

Fig. 4. Intermittent fluid access using DID-MSA does not alter water intake in inbred strains. A) B6 adults (n= 23 males and 25 females) and adolescents (n= 23 males and 24 females) did not show different patterns of water consumption in this paradigm. B)D2 adults and adolescents showed no significant differences in their water intake across the 14 days.

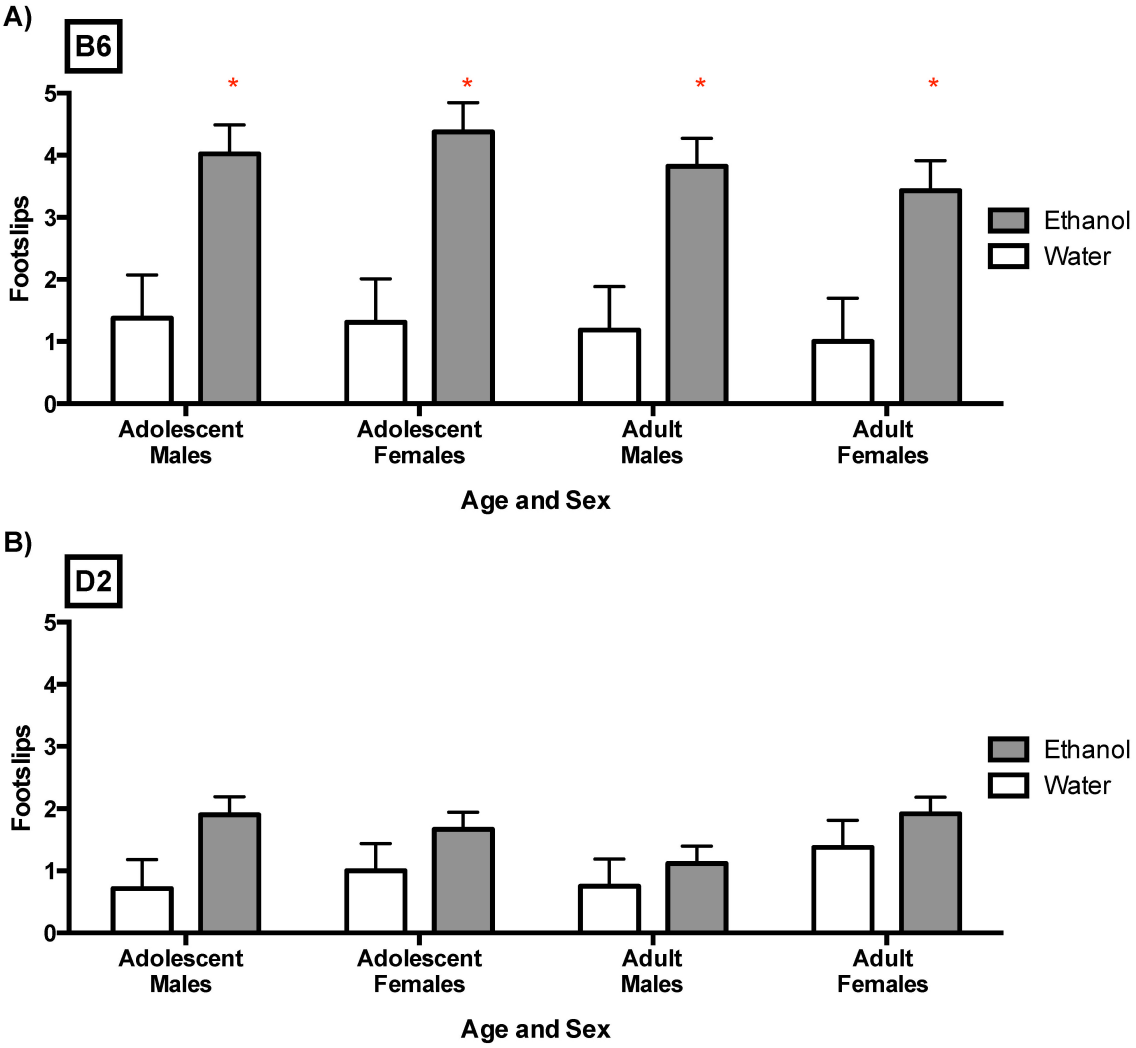

Fig. 5. B6 (A) but not D2 (B) mice show significant intoxication following binge drinking using DIDMSA. All ethanol drinking animals performed significantly worse on the balance beam than water drinking controls (ps<0.05). A) B6 mice that drank ethanol (n=22-25/age/sex) performed significantly worse on the balance beam than water drinking controls (n=8/age/sex). This intoxication did not vary across hour of consumption or across Day 7 and Day 14 and data are collapsed across these variables. B)D2 mice that drank ethanol (n=21-24/age/sex) showed no difference in balance beam performance when compared to those that consumed water (n=8/age/sex).

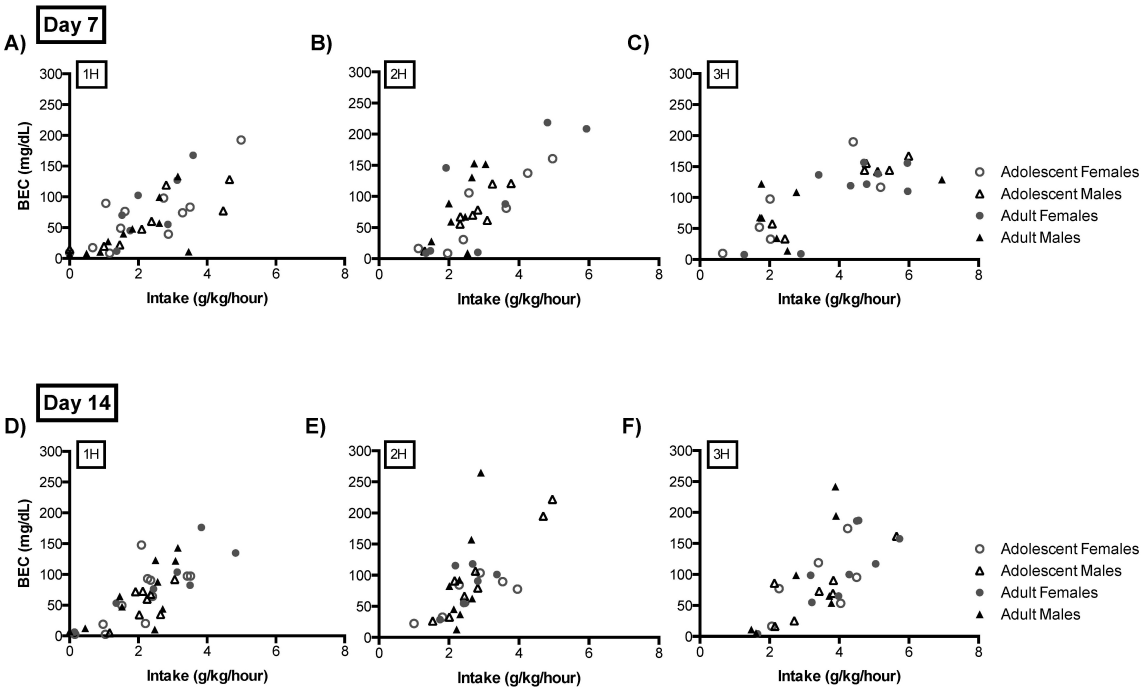

Fig. 6. For B6 mice, ethanol drinking in DID-MSA continues to predict BEC and results in significant behavioral intoxication in B6 mice on Day 7 and Day 14. Levels of ethanol intake during the 1st hour of drinking (A and D), 2nd hour of drinking (B and E) and 3rd hour of drinking (C and F) significantly correlate with respective BECs (n=6-11/age/sex/hour).

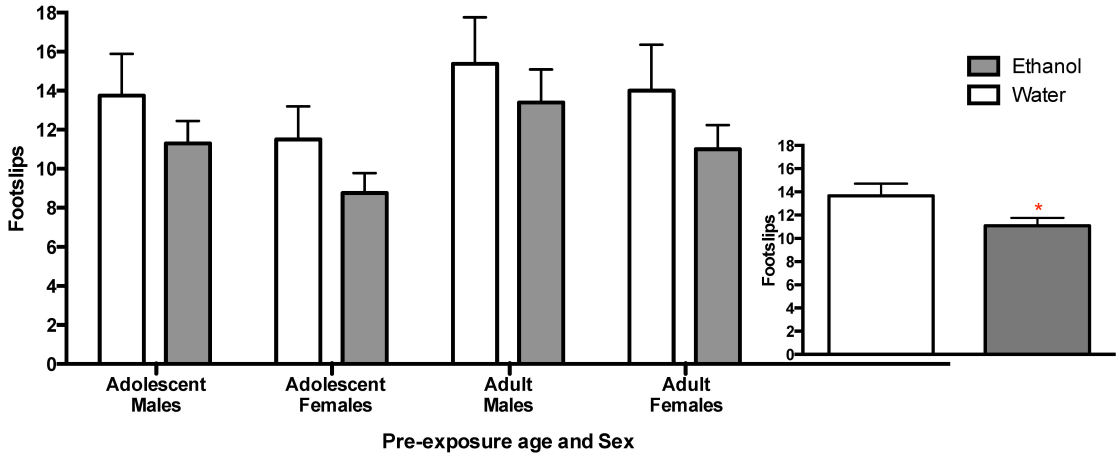

Fig. 7. B6 mice with a history of binge drinking show attenuated ataxic response to ethanol, even following 30 days of abstinence. There was a significant effect of solution consumed on the level of impairment following 1.75 g/kg EtOH ($p<0.05$; inset). There was no effect of age or sex on this display of reduced sensitivity to an ethanol challenge (n=8/age/sex for water and n=23-24/age/sex for ethanol).

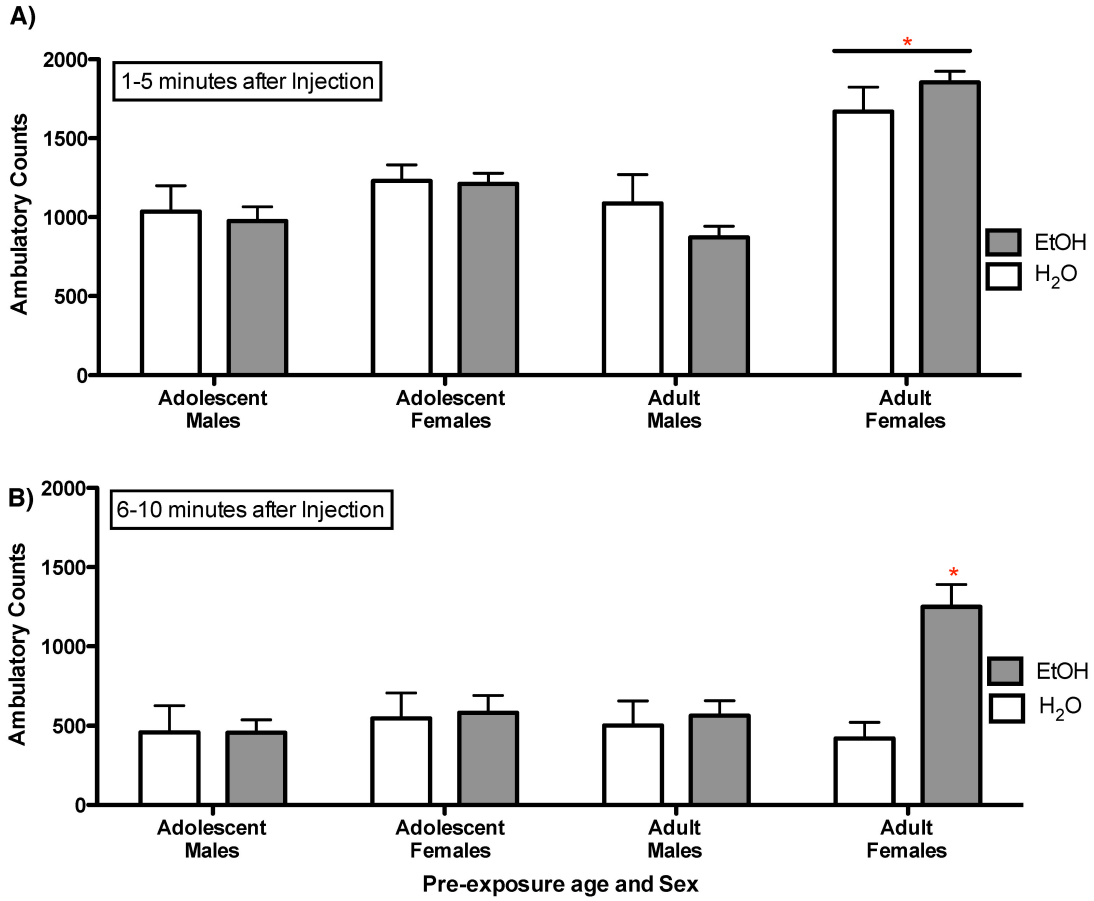

Fig. 8. Binge drinking using DID-MSA significantly altered locomotor response to ethanol for female mice that binged during adulthood. A) During the first 5 minutes following injection, adult B6 females, regardless of binge drinking history, display greater locomotor response to the 1.75g/kg ethanol challenge compared to all other groups($p<0.001$). B) During the final 5 minutes of the ten minute test, adult females with a history of DID-MSA ethanol consumption showed a significantly greater locomotor response to this ethanol challenge, as compared to their water consuming controls ($p<0.01$). This effect was not noted for males or females that binged as adolescents (n=6-7/age/sex for water and n=15-16/age/sex for ethanol).

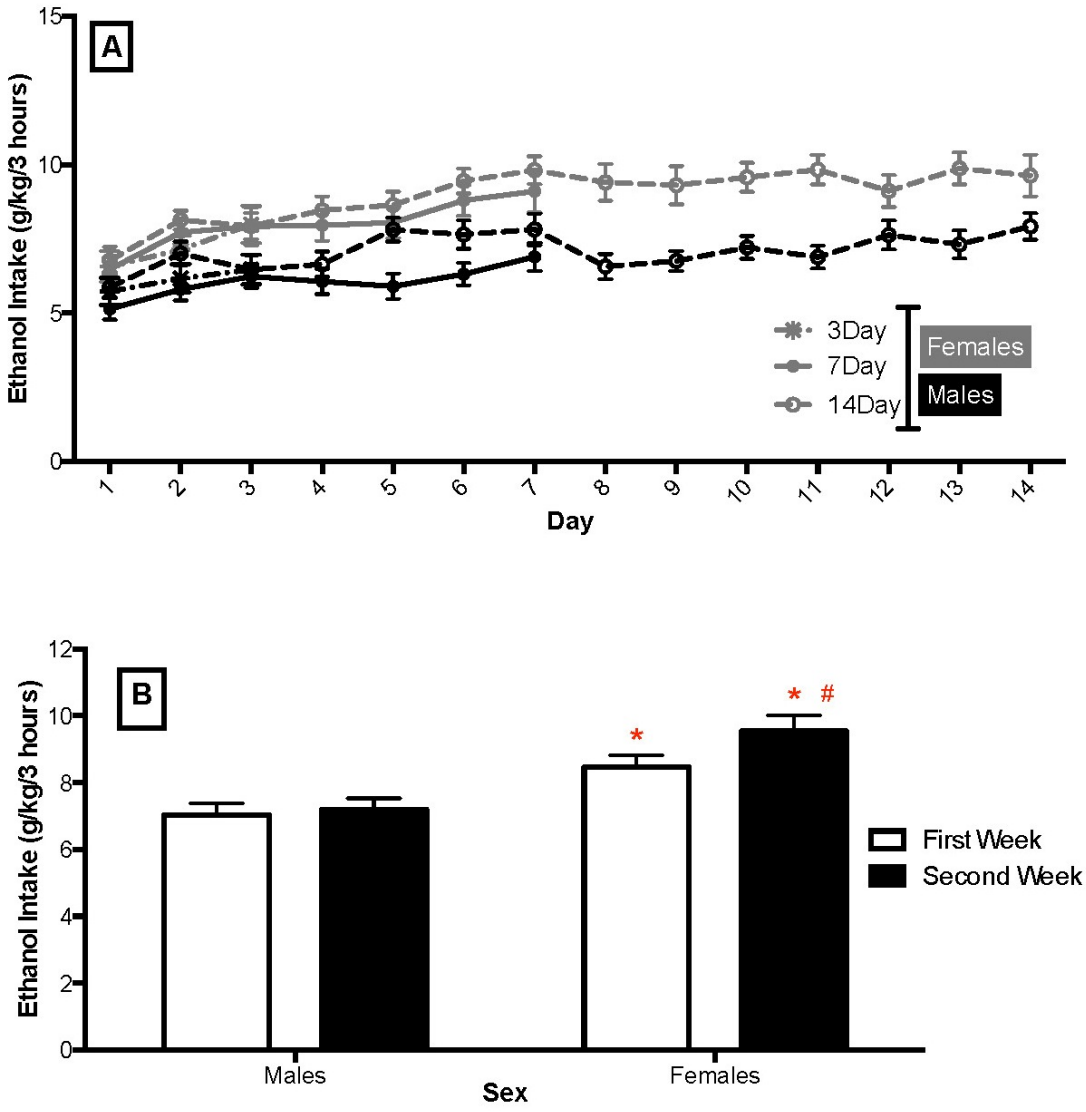

Fig. 9. Females, but not males, consistently increase binge-like ethanol consumption following limited access using DID-MSA. A) Total daily intake across the three hourly binge access periods for (9-11/group) B6 males and females. Mice had access to alcohol for 3, 7 or 14 days. There was a main effect of sex, as female mice consumed significantly more alcohol, across the 3 hours of access, than their male conspecifics ($p<0.01$). B) Females also increased their consumption over time (week 1 vs. week 2; #, $p<0.001$), whereas males show no such acquisition of heavier binge drinking across this protocol. Unlike Chapter 1(Fig. 1), females in this study consumed more than males during both the first and second weeks (, $ps<0.01$).

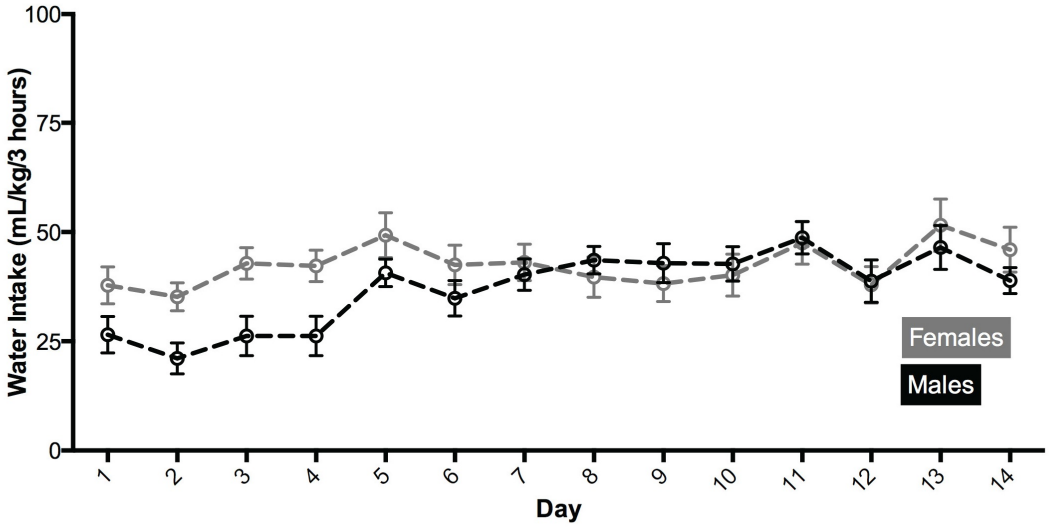

Fig. 10. B6 mice do not steadily increase water intake in the DID-MSA paradigm. There was a significant effect of day on water intake, but it did not interact with any other factor. Pairwise comparisons revealed changes in water consumption across the days that were not systematic.

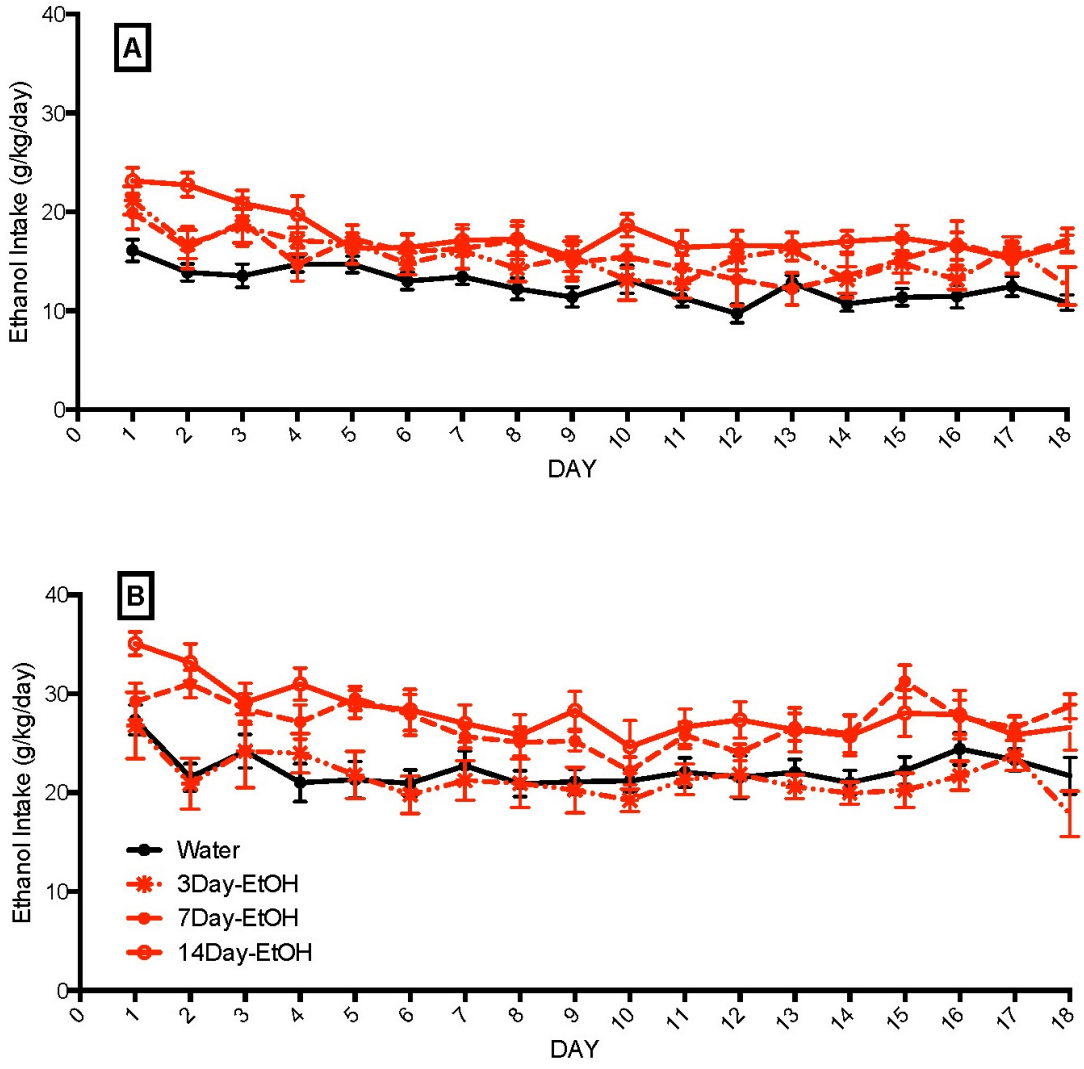

Fig. 11. Both males and females demonstrate a significant effect of binge drinking history on 24hour, 2 bottle choice drinking levels. A) males required 14 days of binge exposure to show any change in their baseline drinking behavior (ps<0.0001). B) For female mice, 14 days of binge exposure also increased intake (p<0.0001) but 7 days of binge drinking was sufficient to augment total free-choice drinking levels(p<0.001)

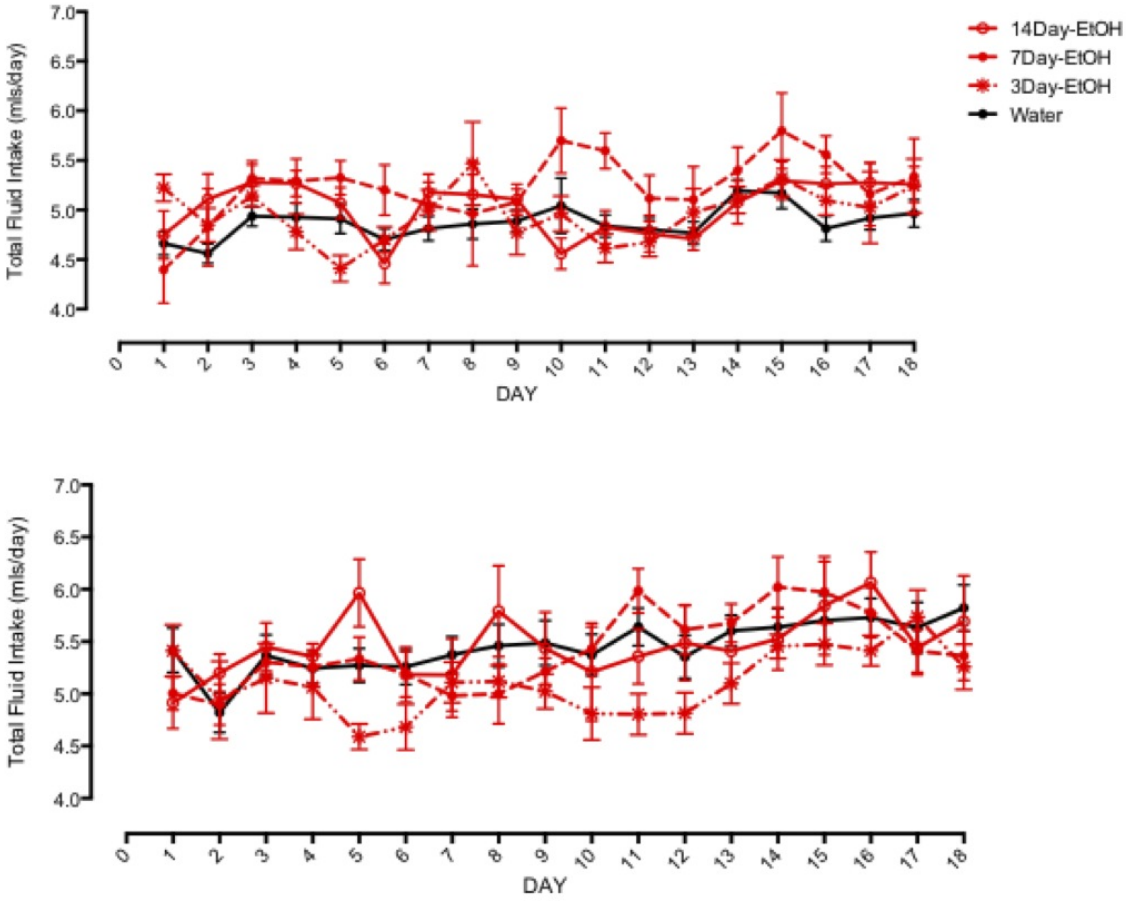

Fig. 12. A) Males showed non-systematic differences in the total fluid consumed by the control mice and 3, 7 and 14 day bingers apparent on three days: 5, 11 and 16. On day 5, 3 day bingers were consuming significantly less fluid than 7 day bingers (p¡0.05), on day 11 the 7 day bingers are consuming significantly more fluid than all other groups (ps¡0.05) and on day 16 these same mice are consuming significantly more fluid than water exposed mice (p¡0.05). B) Females did not show an interaction of day and groupmales required 14 days of binge exposure to show any change in their baseline drinking behavior (ps<0.0001).

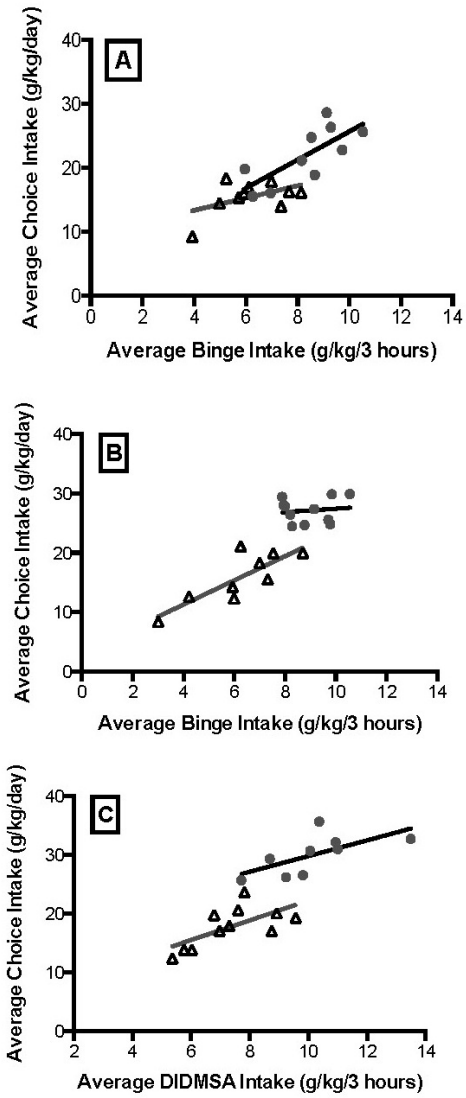

Fig. 13. Binge drinking did not always predict 24hr intakes in DIDMSA. A) DIDMSA intake over 3 days significantly predicted and amount of 2-bottle ethanol intake for females (R2= 0.56; $p<0.05$) only. B) Only males show a significant predictive relationship between the amount they consumed during the binge sessions and the amount they consumed when switched to continuous access after 7 days (R2=0.67; $p<0.01$) C) Again, average amount consumed over 14 days of DIDMSA only predicted 24hr drinking levels for males (R2=0.45; $p<0.05$)

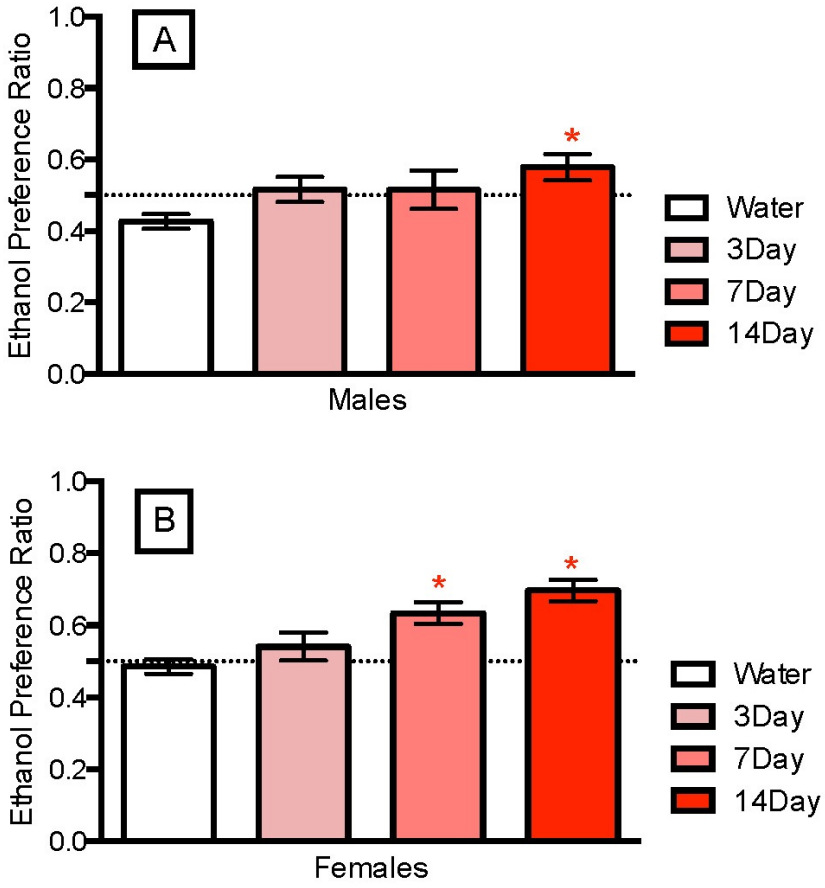

Fig. 14. The average preference ratio over the 18 days of access reveals significant effects of binge drinking on preference ratio for males and females. A) Males required 14 days of limited access binge drinking in order to show an increase in their alcohol preference in this 24hr 2-bottle choice procedure($p<0.01$). B) Females show a significant increase in preference for alcohol in a 24-hour 2 bottle choice paradigm following just 7 days of limited access binge drinking ($p<0.001$). This augmented preference is also displayed following 14 days of binge drinking ($p<0.001$).

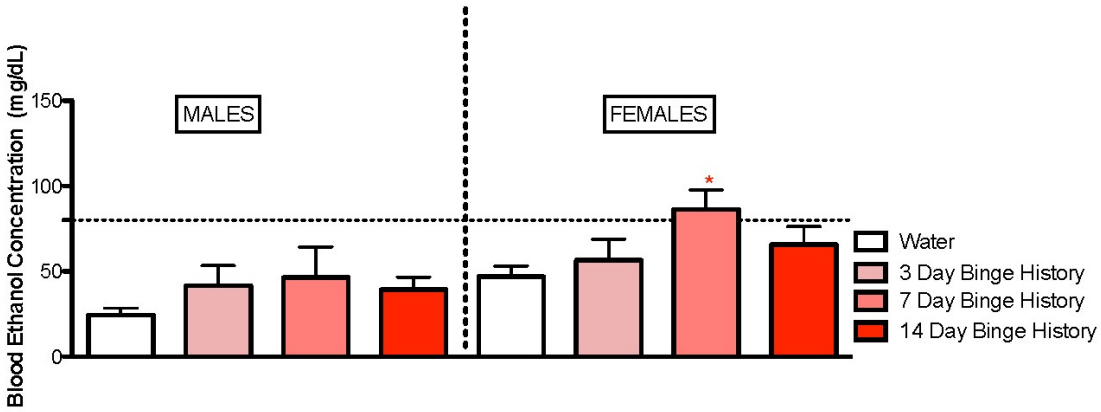

Fig. 15. BEC following the first drinking bout only approaches binge-like for most groups. Blood ethanol level achieved during the first two hours of a 24-hour, 2-bottle choice drinking following lights out on day 20 show that only females with a 7-day binge drinking history display an effect of binge alcohol exposure on the level of BEC achieved during free-choice drinking ($p<0.05$).

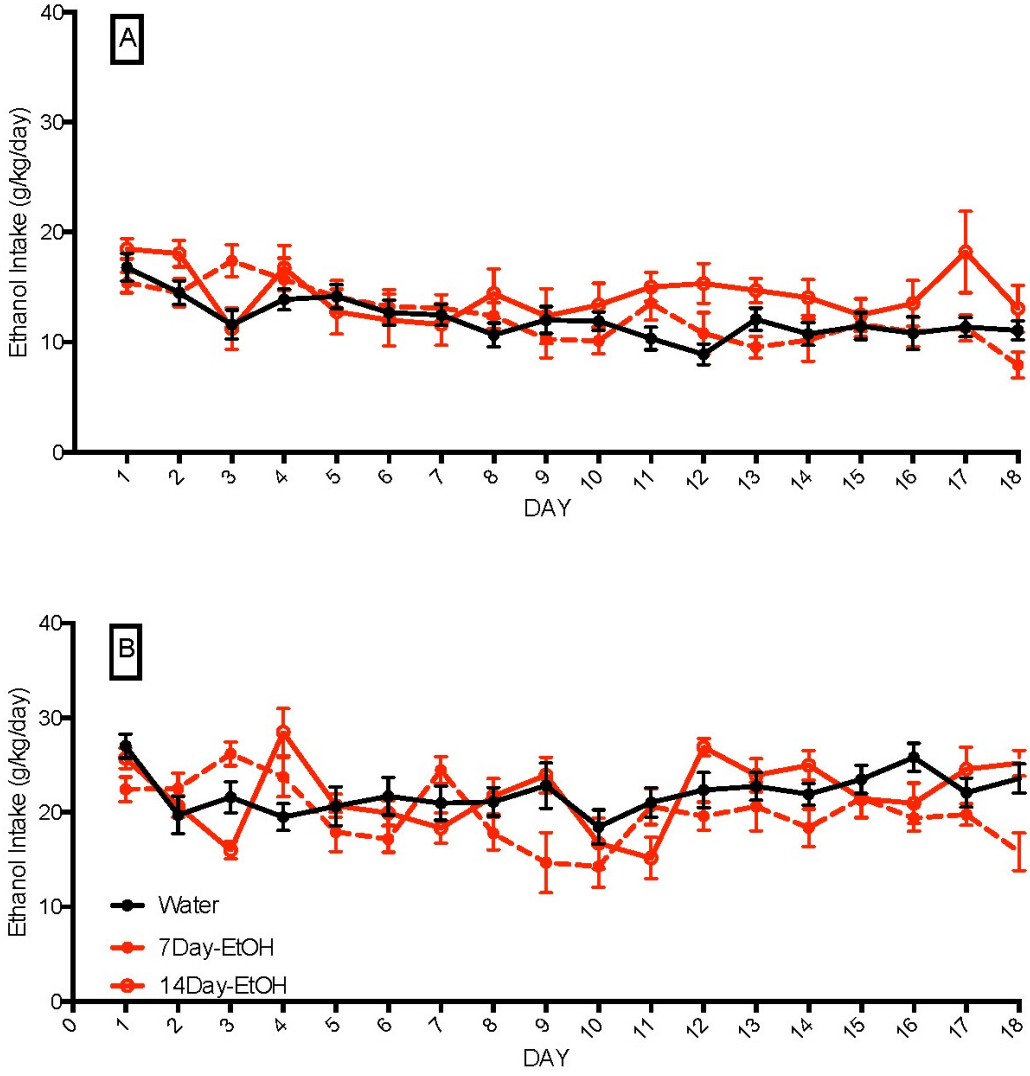

Fig. 16. Two weeks of abstinence reversed the effects of binge drinking on 24hr 2 bottle choice drinking for both males (A) and females (B).

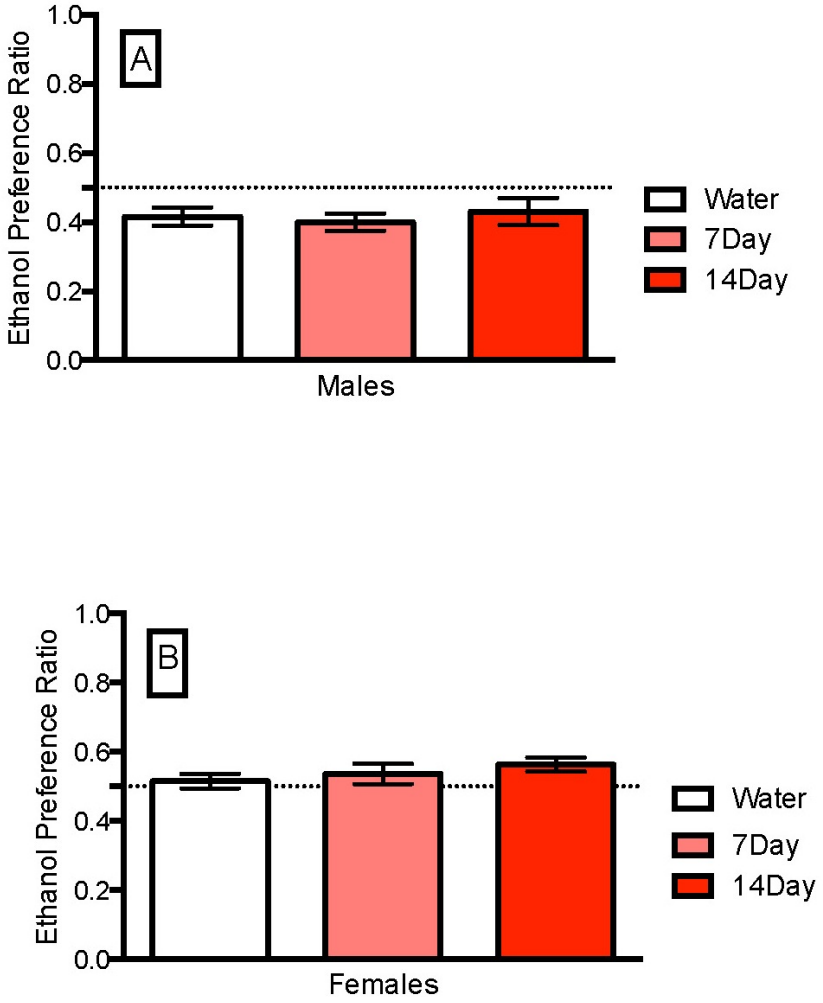

Fig. 17. Two weeks of abstinence reversed the effects of binge drinking on 24hr 2 bottle choice preference ratio for both males (A) and females (B).

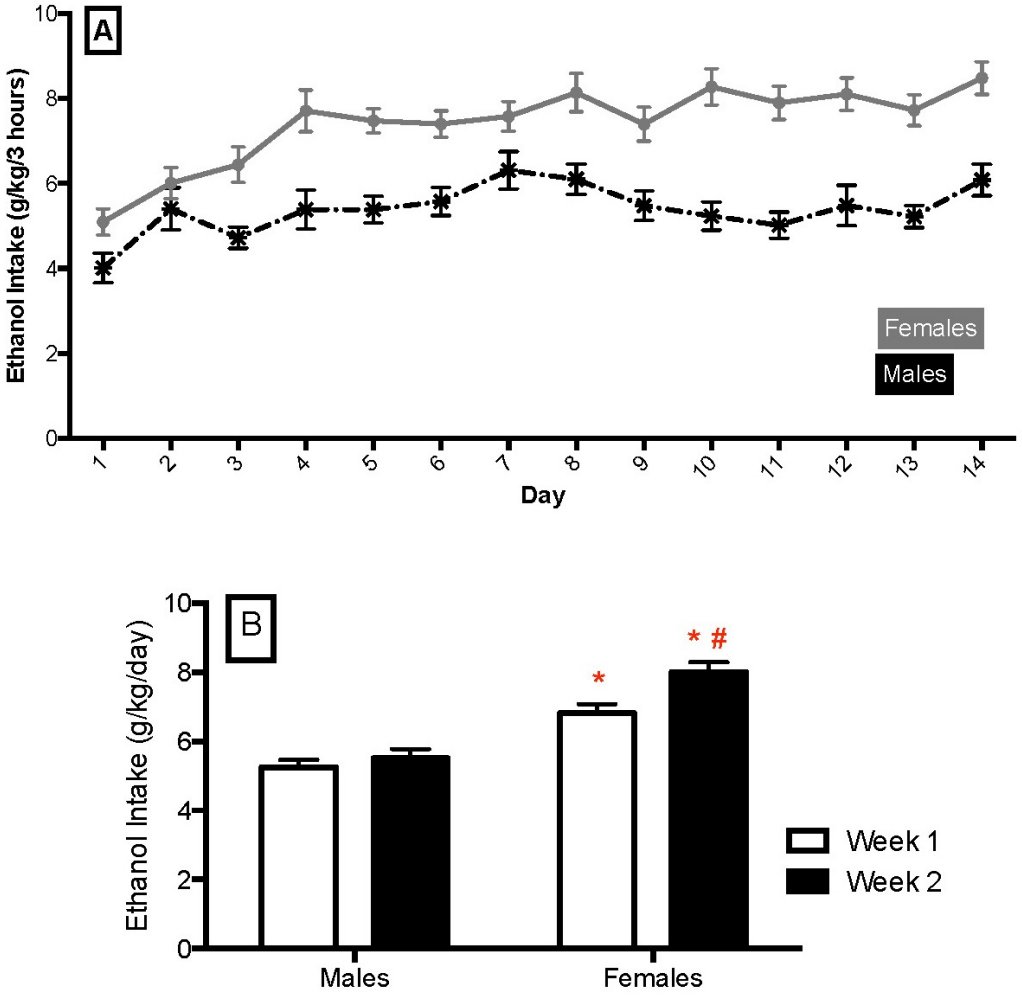

Fig. 18. Female mice escalate their intakes over the course of the two weeks while males do not. A) Daily intakes for males and females across 14 days of DIDMSA showed a significant effect of sex and day (ps<0.00001) B) Average weekly intakes showed that females consumed more ethanol than males during the first (, p<0.001) and second (, p<0.0001) weeks of access but also ramped up their drinking across the weeks (#, p<0.001).

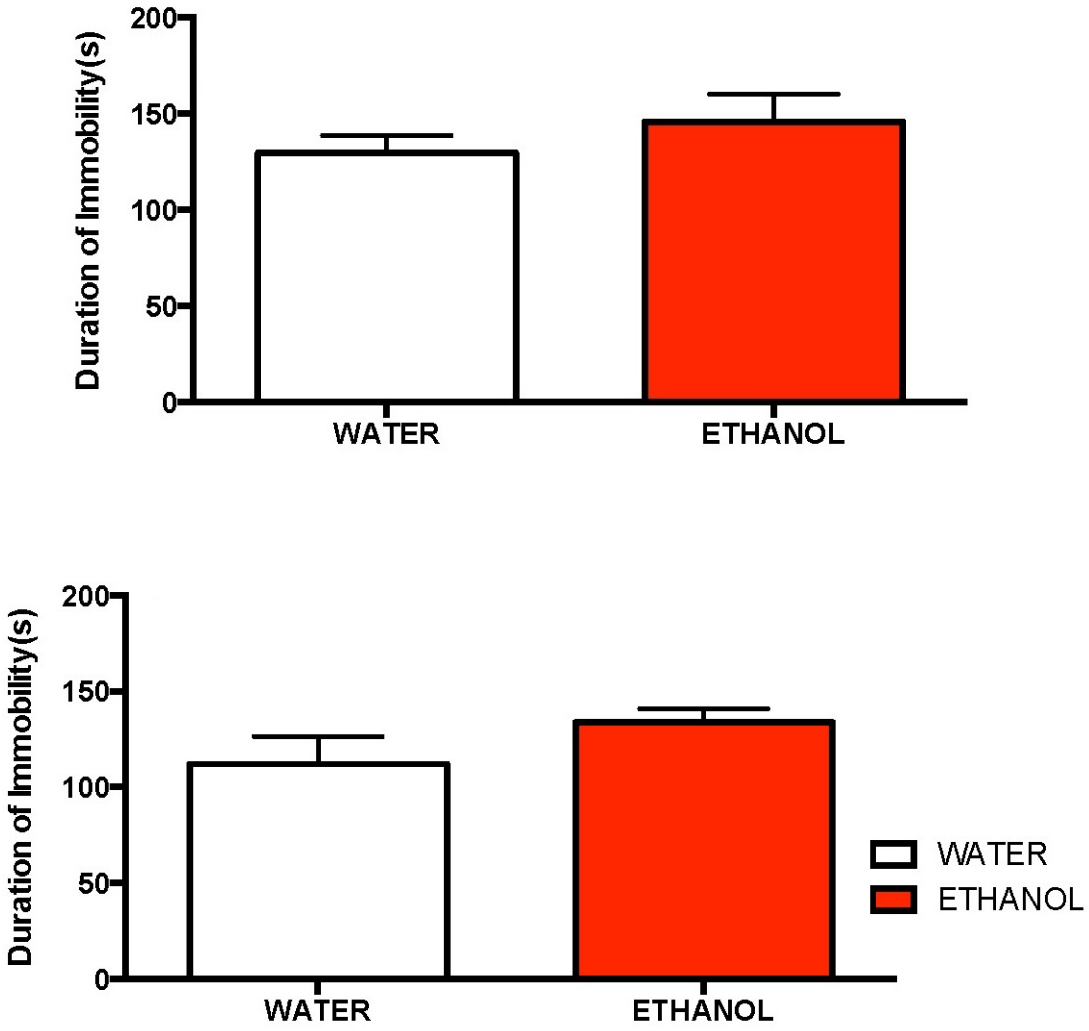

Fig. 19. Duration of immobility was not different for males who binged when compared to water controls during early withdrawal (top panel). During late withdrawal (bottom panel), a trend for bingers to show increased immobility is noted, however ($p<0.08$)

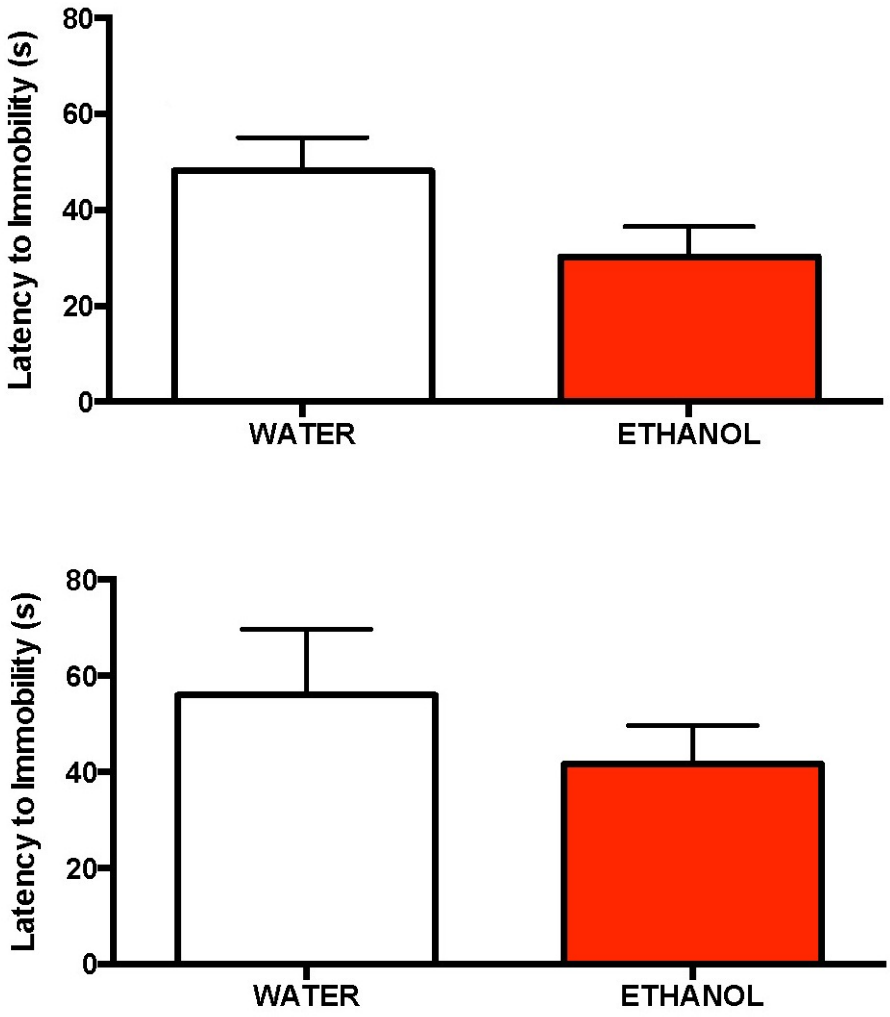

Fig. 20. Early during abstinence from binge drinking males display a marginal effect of binge drinking on latency to display the first bout of immobility(p=0.07; top panel). This trend is not evident during late abstinence (bottom panel).

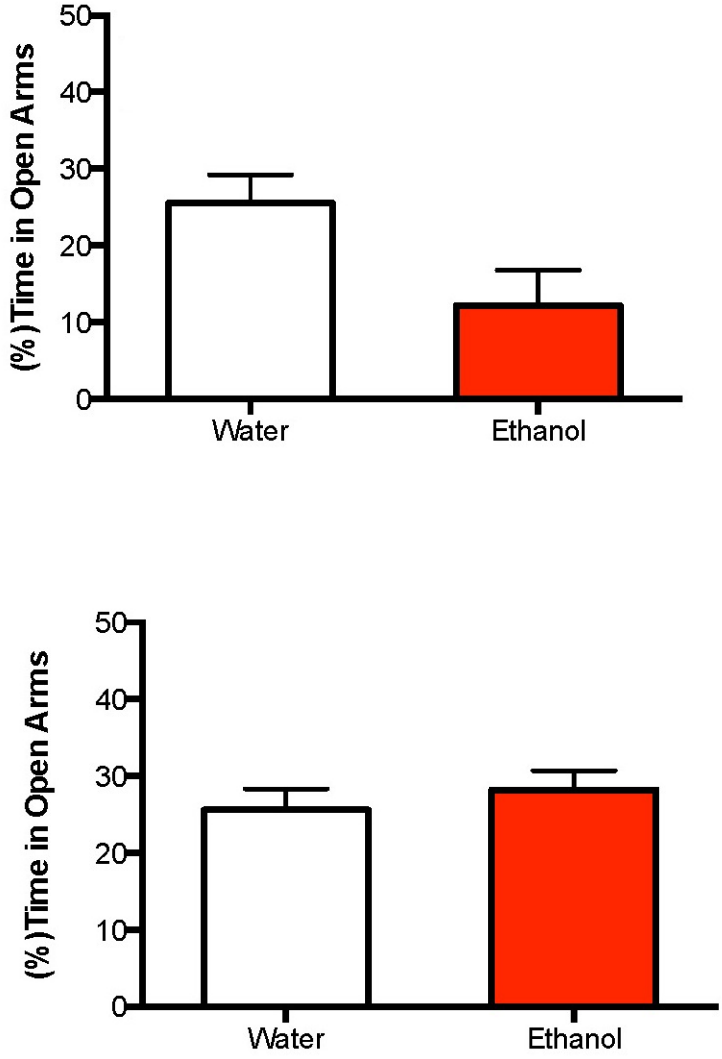

Fig. 21. Binge drinkers show reduced percent of open arm time exploration for males during early abstinence ($p<0.05$; top panel) . This anxiety-like behavior dissipates during protracted abstinence (bottom panel).

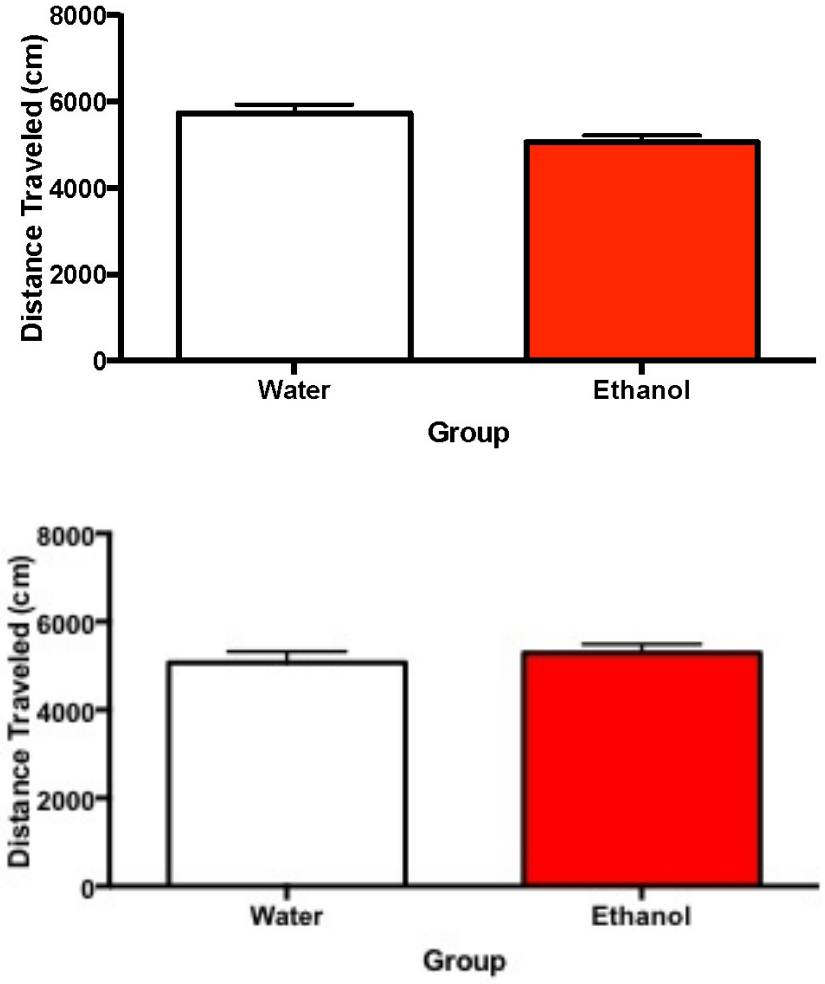

Fig. 22. Males display reduced activity during early abstinence (ps<0.05 ; top panel). This hypolocomotion returns to baseline by protracted abstinence (bottom panel).

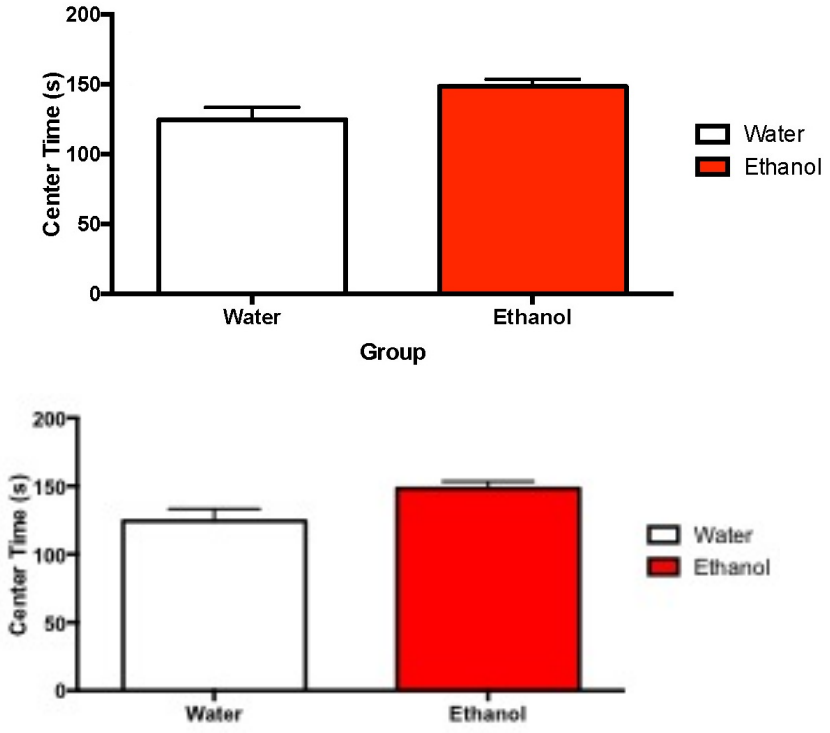

Fig. 23. Males did not show an effect of binge drinking on time spent in the center of the open field during early (top panel) or late (bottom panel) abstinence.

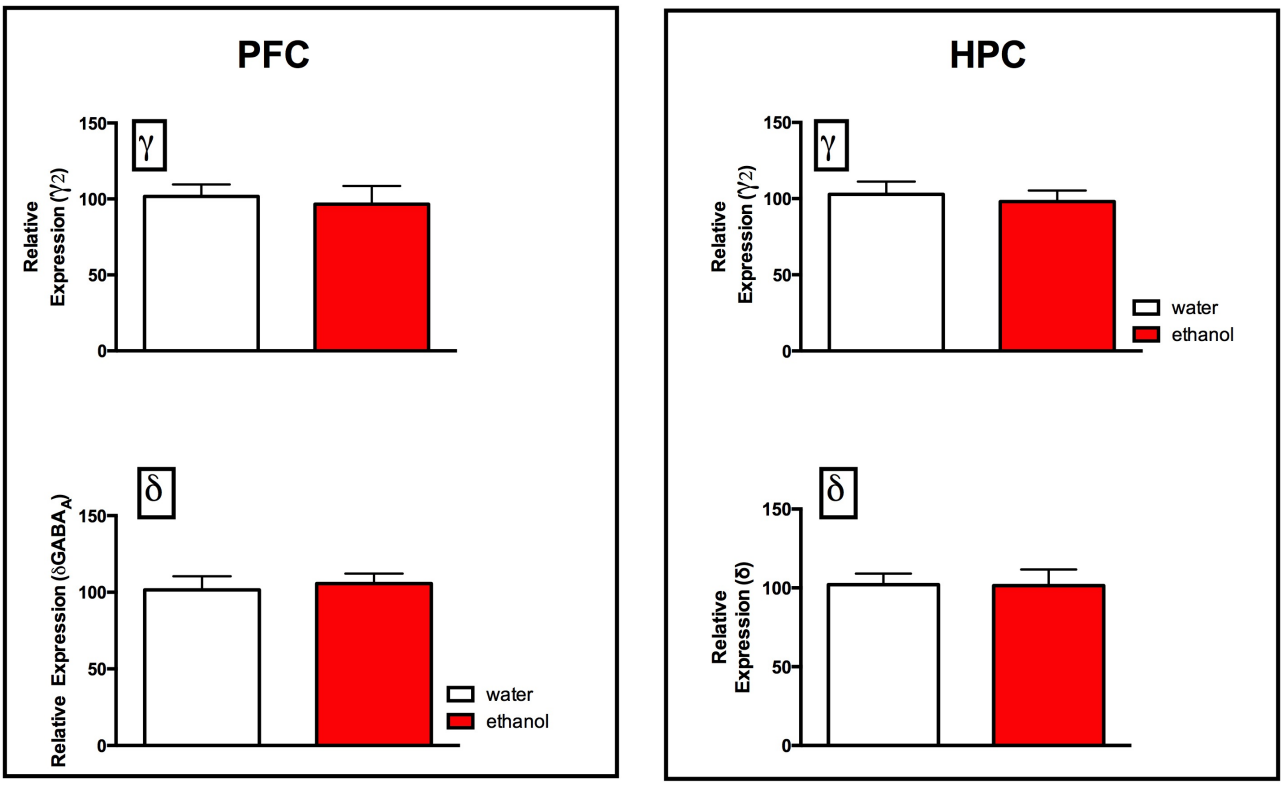

Fig. 24. Binge drinking was not associated with any changes in δ orγ2 subunit expression in the hippocampus or prefrontal cortex for males

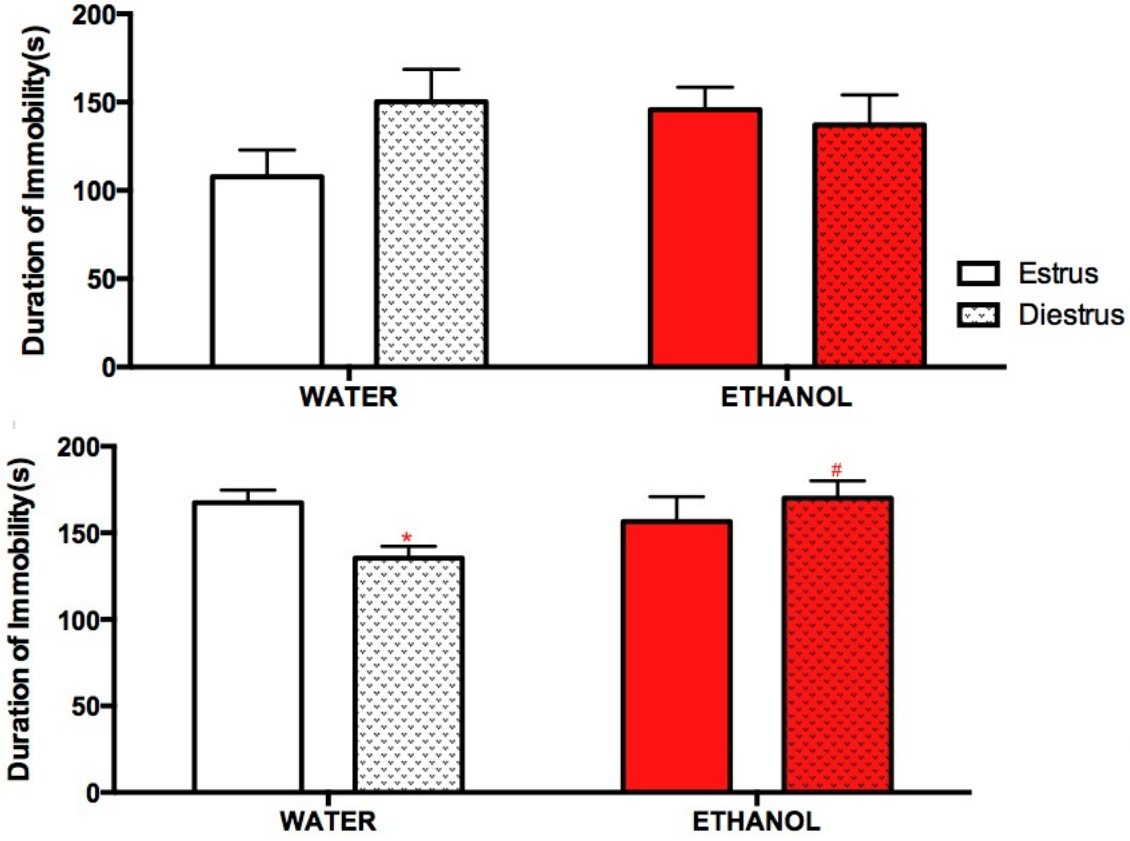

Fig. 25. Duration of immobility was not different for females who binged when compared to water controls during early withdrawal (top panel). After protracted abstinence from binge drinking, the significant effect of estrous on baseline duration of immobility ($\times$ *, $p<0.05$), with estrus females display more depressive-like behavior than diestrus females goes away, as binge exposure significantly increases duration of immobility for diestrus females (#, $p<0.03$).

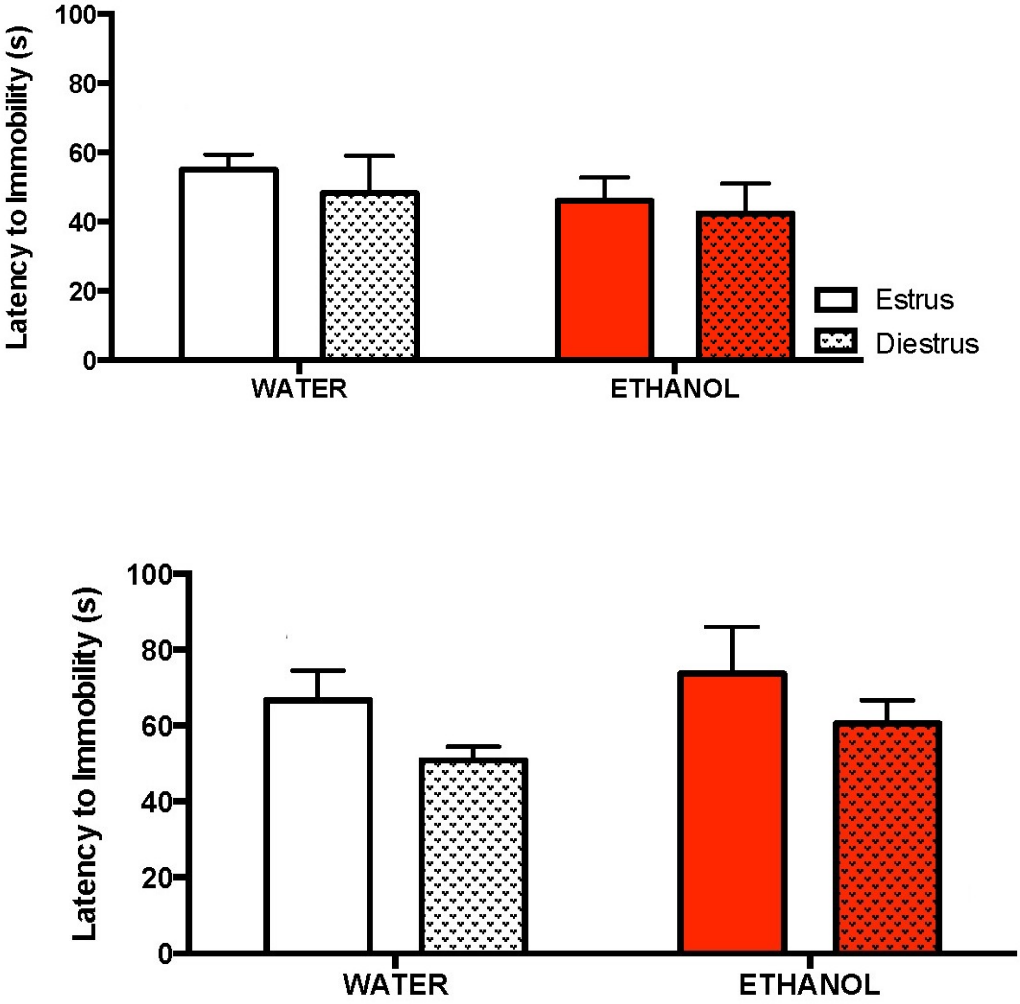

Fig. 26. There was no significant effect of binge history or estrous status on latency to first bout of immobility during either early or protracted abstinence for these females.

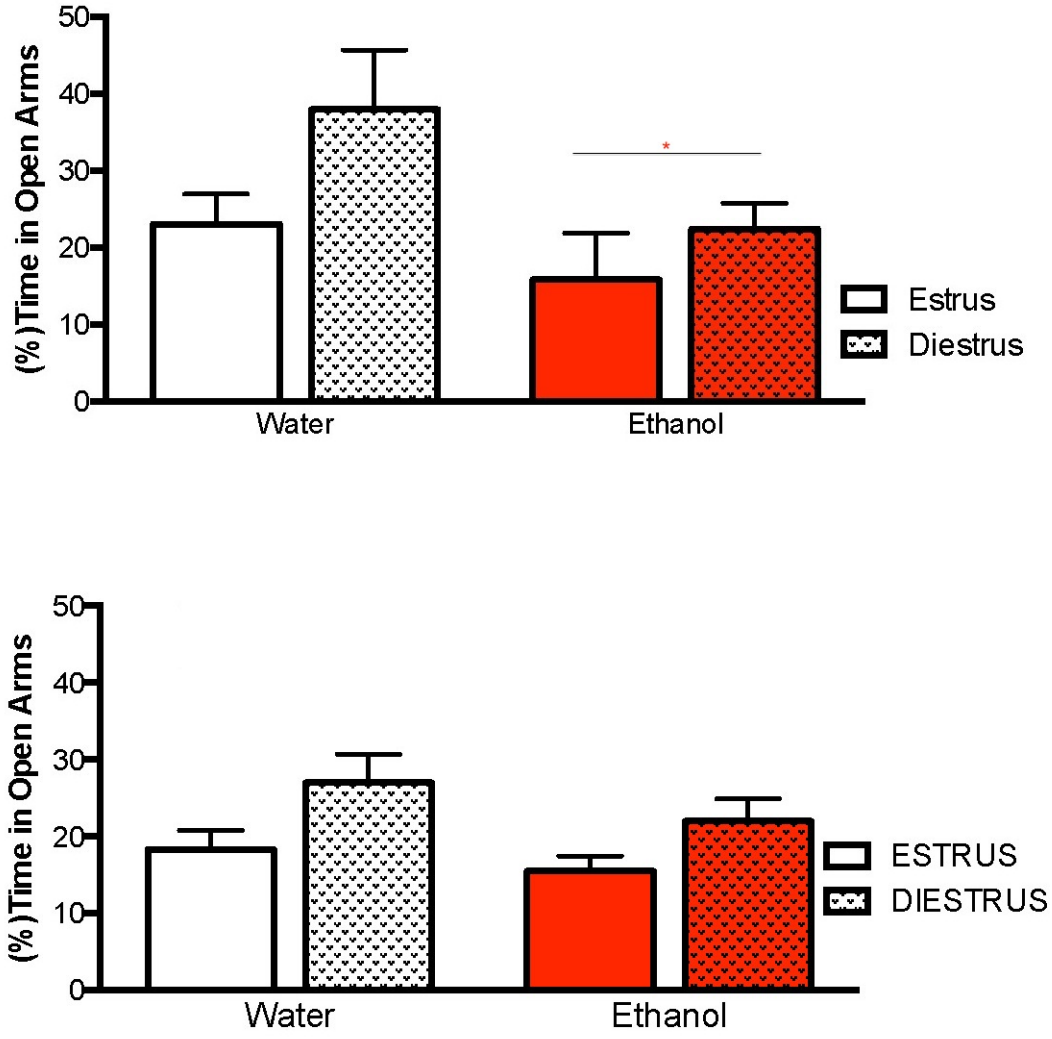

Fig. 27. During early abstinence, females showed reduced percent of open arm time after drinking (*, $p<0.05$). Estrous status marginally ($p=0.056$) affected this measure, with estrus females demonstrating greater anxiety-like behavior than diestrus females (patterned bars) at baseline. This difference is not apparent after binge drinking. Anxiety-like behavior returns to baseline after extended abstinence.

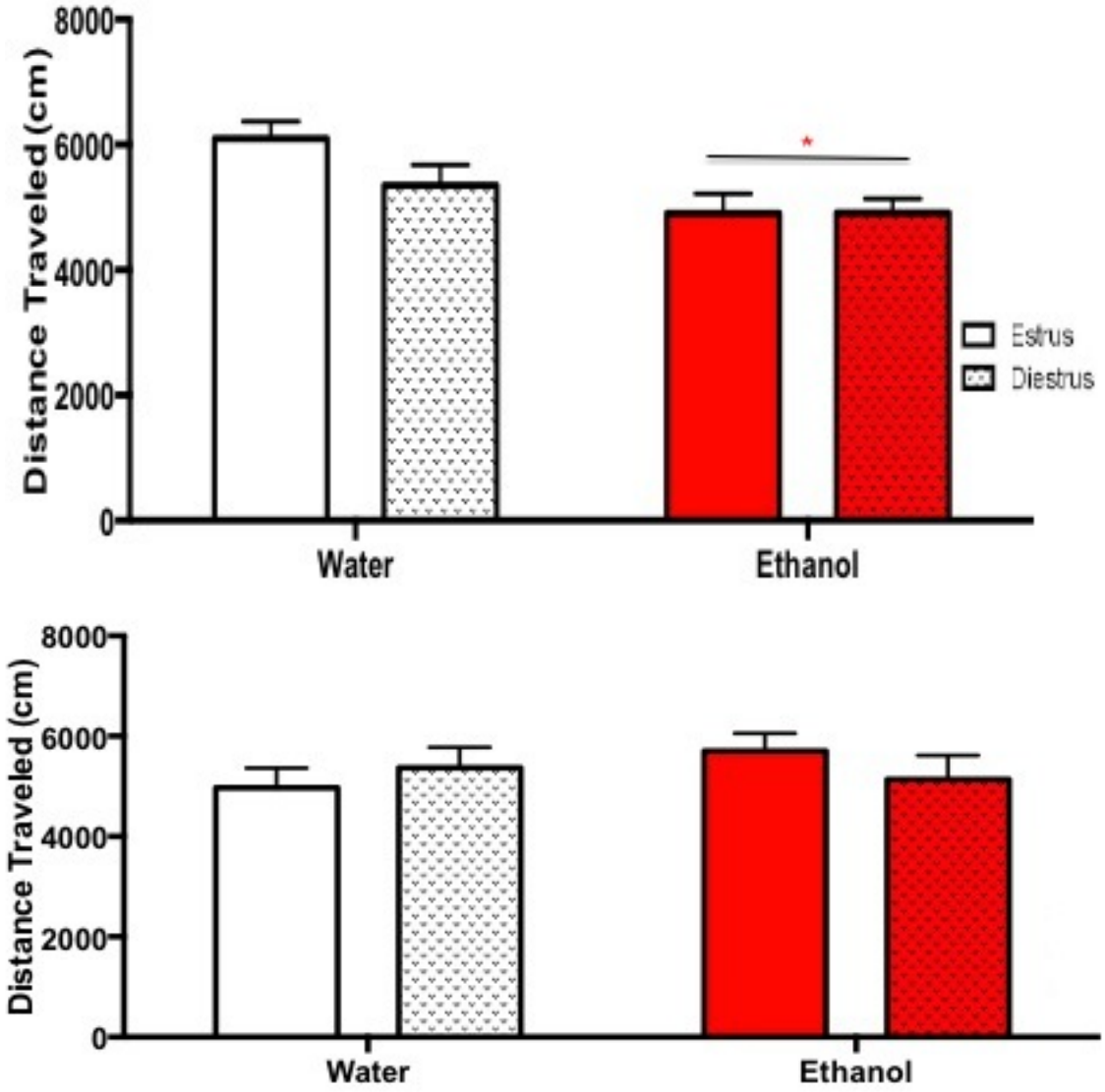

Fig. 28. During early abstinence, females showed hypolocomotion in the open field(*, $p<0.05$), when compared to water drinking controls. This difference is not apparent after binge drinking.

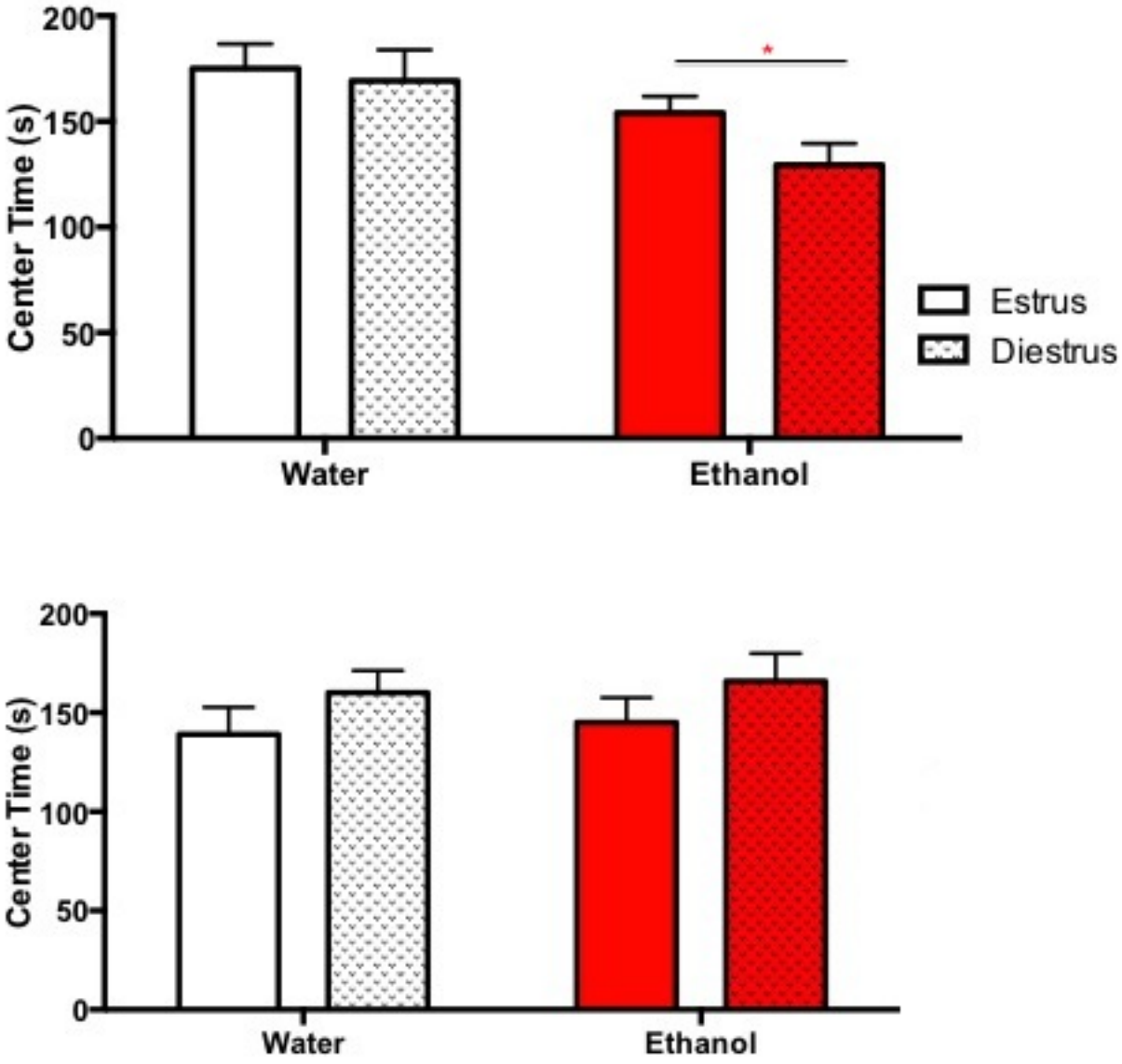

Fig. 29. During early abstinence, females displayed reduced time in the center (*, $p<0.05$). Estrous cycle did not affect baseline levels of center time and did not interact with binge drinking to moderate the main effect on this measureFemales did not show an effect of binge drinking or estrous status on time spent in the center of the open field during late abstinence.

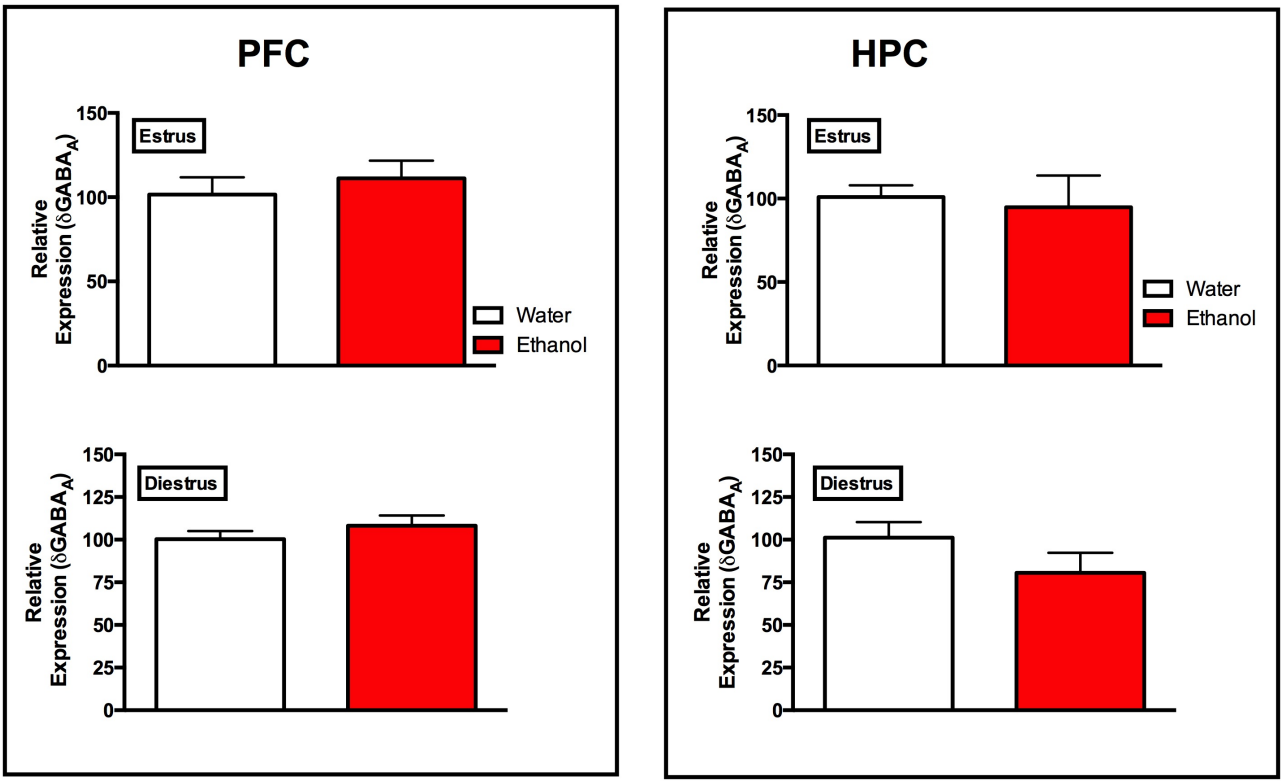

Fig. 30. Binge drinking was not associated with any changes in δ subunit expression for females, nor did this transcript show changes across the estrous cycle in water-drinking controls.

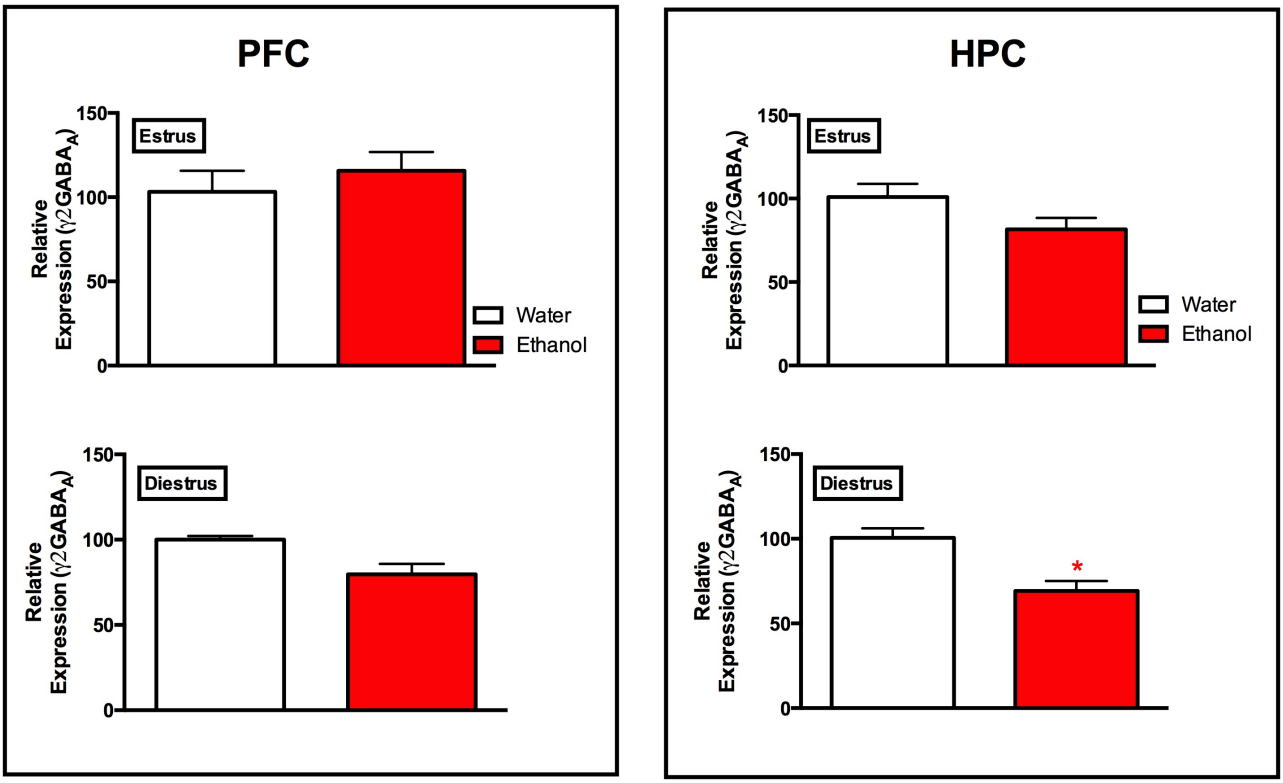

Fig. 31. Binge drinking was associated with a decrease in the expression of $\gamma 2$ subunit expression for diestrus females.

Table 1: Relationship between BEC and intake using DIDMSA

		ADOLESCENTS				ADULTS			
		MALES		FEMALES		MALES		FEMALES	
		D7	D14	D7	D14	D7	D14	D7	D14
1 HR	G/KG ETOH	$2.36^{\pm 0.57}$	$2.19^{\pm 0.20}$	$2.13^{\pm 0.44}$	$2.0^{\pm 0.31}$	$1.77^{\pm 0.37}$	$1.99^{\pm 0.34}$	$2.18^{\pm 0.32}$	$2.53^{\pm 0.56}$
	BEC	$61.2^{\pm 15.66}$	$54.78^{\pm 9.89}$	$67.1^{\pm 15.92}$	$62.21^{\pm 14.28}$	$44.2^{\pm 18.58}$	$66.1^{\pm 16.01}$	$82.77^{\pm 22.20}$	$76.19^{\pm 20.68}$
	R^2	0.70	0.94	0.64	0.59	0.64	0.56	0.38	0.33
	SAMPLE SIZE	8	8	11	11	10	10	8	8
2 HR	G/KG ETOH	$2.7^{\pm 0.26}$	$2.92^{\pm 0.44}$	$2.99^{\pm 0.51}$	$2.56^{\pm 0.38}$	$2.37^{\pm 0.17}$	$2.40^{\pm 0.11}$	$3.13^{\pm 0.66}$	$2.52^{\pm 0.19}$
	BEC	$73.2^{\pm 12.49}$	$102.33^{\pm 25.18}$	$77.2^{\pm 22.87}$	$66.63^{\pm 11.50}$	$85.7^{\pm 20.77}$	$94.12^{\pm 28.82}$	$98.9^{\pm 22.2}$	$58.8^{\pm 16.64}$
	R^2	0.83	0.69	0.81	0.34	0.63	0.50	0.43	0.63
	SAMPLE SIZE	8	8	11	11	8	8	7	7
3 HR	G/KG ETOH	$4.37^{\pm 0.57}$	$3.39^{\pm 0.46}$	$2.67^{\pm 0.71}$	$3.42^{\pm 0.42}$	$2.82^{\pm 0.71}$	$3.02^{\pm 0.41}$	$4.27^{\pm 0.57}$	$4.02^{\pm 0.4}$
	BEC	$120.5^{\pm 19.84}$	$74.49^{\pm 18.12}$	$83.1^{\pm 26.8}$	$89.23^{\pm 22.26}$	$77.5^{\pm 22.2}$	$95.57^{\pm 34.19}$	$106.1^{\pm 19.58}$	$107.88^{\pm 20.61}$
	R^2	0.91	0.69	0.67	0.34	0.62	0.51	0.22	0.63
	SAMPLE SIZE	7	7	6	6	8	8	9	9

LIST OF REFERENCES

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VITA

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Research Interests

Binge drinking, addiction, sex differences, GABA, receptor plasticity, behavioral plasticity, habit formation, depression, anxiety, individual differences

Education

Ph.D., Addiction Neuroscience, ***May 2014***
Indiana University-Purdue University Indianapolis

Dissertation: *Does Binge Drinking Induce PMDD-like Dysfunction for Female C57Bl/6J mice? Implications for Sex Differences in Addiction Vulnerability*
Advisor: Stephen L. Boehm, Ph.D
Additional Committee: Susie Swithers, Ph.D, Cris Czachowski, Ph.D, and Nicholas Grahame, Ph.D

M.S., Behavioral Neuroscience, ***May 2010***
Binghamton University

Topic: *$GABA_A$ receptors in the posterior but not anterior ventral tegmental area mediate RO 15-4513 attenuation of binge-like ethanol consumption in C57BL/6J females*
Advisor: Stephen L. Boehm, Ph.D
Additional Committee: Terry Deak, Ph.D and Norman Spear, Ph.D

B.A., Neuroscience (major)
Women's and Gender studies (minor) ***May 2007***
Middlebury College, Middlebury, VT

Topic: *Effect of ethanol pre-treatment on voluntary consumption and development of tolerance*
Advisor:Kim Cronise, PhD

Citizenship

Dual citizen of the United States of America and the Republic of Trinidad and Tobago

Funding /Awards

Student Merit Award, Research Society on Alcoholism	2012
Predoctoral Fellowship, NIAAA T32 (AA07462)	2011
Graduate STEM Fellow, NSF (I declined due to NIAAA T32)	2011
Student Merit Award, Research Society on Alcoholism	2011

Scholar, Southern Regional Educational Board 2011

NIAAA Travel Award, International Society for Biomedical Research on Alcoholism 2010

Travel Award, IUPUI School of Science Graduate Student Organization 2010

Student Merit Award, Research Society on Alcoholism 2010

Covance Diversity Poster Award, Indianapolis Society of Neuroscience 2010

Student Travel Award, Center for Developmental and Behavioral Neuroscience 2009

Student Merit Award, Research Society on Alcoholism 2009

Clark Fellowship, Binghamton University 2008

Student Merit Award, Research Society on Alcoholism 2008

Summer Research Grant, Posse Foundation 2006

Student Research Award and Stipend, Middlebury College 2006

Full tuition scholarship to Middlebury College (4yrs), Posse Foundation 2003

Research Experience

Graduate Research Assistant July 2009 to present

Alcohol Behavior Laboratory
IUPUI
Supervisor: Stephen Boehm, Ph.D

Graduate Research Assistant Sep 2007 to July 2009

Alcohol Behavior Laboratory
IUPUI
Supervisor: Stephen Boehm, Ph.D

Research Assistant March 2006 to May 2007

Alcohol Behavior Laboratory,
Middlebury College
Supervisor: Kim Cronise, Ph.D

Research Assistant June 2006 to May 2007

Women's and Gender Studies,
University of Minnesota
Supervisor: Sujata Moorti, Ph.D

Research Intern June 2005 to Aug 2005

Neurobiology of Apoptosis Laboratory,
Rutgers University
Supervisor: Wilma Friedman, Ph.D

Research Intern Jan 2002 to May 2003

Cancer Genetics Institute
Columbia University
Supervisors: Riccardo Dalla Favera, Ph.D and Giorgio Cattoretti, Ph.D

Refereed Journal Publications

1. Linsenbardt DN, Moore EM, Gross, CD, Goldfarb, KJ, Blackman LC, Boehm SL 2nd (2009) "Sensitivity and tolerance to the hypnotic and ataxic effects of ethanol in adolescent and adult C57BL/6J and DBA/2J Mice." *Alcoholism: Clinical and Experimental Research*, 33: 1-13.
2. Moore EM, Linsenbardt DN, MelónLC, Boehm SL 2nd (2009) "Adolescent C57BL/6J (but not DBA/2J) mice consume greater amounts of limited-access ethanol compared to adults and display continued elevated ethanol intake into adulthood." *Alcoholism: Clinical and Experimental Research*, 34: 734-742.
3. Moore EM, Linsenbardt DN, MelónLC, Boehm SL 2nd (2010) "Ontogenetic differences in adolescent and adult C57BL/6J and DBA/2J mice: Anxiety-like, locomotor, and consummatory behaviors. *Developmental Psychobiology*, 53: 141156.
4. Melón LC, and Boehm, SL 2nd (2011)" Role of genotype in the development of locomotor sensitization to alcohol in adult and adolescent mice: comparison of the DBA/2J and C57BL/6J inbred mouse strains." *Alcoholism: Clinical and Experimental Research* 35: 13511360.
5. Melón LC and Boehm SL 2nd (2011)" $GABA_A$ receptors in the posterior, but not anterior, ventral tegmental area mediate Ro15-4513-induced attenuation of binge-like ethanol consumption in C57BL/6J female mice." *Behavioural Brain Research*, 220: 230237.
6. Melón LC, Wray KN, Moore EM, Boehm SL 2nd (in press)" Sex and age effects on alcohol responsivity following abstinence." *Pharmacology Biochemistry and Behavior*.

Conference Abstracts

1. Sindel CE, Guarnier M, Blackman L, Cronise K (2006) Expression of sensitization to the locomotor stimulating effect of ethanol is dependent on contextually conditioned cues in DBA/2J mice. Abstract Viewer and Itinerary Planner, Atlanta, GA: Society for Neuroscience, online.
2. Cronise K., Guarnier M, Blackman L, Cook A (2007) Ethanol tolerance may enhance ethanol consumption in a modified drinking in the dark paradigm. Alcoholism: Clinical and Experimental Research, 31:105A.
3. Linsenbardt DN, Moore EM, Blackman L, Boehm SL 2nd (2008) Differential sensitivity to the acute locomotor effects of $GABA_A$ drugs following ethanol-induced sensitization in DBA/2J mice. Alcoholism: Clinical and Experimental Research, 32:158A.
4. Blackman LC, Linsenbardt DN, Boehm SL 2nd (2008) Binge-like ethanol intake alters GABA receptor subunit/subtype expression in C57BL/6J Mice. Alcoholism: Clinical and Experimental Research, 32:229A.
5. Moore EM, Mariani JN, Blackman LC, Linsenbardt DN, Boehm SL 2nd (2008) Binge-like alcohol intake is increased during early adolescence in C57BL/6J and DBA/2J mice. Program No. 257.6. 2008 Abstract Viewer and Itinerary Planner, Washington, DC: Society for Neuroscience, online.
6. Blackman LC, Moore EM, Goldfarb KJ, Mariani JN., Linsenbardt DN, Boehm, SL 2nd (2008) RO15-4513 alters female C57BL/6J consummatory behavior in drinking in the dark model. Program No.158.6. Abstract Viewer and Itinerary Planner, Washington, DC: Society for Neuroscience, online.

7. Blackman LC, Moore EM, Boehm SL 2nd (2009) Acute and sensitized ethanol induced locomotor activity in adult and adolescent mice: A comparison of the DBA/2J and C57BL/6J strains. Alcoholism: Clinical and Experimental Research, 33:212A.
8. Moore EM, Blackman LC, Linsenbardt DN, Boehm SL 2nd (2009) Adolescent C57BL/6J and DBA/2J mice show differential preference to ethanol in a two bottle Drinking in the Dark procedure. Program No. 252.10. 2009 Abstract Viewer and Itinerary Planner, Chicago, IL: Society for Neuroscience, online.
9. Blackman LC and Boehm SL 2nd (2009) Binge drinking in the DID paradigm changes locomotor response to 2.0g/kg challenge of EtOH in C57Bl/6J females Program No. 445.7. 2009 Abstract Viewer and Itinerary Planner, Chicago, IL: Society for Neuroscience, online.
10. Moore EM, Melón LC, K.N. Wray, Boehm SL 2nd (2010) Ethanol induced conditioned taste aversion in adolescent and adult C57BL/6J and DBA/2J mice. Alcoholism: Clinical and Experimental Research, 34(6):240A.
11. Bracken AL, Melón LC, Boehm SL 2nd (2010) Age-dependent reduction of drinking-in-the-dark by the $GABA_A$ partial inverse agonist RO 15-4513 in adolescent and adult B6 mice. Alcoholism: Clinical and Experimental Research, 34(8):91A.
12. Melón LC, EM Moore, K. N. Wray, Boehm SL 2nd (2010) The effect of repeat, forced administration of ethanol during adolescence on voluntary intake and duration of sensitization in DBA/2J and C57Bl/6J Mice. Alcoholism: Clinical and Experimental Research, 34(6):240A.
13. Boehm SL 2nd, Melón LC, Moore EM, Bracken AL, Sissons HT, Wray K, Best C, Grahame NJ (2010) Adolescent and adult High Alcohol Preferring selectively bred mice engage in binge-like alcohol intake using drinking in the dark procedures. International Neurobehavioral Genetics Meeting in Halifax, Canada.
14. Melón LC and Boehm SL 2nd (2010) Posterior (but not anterior) VTA RO 15-4513 decreased binge-like ethanol consumption in C57Bl/6J females. Alcoholism: Clinical and Experimental Research, 32:158A.
15. Moore EM, Melón LC, Schneider BL, Wray KN, and Boehm SL 2nd (2010) Modulation of ethanol induced locomotor stimulation by baclofen in adolescent and adult mice. International Society for Developmental Psychobiology. San Diego, CA.
16. Melón LC, Nolan ZT, Moore EM and Boehm SL 2nd (2010) Intra-VTA microinjection of gaboxadol modulates home-cage activity and has estrous-cycle dependent effects on alcohol intake in C57BL/6J mice. Program No. 66.11 Abstract Viewer and Itinerary Planner, San Diego, CA: Society for Neuroscience, online.
17. Melón LC, Wray KN, Moore EM, Schneider BL, Forrest IV RD, Nolan ZT and Boehm SL 2nd (2011) The effect of adolescent alcohol intake on sensitivity to ethanol-induced motor impairment and locomotion during adulthood. Alcoholism: Clinical and Experimental Research, 32:158A.
18. Melón LC, Stahl RM, Boehm SL 2nd (2012) Sex differences in the effect of binge drinking history on alcohol preference and anxiety-like behavior in C57BL/6J mice. Alcoholism: Clinical and Experimental Research, 36: 112A.
19. Melón LC, Nolan ZT, Boehm SL 2nd (2012) Activation of $GABA_A$ receptors and inhibition of neurosteroid synthesis have separable estrous-

dependent effects on binge drinking in female mice. Alcoholism: Clinical and Experimental Research, 36:184A.

Invited Talks

AGEP Midwest Regional Conference: 2011

Intra-VTA microinjection of gaboxadol modulates home-cage activity and has estrous-cycle dependent effects on alcohol intake in C57BL/6J mice

Indianapolis Society for Neuroscience Annual Meeting: 2012

Estrous status and $GABA_A$ sensitivity: Implications for the pharmacological manipulation of ethanol consumption in female bingers

Research Society on Alcoholism Annual Meeting: 2013

Sex differences in the development of negative affect and anxiety following repeated binge drinking (Paper Session)

Teaching Experience

Teaching Assistant Fall 2006

Physiological Psychology
Middlebury College

Teaching Assistant Fall 2007

Physiological Psychology
Binghamton Unviersity

Teaching Assistant Fall 2007

Learning Lab
Binghamton Unviersity

Instructor on Record Fall 2010 – Spring 2011

Introduction to Psychology
Indiana University-Purdue University Indianapolis

Service

Middlebury College 2006 – 2007

Co-founder and President :MiDDialouges: a presently active organization that develops and facilitates workshops to discuss/create plans of action in response to cultural, racial or gender issues on campus and in the community.

Binghamton University March, 2009

Brain Awareness Week (March, 2009): as a member of a group of graduate students and faculty who helped to organize activities regarding the brain and present those to young students (Ages 5-12) at the African Road Elementary school in Vestal, NY.

IUPUI 2010 – 2012

Co-founder and Co-President (2010-2012), Psychology Graduate Student Organization at IUPUI.

IUPUI March, 2011

Co-organized a trip of psychology graduate students to Arsenal High School in order to discuss our research and interest in the brain with 9-12th graders.

ADDICTION 2013-

Ad hoc reviewer