The relationship of alcohol access in adolescence with omission learning and interneuron numbers

by

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B.S., Missouri State University, 2016

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Psychological Sciences
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

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Abstract

The typical autoshaping and omission contingency procedures consist of initially presenting a cue followed by a reward regardless of responding, a phase in which the subject usually learns to approach the cue (autoshaping), followed by presenting the same cue followed by reward only if the subject does not respond to the cue (omission contingency learning). Our lab has previously found that rats allowed to consume alcohol during adolescence show faster omission contingency learning later in adulthood. Here, we aim to determine neurobiological correlates relating to the behavioral differences in an autoshaping and omission contingency learning task and prior alcohol consumption in rats with or without prior alcohol access. A total of 24 male Long Evans rats were used and underwent the chronic intermittent access (CIA) alcohol exposure paradigm from postnatal days (PND) 26-68. After the end of CIA, rats began behavioral training in which they were trained on an autoshaping task for six days followed by omission contingency learning for six days. Animals were then sacrificed, and brains were stained for Parvalbumin (PV) and choline acetyltransferase (ChAT). Data were analyzed using both binomial and Poisson mixed effects models. Results showed that subjects exposed to alcohol did show faster omission contingency learning using the measure of number of lever presses made, but the measure of the likelihood to press the lever at least once during a trial failed to show this effect. For PV expressing (PV+) neurons there were no group differences between water and alcohol groups. For ChAT expressing (ChAT+) neurons there was a decrease in ChAT+ neurons in the alcohol group compared to the water group in Ch1/Ch2 cholinergic nuclei. There was also an increase in ChAT+ neurons in the alcohol group compared to the water group in the nucleus accumbens shell. However, there were positive correlations between the number of PV+ neurons in the DLS and ACC and levels of alcohol consumption in the alcohol

group. There were also positive correlations between ChAT+ neurons in Ch1/Ch2, Ch3/Ch4, DMS, DLS, and nucleus accumbens core and levels of alcohol consumption in the alcohol group. These correlations showed that, as alcohol consumption in the alcohol group increased, the number of PV+ and ChAT+ neurons also increased in these brain areas. Since the majority of studies assessing the effects of alcohol on PV+ and ChAT+ neurons used forced exposure methods rather than voluntary methods, we were able to assess these correlations where most studies could not. These correlates are novel findings and further expand the scientific literature. Unfortunately, we did not find any differences in PV+ and ChAT+ neurons in the main brain areas believed to be involved in autoshaping and omission contingency (ACC, NAc Core, and DLS). Future research is needed for exploring the relationships between omission contingency learning and the brain areas that did show group differences and to assess the neuronal function within the brain areas associated with omission contingency learning.

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Acknowledgements

I would like to thank my advisor, Charles Pickens, for his guidance and support. I would also like to thank the members of the Comparative Learning and Pharmacology Lab who assisted with data collection, especially Hayley Fisher, Alisa Pajser, Skyler Wikoff (Gilbert), Rachel Boerger, Sydney Zimmerman, and Jing Wu.

Chapter 1 - Introduction

There is an established link between a history of excessive alcohol consumption and cognitive idiosyncrasies in humans (Le Berre et al., 2017), however the causal direction of this relationship is unclear (Khemiri et al., 2015; Malone et al., 2014). Research suggests that the earlier a person begins to consume alcohol, particularly in a binge-drinking fashion, the more a person is susceptible to these cognitive abnormalities and developing alcohol use disorder (AUD) (Marshall, 2014; McCambridge et al., 2011). In particular, humans who begin drinking during adolescence are more vulnerable to changes in behavior, cognition, and neurological functions. This may be due to alcohol's effect on the development of the brain during adolescence, since adolescence is a critical developmental period for many aspects of neurological function (Hiller-Sturmhofel & Spear, 2018). However, the causal nature of this relationship within humans remains unclear since we are unable to directly control environmental upbringing and drug exposure. Research using animal models provides a valuable tool that allows us to investigate the causal relationships between alcohol exposure and behavioral changes. Animal models allow more control over factors such as who is given drug exposure, the amount of drug exposure, age of access/exposure, the sex of the subjects assessed, and environmental upbringing.

In this thesis, I examine effects of voluntary alcohol consumption on the neurobiology of several neurotransmitter systems and behavior in the autoshaping and omission contingency tasks. The typical autoshaping and omission contingency procedures consist of initially presenting a cue followed by a reward regardless of responding, a phase in which the subject usually learns to approach the cue (autoshaping), followed by presenting the same cue followed by reward only if the subject does not respond to the cue (omission contingency learning). These

tasks were chosen because our lab has found them to be the most sensitive behavioral measures to alcohol-related changes in behavior that we have tested. Our lab and others have previously found that alcohol exposure leads to faster omission contingency learning, which occurs when there is a faster decrease in responding to the predictive cue throughout the trials (Pickens et al., 2019; Hellberg et al., 2018; Depoy et al., 2013). For example, male Long-Evans rats given alcohol access during adolescence show faster omission contingency learning in adulthood compared to alcohol naïve rats (Pickens et al., 2019). However, this effect was not found when rats were exposed to alcohol during adulthood rather than adolescence, even though the adult rats voluntarily consumed more alcohol than the adolescents (Pickens et al., 2019). The current proposal aims to further analyze a replication of the faster rate of omission learning found following adolescent alcohol exposure, as well as establish a neurological correlate for this effect of alcohol access on behavior.

The goal of this study is to determine neurobiological correlates relating to the behavioral differences in an autoshaping and omission contingency learning task found between adult rats that have or have not been exposed to alcohol during adolescence. While I report neurobiological data from several brain areas in this thesis, the original focus of the research was the striatum, as 1) alcohol exposure leads to alterations in the striatum (Corbit et al., 2012; O'Tousa & Grahame, 2013) and 2) each of the types of associations potentially implicated in autoshaping and omission contingency learning tasks rely on subregions of the striatum (Yin et al., 2006; Yin et al., 2005; Mannella et al.,2013). Below, I discuss the autoshaping and omission contingency tasks, the relevance and importance of the adolescent timeframe, the alcohol consumption paradigm used, the results of previous neurobiological studies on these behavioral tasks (in terms of brain areas and neurotransmitter systems), and then discuss how alcohol may be affecting these systems.

Autoshaping and omission contingency.

With an autoshaping task followed by omission contingency learning, the autoshaping task often leads to organisms making responses to a cue even though reward is non-contingent upon responding, then after this training the subject must learn to withhold their response (ex: lever press) in order to receive the reward (omission contingency learning). Interestingly, although the autoshaping task is a Pavlovian task that requires no response, the subjects often learn to respond to the cue presentations by contacting the cue, often called sign-tracking. An alternative response (incompatible with sign-tracking) is called goal-tracking, and it occurs when the subject approaches the location of where the reward will appear upon presentation of the conditioned stimulus (CS) predicting reward (Tomie, Grimes, & Pohorecky, 2008; Saunders & Robinson, 2011; Tomie & Sharma, 2013; Robinson et al., 2014; Yager & Robinson, 2015). Both sign-tracking and goal-tracking are often thought to be examples of responses caused by Pavlovian conditioning that elicits not only a reflexive response, but also emotional and motivational responses. The types of responses to these cues are used as indicators of an individual's sensitivity to cues that predict reward.

Although there are individual differences in sign-tracking and goal-tracking, it is possible to bias the response towards sign-tracking behavior, and the autoshaping procedure we employ uses these parameters to bias subject behavior toward sign-tracking. The conditioned response (CR) towards the conditioned stimulus (CS) is able to be biased towards either sign-tracking or goal-tracking through a variety of ways, including the distance between the cue and the reward delivery location, the localizability of the cue itself, and the length of the interval between cue presentations. When the CS is presented in close proximity to the location where the reward, or unconditioned stimulus (US), will appear, then sign-tracking behavior is more likely to occur. In

contrast, if the cue if further away from the point of reward delivery, then the subject is more likely to engage in goal-tracking behavior (Holland, 1980a). Additionally, the cue itself is important in dictating what CR will occur. Cues that have a distinct location, such as a cuelight or lever, are more prone to causing sign-tracking, whereas cues that are less localizable, such as a tone, are more prone to causing goal-tracking (Holland, 1977; Holland, 1980a). Furthermore, the intervals between cue presentations and reward delivery have also been found to bias the CR towards certain types of behaviors. Cue presentations that last 10-30 seconds tend to bias behavior towards sign-tracking, whereas shorter presentations create a competition between cue orientation behavior and US approach and don't allow for much response which leads to neither sign-tracking nor goal-tracking. Longer presentations may lead to more goal-tracking behavior since they allow time for both cue orientation and reward orientation (Holland, 1980b). Our autoshaping procedure manipulates these parameters to bias subject behavior toward sign-tracking with a localizable lever-light close to the foodcup and 12 second trials, and therefore learning to press the lever upon cue presentation.

During omission contingency learning the subject must learn to withhold their sign-tracking response in order to receive the reward. Research has shown that rats will decrease their responses (or presses) to the cue during the omission contingency phase (Atnip, 1977; Depoy et al., 2013; Hellberg et al., 2018; Pickens et al., 2019). This may suggest that the association the rat is creating is not solely between the CS and the US, but also between its responses preceding the US. Omission contingency learning has been previously shown to be sensitive to damage or dysfunction of the dorsolateral striatum (DLS) and is sometimes included in batteries of tests designed to assess DLS function (Depoy et al., 2013), so my research may be considered a test of DLS function. However, many brain areas and neurotransmitter systems are affected by alcohol

exposure (as reviewed in later sections of this introduction) and so I will try to assess these alterations to determine whether DLS changes are a plausible cause of the behavioral changes and/or whether there are other plausible loci of this effect.

Even though this is not crucial to the hypotheses I will be testing in my proposal, in the next two paragraphs I will provide some background information about two psychological frameworks people have used to understand omission contingency findings. These two frameworks are 1) distinguishing whether sign-tracking behavior is Pavlovian or operant conditioning and 2) assessing habitual behavior. However, I primarily chose to examine omission learning for two reasons largely unrelated to these frameworks. The first reason is that previous data suggest that this task is highly sensitive to alterations after alcohol access or exposure (and possibly more sensitive than other assessments such as devaluation, reversal learning, or fear conditioning) (Depoy et al., 2013; Fisher et al., 2017; Hellberg et al., 2018; Pajser et al., 2018; Pajser et al., 2019; Pickens et al., 2019). In a previous study with mice, researchers have found that alcohol exposure from vapor inhalation during adulthood can lead to faster omission contingency learning tested after an alcohol-free interval (Depoy et al., 2013). Hellberg and colleagues (2018) have also previously found that both male and female rats exposed to alcohol in gelatin during adolescence showed a faster rate of omission contingency learning tested alcohol-free in adulthood, but only in a task in which the rewards' delivery was uncertain after the cues during autoshaping. Finally, our lab has found that voluntary intermittent alcohol exposure during adolescence leads to faster omission contingency learning in male rats tested alcohol-free in adulthood (Pickens et al., 2019). Omission contingency learning may be a highly sensitive task towards alcohol-induced alterations due to exposure in adolescence more so than other tasks, since there were no behavioral effects found in go/no-go reversal learning,

devaluation, or fear conditioning using the same alcohol paradigm (Fisher et al., 2017; Pajser et al., 2018; Pajser et al., 2019; Pajser et al., 2021). Second, this increased sensitivity provides a key opportunity to assess the neurobiological underpinnings of behavioral changes after adolescent alcohol consumption. This proposal is therefore primarily testing whether alcohol-induced neurobiological changes are related to alcohol-induced behavioral changes in a task with greater sensitivity using this omission contingency task. Thus, I do not conduct any parametric research to assess these different psychological frameworks about what the behavioral changes in omission contingency task behavior may represent. However, these frameworks will give some idea of the potential underlying associations that may be affected after alcohol consumption.

It is unclear whether the subjects' behavior during the autoshaping procedure is Pavlovian or operant, since no response is required to receive the reward but the subject responds regardless. Procedurally, it is a Pavlovian task as the reward is delivered independent of responding, and the lever-pressing behavior could be an automatic response to the lever that is paired with food, even if the rat does not believe that their behavior causes the delivery of food. During autoshaping, however, the subjects learn to push the lever as though they may believe their action affects the outcome, which would be categorized as operant conditioning. Operant conditioning elicits a behavior upon a presentation of a stimulus that predicts an event will occur based on how the subject responds. Therefore, it is possible that operant conditioning or Pavlovian conditioning occurs during autoshaping. Previous researchers have used the autoshaping and omission contingency learning tasks to try to determine whether autoshaping/sign-tracking is predominantly operant or Pavlovian conditioning and what types of associations are being made, such as stimulus-outcome (S-O) and response-outcome (R-O) learning (Williams & Williams, 1969; Locurto, Terrace, & Gibbon,1976; Atnip, 1977). Once the

rat is consistently pressing the lever during autoshaping it is unclear whether the rat is predominantly focusing on the association between the stimulus and receiving food (S-O) or the association between the response of pressing the lever and receiving food (R-O). Omission contingency learning tasks have often been used following autoshaping in order to determine what is the primary association being used, since in this task any sign-tracking due to S-O associations would conflict with R-O associations (as responses prevent reward delivery). Any R-O driven behavior should decrease, as the response prevents the outcome. In contrast S-O driven behavior could remain, as the presentation of the stimulus still indicates the possibility of reward delivery. Since it has been shown that rats will decrease their responses to the lever during the omission contingency phase (Atnip, 1977; Depoy et al., 2013; Hellberg et al., 2018; Pickens et al., 2019), this could indicate potentially that the lever-pressing is entirely maintained from superstitious behavior during autoshaping, meaning the subjects believe it influences the outcome when it does not. Alternatively or additionally, it is possible that the sign-tracking/autoshaping behavior was supported by S-O associations alone during autoshaping, but the omission learning leads to the addition of an R-O association and this R-O association may be overpowering the S-O association during omission learning. There is evidence that Pavlovian conditioning does also play a role in this autoshaping behavior as well, since rats trained in omission contingency without learning autoshaping previously press the lever at higher rates than training-naïve rats (meaning the lever never previously predicted anything) (Locurto et al., 1976). This may indicate that some reflexive response is elicited upon presentation of the CS. Overall, it is likely there is a mix of associations occurring in which perhaps S-O driven associations supports early behavior however then there is a shift to R-O associations which account for the decrease in responding during omission contingency learning.

There are also researchers who use omission learning as an indicator of goal-oriented or habitual behavior (Corbit & Janak, 2016; Dickinson, Squire et al. 1998, Mangieri, Cofresi et al. 2014). If a rat is able to learn to stop pressing the lever quickly, then this could represent goal-oriented behavior. Goal-directed action is hypothesized to occur when the outcome is encoded and behavior is sensitive to the value of the outcome and the cue's or response's relationship with it (R-O or S-O associations). However, habitual behavior, in which a subject does not exhibit sensitivity to the outcome of a specific response, can be commonly observed in operant conditioning after extended operant responding for an outcome (Adams, 1982; Dickinson et al., 1995). Subjects that show habitual behavior may have the greatest association between the stimulus that indicate a response could be made and the action of making that response (an S-R association), regardless of the outcome (Corbit & Janak, 2016). Subjects may show habitual behavior during omission learning if they continue to press the lever during omission contingency learning even though this results in a loss of reward. It has been shown that extended operant training or training with variable interval schedule decreases omission learning sensitivity possibly due to increased habitual behavior (Dickinson, Squire et al. 1998, Mangieri, Cofresi et al. 2014).

Adolescence.

The period of adolescence seems to be a critical period of time for the effects of alcohol exposure on omission contingency learning to take place. Pickens and colleagues (2019) previously found faster omission learning following adolescent alcohol consumption, but this effect was not found when alcohol access occurred during adulthood. A similar pattern has been found using a concurrent instrumental probability-discounting task, such that rats given alcohol during adolescence showed impairments in this task while those given alcohol during adulthood

did not show impairment (Schindler, Tsutsui, & Clark, 2014). These differences between adolescent and adult alcohol exposure suggest that neurological changes occurring during adolescence are disrupted by alcohol consumption or that adolescents are more sensitive to neurotoxicity. However, adolescents are less sensitive to the sedative properties, motor impairments, and withdrawal effects of alcohol compared to adults (Barron et al., 2005; Spear, 2014). The remainder of this section will continue on to define the time period of adolescence.

In humans, adolescence is a period of development that is primarily defined as a transitional phase from parental dependency to fully independent adulthood. This phase is highlighted by a number of biological changes, many of which are neurological. These neurobiological changes include declines in relative volume in certain cortical and subcortical areas, reduction in number of synaptic connections, increased speed of information flow across distant brain regions, and increased reactivity of certain subcortical brain regions (Hiller-Sturmhofel & Spear, 2018). It is believed that the developing subcortical regions such as the nucleus accumbens (NAc), dorsal striatum, and amygdala play a critical role in these changes in behavior, since these brain regions are involved in responding to rewarding, aversive, and emotional stimuli. This heightened sensitivity to reward and aversion may contribute to the increased likelihood of adolescents to start exploratory alcohol use, especially in binge drinking fashion (Hiller-Sturmhofel & Spear, 2018).

There is some ambiguity and debate about the timeline of when both humans and rats are going through adolescence. What is agreed upon is that adolescence overlaps with sexual maturation and a period of behavioral changes in which individuals show exaggerated behaviors to motivational stimuli, more peer-directed behavior, novelty-seeking, increased consummatory behavior, increased risk taking, and increased experimental alcohol and drug use (Buchanan et

al., 1992; Petersen et al., 1996; Baumrind, 1987; Spear & Brake,1983; Ojeda and Urbanksi, 1994; Spear, 2000; Doremus-Fitzwater et al., 2010; Vetter-O'Hagen & Spear, 2012; Spear, 2016; Hiller-Sturmhofel & Spear, 2018). Some researchers define adolescence within humans as 9 to 18 years of age (Buchanan et al., 1992), whereas others define it as the entire second decade of life (Petersen et al., 1996). Some researchers have even defined late adolescence as being as old as 25 (Baumrind, 1987).

This ambiguity is also found within the rodent literature. Spear and Brake (1983) have defined periadolescence as postnatal day (PND) 30-42. However adolescent-type characteristics have been shown to emerge near PND 28 and last until PND 55 (Ojeda and Urbanksi, 1994). An even broader timeframe of PND 26-65 has also been defined, which stretches across 6 weeks, and is considered to cover the entire spectrum of early adolescence into late adolescence/early adulthood (Spear, 2016). PND 25-42 could be analogous to the human ages of 10-18, while PND 43-65 would be analogous to 18-25 years old in humans (Spear, 2016). This definition encompassing PND 26-65 includes the phase of late adolescence/early adulthood, since some researchers have considered ages in humans up to 25 years old to be considered late adolescence (Petersen et al., 1996; Spear, 2000). These definitions are made on the basis of similar adolescent-like behaviors seen during these ages in rats compared to the adolescent ages in humans. During these stages, rats show exaggerated behaviors to motivational stimuli, more peer-directed behavior, increased novelty-seeking, increased consummatory behavior, increased risk taking, and increased experimental alcohol and drug use, which are the key characteristics of adolescence (Spear, 2000; Vetter-O'Hagen & Spear, 2012; Spear, 2016; Hiller-Sturmhofel & Spear, 2018). Since the 6-week span of PND 26-65 encompasses nearly all definitions of

adolescence, both in rats and the human analog, this will be the definition used for adolescence/early adulthood in rats for this study.

Alcohol consumption.

There are a number of alcohol exposure methods in animal models that can be used to study the direct relationship between adolescent development, alcohol exposure during that development, and how this may impact behavior and the brain. In this section, I explain the reasons why I chose the chronic intermittent access procedure used for my research.

Models vary on whether exposure is forced or voluntary. Forced exposure is a method in which a rat is exposed to a fixed dose of alcohol, which can be via injections, gavage, or vapor inhalation (Knapp & Breese, 2012). These techniques are ideal for purposes of creating strong group effects since each rat within a group of the experiment is exposed to the same amount of alcohol. This method often gives a high dose of alcohol as well, so it can be used for a strong manipulation. However, the methods of forced exposure may induce stress for subjects. Voluntary alcohol consumption models typically provide the subjects with access to alcohol within their homecage. With this free access to alcohol, voluntary alcohol consumption models allow for assessment of drinking behavior. The access can be given either continuously or intermittently. Continuous alcohol exposure occurs consistently 24 hours every day for 7 days/week for an extended amount of time. However, since it is voluntary the subjects do not have to drink the alcohol each day, or at all. Intermittent exposure paradigms introduce breaks between exposure points, such that the subjects have days (or hours) of withdrawal between the access periods. A common binge-withdrawal pattern used is providing alcohol access for 24 hours, followed by a 24-hour withdrawal period, with access to alcohol given three times per week (Wise, 1973; Simms et al., 2008). Studies have found that rats given intermittent access,

but not continuous access, will increase or escalate the level of alcohol consumption over time (Kimbrough et al., 2017; Brancato et al., 2016; Simms et al., 2008).

When attempting to model the binge-withdrawal pattern often found in adolescent drinkers, the chronic intermittent access (CIA) paradigm may simulate the pattern of use and behaviors observed in humans (Wise, 1973; Simms et al., 2008). In the CIA paradigm, two bottles are placed on each subject's cage, one of which will always be filled with water. The other bottle alternates between water and alcohol solution, with alcohol access occurring three days per week for 24 hours. Since this model uses intermittent rather than continuous access, this often leads to an escalation of alcohol consumption over several weeks. The robust effects of this model to escalate drinking over a period of time may be due to how it implements the alcohol deprivation effect (ADE) into its design. ADE is a phenomenon in which there is a robust but temporary increase in alcohol consumption above the previous baseline amount of consumption following a period of deprivation from alcohol (McKinzie et al., 1998; Vengeliene et al., 2014; Melendez et al., 2006; Rodd-Hendricks et al., 2000). Within the CIA model, the repeated cycles of alcohol intake followed by withdrawal may encourage increased drinking each time alcohol is available once again. This effect was shown to escalate outbred Long-Evans rats' consumption to a comparable consumption level of P-rats, which are bred to drink at high levels (Simms et al., 2008). CIA is able to induce an 80 mg/dl BAC 30 minutes after access to alcohol bottles has initially been given in Long Evans rats (Simms et al., 2008). However, our lab has previously collected BAC levels at 2, 4, and 6 weeks of the CIA paradigm using adolescent, non-food restricted rats and only found levels of 6.2 ± 1.3, 11.4 ± 0.7, and 14.3 ± 1.0 mg/dl, respectively (Pajser et al., 2018). Even though forced models obtain a higher BAC than voluntary models, this voluntary consumption peak BAC parallels binge drinking behavior, as 80 mg/dl has been

determined a sufficient BAC level to be considered binge drinking (NIAAA, 2004). In humans, it is most common to see a binge-like drinking pattern during adolescence and young adulthood (Windle, 2016; Spear, 2018). Therefore, this paradigm may adequately model the common way in which adolescents and young adults begin drinking and determine whether this may have lasting neurological and behavioral effects.

In addition to exposing the rats to a binge level of alcohol, the CIA paradigm also allows for a measure of how much a rat will voluntarily drink (and based on the alcohol itself rather than any adulterants or water/caloric needs it meets). Then, we are able to correlate these measures of voluntary consumption with behavioral performance and neurobiological aspects. Linking the amount of consumption with behavior and neurological factors may uncover genetic factors that influence whether an individual drinks more or less alcohol. It also enables the measurement of how alcohol consumption, and only alcohol consumption, affects behaviors and neurobiology. This model avoids the major confounds of other popular models designed to increase alcohol consumption and is still able to promote an increase in drinking over time. An example of one confound that the CIA controls for is the need for an additive to the alcohol solution, such as sucrose or the use of beer (Vargas et al., 2014; Mangieri et al., 2014; Millan et al., 2013). The additives will sweeten and make the solution more palatable, thereby increasing consumption. However, the addition of a sweetener also adds a confound since the subjects may now be consuming the solution for caloric intake rather than the alcohol's effects. The CIA paradigm allows the rodents to have unrestricted access to both food and water while exposed to an alcohol solution without additives while also increasing their consumption over time. This paradigm also avoids confounds in which rats may consume the alcoholic solution for its water or calories if the rats were food or water restricted. Thus, it represents a procedure to

simultaneously assess both long-term effects of alcohol exposure (at moderate levels) on other behaviors and how individual differences in the propensity to consume alcohol may relate to other behavioral traits.

Finally, CIA causes exposure to lower levels of alcohol than forced exposure, but it allows for examination of what effects would still be present at these lower levels and allows for examination of any changes that may occur due to the voluntary nature of the intake (which may differ from those of forced exposure). Forced exposure models allow for controlled and higher blood alcohol concentrations (BACs) that can rise to a level of intoxication (150-250 mg/dl) (Kimbrough et al., 2017). However, previous research shows voluntary and forced exposure of different drugs leads to different long-term neurobiological effects. There is evidence that voluntary intake of other types of drugs tends to have different neurobiological effects compared to forced intake (Jacobs et al. 2003, Stefanski et al, 2007; Wiskerke et al., 2016). Although these differences have been predominantly found for cocaine and opiate exposure, there is a possibility that these mechanisms differ in alcohol as well. Since we are modeling a binge behavior that human adolescents partake in voluntarily, it may be more appropriate to look at the mechanisms affected by voluntary intake.

Potential neural mechanisms.

Although there are multiple brain areas affected by adolescent alcohol exposure (Crews et al. 2016; Spear, 2016), the omission contingency task has only been shown to be sensitive to a function of a subset of them. Although I examine a wide range of brain areas in my immunohistochemistry assays, a main focus is on brain areas previously implicated in autoshaping and the omission contingency task in order to link my behavioral pattern to the

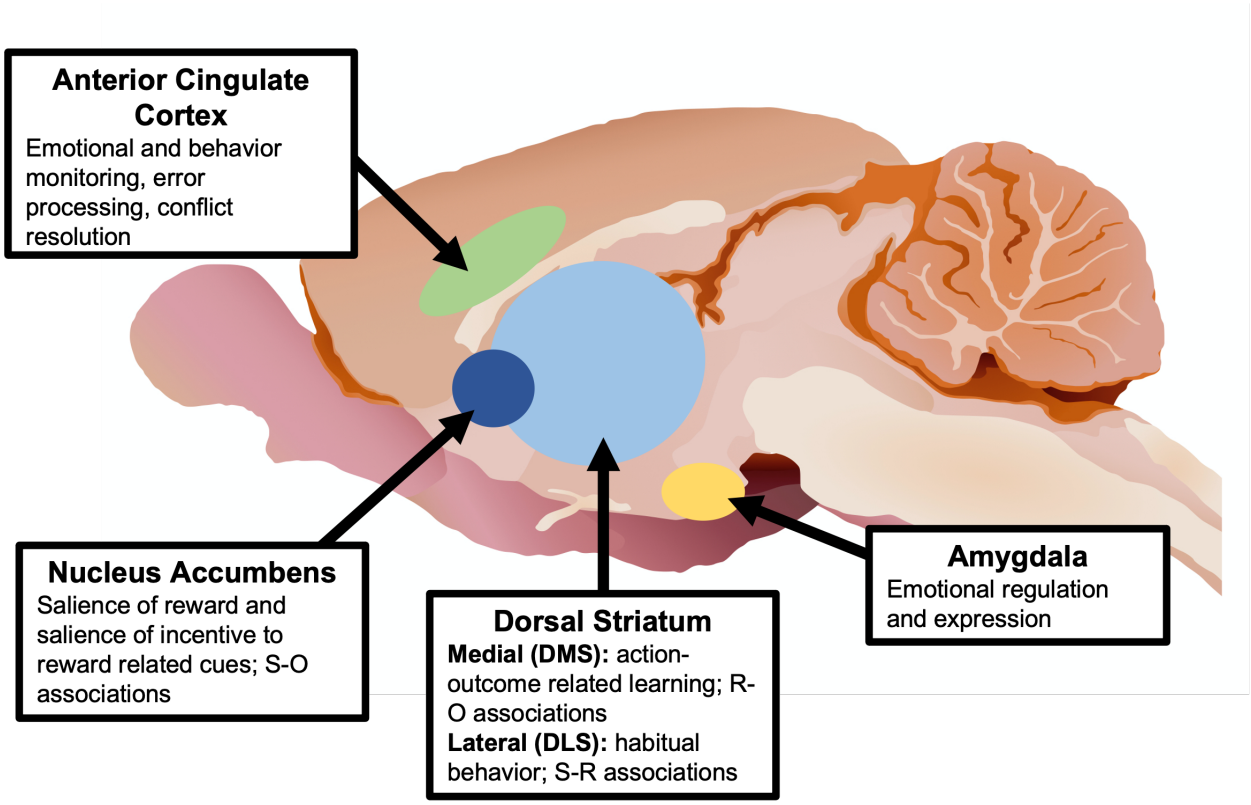
neurobiological changes observed. Below, I review the relevant brain areas for this study (see Figure 1).

Brain Areas Influencing Autoshaping and Omission Contingency Learning.

There are many brain areas that affect autoshaping/sign-tracking and/or omission contingency learning. As adolescent alcohol consumption appears to alter omission learning but not autoshaping/sign-tracking, we can figure out which brain area alterations are likely candidates (or can likely be excluded) as the cause of alcohol induced changes in omission contingency learning but not autoshaping by reviewing the pattern of effects of lesions/inactivations on these tasks. Lesions or inactivation of certain limbic areas have been shown to improve performance during omission contingency learning in rats.

Figure 1.1

The primary brain areas of interest for this study and their key functions.



The lesioning or inactivation of the ACC disrupts both autoshaping and discrimination learning. Bussey and colleagues (1997) aimed to determine the roles three different prefrontal brain regions played in omission contingency. These three regions under investigation were the medial prefrontal cortex, the anterior cingulate cortex (ACC), and the posterior cingulate cortex. They found that lesioning the ACC severely impaired rats' ability to learn the autoshaping task, specifically in a failure to discriminate between the CS+ and CS-, while lesions to the medial prefrontal cortex and posterior cingulate cortex did not affect acquisition of autoshaping. Bussey and colleagues (1997) then looked at effects of lesions made after learning autoshaping but before omission learning. The subjects with ACC lesions made before omission learning showed a trend towards a faster rate of learning to withhold responding, though nonsignificant. However, ACC lesioned subjects failed to discriminate between the omission cue and the control cue, such that approach to the control cue was much higher than in any other group and responding to the omission cue was not significantly different compared to any other group. This effect of ACC lesions on autoshaping and omission learning were further replicated (Parkinson et al., 2000). During omission learning, the medial prefrontal cortex lesions caused slower approach to the stimuli, and the posterior cingulate cortex lesions lowered responses overall, but neither lesion affected discrimination between the CS+/CS-. The ACC could be a particular region of interest in the PFC since it plays key roles in reward-expectancy processing, behavioral control, and error monitoring (Heilbronner & Hayden, 2016; Shenhav et al., 2013).

However, alterations in the ACC may not play direct role in our autoshaping and omission contingency learning paradigm. The current study is using an autoshaping procedure exactly like the one Pickens and colleagues (2019) used, in which there is no discrimination between a CS+ and a CS-. Instead, our study used two levers that both deliver reward (both

CS+'s in the first phase). With this method being used, Pickens and colleagues (2019) found no impairment of autoshaping and only found alterations in omission contingency learning. This may suggest that the ACC is not impaired during this version of the task and does not lead to this behavioral change. Additionally if the ACC was impaired then you might expect poorer discrimination during the omission learning phase between the omission lever and the passive lever. However, that is not the case as pressing is reduced on the omission lever but continues to remain high on the passive lever (Pickens et al., 2019). Therefore, it seems most likely that changes in ACC are not driving the behavioral effects.

Lesioning or inactivation of differing areas of the striatum produce differing effects on autoshaping and omission learning. As with the ACC, both autoshaping acquisition and CS+/CS- discrimination during omission learning were impaired by nucleus accumbens core (NAcCore) lesions (Parkinson et al., 2000; Cardinal et al., 2002). Furthermore, discrimination of the CS+/CS- was also impaired through contralateral lesioning of the ACC and NAcCore (a method of disconnecting circuits between them), which suggests these areas may be part of an important cortico-striatal pathway critical for this learning (Parkinson et al., 2000). These findings are similar to those with the ACC, suggesting that the NAcCore may not play a direct role in the effects of alcohol on omission learning in our task, since there is no impairment of autoshaping or weaker discrimination in omission contingency learning after alcohol access (Pickens et al., 2019).

While inactivation of the ventral striatum, specifically the nucleus accumbens core, completely impaired discrimination of the CS+/CS-, inactivating the dorsolateral portion of the striatum improved omission contingency learning in a different version of the task. Upon inactivation of the dorsolateral striatum (DLS; an area of the striatum) rats show an improvement

in omission learning (Yin et al. 2006). Subjects reduced their rate of responding faster than controls upon inactivation of the DLS via microinjections of the GABA agonist muscimol during omission training (Yin et al., 2006). However, it has been found that lesions of the DLS also reduce lever pressing during an autoshaping task (Naeem & White, 2016). Naeem and White (2016) also found that DMS lesions impaired lever pressing during an autoshaping task, though to a lesser extent than lesioning the DLS. Both lesions to the DLS and DMS not only caused reduced lever pressing (sign-tracking), but caused subjects to approach the food magazine faster than the control group as well (goal-tracking). Because of this impairment to the autoshaping, the DMS and DLS may not be responsible for behavioral effects we have found after alcohol access (Pickens et al., 2019).

Decreased function of any of the brain areas reviewed doesn't quite fit the pattern observed, so maybe there is a complex pattern of more than one brain area or maybe increased function of some other area might be able to explain the effects. In this experiment, I will examine this possibility.

Vulnerable Neurotransmitter Systems.

Now that I have reviewed the literature on what brain areas are implicated in autoshaping/omission learning, I will now review the literature on how alcohol exposure alters neuronal activity and neurochemistry in the particular brain areas implicated. Lesion and inactivation studies provide insight about which brain areas are necessary for a behavior to occur, however they do not fully provide the answer of what neural pathways and neurotransmitter systems are utilized in a given task. One of the most pronounced neurobiological disruptions of adolescent alcohol exposure is alterations of neurotransmitter activity (Spear 2000; Spear, 2010), and alcohol directly or indirectly acts on many

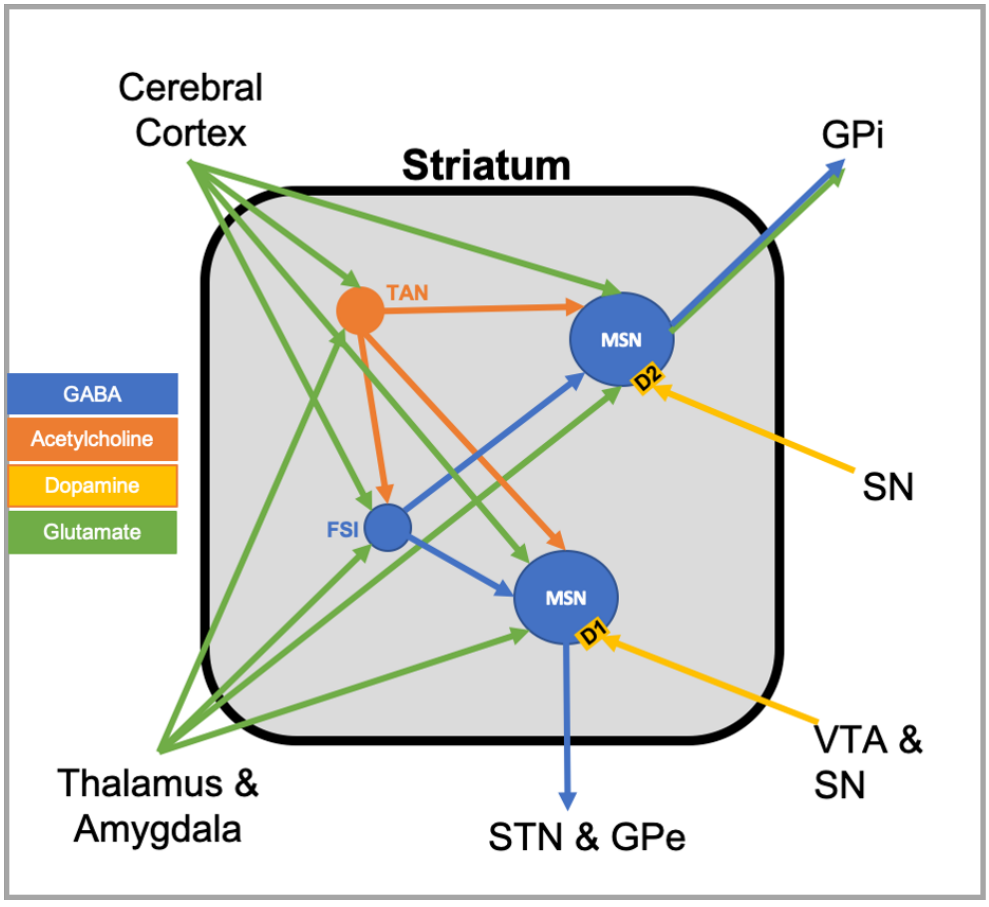
neurotransmitter pathways (Kluge et al., 1979; Costardi et al., 2015). The finding that adolescent alcohol exposure increases the rate of omission learning implies that omission contingency learning may be a behavioral task to allow us to investigate the effects alcohol has on the vulnerable adolescent neurotransmitter pathways and brain areas. The current body of literature suggests dopamine, GABA, and acetylcholine may play important roles within the ACC, DLS, and NAc in learning and prior alcohol exposure can lead to long-term alterations of these neurotransmitter systems, and alterations in these neurotransmitter systems may underlie the behavioral effects of prior alcohol access on behavior.

Understanding the neural transmission, neuronal cell types, and their roles within brain areas is key for knowing how dysregulation can occur. Although I will assess neuronal markers in many brain areas, my initial focus was on potential alterations in the striatum. The striatum is predominantly composed of GABAergic neurons call medium spiny neurons (MSNs), and these neurons are heavily regulated by GABAergic and cholinergic interneurons (Burke et al., 2017; Clarke & Adermark, 2015). A specific type of GABAergic interneuron called parvalbumin expressing (PV+) neurons (also called fast spiking interneurons [FSIs]) are found within areas that facilitate reward-related learning, including the dorsal striatum and prefrontal cortex (Mohila & Onn, 2005; Orduz et al., 2013; Wang et al., 2016; Melon et al., 2016). Additionally, there are tonically active cholinergic interneurons (TANs) that not only regulate MSNs, but also FSIs/PV+ neurons (Burke et al., 2017; Clarke & Adermark, 2015). As Figure 1.2 shows, GABAergic and cholinergic interneurons regulate neuronal activity within the striatum, while dopamine and glutamate are predominantly inputs from other areas (Burke et al., 2017; Yager et al., 2015). Therefore, activity responsible for striatum-specific learning may be more involved with these GABAergic and cholinergic interneurons that regulate input and output signals. The activity of

these neuronal subtypes is being assessed because if the omission contingency learning task is indeed reliant on the striatum (specifically the DLS), then the neurons that regulate the activity of the region responsible may be the ones being altered which is leading to these behavioral changes.

Figure 1.2

The inputs, outputs, and main neuronal cell types of the striatum and what sort of neurotransmitter is predominately transmitted by each. Tonically active cholinergic interneuron (TAN); fast spiking parvalbumin interneuron (FSI); medium spiny neuron (MSN) dopamine receptors for the direct pathway (D1); dopamine receptors for the indirect pathway (D2); internal globus pallidus (GPi); external globus pallidus (GPe); subthalamic nucleus (STN); ventral tegmental area (VTA); substantia nigra (SN).



The Role of GABAergic Interneurons.

Here, I will study GABA-ergic function after alcohol access because our preliminary findings have found that the level of alcohol consumption is related to the number of certain GABAergic interneurons expressing parvalbumin (PV) in both the ACC and DLS. FSIs (PV+ neurons) are found throughout cortical and striatal areas. PV+ interneurons are believed to aid in maintaining the calcium homeostasis and modulating activity of surrounding neurons (Kalus & Senitz, 1996; Orduz et al., 2013). Activity of the ACC, amygdala, and dorsal striatum (both DLS and DMS) are partially regulated by PV+ GABAergic interneurons, and PV+ expressing interneurons within each of these areas show a positive relationship with drug use, such that PV+ neuron density increases with drug intake compared to water only controls (Liu et al., 2005; Mohila, 2004; Melon et al., 2019; Olsen & Liang, 2017; unpublished data).

First, I review the literature on PV+ neurons in the striatum. The striatum is highly regulated by PV+ interneurons, however the effects of alcohol on the neurons in this region have been understudied and should be considered. In the striatum, PV+ neurons play a strong role of feedforward inhibition (taking an excitatory input and turning it into an inhibitory output) from the cortex and synapse onto medium spiny neurons (MSNs) (Burke et al., 2017; Nahar et al., 2021) A single PV+ neuron can synapse onto ~100 MSNs, which may exert profound downstream effects and cause changes in glutamate dysregulation and therefore cognitive and behavioral changes (Nahar et al., 2021).When PV is removed from PV+ neurons within the striatum, there is increased firing of the PV+ neurons and modifications to inhibitory functioning at its synapses. This finding suggests PV specifically plays a role in the PV+ interneurons' orchestration of neuron firing (Orduz et al., 2013). It has also been shown using optogenetic and chemogenetic techniques that PV+ neurons in the DLS influence the initial expression of reward-

conditioned responses, however their contribution to this performance declines following experience (Lee et al., 2017). Together these findings indicate that PV+ neurons are crucial regulators within the striatum for both neural transmission and behavior.

There is also some evidence that PV+ neurons in the dorsal striatum are related to alcohol consumption. Previous work from our data has shown that maintenance errors in reversal learning correlated with PV+ neurons in the DLS and with voluntary alcohol consumption (Fisher et al., 2017; data not published). Preliminary immunohistochemical results from the current study show a significant positive relationship between PV+ neurons within the DLS and level of alcohol consumption, but this effect is not found within the DMS. However, when comparing alcohol-exposed versus alcohol naïve rats, there was no significant difference in PV+ neurons found in either the DMS or DLS between the two groups. The differences in PV+ neurons may be due to individual differences within the PV+ neuron population of the DLS that may predict and/or affect a subject's level of alcohol consumption rather than alcohol changing PV+ expression in the population.

Next, I review the literature on PV+ neurons in the ACC. Although effects of alcohol exposure during adolescence on PV+ neurons are understudied, an increase in ACC PV+ neurons has been observed following adolescent exposure and withdrawal from other drugs of abuse, such as nicotine and amphetamine (Liu et al., 2005; Mohila, 2004). PV+ neurons within the ACC seem to show an age-dependent reactivity following drug use. Specifically, there is evidence that PV+ neurons in the ACC may be differentially affected by alcohol exposure at different developmental periods (Moore et al., 1998; Mitchell et al., 2000; Vongvatcharanon et al., 2010). However, there is evidence that pre-existing individual differences may be associated with PV+ neurons, as one study showed that substance-naïve people with a family history of

alcoholism exhibit differences in GABA-related neurochemistry in ACC (Cohen-Gilbert et al., 2015). This finding suggests that individual differences in PV+ neuron population within the ACC may predict a genetic predisposition for alcohol consumption.

Our preliminary immunohistochemistry work shows a significant relationship between number of PV+ neurons in the ACC and the amount of alcohol a rat consumed, such that rats that consumed higher amounts of alcohol showed a higher count of PV+ neurons within their ACC. However, no difference was found between alcohol exposed rats and rats given water only. The relationship between consumption and PV+ neurons but lack of difference between the different groups may be due to a couple factors. First, this relationship between consumption and PV+ neurons could be a predictor for individual differences that affect alcohol consumption, such that individual rats with specific brain areas containing a higher PV+ neuron count can lead to a susceptibility for drinking more. Conversely, this could be an alcohol-induced effect that was not powerful enough to reach significance because CIA paradigm allows voluntary consumption, which is typically lower than other paradigms and allows for individual differences in consumption between animals. Therefore, with this method we may not have been able to get a large enough effect between alcohol-exposed and alcohol naïve groups due to variability of consumption. The ACC appears to be affected by alcohol consumption and drug intake, and this brain area transmits inputs to the striatum, which could affect learning within the striatum.

Finally, I review the literature on PV+ neurons in the amygdala. The amygdala also projects to the striatum, and recent literature shows a positive relationship between PV+ neurons within the amygdala and alcohol exposure, however this relationship is caused more strongly by alcohol withdrawal (Melon et al., 2019). Exposure to binge drinking paradigms has been found to cause up-regulation of GABA-A receptors on PV+ neurons within the basolateral amygdala

(BLA) (Olsen & Liang, 2017). Additionally, with this upregulation in the GABAergic neurons, upon withdrawal of alcohol rats show heightened negative affect (such as symptoms of withdrawal) and reinforcement sensitivity accompanied by dysregulation of neurotransmitter levels within the amygdala. In addition, PV+ neurons within the amygdala seem to play key factors in withdrawal expression and negative affect, since loss of GABA-A receptors in PV+ neurons within the amygdala has been shown to increase binge drinking behavior, decrease sensitivity to alcohol-induced motor incoordination, enhance alcohol-induced locomotor activity, and decrease expression of withdrawal symptoms (Melon et al., 2019). However, it is unclear if PV+ neuronal expression is altered following alcohol consumption, or if this is correlated with behavioral differences in omission contingency learning. If the PV+ neuron population in the amygdala is altered, it could indicate that the binge-withdrawal aspect of the CIA paradigm could be a key component for our behavioral effects since this paradigm is designed with repeated periods of binges followed by withdrawals.

In conclusion, PV+ neurons play important roles in the ACC, amygdala, and the striatum. From lesion and inactivation studies, there is indication that the ACC and DLS are involved in autoshaping and/or omission contingency learning. It is possible that since PV+ neurons regulate these areas, they may be involved with the behavioral changes found following adolescent alcohol exposure. Therefore, PV may be essential for proper regulation of GABAergic neurotransmission and potentially learning strategies, and I will test whether neurobiological changes in this system in these areas accompany the behavioral changes.

The Role of Cholinergic Nuclei and Interneurons.

Acetylcholine plays an important role in many learning tasks. There is a hypothesis that the balance between acetylcholine and dopamine within the striatum has strong effects on

striatal-dependent learning (Cragg, 2006; Aosaki et al., 2010; Pitchers et al., 2017; Augustin et al., 2018). It is thought that dopamine plays more of a motivational role for action, while acetylcholine is vital for cognition and goal-oriented behavior (Pitchers et al., 2017). One piece of evidence for this is that during learning, a presentation of a CS predicting reward is associated with a 'pause' in a spike in cholinergic interneurons' firing, such that they have an initial excitation, followed by a pause in firing, and then subsequent rebound excitation. During the pause of the cholinergic interneurons' firing, there is a simultaneous surge in phasic dopamine firing (Aosaki et al., 2010). This synchronization of acetylcholine and dopamine indicates that the temporal acetylcholine and dopamine firing pattern is an important function for reward-related learning within the striatum.

The balance between acetylcholine and dopamine may have a very important role in the learning strategies utilized and types of associations made. During a Pavlovian conditioned approach task for intravenous injections of cocaine, sign-trackers show increased dopamine levels upon stimulus presentation, whereas goal-trackers instead showed increased acetylcholine levels upon stimulus presentation in a microdialysis measure (Pitchers et al., 2017). The cholinergic neurons react more to the context and location of stimuli during learning, whereas the dopamine neurons firing is based on the strength of desire for the reward (Aosaki et al., 2010). This idea maps on well to the theory of sign-tracking vs. goal-tracking, since sign-trackers have shown to be more motivated towards cues that predict reward, whereas goal-trackers are more context oriented (Saunders et al., 2012; Robinson et al., 2013). Since they each react to different reward related events, this acetylcholine-dopamine balance in the striatum may be a crucial piece of learning strategies used in a given task. Based on this evidence, increased dopamine firing may cause more action-oriented responses, while increased acetylcholine firing

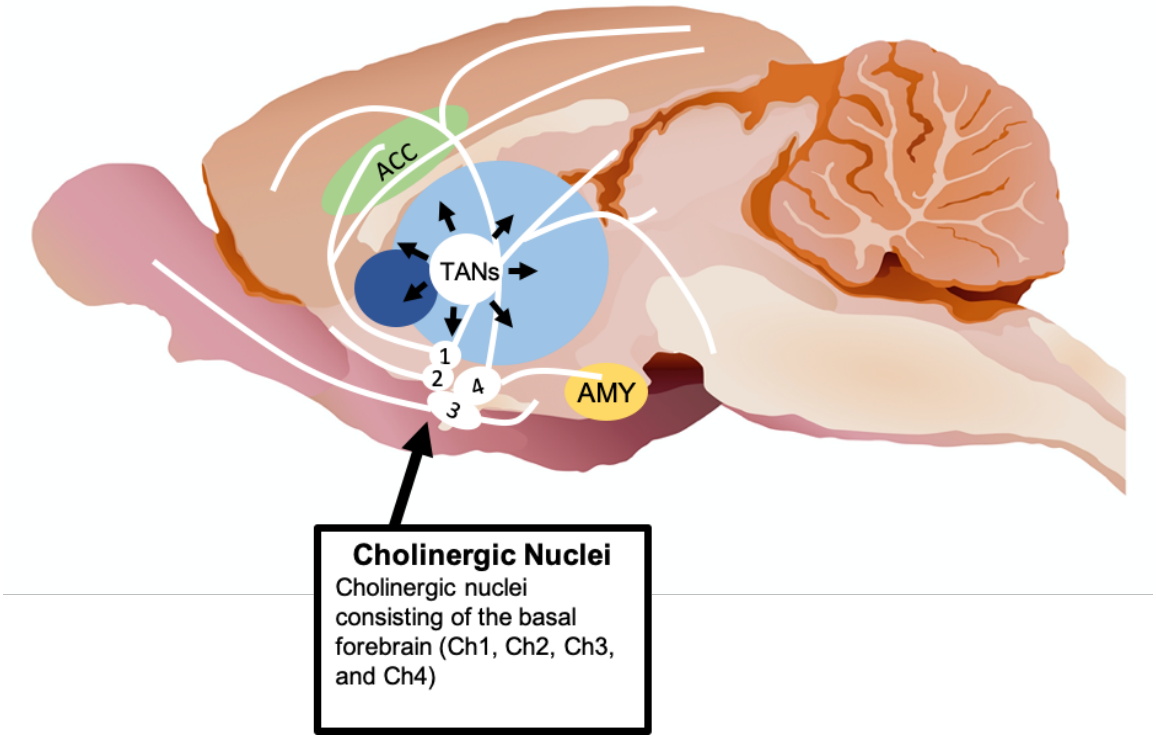
may cause increased stimuli-oriented responses. Therefore, a reduction in acetylcholine levels might shift a bias toward sign-tracking behavior, however it is unclear what the effect would be during autoshaping and omission learning. The task used in this study is biased towards sign-tracking, so some of these individual differences might not play out in the data.

Here, I will study cholinergic function after alcohol access because several studies have found a decrease in the number of neurons expressing choline acetyltranferase (ChAT), which is an enzyme required for the synthesis of acetylcholine, following intermittent alcohol exposure (Vetreno et al., 2014; Coleman Jr. et al., 2011; Boutros et al., 2015). Importantly, this effect has only been found following adolescent alcohol exposure and not found with adult exposure. As Figure 1.3 shows, the cholinergic nuclei of the basal forebrain lie adjacent to the striatum, and within the striatum there are also many cholinergic interneurons providing input as well. Cholinergic neurons are found throughout the cortex and the striatum, however I am focusing on the areas of the striatum and cholinergic nuclei for this experiment because there is previous research showing that adolescent alcohol consumption affects these cholinergic nuclei throughout these areas (Vetreno et al., 2014; Boutros et al., 2015). The ACC is additionally going to be assessed since the prefrontal cortex is also an area commonly of interest for ChAT+ neurons in relation to alcohol exposure (Coleman Jr. et al., 2011; Boutros et al., 2015), however the amygdala will not be assessed since there is no known link between ChAT+ neurons in the amygdala and alcohol consumption.

For one key example, there is evidence that adolescent intermittent intragastric (5g/kg with a 2 days on/ 2 day off pattern) alcohol access causes a reduction of ChAT expressing (ChAT+) neurons within the basal forebrain cholinergic nuclei (Ch1-4), the brainstem cholinergic nuclei (Ch5-6), the medial habenula (Ch7), and the striatum in adulthood. The only

Figure 1.3

The cholinergic pathways and their flow through relevant brain areas for this study (not all cholinergic projections or nuclei included). Tonically active cholinergic interneurons (TANs) are the dominate cholinergic input for the striatum, while Ch1 provides acetylcholine to the anterior cingulate cortex (ACC) and Ch4 provides acetylcholine to the amygdala (AMY).



cholinergic nucleus not shown to be affected by adolescent alcohol exposure was the parabigeminal nucleus (Ch8) (Vetreno et al., 2014). These findings strongly suggest that alcohol during adolescence leads to dysregulation of the cholinergic system, which can have lasting effects through adulthood.

Other studies looking at both acetylcholine and dopamine levels found primarily large reductions in the cholinergic levels following alcohol exposure. Coleman Jr. and colleagues (2011) observed reduced cholinergic and dopaminergic gene expression 24 hours after

intragastric alcohol exposure (5 g/kg daily for 10 days), and gene expression was reduced even more so into adulthood. Additionally, they found smaller basal forebrain areas with fewer cholinergic neurons in mice exposed to alcohol. These mice also showed impairment in a reversal learning task, which may implicate these neurons as important for such learning. Relatedly, Boutros and colleagues (2015) administered alcohol intragastrically (5 g/kg 3 times/day with a 2 days on/ 2 day off pattern) throughout adolescence, followed by a delay discounting task with altered probabilities of reward to measure risky choice, and then double-labelled brains for ChAT and tyrosine hydroxylase (a common marker for dopamine). They found that rats exposed to alcohol had a significantly decreased ChAT+ neurons in Ch1-4. The tyrosine hydroxylase (TH) expressing neurons were only decreased within the prelimbic cortex of the PFC. Although there was no relationship between risky choice behavior and alcohol exposure, there was a negative correlation between risky choice and ChAT+ neurons. These results show strong support for the hypothesis that adolescent alcohol exposure alters cholinergic functioning within the basal forebrain and striatum. However, these studies used high doses that are not seen in voluntary consumption, so I determined in the current study if this effect would still occur at the lower exposure level associated with voluntary intake.

Goal of the Experiment.

The goal of this experiment is to assess which brain areas may be altered after adolescent alcohol access and, thus, may be involved in the altered omission contingency learning found. In the behavioral aspect of my study, we have replicated previous findings that show this effect of faster omission learning, but this previous study did not assess an underlying neurobiological substrate. Since alcohol exposure during adulthood does not cause this effect, we aim to find the long-term neurological alterations that occur to adults with previous adolescent alcohol access. I

assessed the role that cholinergic and GABAergic neurons within the ACC, DLS, DMS, NAc, and amygdala, and how this may inform our findings of voluntary alcohol access during adolescence/early adulthood leading to faster omission contingency learning. I stained for PV+ and ChAT+ interneurons, as there is evidence these highly influence the modulation of functioning within several brain regions, and I primarily focused on brain areas previously shown to play roles in sign-tracking or omission learning. I assessed my findings using statistical analysis that is more nuanced analyses of binomial and Poisson mixed effect models. I hypothesized that similar to our finding within the ACC, we would find a relationship between PV+ neurons and alcohol consumption within the amygdala. I predicted that PV+ neurons in the amygdala will increase as a rat's voluntary alcohol consumption increases, and that there would be a greater PV+ neuron population in alcohol exposed rats compared to alcohol naïve rats. I further hypothesized that there would be a reduction of ChAT+ neurons in the basal forebrain cholinergic nuclei (Ch1-4) and within the striatum, particularly in the DLS, of rats exposed to alcohol during adolescence, and this would correlate with their level of alcohol consumption.

Chapter 2 - Methods

Animals.

A total of 24 male Long Evans rats from Charles River Laboratories (Raleigh, NC) were used in this experiment and arrived at PND 21. All animals were kept in a temperature and humidity controlled environment on a 12-hour reverse light-dark cycle with lights off at 7:00 AM. Subjects were given five days to habituate to the colony room, and started alcohol access on PND 26. All subjects were given food and water *ad libitum* during habituation and alcohol access periods. Upon completion of the alcohol access phase, rats were food restricted to 85% of their body weight on the last day of the CIA paradigm. Once rats reached the initial 85% target weight, 1 gram/day was added to their weight, such that each rat gained 7 grams/week for the rest of the experiment. During food restriction, free access to water remained available. All procedures and animal care were in accordance with the Kansas State University Institutional Animal Care and Use Committee guidelines (IACUC), the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and United States federal law.

Alcohol access.

Rats received six weeks of either alcohol access (Alcohol Group; n = 12) or water-only access (Water Group; n = 12), with the first four weeks occurring during adolescent development (PND: 26-54) and the remaining two weeks occurring during early adulthood (PND 54-68). The alcohol access paradigm utilized in this study was the chronic intermittent access (CIA) paradigm (Simms et al., 2008; Wise, 1973), in which two bottles were available to all rats on their home cages at all times. For the Water Group, both bottles provided to the subjects always contained tap water. For the Alcohol Group, one bottle provided on the home cage consistently was filled with water while the second bottle would rotate between water and a 20% v/v alcohol

solution. The 20% alcohol solution was provided in one of the bottles 3x per week for 24 hours, with a 24- or 48-hour period of water only access in both bottles between exposures. The alcohol and water bottles were weighed daily between 11:00am and 12:30pm in order to determine amount of consumption within a 24-hour period. Upon completion of the 6-week CIA paradigm, rats were given one water bottle for the remainder of the experiment.

Behavioral apparatus.

This experiment was conducted in standard self-administration chamber (Med Associates, St. Albans, VT). These chambers contained two retractable levers on either side of the food cup at a height around one-third of the total chamber. A white cuelight was located above each of the retractable levers. A photocell beam was located at the entrance of the food cup was to record head entries during the autoshaping training. A red houselight was centrally mounted on the top part of the back wall, opposite that of the food cup. A speaker was also located on the left side of this wall for the purpose of delivering auditory stimuli. A Dell Optiplex computer equipped with Med-PC for Windows controlled the mechanics of the chambers and recorded lever presses and head entries.

Behavioral training.

Autoshaping learning.

Behavioral training began 10 days after the last day of alcohol access, and each rat was run in the same operant chamber throughout testing. First, rats received two once-daily sessions of magazine training where there were no levers available or exposure to operant training. This was done in order to expose the rats to the sound the pellet dispenser makes when delivery of a pellet occurs in order to reverse effects of neophobia and allow them to know this sound means

they have just been rewarded. Both sessions lasted 45 minutes with a food pellet being delivered every 305 seconds.

Following magazine training, rats received 6 once-daily sessions of autoshaping training, which lasted for 45 minutes each. During this training, the presentation of each lever with its paired cuelight would separately be paired with delivery of two food pellets regardless of responding. Each lever and its corresponding cuelight co-occurred and co-terminated during each trial. There were 24 total trials of cuelight+lever presentations, with 12 trials presented for the left lever and 12 presented for the right lever. The cuelight+lever pairings were presented one at a time, with the order pseudo-randomly selected such that every block of 6 trials would contain 3 presentations of each lever. All trials lasted 12 seconds and began by an extension of one of the levers and a steady illumination of the cuelight above it. The trial ended by the retraction of the lever and termination of the cuelight, with one food pellet being delivered as the lever retracted and the cuelight was extinguished followed by another pellet dispensed a second later. During each trial, the number of lever presses and number of head entries were recorded. In the preliminary results section, the levers are labeled based on their consequences during the omission contingency phase, such that the left lever is called the "Omission Lever" and the right lever is called the "Passive Lever." However, during this phase of training there was no difference in outcome between the two lever presentations.

Omission contingency learning.

Following the autoshaping training, rats received 6 once-daily sessions of omission contingency training. The number of trials, timing of trials, and cue presentations remained the same as they were in the autoshaping phase. However, the left lever (the "Omission Lever") during this training only resulted in food reinforcement if the rats withheld from making any

lever presses during that trial. If no response was made during left cuelight+lever presentations, then the rats received their usual two-pellet reward. Any trial with a left cuelight+lever presentation in which a rat did respond with a lever press ended with a 0.5-tone but no food reward. Trials that had presentations of the right lever (the "Passive Lever") continued to end in the food reinforcement regardless of responding, just like in the autoshaping training sessions. Lever presses were recorded during each trial. Due to experimenter error, head entries were not recorded during this phase.

Immunohistochemistry.

Two days after completion of behavioral testing, rats were euthanized with a pentobarbital and then perfused with 0.1-M PBS followed by 4% paraformaldehyde. Brains were extracted and stored in 4% paraformaldehyde for 48 hours followed by a 20% sucrose solution for five days at 4° Celsius. Brains were then frozen at -80° C, and slices (40μm) were collected from regions of interest (Coordinates relative to Bregma: ACC at 2.7 ± .1 and 3.2 ± .1; NAc Core and Shell at 1.6 ± .1; DMS and DLS at 0.8 ± .1 and 0.4 ± .1; cholinergic nuclei Ch1-Ch4 at 0.6 ± .1; Amygdala at -3.0 ± .1 and -3.3 ± .1).

Parvalbumin staining.

These slices were rinsed 3 X10 minutes in PBS. Then they were washed in PBS containing 5% normal horse serum (NHS) for 30 minutes, and afterwards are incubated for 48 hours in a solution of PBS, 2% NHS, .2% Triton X, and a ratio of 1:40,000 of the primary antibody mouse anti-PV (Sigma P-3088). Then slices were washed 3 x 10 minutes in PBS, and incubate in a solution of PBS, 2% NHS, .2% Triton X, and a ratio of 1:1000 of the secondary antibody donkey anti-mouse (Vector Labs BA-2000). Then slices were once again rinsed 3 x 10 minutes in PBS, and then incubated in an avidin-biotin complex. Slices were again rinsed 3 x 10

minutes in PBS. The chromogen 3,3'-Diaminobenzidine (DAB) was used for immunohistochemical staining. Brain sections were mounted on microscope slides and cover-slipped before imaging. ImageJ® _software was used to identify and count the number of positively stained PV+ neurons.

Acetylcholine transferase staining.

The same rats and brains areas were also used to stain for ChAT+ neurons, however a different series of slices were used. These slices were rinsed 3 X10 minutes in PBS. Then they were washed in PBS containing 5% normal goat serum (NGS) for 30 minutes, and afterwards were incubated for 48 hours in a solution of PBS, 2% NGS, .2% Triton X, and a ratio of 1:40,000 of the primary polyclonal antibody rabbit anti-ChAT (FisherSci NBP233530). Then slices were washed 3 x 10 minutes in PBS, and incubate in a solution of PBS, 2% NGS, .2% Triton X, and a ratio of 1:200 of the biotinylated secondary antibody goat anti-rabbit (FisherSci PI31460). Then slices were once again rinsed 3 x 10 minutes in PBS, and then incubated in an avidin-biotin complex. Slices were again rinsed 3 x 10 minutes in PBS. The chromogen 3,3'-Diaminobenzidine (DAB) was used for immunohistochemical staining. Brain sections were mounted on microscope slides and cover-slipped before imaging. The same imaging technique used for the PV+ neuron staining was used here as well for ChAT.

Statistics.

This data was analyzed using multiple mixed effects modeling in order to visualize the effects in multiple ways and fully determine the relationship between adolescent alcohol exposure and omission contingency learning. All significant interactions have been followed up with a pairwise comparison with a Tukey's adjustment.

Autoshaping learning.

Poisson mixed effects models were additionally used to assess the effects of variables of interest on the total number of lever presses and head entries made per trial. All Poisson models were checked for overdispersion and minimal overdispersion was found, therefore alternative models were deemed unnecessary. Binomial mixed effects models were used to analyze the likelihood of whether a lever press or head entry would be made per trial. For all of the Poisson and Binomial models, there was a fixed effects structure of the Day x Group x Lever factorial, but Trial was also added as a covariate to account for additional variance. The random effects structures of all of these models were that the intercept, slope of day, slope of lever, and slope of trial were allowed to vary by subject.

Additionally a Pavlovian Conditioned Approach (PCA) ratio was calculated using these previous dependent variables (See Equation 1), where N_{lever} is the total number of lever presses, $N_{foodcup}$ is the total number of head entries, T_{lever} is the number of trials with a lever press, and $T_{foodcup}$ is the number of trials with a head entry. This ratio could possibly range from a positive 1, which indicates exclusive sign-tracking behavior, to a negative 1, which indicates exclusive goal-tracking behavior. The PCA index is beneficial in that it provides a single measure of bias rather than needing to extrapolate from multiple graphs and numbers. A linear mixed effects model was used to assess the Day x Group x Lever interaction of the PCA ratio, which utilized a random effects structure that allowed the intercept, slope of day, and slope of lever to vary by subject.

Equation 2.1.

PCA Index Equation.

$$\text{PCA Index} = [\{(N_{lever} - N_{foodcup})/(N_{lever} + N_{foodcup})\} + \{(T_{lever} - T_{foodcup})/(T_{lever} + T_{foodcup})\}]/2$$

Omission contingency learning.

A Poisson mixed effects model was used to analyze the effects of predictors on the total number of lever presses per trial. A binomial mixed effects model was used to analyze the likelihood of whether a lever press would be made per trial. For both the Poisson and Binomial models, there was a fixed effects structure of the Day x Group x Lever factorial, but Trial was also added in as a covariate to account for additional variance. The random effects structures of these models were that the intercept, slope of day, slope of lever, and slope of trial were allowed to vary by subject. The PCA ratio could not be analyzed, due to a programming error which caused head entries to not be recorded for these sessions.

Immunohistochemistry.

To assess group differences a Poisson mixed effects model was used to assess the number of PV+ or ChAT+ neurons with the fixed effects of Group x Brain Area. The random structure of this model will allow the intercept and slope of brain area to vary.

To assess individual differences consistently with previous data within the alcohol consumption group, a Poisson mixed effects model was used to assess the number of PV+ or ChAT+ neurons with the fixed effects of Alcohol Consumption x Brain Area. The random structure of this model will allow the intercept and slope of brain area to vary.

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Chapter 3 - Results

Alcohol consumption.

A linear mixed effects model confirmed a steady alcohol escalation occurred throughout the six weeks of CIA treatment, $F(1, 215) = 19.01$, $p < .001$ (See Figure 3.1a) . Although there was a slight decline in week 4's average consumption from week 3, there was an overall trend for increase in consumption across weeks. The standard deviations are quite large for the average amount consumed for each week due to the fact that there were considerable individual differences in alcohol consumption between subjects, which are shown in Figure 3.1b. I will be using the average alcohol consumption in g/kg/24hr of the last two week of the CIA paradigm for analyzing the relationship between behavioral effects and alcohol consumption. In weeks 5 and 6, rats have reached young adulthood in which their body weight and alcohol consumption tend to stabilize into a steady pattern.

Autoshaping.

Figure 3.2a shows the results of the binomial mixed effects model of autoshaping behavior. When assessing the likelihood of a response per trial using a binomial mixed effects model, there were three main effects. There was a main effect of group (Alcohol vs. Water), $F(1, 3413) = 5.76$, $p = .011$, such that the water group was more likely to press than the alcohol group. This effect was predominantly influenced by the initial lower likelihoods of responding on days 1 and 2 of training for the alcohol group. However, by day three the alcohol group no longer differed from the water group due to a ceiling effect. There was a main effect of day, $F(1, 3413) = 30.63$, $p < .001$, such that as the days of training progressed the likelihood of lever presses increased. There was also a main effect of trial, $F(1, 3413) = 17.42$, $p < .001$, such that as trials progressed within a single session (day) the likelihood of lever presses increased. These

two main effects were expected. There was not a main effect of lever and there were no significant interactions between variables.

Figure 3.1

Alcohol consumption data. A) The average consumption of all subjects over the entire six weeks. B) The individual levels of consumption for each subject averaged over weeks 5 and 6.

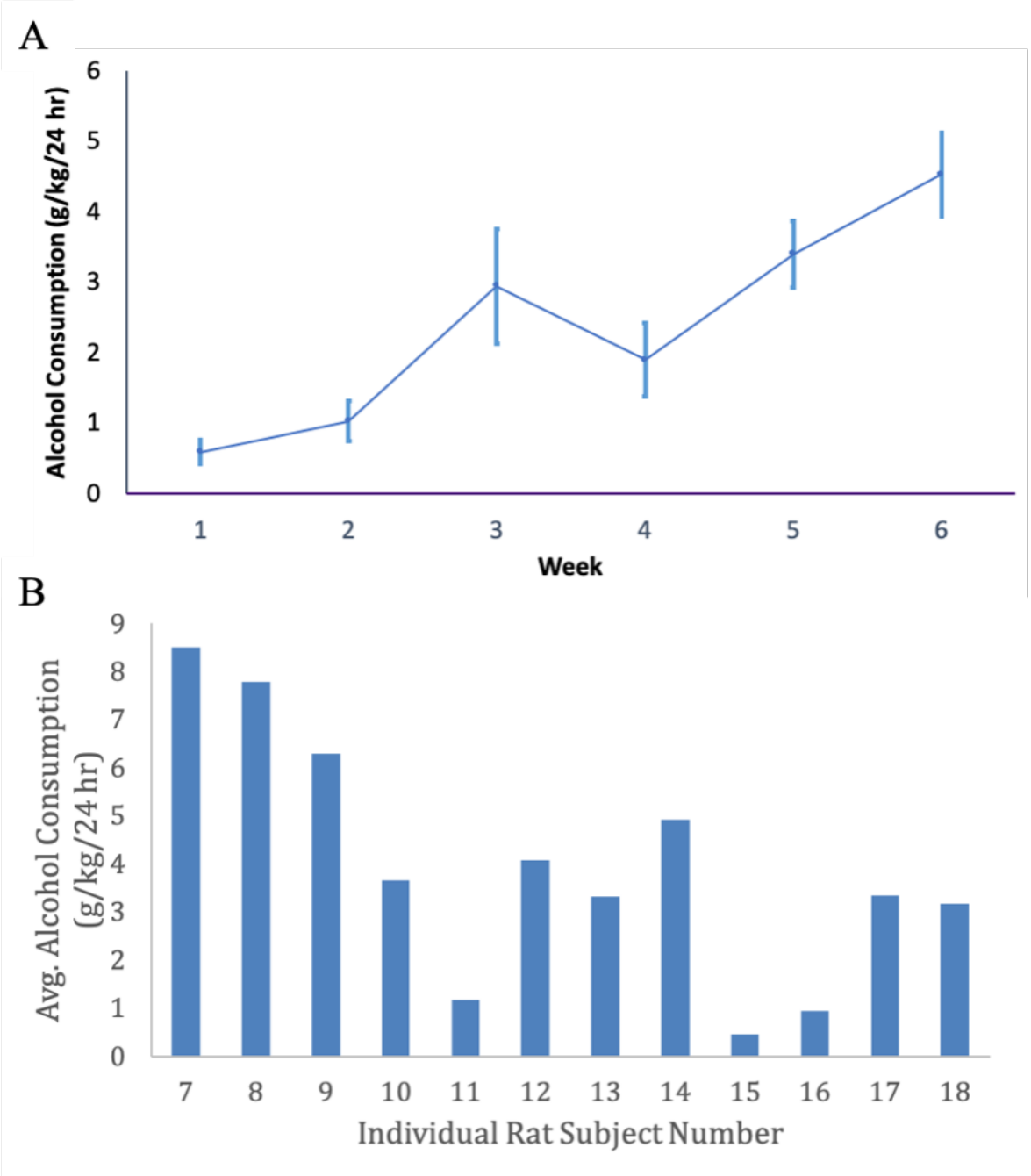


Figure 3.2

There are consistent increases for both levers (omission lever: left, passive lever: right) of lever presses through autoshaping sessions in A) the likelihood of responding per trial and B) the number of lever presses per trial.

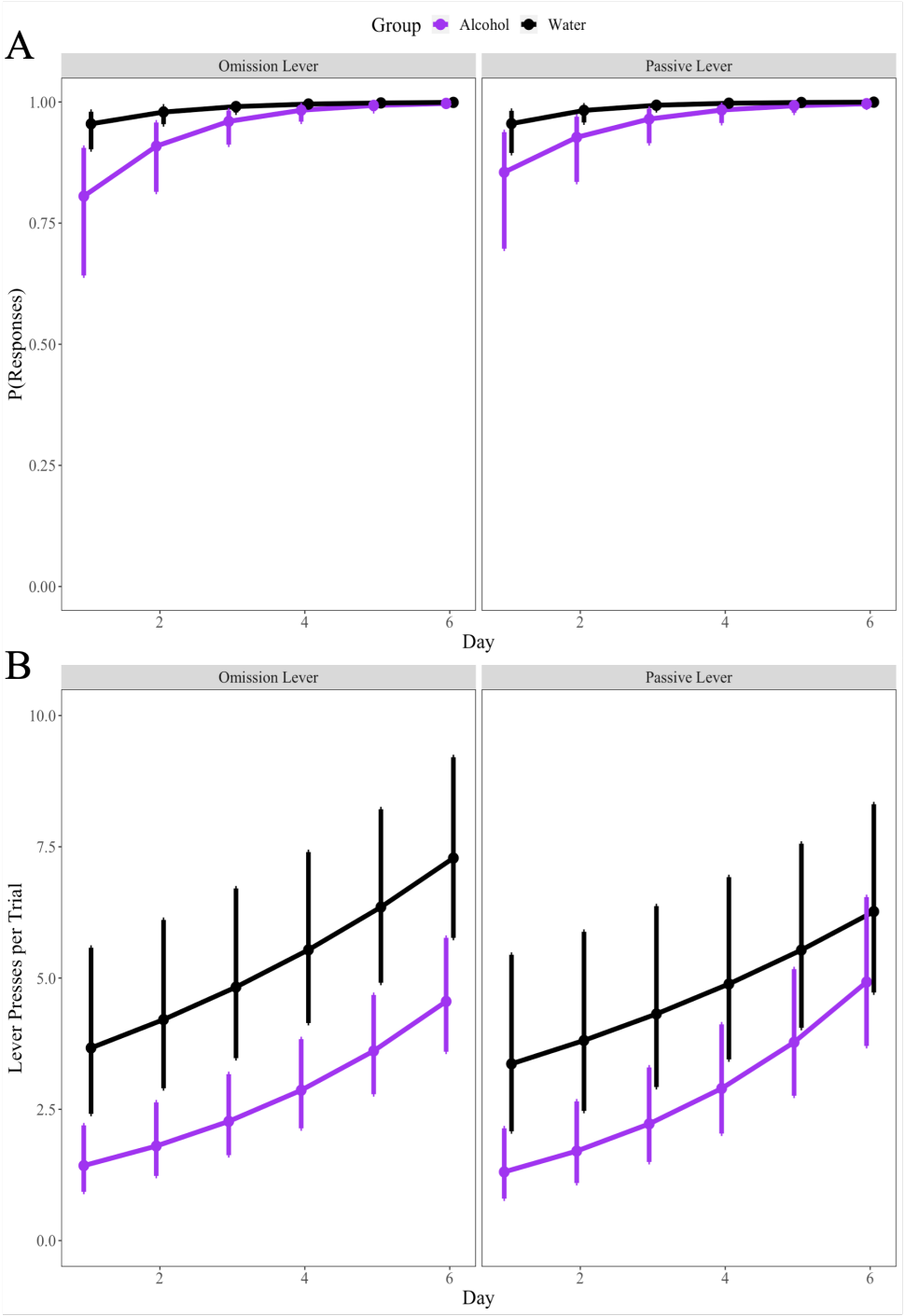


Figure 3.2b shows a Poisson mixed effects model assessing the number of lever presses per trial found a main effect of day, $F(1, 3413) = 23.50$, $p < .001$. This indicates that the number of lever presses increased as the days of autoshaping progressed. There was also a Group x Day x Lever interaction, $F(1, 3413) = 5.82$, $p = .016$. However, pairwise comparisons across day showed no significance between any groups or levers. No other main effects or interactions were significant. This model and all other Poisson models conducted were checked for overdispersion and minimal overdispersion was found.

The analysis of the likelihood of a head entry per trial showed a main effect of Day, $F(1, 3413) = 26.04$, $p < .001$, and Trial, $F(1, 3413) = 18.28$, $p < .001$ (See Figure 3.3a). These both indicate that as the days and trials within each day progressed, the likelihood of head entries per trial decreased. This analysis also showed an interaction of Day x Lever, $F(1, 3413) = 6.07$, $p = .018$, which shows that the passive level initially had more head entries on day 1 and likelihood of entries decreased faster on the Passive lever because of this on days 2 and 3. No other effects of this analysis were significant. The number of head entries per trial was additionally analyzed, which found significant main effects of Day, $F(1, 3413) = 45.41$, $p < .001$, and of Trial, $F(1, 3413) = 17.07$, $p < .001$ (See Figure 3.3b). Similarly, these both indicate that as the days and trials within each day progressed, the number of head entries per trial decreased. No other effects for the analysis of numbers of head entries were found to be significant.

The PCA index (or sign-tracking ratio) was also analyzed using a linear mixed-effects model predicted by Group x Day x Lever (see Figure 3.4). The analysis of this ratio only indicated a main effect of Day, $F(1, 3413) = 44.30$, $p < .001$, such that as the days progressed rats began to sign-track more than goal-track. No other effects were found to be significant.

Figure 3.3

There are consistent decreases for both levers (omission lever: left, passive lever: right) through autoshaping sessions in A) the likelihood of head entries per trial and B) the number of head entries per trial.

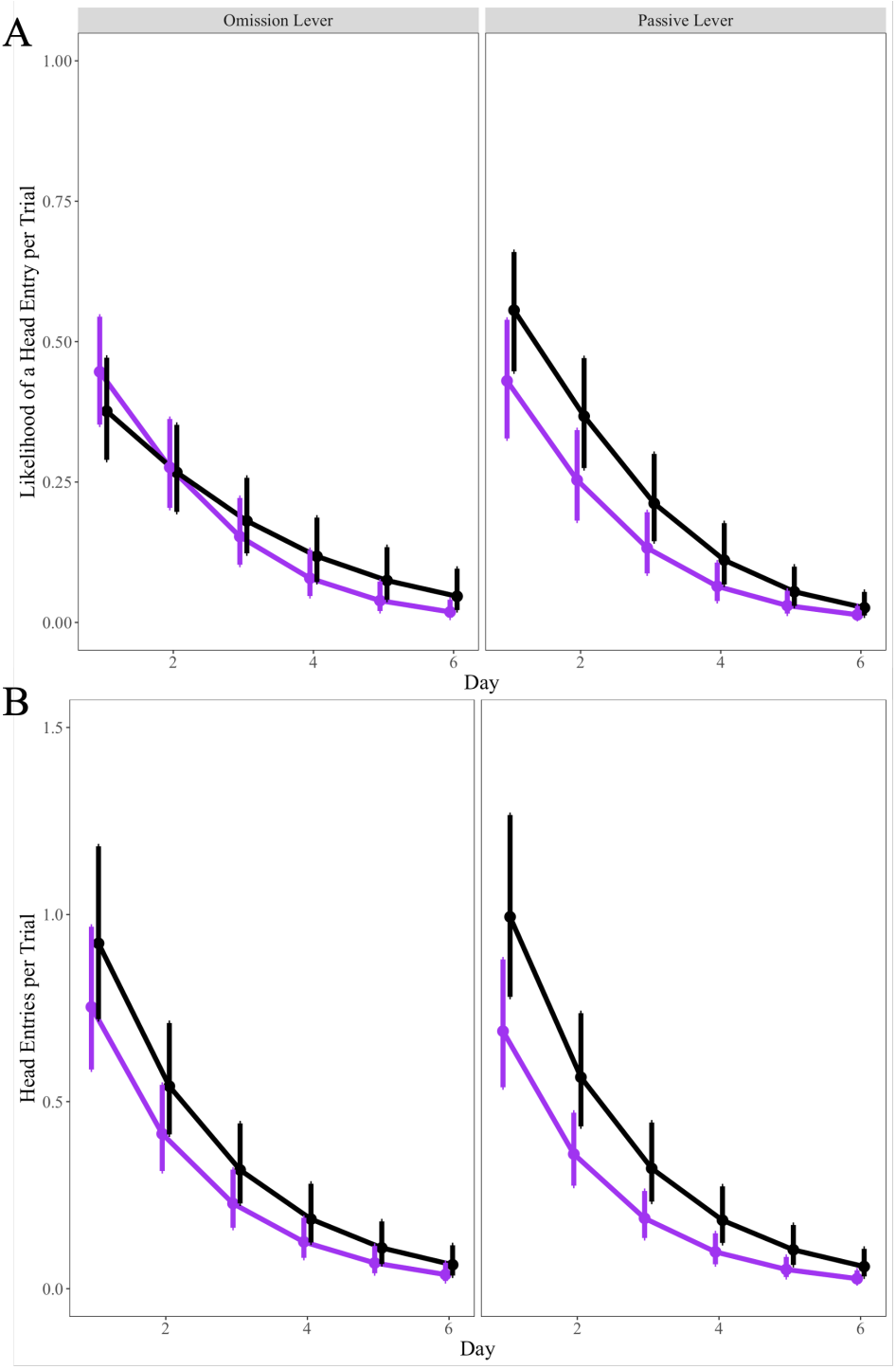
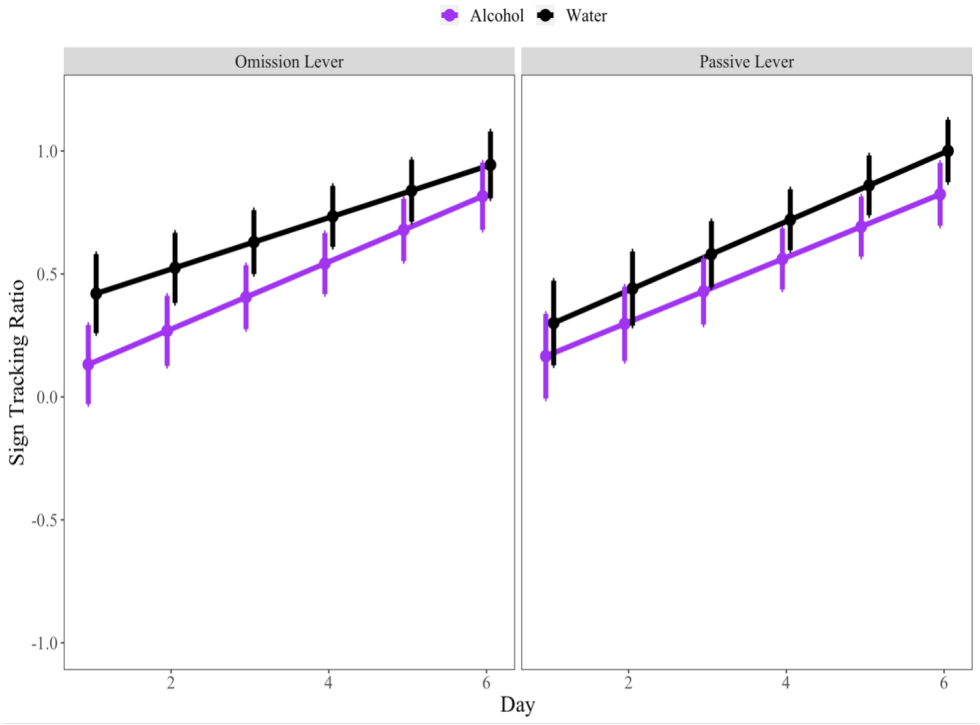


Figure 3.4

The sign-tracking ratio (or PCA index) as calculated by Equation 1 during autoshaping for both levers (Omission lever: left and passive lever: right) and each group (alcohol: purple, water: black).

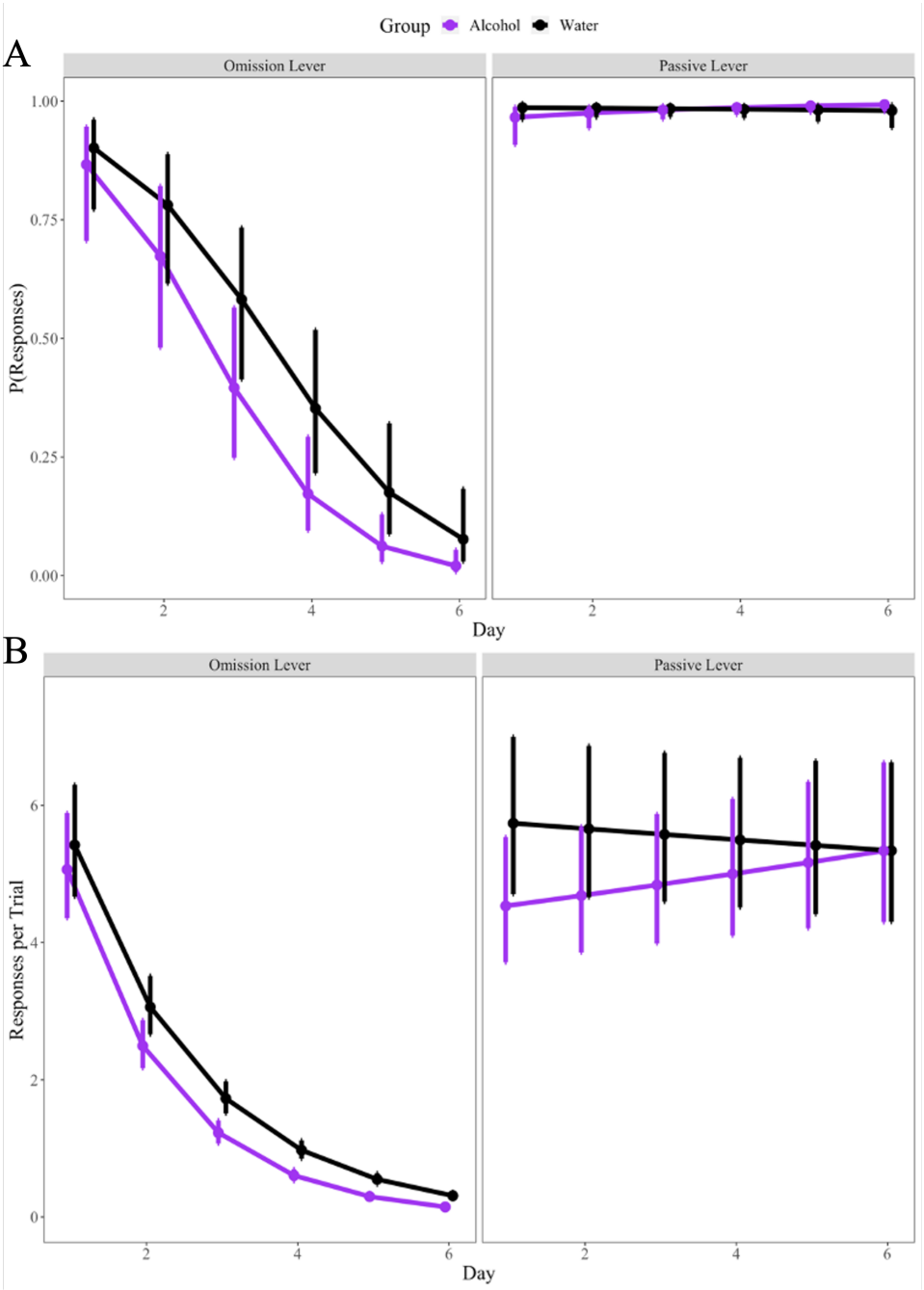


Omission contingency learning.

For the omission contingency learning the likelihood of a response per trial was analyzed using a binomial mixed-effects model, which is shown in Figure 3.5a. This analysis found main effects of Day, Trial, and Lever (all $p < .001$). The main effects of Day and Trial both show a decrease in likelihood of responding as days and trials progress. The main effect of Lever shows that the likelihood of a response per trial on the Omission Lever was significantly less than the likelihood for the Passive Lever. There was also a Day x Lever interaction, $F(1, 3437) = 78.96$, $p < .001$. This interaction indicates that as the days of omission contingency learning progressed the likelihood of a response per trial decreased on the Omission Lever, while the likelihood did

Figure 3.5

There is a consistent decrease for the omission lever through autoshaping sessions in A) the likelihood of responding per trial and B) the number of lever presses per trial. Passive lever is largely unchanged. The alcohol group (purple) shows are steeper decline in the number of lever presses per trial.



not change on the Passive Lever. The three-way interaction of Group x Day x Lever was also significant, $F(1, 3437) = 5.58$, $p = .019$. However, pairwise comparisons across day showed that the groups of interest (Water vs. Alcohol Group on Omission Lever; Water vs. Alcohol Group on Passive Lever) were not significantly different. No other fixed effects were significant.

The analysis of number of responses per trial during omission contingency learning utilized a Poisson mixed-effect model, which is shown in Figure 3.5b. This analysis showed main effects of Day, Trial, and Lever (all $p < .001$). The main effects of Day and Trial both show a decrease in number in responses per trial as days and trials progress. The main effect of Lever shows that the number of responses per trial on the Omission Lever was significantly less than the Passive Lever. This analysis also found a Day x Lever interaction, $F(1, 3437) = 1960.08$, $p < .001$. This indicated similar findings to the binomial analysis, such that the number of responses per trial decreased on the Omission Lever across days and did not change across days for the Passive Lever. The Group x Day x Lever three-way interaction was also found, $F(1, 3437) = 39.54$, $p < .001$. Pairwise comparisons revealed the Alcohol Group significantly decreased their number of responses by day compared to the Water Group on the Omission Lever ($p = .004$).

Immunohistochemistry.

Overall, the counts acquired from our brain regions in both the PV and ChAT immunohistochemical staining were lower than counts found in previous studies. This could be due to a number of reasons. One possible reason for this difference of counts could be due to differences in imaging methods. This study imaged subregions of the regions of interest in the same area of space for each subject, whereas other studies may have imaged the entire regions of interest and therefore produced a higher count. Additionally, this study may have been using a higher threshold to determine how darkly stained a neuron must be in order to count as a neuron.

This, too, could result in our lower count. And finally, there may potentially be less signal in our staining within this study compared to other studies. With less signal in the immunohistochemical staining, not as many PV+ or ChAT+ neurons would reach threshold to be counted in our brain regions of interest and could therefore lead to lower counts. However, during the immunohistochemistry and imaging process of this study, the images appeared to have an adequate number of neurons developed.

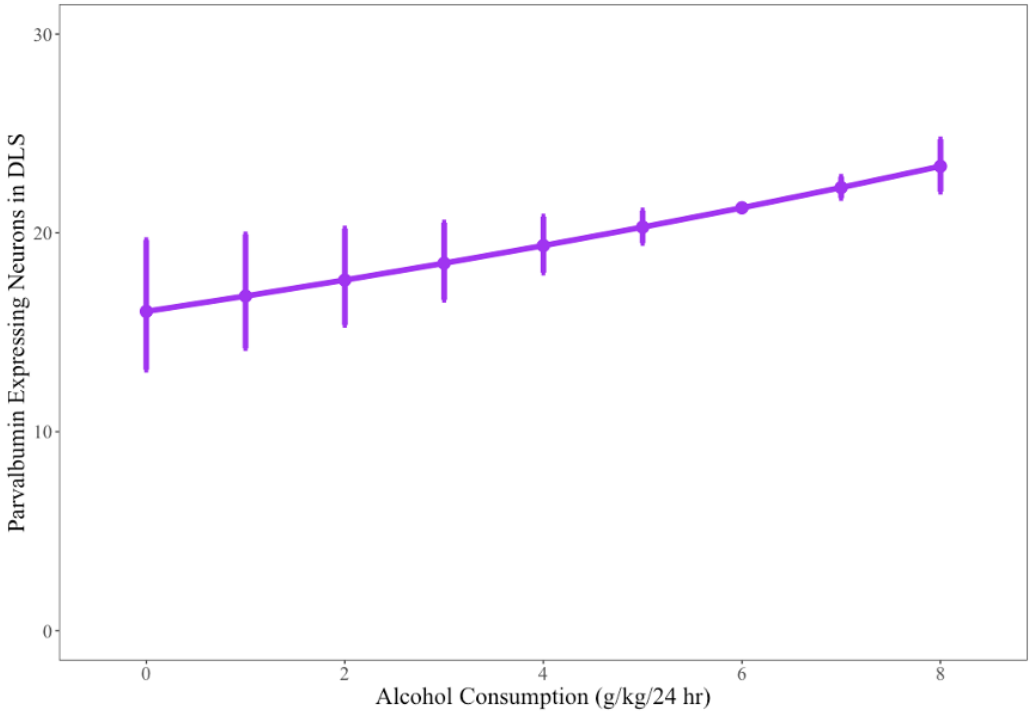
Parvalbumin Expressing Neurons.

The number of PV+ neurons was assessed in the DLS and DMS (+0.8 ± .1 & +0.4 ± .1mm relative to Bregma) using Poisson mixed-effects models. There were no significant differences found between alcohol and water groups within either the DMS or DLS (all effects $p > .05$). Individual differences in alcohol consumption and the relationship with PV+ neurons both the DLS and DMS were also analyzed. No significant relationship between alcohol consumption was found within the DMS, however there was a significant main effect of alcohol consumption within the DLS, $F(1, 39) = 3.42$, $p = .034$. This indicates that as subjects consumed more alcohol, they expressed more PV+ neurons within the DLS (see Figure 3.6).

The number of PV+ neurons was also assessed in two regions of the ACC (+3.2 ± .1 & +2.7 ± .1 relative to Bregma) using a Poisson mixed-effects model (see Figure 3.7). To assess group differences, the fixed effects of Group x ACC Region predicted the number of PV+ neurons. This showed a main effect of ACC region, $F(1, 85) = 83.12$, $p < .001$, such that the +3.2 region had more neurons overall than the +2.7 region. There was not a main effect of Group, nor

Figure 3.6

The model of level of alcohol consumed (g/kg/24 hr) by each individual rat in relation to number of parvalbumin expressing neurons within the dorsolateral striatum. The average consumption through weeks 5 and 6 for each rat is their individual consumption measure.



an interaction. Individual differences in alcohol consumption and the relationship with PV+ neurons in the ACC were also analyzed, as shown in Figure 3.8. This analysis showed a significant main effect of alcohol consumption, $F(1, 39) = 18.21$, $p = .001$. This main effect shows that the more alcohol a rat consumed, the more PV+ neurons they would express in their ACC. The main effect of ACC region was not significant, and neither was the interaction between alcohol consumption and ACC region.

Figure 3.7

Bar chart depicting the group differences of the number of parvalbumin expressing neurons between the two regions of the anterior cingulate cortex (ACC) imaged, and the lack of group difference between alcohol rats (purple) and water rats (black).

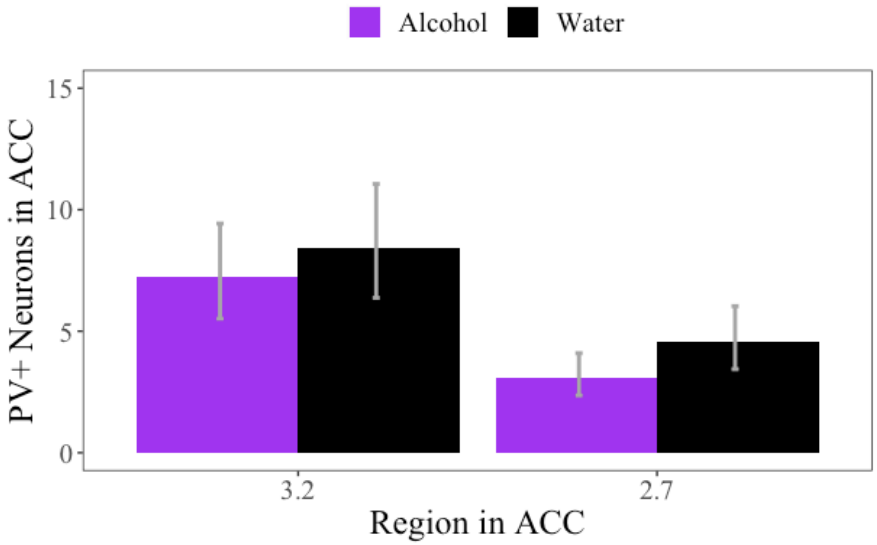
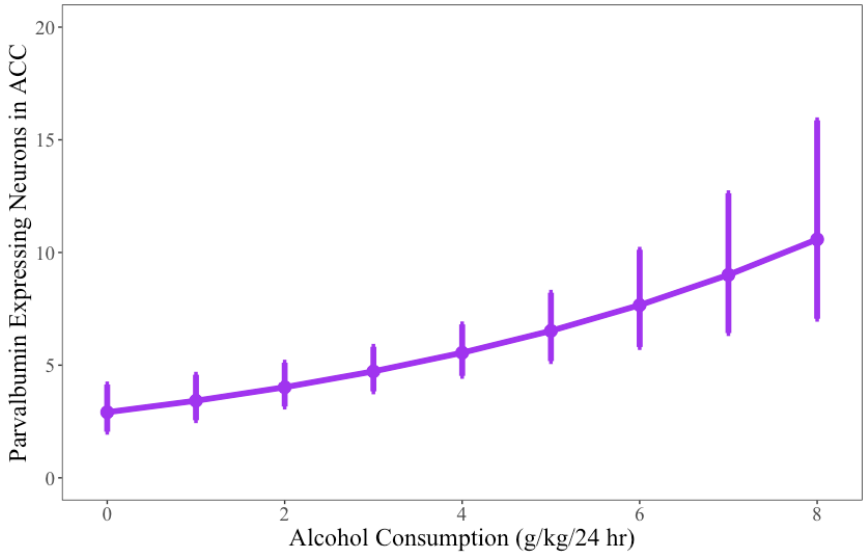


Figure 3.8

The model of level of alcohol consumed (g/kg/24 hr) by each individual rat in relation to number of parvalbumin expressing neurons within the anterior cingulate cortex (ACC). The average consumption through weeks 5 and 6 for each rat is their individual consumption measure.



Poisson mixed-effects models analyzed PV+ neurons within the NAc for both NAc core and shell (+1.6 relative to Bregma) in relation to alcohol exposure group. No significant effects were found between the water and alcohol group's PV+ neuron expression (all effects $p > .05$). Additionally, upon analyzing individual differences in alcohol consumption within the alcohol group and PV+ neuron expression, no significant effects were found ($p > .05$).

PV+ neurons within the amygdala (BLA; -3.0 ± .1 & -3.3 ± .1mm relative to Bregma) were analyzed using Poisson mixed-effects models as well. There was a main effect of amygdala coordinates, $F(1, 68) = 5.66$, $p = .031$, indicating that there were significantly fewer neurons expressed in the -3.0 region compared to the -3.3 region relative to Bregma. This is qualified by a group x region interaction, $F(1, 68) = 5.86$, $p = .020$. However, pairwise comparisons reveal that there is only a trending difference between the alcohol and water group in the -3.0 region of the amygdala ($p = .064$; See Figure 3.9). Additionally, upon analyzing individual differences in alcohol consumption within the alcohol group and PV+ neuron expression, no significant effects were found (all effects $p > .05$).

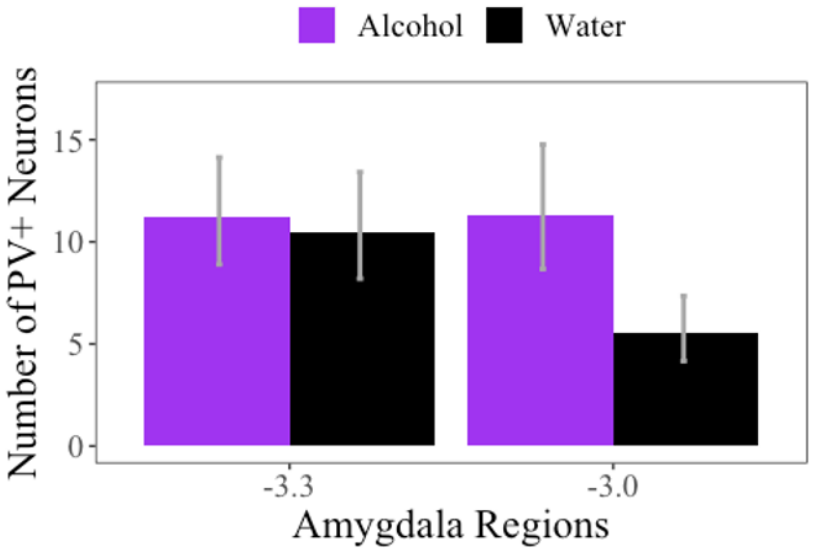
ChAT Expressing Neurons.

The number of ChAT+ neurons was assessed in the DLS and DMS (+0.8 ± .1 & +0.4 ± .1 mm relative to Bregma) using Poisson mixed-effects models. There were no significant differences found between alcohol and water groups within either the DMS or DLS (all effects $p > .05$). Individual differences in alcohol consumption and the relationship with PV+ neurons both the DLS and DMS were also analyzed. These analyses showed a significant main effect of alcohol consumption in both the DMS, $F(1, 30) = 10.65$, $p = .002$, and the DLS, $F(1, 32) = 28.51$, $p < .001$. As shown in Figure 3.10, this indicates that as alcohol consumption increased within the

alcohol exposure group, the number of ChAT+ neurons also increased in both the DMS and the DLS.

Figure 3.9

Bar chart depicting the group differences of the number of parvalbumin expressing neurons between the two regions of the basolateral amygdala (BLA) imaged.



Poisson mixed-effects models were used to analyze ChAT+ neurons in the NAc (+1.6 ± .1 mm relative to Bregma) in relation to alcohol exposure group and alcohol consumption levels. There was no significant group effect in the NAc core ($p > .05$). However, there was a significant group effect found in the NAc shell, $F(1, 29) = 14.71$, $p < .001$, such that the alcohol group showed more ChAT+ neurons compared to the water only group (see Figure 3.11). Additionally, upon analyzing individual differences in alcohol consumption within the alcohol group and PV+ neuron expression, no significant effects were found in the NAc shell ($p > .05$). However, there was a significant correlation found in the the NAc core, $F(1, 12) = 5.71$, $p = .017$, such that as alcohol consumption increased PV+ neurons in the NAc core increased (See Figure 3.12).

Figure 3.10

The correlations between alcohol consumption and numbers of ChAT+ neurons per unit area in the DMS (A) and DLS (B) regions imaged.

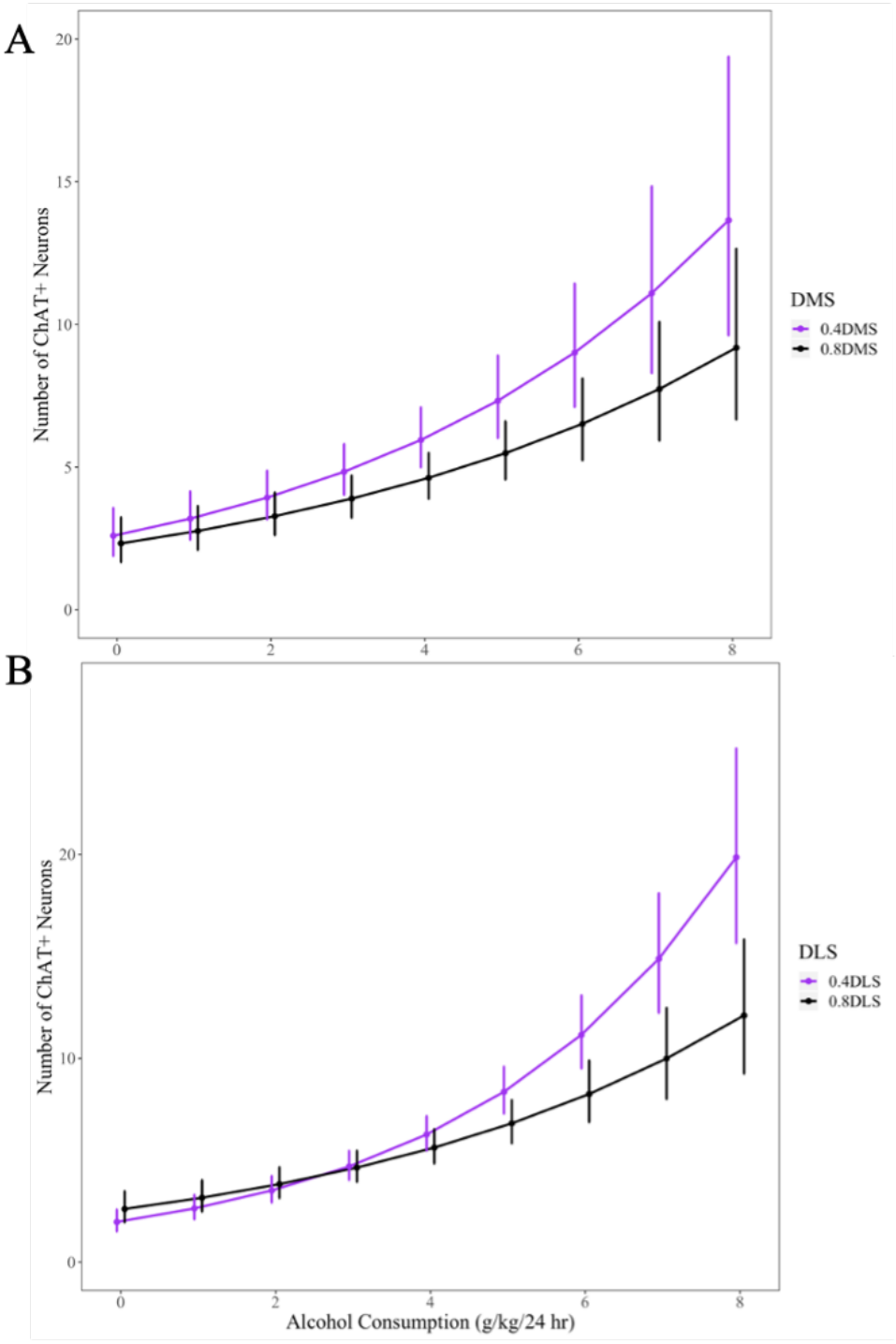


Figure 3.11

Bar chart depicting the group differences of the number of ChAT+ neurons between the nucleus accumbens (NAc) core (A) and shell (B) imaged.

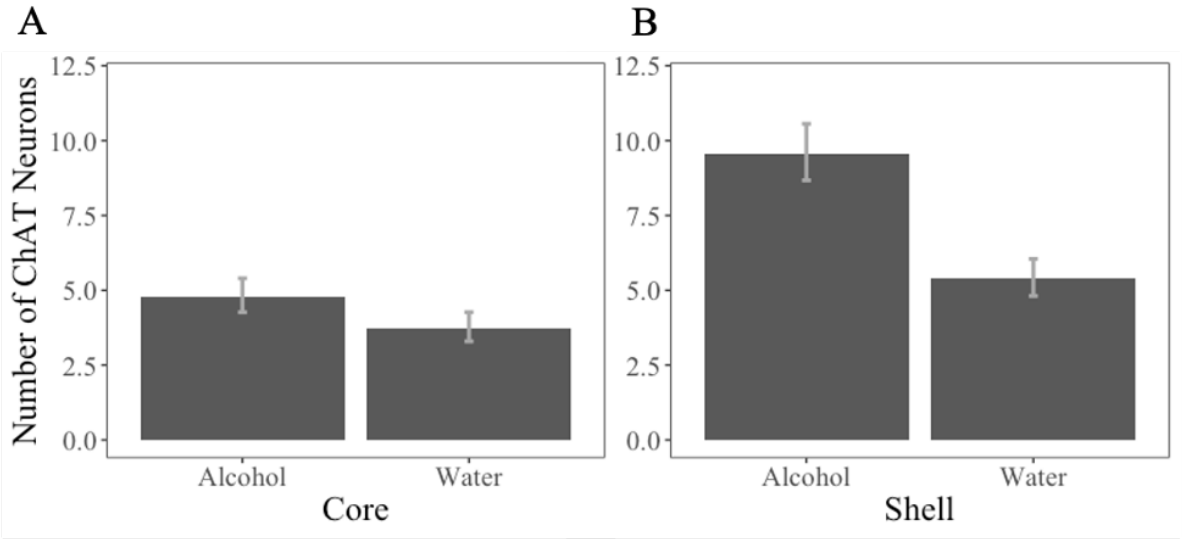
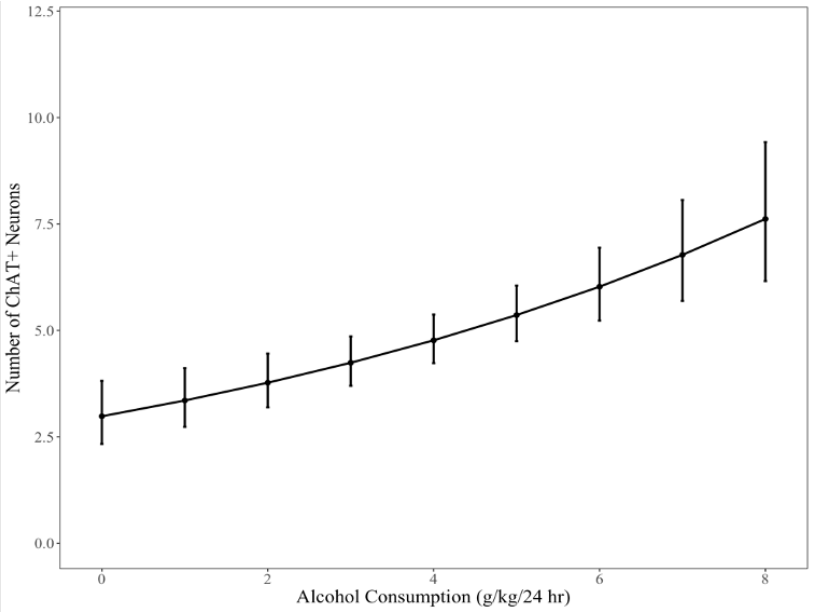


Figure 3.12

The correlation between alcohol consumption and the number of ChAT+ neurons in the NAc core.



The number of ChAT+ neurons was also assessed in two regions of the ACC (+3.2 ± .1 & +2.7 ± .1 relative to Bregma) using Poisson mixed-effects models. To assess group differences, the fixed effects of Group x ACC Region predicted the number of ChAT+ neurons. No significant effects were found between the water and alcohol group's expression (all effects $p > .05$). Additionally, upon analyzing individual differences in alcohol consumption within the alcohol group and ChAT+ neuron expression, no significant effects were found (all effects $p > .05$).

ChAT+ neurons in the cholinergic nuclei (Ch1-4; +0.6 ± .1 relative to Bregma) and their relationship to alcohol exposure was assessed using Poisson mixed-effects models. Upon assessing group differences of alcohol exposure, the cholinergic nuclei Ch1 and Ch2 showed a significant effect of group, $F(1, 75) = 7.70$, $p = .006$, indicating that the water group showed a higher expression of ChAT+ neurons compared to the alcohol group (See Figure 3.13a). For Ch3 and Ch4, the effect of group was trending but not significant (See Figure 3.13b). Upon analysis of the relationship of alcohol consumption within the alcohol group and ChAT+ neurons, a main effect of alcohol consumption was found for Ch1 and Ch2, $F(1, 36) = 10.11$, $p = .001$, and for Ch3 and Ch4, $F(1, 36) = 5.24$, $p = .022$. Both main effects indicate that as alcohol consumption increased, the number of ChAT+ neurons also increased (See Figure 3.14).

Figure 3.13

Bar charts depicting the group differences of the number of ChAT+ neurons between the cholinergic nuclei Ch1/Ch2 (A) and Ch3/Ch4 (B).

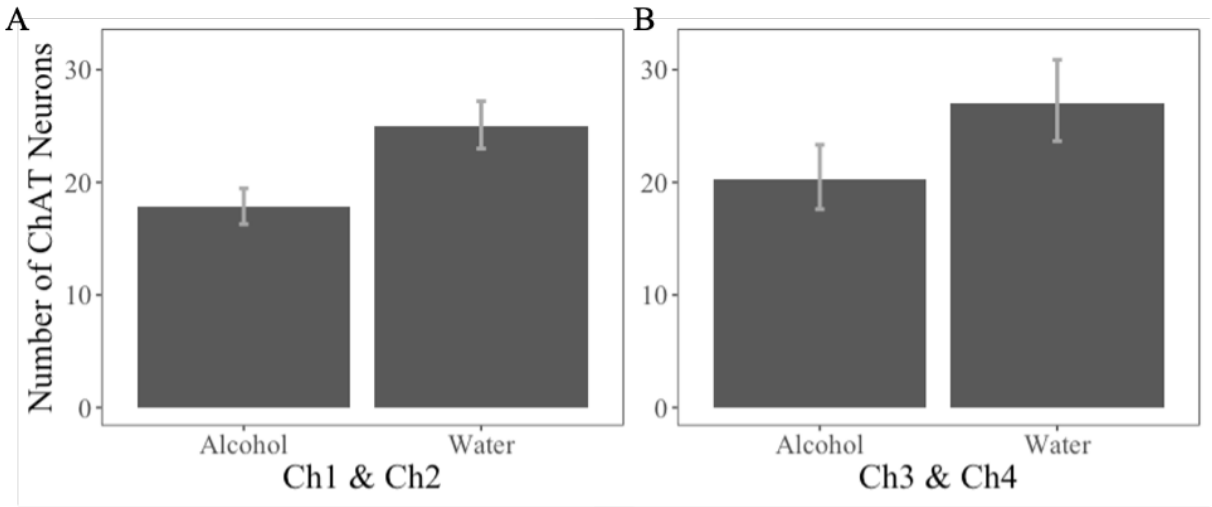
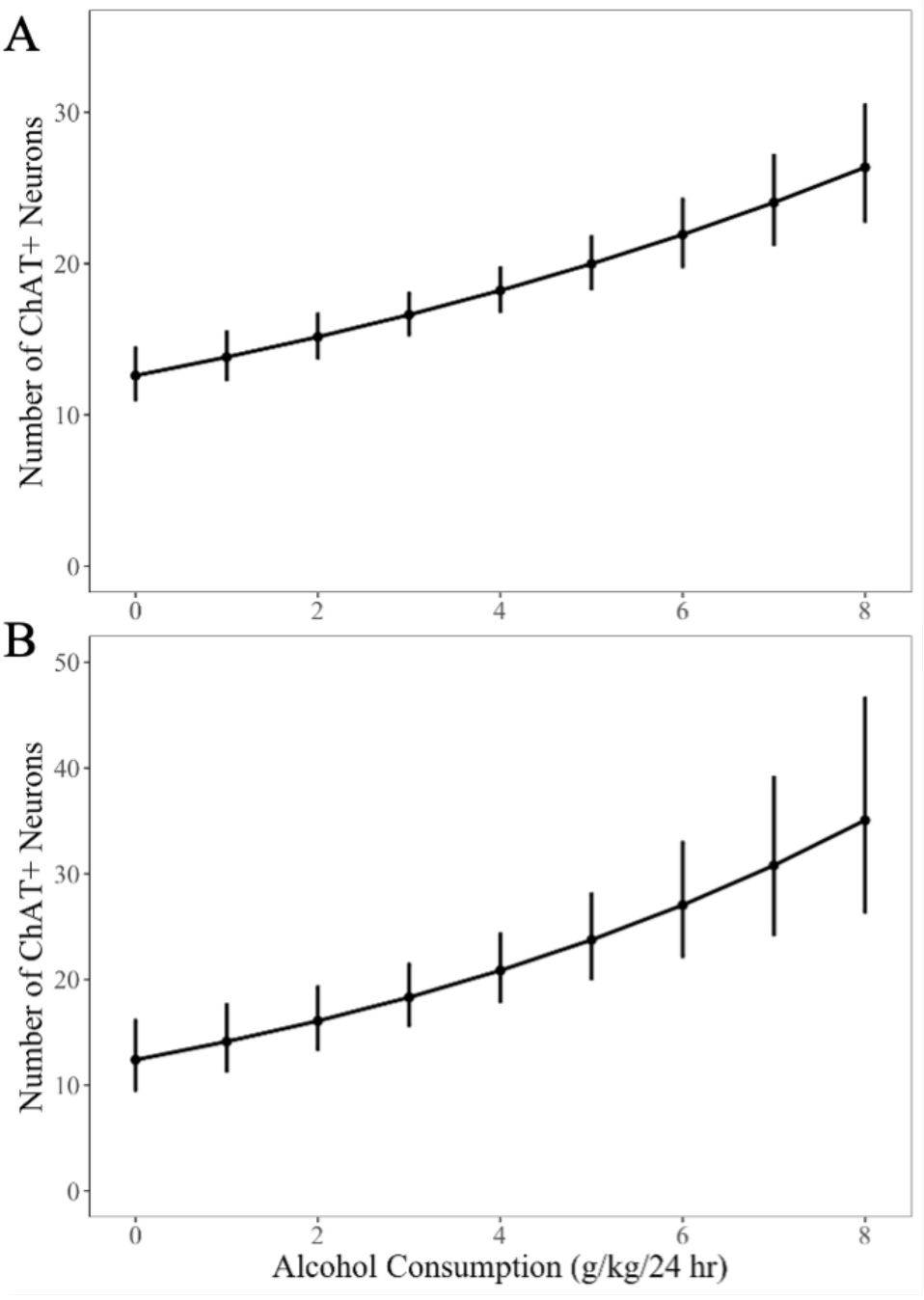


Figure 3.14

Bar chart depicting the group differences of the number of ChAT+ neurons between the cholinergic nuclei Ch1/Ch2 (A) and Ch3/Ch4 (B).



Chapter 4 - Discussion

Summary.

This study aimed to determine the relationship between adolescent alcohol, altered omission contingency learning, and the neural components that are affected. In this experiment, rats that underwent adolescent voluntary alcohol access showed an increase in overall consumption (g/kg) across the 6-week period. Although several studies find that adolescent rats do not always follow this increase in overall consumption pattern (Schramm-Sapyta et al., 2014; diLeo et al., 2015; Fisher et al., 2017; Pajser et al., 2018; Pajser et al., 2019; Pickens et al., 2019), this escalation of consumption is generally the pattern that is found in the CIA paradigm in adults (Simms et al., 2008). It is not known as to why there is a differing pattern here in the adolescent rats compared to previous studies, however it does follow the alcohol escalation pattern intended by the design of the alcohol exposure paradigm.

We were able to replicate previous findings showing adolescent alcohol consumption leading to faster omission contingency learning (Pickens et al., 2019), and the behavioral differences were observed in a different measure than the previous paper. Different analyses were conducted in order to increase the power of the analyses and control for heteroscedasticity issues that the previously used analyses were not able to control for. Two generalized mixed-effects models were used and compared in order to conclude that the results did show faster omission contingency learning in the alcohol exposure group compared to the water only group. The measure of a likelihood to respond per trial did not support this conclusion, however the measure of number of responses per trial supported the hypothesis that adolescent alcohol consumption leads to faster omission contingency learning. These analyses and their results will be further discussed in the subsequent section below.

Our data shows that parvalbumin-positive (PV+) neurons may have some relation to alcohol consumption, however the specific nature of the relationship is unclear. Upon investigation of all of the brain areas of interest, no significant group findings (meaning significant differences between the alcohol and water groups as a whole) were found, indicating that alcohol-induced alterations in the number of parvalbumin-positive neurons in the areas I examined aren't responsible for altered omission contingency learning. It is possible that my analysis focused on regions that do not show this change or it is possible that these neurons changed their function while the overall number doesn't change. It is also possible that PV+ neurons play no role in altered omission contingency learning. However, we did find correlations of alcohol consumption and the number of PV+ neurons within the ACC and DLS. This could suggest that a neurotoxic effect does exist, but is not strong enough for a group effect to become statistically significant. On the other hand, these correlations could also suggest a neurological marker of individual differences between higher and lower drinking rats independent of whether the rats are ever exposed to alcohol. I will discuss these findings further in a subsequent section.

The findings of the ChAT expression in relation to alcohol exposure (alcohol exposure vs. water only) did show significant group differences within the cholinergic nuclei similarly to what has been found in previous research. Previous research has found a decrease in ChAT expression in adolescent alcohol exposed subjects compared to subjects naïve to alcohol in both Ch1/Ch2 and Ch3/Ch4 cholinergic nuclei areas (Vetreno et al., 2014). The alcohol exposure group showed a significant decrease in Ch1/Ch2 ChAT expression compared to the water only group. For Ch3/Ch4 there was not a significant difference found. This is not a direct replication of Vetreno and colleagues' (2014) findings, but did replicate their Ch1/Ch2 findings. Unlike previous findings, there was no group difference in ChAT expression found in the DMS or DLS.

The lack of a full replication could be due to differences in alcohol exposure methods, as previous research used forced exposure methods while this study used voluntary alcohol access. Similarly to the PV expression results, this could be due to not achieving a high enough BAC level in the subjects to show strong enough group effects in all areas. Previous work in our lab has only found the levels of 6.2 ± 1.3, 11.4 ± 0.7, and 14.3 ± 1.0 mg/dl during weeks 2, 4, and 6 of the CIA paradigm, respectively, so it is possible we achieved similarly low numbers during this experiment (Pajser et al., 2018). ChAT expression in the NAc shell showed the opposite relationship to the cholinergic nuclei, such that alcohol exposed subjects had increased ChAT expression in the NAc Shell compared to the water only subjects. This increased expression in the NAc shell could potentially be due to a compensatory response of decreased activity of the cholinergic nuclei.

What previous research did not explore (that this study did assess) is the relationship between ChAT+ neurons and level of alcohol consumption within the alcohol exposure group. We found an unexpected positive correlation of the number of ChAT+ neurons and alcohol consumption in the cholinergic nuclei (both Ch1/Ch2 and Ch3/Ch4), NAc core, the DMS, and the DLS. These findings seem somewhat contradictory to the group effects found in the cholinergic nuclei of Ch1/Ch2, however there are possible explanations. One possible explanation is that there is a compensatory mechanism in a compensatory changes in lower drinkers to lower the amount of alcohol consumed. Another explanation may be that these individual differences found are not at all related to the group effects found and could be an additional neural mechanism that distinguishes higher drinking subjects from lower drinking subjects independently of the effect of alcohol on these neurons. These speculations will be further discussed in a later section.

Analysis selection for alcohol exposure and omission learning.

This study used various analytical techniques in order to assess the relationship between alcohol consumption and omission contingency learning, and the overall consensus can be determined that alcohol consumption during adolescence does lead to a faster rate of omission contingency learning. The data were analyzed using mixed effects modeling, also known as multilevel modeling, because the data showed heteroscedasticity issues that standard linear models would not be able to control for. Mixed effects models allow for the use of all of the data available, can handle uneven datasets, and have a structure that allows group variables to be influenced by each individual subject and vice versa. This technique adds power to the analysis and removes the need to average across datapoints. This study specifically used two different types of Generalized mixed effects models, binomial and Poisson, to analyze different measures. There a different theoretical and practical implications for using either analysis.

Ultimately, which analysis to favor depends on the theoretical question proposed. Whether a response was made or how responsive the subjects were are the fundamental distinctive questions that differentiate the theoretical relevance of these two analyses. Whether a response was made (which is what the binomial model analyzes) does answer the ultimate question of whether a subject succeeded in omitting their response on a given trial. However, how responsive the subjects were (which is what the Poisson model analyzes) can further show the learning curve of responding throughout training which can paint a clearer picture of which subjects are learning to respond less and less. The number of responses made per trial may also show the level of motivation and slope of learning more clearly than just simply whether there was a response made or not, even though all responses after the first one on a trial had no effect on the outcome of that trial. The data obtained from the omission contingency task is count data

of number of lever presses per trial, and a Poisson generalized model is designed to account for this type of count data. To use a binomial model instead requires aggregation of the original data into probabilities rather than using raw data to determine whether a relationship exists or not. Overall, between a binomial and Poisson analysis, the Poisson seems to be a better analysis from a theoretical standpoint because it can analyze the count data of the number of lever presses and provide more information about the omission contingency learning, although it actually isn't as accurate in terms of telling you whether their behavioral pattern was adequate to still get a food reward or not. Upon the occurrence of overdispersion within a Poisson mixed-effects model, a negative binomial model or a zero-inflated model can be run in order to decrease the overdispersion found but maintain the same theoretical implications. There was not severe overdispersion with this data, so a Poisson mixed effects model was a sufficient analysis to be used.

The findings using the binomial mixed-effects model did not quite support the replication of Pickens and colleagues (2019). It is important to note that the binomial analyses analyzed the probability of a response per trial, which only uses the data of whether a response was or was not made on each trial. This removes information from the original count data about how many responses were made and censors the amount of information able to be retrieved from the data available to be analyzed, although it does provide more information about whether their behavior was sufficient to get a reward. The Day x Lever interaction indicates successful omission contingency learning towards the Omission lever and continued sign-tracking behavior to the Passive lever. While the three-way interaction was significant, pairwise comparisons showed that the groups of interest (Water vs. Alcohol Group on Omission Lever; Water vs. Alcohol Group on Passive Lever) were not significantly different from each other. Therefore, the results of the

pairwise comparisons do not support the hypothesis of faster omission contingency learning in the alcohol group in terms of likelihood of whether the lever would be pressed each trial or not.

Although the binomial mixed effects model did not yield a significant replication, our Poisson mixed effects model of the data did show a group effect of alcohol exposure causing faster omission contingency learning. Poisson mixed effects models are able to provide more information than the binomial because a Poisson analysis retains information about the specific number of responses made in each trial rather than simply whether a response was made or not each trial. Of the Poisson mixed effects model, the Group x Day x Lever was found to be significant and pairwise comparisons confirmed that the subjects exposed to alcohol significantly decreased their number of responses on the omission lever throughout the days compared to the subjects that only received water. This does support the hypothesis that adolescent alcohol exposure causes faster omission contingency learning, since the alcohol exposure group significantly decreased their number of responses per trial faster than the water exposure only group. However, the lack of a binomial three-way interaction does mean the probability of responding at all on a given trial was not significantly different throughout the days between the two groups. Therefore, there is faster omission contingency learning in terms of number of lever presses, but there is not faster learning in terms of the probability of at least once pressing per trial.

Adolescent alcohol exposure causes faster omission contingency learning.

The results showed that adolescent alcohol exposure causes faster omission contingency learning when analyzing the data using a Poisson mixed effects model, however we failed to see the same effect using the binomial mixed effects model. This study does show that the alcohol exposure group decreased their number of lever presses faster over session days compared to the

water only group, however we fail to see this relationship in the likelihood of a lever press per trial. This discrepancy in findings could be due to a few reasons. First, the Poisson analysis most likely has more power than the binomial analysis since it uses all of the lever-press data rather than an aggregate of the data. Since the Poisson analysis shows all of the lever-presses per trial, this may also illuminate the motivation of the subjects more in depth than whether a press was made or not during a trial. The reasons why subjects may still have made lever presses throughout trials could be because autoshaping behavior does not fully extinguish (Locurto et al., 1976). The motivation to press the lever learned from the autoshaping phase may still be partially present, which may affect the number of times a rat is willing to press the lever for a reward.

The support of our hypothesis that adolescent alcohol exposure causes faster omission contingency learning does further support previous findings in the literature (Pickens et al., 2019; Depoy et al., 2013; Hellberg et al., 2018). From this corroboration of studies, it is clear that a true relationship exists between alcohol consumption and omission contingency learning and that this is not just a coincidental finding. However, each study that analyzed these effects of alcohol exposure on omission learning used different techniques including difference scores (Hellberg et al., 2019), count of whether a response was made (Pickens et al., 2019), sign-tracking response bias (Depoy et al., 2013), and number of lever presses (this study), so it would be beneficial to replicate a study using the same analyses. However, it is worth noting that an exploratory analysis was conducted on this study's current data using the same analysis as Pickens and colleagues (2019), and no significant interaction was found. It is also known that this effect of faster learning is only specifically found from adolescent alcohol consumption and not from adulthood (Pickens et al., 2019). However, this is specifically for voluntary alcohol consumption, since vapor chambers during adulthood can also lead to faster omission learning in mice (Depoy

et al., 2013). Therefore, this could be a unique behavioral task to utilize in order to study the effects of adolescent voluntary alcohol exposure's effects on various neurological mechanisms.

Why faster omission contingency learning occurs following adolescent alcohol exposure is not fully understood and is up for speculation. My primary theory is that motivation processes are disrupted and/or imbalanced due to alcohol exposure in the adolescent brain. Rats show exaggerated behaviors to motivational stimuli during adolescence (Doremus-Fitzwater et al., 2010), and alcohol consumption has been shown to keep rats in the adolescent motivational cognitive capacity throughout adulthood (Spear, 2016; Hiller-Sturmhofel & Spear, 2018). This hypothesis is supported by the findings that after intermittent alcohol exposure during adolescence high-drinking rats showed more sign-tracking behavior compared to low-drinking rats, suggesting a higher association of the cue to making a response (Spoelder et al., 2017). Additionally, it has been found that rats exposed to alcohol during adolescence show a higher sensitivity to unexpected variation in reward outcomes (Spoelder et al., 2015), which may aid their learning in an omission contingency task since the withholding of a reward following a response is highly unexpected following the autoshaping procedure. The increased sensitivity to motivational cues and unexpected outcomes could be the key reasons as to why rats exposed to alcohol during adolescence show faster omission contingency learning.

In our immunohistochemical findings, none of the DLS, DMS, ACC, and NAc Core areas were altered between alcohol exposed and water only subjects (as far as they were measured) so the number of PV+ or ChAT+ neurons in these areas cannot be concluded to be related to omission contingency learning, but may play some role. It is still possible that their function changes and the number of neurons stays the same. Some of the cholinergic nuclei (Ch1/Ch2) were altered in their ChAT+ levels and so areas receiving projections from these

areas (such as the cingulate cortex, hippocampus, ventral pallidum, lateral septum, ventral pallidum, medial prefrontal cortex, motor cortex, and amygdala [Dautan et al., 2016]) might still responsible, but this cannot be detected by the immunohistochemistry results. It would have been better to measure neuronal activity in several of these regions during the omission contingency test (using Fos or Arc or some other IEG measure of neuronal activity). Unfortunately, the rats in the current study were not sacrificed at the proper time-point after test to assess this.

We did find significant correlations in the ACC and DLS between the number of PV+ neurons and levels of alcohol consumption in the alcohol group, along with correlations in the DMS, DLS, NAc Core, Ch1/Ch2, and Ch3/Ch4 between ChAT+ neurons and levels of alcohol consumption in the alcohol group. These are novel findings that may further shed a light on the relationship between these neurological markers and alcohol consumption. PV and ChAT expression within these areas may be a phenotypic marker of a subjects' propensity to drinking alcohol, with an increase in expression in these areas increasing the likelihood of an individual to consume more alcohol. Studies have shown genetic variation in subjects that are more or less likely to consume alcohol (Saba et al., 2011; Spence et al., 2020), and these may be a neurological markers that coincide with these differences found.

Parvalbumin expression and alcohol consumption.

This study did not find any group differences between alcohol exposure (alcohol exposed vs. water only groups) and number of PV+ neurons in any brain area assessed, however there is some evidence of a correlation between alcohol consumption and PV+ neuron numbers in certain brain areas. These correlations that we have found are new information that has not been previously discovered before to our knowledge.

There have been previous findings of group differences between alcohol exposure and PV+ neurons throughout several brain areas. Previous studies have found that neonatal alcohol exposure decreases PV density later in adulthood in the cortex and hippocampus in mice (Coleman et al. 2012; Sadrian et al. 2014; Smiley et al. 2015). Additionally, it has been found that forced alcohol exposure in adulthood for an extended period of time led to a reduction in PV+ neurons across the cerebral cortex (Udomuksorn et al., 2011). In the current study, the lack of a difference between alcohol exposed and water only groups in the ACC is in contrast to previous research in prenatal, neonatal, and adult rats. In prenatal, neonatal, and adult subjects PV+ neurons were shown to decrease following alcohol exposure (Moore et al., 1998; Hamilton et al., 2017; Vongvatcharanon et al., 2010). However, similar to our findings, there was one study that failed to find a significant difference in PV+ neuron expression between alcohol exposed and control groups in neonatal rats (Mitchell et al., 2000). The only study assessing alcohol's effects on PV+ neurons within the striatum focused on group differences and used forced exposure methods via vapor inhalation in neonatal rats, and there has been shown to be reduced dendritic trees on PV+ neurons (De Giorgio et al., 2012). These studies did not assess correlations between amount of alcohol consumption, since they used forced exposure methods. These findings from forced exposure methods do not coincide with the findings of the current study, since I found that PV+ neuron expression increases as alcohol consumption increases but there is no overall difference between the alcohol and water exposed groups. The discrepancy in findings may be due to the differences in exposure methods, as it has been shown that forced exposure causes different neurological changes compared to voluntary exposure (Jacobs et al., 2003). The lack of group differences found in this study may also be due to alcohol exposure method or the exposure dosage used, since it used an intermittent voluntary paradigm that does

not reach the BAC levels of other exposure paradigms. However, one of the advantages of the CIA paradigm used in this study is that it allows for investigation into the differences in higher and lower drinking subjects.

In the ACC, no group differences were found but PV+ neurons were positively correlated with amount of alcohol consumed within the alcohol exposed rats. This is a unique finding for our study, as our methods of alcohol exposure allow for correlations to be conducted. Forced exposure methods miss this relationship since a set dose is given to the subjects and the subjects are not allowed to consume at voluntary levels. This correlation found may indicate a phenotypic marker of one's likelihood to consume alcohol. As alcohol consumption increased in the subjects, the number of PV+ neurons also increased, which may indicate that an endogenous level of PV in the ACC may be associated with one's incentive to drink alcohol. In contrast, it is possible that a group difference does exist, but our alcohol exposure manipulation was not strong enough for a group effect to become statistically significant except in rats with higher alcohol consumption. Many studies used forced alcohol exposure methods in order to reach BACs of 150-250 mg/dl (Kimbrough et al., 2017), which is much higher than a rat will voluntarily drink. The CIA paradigm used in this study can produce BAC levels of up to ~80 mg/dl (Simms et al., 2008). However, upon measuring BACs in our lab with adolescent non-food restricted rats, we found the levels of 6.2 ± 1.3, 11.4 ± 0.7, and 14.3 ± 1.0 mg/dl during weeks 2, 4, and 6 of the CIA paradigm, respectively (Pajser et al., 2018). The CIA paradigm used in this study of voluntary drinking may not be able to reach the level of consumption needed in order to see the neurological effects because the subjects did not consume enough alcohol voluntarily to create sufficient intoxication, however they may have reached sufficient levels of intoxication to see a correlation between alcohol consumption and PV+ neurons. However, this correlation

contradicts human research studying people with AUD compared to low drinkers. Human studies find that people with AUD have lower levels of GABA in the ACC compared to lower drinkers (Silveri et al., 2014; Prisciandaro et al., 2019), whereas we have found a higher concentration of PV+ neurons (a GABAergic interneuron) in higher drinkers.

There was no relationship found between PV+ neurons and alcohol consumption in the DMS in my study, however there was a positive correlation between PV+ neurons within the DLS and alcohol consumed by the alcohol exposure group. Similarly to the correlation found in the ACC, this could indicate a phenotypic marker of one's propensity to consume alcohol or could be due to BAC levels not reaching high enough levels to result in a group effect, but an effect in rats with higher alcohol consumption. Why a correlation was found in the DLS but not the DMS is not fully understood. It has been suggested that the PV population within the dorsal striatum is not homogeneous, as PV+ neurons within the DMS show an increased intrinsic excitability compared to DLS PV+ neurons (Monteiro et al., 2018). This heterogeneity of PV+ neurons could explain why there is a correlation of alcohol consumption with these neurons in the DLS but not in the DMS and could play a potential role in how these two areas play opposing roles in goal-directed learning. Conversely, the PV+ neuron population increases laterally throughout the striatum, so it possible that there is simply a higher population to be manipulated within the DLS (Schlösser et al., 1999; Luk & Sadikot, 2001). This positive correlation in the DLS has been previously found by our lab (data not published), using the CIA paradigm during adolescence and a reversal learning task in adulthood. How the correlation found within the DLS but not in the DMS relates to this task could be related to the brain areas' functions. The DMS is primarily associated with action-outcome, or R-O, learning, while the DLS is associated with habitual, or S-R, learning (Penner & Mizumori, 2012). The increased expression in the DLS

following increased alcohol consumption may be part of a mechanism to disinhibit the habitual behavior that often comes from using substances of abuse.

This DLS correlation does not quite fit with findings from human studies, although there are some mixed results in the human studies. One study found that in post-mortem tissue of alcoholic brains that the GABA-synthesizing enzyme GAD was decreased in the caudate nucleus (part of the dorsal striatum) compared to controls (Abdul Kashem et al., 2016). Another post-mortem study looking at the mRNA expression of various neurotransmitters in alcoholic brains found that there was a decrease in three types of GABAergic receptors (δ, ε, and ρ2 GABA-A), but an increase in a different GABAergic receptor (GABA-A γ1) in the caudate nucleus (Bhandage et al., 2014). However, it should be noted that these studies are looking at overall GABAergic population, and not specifically PV interneurons.

ChAT expression and alcohol consumption.

ChAT expression within this study did show both significant group differences between the alcohol and water groups as well as correlations with alcohol consumption within the alcohol exposed group. We replicated previous findings that alcohol exposure leads to decreased ChAT expression in Ch1/Ch2 (Vetreno et al., 2014). We found a group difference in the opposing direction within the nucleus accumbens shell in which alcohol exposure led to increased ChAT expression. We also expanded the literature by discovering correlations between the number of ChAT+ neurons and alcohol consumption within Ch1/Ch2, Ch3/Ch4, DMS, and DLS. Whether these group differences and correlations are related to each other is unknown.

We found that alcohol exposure significantly reduced ChAT+ neurons in Ch1/Ch2. This is an incomplete replication of the findings of previous research (Vetreno et al., 2014), in which previous studies found reduction of ChAT expression in Ch1/Ch2 and Ch3/Ch4. The incomplete

replication could be due to the differences in alcohol exposure methods, since previous research used higher dosage forced exposure methods compared to our lower dosage voluntary consumption methods. The BAC levels might not have reached high enough levels to produce full effects in order to show this result. However, still finding this effect in Ch1/Ch2 using voluntary alcohol consumption shows the robustness of the effect at hand and provides additional support that alcohol exposure does indeed affect the number of ChAT+ neurons. Specifically, alcohol exposure during adolescence reduces ChAT expression with cholinergic nuclei within the basal forebrain. This reduction in ChAT expression leads to a disruption in neuronal function in the basal forebrain, which may be a contributing factor to the altered omission contingency learning found within our study.

However, the number of ChAT+ neurons was increased in the alcohol exposure group compared to the water only group within the nucleus accumbens shell. A previous study looking at alcohol exposure in relation to ChAT expression in the nucleus accumbens found no change in ChAT expression following alcohol exposure and withdrawal (Pereira et al., 2014). Another study using intermittent forced alcohol exposure during adolescence found decreased ChAT expression in the nucleus accumbens following alcohol treatment (Galaj et al., 2019). The discrepancies in findings may be due to the differing natures of forced versus voluntary exposure, as it as been shown that forced and voluntary exposure of other drugs use have different neurobiological effects (Jacobs et al., 2003). Voluntary alcohol intake has been shown to increase extracellular acetylcholine levels in the ventral tegmental area (VTA), which correlates with dopamine levels in the NAc (Larsson et al., 2005). However, there is also evidence that dopamine levels in response to reward-related stimuli in the NAc are regulated by cholinergic interneurons within the NAc (Hanada et al., 2018), therefore it is possible that the

increase in dopamine expression could be due to an additional increase in cholinergic expression within the NAc. This finding of increased ChAT expression within the NAc Shell is a novel finding and may contribute to our findings of altered omission contingency learning.

What has been understudied is the individual differences of ChAT expression within a voluntary alcohol consumption group. This study additionally looked at how the levels of alcohol consumption within the alcohol exposed group relate to ChAT+ neurons within the brain regions of interest. We found positive correlations between ChAT+ neurons and alcohol consumption in the DMS, DLS, Ch1/Ch2, and Ch3/Ch4 such that there was greater expression of ChAT+ neurons as alcohol consumption increased. This relationship seems counterintuitive since the group difference in Ch1/2 showed decreased ChAT expression in the alcohol group compared to the water only group. However, this finding could be due to a compensatory changes in lower drinkers to lower the amount of alcohol consumed. Theoretically, if the lower drinkers showed decreased ChAT expression due to alcohol, then there would also be a decrease in dopamine expression since the level of acetylcholine regulate the levels of dopamine released in the striatum (Gonzales & Smith, 2015). This decrease in dopamine would make drinking less rewarding, since there would be less of a dopamine "hit" while drinking, and therefore cause them to consume less alcohol in the future. In contrast, rats that have higher levels of acetylcholine would also show higher levels of dopamine, which would cause a greater rewarding effect to the alcohol intake. This hypothesis is supported by evidence that when both rats and humans are given an acetylcholine antagonist their level of alcohol consumption decreases (Srisontiyakul et al., 2016; Quiroz et al., 2018; Miller & Kamens, 2020). Another possibility is that these individual differences found may also be a phenotypic marker of a subjects' propensity to drinking alcohol as could be the PV+ neurons in the ACC and DLS.

Perhaps having a higher amount of ChAT+ neurons may increase the rewarding effects of alcohol, and therefore cause a subject to increase their drinking while those subjects who have lower levels of ChAT expression do not feel the rewarding effects of alcohol as much and therefore do not drink as much.

Brain areas previously associated with autoshaping and omission contingency learning do not exhibit altered PV+ or ChAT+ neuron numbers after alcohol.

Previous lesion and inactivation studies have provided guidance into which brain areas may be responsible for altered omission contingency learning. The ACC, NAc Core, and DLS were particular regions of interest based on previous studies. Lesioning the ACC severely impaired rats' ability to learn the autoshaping task, specifically in a failure to discriminate between the CS+ and CS- (Bussey et al., 1997; Parkinson et al., 2000). Lesions of the NAc Core impaired both autoshaping acquisition and CS+/CS- discrimination during omission learning (Parkinson et al., 2000; Cardinal et al., 2002). However, since work in our lab previously has found alterations in omission contingency learning without alterations in autoshaping (Pickens et al., 2019), it was possible that the ACC and NAc Core did not play roles in our task since they altered autoshaping behavior. However, our autoshaping task was different in that both levers were autoshaping (CS+) levers, whereas previous studies used a CS+ lever and a CS- lever. Therefore it is possible that the ACC and NAc Core may still play a role if what is being affected is the ability to discriminate during the task. Upon inactivation of the DLS, however, rats show an improvement in omission learning and reduced their rate of responding faster than controls upon inactivation of the DLS via microinjections of the GABA agonist muscimol during omission training (Yin et al., 2006). This pattern is similar to the pattern found by Pickens and

colleagues (2019), using the same alcohol exposure method as the current study. The behavioral pattern is also similar to the pattern found in the current study, so therefore the DLS was the primary region of interest expected to see a group difference.

However, there were no group differences found within these brain regions within this study. In the PV analysis, no group differences were found in any of the brain regions, including the ones thought to be involved in autoshaping and omission contingency learning. In the ChAT analysis, the regions thought to be involved in autoshaping and omission contingency learning were also not affected, however there were some other group differences. Ch1/Ch2 ChAT+ neurons decreased in the alcohol group compared to the water group, and NAc Shell ChAT+ neurons increased in the alcohol group compared to the water group. These regions have not been studied on their relationships towards autoshaping and omission contingency learning. Ch1/Ch2 does, however, project to the ACC (Dautan et al., 2016), so it is possible that there may be some cholinergic alterations in the ACC that could alter omission contingency learning and that this paradigm was simply not a strong enough manipulation to show these results. It is also possible there was a change in activity that wasn't apparent in ChAT+ or PV+ cell numbers.

There is no evidence that the brain regions previously studied by lesion and inactivation studies (ACC, NAc Core, and DLS) are responsible for the altered omission contingency learning found in this study. It is possible that previous studies were incorrect in determining which areas may be responsible in omission contingency learning due to type one error and lack of power. However, it is also possible that the current study was underpowered to detect these effects. Also, in this study we measured two types of neuronal markers and did not measure neuronal function. It is possible that the number of PV+ and ChAT+ neurons may have stayed the same in these regions, but that they show altered function. Future studies should assess the

functionality of these brain areas examined measuring neuronal activity in several of these regions during the omission contingency test (using Fos or Arc or some other IEG measure of neuronal activity). This could prove useful in order to determine whether these brain areas were indeed altered or whether other brain areas are responsible for the altered omission contingency learning found in this study.

Conclusion.

The goal of this experiment was to elucidate the neural components that may be responsible for altered omission contingency learning following adolescent alcohol exposure. This study furthered the research in the field by exploring correlates of alcohol-related behavioral alterations through two different neuronal markers. We primarily focused on the striatum. The striatum has three main parts, the NAc, DMS, and DLS, and each have their unique role in reward-related learning. We additionally examined the roles played by the ACC, amygdala, and cholinergic nuclei within the basal forebrain in omission contingency learning and adolescent alcohol consumption. ChAT expression was decreased in Ch1/Ch2 in the alcohol exposed rats compared to water only controls, which suggests that alcohol does reduce cholinergic expression within the basal forebrain even in low level voluntary consumption. ChAT expression, however, was increased in the nucleus accumbens shell which suggests that voluntary alcohol consumption may cause different neurobiological changes in the cholinergic system compared to forced alcohol exposure. However, no other regions exhibited differences in the markers assessed between the water and alcohol groups. We found individual differences of PV expression in the alcohol exposure group in the ACC and DLS, which may suggest either a phenotypic marker of alcohol consumption or group difference that was not strong enough to reach significance. We also found individual differences of ChAT expression in the alcohol

exposure group in the DMS, DLS, Ch1/Ch2, and Ch3/Ch4, which may suggest either a phenotypic marker of alcohol consumption or a compensatory response to alcohol consumption. These individual differences have not previously been found before and further our scientific knowledge about the impacts of voluntary alcohol consumption's effects on the brain.

My hypothesis is that we would not expect to see the same results found in this study if this study had used forced alcohol exposure methods with differing levels of exposure for subjects to simulate a range of voluntary drinking levels. In particular, the correlations between PV+ and ChAT+ neurons in brain regions of interest and alcohol exposure would most likely not show the same relationship. This is because there is a motivational component of voluntary drinking that forced exposure lacks. Previous studies have found in other types of drugs that voluntary intake has differing neurobiological effects compared to forced exposure (Jacobs et al. 2003, Stefanski et al, 2007; Wiskerke et al., 2016). Even though these differing neurobiological effects have been predominantly found for cocaine and opiate exposure, there is a possibility that differences occur in alcohol exposure as well.

This study did not find evidence that brain regions previously associated with autoshaping and omission contingency learning (ACC, NAc Core, and DLS) are related to PV+ and ChAT+ neurons. However, we did find that ChAT+ neurons in the NAc Shell increased in alcohol rats compared to water rats. Future research should further explore this relationship and how it pertains to omission contingency learning. We also found that ChAT+ neurons in Ch1/Ch2 decreased in alcohol rats compared to water rats. Ch1/Ch2 project to many areas including the ACC. Future research should explore the projections of Ch1/Ch2 and their relationship to omission contingency learning. Additionally, future research should assess neuronal function of these brain areas associated with autoshaping and omission contingency

learning in order to be able to have a more complete picture of whether alterations have occurred. It is possible that neuronal activity is altered within these brain regions, however PV+ and ChAT+ neuron numbers remain the same.

In summary, voluntary drinking can uncover interesting behavioral and neurological associations, however the particular neuronal markers we examined were not found to be responsible for the behavioral changes found in the study. We found that voluntary drinking can lead to faster omission contingency learning, which is a replication of previous work from our lab (Pickens et al., 2018). The markers we looked at did not find significant group differences in the areas associated with omission contingency learning, so it is possible that neuronal activity is changing in these areas, but these specific neuron populations remain the same following alcohol exposure. We have strong candidates for future research in this domain, such as the markers Fos or Arc as measures of neuronal activity. Finally, this study further confirms that low level voluntary drinking can cause behavioral changes, andbrings us one step closer to determining the underlying neurological changes.

References

Adams CD (1982). Variations in the sensitivity of instrumental responding to reinforcer devaluation. *Q J Exp Psychol Comp Physiol Psychol* 34B:77–98.

Aosaki, T., Miura, M., Suzuki, T., Nishimura, K., & Masuda, M. (2010). Acetylcholine–dopamine balance hypothesis in the striatum: An update. *Geriatrics & Gerontology International*, *10*(s1), S148–S157. doi:10.1111/j.1447-0594.2010.00588.x

Atnip, G. W. (1977). Stimulus- and response-reinforcer contingencies in autoshaping, operant, classical, and omission training procedures in rats. *Journal of the Experimental Analysis of Behavior*, *28*(1), 59–69. https://doi.org/10.1901/jeab.1977.28-59

Baik, J.-H. (2013). Dopamine Signaling in reward-related behaviors. *Frontiers in Neural Circuits*, *7*, 152. https://doi.org/10.3389/fncir.2013.00152

Barron, S., White, A., Swartzwelder, H. S., Bell, R. L., Rodd, Z. A., Slawecki, C. J., Ehlers, C. L., Levin, E. D., Rezvani, A. H., & Spear, L. P. (2005). Adolescent vulnerabilities to chronic alcohol or nicotine exposure: findings from rodent models. *Alcoholism, clinical and experimental research*, *29*(9), 1720–1725. https://doi.org/10.1097/01.alc.0000179220.79356.e5

Baumrind, D. (1987) A developmental perspective on adolescent risk taking in contemporary America. In: Irwin, Jr., C.E., ed. *Adolescent Social Behavior and Health*. San Francisco: Jossey-Bass, pp. 93– 125.

Bhandage, A. K., Jin, Z., Bazov, I., Kononenko, O., Bakalkin, G., Korpi, E. R., & Birnir, B. (2014). GABA-A and NMDA receptor subunit mRNA expression is altered in the

caudate but not the putamen of the postmortem brains of alcoholics. *Frontiers in Cellular Neuroscience*, *8*, 415. https://doi.org/10.3389/fncel.2014.00415

Boutros, N., Semenova, S., Liu, W., Crews, F., & Markou, A. (2015). Adolescent intermittent ethanol exposure is associated with increased risky choice and decreased dopaminergic and cholinergic neuron markers in adult rats. *International Journal of Neuropsychopharmacology*, *18*(2). doi:10.1093/ijnp/pyu003

Brown PL, Jenkins HM. (1967). Conditioned inhibition and excitation in operant discrimination learning. *J Exp Psychol.* 75(2):255-66. doi: 10.1037/h0024985. PMID: 6062966.

Buchanan, C.M., Eccles, J.S., Becker, J.B. (1992) Are adolescents the victims of raging hormones? Evidence for activational effects of hormones on moods and behavior. *Psychology Bulletin* 111:62– 107

Burke, D. A., Rotstein, H. G., & Alvarez, V. A. (2017). Striatal local circuitry: a new framework for lateral inhibition. *Neuron*, *96*(2), 267–284. https://doi.org/10.1016/j.neuron.2017.09.019

Chang, S., & Smith, K. (2016). An omission procedure reorganizes the microstructure of sign-tracking while preserving incentive salience. *Learn Memory*, *23*(4), 151–155. https://doi.org/10.1101/lm.041574.115

Clarke, R., & Adermark, L. (2015). Dopaminergic regulation of striatal interneurons in reward and addiction: focus on alcohol. *Neural Plasticity*, 1–11. doi:10.1155/2015/814567

Cohen-Gilbert, J. E., Sneider, J. T., Crowley, D. J., Rosso, I. M., Jensen, J. E., & Silveri, M. M. (2015). Impact of family history of alcoholism on glutamine/glutamate ratio in anterior cingulate cortex in substance-naïve adolescents. *Developmental Cognitive Neuroscience*, *16*, 147–154. doi: 10.1016/j.dcn.2015.04.005

Coleman Jr, L., He, J., Lee, J., Styner, M., & Crews, F. (2011). Adolescent binge drinking alters adult brain neurotransmitter gene expression, behavior, brain regional volumes, and neurochemistry in mice. *Alcoholism: Clinical and Experimental Research*, *35*(4), 671–688. doi:10.1111/j.1530-0277.2010.01385.x

Coleman, L. G., Jr, Oguz, I., Lee, J., Styner, M., & Crews, F. T. (2012). Postnatal day 7 ethanol treatment causes persistent reductions in adult mouse brain volume and cortical neurons with sex specific effects on neurogenesis. *Alcohol (Fayetteville, N.Y.)*, *46*(6), 603–612.

Corbit, L., & Janak, P. (2010). Posterior dorsomedial striatum is critical for both selective instrumental and Pavlovian reward learning. *European Journal of Neuroscience*, *31*(7), 1312–1321. doi:10.1111/j.1460-9568.2010.07153.x

Costardi, J. V. V., Nampo, R. A. T., Silva, G. L., Ribeiro, M. A. F., Stella, H. J., Stella, M. B., & Malheiros, S. V. P. (2015). A review on alcohol: from the central action mechanism to chemical dependency. *Revista Da Associação Médica Brasileira*, *61*(4), 381–387. https://doi.org/10.1590/1806-9282.61.04.381

Cragg, S. (2006). Meaningful silences: how dopamine listens to the ACh pause. *Trends in Neurosciences*, *29*(3), 125–131. doi:10.1016/j.tins.2006.01.003

Crews, F. T., Vetreno, R. P., Broadwater, M. A., & Robinson, D. L. (2016). Adolescent alcohol exposure persistently impacts adult neurobiology and behavior. *Pharmacological reviews*, *68*(4), 1074–1109. https://doi.org/10.1124/pr.115.012138

Dautan, D., Bay, H. H., Bolam, J. P., Gerdjikov, T. V., & Mena-Segovia, J. (2016). Extrinsic sources of cholinergic innervation of the striatal complex: a whole-brain mapping analysis. *Frontiers in Neuroanatomy*, *10*, 1. https://doi.org/10.3389/fnana.2016.00001

Depoy, L., Daut, R., Brigman, J. L., Macpherson, K., Crowley, N., Gunduz-Cinar, O., … Holmes, A. (2013). Chronic alcohol produces neuroadaptations to prime dorsal striatal learning. *Proceedings of the National Academy of Sciences*, *110*(36), 14783–14788. doi: 10.1073/pnas.1308198110

Dickinson A, Balleine B, Watt A, Gonzalez F, Boakes RA (1995). Motivational control after extended instrumental training. *Anim Learn Behav* 23:197–206.

Dickinson, A., Squire, S., Varga, Z., & Smith, J. W. (1998). Omission learning after instrumental pretraining. *The Quarterly Journal of Experimental Psychology B: Comparative and Physiological Psychology, 51B*(3), 271–286.

Doremus-Fitzwater, T., Varlinskaya, E., & Spear, L. (2010). Motivational systems in adolescence: Possible implications for age differences in substance abuse and other risk-taking behaviors. *Brain Cognition*, *72*(1), 114–123. https://doi.org/10.1016/j.bandc.2009.08.008

Doremus, T., Brunell, S., Rajendran, P., & Spear, L. (2005). Factors influencing elevated ethanol consumption in adolescent relative to adult rats. *Alcoholism: Clinical and Experimental Research*, *29*(10), 1796–1808. doi:10.1097/01.alc.0000183007.65998.a

Everitt, B. J., & Robbins, T. W. (2005). Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nature Neuroscience*, *8*(11), 1481–9. doi:10.1038/nn1579

Fisher, H., Bright, N., Gallo, M., Pajser, A., & Pickens, C. (2017). Relationship of low doses of alcohol voluntarily consumed during adolescence and early adulthood with subsequent behavioral flexibility. *Behavioural Pharmacology*, *28*(7), 531. doi:10.1097.

Galaj, E., Kipp, B. T., Floresco, S. B., & Savage, L. M. (2019). Persistent alterations of accumbal cholinergic interneurons and cognitive dysfunction after adolescent intermittent ethanol exposure. *Neuroscience*, *404*, 153–164. https://doi.org/10.1016/j.neuroscience.2019.01.062

Gmaz, J. M., Carmichael, J. E., & Meer, M. A. van der. (2018). Persistent coding of outcome-predictive cue features in the rat nucleus accumbens. *ELife*, *7*, e37275. https://doi.org/10.7554/elife.37275

Hanada, Y., Kawahara, Y., Ohnishi, Y. N., Shuto, T., Kuroiwa, M., Sotogaku, N., Greengard, P., Sagi, Y., & Nishi, A. (2018). p11 in cholinergic interneurons of the nucleus accumbens is essential for dopamine responses to rewarding stimuli. *eNeuro*, *5*(5), ENEURO.0332-18.2018.

Heilbronner, S. R., & Hayden, B. Y. (2016). Dorsal anterior cingulate cortex: a bottom-up view. *Annual Review of Neuroscience*, *39*(1), 149–170. doi: 10.1146/annurev-neuro-070815-013952

Hellberg, S. N., Levit, J. D., & Robinson, M. J. (2018). Under the influence: Effects of adolescent ethanol exposure and anxiety on motivation for uncertain gambling-like cues in male and female rats. *Behavioural Brain Research*, *337*, 17–33. doi: 10.1016/j.bbr.2017.09.036

Hiller-Sturmhofel, S., & Spear, L.P. (2018). Binge drinking's effects on the developing brain - animal models. *Alcohol Research, 39*(1), 77-86.

Holland, P. C. (1977). Conditioned stimulus as a determinant of the form of the Pavlovian conditioned response. *Journal of Experimental Psychology: Animal Behavior Processes, 3*(1), 77–104. https://doi-org.er.lib.k-state.edu/10.1037/0097-7403.3.1.77

Holland, P. C. (1980). CS–US interval as a determinant of the form of Pavlovian appetitive conditioned responses. *Journal of Experimental Psychology: Animal Behavior Processes, 6*(2), 155–174. https://doi-org.er.lib.k-state.edu/10.1037/0097-7403.6.2.155

Holland, P. C. (1980). Influence of visual conditioned stimulus characteristics on the form of Pavlovian appetitive conditioned responding in rats. *Journal of Experimental Psychology: Animal Behavior Processes, 6*(1), 81–97. https://doi-org.er.lib.k-state.edu/10.1037/0097-7403.6.1.81

Jacobs, E. H., Smit, A. B., Vries, T. J. de, & Schoffelmeer, A. N. M. (2003). Neuroadaptive effects of active versus passive drug administration in addiction research. *Trends in Pharmacological Sciences*, *24*(11), 566–573. https://doi.org/10.1016/j.tips.2003.09.006

Kashem, M. A., Ahmed, S., Sultana, N., Ahmed, E. U., Pickford, R., Rae, C., Šerý, O., McGregor, I. S., & Balcar, V. J. (2016). Metabolomics of neurotransmitters and related metabolites in post-mortem tissue from the dorsal and ventral striatum of alcoholic human brain. *Neurochemical Research*, *41*(1–2), 385–397. https://doi.org/10.1007/s11064-016-1830-3

Keiflin, R., & Janak, P. H. (2015). Dopamine prediction errors in reward learning and addiction: from theory to neural circuitry. *Neuron*, *88*(2), 247–263. https://doi.org/10.1016/j.neuron.2015.08.037

Khemiri, L., Kuja-Halkola, R., Larsson, H., & Jayaram-Lindström, N. (2015). Genetic overlap between impulsivity and alcohol dependence: a large-scale national twin study. *Psychological Medicine*,*46*(5), 1091–1102. doi:10.1017/S0033291715002652.

Knapp, D. J., & Breese, G. R. (2012). Models of chronic alcohol exposure and dependence. *Methods in molecular biology (Clifton, N.J.)*, *829*, 205–230. https://doi.org/10.1007/978-1-61779-458-2_13

Larsson, A., Edström, L., Svensson, L., Söderpalm, B., & Engel, J. A. (2005). Voluntary ethanol intake increases extracellular acetylcholine levels in the ventral tegmental area in the rat. *Alcohol and alcoholism (Oxford, Oxfordshire)*, *40*(5), 349–358. https://doi.org/10.1093/alcalc/agh180

Le Berre, A.P., Fama, R., Sullivan, E.V., (2017). Executive Functions, Memory, and social cognitive deficits and recovery in chronic alcoholism: a critical review to inform future research, *Alcohol. Clin. Exp. Res.,* 41 (8) 1432–1443.

Liu, J.-J., Mohila, C. A., Gong, Y., Govindarajan, N., & Onn, S.-P. (2005). Chronic nicotine exposure during adolescence differentially influences calcium-binding proteins in rat anterior cingulate cortex. *European Journal of Neuroscience*, *22*(10), 2462–2474. doi: 10.1111/j.1460-9568.2005.04423.x

Locurto C, Terrace HS, Gibbon J. (1976). Autoshaping, random control, and omission training in the rat. *J Exp Anal Behav.* 26(3):451-62. doi: 10.1901/jeab.1976.26-451. PMID: 16811960; PMCID: PMC1333535.

Luk, K. C., & Sadikot, A. F. (2001). GABA promotes survival but not proliferation of parvalbumin-immunoreactive interneurons in rodent neostriatum: an in vivo study with stereology. *Neuroscience*, *104*(1), 93–103. https://doi.org/10.1016/s0306-4522(01)00038-0

Malone, S., Luciana, M., Wilson, S., Sparks, J., Hunt, R., Thomas, K., & Iacono, W. (2014). Adolescent Drinking and Motivated Decision-Making: A Cotwin-Control Investigation with Monozygotic Twins. *Behavior Genetics*, *44*(4), 407–418. doi:10.1007/s10519-014-9651-0. PMCID: PMC4058380

Mangieri, R. A., Cofresí, R. U., & Gonzales, R. A. (2014). Ethanol exposure interacts with training conditions to influence behavioral adaptation to a negative instrumental

contingency. *Frontiers in behavioral neuroscience*, *8*, 220. https://doi.org/10.3389/fnbeh.2014.00220

Mangieri, R., Cofresi, R., & Gonzales, R. (2014). Ethanol exposure interacts with training conditions to influence behavioral adaptation to a negative instrumental contingency. *Front Behav Neurosci*, *8*, 220. https://doi.org/10.3389/fnbeh.2014.00220

Mannella, F., Gurney, K., & Baldassarre, G. (2013). The nucleus accumbens as a nexus between values and goals in goal-directed behavior: a review and a new hypothesis. *Frontiers in Behavioral Neuroscience*, *7*, 135. https://doi.org/10.3389/fnbeh.2013.00135

Marshall, E. (2014). Adolescent Alcohol Use: Risks and Consequences. *Alcohol Alcoholism*, *49*(2), 160–164. https://doi.org/10.1093/alcalc/agt180

McCambridge J, McAlaney J, Rowe R. (2011) Adult consequences of late adolescent alcohol consumption: a systematic review of cohort studies. *PLoS Med* 8:e1000413. doi:10.1371/journal. pmed.1000413.

Millan, E. Z., Milligan-Saville, J., & McNally, G. (2013). Memory retrieval, extinction, and reinstatement of alcohol seeking. *Neurobiol Learn Mem*, *101*, 26–32. https://doi.org/10.1016/j.nlm.2012.12.010

Miller, C. N., & Kamens, H. M. (2020). The role of nicotinic acetylcholine receptors in alcohol-related behaviors. *Brain Research Bulletin*, *163*, 135–142. https://doi.org/10.1016/j.brainresbull.2020.07.017

Mitchell, J., Paiva, M., & Heaton, M. B. (2000). Effect of neonatal ethanol exposure on parvalbumin-expressing GABAergic neurons of the rat medial septum and cingulate cortex. *Alcohol*, *21*(1), 49–57. doi: 10.1016/s0741-8329(99)00101-9

Mohila, C. A. (2004). Increases in the density of parvalbumin-immunoreactive neurons in anterior cingulate cortex of amphetamine-withdrawn rats: evidence for corticotropin-releasing factor in sustained elevation. *Cerebral Cortex*, *15*(3), 262–274. doi: 10.1093/cercor/bhh128

Moore, D., Quintero, M. A., Ruygrok, A. C., Walker, D. W., & Heaton, M. B. (1998). Prenatal ethanol exposure reduces parvalbumin-immunoreactive GABAergic neuronal number in the adult rat cingulate cortex. *Neuroscience Letters*, *249*(1), 25–28. doi: 10.1016/s0304-3940(98)00378-4

Olsen, R., & Liang, J. (2017). Role of GABAA receptors in alcohol use disorders suggested by chronic intermittent ethanol (CIE) rodent model. *Molecular Brain*, *10*(1), 45. doi:10.1186/s13041-017-0325-8

Orduz, D., Bischop, D., Schwaller, B., Schiffmann, S., & Gall, D. (2013). Parvalbumin tunes spike-timing and efferent short-term plasticity in striatal fast spiking interneurons. *The Journal of Physiology*, *591*(13), 3215–3232. doi:10.1113/jphysiol.2012.250795

Pajser, A., Breen, M., Fisher, H., & Pickens, C. (2018). Individual differences in conditioned fear are associated with levels of adolescent/early adult alcohol consumption and instrumental extinction.*Behavioural Brain Research*. doi:10.1016/j.bbr.2018.04.020

Pajser, A., Fisher, H. & Pickens, C. L. (2021). Pre-training naltrexone increases conditioned fear learning independent of adolescent alcohol consumption history. *Physiol Behav* **229**, 113212.

Pajser, A., Limoges, A., Long, C. & Pickens, C. L. (2019). Individual differences in voluntary alcohol consumption are associated with conditioned fear in the fear incubation model. *Behav Brain Res* **362**, 299–310.

Pajser, A., Limoges, A., Long, C., & Pickens, C. L. (2019). Individual differences in voluntary alcohol consumption are associated with conditioned fear in the fear incubation model. *Behavioural Brain Research*, *362*, 299–310. doi: 10.1016/j.bbr.2019.01.027

Penner, M. R., & Mizumori, S. J. Y. (2012). Neural systems analysis of decision making during goal-directed navigation. *Progress in Neurobiology*, *96*(1), 96–135. https://doi.org/10.1016/j.pneurobio.2011.08.010

Peterson, A.C., Silbereisen, R.K., Sorenson, S. (1996) Adolescent development: A global perspective. In: Hurrelmann, K., and Hamilton, S.F., eds. *Social Problems and Social Contexts in Adolescence*. New York: Aldine de Gruyter, pp. 3–37.

Pickens, C. L., Kallenberger, P., Pajser, A., & Fisher, H. (2019). Voluntary alcohol access during adolescence/early adulthood, but not during adulthood, causes faster omission contingency learning. *Behavioural Brain Research*, *370*, 111918. doi: 10.1016/j.bbr.2019.111918

Prisciandaro, J. J., Schacht, J. P., Prescot, A. P., Renshaw, P. F., Brown, T. R., & Anton, R. F. (2019). Brain Glutamate, GABA, and Glutamine Levels and Associations with Recent

Drinking in Treatment-Naïve Individuals with Alcohol Use Disorder Versus Light Drinkers. *Alcoholism: Clinical and Experimental Research*, *43*(2), 221–226. https://doi.org/10.1111/acer.13931

Quiroz, G., Guerra-Díaz, N., Iturriaga-Vásquez, P., Rivera-Meza, M., Quintanilla, M. E., & Sotomayor-Zárate, R. (2018). Erysodine, a competitive antagonist at neuronal nicotinic acetylcholine receptors, decreases ethanol consumption in alcohol-preferring UChB rats. *Behavioural Brain Research*, *349*, 169–176. https://doi.org/10.1016/j.bbr.2018.04.038

Robinson, T., Yager, L., Cogan, E. & Saunders, B. (2014). On the motivational properties of reward cues: Individual differences. *Neuropharmacology* **76**, 450–459

Saba, L. M., Bennett, B., Hoffman, P. L., Barcomb, K., Ishii, T., Kechris, K., & Tabakoff, B. (2011). A systems genetic analysis of alcohol drinking by mice, rats and men: Influence of brain GABAergic transmission. *Neuropharmacology*, *60*(7–8), 1269–1280. https://doi.org/10.1016/j.neuropharm.2010.12.019

Sadrian, B., Lopez-Guzman, M., Wilson, D. A., & Saito, M. (2014). Distinct neurobehavioral dysfunction based on the timing of developmental binge-like alcohol exposure. *Neuroscience*, *280*, 204–219. https://doi.org/10.1016/j.neuroscience.2014.09.008

Saunders BT, Robinson TE. (2011). Individual variation in the motivational properties of cocaine. *Neuropsychopharmacology.* 36(8):1668-76. doi: 10.1038/npp.2011.48. Epub 2011 Apr 6. PMID: 21471956; PMCID: PMC3138662.

Schindler, A. G., Tsutsui, K. T., & Clark, J. J. (2014). Chronic alcohol intake during adolescence, but not adulthood, promotes persistent deficits in risk-based decision making. *Alcoholism, clinical and experimental research*, *38*(6), 1622–1629. https://doi.org/10.1111/acer.12404

Schlösser, B., Klausa, G., Prime, G., & Ten Bruggencate, G. (1999). Postnatal development of calretinin- and parvalbumin-positive interneurons in the rat neostriatum: an immunohistochemical study. *The Journal of comparative neurology*, *405*(2), 185–198. https://doi.org/10.1002/(sici)1096-9861(19990308)405:2<185::aid-cne4>3.0.co;2-b

Shenhav, A., Botvinick, M. M., & Cohen, J. D. (2013). The expected value of control: an integrative theory of anterior cingulate cortex function. *Neuron*, *79*(2), 217–240. doi: 10.1016/j.neuron.2013.07.007

Silveri, M. M., Cohen-Gilbert, J., Crowley, D. J., Rosso, I. M., Jensen, J. E., & Sneider, J. T. (2014). Altered anterior cingulate neurochemistry in emerging adult binge drinkers with a history of alcohol-induced blackouts. *Alcoholism: Clinical and Experimental Research*, *38*(4), 969–979. https://doi.org/10.1111/acer.12346

Simms, J., Steensland, P., Medina, B., Abernathy, K., Chandler, L., Wise, R., & Bartlett, S. (2008). Intermittent access to 20% ethanol induces high ethanol consumption in long–evans and wistar rats. *Alcoholism: Clinical and Experimental Research*,*32*(10), 1816–1823. doi:10.1111. PMCID: 3151464

Smiley, J. F., Saito, M., Bleiwas, C., Masiello, K., Ardekani, B., Guilfoyle, D. N., Gerum, S., Wilson, D. A., & Vadasz, C. (2015). Selective reduction of cerebral cortex GABA

neurons in a late gestation model of fetal alcohol spectrum disorder. *Alcohol (Fayetteville, N.Y.)*, *49*(6), 571–580.

Spear, L. P. (2016). Consequences of adolescent use of alcohol and other drugs: Studies using rodent models. *Neuroscience & Biobehavioral Reviews*, *70*, 228–243. https://doi.org/10.1016/j.neubiorev.2016.07.026

Spence JP, Lai D, Reiter JL, Cao S, Bell RL, Williams KE, Liang T. (2020). Epigenetic changes on rat chromosome 4 contribute to disparate alcohol drinking behavior in alcohol-preferring and -nonpreferring rats. *Alcohol.* 89:103-112. doi: 10.1016/j.alcohol.2020.08.004.

Srisontiyakul, J., Kastman, H. E., Krstew, E. V., Govitrapong, P., & Lawrence, A. J. (2016). The nicotinic α6-subunit selective antagonist bPiDI reduces alcohol self-administration in alcohol-preferring rats. *Neurochemical Research*, *41*(12), 3206–3214. https://doi.org/10.1007/s11064-016-2045-3

Tomie A, Grimes KL, Pohorecky LA. (2008). Behavioral characteristics and neurobiological substrates shared by Pavlovian sign-tracking and drug abuse. *Brain Res Rev.* 58(1):121-35. doi: 10.1016/j.brainresrev.2007.12.003.

Tomie A, Sharma N. (2013). Pavlovian sign-tracking model of alcohol abuse. *Curr Drug Abuse Rev.* 6(3):201-19. doi: 10.2174/18744737113069990023. PMID: 24694108.

Udomuksorn, W., Mukem, S., Kumarnsit, E., Vongvatcharanon, S., & Vongvatcharanon, U. (2011). Effects of alcohol administration during adulthood on parvalbumin and glial

fibrillary acidic protein immunoreactivity in the rat cerebral cortex. *Acta histochemica*, *113*(3), 283–289.

Vargas, W., Bengston, L., Gilpin, N., Whitcomb, B., & Richardson, H. (2014). Alcohol binge drinking during adolescence or dependence during adulthood reduces prefrontal myelin in male rats. *J Neurosci*, *34*(44), 14777–14782. https://doi.org/10.1523/jneurosci.3189-13.2014

Vetreno, R., Broadwater, M., Liu, W., Spear, L., & Crews, F. (2014). Adolescent, but Not Adult, Binge Ethanol Exposure Leads to Persistent Global Reductions of Choline Acetyltransferase Expressing Neurons in Brain. *PLoS ONE*, *9*(11), e113421. doi:10.1371/journal.pone.0113421

Vongvatcharanon, U., Mukem, S., Udomuksorn, W., Kumarsit, E., & Vongvatcharanon, S. (2010). Alcohol administration during adulthood induces alterations of parvalbumin and glial fibrillary acidic protein immunoreactivity in rat hippocampus and cingulate cortex. *Acta Histochemica*, *112*(4), 392–401. doi: 10.1016/j.acthis.2009.04.001

Williams DR, Williams H. (1969). Auto-maintenance in the pigeon: sustained pecking despite contingent non-reinforcement. *J Exp Anal Behav.*12(4):511-20. doi: 10.1901/jeab.1969.12-511. PMID: 16811370; PMCID: PMC1338642.

Wise, R. (1973). Voluntary ethanol intake in rats following exposure to ethanol on various schedules. *Psychopharmacology*, 29(3), 203-210.

Wise, R. A. (2004). Dopamine, learning and motivation. *Nature Reviews Neuroscience*, *5*(6), 483–494. https://doi.org/10.1038/nrn1406

Yager LM, Robinson TE. (2015). Individual variation in the motivational properties of a nicotine cue: sign-trackers vs. goal-trackers. *Psychopharmacology (Berl).* 232(17):3149-60. doi: 10.1007/s00213-015-3962-6. Epub 2015 May 19. PMID: 25980485; PMCID: PMC4536151.

Yager, L. M., Garcia, A. F., Wunsch, A. M., & Ferguson, S. M. (2015). The ins and outs of the striatum: Role in drug addiction. *Neuroscience*, *301*, 529–541. https://doi.org/10.1016/j.neuroscience.2015.06.033

Yin, H. H., Ostlund, S. B., Knowlton, B. J., & Balleine, B. W. (2005). The role of the dorsomedial striatum in instrumental conditioning. *European Journal of Neuroscience*, *22*(2), 513–523. https://doi.org/10.1111/j.1460-9568.2005.04218.x

Yin, H., Knowlton, B., & Balleine, B. (2006). Inactivation of dorsolateral striatum enhances sensitivity to changes in the action–outcome contingency in instrumental conditioning. *Behavioural Brain Research*, *166*(2), 189–196. doi:10.1016/j.bbr.2005.07.012

Appendix A - R Code

```
#### Paige4 alcohol consumption anaylsis ####

myd <- Paige4.alcohol.analysis

is.na(NA)
na.action(na.exclude)

## centering continuous variables
myd$week = myd$week - mean(myd$week)

myd$rat = as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(12)



mod1 <- lmer(etoh~week+(week|rat),
             data = myd,
             control=lmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e7)))
summary(mod1)

anova(mod1)
anova(mod1, ddf="Kenward-Roger")
coefs <- data.frame(coef(summary(mod1)))
coefs$p.z <- 2 * (1-pnorm(abs(coefs$t.value)))

coefs$df <- coef(summary(mod1))[, 3]

coefs
```

```
## ggplot ratio model
newdat <- expand.grid(
  week=c(1,2,3,4,5,6)-3.5,
  sex=c("Female","Male"),
  etoh = 0
)


res <- mod1

mm <- model.matrix(terms(res), newdat)
newdat$etoh <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo = (newdat$etoh - sqrt(pvar1)),
  phi = (newdat$etoh + sqrt(pvar1))
)

newdat

## uncenter day
newdat$week <- newdat$week+3.5

library(ggplot2)

pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=week, y=etoh,
                         col=sex))+ geom_line(position=pd,
size=2)+
```

```
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=4) +ylim(0,10)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position="top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo, ymax = phi),position=pd,
size=2,
                   linetype=1,
width=.04)+xlab("Week")+ylab("Alcohol Consumption (g/kg/24 hr)")



     ##### Paige4 Autoshaping analyses #####


     myd <- Paige4.autoshape.ST.data


     is.na(NA)
     na.action(na.exclude)


     ## renaming, centering, effect coding, etc.


     myd$Tpresses <- myd$Trials.w.presses
     myd$Tentries <- myd$Trials.w.entry
```

```
myd$Npresses <- myd$Total.presses
myd$Nentries <- myd$Total.entries
myd$ratio <- myd$ST.Ratio

myd$Day <- as.factor(myd$Day)
contrasts(myd$Day)=contr.sum(6)
contrasts(myd$Day)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

contrasts(myd$Lever)=contr.sum(2)
contrasts(myd$Lever)

myd$rat = as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(24)

### Let's start with an ANOVA

myd2 = subset(myd, rat != "14", na.action=na.exclude,
select = c(Group, Day, Lever, Tpresses, Tentries, Npresses,
Nentries, ratio, rat))

myd2$rat <- as.integer(myd2$rat)
```

```
myd2$rat <- as.factor(myd2$rat)


contrasts(myd2$rat)=contr.sum(23)


myd3 <- subset(myd2, rat != "1", na.action=na.exclude,
select = c(Group, Day, Lever, Tpresses, Tentries, Npresses,
Nentries, ratio, rat))

myd3$rat <- as.integer(myd3$rat)
myd3$rat <- as.factor(myd3$rat)


contrasts(myd3$rat)=contr.sum(22)

## total lever presses
mod1 = ezANOVA(data = myd3,
               wid = rat,
               between = Group,
               within = c(Day, Lever),
               dv = Npresses,
               type = 3)
mod1
```

```
## total head entries
mod2 = ezANOVA(data = myd3,
               wid = rat,
               between = Group,
               within = c(Day, Lever),
               dv = Nentries,
               type = 3)
mod2

## me figuring out how to check assumptions
tapply(myd3$Npresses, myd3$Day, mean)
tapply(myd3$Npresses, myd3$Day, var)
tapply(myd3$Npresses, myd3$Day, length)

tapply(myd3$Nentries, myd3$Day, mean)
tapply(myd3$Nentries, myd3$Day, var)
tapply(myd3$Nentries, myd3$Day, length)

boxplot(myd3$Npresses ~ myd3$Group)
boxplot(myd3$Npresses ~ myd3$Day)
boxplot(myd3$Npresses ~ myd3$Day*myd3$Group)
boxplot(myd3$Npresses ~ myd3$Day*myd3$Lever)
```

```
boxplot(myd3$Nentries ~ myd3$Group)
boxplot(myd3$Nentries ~ myd3$Day)
boxplot(myd3$Nentries ~ myd3$Day*myd3$Group)
boxplot(myd3$Nentries ~ myd3$Day*myd3$Lever)

# test homogeneity of variance
bartlett.test(Npresses ~ Group, data=myd3)
bartlett.test(Npresses ~ Day, data=myd3)
bartlett.test(Npresses ~ Lever, data=myd3)


xyplot(fitted(mod1)+Npresses~Day, myd3, type=c("a", "p"))


## trials with lever presses
mod3 = ezANOVA(data = myd3,
               wid = rat,
               between = Group,
               within = c(Day, Lever),
               dv = Tpresses,
               type = 3)
mod3

tapply(myd3$Tpresses, myd3$Day, mean)
```

```
tapply(myd3$Tpresses, myd3$Day, var)
tapply(myd3$Tpresses, myd3$Day, length)


boxplot(myd3$Tpresses ~ myd3$Group)
boxplot(myd3$Tpresses ~ myd3$Day)
boxplot(myd3$Tpresses ~ myd3$Day*myd3$Group)
boxplot(myd3$Tpresses ~ myd3$Day*myd3$Lever)



## trials with head entries
mod4 = ezANOVA(data = myd3,
               wid = rat,
               between = Group,
               within = c(Day, Lever),
               dv = Tentries,
               type = 3)
mod4

tapply(myd3$Tentries, myd3$Day, mean)
tapply(myd3$Tentries, myd3$Day, var)
tapply(myd3$Tentries, myd3$Day, length)
```

```
boxplot(myd3$Tentries ~ myd3$Group)
boxplot(myd3$Tentries ~ myd3$Day)
boxplot(myd3$Tentries ~ myd3$Day*myd3$Group)
boxplot(myd3$Tentries ~ myd3$Day*myd3$Lever)




#######

##### Ok now let's do it with the ST RATIO! #####

mod5 = ezANOVA(data = myd3,
               wid = rat,
               between = Group,
               within = c(Day, Lever),
               dv = ratio,
               type = 3)
mod5

## me figuring out how to check assumptions
tapply(myd3$ratio, myd3$Day, mean)
```

```
tapply(myd3$ratio, myd3$Day, var)
tapply(myd3$ratio, myd3$Day, length)


boxplot(myd3$ratio ~ myd3$Group)
boxplot(myd3$ratio ~ myd3$Day)
boxplot(myd3$ratio ~ myd3$Day*myd3$Group)
boxplot(myd3$ratio ~ myd3$Day*myd3$Lever)
boxplot(myd3$ratio ~ myd3$Day*myd3$Lever*myd3$Group)


# test homogeneity of variance
bartlett.test(myd3$ratio ~ Group, data=myd3)
bartlett.test(myd3$ratio ~ Day, data=myd3)
bartlett.test(myd3$ratio ~ Lever, data=myd3)



###### let's try an lmer with the ratio...? ######

myd$Day <- as.numeric(myd$Day)
myd$Day <- myd$Day - mean(myd$Day)

mlm <- lmer(ratio~Group*Day*Lever+(Day+Lever|rat),
```

```
                    data = myd,
     control=lmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e7)))
     summary(mlm)
     mlm

     anova(mlm)
     anova(mlm, ddf="Kenward-Roger")
     coefs <- data.frame(coef(summary(mlm)))
     coefs$p.z <- 2 * (1-pnorm(abs(coefs$t.value)))

     coefs$df <- coef(summary(mlm))[, 3]

     coefs

     step_mlm <- step(mlm)

     plot(resid(mlm))
     hist(resid(mlm))


     ## ggplot ratio model
     newdat <- expand.grid(
       Group=c("Alcohol", "Water"),
```

```
  Day=c(1,2,3,4,5,6)-3.5,
  Lever=c("Omission Lever", "Passive Lever"),
  ratio = 0
)

contrasts(newdat$Group)=contr.sum(2)
contrasts(newdat$Lever)=contr.sum(2)



res <- mlm

mm <- model.matrix(terms(res), newdat)
newdat$ratio <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo = (newdat$ratio - sqrt(pvar1)),
  phi = (newdat$ratio + sqrt(pvar1))
)

newdat

## uncenter day
```

```
     newdat$Day <- newdat$Day+3.5


     library(ggplot2)


     pd<-position_dodge(width=.2)
     g0 <- ggplot(newdat, aes(x=Day, y=ratio,
                              col=Group))+
geom_line(position=pd, size=2)+
       scale_color_manual(values = c("purple", "black")) +
       geom_point(position=pd, size=4) +
       facet_grid(. ~ Lever)+ylim(-1,1.2)+
       theme(panel.background = element_rect(fill = "white",
colour = "black", size = .5, linetype = "solid"),
             panel.grid.major = element_blank(),
panel.grid.minor = element_blank(), text = element_text(size=18,
family="Times New Roman"),
             legend.position="top", legend.title =
element_blank())
     g0
     g0 + geom_errorbar(aes(ymin = plo, ymax = phi),position=pd,
size=2,
                        linetype=1,
width=.04)+xlab("Day")+ylab("Sign Tracking Ratio") +
       facet_grid(. ~ Lever)
```

```
#### okay moving on to glmer analyses



#####

paige <- Paige4.autoshaping.by.trial.long

is.na(NA)
na.action(na.exclude)

## centering continuous variables
paige$Day = paige$Day - mean(paige$Day)

paige$Trial = paige$Trial - mean(paige$Trial)

## effect coding categorical variables
contrasts(paige$Group)=contr.sum(2)
contrasts(paige$Group)

contrasts(paige$Lever)=contr.sum(2)
contrasts(paige$Lever)
```

```
paige$rat = as.factor(paige$rat)
contrasts(paige$rat)=contr.sum(24)

### Binomial models


## first lever presses
binom <-
glmer(BinomPresses~Group*Day*Lever+Trial+(Day+Trial+Lever|rat),
                      family = binomial(link = "logit"), data =
paige,
                      control=glmerControl(optimizer =
"bobyqa", optCtrl=list(maxfun=2e7)))
summary(binom)

anova(binom)


emmeans(binom, pairwise~Group)

emtrends(binom, ~Group, var="Day")
emtrends(binom, pairwise~Group, var="Day")
emtrends(binom, pairwise~Group*Lever, var="Day",
adjust="none")
```

```
## ggplot
newdat4 <- expand.grid(
  Group=c("Alcohol", "Water"),
  Day=c(1,2,3,4,5,6)-3.5,
  Lever=c("Omission Lever", "Passive Lever"),
  Trial=6.5,
  BinomPresses = 0
)


contrasts(newdat4$Group)=contr.sum(2)
contrasts(newdat4$Group)

contrasts(newdat4$Lever)=contr.sum(2)
contrasts(newdat4$Lever)

res4 <- binom

mm4 <- model.matrix(terms(res4), newdat4)
newdat4$BinomPresses <- mm4 %*% fixef(res4)
pvar4 <- diag(mm4 %*% tcrossprod(vcov(res4),mm4))
```

```
      newdat4 <- data.frame(
        newdat4,
        plo = inv.logit(newdat4$BinomPresses - sqrt(pvar4)),
        phi = inv.logit(newdat4$BinomPresses + sqrt(pvar4))
      )

      newdat4

      ## uncenter day
      newdat4$Day <- newdat4$Day+3.5


      g3 <- ggplot(newdat4, aes(x=Day, y=inv.logit(BinomPresses),
                                col=Group))+
geom_line(position=pd, size=2)+
        scale_color_manual(values = c("purple", "black")) +
        geom_point(position=pd, size=4) +
        facet_grid(. ~ Lever)+ylim(0,1)+
        theme(panel.background = element_rect(fill = "white",
colour = "black", size = .5, linetype = "solid"),
              panel.grid.major = element_blank(),
panel.grid.minor = element_blank(), text = element_text(size=18,
family="Times New Roman"),
              legend.position = "top")
```

```
     g3
     g3 + geom_errorbar(aes(ymin = plo, ymax = phi),position=pd,
size=2,
                        linetype=1,
width=.04)+xlab("Day")+ylab("P(Responses)") +
       facet_grid(. ~ Lever)




     ## now head entries
     binom2 <-
glmer(BinomEntries~Group*Day*Lever+Trial+(Day+Trial+Lever|rat),
                   family = binomial(link = "logit"), data =
paige,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e7)))
     summary(binom2)


     anova(binom2)


     emmeans(binom2, pairwise~Group)
```

```
      emtrends(binom2, ~Group*Lever, var="Day")
      emtrends(binom2, pairwise~Lever, var="Day")
      emtrends(binom2, pairwise~Group*Lever, var="Day",
adjust="none")

      anova(binom2)



      ## ggplot
      newdat5 <- expand.grid(
        Group=c("Alcohol", "Water"),
        Day=c(1,2,3,4,5,6)-3.5,
        Lever=c("Omission Lever", "Passive Lever"),
        Trial=6.5,
        BinomEntries = 0
      )



      contrasts(newdat5$Group)=contr.sum(2)
      contrasts(newdat5$Group)

      contrasts(newdat5$Lever)=contr.sum(2)
      contrasts(newdat5$Lever)
```

```
res5 <- binom2

mm5 <- model.matrix(terms(res5), newdat5)
newdat5$BinomEntries <- mm5 %*% fixef(res5)
pvar5 <- diag(mm5 %*% tcrossprod(vcov(res5),mm5))

newdat5 <- data.frame(
  newdat5,
  plo4 = inv.logit(newdat5$BinomEntries - sqrt(pvar5)),
  phi4 = inv.logit(newdat5$BinomEntries + sqrt(pvar5))
)

newdat5

## uncenter day
newdat5$Day <- newdat5$Day+3.5

pd<-position_dodge(width=.2)
g4 <- ggplot(newdat5, aes(x=Day, y=inv.logit(BinomEntries),
                          col=Group))+
geom_line(position=pd, size=2)+
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=4) +
  facet_grid(. ~ Lever)+ylim(0,1)+
```

```
       theme(panel.background = element_rect(fill = "white",
colour = "black", size = .5, linetype = "solid"),
             panel.grid.major = element_blank(),
panel.grid.minor = element_blank(), text = element_text(size=18,
family="Times New Roman"),
             legend.position = "none")
     g4
     g4 + geom_errorbar(aes(ymin = plo4, ymax =
phi4),position=pd, size=2,
                        linetype=1,
width=.04)+xlab("Day")+ylab("Likelihood of a Head Entry per
Trial") +
       facet_grid(. ~ Lever)



     ## le poisson

     PoissMod <-
glmer(Presses~Group*Day*Lever+Trial+(Day+Trial+Lever|rat),
                        family = poisson, data = paige,
                        control=glmerControl(optimizer =
"bobyqa", optCtrl=list(maxfun=2e5)))
     summary(PoissMod)
```

```
coefs2 <- data.frame(coef(summary(PoissMod)))
coefs2$p.z <- 2 * (1-pnorm(abs(coefs2$z.value)))

coefs2$df <- coef(summary(PoissMod))[, 3]
#rerun mod

coefs2

anova(PoissMod)

## checking for overdispersion
hist(resid(PoissMod))
deviance(PoissMod)/df.residual(PoissMod)

emmeans(PoissMod, pairwise~Group*Lever, type = "response")
emmeans(PoissMod, pairwise~Group*Lever, type = "response",
adjust="none")

emtrends(PoissMod, pairwise~Group, var="Day",
adjust="none")
emtrends(PoissMod, pairwise~Group, var="Day")
```

```
     emtrends(PoissMod, ~Group*Lever, var="Day")
     emtrends(PoissMod, pairwise~Group*Lever, var="Day",
adjust="none")
     emtrends(PoissMod, pairwise~Group*Lever, var="Day")

     ## ggplot Poisson model
     newdat2 <- expand.grid(
       Group=c("Alcohol", "Water"),
       Day=c(1,2,3,4,5,6)-3.5,
       Lever=c("Omission Lever", "Passive Lever"),
       Trial=6.5,
       Presses = 0
     )


     contrasts(newdat2$Group)=contr.sum(2)
     contrasts(newdat2$Group)

     contrasts(newdat2$Lever)=contr.sum(2)
     contrasts(newdat2$Lever)

     res2 <- PoissMod

     mm2 <- model.matrix(terms(res2), newdat2)
```

```
      newdat2$Presses <- mm2 %*% fixef(res2)
      pvar2 <- diag(mm2 %*% tcrossprod(vcov(res2),mm2))

      newdat2 <- data.frame(
        newdat2,
        plo1 = exp(newdat2$Presses - sqrt(pvar2)),
        phi1 = exp(newdat2$Presses + sqrt(pvar2))
      )

      newdat2

      ## uncenter day
      newdat2$Day <- newdat2$Day+3.5



      pd<-position_dodge(width=.2)
      g1 <- ggplot(newdat2, aes(x=Day, y=exp(Presses),
                                col=Group))+
geom_line(position=pd, size=2)+
        scale_color_manual(values = c("purple", "black")) +
        geom_point(position=pd, size=4) +
        facet_grid(. ~ Lever)+ylim(0,10)+
        theme(panel.background = element_rect(fill = "white",
colour = "black", size = .5, linetype = "solid"),
```

```
            panel.grid.major = element_blank(),
panel.grid.minor = element_blank(), text = element_text(size=18,
family="Times New Roman"),
            legend.position = "none")
     g1
     g1 + geom_errorbar(aes(ymin = plo1, ymax =
phi1),position=pd, size=2,
                        linetype=1,
width=.04)+xlab("Day")+ylab("Lever Presses per Trial") +
       facet_grid(. ~ Lever)



     ### head entries poisson

     paige$Entries <- paige$Head.entries

     PoissMod2 <-
glmer(Entries~Group*Day*Lever+Trial+(Day+Trial+Lever|rat),
                        family = poisson, data = paige,
                        control=glmerControl(optimizer =
"bobyqa", optCtrl=list(maxfun=2e5)))
     summary(PoissMod2)
```

```
anova(PoissMod2)


## checking for overdispersion
hist(resid(PoissMod2))
deviance(PoissMod2)/df.residual(PoissMod2)


## ggplot
newdat3 <- expand.grid(
  Group=c("Alcohol", "Water"),
  Day=c(1,2,3,4,5,6)-3.5,
  Lever=c("Omission Lever", "Passive Lever"),
  Trial=6.5,
  Entries = 0
)



contrasts(newdat3$Group)=contr.sum(2)
contrasts(newdat3$Group)

contrasts(newdat3$Lever)=contr.sum(2)
contrasts(newdat3$Lever)

res3 <- PoissMod2
```

```
      mm3 <- model.matrix(terms(res3), newdat3)
      newdat3$Entries <- mm3 %*% fixef(res3)
      pvar3 <- diag(mm3 %*% tcrossprod(vcov(res3),mm3))

      newdat3 <- data.frame(
        newdat3,
        plo2 = exp(newdat3$Entries - sqrt(pvar3)),
        phi2 = exp(newdat3$Entries + sqrt(pvar3))
      )

      newdat3

      ## uncenter day
      newdat3$Day <- newdat3$Day+3.5


      g2 <- ggplot(newdat3, aes(x=Day, y=exp(Entries),
                                col=Group))+
geom_line(position=pd, size=2)+
        scale_color_manual(values = c("purple", "black")) +
        geom_point(position=pd, size=4) +
        facet_grid(. ~ Lever)+ylim(0,1.5)+
        theme(panel.background = element_rect(fill = "white",
colour = "black", size = .5, linetype = "solid"),
```

```
            panel.grid.major = element_blank(),
panel.grid.minor = element_blank(), text = element_text(size=18,
family="Times New Roman"),
            legend.position = "none")
     g2
     g2 + geom_errorbar(aes(ymin = plo2, ymax =
phi2),position=pd, size=2,
                        linetype=1,
width=.04)+xlab("Day")+ylab("Head Entries per Trial") +
       facet_grid(. ~ Lever)

summary(paige)




### Paige4 Omission Contingency Analyses Clean ###


paige <- Paige4.trial.responses.lever.stacked

is.na(NA)
na.action(na.exclude)

## centering continuous variables
paige$Day = paige$Day - mean(paige$Day)
```

```
paige$Trial = paige$Trial - mean(paige$Trial)

## effect coding categorical variables
contrasts(paige$Group)=contr.sum(2)
contrasts(paige$Group)

contrasts(paige$Lever)=contr.sum(2)
contrasts(paige$Lever)

paige$rat = as.factor(paige$rat)
contrasts(paige$rat)=contr.sum(24)



### Binomial Model

TrialME2 <-
glmer(BinomResponse~Group*Day*Lever+Trial+(Day+Trial+Lever|rat),
                  family = binomial(link = "logit"), data =
paige,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e10)))
summary(TrialME2)



coefs <- data.frame(coef(summary(TrialME2)))
coefs$p.z <- 2 * (1-pnorm(abs(coefs$z.value)))

coefs$df <- coef(summary(TrialME2))[, 3]
#rerun mod
```

```
coefs

anova(TrialME2)



#emmeans testing
library(emmeans)
emmeans(TrialME2, ~Group, type = "response")
emmeans(TrialME2, ~Lever, type = "response")
emmeans(TrialME2, ~Group*Lever, type = "response")
emmeans(TrialME2, pairwise~Group*Lever, type = "response",
adjust="none")

emtrends(TrialME2, ~Group*Lever, var="Day")
emtrends(TrialME2, pairwise~Group*Lever, var="Day")
emtrends(TrialME2, pairwise~Group*Lever, var="Day",
adjust="none")


##### ggplot of binomial model
newdat2 <- expand.grid(
  Group=c("Alcohol", "Water"),
  Day=c(1,2,3,4,5,6)-3.5,
  Lever=c("Omission Lever", "Passive Lever"),
  Trial=2.5,
  BinomResponse = 0
)

contrasts(newdat2$Group)=contr.sum(2)
contrasts(newdat2$Lever)=contr.sum(2)
```

```
res2 <- TrialME2

mm <- model.matrix(terms(res2), newdat2)
newdat2$BinomResponse <- mm %*% fixef(res2)
pvar2 <- diag(mm %*% tcrossprod(vcov(res2),mm))

newdat2 <- data.frame(
  newdat2,
  plo = inv.logit(newdat2$BinomResponse - sqrt(pvar2)),
  phi = inv.logit(newdat2$BinomResponse + sqrt(pvar2))
)

newdat2

## uncenter day
newdat2$Day <- newdat2$Day+3.5

pd2<-position_dodge(width=.2)
g1 <- ggplot(newdat2, aes(x=Day, y=inv.logit(BinomResponse),
                          col=Group))+ geom_line(position=pd2,
size=2)+
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd2, size=4) +
  facet_grid(. ~ Lever)+ylim(0,1)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top")
g1
```

```
g1 + geom_errorbar(aes(ymin = plo, ymax = phi),position=pd2,
size=2,
                    linetype=1,
width=.04)+xlab("Day")+ylab("P(Responses)") +
  facet_grid(. ~ Lever)




##### Poisson Model

PoissMod <-
glmer(Responses~Group*Day*Lever+Trial+(Day+Trial+Lever|rat),
                  family = poisson, data = paige,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)



coefs2 <- data.frame(coef(summary(PoissMod)))
coefs2$p.z <- 2 * (1-pnorm(abs(coefs2$z.value)))

coefs2$df <- coef(summary(PoissMod))[, 3]
#rerun mod

coefs2

anova(PoissMod)

## checking for overdispersion
hist(resid(PoissMod))
```

```
deviance(PoissMod)/df.residual(PoissMod)

emmeans(PoissMod, ~Group, type = "response")
emmeans(PoissMod, ~Lever, type = "response")
emmeans(PoissMod, ~Group*Lever, type = "response")
emmeans(PoissMod, pairwise~Group*Lever, type = "response")
emmeans(PoissMod, pairwise~Group*Lever, type = "response",
adjust="none")

emtrends(PoissMod, ~Group*Lever, var="Day")
emtrends(PoissMod, pairwise~Group*Lever, var="Day",
adjust="none")
emtrends(PoissMod, pairwise~Group*Lever, var="Day")

plot(effect("Group:Day:Lever", PoissMod, ylab="Number of
responses"),  rug=FALSE, confint=list(style="bands"),
multiline=TRUE, type="response")
plot(effect("Group:Day:Lever", TrialME2, ylab="Number of
responses"), se.prob=T, rug=FALSE, multiline=T, rescale.axis =
F)

plot(effect("Group:Day:Lever", PoissMod, ylab="Number of
responses"), rug=FALSE, multiline=T, type="response")

xyplot(fitted(TrialME2)~Day|Lever, group=Group, data=paige,
type="a")
xyplot(fitted(PoissMod)~Day|Lever, group=Group, data=paige,
type="a", ylim=c(0,8))


## attempting ggplot
newdat <- expand.grid(
```

```
  Group=c("Alcohol", "Water"),
  Day=c(1,2,3,4,5,6)-3.5,
  Lever=c("Omission Lever", "Passive Lever"),
  Trial=2.5,
  Responses = 0
)

contrasts(newdat$Group)=contr.sum(2)
contrasts(newdat$Lever)=contr.sum(2)



res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$Responses <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo = exp(newdat$Responses - sqrt(pvar1)),
  phi = exp(newdat$Responses + sqrt(pvar1))
)

newdat

## uncenter day
newdat$Day <- newdat$Day+3.5

library(ggplot2)
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=Day, y=exp(Responses),
```

```
                        col=Group))+ geom_line(position=pd,
size=2)+
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=4) +
  facet_grid(. ~ Lever)+ylim(0,7.5)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "none")
g0
g0 + geom_errorbar(aes(ymin = plo, ymax = phi),position=pd,
size=2,
                   linetype=1,
width=.04)+xlab("Day")+ylab("Responses per Trial") +
  facet_grid(. ~ Lever)



### neg binomal


histogram(~Responses|Lever*Group, data=paige)

Sys.time()
NBMod <-
glmer.nb(Responses~Group*Day*Lever+Trial+(Day+Trial+Lever|rat),
                  data = paige,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(NBMod)
```

```
Sys.time()

emmeans(NBMod, ~Group, type = "response")
emmeans(NBMod, ~Lever, type = "response")
emmeans(NBMod, ~Group*Lever, type = "response")
emmeans(NBMod, pairwise~Group*Lever, type = "response")
emmeans(NBMod, pairwise~Group*Lever, type = "response",
adjust="none")

emtrends(NBMod, pairwise~Group*Lever, var="Day", adjust="none")

## checking for overdispersion
hist(resid(NBMod))
deviance(NBMod)/df.residual(NBMod)



## attempting ggplot
newdat <- expand.grid(
  Group=c("Alcohol", "Water"),
  Day=c(1,2,3,4,5,6)-3.5,
  Lever=c("Omission Lever", "Passive Lever"),
  Trial=2.5,
  Responses = 0
)

contrasts(newdat$Group)=contr.sum(2)
contrasts(newdat$Lever)=contr.sum(2)


res <- NBMod
```

```
mm <- model.matrix(terms(res), newdat)
newdat$Responses <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo = exp(newdat$Responses - sqrt(pvar1)),
  phi = exp(newdat$Responses + sqrt(pvar1))
)

newdat

## uncenter day
newdat$Day <- newdat$Day+3.5

library(ggplot2)
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=Day, y=exp(Responses),
                         col=Group))+ geom_line(position=pd,
size=2)+
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=4) +
  facet_grid(. ~ Lever)+ylim(0,7.5)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "none")
g0
g0 + geom_errorbar(aes(ymin = plo, ymax = phi),position=pd,
size=2,
```

```
                    linetype=1,
width=.04)+xlab("Day")+ylab("Responses per Trial") +
  facet_grid(. ~ Lever)









## zero inflated

install.packages("R2admb")
install.packages("glmmADMB",
                 repos=c("http://glmmadmb.r-forge.r-
project.org/repos",
                         getOption("repos")),
                 type="source")
library(glmmADMB)

ZIMod <-
glmmadmb(Responses~Group*Day*Lever+Trial+(Trial+Day+Lever|rat),
                  family = "poisson", data = paige,
zeroInflation = TRUE,
                  mcmc=TRUE, mcmc.opts=mcmcControl(mcmc=50000))
summary(ZIMod)

ZIMod2 <-
glmmadmb(Responses~Group*Day*Lever+Trial+(Trial+Day+Lever|rat),
```

```
                  family = "poisson", data = paige,
zeroInflation = FALSE,
  mcmc=TRUE, mcmc.opts=mcmcControl(mcmc=50000))
summary(ZIMod2)

## checking for overdispersion
hist(resid(ZIMod))
deviance(ZIMod)/df.residual(ZIMod)
residuals(ZIMod)/df(ZIMod)

overdisp_fun <- function(model) {
  ## number of variance parameters in
  ##   an n-by-n variance-covariance matrix
  vpars <- function(m) {
    nrow(m)*(nrow(m)+1)/2
  }
  model.df <-
sum(sapply(VarCorr(model),vpars))+length(fixef(model))
  rdf <- nrow(model.frame(model))-model.df
  rp <- residuals(model,type="pearson")
  Pearson.chisq <- sum(rp^2)
  prat <- Pearson.chisq/rdf
  pval <- pchisq(Pearson.chisq, df=rdf, lower.tail=FALSE)
  c(chisq=Pearson.chisq,ratio=prat,rdf=rdf,p=pval)
}

overdisp_fun(ZIMod)
overdisp_fun(NBMod)
overdisp_fun(PoissMod)

anova(ZIMod, ZIMod2)
```

```
emmeans(ZIMod, ~Group, type = "response")
emmeans(ZIMod, ~Lever, type = "response")
emmeans(ZIMod, ~Group*Lever, type = "response")
emmeans(ZIMod, pairwise~Group*Lever, type = "response")
emmeans(ZIMod, pairwise~Group*Lever, type = "response",
adjust="none")


## attempting ggplot
newdat <- expand.grid(
  Group=c("Alcohol", "Water"),
  Day=c(1,2,3,4,5,6)-3.5,
  Lever=c("Omission Lever", "Passive Lever"),
  Trial=2.5,
  Responses = 0
)

contrasts(newdat$Group)=contr.sum(2)
contrasts(newdat$Lever)=contr.sum(2)


res <- ZIMod

mm <- model.matrix(terms(res), newdat)
newdat$Responses <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo = exp(newdat$Responses - sqrt(pvar1)),
  phi = exp(newdat$Responses + sqrt(pvar1))
```

```
)

newdat

## uncenter day
newdat$Day <- newdat$Day+3.5

library(ggplot2)
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=Day, y=exp(Responses),
                         col=Group))+ geom_line(position=pd,
size=2)+
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=4) +
  facet_grid(. ~ Lever)+ylim(0,7.5)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "none")
g0
g0 + geom_errorbar(aes(ymin = plo, ymax = phi),position=pd,
size=2,
                   linetype=1,
width=.04)+xlab("Day")+ylab("Responses per Trial") +
  facet_grid(. ~ Lever)
```

```
### Paige4 DMS all counts analyses ###

myd <- Paige4.DMS.PV.long

summary(myd)

is.na(NA)
na.action(na.exclude)

myd$DMS <- myd$DMS.Region
contrasts(myd$DMS)=contr.sum(2)
contrasts(myd$DMS)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

myd$rat <- myd$Rat
myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(24)


contrasts(myd$Hemisphere)=contr.sum(2)
contrasts(myd$Hemisphere)

myd$c.ETOH <- myd$etoh - mean(myd$etoh)



PoissMod <- glmer(PV~Group*DMS+(DMS|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
```

```
summary(PoissMod)

anova(PoissMod)


emmeans(PoissMod, pairwise~Group*DMS, type = "response")
emmeans(PoissMod, pairwise~Group*DMS, type = "response",
adjust="none")



### ggplot
newdat <- expand.grid(
  Group = c("Alcohol", "Water"),
  DMS=c("0.4DMS","0.8DMS"),
  PV = 0
)

contrasts(newdat$ACC)=contr.sum(2)
contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
```

```
)

newdat


## interaction
pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=DMS, y=exp(PV),
                         fill=Group)) +
geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,15)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Region in ACC")+
  ylab("PV+ Neurons in ACC")



### collapsed across brain regions
PoissMod3 <- glmer(PV~Group+(1|rat),
                   family = poisson, data = myd,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
```

```
summary(PoissMod3)


### ggplot
newdat <- expand.grid(
  Group = c("Alcohol", "Water"),
  PV = 0
)

contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod3

mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)

newdat


pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=Group, y=exp(PV)
                         )) + geom_bar(stat="identity",
position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
```

```
  ylim(0,22)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Group")+
  ylab("PV+ Neurons in DMS")




#### all counts of ACC with ETOH ####



etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH, PV,
DMS, rat, Hemisphere))


PoissMod2 <- glmer(PV~DMS*c.ETOH+(DMS|rat),
                   family = poisson, data = etoh,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
```

```
summary(PoissMod2)

anova(PoissMod2)


summary(etoh)

### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.6125,
  DMS=c("0.4DMS","0.8DMS"),
  PV = 0
)

contrasts(newdat$DMS)=contr.sum(2)


res <- PoissMod2

mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)

newdat

## uncenter day
```

```
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## interaction
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(PV),
                         col=DMS))+ geom_line(position=pd,
size=1) +
  geom_point(position=pd, size=2) +
  ylim(0,30)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr")+
  ylab("PV+ Neurons in DMS")


## collapsed across brain regions
PoissMod4 <- glmer(PV~c.ETOH+(1|rat),
                   family = poisson, data = etoh,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod4)

### ggplot
newdat <- expand.grid(
```

```
  c.ETOH=c(0:8.5064)-1.6125,
  PV = 0
)



res <- PoissMod4

mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## interaction
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(PV)
                         ))+ geom_line(position=pd, size=1) +
  geom_point(position=pd, size=2) +
  ylim(0,30)+
```

```
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr")+
  ylab("PV+ Neurons in DMS")




### Paige4 DLS all counts analyses ###

myd <- Paige4.DLS.PV.long

summary(myd)

is.na(NA)
na.action(na.exclude)

myd$DLS <- myd$DLS.Region
contrasts(myd$DLS)=contr.sum(2)
contrasts(myd$DLS)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)
```

```
myd$rat <- myd$Rat
myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(24)


contrasts(myd$Hemisphere)=contr.sum(2)
contrasts(myd$Hemisphere)

myd$c.ETOH <- myd$etoh - mean(myd$etoh)



PoissMod <- glmer(PV~Group*DLS+(DLS|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)


emmeans(PoissMod, pairwise~Group*DLS, type = "response")
emmeans(PoissMod, pairwise~Group*DLS, type = "response",
adjust="none")



### ggplot
newdat <- expand.grid(
  Group = c("Alcohol", "Water"),
  DLS=c("3.2","2.7"),
```

```
  PV = 0
)

contrasts(newdat$ACC)=contr.sum(2)
contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)

newdat


## interaction
pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=DLS, y=exp(PV),
                         fill=Group)) +
geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,25)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
```

```
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Region in DLS")+
  ylab("PV+ Neurons in DLS")



### collapsed across brain regions
PoissMod3 <- glmer(PV~Group+(1|rat),
                   family = poisson, data = myd,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod3)

##ggplot
newdat <- expand.grid(
  Group = c("Alcohol", "Water"),
  PV = 0
)

contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod3

mm <- model.matrix(terms(res), newdat)
```

```
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)

newdat



pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=Group, y=exp(PV)
                         )) + geom_bar(stat="identity",
position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,25)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Group")+
  ylab("PV+ Neurons in DLS")
```

```
#### all counts of ACC with ETOH ####



etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH, PV,
DLS, rat, Hemisphere))


PoissMod2 <- glmer(PV~DLS*c.ETOH+(DLS|rat),
                   family = poisson, data = etoh,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod2)

anova(PoissMod2)

xyplot(fitted(PoissMod2)+PV~c.ETOH, data=etoh, type=c("a", "p"),
auto.key = TRUE)
#ew

summary(etoh)

### ggplot
```

```
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.6125,
  DLS=c("0.4DLS","0.8DLS"),
  PV = 0
)

contrasts(newdat$DLS)=contr.sum(2)


res <- PoissMod2

mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## interaction
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(PV),
```

```
                          col=ACC))+ geom_line(position=pd,
size=1) +
  geom_point(position=pd, size=2) +
  ylim(0,30)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr")+
  ylab("PV+ Neurons in ACC")


## alcohol main effect
head(PoissMod2)

newdat2 <- summarySE(newdat, measurevar="PV",
groupvars="c.ETOH", na.rm=FALSE, conf.interval = 0.95,
.drop=TRUE)
newdat2

newdat2$c.ETOH <- newdat2$c.ETOH+1.6125
newdat2


g1 <- ggplot(newdat2, aes(x=c.ETOH, y=exp(PV)))+
  geom_line(size=2, color = "purple") + ylim(0,30) +
  geom_point(position=pd, size=4, color="purple") +
```

```
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"))
g1
g1 + geom_errorbar(aes(ymin = exp(PV-se), ymax =
exp(PV+se)),size=2,
                   linetype=1, width=.04, color="purple") +
  xlab("Alcohol Consumption (g/kg/24 hr)")+ylab("Parvalbumin
Expressing Neurons in DLS")




### Paige4 ACC all counts analyses ###

myd <- Paige4.allACC.ETOH.long

summary(Paige4.allACC.ETOH.long)

is.na(NA)
na.action(na.exclude)

myd$ACC <- as.factor(myd$ACC)
contrasts(myd$ACC)=contr.sum(2)
contrasts(myd$ACC)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)
```

```
myd$rat <- myd$Rat
myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(23)

PoissMod <- glmer(PV~Group*ACC+(1|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)


emmeans(PoissMod, pairwise~Group*ACC, type = "response")
emmeans(PoissMod, pairwise~Group*ACC, type = "response",
adjust="none")



### ggplot
newdat <- expand.grid(
  Group = c("Alcohol", "Water"),
  ACC=c("3.2","2.7"),
  PV = 0
)

contrasts(newdat$ACC)=contr.sum(2)
contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod
```

```
mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)

newdat


## interaction
pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=ACC, y=exp(PV),
                     fill=Group)) + geom_bar(stat="identity",
position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,15)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Region in ACC")+
```

```
  ylab("PV+ Neurons in ACC")
```

```
#### all counts of ACC with ETOH ####

myd2 <- Paige4.allACC.ETOH.long

is.na(NA)
na.action(na.exclude)

myd2$ACC <- as.factor(myd2$ACC)
contrasts(myd2$ACC)=contr.sum(2)
contrasts(myd2$ACC)

contrasts(myd2$Hemisphere)=contr.sum(2)
contrasts(myd2$Hemisphere)

contrasts(myd2$Group)=contr.sum(2)
contrasts(myd2$Group)

myd2$rat <- myd2$Rat
myd2$rat <- as.factor(myd2$rat)
```

```
contrasts(myd2$rat)=contr.sum(23)

myd2$c.ETOH <- myd2$ETOH - mean(myd2$ETOH)



etoh = subset(myd2, Group == "Alcohol", select = c(c.ETOH, PV,
ACC, rat, Hemisphere))


PoissMod2 <- glmer(PV~ACC*c.ETOH+(ACC|rat),
                   family = poisson, data = etoh,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod2)

anova(PoissMod2)

xyplot(fitted(PoissMod2)+PV~c.ETOH, data=etoh, type=c("a", "p"),
auto.key = TRUE)
#ew

PoissMod3 <- glmer(PV~c.ETOH+(1|rat),
                    family = poisson, data = etoh,
                    control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod3)

summary(etoh)

### ggplot
newdat <- expand.grid(
```

```
  c.ETOH=c(0:8.5064)-1.6125,
  ACC=c("3.2","2.7"),
  PV = 0
)

contrasts(newdat$ACC)=contr.sum(2)


res <- PoissMod2

mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## interaction
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(PV),
                         col=ACC))+ geom_line(position=pd,
size=1) +
```

```
  geom_point(position=pd, size=2) +
  ylim(0,30)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr")+
  ylab("PV+ Neurons in ACC")


## alcohol main effect
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.6125,
  PV = 0
)


res <- PoissMod3

mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)
```

```
newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125


g1 <- ggplot(newdat, aes(x=c.ETOH, y=exp(PV)))+
  geom_line(size=2, color = "purple") + ylim(0,20) +
  geom_point(position=pd, size=4, color="purple") +
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"))
g1
g1 + geom_errorbar(aes(ymin = plo1, ymax = phi1),size=2,
                   linetype=1, width=.04, color="purple") +
                  xlab("Alcohol Consumption (g/kg/24
hr)")+ylab("Parvalbumin Expressing Neurons in ACC")




correl = subset(etoh, ACC == "3.2", select = c(c.ETOH, PV))
correl$PV <- as.numeric(correl$PV)

cor(correl, y = NULL, method = "pearson")

is.na(NA)
na.action(na.exclude(NA))
```

```
correl$PV <- as.numeric(correl$PV)
correl2 <- subset(etoh, select = c(c.ETOH, PV))
correl2 <- na.omit(correl2)

allcorrel <- cor(correl2, y = NULL, method = "pearson")
allcorrel
plot(allcorrel)




#### Paige4 PV NAc analysis ####

myd <- Paige4.PV.NAc.long

summary(myd)

is.na(NA)
na.action(na.exclude)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(24)
```

```
contrasts(myd$Nac)=contr.sum(2)
contrasts(myd$Nac)

myd$c.ETOH <- myd$etoh - mean(myd$etoh)


### NAc analysis
PoissMod <- glmer(PV~Group*Nac+(Nac|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
emmeans(PoissMod, pairwise~Group*Nac, type = "response",
adjust="none")

## core alone

myd <- Paige4.NAc.Core

summary(myd)

is.na(NA)
na.action(na.exclude)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

myd$rat <- as.factor(myd$rat)
```

```
contrasts(myd$rat)=contr.sum(24)


myd$c.ETOH <- myd$etoh - mean(myd$etoh)


### NAc core analysis
PoissMod <- glmer(core~Group+(1|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
emmeans(PoissMod, pairwise~Group*Nac, type = "response",
adjust="none")

##ggplot
newdat <- expand.grid(
  Group = c("Alcohol", "Water"),
  core = 0
)

contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$core <- mm %*% fixef(res)
```

```
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$core - sqrt(pvar1)),
  phi1 = exp(newdat$core + sqrt(pvar1))
)

newdat



pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=Group, y=exp(core)
)) + geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,5)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Group")+
  ylab("PV+ Neurons in NAc Core")
```

```
## shell alone

myd <- Paige4.PV.NAc.shell

summary(myd)

is.na(NA)
na.action(na.exclude)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(24)


myd$c.ETOH <- myd$etoh - mean(myd$etoh)


### NAc shell analysis
PoissMod <- glmer(shell~Group+(1|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
```

```
emmeans(PoissMod, pairwise~Group*Nac, type = "response",
adjust="none")


##ggplot
newdat <- expand.grid(
  Group = c("Alcohol", "Water"),
  shell = 0
)

contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$shell <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$shell - sqrt(pvar1)),
  phi1 = exp(newdat$shell + sqrt(pvar1))
)

newdat



pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=Group, y=exp(shell)
)) + geom_bar(stat="identity", position="dodge") +
```

```
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,5)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Group")+
  ylab("PV+ Neurons in NAc Shell")



### etoh correl

etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH, PV,
Nac, rat))


PoissMod <- glmer(PV~c.ETOH*Nac+(Nac|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)
```

```
### core correl

etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH, core,
rat))


PoissMod <- glmer(core~c.ETOH+(1|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.6125,
  core = 0
)



res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$core <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$core - sqrt(pvar1)),
  phi1 = exp(newdat$core + sqrt(pvar1))
```

```
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## interaction
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(core)
))+ geom_line(position=pd, size=1) +
  geom_point(position=pd, size=2) +
  ylim(0,5)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr")+
  ylab("PV+ Neurons in NAc Core")
```

```
### shell correl

etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH, shell,
rat))


PoissMod <- glmer(shell~c.ETOH+(1|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.6125,
  shell = 0
)



res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$shell <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$shell - sqrt(pvar1)),
```

```
  phi1 = exp(newdat$shell + sqrt(pvar1))
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## interaction
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(shell)
))+ geom_line(position=pd, size=1) +
  geom_point(position=pd, size=2) +
  ylim(0,5)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr")+
  ylab("PV+ Neurons in NAc Shell")
```

```
#### Paige4 PV amygdala analysis ####

myd <- Paige4.amygdala.analysis

summary(myd)

is.na(NA)
na.action(na.exclude)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(23)

contrasts(myd$amy)=contr.sum(2)
contrasts(myd$amy)

myd$c.ETOH <- myd$etoh - mean(myd$etoh)


### amygdala analysis
PoissMod <- glmer(PV~Group*amy+(amy|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
```

```
emmeans(PoissMod, pairwise~Group*amy, type = "response",
adjust="none")

### ggplot
newdat <- expand.grid(
  Group=c("Alcohol", "Water"),
  amy=c("-3.3","-3.0"),
  PV = 0
)

contrasts(newdat$Group)=contr.sum(2)
contrasts(newdat$amy)=contr.sum(2)

res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)

newdat



library(ggplot2)

## interaction
```

```
pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=amy, y=exp(PV),
                         fill=Group)) +
geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,17)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Amygdala Regions")+
  ylab("Number of PV+ Neurons")


### etoh correl

etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH, PV,
amy, rat))


PoissMod <- glmer(PV~c.ETOH*amy+(amy|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)
```

```
anova(PoissMod)


## collapsed across brain region
PoissMod2 <- glmer(PV~c.ETOH+(1|rat),
                   family = poisson, data = etoh,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod2)

### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.6125,
  PV = 0
)



res <- PoissMod2

mm <- model.matrix(terms(res), newdat)
newdat$PV <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$PV - sqrt(pvar1)),
  phi1 = exp(newdat$PV + sqrt(pvar1))
)

newdat
```

```
## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## interaction
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(PV)
))+ geom_line(position=pd, size=1) +
  geom_point(position=pd, size=2) +
  ylim(0,20)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr")+
  ylab("PV+ Neurons in Amygdala")




#### Paige4 ChAT DMS and DLS analysis ####
```

```
myd <- Paige4.ChAT.DMSDLS.long

summary(myd)

is.na(NA)
na.action(na.exclude)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(24)

contrasts(myd$DMS)=contr.sum(2)
contrasts(myd$DMS)

contrasts(myd$DLS)=contr.sum(2)
contrasts(myd$DLS)

myd$c.ETOH <- myd$etoh - mean(myd$etoh)


### DMS analysis
PoissMod <- glmer(chatDMS~Group*DMS+(DMS|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
```

```
emmeans(PoissMod, pairwise~Group*DMS, type = "response",
adjust="none")


### collapsed across brain regions
PoissMod2 <- glmer(chatDMS~Group+(1|rat),
                   family = poisson, data = myd,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod2)

##ggplot
newdat <- expand.grid(
  Group = c("Alcohol", "Water"),
  chatDMS = 0
)

contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod2

mm <- model.matrix(terms(res), newdat)
newdat$chatDMS <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chatDMS - sqrt(pvar1)),
  phi1 = exp(newdat$chatDMS + sqrt(pvar1))
)
```

```
newdat



pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=Group, y=exp(chatDMS)
)) + geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,10)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Group")+
  ylab("ChAT+ Neurons in DMS")




## etoh correl
etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH,
chatDMS, DMS, rat, chatDLS, DLS))
```

```
PoissMod <- glmer(chatDMS~c.ETOH*DMS+(1|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)


### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.6125,
  DMS=c("0.4DMS","0.8DMS"),
  chatDMS = 0
)

contrasts(newdat$DMS)=contr.sum(2)


res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$chatDMS <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chatDMS - sqrt(pvar1)),
  phi1 = exp(newdat$chatDMS + sqrt(pvar1))
)

newdat
```

```
## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## main
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(chatDMS),
                         col=DMS))+ geom_line(position=pd,
size=1) +
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=2) +
  ylim(0,20)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(),   text = element_text(size=18, family="Times
New Roman"))
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr)")+
  ylab("Number of ChAT+ Neurons")
```

```
### DLS analysis

PoissMod3 <- glmer(chatDLS~Group*DLS+(DLS|rat),
                   family = poisson, data = myd,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod3)

anova(PoissMod3)

# collapse across brain regions
PoissMod4 <- glmer(chatDLS~Group+(1|rat),
                    family = poisson, data = myd,
                    control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod4)

##ggplot
newdat <- expand.grid(
  Group = c("Alcohol", "Water"),
  chatDLS = 0
)

contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod4

mm <- model.matrix(terms(res), newdat)
newdat$chatDLS <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))
```

```
newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chatDLS - sqrt(pvar1)),
  phi1 = exp(newdat$chatDLS + sqrt(pvar1))
)

newdat



pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=Group, y=exp(chatDLS)
)) + geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,10)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Group")+
  ylab("ChAT+ Neurons in DLS")



## etoh correl
```

```
PoissMod <- glmer(chatDLS~c.ETOH*DLS+(DLS|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)




### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.6125,
  DLS=c("0.4DLS","0.8DLS"),
  chatDLS = 0
)

contrasts(newdat$DLS)=contr.sum(2)


res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$chatDLS <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chatDLS - sqrt(pvar1)),
  phi1 = exp(newdat$chatDLS + sqrt(pvar1))
)
```

```
newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## main
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(chatDLS),
                         col=DLS))+ geom_line(position=pd,
size=1) +
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=2) +
  ylim(0,28)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(),  text = element_text(size=18, family="Times New
Roman"))
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr)")+
  ylab("Number of ChAT+ Neurons")
```

```
#### Paige4 ChAT NAc analysis ####

myd <- Paige4.ChAT.NAc.long

summary(myd)

is.na(NA)
na.action(na.exclude)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(24)

contrasts(myd$Nac)=contr.sum(2)
contrasts(myd$Nac)

myd$c.ETOH <- myd$etoh - mean(myd$etoh)


### NAc analysis
PoissMod <- glmer(chat~Group*Nac+(Nac|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
```

```
emmeans(PoissMod, pairwise~Group*Nac, type = "response",
adjust="none")


### ggplot
newdat <- expand.grid(
  Group=c("Alcohol", "Water"),
  Nac=c("core","shell"),
  chat = 0
)

contrasts(newdat$Group)=contr.sum(2)
contrasts(newdat$Nac)=contr.sum(2)

res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$chat <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chat - sqrt(pvar1)),
  phi1 = exp(newdat$chat + sqrt(pvar1))
)

newdat



library(ggplot2)
```

```
## interaction
pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=Nac, y=exp(chat),
                         fill=Group)) +
geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,12)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Nucleus Accumbens Region")+
  ylab("Number of ChAT Neurons")



### core only

myd <- Paige4.ChAT.NAc.core

summary(myd)

is.na(NA)
na.action(na.exclude)

contrasts(myd$Group)=contr.sum(2)
```

```
contrasts(myd$Group)

myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(24)

myd$c.ETOH <- myd$etoh - mean(myd$etoh)


### core analysis
PoissMod <- glmer(core~Group+(1|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
emmeans(PoissMod, pairwise~Group*Nac, type = "response",
adjust="none")

### ggplot
newdat <- expand.grid(
  Group=c("Alcohol", "Water"),
  core = 0
)

contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod
```

```
mm <- model.matrix(terms(res), newdat)
newdat$core <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$core - sqrt(pvar1)),
  phi1 = exp(newdat$core + sqrt(pvar1))
)

newdat



library(ggplot2)

## interaction
pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=Group, y=exp(core)
)) + geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,12)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
```

```
                    linetype=1, width=.04,
color="darkgray")+xlab("Core")+
  ylab("Number of ChAT Neurons")




### shell only

myd <- Paige4.ChAT.NAc.shell

summary(myd)

is.na(NA)
na.action(na.exclude)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(24)

myd$c.ETOH <- myd$etoh - mean(myd$etoh)


### shell analysis
PoissMod <- glmer(shell~Group+(1|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
```

```
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
emmeans(PoissMod, pairwise~Group*Nac, type = "response",
adjust="none")

### ggplot
newdat <- expand.grid(
  Group=c("Alcohol", "Water"),
  shell = 0
)

contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$shell <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$shell - sqrt(pvar1)),
  phi1 = exp(newdat$shell + sqrt(pvar1))
)

newdat
```

```
library(ggplot2)

## interaction
pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=Group, y=exp(shell)
                         )) + geom_bar(stat="identity",
position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,12)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Shell")+
  ylab("Number of ChAT Neurons")









#### etoh correl
```

```
etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH, chat,
Nac, rat))


PoissMod <- glmer(chat~c.ETOH*Nac+(Nac|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)




### core correl

etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH, core,
rat))


PoissMod <- glmer(core~c.ETOH+(1|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

mean(myd$etoh)
myd$etoh
```

```
### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.51)-1.9858,
  core = 0
)



res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$core <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$core - sqrt(pvar1)),
  phi1 = exp(newdat$core + sqrt(pvar1))
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.9858

library(ggplot2)

## main
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(core)
                         ))+ geom_line(position=pd, size=1) +
```

```
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=2) +
  ylim(0,12)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr)")+
  ylab("Number of ChAT+ Neurons")




### shell correl
etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH, shell,
rat))


PoissMod <- glmer(shell~c.ETOH+(1|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)
```

```
### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.51)-1.9858,
  shell = 0
)



res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$shell <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$shell - sqrt(pvar1)),
  phi1 = exp(newdat$shell + sqrt(pvar1))
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.9858

library(ggplot2)

## main
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(shell)
))+ geom_line(position=pd, size=1) +
```

```
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=2) +
  ylim(0,15)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr)")+
  ylab("Number of ChAT+ Neurons")




##### Paige4 ChAT ACC analysis ####

myd <- Paige4.ChAT.ACC.long

summary(myd)

is.na(NA)
na.action(na.exclude)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)
```

```
myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(24)

contrasts(myd$acc)=contr.sum(2)
contrasts(myd$acc)

myd$c.ETOH <- myd$etoh - mean(myd$etoh)


### acc analysis
PoissMod <- glmer(chat~Group*acc+(acc|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
emmeans(PoissMod, pairwise~Group*acc, type = "response",
adjust="none")

## collapse across brain regions
PoissMod2 <- glmer(chat~Group+(1|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod2)

##ggplot
newdat <- expand.grid(
```

```
  Group = c("Alcohol", "Water"),
  chat = 0
)

contrasts(newdat$Group)=contr.sum(2)


res <- PoissMod2

mm <- model.matrix(terms(res), newdat)
newdat$chat <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chat - sqrt(pvar1)),
  phi1 = exp(newdat$chat + sqrt(pvar1))
)

newdat



pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=Group, y=exp(chat)
)) + geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,2)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
```

```
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Group")+
  ylab("ChAT+ Neurons in ACC")




#### etoh correl

etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH, chat,
acc, rat))


PoissMod <- glmer(chat~c.ETOH*acc+(acc|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)


## collapse across brain region
PoissMod3 <- glmer(chat~c.ETOH+(1|rat),
                  family = poisson, data = etoh,
```

```
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod3)

mean(myd$etoh)

### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.9858,
  chat = 0
)



res <- PoissMod3

mm <- model.matrix(terms(res), newdat)
newdat$chat <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chat - sqrt(pvar1)),
  phi1 = exp(newdat$chat + sqrt(pvar1))
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.9858

library(ggplot2)
```

```
## main
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(chat)
                         ))+ geom_line(position=pd, size=1) +
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=2) +
  ylim(0,1)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(),   text = element_text(size=18, family="Times
New Roman"))
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr)")+
  ylab("Number of ChAT+ Neurons")




### Paige4 Cholinergic Nuclei Analysis ###

myd <- paige4.ChATnuclei.long

summary(myd)

is.na(NA)
na.action(na.exclude)
```

```
contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(21)


myd$c.ETOH <- myd$etoh - mean(myd$etoh)


### ch1 analysis
PoissMod <- glmer(ch1~Group+(1|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
emmeans(PoissMod, pairwise~Group, type = "response",
adjust="none")

### ch2 analysis
PoissMod2 <- glmer(ch2~Group+(1|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod2)

anova(PoissMod2)
```

```
emmeans(PoissMod2, pairwise~Group, type = "response")
emmeans(PoissMod2, pairwise~Group, type = "response",
adjust="none")

### ch3 analysis
PoissMod3 <- glmer(ch3~Group+(1|rat),
                   family = poisson, data = myd,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod3)

anova(PoissMod3)

emmeans(PoissMod3, pairwise~Group, type = "response")
emmeans(PoissMod3, pairwise~Group, type = "response",
adjust="none")


#### ch4 analysis
PoissMod4 <- glmer(ch4~Group+(1|rat),
                   family = poisson, data = myd,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod4)

anova(PoissMod4)

emmeans(PoissMod4, pairwise~Group, type = "response")
emmeans(PoissMod4, pairwise~Group, type = "response",
adjust="none")
```

```
#### how Vetreno did it

myd <- paige4.ChATnuclei.ch12.ch34.long

summary(myd)

is.na(NA)
na.action(na.exclude)

contrasts(myd$Group)=contr.sum(2)
contrasts(myd$Group)

myd$rat <- as.factor(myd$rat)
contrasts(myd$rat)=contr.sum(21)


myd$c.ETOH <- myd$etoh - mean(myd$etoh)

contrasts(myd$ch12)=contr.sum(2)
contrasts(myd$ch12)

contrasts(myd$ch34)=contr.sum(2)
contrasts(myd$ch34)


### ch1 and ch2 analysis
```

```
PoissMod <- glmer(chat12~Group*ch12+(ch12|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)

emmeans(PoissMod, pairwise~Group, type = "response")
emmeans(PoissMod, pairwise~Group, type = "response",
adjust="none")


PoissMod2 <- glmer(chat12~Group+(1|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod2)

anova(PoissMod2)


### ggplot
newdat <- expand.grid(
  Group=c("Alcohol", "Water"),
  ch12=c("ch1","ch2"),
  chat12 = 0
)

contrasts(newdat$Group)=contr.sum(2)
contrasts(newdat$ch12)=contr.sum(2)
```

```
res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$chat12 <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chat12 - sqrt(pvar1)),
  phi1 = exp(newdat$chat12 + sqrt(pvar1))
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## interaction
pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=ch12, y=exp(chat12),
                         fill=Group)) +
geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,32)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
```

```
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Cholinergic Nuclei")+
  ylab("Number of ChAT Neurons")


## main effect of group
newdat2 <- expand.grid(
  Group=c("Alcohol", "Water"),
  chat12 = 0
)

contrasts(newdat2$Group)=contr.sum(2)

res2 <- PoissMod2

mm2 <- model.matrix(terms(res2), newdat2)
newdat2$chat12 <- mm2 %*% fixef(res2)
pvar1 <- diag(mm2 %*% tcrossprod(vcov(res2),mm2))

newdat2 <- data.frame(
  newdat2,
  plo = exp(newdat2$chat12 - sqrt(pvar1)),
  phi = exp(newdat2$chat12 + sqrt(pvar1))
)

newdat2
```

```
pd<-position_dodge(width=.87)
g0 <- ggplot(newdat2, aes(x=Group, y=exp(chat12)
                      )) + geom_bar(stat="identity",
position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,32)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo, ymax = phi),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Ch1 & Ch2")+
  ylab("Number of ChAT Neurons")



### ch3 and ch4

PoissMod <- glmer(chat34~Group*ch34+(ch34|rat),
                  family = poisson, data = myd,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)
```

```
emmeans(PoissMod, pairwise~Group, type = "response")
emmeans(PoissMod, pairwise~Group*ch34, type = "response",
adjust="none")


PoissMod2 <- glmer(chat34~Group+(1|rat),
                   family = poisson, data = myd,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod2)


### ggplot
newdat <- expand.grid(
  Group=c("Alcohol", "Water"),
  ch34=c("ch3","ch4"),
  chat34 = 0
)

contrasts(newdat$Group)=contr.sum(2)
contrasts(newdat$ch34)=contr.sum(2)

res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$chat34 <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chat34 - sqrt(pvar1)),
  phi1 = exp(newdat$chat34 + sqrt(pvar1))
```

```
)

newdat



library(ggplot2)

## interaction
pd<-position_dodge(width=.87)
g0 <- ggplot(newdat, aes(x=ch34, y=exp(chat34),
                         fill=Group)) +
geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,45)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Cholinergic Nuclei")+
  ylab("Number of ChAT Neurons")


## main effect of group
newdat2 <- expand.grid(
  Group=c("Alcohol", "Water"),
```

```
  chat34 = 0
)

contrasts(newdat2$Group)=contr.sum(2)

res2 <- PoissMod2

mm2 <- model.matrix(terms(res2), newdat2)
newdat2$chat34 <- mm2 %*% fixef(res2)
pvar1 <- diag(mm2 %*% tcrossprod(vcov(res2),mm2))

newdat2 <- data.frame(
  newdat2,
  plo = exp(newdat2$chat34 - sqrt(pvar1)),
  phi = exp(newdat2$chat34 + sqrt(pvar1))
)

newdat2



pd<-position_dodge(width=.87)
g0 <- ggplot(newdat2, aes(x=Group, y=exp(chat34)
)) + geom_bar(stat="identity", position="dodge") +
  scale_fill_manual(values = c("purple", "black")) +
  ylim(0,32)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.position = "top", legend.title = element_blank())
```

```
g0
g0 + geom_errorbar(aes(ymin = plo, ymax = phi),position=pd,
size=1,
                   linetype=1, width=.04,
color="darkgray")+xlab("Ch3 & Ch4")+
  ylab("Number of ChAT Neurons")




### etoh correlations
etoh = subset(myd, Group == "Alcohol", select = c(c.ETOH,
chat12, ch12, rat, chat34, ch34))



## ch12
PoissMod <- glmer(chat12~c.ETOH+(1|rat),
                  family = poisson, data = etoh,
                  control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod)

anova(PoissMod)


### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.6125,
  chat12 = 0
)
```

```
contrasts(newdat$ch12)=contr.sum(2)


res <- PoissMod

mm <- model.matrix(terms(res), newdat)
newdat$chat12 <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chat12 - sqrt(pvar1)),
  phi1 = exp(newdat$chat12 + sqrt(pvar1))
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## main
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(chat12)
                         ))+ geom_line(position=pd, size=1) +
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=2) +
  ylim(0,35)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
```

```
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
        legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr)")+
  ylab("Number of ChAT+ Neurons")




PoissMod2 <- glmer(chat34~c.ETOH+(1|rat),
                   family = poisson, data = etoh,
                   control=glmerControl(optimizer = "bobyqa",
optCtrl=list(maxfun=2e5)))
summary(PoissMod2)

anova(PoissMod2)

### ggplot
newdat <- expand.grid(
  c.ETOH=c(0:8.5064)-1.6125,
  chat34 = 0
)

contrasts(newdat$ch34)=contr.sum(2)
```

```
res <- PoissMod2

mm <- model.matrix(terms(res), newdat)
newdat$chat34 <- mm %*% fixef(res)
pvar1 <- diag(mm %*% tcrossprod(vcov(res),mm))

newdat <- data.frame(
  newdat,
  plo1 = exp(newdat$chat34 - sqrt(pvar1)),
  phi1 = exp(newdat$chat34 + sqrt(pvar1))
)

newdat

## uncenter day
newdat$c.ETOH <- newdat$c.ETOH+1.6125

library(ggplot2)

## main
pd<-position_dodge(width=.2)
g0 <- ggplot(newdat, aes(x=c.ETOH, y=exp(chat34)
                         ))+ geom_line(position=pd, size=1) +
  scale_color_manual(values = c("purple", "black")) +
  geom_point(position=pd, size=2) +
  ylim(0,50)+
  theme(panel.background = element_rect(fill = "white", colour =
"black", size = .5, linetype = "solid"),
        panel.grid.major = element_blank(), panel.grid.minor =
element_blank(), text = element_text(size=18, family="Times New
Roman"),
```

```
        legend.title = element_blank())
g0
g0 + geom_errorbar(aes(ymin = plo1, ymax = phi1),position=pd,
size=1,
                   linetype=1, width=.04)+xlab("Alcohol
Consumption (g/kg/24 hr)")+
  ylab("Number of ChAT+ Neurons")
```

Appendix B - Representative Images

Figure B. 1

Representative images for the DMS with parvalbumin staining. (A) 0.8 DMS at 2x magnification. (B) 0.8 DMS at 10x magnification. (C) 0.4 DMS at 2x magnification. (D) 0.4 DMS at 10x magnification.

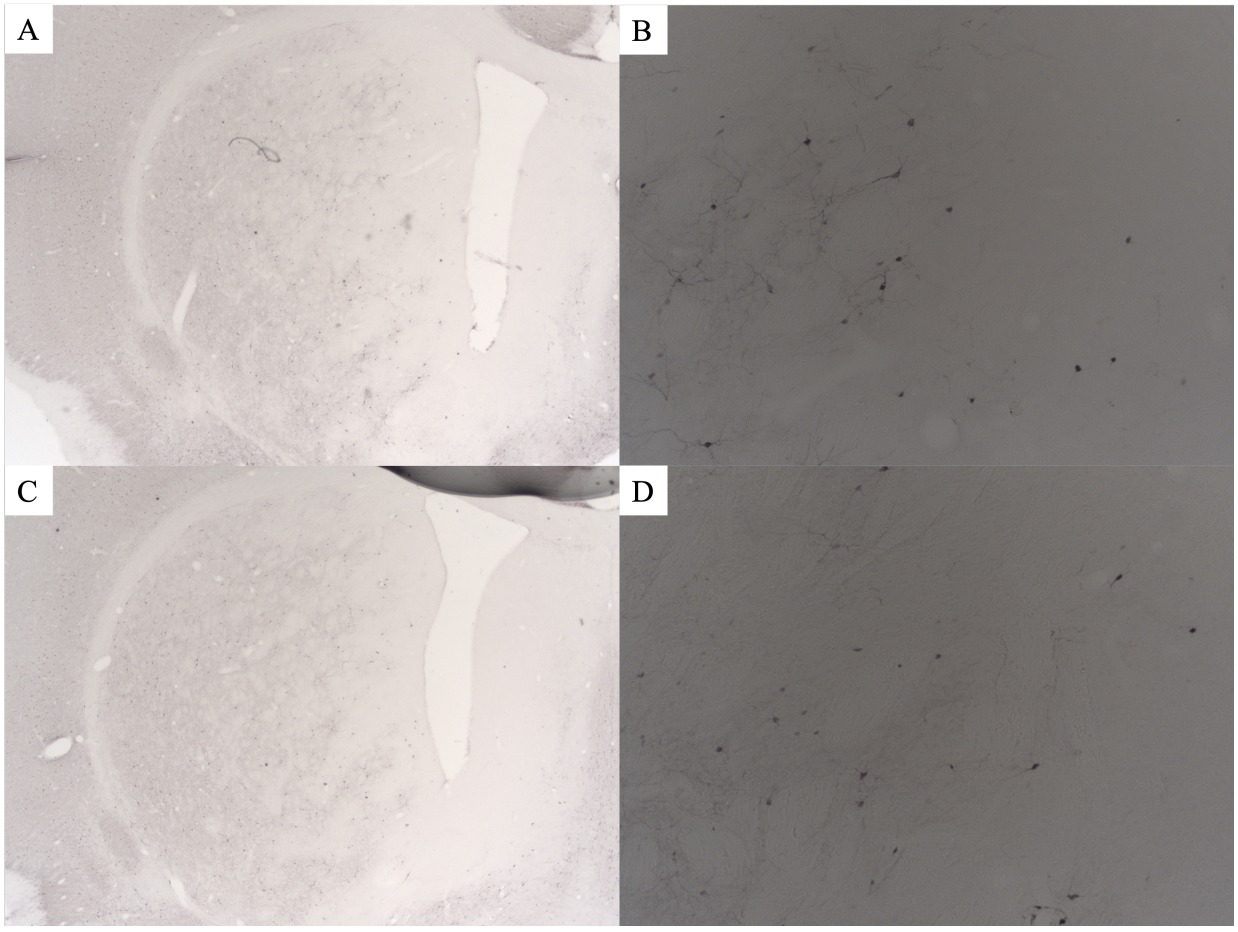


Figure B. 2

Representative images for the DLS with parvalbumin staining. (A) 0.8 DLS at 2x magnification. (B) 0.8 DLS at 10x magnification. (C) 0.4 DLS at 2x magnification. (D) 0.4 DLS at 10x magnification.

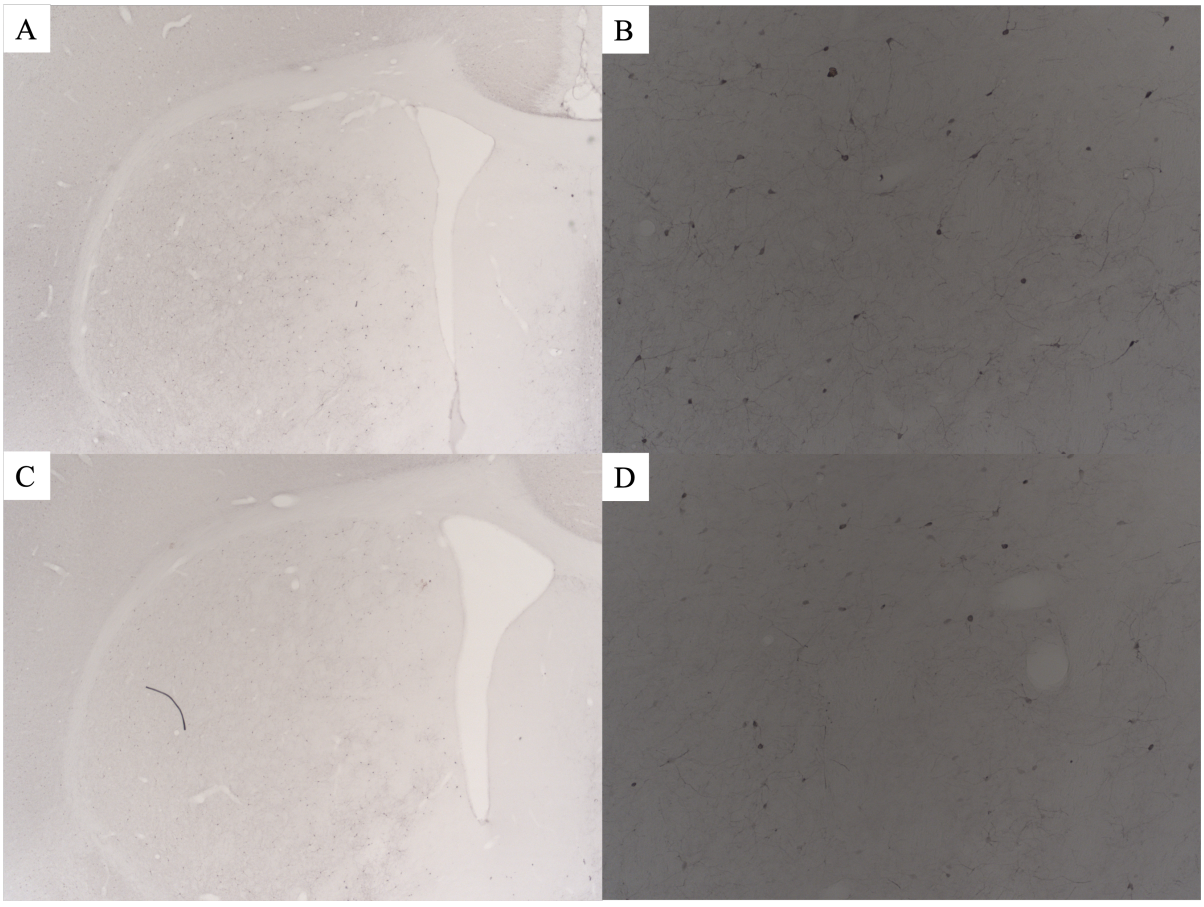


Figure B. 3

Representative images for the ACC with parvalbumin staining. (A) 3.2 ACC at 2x magnification. (B) 3.2 ACC at 10x magnification. (C) 2.7 ACC at 2x magnification. (D) 2.7 ACC at 10x magnification.

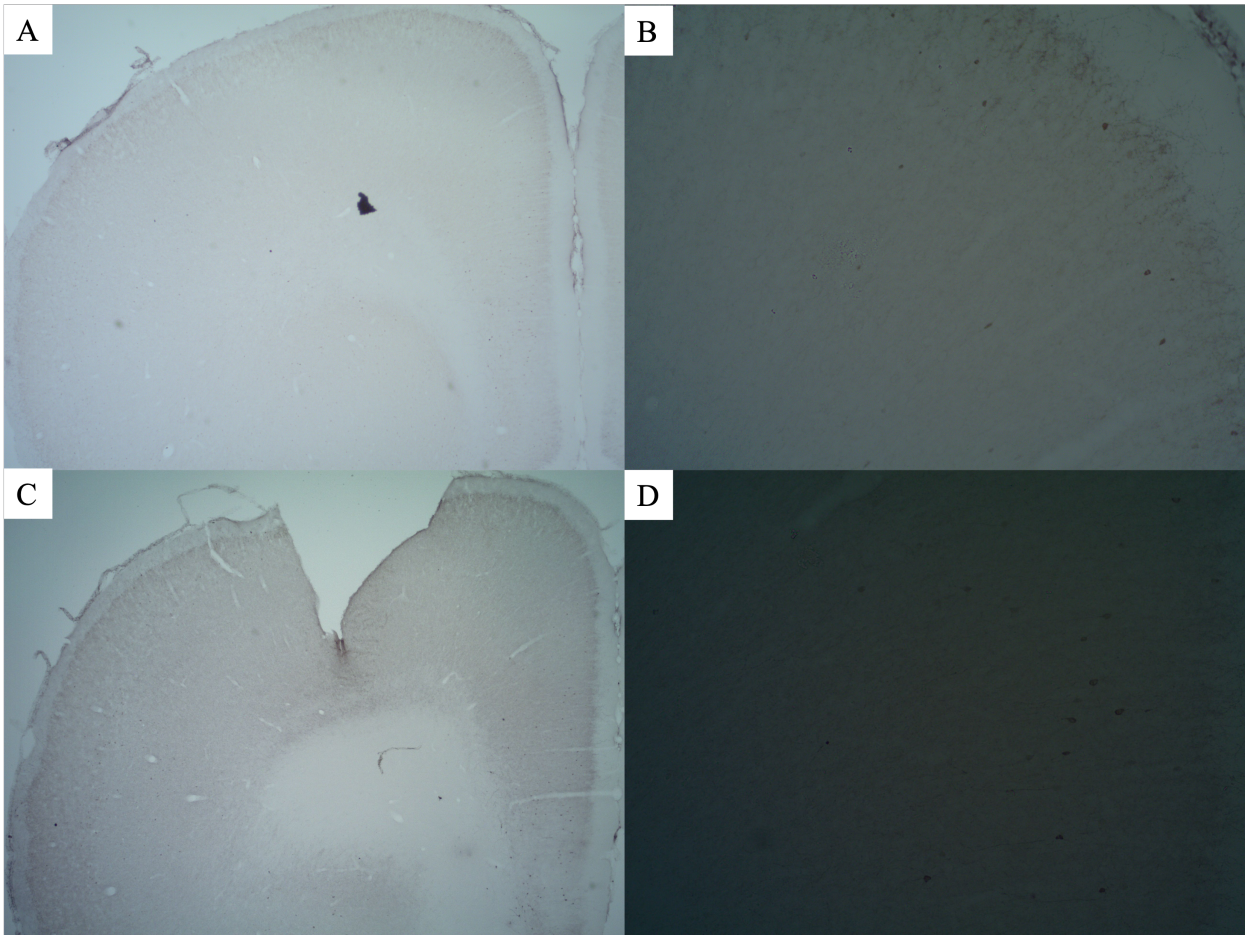


Figure B. 4

Representative images for the NAc Core and Shell with parvalbumin staining. (A) 1.6 NAc at 2x magnification. (B) 1.6 NAc Core at 20x magnification. (C) 1.6 NAc Shell at 20x magnification.

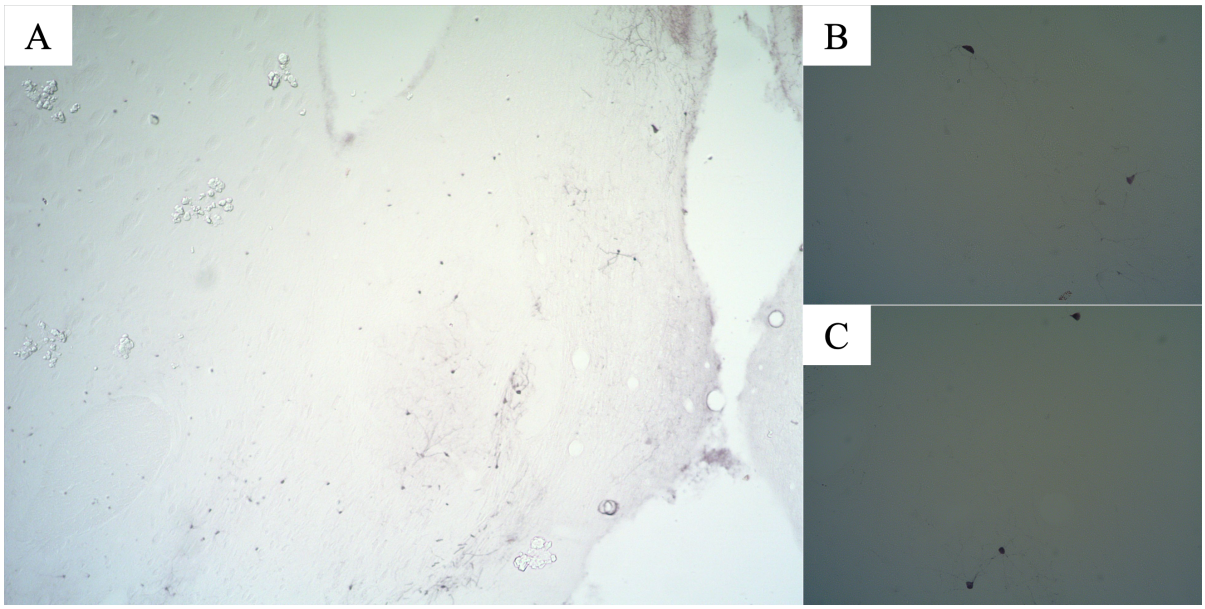


Figure B. 5

Representative images for the DMS with ChAT staining. (A) 0.8 DMS at 2x magnification. (B) 0.8 DMS at 10x magnification. (C) 0.4 DMS at 2x magnification. (D) 0.4 DMS at 10x magnification.

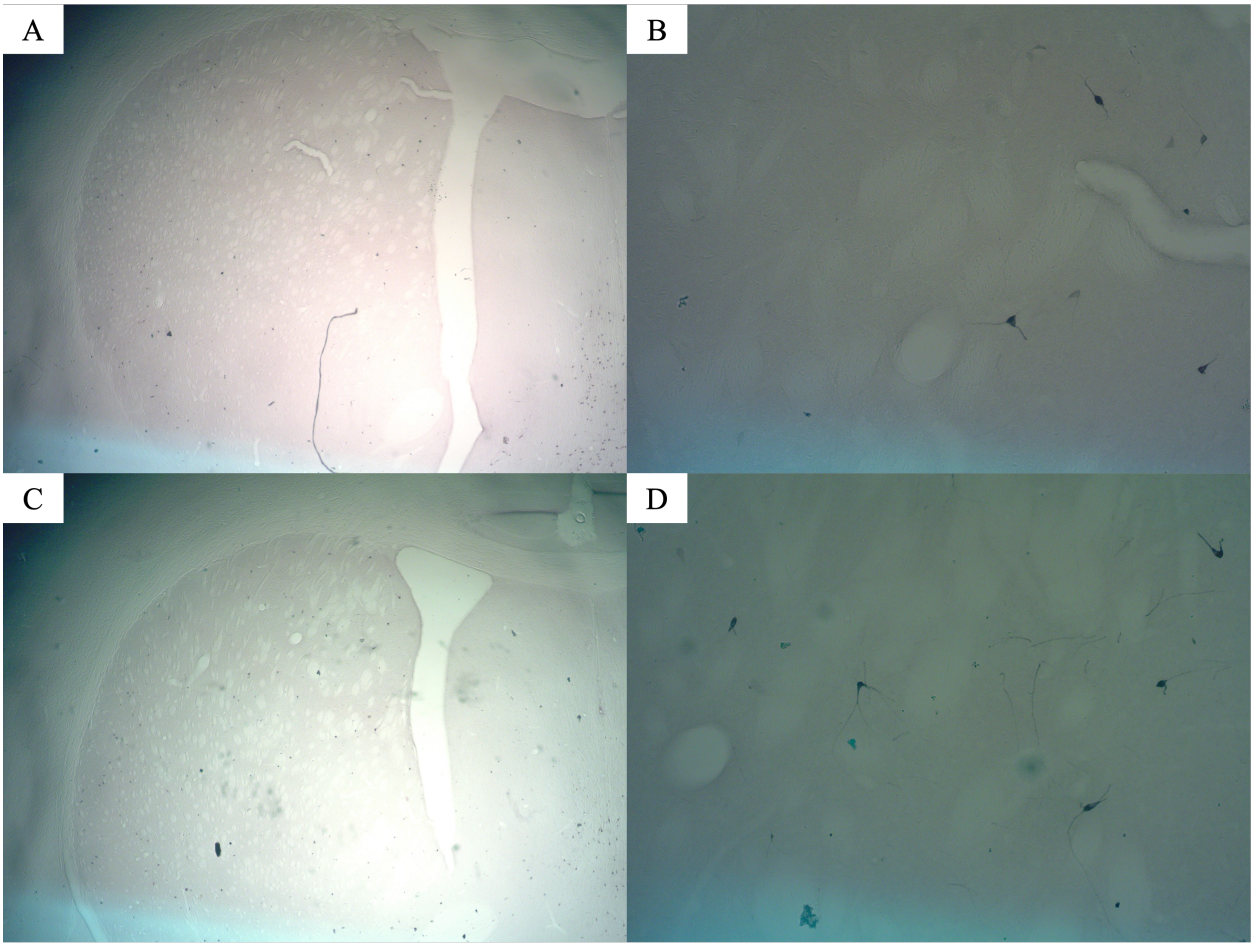


Figure B. 6

Representative images for the DLS with ChAT staining. (A) 0.8 DLS at 2x magnification. (B) 0.8 DLS at 10x magnification. (C) 0.4 DLS at 2x magnification. (D) 0.4 DLS at 10x magnification.

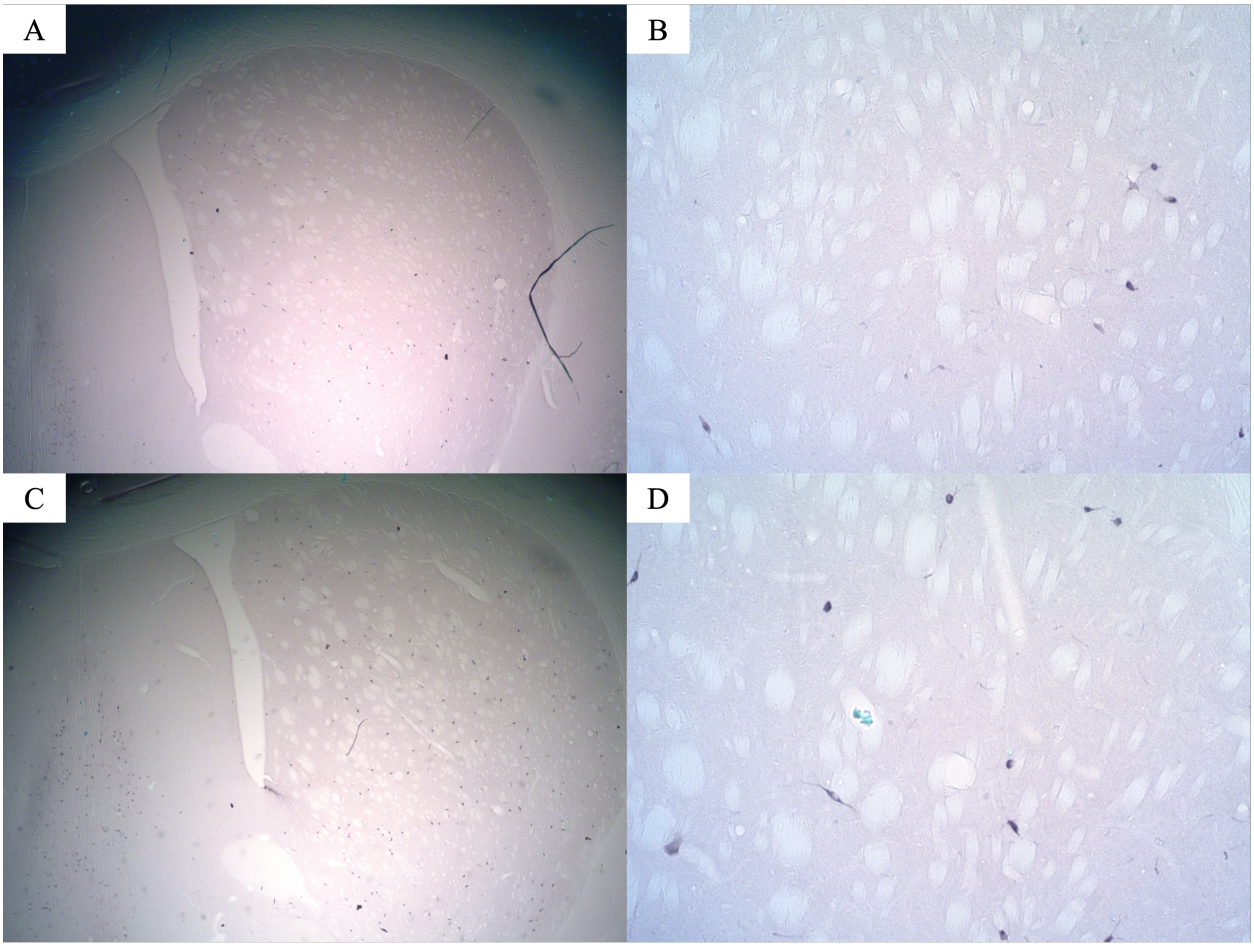


Figure B. 7

Representative images for the NAc Core and Shell with ChAT staining. (A) 1.6 NAc at 2x magnification. (B) 1.6 NAc Core at 20x magnification. (C) 1.6 NAc Shell at 20x magnification.

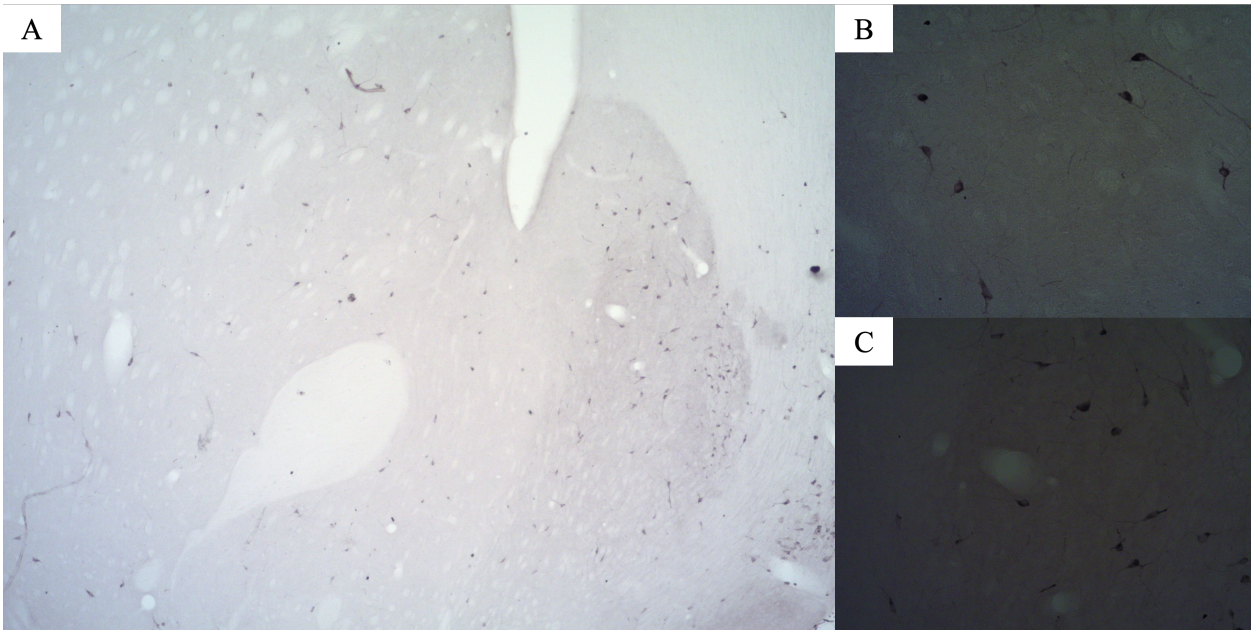


Figure B. 8

Representative images for the ACC with ChAT staining. (A) 2.7 ACC at 2x magnification. (B) 2.7 ACC at 10x magnification. (C) 3.2 ACC at 2x magnification. (D) 3.2 ACC at 10x magnification.

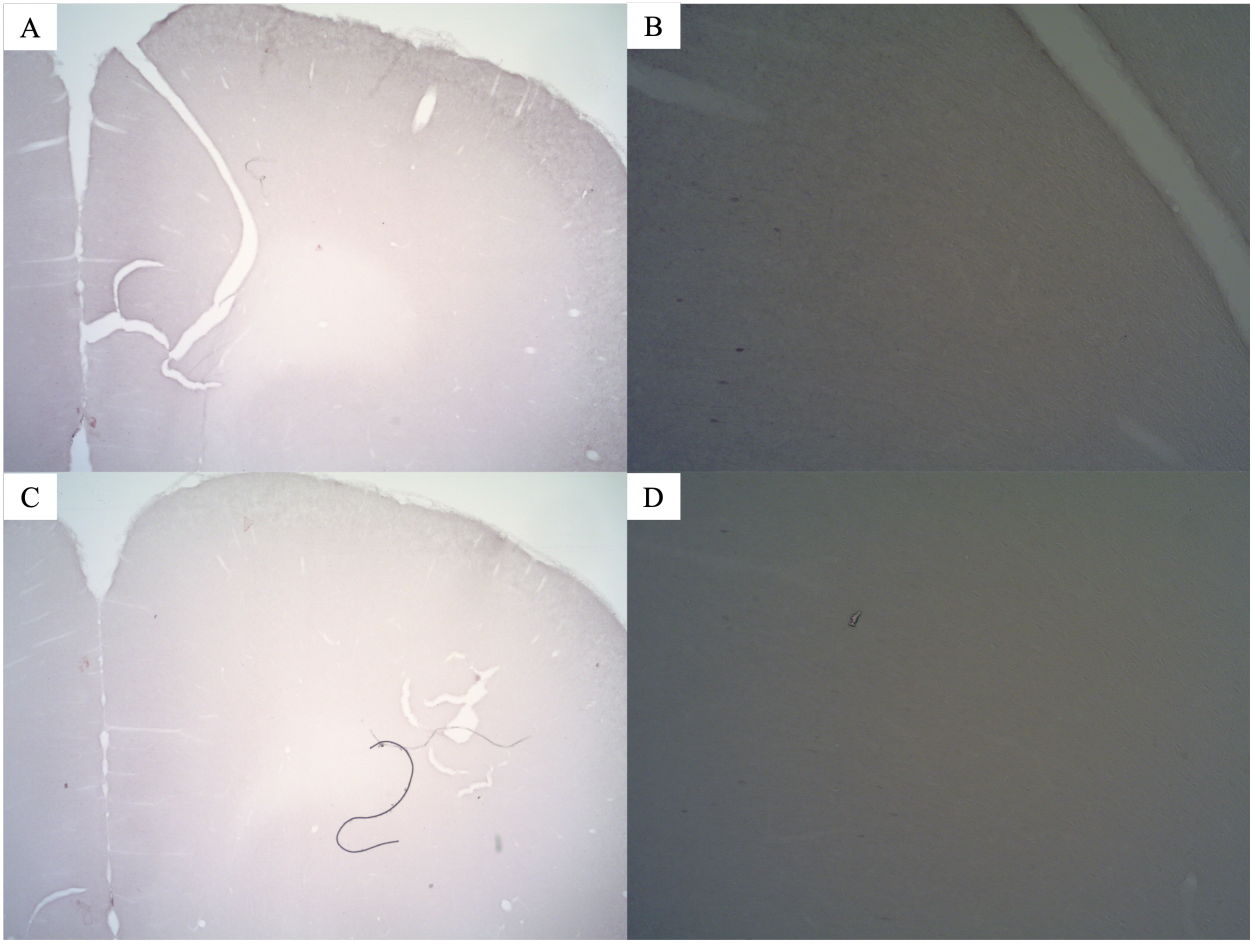


Figure B. 9

Representative images for the Ch1 and Ch2 cholinergic nuclei with ChAT staining. (A) 0.6 cholinergic nuclei at 2x magnification. (B) 0.6 Ch1 at 20x magnification. (C) 0.6 Ch2 at 20x magnification.

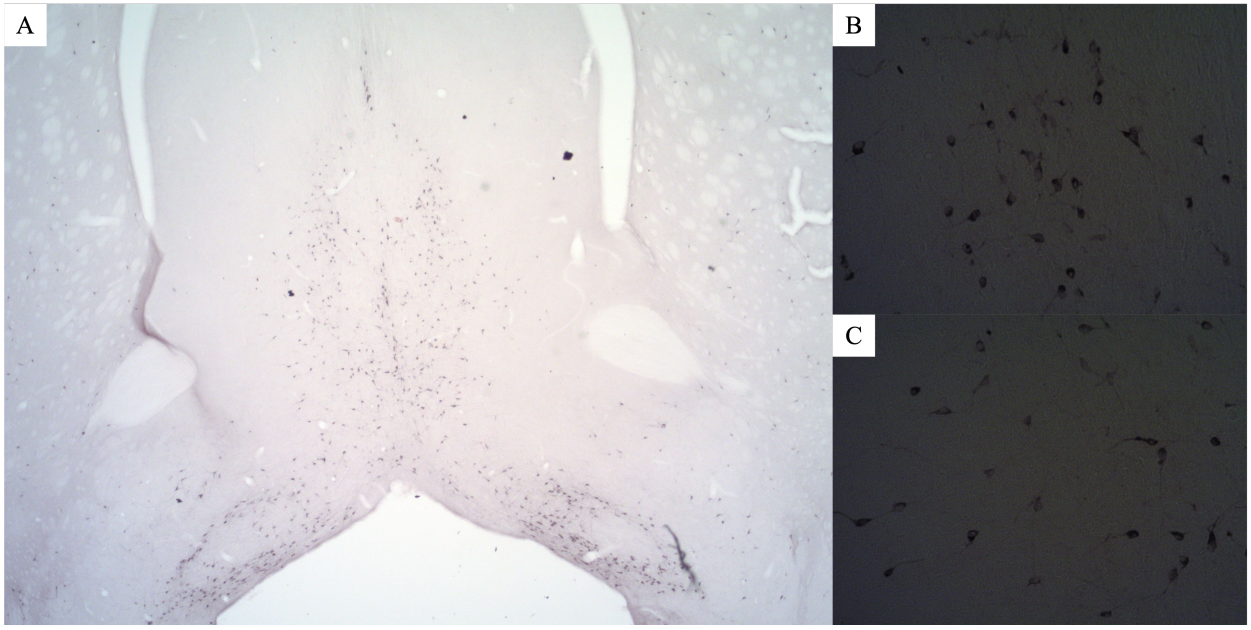
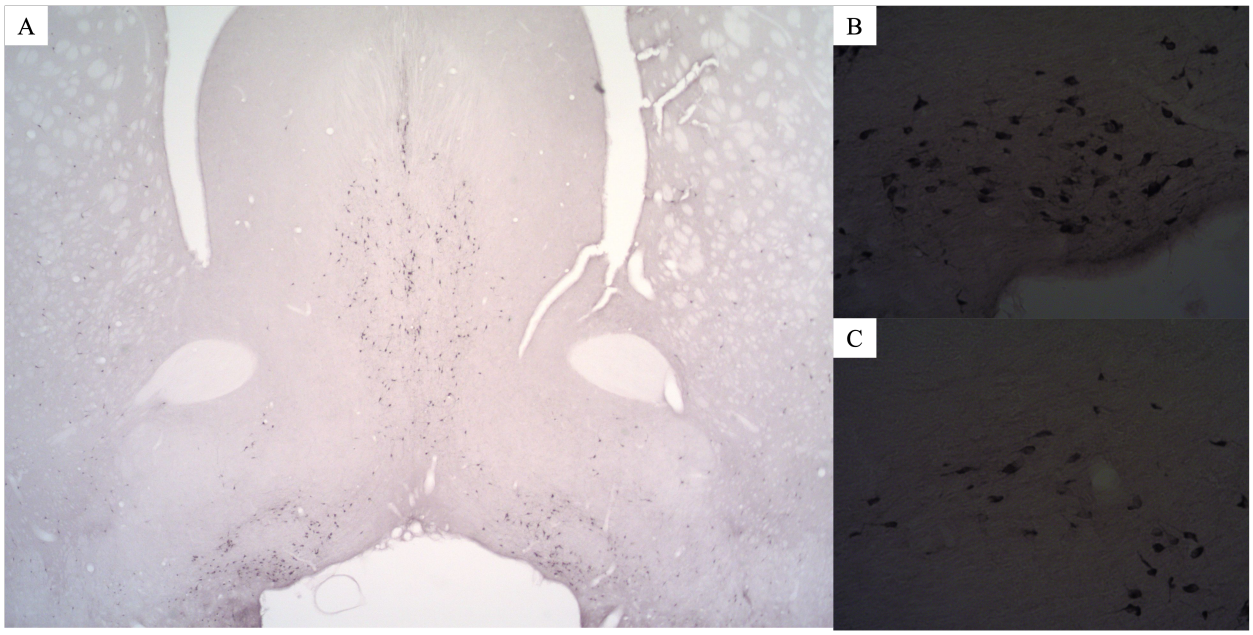


Figure B. 10

Representative images for the Ch3 and Ch4 cholinergic nuclei with ChAT staining. (A) 0.6 cholinergic nuclei at 2x magnification. (B) 0.6 Ch3 at 20x magnification. (C) 0.6 Ch4 at 20x magnification.



Appendix C - Additional Figures

Figure C. 1

Bar charts depicting the lack of group differences between PV+ neurons in the DMS (A) and DLS (B) in alcohol and water groups.

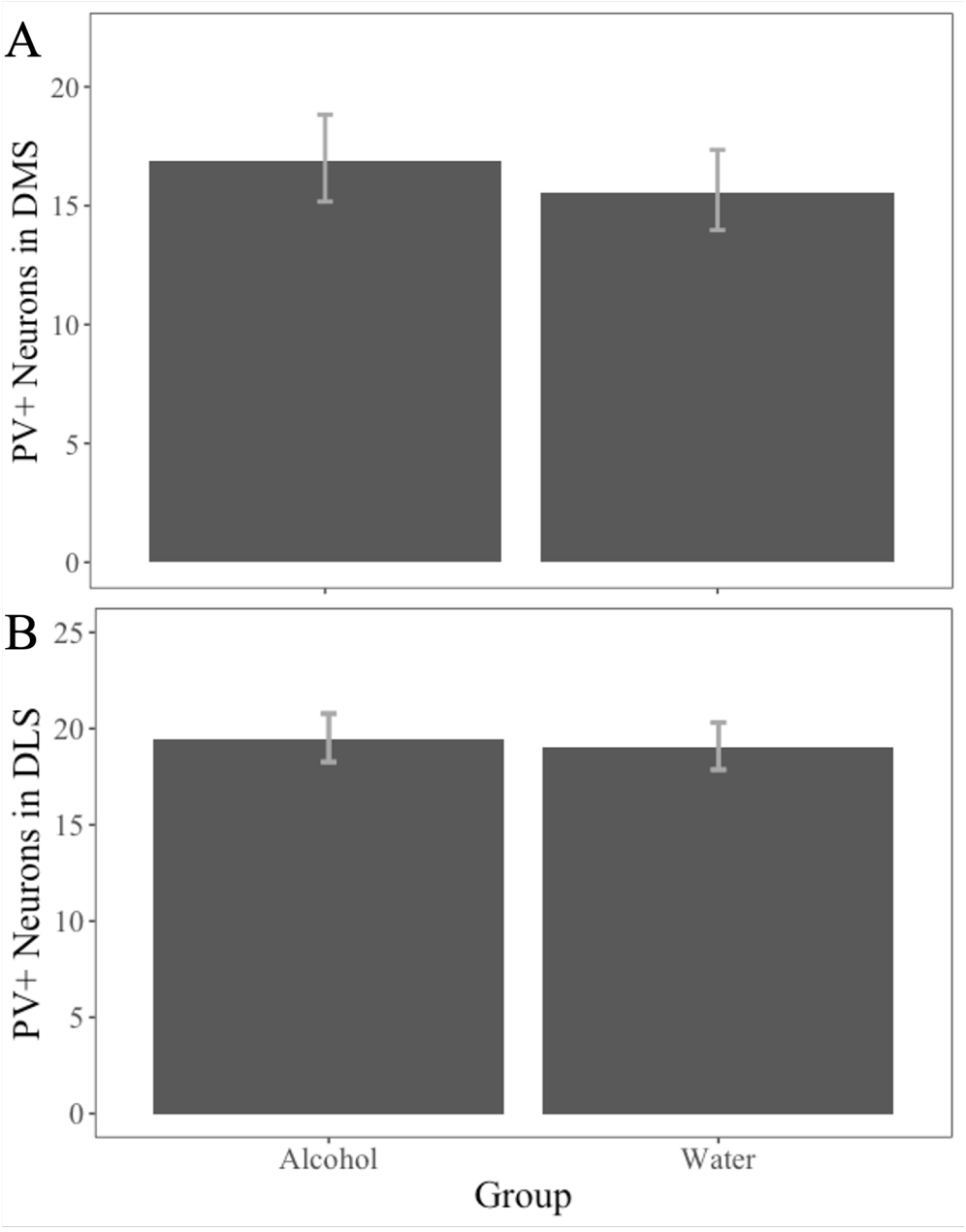


Figure C. 2

The correlation between alcohol consumption and the number of PV+ neurons in the DMS.

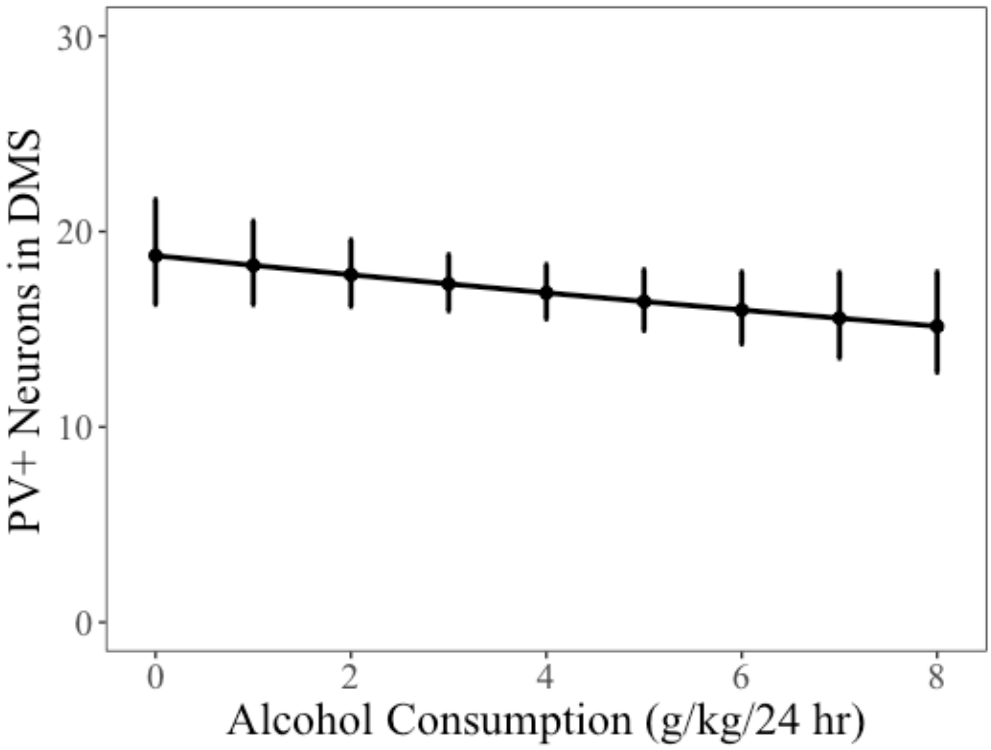


Figure C. 3

Bar charts depicting the lack of group differences between PV+ neurons in the ACC in alcohol and water groups.

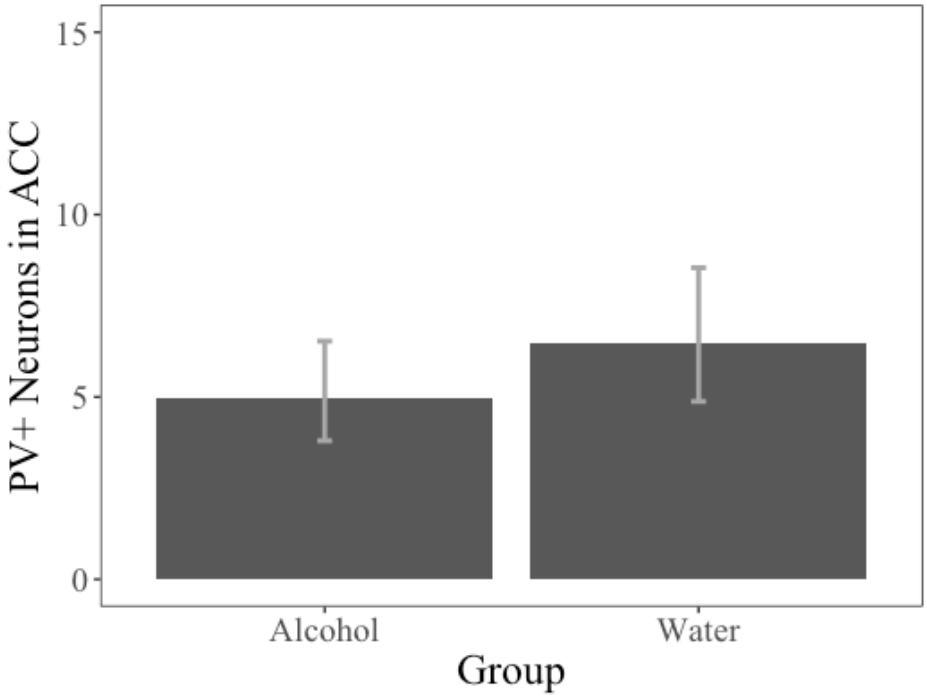


Figure C. 4

Bar charts depicting the lack of group differences between PV+ neurons in the NAc Core (A) and Shell (B) in alcohol and water groups.

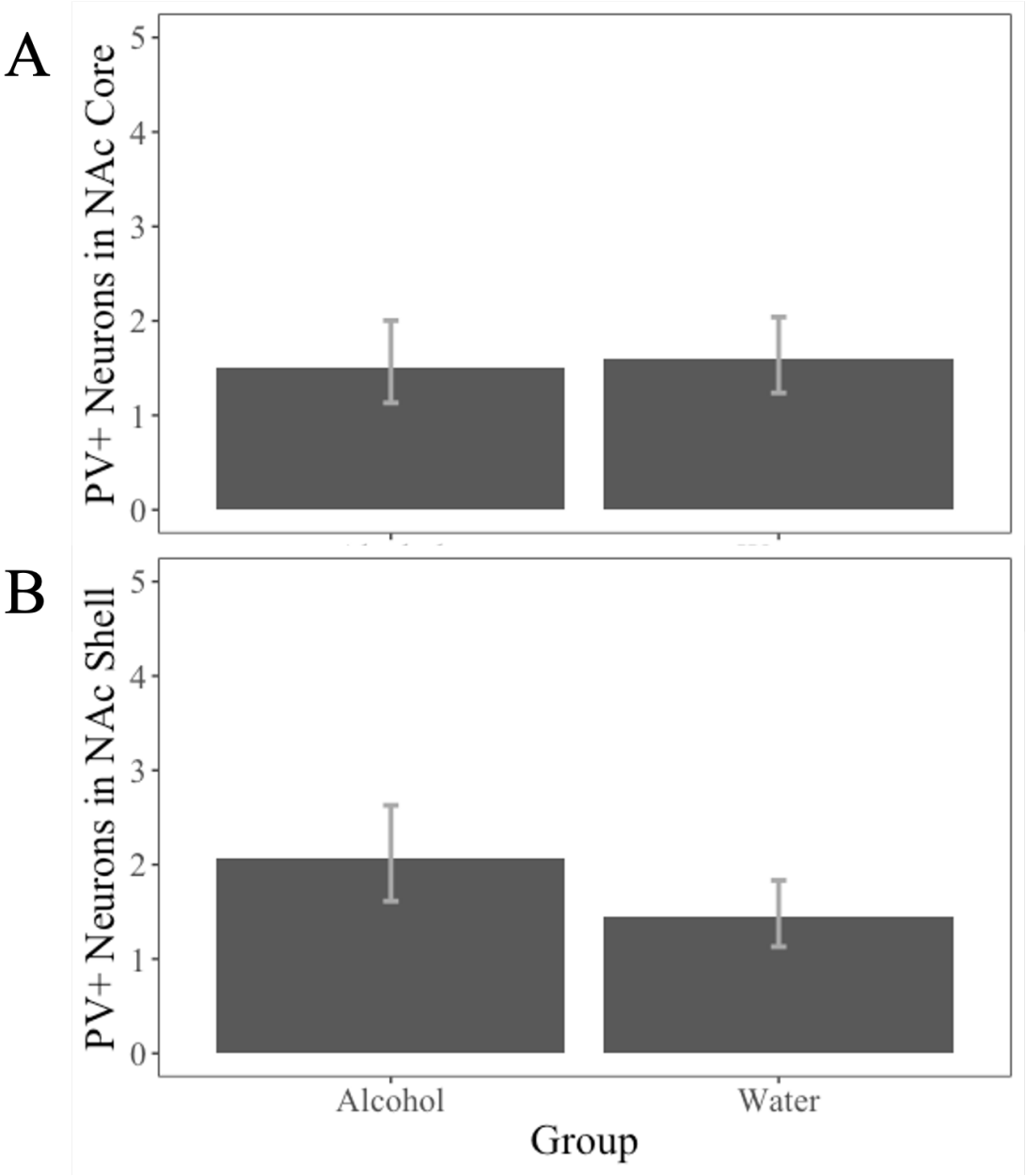


Figure C. 5

The correlation between alcohol consumption and the number of PV+ neurons in the NAc Core (A) and NAc Shell (B).

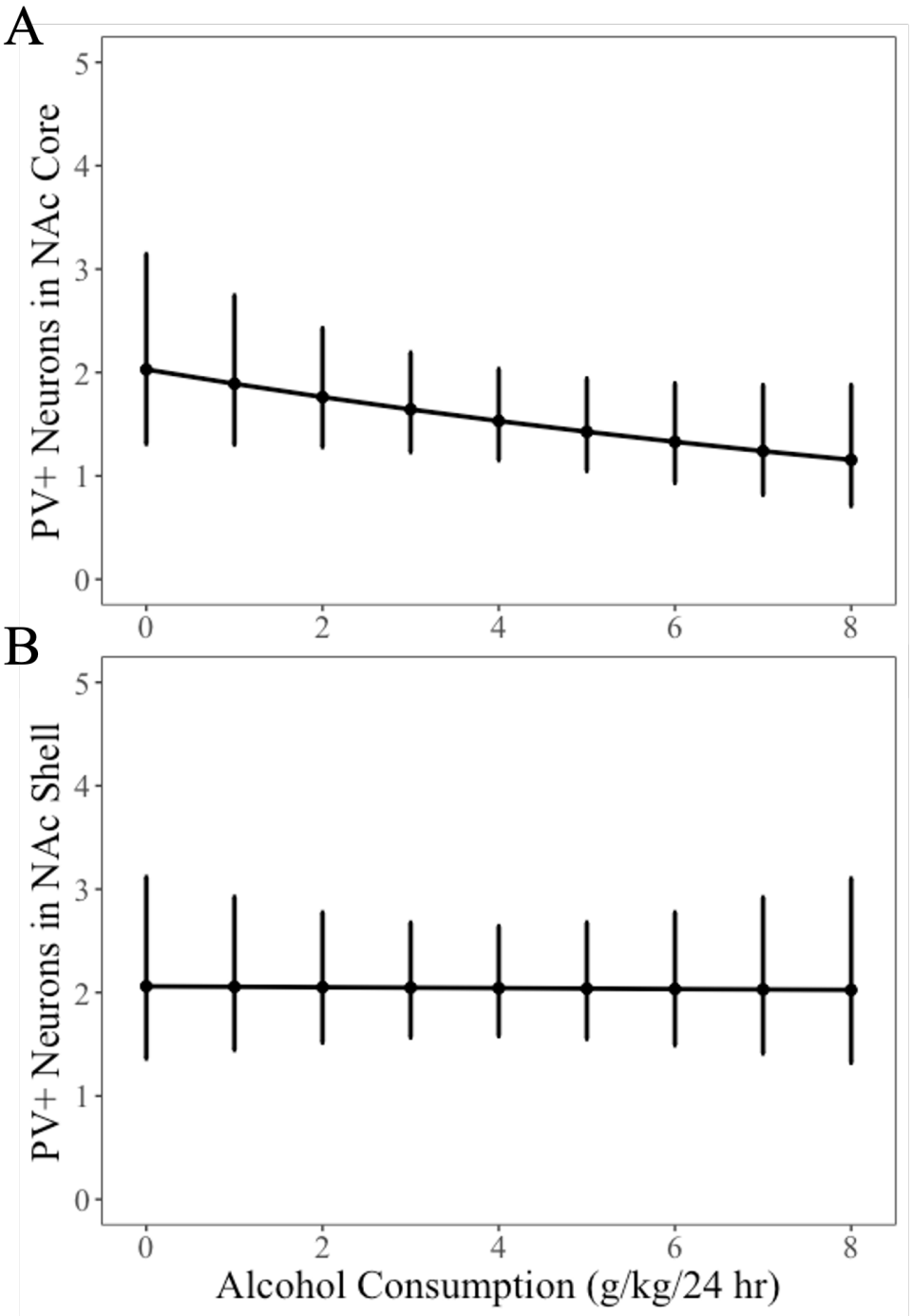


Figure C. 6

Bar chart depicting the lack of group difference between PV+ neurons in the Amygdala (A) and the correlation between alcohol consumption and the number of PV+ neurons in the Amygdala (B).

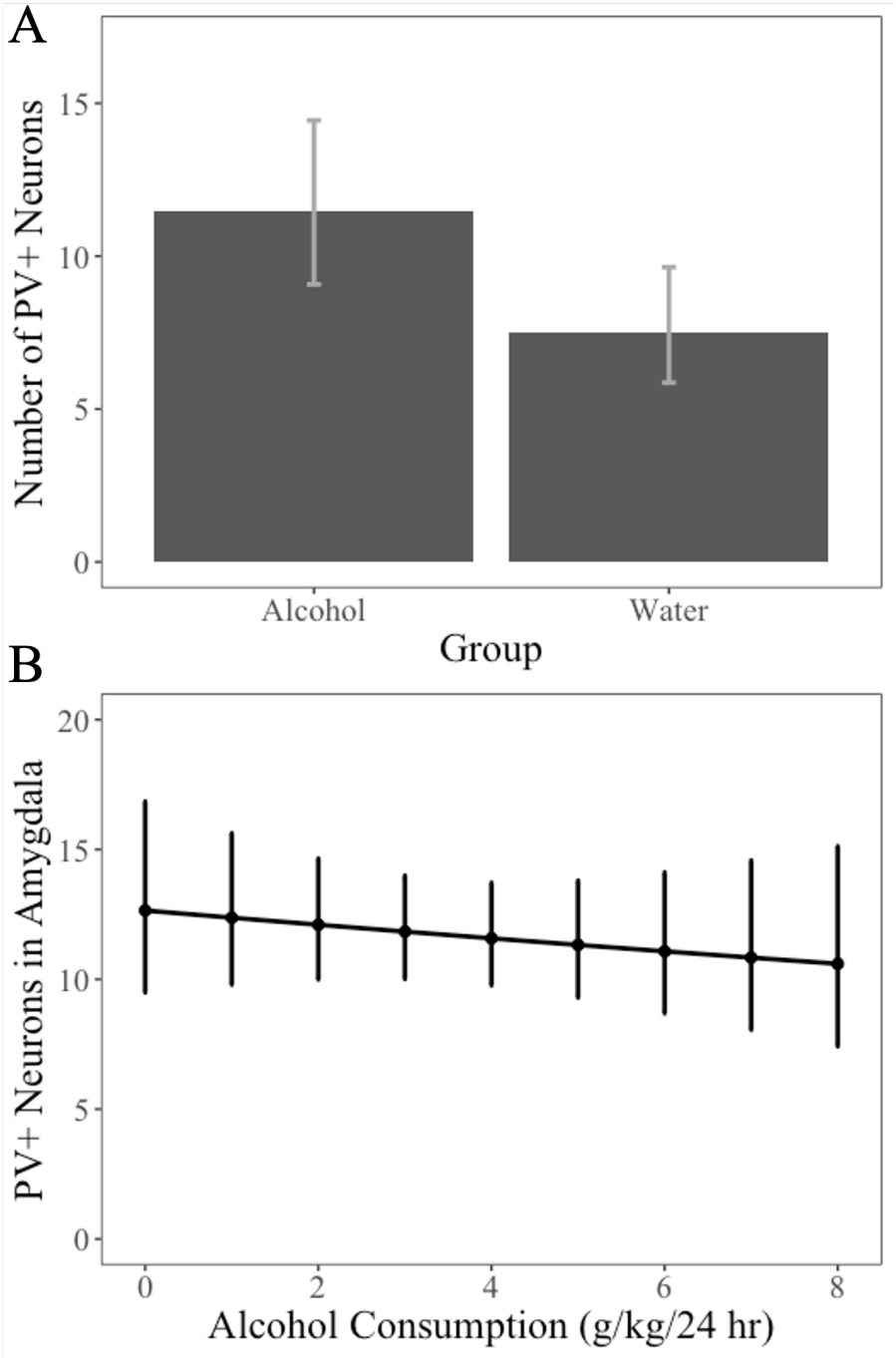


Figure C. 7

Bar charts depicting the lack of group differences between ChAT+ neurons in the DMS (A) and DLS (B) in alcohol and water groups.

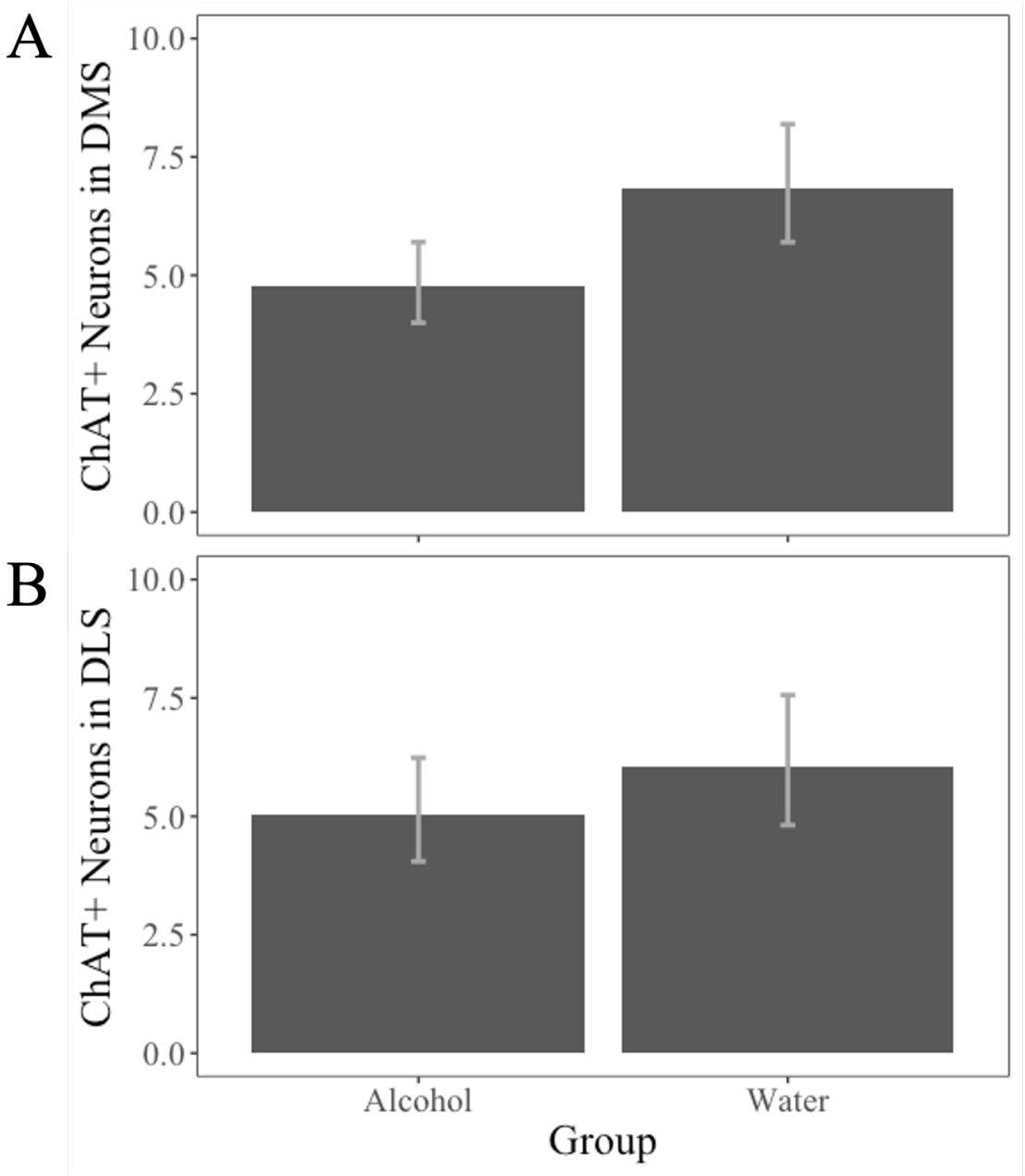


Figure C. 8

The correlation between alcohol consumption and the number of ChAT+ neurons in the DMS(A) and DLS (B).

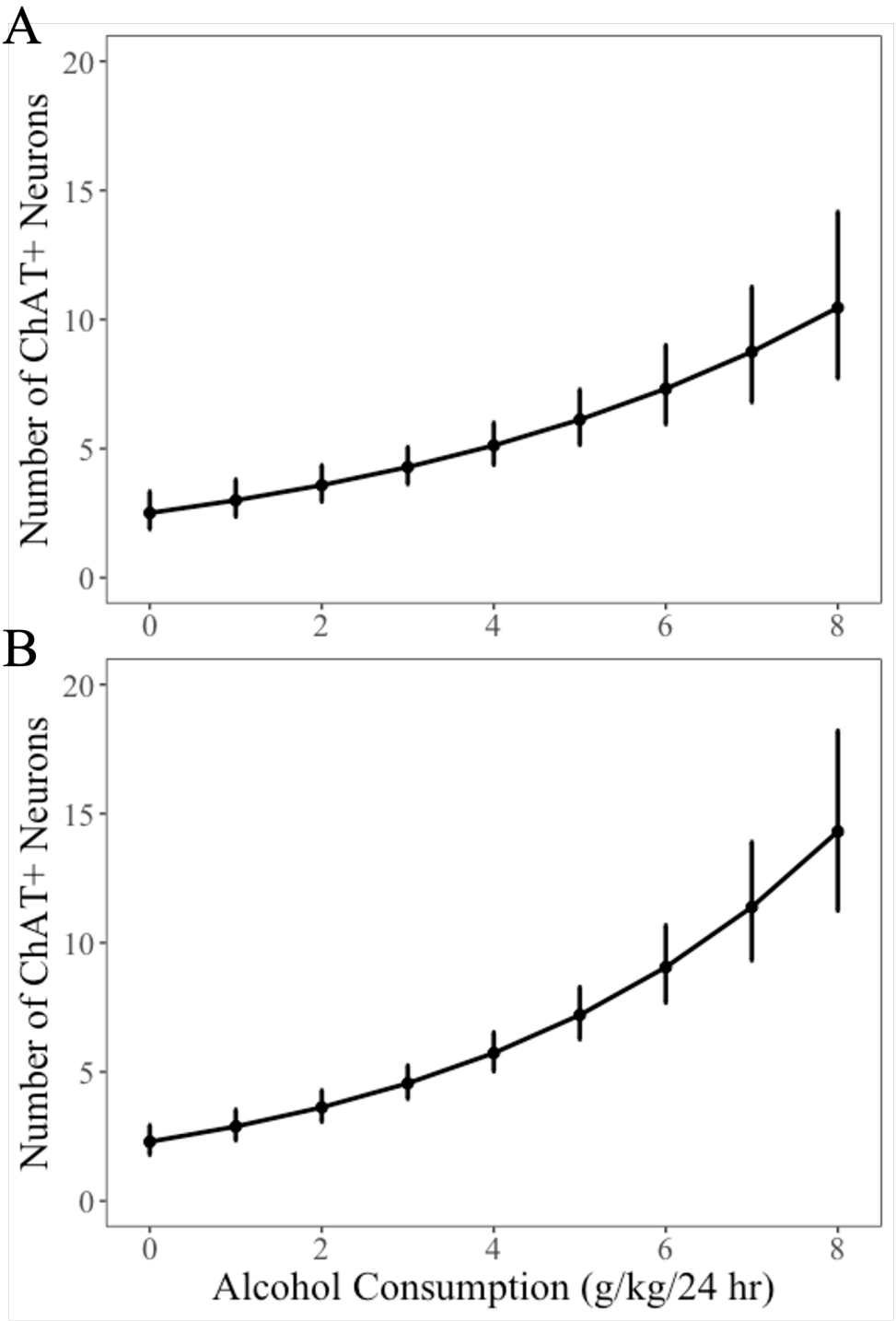


Figure C. 9

The correlation between alcohol consumption and the number of ChAT+ neurons in the NAc Shell.

Figure C. 10

Bar chart depicting the lack of group difference between ChAT+ neurons in the ACC (A) and the correlation between alcohol consumption and the number of ChAT+ neurons in the ACC (B).