THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

Adolescent Alcohol's Effects on Adult Drinking and Somatostatin Neurons

MATT SPRINGER
SPRING 2024

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biology
with honors in Biology

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ABSTRACT

Adolescence serves as a vital period of brain development. Reorganization of the prefrontal cortex is one of the most important and prominent biological changes during this time. In this region, the subclass of neurons expressing somatostatin play an important role. These adolescent brain changes lead to complex changes in behavior, particularly in the area of risk-taking. These effects have been shown to be modulated by substances such as alcohol, which is one of the most prevailing substances of abuse in society today. The effects of alcohol on brain circuitry are often modeled in mice, as in this research. Alcohol is most dangerous when consumed in high quantities, such as during bouts of binge drinking. Literature in humans and animal models has shown that adolescent exposure to alcohol generally increases alcohol consumption in adulthood. Other literature has shown that SST neurons in the prelimbic cortex are implicated in binge drinking behavior. This thesis examines the effects of adolescent alcohol exposure in mice through a Drinking in the Dark (DID) model before repeating the DID model in adulthood or diverting mice into an alternative 2 Bottle Choice (2BC) model. Electrophysiology at the time of adult exposure was conducted to examine the intrinsic excitability of SST neurons in the prelimbic cortex prior to adult alcohol exposure in adolescent exposure mice. Results showed that SST neurons in the prelimbic cortex were hyperexcitable at the time of adult drinking models in adolescent start drinking mice. No discernible changes were seen in the DID and 2BC models between mice that began drinking in adolescence and mice that began drinking in adulthood. Taken together, this suggests that in mouse models, biological adaptations driven by adolescent alcohol consumption do not drive increased consumption of alcohol in adulthood, and other social and environmental factors should be considered in humans.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank Dr. Crowley and Dr. Assmann for serving as the committee members for this thesis. I would also like to thank Avery Sicher for her years as a research mentor. Thanks also go to Keith Griffith, David Starnes, and the rest of the Crowley lab team for providing training and support.

This work was funded by the National Institutes of Health (P50AA017823, R01AA029403 to NAC, F31AA0304550 to ARS), a Penn State's Erickson Award (to MS) and The Huck Institutes of the Life Sciences Endowment Funds (to NAC).

Chapter 1

Literature Review

Adolescent Brain Development

Adolescence is defined broadly as the period of development between childhood and adulthood (Spear, 2000). This timeframe encompasses the time before, during, and after puberty, which is defined more clearly by physical markers of sexual maturation. During adolescence, numerous changes happen throughout the brain that contribute to physical maturation. Some of the most significant changes occur within the prefrontal cortex. These changes manifest in adolescents through increases in reward-seeking and risk-related behaviors (Spear, 2000).

Inhibitory neurotransmission undergoes significant development and expansion. Pyramidal neurons act as the basic excitatory cells in the prefrontal cortex. These cells receive inputs, including inhibition, onto their dendritic spines. Throughout early development, the high density of spines allows for continual reorganization of circuity based on experience. As the brain develops, synaptic pruning occurs, which greatly reduces density at spines and delivers more refined circuits and removes redundant or underused synapses (Afroz et al. 2016). Pruning also has great energetic benefits, as maintaining the level of synapses present during childhood would be costly (Spear, 2013).

Throughout adolescence, the inputs onto pyramidal cells trend from being largely excitatory (i.e., from other pyramidal cells) towards inhibitory. Inhibitory cells expressing parvalbumin and somatostatin mature and integrate into cortical circuits and influence changes in behavior (Rudy et al. 2011). Parvalbumin (PV) is a calcium binding protein that serves as a

buffer regulating intracellular calcium levels. Somatostatin (SST) is a neuropeptide expressed across the body and in the brain in subsets of neurons. PV and SST can be used to distinguish largely non-overlapping cell types (Ferrer & Garcia, 2022). Levels of these proteins and their respective cells change throughout development and are shown to have sex-dependent and species-dependent interactions (Joffe et al. 2020). For example, while PV cell density only increases in male mice, it increases in both sexes of rat. PV protein expression increases in both male and female mice as well (Binette et al. 2023). For SST neurons, protein levels were only found to increase in female mice (Girgenti et al, 2019) The interplay between the changes seen in these neuronal subtypes and pyramidal cells expands the control exerted by inhibitory circuits. Importantly, these developmental trajectories likely differ across both cortical regions, and non-cortical structures.

Somatostatin

SST is a peptide expressed throughout the body with many important physiological roles. The two forms of somatostatin, labelled SST-14 and SST-28, are produced from the same precursor molecule, protosomatostatin (Yamada et al. 1992). SST has five receptors (canonically called SSTR-1 to SSTR-5). SST-14 and SST-28 differ in where they are most expressed (O'Carroll et al 1992). While SST expression within the central nervous system as a neuropeptide is most relevant here, it is expressed in organs diffusely. SST-28 is primarily found within the periphery and organs (Shamsi et al. 2021). The various receptors of somatostatin bind each form with different affinities. In the pancreas, SST inhibits the release of insulin and

glucagon. Within the gastrointestinal tract, somatostatin regulates gastric secretion, serving to reduce hormone release (Strowski et al. 2000).

In the brain, SST serves as an identifier for a subclass of inhibitory neurons throughout the brain, including the cortex (Isaacson et al. 2011). The primary isoform of SST located within these cells is SST-14, but both forms are expressed. SST neurons in the cortex serve a variety of important functions in maintaining brain function, including suppression of sensory neurons projecting to the sensory cortex, modulation to the visual and auditory cortexes, and generation of cortical slow waves critical to non-rapid eye movement sleep (Funk et al. 2013). In the prefrontal cortex, γ-Aminobutyric acid (GABA) neurons expressing SST synapse on pyramidal cells, which are the primary source of excitatory activity within the prefrontal cortex (Song et al. 2021), as well as on non-pyramidal GABAergic cells, allowing for robust modulation of prefrontal circuits (Cummings and Clem 2020; Dao et al., 2021). These inhibitory cells are involved in modulating cortical circuits including a microcircuit implicated in alcohol-binge consumption (Dao et al. 2021). The neuropeptide can exert inhibitory effects on pyramidal neurons independently of the co-expressed neurotransmitter GABA, as seen in bath application experiments (Brockway et al. 2023).

At the synapse level, SST can be released via exocytosis of dense core vesicles into the synaptic cleft. This process has been shown to be calcium-dependent and can occur with simultaneous release of GABA from the same cell (Patel, 1999). In this case, SST can bind to SSTRs and promote downstream inhibition of excitatory activity. While this is produced via an increase in calcium activity in the presynaptic cell, the effect in the postsynaptic cell is to decrease calcium influx and thus reduce cyclic AMP activity in turn. When coupled to the

traditional inhibitory GABAergic input, this can produce a strong modulatory effect on excitatory activity in the cortex (Song et al. 2021).

The importance of furthering understanding of SST neurons is not limited to their role in binge-drinking behavior but extends to many of the most prevalent and deadly neurodegenerative diseases (Song et al., 2021). In Parkinson's disease, reduced levels of SST cells in cortical regions of the PARK2 subtype may contribute to the extreme motor dysfunction that is the hallmark symptom of that disease (McGregor & Nelson, 2019). In Schizophrenia, SST neuron loss in brain regions projecting to the ventral tegmental area dopaminergic system, such as the hippocampus, has been postulated to play a role in driving symptoms. Even Alzheimer's Disease, the most common neurodegenerative disease, has shown changes in SST neuron expression. Reduced SST protein expression in areas including the cortex is associated with Alzheimer's (Terry & Katzman, 1983). Further research into the mechanistic effects of the SST peptide within this disease is needed, but previous research has shown potential relationships between SST and beta-amyloid clearance as well as SST and downregulation of tau hyperphosphorylation (Ramos et al., 2006). As beta-amyloid and tau hyperphosphorylation are heavily implicated in Alzheimer's pathology, SST presents an area for future research in this field.

SST and its role in risk taking and binge drinking behavior has been investigated extensively by the Crowley lab. SST neurons were shown to control a binge-drinking microcircuit in the prelimbic cortex of mice. The intrinsic excitability and excitatory activity of prelimbic (PL) cortical SST neurons was altered by the exposure to binge consumption of alcohol (Dao et al, 2021). These effects contributed to an observed hypoactive state of these neurons in the binge-drinking circuit. Targeted manipulation of this population of neurons via both excitatory and inhibitory DREADDs (*D*esigner *R*eceptors *E*xclusively *A*ctivated by

*D*esigner *D*rugs, a behavioral pharmacology tool for in vivo circuit manipulation) was able to cause a reduction in the binge-drinking behavior (Dao et al, 2021). Somatostatin neurons in the bed nucleus of the stria terminalis, a subregion of the amygdala and a locus of fear, anxiety, depression, and alcohol-withdrawal, were also shown to play a role in binge-drinking behavior. Activation of SST neurons here reduced binge-drinking behavior in only female mice (Nair et al, 2022). Research has also shown that the SST peptide modulates cortical circuitry and is able to promote exploratory behaviors including in an elevated plus maze and open field test (Brockway et al. 2023).

Adolescent Behavioral Changes

The adolescent period is marked by complex behavioral changes. Adolescence is marked by increased socialization with peers, risk-taking behavior, and novelty-seeking (Spear, 2000). Many of the social skills learned by humans as well as in rodent models are gained during adolescence. In rodents, there is an increase in time spent performing social actions such as play fighting and mock mating (Primus et al. 1989). This is similarly true for human adolescents. Human adolescents perceive time spent with peers as positive experiences. The increase in time spent with similarly aged companions is coupled with an increase in reported number of conflicts with parents (Steinberg et al. 1989).

Risk-taking behavior is an important area of behavioral change in adolescents. Hypothesized as potentially increasing reproductive success within a competitive environment, both humans and rodent models are more willing to take risks in adolescence than in any other stage of life (McCormick & Telzer, 2017). Rodent models show increased activity in new

environments during their analogous adolescence when compared to mature rodents. These adolescent rats also show hyper-reactivity to startling stimuli (Spear, 2000). These patterns of behavior are logical when examining the lifespan of the rodent. Rats first start their move towards independence when they eat solid food at approximately 18 days. By 28 days, they often have left the burrow for the first time and are proficiently exploring the near surroundings by 34 days. By engaging in more risky behaviors such as exploration of a novel environment, these young rats are able to accelerate the speed at which they learn about their surroundings and the world at large. This behavior includes the tradeoff that they face larger chances of predation and injury (Spear, 2000).

Humans also seek out novel experiences, many of which include exploration of their environment. These behaviors include recreational drug use, sexual activity without protection or with multiple partners, and engaging in minor crimes including theft and trespassing (Spear, 2000). While these activities occur at elevated rates indicating a normal progression of development, they bring with them increased danger and risk. 30% of adolescent deaths arise from accidents while 15% from homicides and another 12% from suicides (Balocchini et al. 2013). These produce a substantial increase in mortality risk during the adolescent period. Beyond mortality, approximately half of the diagnosed sexually transmitted infections for this group come from sexual behavior classified as having been risky (Balocchini et al. 2013).

However, risk taking behavior is also a normal and important process in development. Seeking out novel experiences acts as a strong reinforcer during adolescence, contributing to increased occurrences of risky behaviors. These processes can create positive feelings of inclusivity and independence, which can lead to higher senses of self-esteem. There is also research that shows risk taking behavior leads to stronger social skills (Duell et al. 2021). While

risk-taking is widely seen in development, some manifestations such as alcohol use can have serious acute and chronic effects.

Prevalence of Alcohol Exposure

In humans, data show the substantial risks posed by alcohol consumption and its widespread use during adolescence. While the percentage of 8th, 10th, and 12th graders having used alcohol in the past 45 years has declined steadily, the percentage of adolescents drinking remains high. Among 8th graders, 15.2% have used alcohol within the last year. For 10th graders, this number doubles to 31.3%. By the time these adolescents reach 12th grade, 51.9% have used alcohol in the last year. Numbers for other substances with the potential to disrupt neural circuitry in the PFC, such as nicotine vaping and narcotic consumption, are present at much lower levels but nonetheless are a serious concern (*Monitoring the Future Survey*, 2022). The widespread prevalence and ease of access attributed to alcohol, combined with its serious potential for disruptive effects in the adolescent brain, make it vital to understand its interactions with the brain.

Beyond exposure, binge-like drinking presents a more substantial risk to adolescents. At a basic level, binge-drinking is defined as the consumption of alcohol in a pattern that leads to a blood alcohol concentration above 0.08%, which typically occurs by drinking 5 drinks within a 2-hour period for men and 4 drinks for women. Binge drinking presents serious risks from an individual and societal standpoint. Individuals who binge drink are more likely to face acute bodily harm such as from a motor vehicle collision, poisoning from alcohol, or an accident such as a burn or fall. Over the long term, binge drinking can lead to a swathe of risks including

various cancers, heart and liver disease, and cognitive deficits (Chung et al. 2018). Moving to a societal level, costs associated with increased expenditures due to binge drinking reached $191 billion in 2010. This value came from diminished ability to work, medical expenses, and legal costs. (*CDC Fact Sheet: Binge Drinking,* 2024).

Binge drinking peaks during adulthood, but substantial amounts of high school aged individuals and young adults partake in this heavy drinking behavior, with 14% and 21% having self-reported partaken in binge drinking respectively (*Monitoring the Future Survey*, 2022). While binge drinking is defined as 5 and 4 drinks for men and women respectively, it is important to consider adolescent physiology and behavior when applying this definition to this subpopulation. Adolescents are able to reach significant levels of intoxication with fewer drinks than adults (Spear, 2013). Further, 25% of adults who partake in a drinking binge consume 8 or more drinks in a period while this value rose to 44% among high school binge-drinkers (*Monitoring the Future Survey*, 2022).

Studies of Human Alcohol Exposure

In the adolescent brain, exposure to alcohol can manifest in a multitude of physiological changes. One of the substantial areas of change includes the reorganization of white matter development. Under normal conditions, white matter increases from childhood into young adulthood before declining in later adulthood. During this time, grey matter is being pruned away, having reached peak levels. As these changes occur, the introduction of alcohol can lead to impairments in structure development. Comparisons of white and grey matter volumes among adolescents with alcohol use disorders and controls show that alcohol contributed to significantly

altered white matter volumes and decreased grey matter volumes in the cortices (Squeglia et al. 2015). White matter is linked with executive functioning, so this alteration to its development during adolescence proves to be an important area for study. The primary white matter tracts that connect hemispheres at the corpus callosum are an area of study for the interplay between alcohol use and cognitive functions. Studies show that a reduction in volume in the corpus callosum, a key fiber tract in the brain, is linked with decreased cognitive function (Huang et al. 2015). These changes contribute to an overall decrease in volume of the prefrontal cortex among adolescents with alcohol use (Sicher et al. 2022).

Many studies have also been conducted examining adolescent alcohol exposure and risk of later development of an alcohol use disorder. Across the past 20 years, cross-sectional studies of age of first alcohol use and later alcohol use disorders have consistently shown a clear relationship between these variables. An early study using data from the National Longitudinal Alcohol Epidemiologic Survey showed that drinking starting at 14 or younger led to a 7% increase in likelihood of lifetime abuse from 4% among those who started drinking at 20 or older versus 11% for the early group. The rate of lifetime alcohol dependence for these groups rose from 10% for the late-start versus 40% for the early-start (Grant & Dawson, 1997). Additional studies have shown the relationship between binge drinking among adolescents and the development of an alcohol use disorder (AUD). The gap between those who reported binge drinking across the past year and those who did not who went on to develop an AUD was 11.6% for the binge group and 0.9% for the non-binge group (Addolorato et al. 2018). These studies show that adolescents who drink, particularly those who engage in binge-like patterns of consumption, face much higher risks of developing an AUD during their lives.

Conclusions

Adolescence serves as a vital time for brain development, with substantial changes occurring in the prefrontal cortex. These changes give rise to alterations in behavior, where risk-taking behavior becomes more frequent. Substance misuse begins to occur more substantially during this timeframe, with alcohol misuse being a widely studied example. Previous research has shown that SST neurons are modified by binge-drinking (Dao et al. 2021).

In this report, adolescent alcohol exposure effect on adulthood alcohol use is examined with respect to alterations in the excitability of somatostatin neurons. Cohorts of mice were run through an adolescent drinking model or water control before undergoing adult drinking in two models of consumption. These models examine patterns of consumption and preference in binge-drinking conditions. Electrophysiology was also conducted on age-matched adolescent drinking mice to examine alterations in SST neurons of the prelimbic cortex, where these neurons have been shown to have a role in binge-drinking behavior. From the previous literature across the field and work within the Crowley lab, I hypothesized that adolescent consumption would modulate these SST-expressing neurons and lead to changes in preference and consumption during adulthood exposure to alcohol.

Chapter 2

Methods

Mice

For experiments, male and female SST-Cre:Ai9 and SST-Cre:Ai32 mice were used. SST-SST-Cre:Ai9 mice express tdTomato fluorescence in response to Cre-based recombination in SST cells (Chen et Al. 2023). SST-Cre:Ai32 express channel rhodopsin in response to Cre-based recombination in SST cells (Wilson et Al. 2023). All experiments were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Mice were weaned at post-natal day 21 (PND 21) and placed into a reverse light cycle in single housing for the duration of experimentation.

Adolescent Drinking in the Dark (DID)

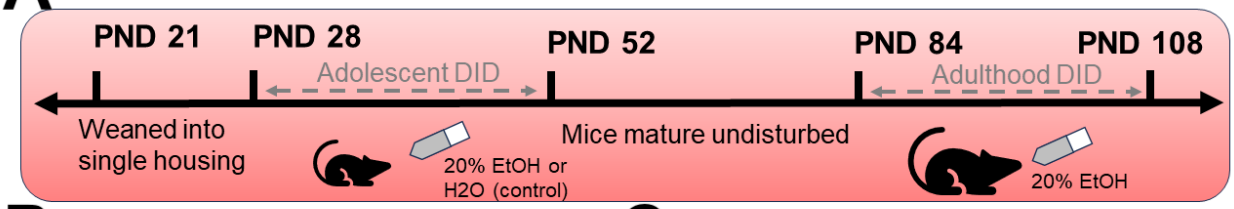
SST-Cre:Ai9 mice were weaned at PND 21 and separated into single housing. Adolescent Drinking in the Dark (DID) began at PND 28 and ran through PND 52. DID is a well-validated model of voluntary binge drinking (Rhodes, 2005), which allows for both high levels of alcohol consumption and individual variability (Crowley et al., 2019). Over four weeks, mice were provided access to 20% ethanol (EtOH) in tap water. Each week, mice received 2 hours of access to their substance over the first 3 days. On the fourth day, mice were given access for a 4-hour period. This longer period of access represented the binging day. For days 5-7, the mice were not provided access to alcohol. This pattern was repeated until the conclusion of the 4-week period. The amount of EtOH consumed was measured at the conclusion of the 2-hour

session and at the halfway point and end of the 4-hour session by comparing the weight prior to the session to after the drinking period. Mice had access to food ad libitum throughout the study, and access to water with the exception of the 2-hour and 4-hour EtOH sessions. Values for daily drinking were excluded if bottle weights changed by more than 2g in 2 hours, which indicated a substantial leak. For total EtOH, excluding first exposure or first binge calculations, the average consumption on the other days that week was used so that equal numbers of drinking sessions for total drinking were compared.

Drinking in the Dark in Adulthood

In order to compare how adolescent drinking influences adulthood drinking, a subset of mice underwent a "double drinking" exposure paradigm. For the double drinking cohort, mice that underwent adolescent DID were then allowed to mature undisturbed in single housing with no access to alcohol (i.e., from PND 52 through PND 83 the mice received only water and food). Starting at PND 84, the mice underwent the same 4-week cycle of DID as they had previously during their adolescence. An adolescent water only control group was provided EtOH in adulthood to examine the effects of adolescent drinking (Figure 1).

A



B

Adolescent DID

	Day 1	Day 2	Day 3	Day 4
Week 1 PND 28-31	2H	2H	2H	**4H**
Week 2 PND 35-38	2H	2H	2H	**4H**
Week 3 PND 42-45	2H	2H	2H	**4H**
Week 4 PND 49-52	2H	2H	2H	**4H**

C

Adulthood DID

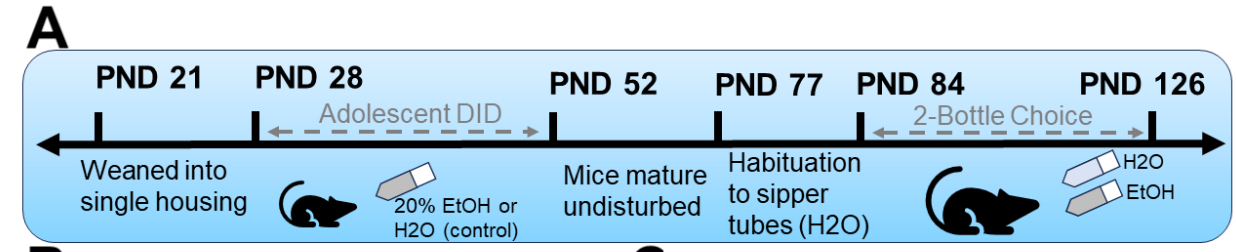
	Day 1	Day 2	Day 3	Day 4
Week 1 PND 84-87	2H	2H	2H	**4H**
Week 2 PND 91-95	2H	2H	2H	**4H**
Week 3 PND 98-101	2H	2H	2H	**4H**
Week 4 PND 105-108	2H	2H	2H	**4H**

Figure 1. The timeline and breakdown for the double drinking in the dark drinking model. Figure 1A shows the timeline with a weaning period, first drinking session in adolescence, maturation period, and second drinking period in adulthood. Figure 1B shows the 4-week period of drinking in adolescence, where mice are given 3 days of 2-hour access and a fourth day of 4-hour access. Figure 1C shows the 4-week period of drinking in adulthood with the same level of access.

Two Bottle Choice (2BC)

We also assessed adolescent binge drinking-induced changes in a non-binge model of alcohol consumption. The 2-bottle choice (2BC) cohort that underwent adolescent DID were then allowed to mature undisturbed in single housing with no access to alcohol (i.e., from PND 52 through PND 83 the mice received only water and food). From PND 28 through PND 76, the mice were allowed to mature undisturbed in single housing (Dao et al. 2020). Starting on PND 77, mice were given a week of habituation to sipper tubes for the 2-bottle choice cycle. Starting at PND 84, the mice were given 24-hour access to a water sipper tube and an EtOH sipper tube.

For PND 84-85, the concentration of EtOH was 3%. From PND 86-91, the concentration of EtOH was increased to 7%. At PND 92, the concentration was increased to the final 10%. The amount of both water and EtOH consumed were measured every 48 hours. The drinking paradigm concluded on PND 126 (Figure 2). By providing both water and EtOH at the same time, this experiment allowed the assessment of both non-binge alcohol consumption and preference for alcohol over water.



B **Adolescent DID**

	Day 1	Day 2	Day 3	Day 4
Week 1 PND 28-31	2H	2H	2H	**4H**
Week 2 PND 35-38	2H	2H	2H	**4H**
Week 3 PND 42-45	2H	2H	2H	**4H**
Week 4 PND 49-52	2H	2H	2H	**4H**

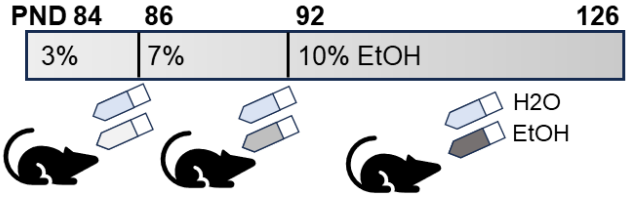


Figure 2. The timeline for breakdown for the 2-Bottle Choice drinking model. Figure 2A shows the timeline beginning with the weaning period followed by adolescent DID, a maturation period, a habituation period, and the 2-Bottle Choice drinking. Figure 2B shows the adolescent drinking in the dark period of 4 weeks with each week consisting of 3 days of 2-hour access and a 4th day of 4-hours of access. Figure 2C shows the change in concentration of EtOH that occurs across the 2-Bottle Choice model.

Electrophysiology

Whole-cell patch-clamp electrophysiology was conducted at 24 hours and 30 days post-adolescent alcohol. Mice were anesthetized via isoflurane inhalation and rapidly decapitated to allow for brain removal. Removed brains were transferred to oxygenated N-methyl-d-glucamine (NMDG) cutting solution consisting of 93 mM NMDG, 2.5 mM KCl, 1.2 mM NaH_2PO4, 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM dextrose, 5 mM ascorbic acid, 2 mM thiourea, 3 mM sodium pyruvate, 10 mM $MgSO_4$-7 H2O, 0.5 mM $CaCl_2$-2 H_2O, 306–310 mOsm, pH to 7.4. Prelimbic cortex slices 300 μm were prepared with a Compresstome vibrating microtome (Precision Instruments). Slices were placed in 31 °C heated oxygenated NMDG for recovery for ten minutes. Slices were transferred to heated, oxygenated artificial cerebrospinal fluid (aCSF) consisting of 124 mM NaCl, 4.0 mM KCl, 1.2 mM MgSO4-7 H_2O, 2.0 mM $CaCl_2$–$2H_2O$, 1 mM NaH_2PO4–H_2O, 305–308 mOsm. Slices rested in aCSF for a minimum of an hour before experimentation began. During the experiment, slices were placed in a submerged chamber and supplied with heated, oxygenated aCSF at 2 mL/min. SST neurons in the prelimbic cortex were identified via fluorescent tdTomato reporter with 565 nm LED excitation. Recording electrodes were pulled from thin-walled borosilicate glass capillaries with a Narishige P-100 Puller. Electrodes were filled with a potassium gluconate-based intracellular recording solution consisting of 135 mM potassium gluconic acid, 5 mM NaCl, 2 mM $MgCl_2$–$6H_2O$, 10 mM HEPES, 0.6 mM EGTA, 4 mM Na_2ATP, and 0.4 mM Na_2-GTP, 287–290 mOsm, pH 7.35. Intrinsic excitability was measured by examining the number of action potentials fired during a voltage-current (VI) protocol. The protocol consisted of stepwise increases in current injected into the target cell (0-200 pA, 10 pA per step per 300 ms). The protocol included negative

current injection controls and was carried out at the resting membrane potential of each cell followed by a uniform -70 mV resting potential.

Data Analysis

Data were analyzed in GraphPad Prism 7.0 (San Diego, CA, United States). Measurements of consumption are expressed in terms of grams of EtOH per kilogram of body weight (g/kg). For adolescent drinking, a two tailed unpaired t-test comparing total consumption between males and females was used. A mixed effects ANOVA was also produced to examine the changes in drinking across the 4-week cycle between the sexes. A mixed-effects ANOVA test was carried out for the adult DID group examining the sex, adolescent drinking, and sex by adolescent drinking effects. The alcohol consumption during the first 2-hour drinking period of adulthood, the first 4-hour period of adulthood, and total consumption during adulthood were examined. A two tailed unpaired t-test was produced for the 2BC group total fluid consumption among females during the habituation week. Two mixed effects ANOVA were conducted for the 2BC group, one examining daily preference and one examining daily consumption. The daily consumption ANOVA compared consumption across the 6 days of the 2BC protocol. The factors for the test were day of drinking and adolescent drinking. The daily preference ANOVA measured water versus EtOH preference by dividing the EtOH consumed by the total fluid consumed. The factors were day of drinking, adolescent drinking, and day of drinking by adolescent drinking. A mixed-effects ANOVA was used for the VI electrophysiology experiment. The factors for this ANOVA were sex, adolescent drinking, and current step.

Chapter 3

Results

Adolescent DID Results

For adolescent consumption, the total consumption and daily consumption were analyzed. For the total consumption between sexes, there was no significant difference in consumption (Figure 3B; $t(32) = 0.05595$, $p = 0.9557$). Across the 4 weeks of the model, there was no effect of the binge-day (Figure 3C; $F(2.500, 76.65) = 0.9729$, $p = 0.3981$). There was an effect of sex ($F(1, 32) = 6.281$, $p = 0.0175$). There was no interaction between sex and the binge day $F(3, 92) = 1.055$, $p = 0.3677$).

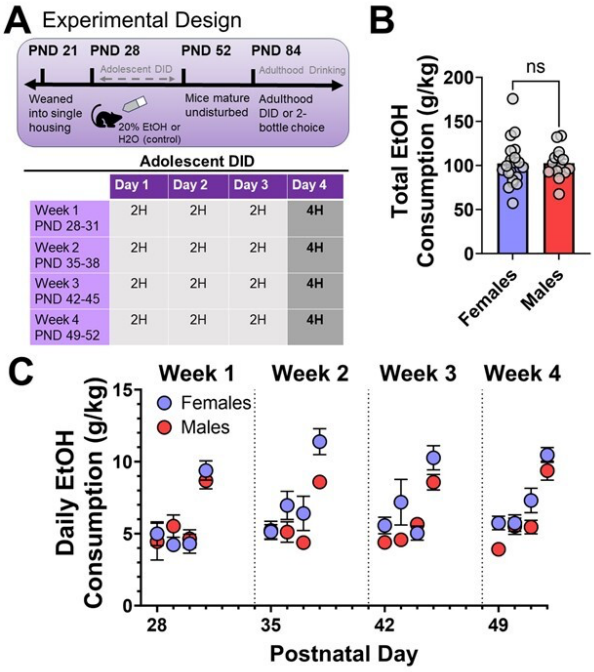


Figure 3. The results of the adolescent DID. Figure 3A shows the experimental timeline. Figure 3B shows the total consumption by sex during the 4-week model. Figure 3C shows the daily consumption across the model. Data are displayed as the mean +/- SEM (n=12 F, n=11 M).

Double Drinking Results

For the Double Drinking experiment, the first two hours of drinking in adulthood, the first 4-hour binge day, and the total EtOH consumed were all measured and analyzed. For the first two hours of adult exposure on PND 84, there was no effect of sex (Figure 4A; *(F(*1, 39) = 1.055, p = 0.3107). There was also no effect of adolescent DID *(F(*1, 39) = 0.005556, p = 0.9410). Further, there was no effect from the interaction of sex and adolescent DID *(F(*1, 42) = 1.305, p = 0.2598). For the first binge day on PND 87, there was no interaction between sex and adolescent DID (Figure 4B; *(F(*1, 42) = 0.9011, p = 0.3479). There was an effect of sex (*F*(1, 42) = 6.098, p = 0.0177). There was no main effect of adolescent DID (*F*(1, 42) = 1.305, p = 0.2598). For the total EtOH consumed during adulthood, there was an effect of sex (Figure 4C; (*F*(1, 43) = 36.29, p < 0.0001). There was also an effect of adolescent DID (*F*(1, 43) = 5.571, p = 0.0229). There was no effect from the interaction of adolescent DID and sex (*F*(1, 43) = 0.2673, p = 0.6078).

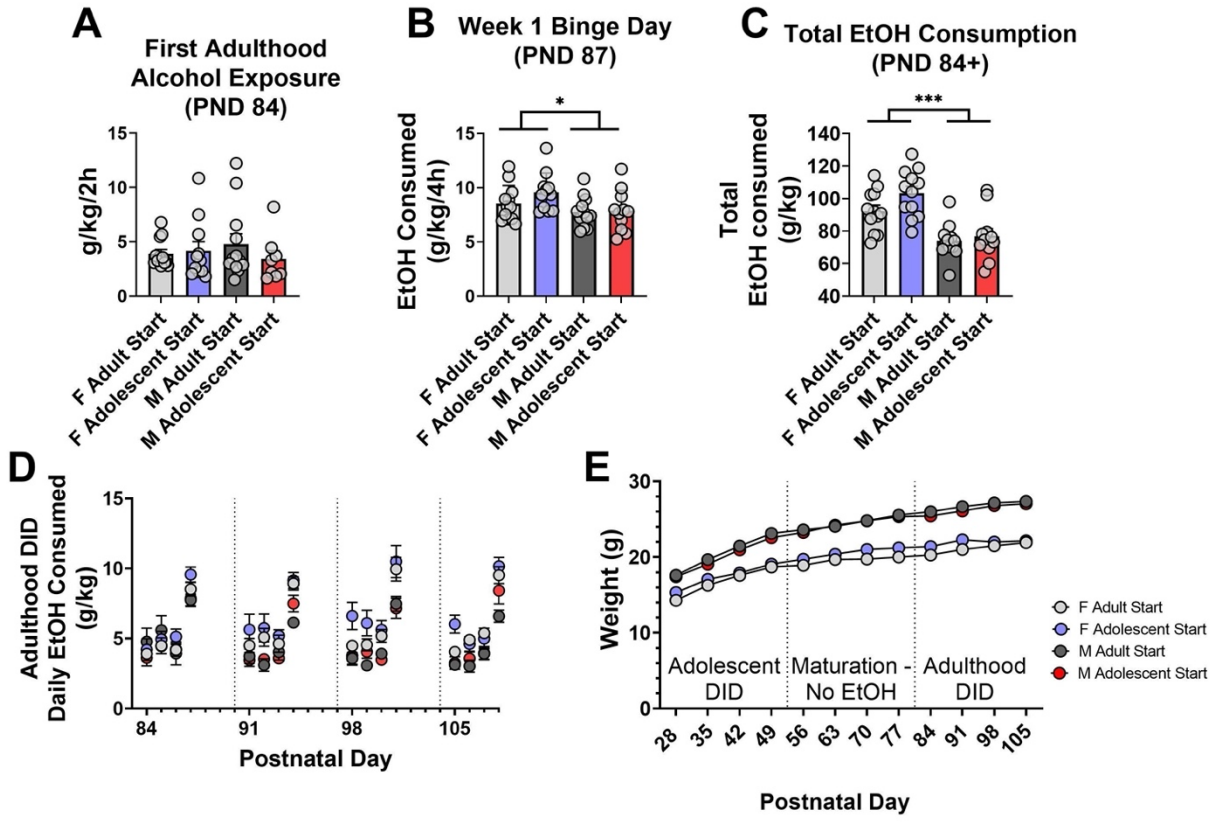


Figure 4. The results of the double drinking experiment. Each figure shows 4 groups representing males and females that are divided into groups based on whether they drank alcohol or water during the adolescent period. Figure 4A shows the amount of EtOH consumed during the first 2-hour exposure of the adult drinking period in grams of liquid per kilogram of body weight per hour. Figure 4B shows the amount of EtOH consumed during the first binge day of the adult period. Figure 4C shows the total amount of EtOH consumed in adulthood. Figure 4D shows the daily EtOH consumed by the 4 groups over the 4-week period. Each week is represented by 3 2-hour access periods and the 4-hour binge period. Figure 4E shows the change in body weight in grams of the mice of the 4 groups over the entirety of the experiment. Data are displayed as the mean +/- SEM (n=12 F Adult, F Adolescent, and M Adult, n=11 M Adolescent). * Indicates $p < 0.05$, * indicates $p < 0.0001$.**

2BC Results

For the 2-bottle choice experiment, the daily EtOH consumption and preference were measured and analyzed. First, the difference in the amount of water consumed during the habituation week between adult and adolescent start female mice was found to be nonsignificant (Figure 5B; ($t(8) = 1.385$, $p = 0.2035$). For the daily consumption, there was an effect from the day of drinking (Figure 5C; ($F(3.205, 25.48) = 23.87$, $p < 0.0001$). There was no effect from the exposure to adolescent DID ($F(1, 8) = 1.428$, $p = 0.2663$). There was also no effect from the interaction of adolescent DID and day of drinking ($F(20, 159) = 1.211$, $p = 0.2517$). For the daily preference, there was an effect from day of drinking (Figure 5D; $F(20, 159) = 7.831$, $p < 0.0001$). There was also no effect from adolescent DID exposure ($F(1, 8) = 0.2328$, $p = 0.6424$). There was also no effect from the interaction of adolescent DID and day in the protocol ($F(20, 159) = 1.451$, $p = 0.1066$).

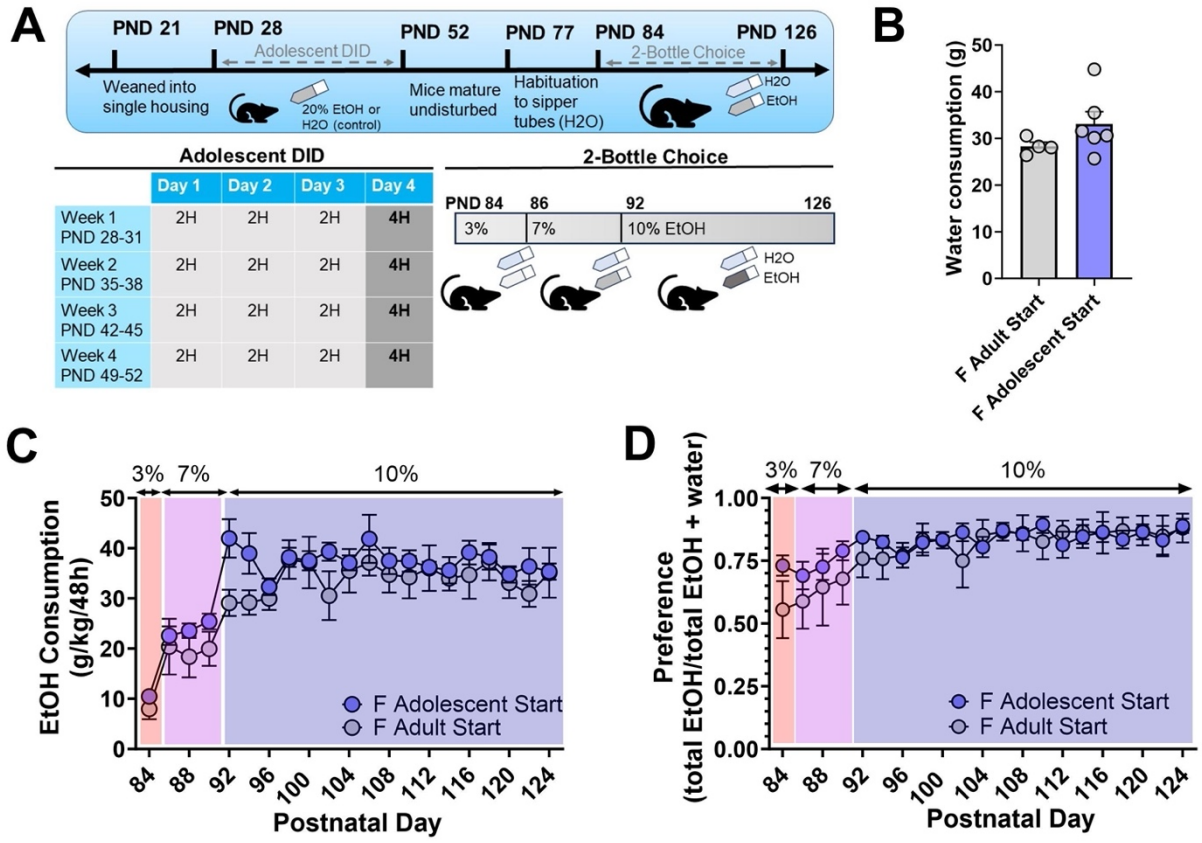


Figure 5. The results of the 2-bottle choice experiment. Figure 5A shows the timeline and weekly protocol for the 2-bottle choice experiment. Figure 5B shows the water consumption during the habituation week. Figure 5C shows the EtOH consumption across the 2-bottle choice experiment by week between adolescent and adult start female mice. Figure 5D shows the preference for EtOH versus water by week between adolescent and adult start female mice. Data are displayed as the mean +/- SEM (n=6 F Adolescent, n=4 F Adult).

Electrophysiology Results

The intrinsic excitability of prelimbic cortex SST neurons was assessed by examining their action potentials fired at increasing current injections (Sicher et al. 2023). These results were stratified by sex. For the males, there was a main effect of current injection step (Figure 6; $F(20, 441) = 31.25$, $p < 0.0001$). There was also a main effect of adolescent DID ($F(1, 441) =$

43.39, p < 0.0001). There was no interaction between adolescent DID and current injection step ($F(20, 441) = 1.502$, p = 0.0758). For the females, there was a main effect of current injection step (Figure 7; $F(20, 462) = 24.02$, p < 0.0001). There was also a main effect of adolescent DID ($F(1, 462) = 46.84$, p < 0.0001). There was no interaction between current and DID ($F(20, 462) = 0.5528$, p = 0.9426) (Sicher et al. 2023).

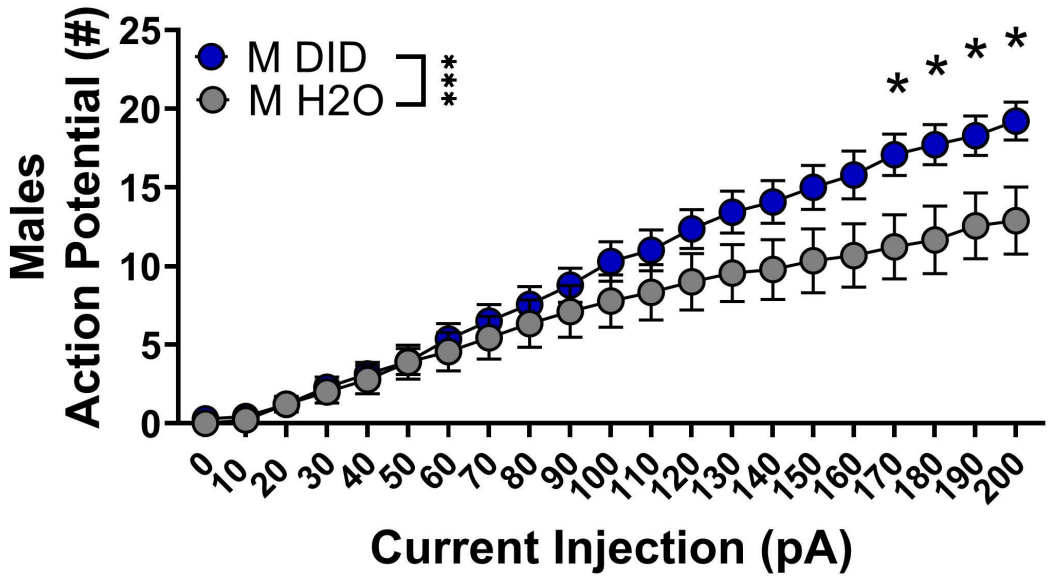


Figure 6. Current injection in picoamps (pA) versus action potentials fired by male mice at the same age as 2BC and double DID experiments were run (PND 84). Data are displayed as the mean +/- SEM (n = 14 cells from 7 mice M DID, 9 cells from 5 mice M H2O). * Indicates p < 0.05, * indicates p < 0.0001.**

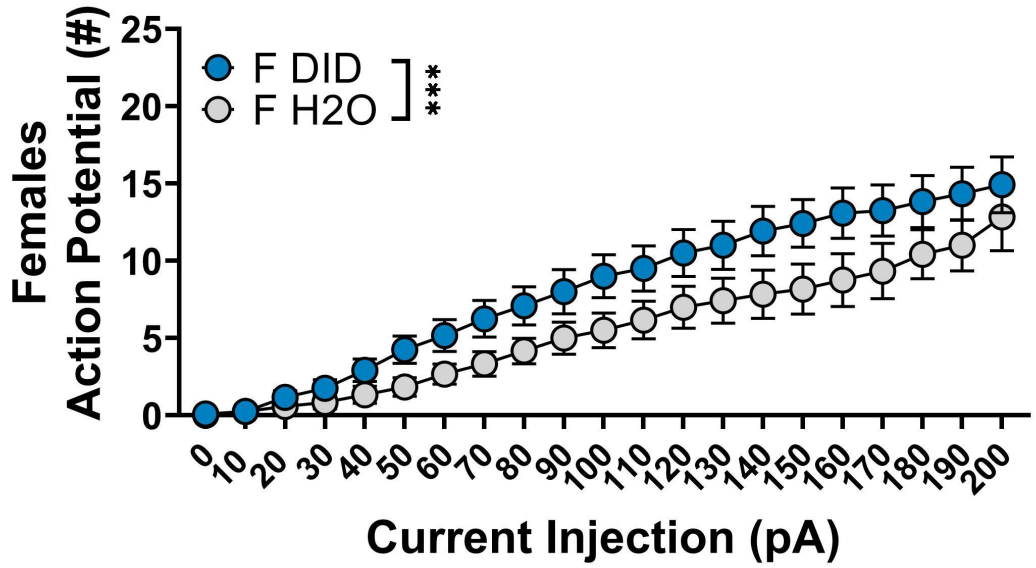


Figure 7. Current injection in picoamps (pA) versus action potentials fired by female mice at the same age as 2BC and double DID experiments were run (PND 84).). Data are displayed as the mean +/- SEM (n=12 cells from 6 mice F DID, 12 cells from 6 mice F H2O). * Indicates $p < 0.05$, * indicates $p < 0.0001$.**

Chapter 4

Discussion

From the double DID model, there was no indication of adolescent alcohol exposure contributing to increases in alcohol consumption from the metrics analyzed. The expected result of female mice consuming more alcohol standardized to body weight than males is seen in this model and has been shown in previous experiments. The first alcohol exposure on PND84 is analogous to the first exposure to alcohol in adulthood. During this first session, the mice with adolescent alcohol exposure drank similarly to their non-exposed peers. Later, for their first binge-session on PND87, the amount of alcohol consumed was also similar. Across the 4 weeks of drinking in the adult stage, there was no significant difference in alcohol consumed among the adolescent and adult start mice. These findings suggest that the exposure to alcohol in adolescence does not produce meaningful changes in consumption at the adult stage based on a model of limited access in rodent models.

The 2BC model showed similar results as the DID model, but an important note is that the results currently pertain only to a female mouse sample. The model showed that both adolescent start and adult start mice showed similar preferences for alcohol and water, which increased with increasing concentration of the EtOH in water solution. Similarly, the difference in total alcohol consumed was not significant between the groups. These results indicate that the preference for alcohol versus water is not altered through adolescent exposure.

In our model, there was no significant difference in the total alcohol consumed between male and female adolescent mice. In adult drinking across virtually all mammals, there is often an increased consumption by females. Despite the similarities in total consumption, there is an effect where the female adolescent mice escalate their binge-drinking throughout the model. In

order for this to be true while the total consumption remains the same, some sort of offset must be present.

Male and female mice showed hyperexcitability in SST neurons 30 days post-adolescent drinking. With larger current injections into cells, the elicited number of action potentials increased significantly. While this showed that SST neurons continued to have altered properties during the start of the adult drinking period compared with water controls, this alteration did not manifest in any changes in adult drinking preference or consumption by the Double Drinking or 2BC models. Although previous research has shown that the modification of these SST cells in the prelimbic cortex contributes to changes in binge-drinking, there are clearly other factors at play (Dao et al. 2021).

One possible explanation for these results is the potential presence of a ceiling effect being produced by the environment where the mice drink. While mice are social animals and are traditionally group housed, this drinking model single houses mice in order to accurately quantify the amount of alcohol consumed by each mouse. This single housing is a substantial stressor on the mouse. Previous research has shown that stressors ranging from acute to chronic can have impacts on alcohol consumption (Becker et al. 2011). Isolation for extended periods of time has been shown to increase consumption of alcohol by both mice and rats. This effect starts in early development (Lopez et al. 2011). The effect also extends into adolescence (Advani et al. 2007). However, unpublished work from our collaborators shows that group housing mice does not change the interaction between adolescent alcohol consumption and adulthood alcohol consumption (personal correspondence and joint paper forthcoming, Sicher and Liss, 2024).

Another explanation for the results seen here versus in human literature is the presence of numerous other variables that alter human interactions with alcohol (Gilbertson et al. 2008).

Social pressures can cause sex-dependent alterations in seeking treatment for alcohol use disorders (Randall et al. 1999). There have also been studies that show a sex-dependent effect of age of onset of initial alcohol consumption (Chou and Dawson, 1994). Comorbidity with other factors has also been linked with alcohol consumption. One of the most studied examples is comorbidity with other substance use disorders (Hasin et al. 2007).

In the near future, the 2BC data will be expanded to include a complete sample of males. This was delayed by breeding issues and this additional data could provide further insight into the effects of this choice-based model. Another potential change in methodology that could yield interesting results would be to utilize paired housing alongside a different method of tracking fluid consumption. One such option would be to pair housed mice and utilize a lickometer and camera in order to track consumption to each individual mouse. A simpler method could be to pair-house mice and average consumption per mouse. This change would require that the mice in each group drink an approximately similar amount. If there is a potential ceiling effect produced by the high chronic stress of this model, then these changes could yield entirely different results. While there were not substantial changes based on adolescent exposure in either adult model, the alteration to the binge-drinking susceptible prelimbic SST neurons is still present. Further experimentation to determine the role and changes of these neurons throughout the adult models could also produce interesting results.

Chapter 5

Conclusions

Prelimbic cortex SST neurons show hyperexcitability 30 days following adolescent alcohol exposure. As changes to these circuits previously have been shown to be important in binge drinking behaviors, this change was expected to modulate consumption in adults with adolescent exposure relative to adults without exposure. In two models of alcohol consumption, a drinking in the dark model and a two-bottle choice model, no significant changes resulted in alcohol consumption across the most relevant measures of consumption, including total consumption, preference, and binging behavior. These results indicate that the often-discussed impact of adolescent alcohol consumption on adult drinking behavior seen in human studies may not be similarly present in a mouse model. Many factors beyond the scope of this study could play into the development of that relationship. Future work in this area will look to expand on this data set and mitigate the potential presence of a ceiling effect brought on by the stressful housing situation.

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