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# Effects of Mild Traumatic Brain Injury on Ethanol Consumption and the Combined Effects on Neuroinflammation, Cognition, and Behavior in Mice

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# Effects of Mild Traumatic Brain Injury on Ethanol Consumption and the Combined Effects on Neuroinflammation, Cognition, and Behavior in Mice

by

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of the requirements for the degree of
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## ABSTRACT

The relationship between alcohol consumption and traumatic brain injury (TBI) often focuses on alcohol consumption increasing the likelihood of incurring a TBI, rather than alcohol use outcomes after TBI. This focus is in part due to the large numbers of TBI patients visiting emergency rooms notable levels of alcohol in their blood. Additionally, increases in alcohol use disorders following TBI can be predicted by previous history of alcohol use. However, studies have also shown patients without a history of an alcohol use disorder can experience increases in problem drinking after single or multiple TBIs. Due to the diffuse impact of alcohol consumption and mild TBI on the brain, it is likely that an interaction exists between TBI outcomes and problematic alcohol use after TBI. To examine the impact of mild repetitive TBI (rmTBI) on voluntary alcohol consumption, male mice were subjected to four mild TBI or sham procedures over a two week period, then offered ethanol (20% v/v) for 2, 4, 6 or 8 weeks using the two-bottle choice, drinking in the dark paradigm. Following the drinking period, mice were sacrificed and brains were extracted to examine expression of the pro-inflammatory cytokine TNF-α, a possible shared mechanism of neuronal damage. An additional cohort of mice was subjected to the same rmTBI and voluntary ethanol paradigm and tested for cognitive and behavioral deficits following the set drinking period. Results indicate there is a temporary decrease in ethanol consumption following rmTBIs compared to Sham mice in this model. Results also suggest an attenuated expression of TNF-α in rmTBI, ethanol drinking groups compared to ethanol exposed mice after the Sham procedure. The outcomes of the cognitive and behavioral tasks suggest that ethanol consumption after rmTBI can cause transient cognitive dysfunction and increased novelty preference.

# INTRODUCTION

## Traumatic Brain Injury

Each year, in the United States, approximately 1.5-2.5 million Americans are hospitalized or seek medical treatment for a traumatic brain injury (TBI) (Faul M, Xu L, Wald MM, 2010; Niska, Bhuiya, & Xu, 2010). Although this is already a substantial number of people, there are also individuals who may not seek treatment at all making the overall prevalence of TBI grossly underestimated. In addition to civilian injuries, nearly 20% of soldiers are estimated to have experienced a TBI during deployment accounting for a significant additional number of injuries and they are at a much higher risk than the general public (Tanielian, T., Jaycox, 2008).

Brain injuries vary in severity, and can have a significant impact on cognitive and motor function, sensation, and emotion (Ashman, Gordon, Cantor, & Hibbard, 2006; Thurman, Alverson, Dunn, Guerrero, & Sniezek, 1999). The majority of TBI cases are concussions, also called closed head injuries, which are classified as ‘mild’ (mTBI). This type of injury is identified by transient confusion, disorientation, or impaired consciousness; dysfunction of memory around the time of injury; and/or loss of consciousness lasting less than 30 minutes (National Center for Injury Prevention and Control, 2003). Despite the fact that up to 90% of TBIs are estimated to be mild (Holm, Cassidy, Carroll, & Borg, 2005; National Center for Injury Prevention and Control, 2003), currently, over 5 million Americans are living with a permanent TBI-related disability (Ashman et al., 2006; Thurman et al., 1999).

One of the challenges of mTBI is that it can be difficult or impossible to observe tissue damage using standard brain imagining techniques such as magnetic resonance imaging (MRI) or computerized tomography (CT) as the physical damage is often limited to microstructures like

cell membranes and ion pumps (Delouche et al., 2015; Honce, Nyberg, Jones, & Nagae, 2016). Although it is difficult to visualize physical brain damage, long-term negative consequences of repetitive mTBIs have recently become increasingly apparent as more is learned about long-term conditions that develop after TBI like post-concussion syndrome or chronic traumatic encephalopathy (CTE; Solomon & Zuckerman, 2015). In an extreme disorder like CTE, neurodegeneration, and development of tau protein tangles (Solomon & Zuckerman, 2015), are not the only possible long-term negative consequences. There is also an increased risk for excessive alcohol consumption following TBI which can lead to neurophysiological and behavioral consequences in civilian (J. D. Corrigan, 1995; Jorge et al., 2005) and military populations (Herrold A. et al., 2014; Miller & Baktash, 2013).

**Alcohol Use**

Alcohol use in America is widespread with more than half of people aged 12 and up (139 million) consuming alcohol at least once in the past month. Many of these people engage in problematic drinking behaviors such a binge drinking (60.9 million), defined as five or more drinks on one occasion, or heavy drinking (16.3 million), defined as five or more drinks on one occasion for five days in the past month (Center for Behavioral Health Statistics and Quality, 2015). In a subset of these populations, problem drinking can develop into an Alcohol Use Disorder (AUD) which is a mental health disorder characterized by the inability to stop drinking ("Substance-Related and Addictive Disorders," 2013). This disorder can be devastating to a person's health, finances, and interpersonal relationships and can co-occur with other mental health disorders (Center for Behavioral Health Statistics and Quality, 2015). In addition, AUD is associated with long-term cognitive deficits including reductions in executive functions like decision making (Brevers et al., 2014; Le Berre et al., 2014), response inhibition (Naim-Feil, Fitzgerald, Bradshaw, Lubman, & Sheppard, 2014), attentional shifting (Goldman, Klisz, &

Williams, 1985; Rourke & Grant, 1999), reversal learning (Gladwin & Wiers, 2012; Trick, Kempton, Williams, & Duka, 2014), as well as memory impairment (Loeber et al., 2009) with some of these deficits extending into protracted abstinence.

A number of animal models of voluntary ethanol consumption have been developed to study these and other alcohol related effects each with their own merits; for a review see Griffin, 2014. The Drinking in the Dark (DID) paradigm is especially practical when modeling drinking behavior without the use of sucrose fading or ethanol vapor inhalation chambers. In this paradigm, mice are allowed to voluntarily consume ethanol and do so at levels considered to be mild intoxication or higher (80 mg/dl; Rhodes et al., 2007; Thiele, Crabbe, & Boehm, 2014; Thiele & Navarro, 2014) making it a highly translatable model for human drinking.

**TBI and Alcohol**

There has long been a relationship between alcohol consumption and TBI, but the causal relationship between TBIs and AUDs is difficult to discern. A large percentage of patients visiting emergency rooms with TBIs have notable levels of alcohol in their blood (Dikmen, Machamer, Donovan, Winn, & Temkin, 1995; Kraus, Morgenstern, Fife, Conroy, & Nourjah, 1989; Weil, Corrigan, & Karelina, 2016). Likewise, increases in AUD following TBI can be predicted by previous history of alcohol use (Bombardier, Temkin, Machamer, & Dikmen, 2003; Dikmen et al., 1995; Horner et al., 2005; Rogers & Read, 2007). However, numerous studies have also shown patients without a history of AUD can experience increases in problem drinking after TBI (J. D. Corrigan, 1995; Hibbard, Uysal, Kepler, Bogdany, & Silver, 1998; Massagli et al., 2004; Silver, Kramer, Greenwald, & Weissman, 2001). Due to the global impacts of both alcohol consumption and TBI on the brain, it is likely that an interaction exists between TBI outcomes and problematic alcohol use rather than a simple, unidirectional causal association.

This series of three experiments further the existing literature by examining the effects of repetitive mTBI on voluntary ethanol consumption using mouse models. Then investigate the combined effects of rmTBI and voluntary ethanol consumption on neuroinflammation, cognition, and behavior. The goal of the first experiment was to investigate the capacity of a repetitive mTBI model to induce excessive ethanol consumption post-injury as observed in portions of clinical populations. The model was also assessed for reliability by comparing ethanol consumption of multiple cohorts over the course of several weeks. The second experiment investigated the long-term consequences of ethanol consumption following repetitive mTBI on neuroinflammation in effort to understand possible shared mechanisms leading to neuronal damage. The final experiment explored the protracted effects of repetitive mTBI and ethanol consumption on neuronal recovery with a series of cognitive and behavioral performance measures.

## EXPERIMENT 1: EFFECTS OF REPETITIVE TRAUMATIC BRAIN INJURY ON VOLUNTARY ETHANOL CONSUMPTION

Alcohol use disorders and TBI independently represent major health problems with 17 million people in the United States diagnosed with AUDs (Center for Behavioral Health Statistics and Quality, 2015) and over 1.5 million patients suffering a TBI annually (Faul M, Xu L, Wald MM, 2010; Niska et al., 2010). Research demonstrates that these mental health concerns are often comorbid. For example, incurring a TBI increases the likelihood of developing an AUD (J. D. Corrigan, 1995; Weil et al., 2016). On the other hand, the consumption of alcohol greatly increases the chances of sustaining a TBI and as many as 50% of TBI emergency room patients have levels of alcohol that surpass the legal limit for most states (Dikmen et al., 1995; Kraus et al., 1989).

In clinical populations, mild TBI (mTBI) is more difficult to diagnose compared to moderate and severe TBIs and can even go untreated altogether because of the lack of visible symptoms. Nevertheless, mTBI can result in significant neurological symptoms such as headache, confusion, loss of consciousness, amnesia, chronic pain, and impaired cognitive function (Ashman et al., 2006; Gessel, Fields, Collins, Dick, & Comstock, 2007; Harmon et al., 2013; National Center for Injury Prevention and Control, 2003). For most patients, symptoms appear to resolve spontaneously within 1-2 weeks, but data suggest there could be long-term consequences especially when the mTBIs are incurred repeatedly (Ashman et al., 2006; Harmon et al., 2013; Solomon & Zuckerman, 2015). Of these long-term consequences, post-concussive syndrome and chronic traumatic encephalopathy tend to garner more attention from media sources; however, some evidence suggests that there can be a significant increase in the

risk of excessive drinking following mTBI which can also lead to troublesome outcomes in terms of health, interpersonal relationships, and psychological well-being (J. D. Corrigan, 1995).

Increased alcohol use following TBI is a substantial concern as it can greatly impact an individual's recovery from TBI and lead to greater risk of incurring additional TBIs (J. D. Corrigan et al., 2013). Military personnel are at an increased risk for TBI compared to the general population with nearly 20% of soldiers experiencing at least one TBI during their deployment. Similar to civilian populations, military personnel with or without a prior history of alcohol misuse can experience a greater risk of developing an AUD after incurring a TBI (Adams, Larson, Corrigan, Ritter, & Williams, 2013; Herrold A. et al., 2014; Miller & Baktash, 2013; Tanielian, T., Jaycox, 2008). There is a paucity of research investigating the relationship between severity of injury or mechanism of injury (e.g. fall, motor vehicle accident) and increased drinking (Horner et al., 2005). Some patients with severe TBI decrease alcohol consumption post-injury possibly due to a reduction in ability or access and greater supervision (Bombardier et al., 2003; Dikmen et al., 1995). However, one interesting pattern in drinking behavior following TBI identifies a short period of reduced alcohol consumption immediately following the injury followed by escalations to problematic alcohol use (Ponsford, Whelan-Goodinson, & Bahar-Fuchs, 2007).

An inherent issue when examining TBI patients using simple observation is the variable nature of the injuries. Brain area(s) affected, the severity of the injury, and whether the injury is local or diffuse can all lead to variation in symptoms and outcomes. Fortunately, these and other factors including past experience with drugs or alcohol and previous or current stressors can be controlled in the laboratory. Animal models of TBI make use of several mechanisms to induce TBI; these include an impactor piston directly to the brain or through the skull, fluid percussion, indirect/blast, and gravity assisted weight drop. Many experiments are designed to model severe trauma but more recently there have been modifications to mimic milder injuries. It is

important that models of mTBI avoid causing skull fractures, hemorrhaging, and major disruption of the vasculature as these would be considered more severe injuries.

One such model utilizes the weight drop mechanism and has successfully generated mild to moderate TBIs modeled in mice. The variability in severity observed in this model is similar to the variability observed in medical settings and may be useful to test generalized treatment, but the variability can also limit the use of this model to understand mechanisms of mTBI specifically (Albert-Weißenberger, Várrallyay, Raslan, Kleinschnitz, & Sirén, 2012). A model of repetitive mTBI using the fluid percussion mechanism has also demonstrated exacerbated memory impairment, axonal damage, and astrocytic reactivity after a second mTBI was administered (Prins, Hales, Reger, Giza, & Hovda, 2011). In an attempt to study the causal relationship between repetitive mTBI and CTE, Petraglia and colleagues (2014) used an impactor model and induced 6 mTBI per day for 7 days. This model was successful in replicating a number of the behavioral and neurological symptoms characteristic of CTE, but with 42 cumulative mTBIs (Petraglia et al., 2014), it is more extreme than necessary to model repetitive mTBI for most populations. Another model of repetitive mTBI also uses the impactor piston mechanism to induce repetitive mild closed head injuries (rmCHI), but is more conservative with only 4 injuries over 2 weeks and has been able to replicate morphological and neurological outcomes of repetitive mTBI in humans (Yang et al., 2015). Similar to the observations of human mTBI, this model of rmCHI does not induce histological damage in mice that can be visualized using standard MRI, but instead some minor damage is observable when using diffusion imaging (Yang et al., 2015).

The literature using animal models to examine the relationship between mTBI and alcohol consumption is sparse and there are very few studies at this time investigating the effect of repetitive mTBI on alcohol drinking behaviors. One study using an impactor mechanism found increased drinking in mice after an acute TBI during adolescence (Weil, Karelina, Gaier,

Corrigan, & Corrigan, 2015). Another study used an acute blast model with rats, and found increases in drinking for injured animals compared to controls following initial access (Lim et al., 2015). In this study, the researchers purposefully chose Sprague Dawley rats due to their naturally low preference for alcohol and the rats never drank to intoxication; therefore, it may be difficult to discern differences in drinking patterns with such low overall consumption. Unlike rats, mice drink to pharmacologically relevant blood ethanol concentrations (80 ml/dl) readily in the DID paradigm (Thiele & Navarro, 2014). Consequently, this longitudinal experiment aims to investigate the effects of rmCHI on ethanol consumption over the course of several weeks using a well characterized model of repetitive mTBI (Yang et al., 2015) and a well-established mouse voluntary drinking model (Rhodes, Best, Belknap, Finn, & Crabbe, 2005; Thiele et al., 2014; Thiele & Navarro, 2014).

**Hypotheses**

Despite patients with TBI reporting an increased prevalence of alcohol consumption following their injury, there is sparse preclinical data investigating the impact of rmTBI on alcohol consumption patterns. Therefore the aim of Experiment 1 was to establish the rmCHI model as an acceptable model to investigate the excessive ethanol consumption post-injury observed in clinical populations. It was expected that all mice (sham and rmTBI) would consume enough ethanol during the drinking periods each day to induce pharmacologically relevant blood ethanol concentrations (BECs, Rhodes et al., 2007; Thiele & Navarro, 2014). Average drinking levels are expected to be greater than 6 g/kg after the 4hr drinking period at the end of each week with corresponding BECs of approximately 80 mg/dl (Rhodes et al., 2007). It was probable that an initial period of reduced drinking would be observed in rmTBI mice, but because the induced mTBIs did not physically debilitate the mice, this effect on ethanol consumption was expected to be minimal compared to observations of humans after more severe TBI (Ponsford et al., 2007).

It was also hypothesized that mTBI mice would exhibit a greater preference for ethanol compared to the sham mice which could be attributed to a number of mechanisms including reduced frontal lobe inhibition or damage to the dopaminergic reward pathways which will be considered for exploration in future experiments (Crews et al., 2006; Jorge et al., 2005; Weil et al., 2016).

## Methods

### Subjects

Ninety-six adult (8-12 weeks old) male C57BL/6J mice (Envigo Laboratories, Indianapolis, IN, USA) were used for a total of 12 mice per condition (Weil et al., 2015; Yang et al., 2015). Food and water were freely available throughout the duration of the experiment and all mice were maintained on a 12hr reverse light/dark cycle (6 pm/6 am) in a temperature- and humidity-controlled animal facility. Mice were transported to a separate room prior to rmTBI induction and all drinking measures occurred in the home cage. This experiment was conducted according to a protocol approved by the Institutional Animal Care and Use Committee of the University of South Florida, and was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Repetitive Mild Traumatic Brain Injury

To model the repetitive mTBI observed in humans, a conservative, but a validated model of rmCHI was used (Yang et al., 2015). Mice were anesthetized with 4% isoflurane and maintained at 2–3% isoflurane throughout the procedure. Using a PSI TBI-0310 Impactor (Precision systems and instrumentation, LLC, USA) fitted with a custom silicon rubber tip (1 cm in thickness, 9 mm in diameter), four brain traumas were induced on the center point of the

sagittal suture with 72hrs of recovery time between impacts. Each impact was made at 4.0 m/s, 3.8 mm compression depth and a 200ms dwell time (compression duration). Sham animals were anesthetized and placed in the impactor for a similar duration, but did not experience the impact. Mice were returned to home cages immediately after the injury procedure where they had access to ad libitum water and food and were monitored for health. After the four injuries or sham procedures, the animals would have an additional two days of recovery prior to ethanol exposure.

### Voluntary Ethanol Consumption

Voluntary ethanol consumption was determined using a well-established DID paradigm (Rhodes et al., 2005; Thiele et al., 2014; Thiele & Navarro, 2014). Ethanol solutions were made daily by diluting a 95% (v/v) ethanol with tap water to a 20% (v/v) concentration. Solutions were presented using sipper tubes featuring a ball bearing nozzle (Thiele et al., 2014). Ethanol was available 3hrs after the dark cycle had started and remained available for 2hrs on Days 1-3 and 4hrs on Day 4, and the mice remained abstinent on Days 5-7; see Figure 1 for illustration of the timeline.

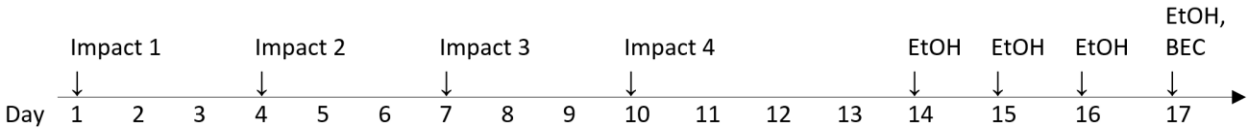



**Figure 1.** Experiment 1 Timeline
Partial timeline for experiment 1 depicting rmTBI schedule and one week of ethanol (EtOH) consumption. Ethanol consumption continued for 2, 4, 6, or 8 weeks.

Separate cohorts were able to consume ethanol for 2, 4, 6, or 8 weeks to measure changes in drinking patterns longitudinally. During the intake period, mice were presented with a sipper filled with 20% ethanol solution and regular drinking water. Prior to placing the sippers on the cages and at the conclusion of the drinking period, the total volume of each solution (ml) consumed was recorded. The position of the bottles alternated daily to negate any side preferences. Drinking was voluntary as water was available at all times during the experiments. The amount of water and ethanol were recorded daily and blood samples were taken once per week on immediately following the Day 4 drinking session via a standard submandibular bleed procedure. After the final drinking session, the mice were given a lethal dose of isoflurane, so that the brains could be cleared of blood via transcardial perfusion with phosphate buffered solution and extracted for use in additional experiments.

The two-bottle choice variant of the DID paradigm was chosen as it allows for ethanol preference to be determined and mice maintain similar amounts of voluntary ethanol consumption (g/kg), though it is likely that the results produced reflect a slight decrease in blood ethanol concentrations (BEC) compared to the single bottle option (Rhodes et al., 2007). Still, researchers using this model have been able to demonstrate pharmacologically meaningful BECs in C57BL/6J non-dependent mice and even binge-like drinking levels of more than 80 mg of ethanol/dl of blood (Thiele et al., 2014).

Blood samples were centrifuged (1,500 x g) following collection and the ethanol concentration measured using an Analox Instrument analyzer (Lunenburg, MA, USA). Plasma samples were analyzed immediately or stored at -20 degrees Celsius until analyzed.

**Analyses**

All analyses were conducted with SPSS 25.0 (IBM). A p-value less than 0.05 was set to evaluate significance unless otherwise indicated. Outliers were identified and removed following

Tukey's method creating boxplots and removing data points falling outside the ±1.5 interquartile range. Results of analyses that are not mentioned specifically are non-significant.

In order to test the prediction of increased ethanol consumption in rmTBI mice compared with sham controls each week of the drinking period, two-way mixed ANOVAs, Procedure (rmTBI vs sham) x Week were conducted comparing the average daily ethanol consumption (g/kg) over the set drinking time (2, 4, 6 or 8 weeks of drinking) for each cohort. To better understand any significant changes in ethanol consumption, two-way mixed ANOVAs were run to assess decreases in total fluid (water and ethanol) consumed. In order to look at variance in consumption within weeks while accounting for possible bottle placement preference, consumption patterns (g/kg) for Days 1 & 2 and Days 3 & 4 were analyzed using mixed ANOVA to compare rmTBI, Week, and Day. These analyses were repeated for ethanol preference. Ethanol (g) intake was calculated by multiplying the volume (ml) of ethanol consumed by the specific density of 20% v/v concentration of ethanol by 1000 and then dividing by the mouse's weight in grams for a g/kg measure. Ethanol preference was calculated by dividing total (ml) of EtOH by the total amount of fluid (water and ethanol) consumed. Any significant ANOVA main effects or interactions were followed with Bonferroni adjusted pairwise comparisons to determine which simple effects were statistically significant.

## Results

### 2 Week Cohort

A 2 x 2 Procedure (rmTBI, Sham) x Week (1, 2) mixed ANOVA was conducted to determine if there were any differences in average ethanol consumption (g/kg) and an additional analysis was conducted to examine ethanol preference in the 2 Week cohort. As shown in Figure 2, Panel A, results indicated no significant differences in ethanol drinking related to

rmTBI or between Weeks 1 and 2. However, mice showed a decreased ethanol preference related to Procedure, $F(1, 34) = 5.416$, $p < .05$. As illustrated in Figure 2, Panel B, Bonferroni pairwise comparison showed a decrease in the rmTBI group (M = 76.7%) compared to Sham (M = 86.3%). Mice also showed an increase in preference from Week 1 (M = 75.5%) to Week 2 (M = 87.5%), $F(1, 34) = 8.487$, $p < .05$.

A 2 x 2 x 2 Procedure (rmTBI, Sham) x Day (Days 1 & 2, Days 3 & 4) x Week (1, 2) mixed ANOVA was conducted to examine changes in daily consumption patterns and an additional analysis was used to examine ethanol preference patterns. As shown in Figure 2, Panel C, results indicated that mice drank more ethanol on Days 3 & 4 (M = 1.47g/kg) compared to Days 1 & 2 (M = 1.17g/kg), $F(1, 40) = 4.670$, $p < .05$. Similarly, mice showed a significant increase in ethanol preference during Days 3 & 4 (M = 85.3%) compared to Days 1 & 2 (M = 77.7%), $F(1, 40)$ 4.608, $p < .05$ as shown Figure 2, Panel D.

In summary, there is insufficient evidence to support the hypothesis of increased ethanol intake following rmTBI, in fact, the data suggest a slight decrease in ethanol preference due to rmTBI despite an increase in ethanol preference from Week 1 to Week 2. Daily patterns of ethanol intake and preference support increased drinking and preference of ethanol on Days 3 & 4 of the four-day drinking paradigm. The variation observed between Days 1 & 2 and Days 3 & 4 imply that day to day voluntary drinking has not yet reached a stable consumption pattern which can sometimes take a few weeks.

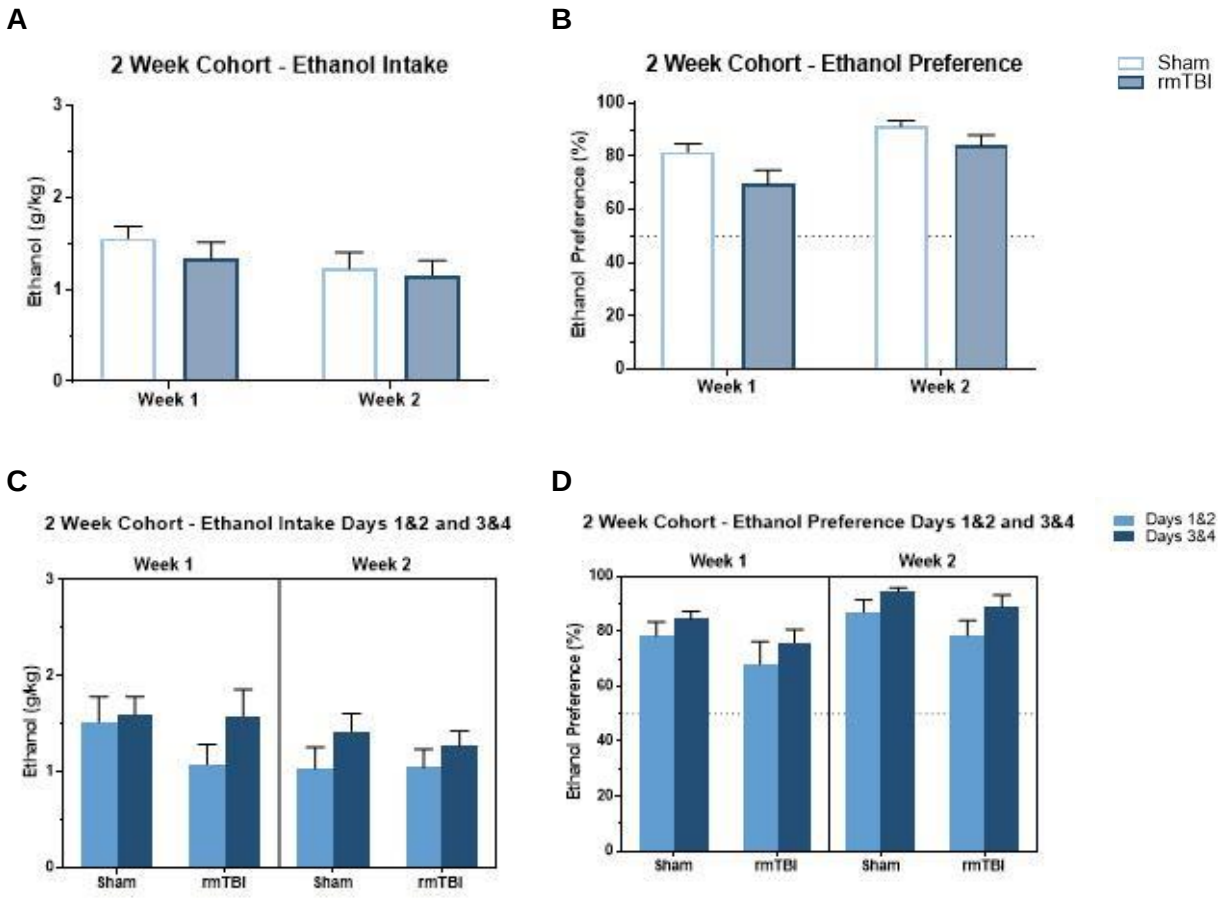



**Figure 2.** Ethanol consumption and preference in Week 2 Cohort.
A) Average daily voluntary ethanol consumption and B) average daily ethanol preference C) ethanol intake by Days 1 & 2 and Days 3 & 4 D) ethanol preference by Days 1 & 2 and Days 3 & 4.

**4 Week Cohort**

A 2 x 4 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4) mixed ANOVA was conducted to determine if there were any differences in average ethanol consumption (g/kg) in the 4 Week cohort. As shown in Figure 3, Panel A, results indicated significant main effects of Week, $F(3, 81) = 6.71$, $p < .05$ and Procedure, $F(1, 81) = 30.570$, $p < .05$. These effects were qualified by a significant Procedure x Week interaction, $F(3, 81) = 4.060$, $p < .05$. Corrected pairwise comparisons specifically showed rmTBI mice consumed less ethanol than Sham mice in Week 2 (Sham, M = 1.49g/kg; rmTBI, M = 0.76g/kg), Week 3 (Sham, M = 2.19g/kg; TBI, M = 1.15g/kg), and Week 4 (Sham, M = 1.78g/kg; rmTBI, M =1.04g/kg). The decrease in ethanol

consumption observed in Week 2 appears to be mediated by an overall decrease in fluid intake as revealed by two-way ANOVA main effect of Week, $F(3, 79) = 9.926$, $p < .05$ as well as main effect of Procedure, $F(1, 79) = 23.639$, $p < .05$. Specifically, there was a decreased total fluid consumption in Week 2 (M = .320) compared to Week 1 (M = .536ml), Week 3 (M = .489ml), and Week 4 (M = .500) and decreased fluid intake (ml) in rmTBI mice (M = .390) compared to Sham mice (M = .533). A 2 x 2 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4) mixed ANOVA was conducted to determine if there were any differences in average ethanol preference in the 4 Week cohort. As shown in Figure 3, Panel B, results indicated a significant decrease in ethanol preference across Week $F(3, 63) = 4.597$, $p < .05$, as well as, a significant decrease in ethanol consumption due to Procedure $F(1, 63) = 4.249$, $p < .05$. Bonferroni pairwise comparison indicated the decrease in ethanol preference across weeks was related to mice drinking less in Week 4 (M = 73.7%) compared to Week 2 (M = 86.3%) and Week 3 (M = 86.3%). Across all four weeks, Sham mice showed higher ethanol preference (M = 85.6%) compared to mice exposed to rmTBI (M = 79.4%).

A 2 x 2 x 4 Procedure (rmTBI, Sham) x Day (Days 1 & 2, Days 3 & 4) x Week (1, 2, 3, 4) mixed ANOVA was conducted to examine changes in daily ethanol consumption patterns between Days 1 & 2 and Days 3 & 4 in the 4 Week cohort. As shown in Figure 3, Panel C, mice drank more on Days 3 & 4 (M = 1.70g/kg) compared to Days 1 & 2 (M = 1.31g/kg), $F(1, 81) = 25.02$, $p < .05$. A 2 x 2 x 2 Procedure (rmTBI, Sham) x Day (Days 1 & 2, Days 3 & 4) x Week (1, 2, 3, 4) mixed ANOVA was used to examine changes in daily ethanol preference patterns between Days 1 & 2 and Days 3 & 4. As shown in Figure 3, Panel D, results indicated a significant Day x Week interaction, $F(3, 63) = 4.878$, $p < .05$. Bonferroni corrected pairwise comparisons showed mice displayed an increase in preference from Days 1 & 2 (M = 80.4%) to Days 3 & 4 (M = 92.0%) during Week 2, but in Week 3, mice showed a decrease in preference from Days 1 & 2 (M = 91.8%) to Days 3 & 4 (M = 80.8%).

In summary, results indicate a rmTBI-dependent decrease in consumption of ethanol during Week 3 and Week 4 accompanied by a decrease in ethanol preference in Week 4. The day to day consumption patterns still indicated lack of stable intake which is corroborated by the observed variation of ethanol preference in the daily preference patterns.

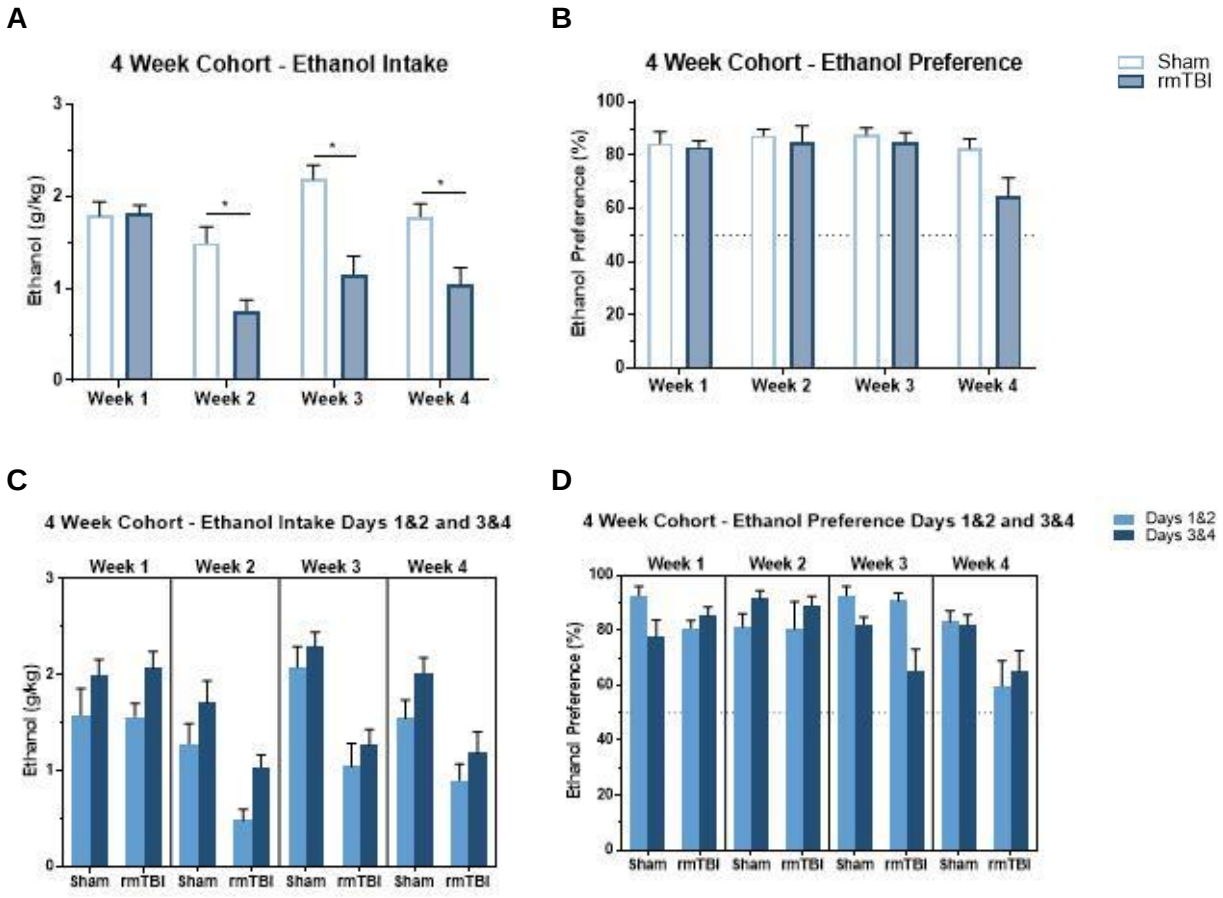



**Figure 3.** Ethanol consumption and preference in Week 4 Cohort.
A) Average daily voluntary ethanol consumption and B) average daily ethanol preference C) ethanol intake by Days 1 & 2 and Days 3 & 4 D) ethanol preference by Days 1 & 2 and Days 3 & 4.

### 6 Week Cohort

A 2 x 6 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4, 5, 6) mixed ANOVA was used to determine if any differences in average ethanol consumption (g/kg) or ethanol preference were present in the 6 Week cohort. As shown in Figure 4, Panel A, results of the ethanol intake

comparison revealed a significant main effect for Week, $F(5, 124) = 5.659$, $p < .05$. Bonferroni adjusted pairwise comparisons showed mice drank more ethanol in Week 4 (M = 3.15g/kg) compared to Week 2 (M = 2.30g/kg), Week 3 (M = 2.25g/kg), Week 5 (M = 2.40g/kg), and Week 6 (M = 2.10g/kg). As shown in Figure 4, Panel B, there were no differences in ethanol preference due to rmTBI or across Weeks in the 6 Week cohort.

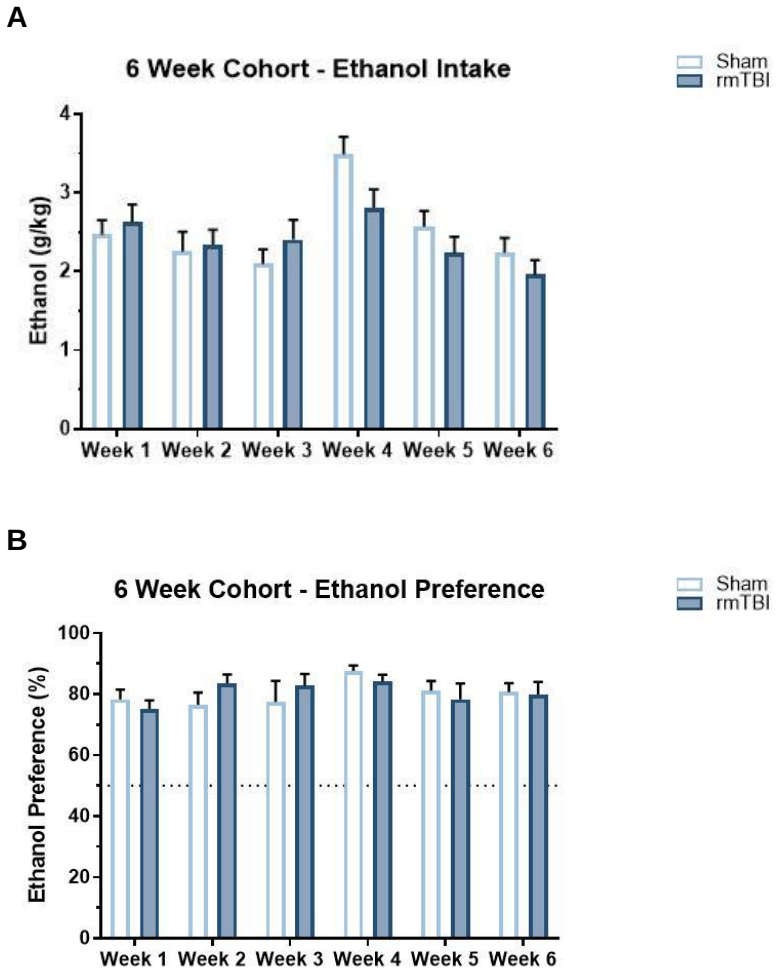
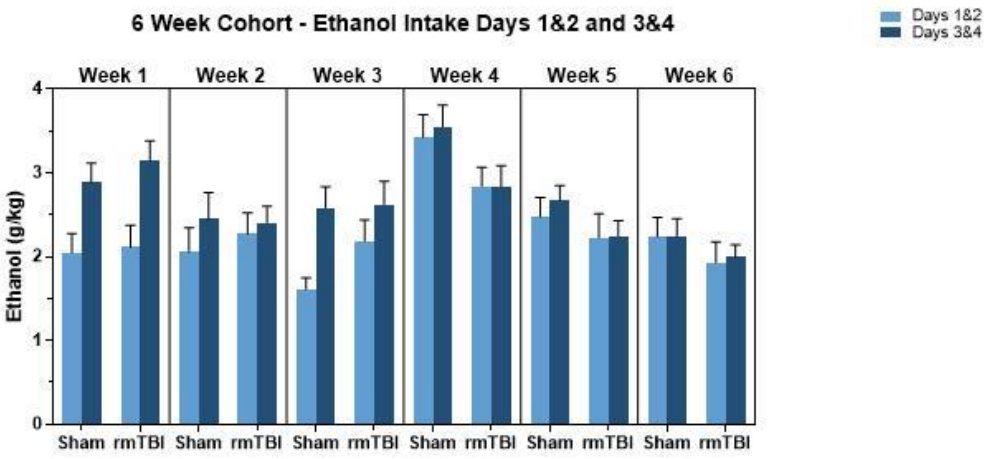



**Figure 4.** Average ethanol consumption and preference in Week 6 Cohort.
A) Average daily voluntary ethanol consumption and B) average daily ethanol preference.

A 2 x 2 x 6 Procedure (rmTBI, Sham) x Day (Days 1 & 2, Days 3 & 4) x Week (1, 2, 3, 4, 5, 6) mixed ANOVA was conducted to examine changes in daily consumption patterns and an

additional analysis was conducted to examine differences in ethanol preference patterns between Days 1 & 2 and Days 3 & 4 in the 6 Week cohort. As shown in Figure 5, Panel A, results a significant main effect of Day, $F(1, 124) = 26.596$; however, this effect was qualified by a significant Day x Week interaction, $F(5, 124) = 5.626$, $p < .05$. Mice drank more in Days 3 & 4 (M = 3.02 g/kg) compared to Days 1 & 2 (M = 2.08g/kg) in Week 1. Similarly, in Week 3, mice drank more during Days 3 & 4 (M = 2.61 g/kg) compared to Days 1 & 2 (M = 2.08g/kg). As shown in Figure 5, Panel B, there was also a significant Day x Week interaction, $F(5, 123) = 7.748$, $p < .05$. During Week 1, mice showed an increased ethanol preference from Days 1 & 2 (M = 66.7%) to Days 3 & 4 (M = 86.7%) while during Week 3, mice displayed a decreased preference from Days 1 & 2 (M = 85.2%) to Days 3 & 4 (M = 75.2%).

**A**




**B**

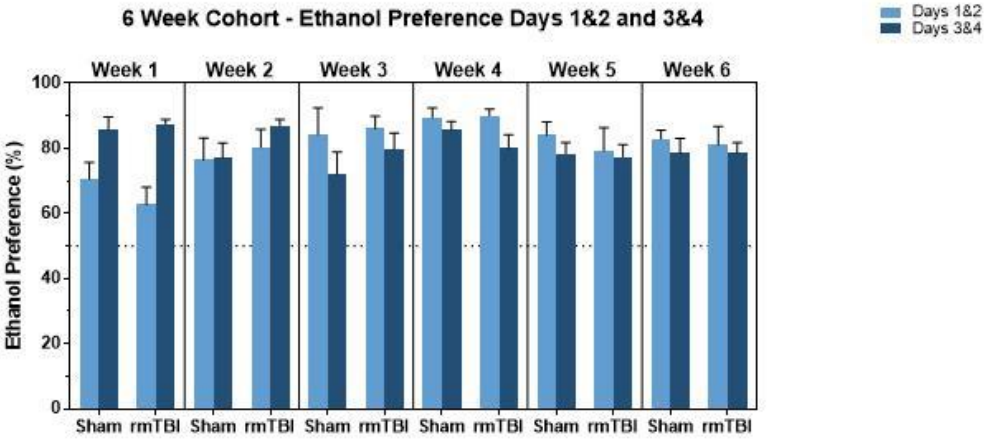


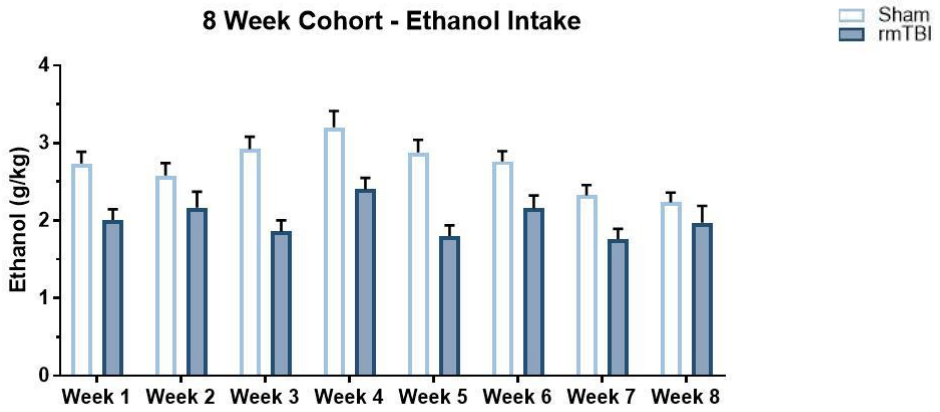

**Figure 5.** Days 1 & 2 and Days 3 & 4 Ethanol consumption and preference in Week 6 Cohort. A) Ethanol intake by Days 1 & 2 and Days 3 & 4 B) ethanol preference by Days 1 & 2 and Days 3 & 4.

In summary, an overall increase in ethanol consumption was observed in Week 4 of the 6 Week cohort for both rmTBI and Sham conditions. However, this change was not accompanied by any changes in average ethanol preference. The observations of daily ethanol intake and preference suggest drinking has stabilized towards the later weeks of the drinking period as differences in Day 1 & 2 vs Day 3 & 4 are no longer evident after Week 3.

**8 Week Cohort**

A 2 x 8 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4, 5, 6, 7, 8) mixed ANOVA was used to determine if any differences in average ethanol consumption (g/kg) were present in the 8 Week cohort. As shown in Figure 6, Panel A, results revealed a significant main effect of Week $F(7, 163) = 4.032$, $p < .05$, as well as a significant main effect of Procedure $F(1, 163) = 72.826$, $p < .05$. Bonferroni pairwise comparison showed mice drank more ethanol in Week 4 (M = 2.81g/kg) compared to Week 7 (M = 2.05g/kg) and Week 8 (M = 2.10g/kg). Across the 8 Week drinking period, mice also showed a decrease in ethanol consumption due to rmTBI (M = 2.02g/kg) compared to Sham (M = 2.71g/kg). A 2 x 6 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4, 5, 6, 7, 8) mixed ANOVA was used to determine if the decreased ethanol consumption was related to decreased total fluid intake. Results indicated main effects of Week, $F(7, 163) = 4.850$, $p < .05$ and Procedure, $F(1, 163)$ 34.381, $p < .05$. Corrected pairwise comparisons showed mice drank less total fluid during Week 7 (M = .553ml), and Week 8 (M = .570ml) compared to Week 1 (M = .759), and drank less after rmTBI (M = .588ml) when compared with Sham (M = .708ml) across weeks. However, these changes in total fluid intake do not fully account for the specific decrease in ethanol consumption (g/kg) previously described. A 2 x 8 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4, 5, 6, 7, 8) mixed ANOVA was used to examine differences in average ethanol preference. As shown in Figure 6, Panel B, results revealed a significant interaction of Week and Procedure $F(7, 161) = 2.403$, $p < .05$. Corrected pairwise comparisons indicated that mice in the rmTBI group (M = 69.1%) displayed a decreased ethanol preference during Week 5 compared to Sham (M = 90.4%).

A




B

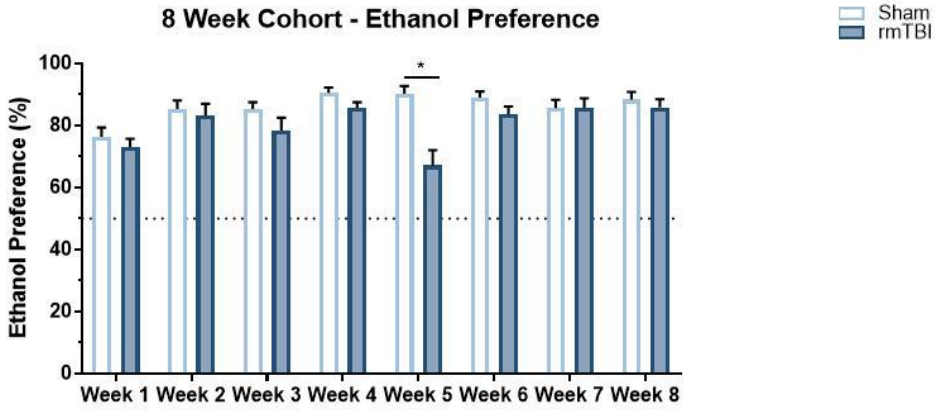



**Figure 6.** Average ethanol consumption and preference in Week 8 Cohort.
A) Average daily voluntary ethanol consumption and B) average daily ethanol preference.

A 2 x 2 x 8 Procedure (rmTBI, Sham) x Day (Days 1 & 2, Days 3 & 4) x Week (1, 2, 3, 4, 5, 6, 7, 8) mixed ANOVA was conducted to examine changes in daily consumption patterns between Days 1 & 2 and Days 3 & 4 in the 8 Week cohort. As shown in Figure 7, Panel A, results indicated a significant Day x Week interaction, $F(7, 163) = 3.584$, $p < .05$. Bonferroni adjusted pairwise comparisons showed mice drank more on Days 3 & 4 (M = 2.67g/kg) than on day Days 1 & 2 (M = 2.07g/kg) in Week 1. Likewise, in Week 2, mice drank more on Days 3 & 4 (M = 2.57g/kg) compared to Days 1 & 2 (M = 2.18g/kg), and again in Week 3, mice showed

elevated intake on Days 3 & 4 (M = 2.71g/kg) compared to Days 1 & 2 (M = 2.07g/kg). After week 4, drinking levels stabilized across days. A 2 x 2 x 8 Procedure (rmTBI, Sham) x Day (Days 1 & 2, Days 3 & 4) x Week (1, 2, 3, 4, 5, 6, 7, 8) mixed ANOVA was conducted to determined differences in ethanol preference patterns between Days 1 & 2 and Days 3 & 4 in the 8 Week cohort. As shown in Figure 7, Panel B, results indicated a significant Day x Week x Procedure interaction, $F(7, 161) = 3.734$, $p < .05$. Specifically, in Week 1, rmTBI mice displayed a decrease in ethanol preference from Days 1 & 2 (M = 85.4%) to Days 3 & 4 (M = 57.0%). Similarly, in Week 3, rmTBI mice showed a decrease of ethanol preference from Days 1 & 2 (M = 84.8%) to Days 3 & 4 (M = 72.3%).

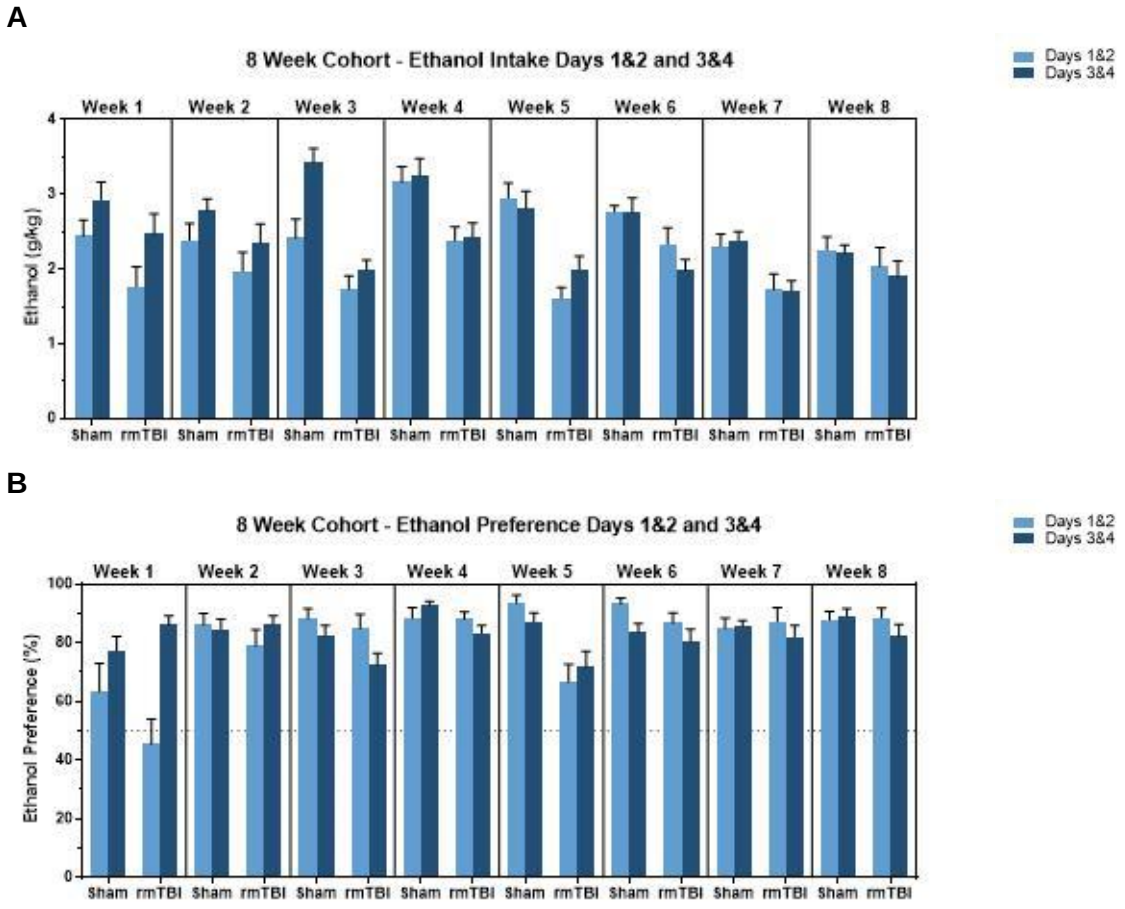



**Figure 7.** Days 1 & 2 and Days 3 & 4 Ethanol consumption and preference in Week 8 Cohort. A) Ethanol intake by Days 1 & 2 and Days 3 & 4 B) ethanol preference by Days 1 & 2 and Days 3 & 4.

In summary, an overall increase in ethanol consumption was again observed in Week 4 for both rmTBI and Sham conditions. However, there was also a rmTBI-dependent decrease in consumption most notably in Weeks 3, 4, and 5 of the 8 Week cohort. Average ethanol preference was generally observed to be similar between groups with the exception of a notable rmTBI decrease in preference during Week 5. The observations of daily ethanol intake and preference support the previous observation in the 6 Week cohort of stabilized ethanol drinking and preference after Week 3.

**Blood Ethanol Concentration and Day 4 Ethanol Consumption**

Blood samples were taken after the 4hr drinking session on Day 4 each week of the experiment for all cohorts. A simple linear regression was used to investigate the relationship between the total volume of ethanol consumed (g/kg) on Day 4 with BEC (mg/dl). As expected, the amount of ethanol consumed (g/kg) significantly predicted BEC, $F(1, 444) = 250.20$, $p < .05$ showing a moderate relationship, $r^2 = .36$; results are illustrated in Figure 8. This observation provides support of the DID drinking paradigm applied following rmTBI as ethanol intake (g/kg) values yielded proportional BECs values that would be considered intoxicating in humans (J. S. Rhodes et al., 2007). Group means of BEC and Day 4 ethanol consumption (g/kg) are included below in Table 1.

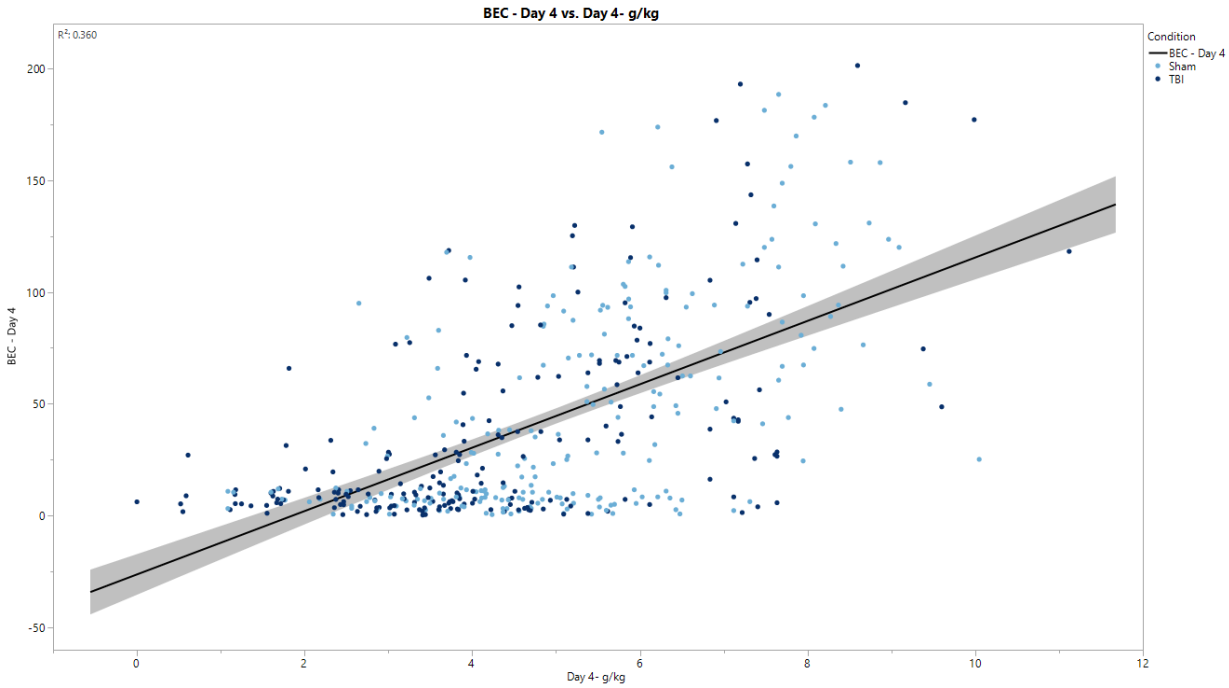



**Figure 8.** Blood ethanol levels as a factor of EtOH on Day 4 across cohorts.

**Table 1**. Blood Ethanol Concentration and Ethanol Consumption by Group and Week

| Procedure | Week | N | BEC(mg/dl) | | SEM | N | 4hr Intake g/kg | | SEM |
|---|---|---|---|---|---|---|---|---|---|
| Sham | 1 | 12 | 49.59 | ± | 13.94 | 12 | 4.04 | ± | 0.54 |
| | 2 | 12 | 32.70 | ± | 10.40 | 12 | 3.32 | ± | 0.40 |
| TBI | 1 | 12 | 22.28 | ± | 5.00 | 12 | 2.87 | ± | 0.43 |
| | 2 | 12 | 22.16 | ± | 6.12 | 12 | 2.78 | ± | 0.30 |
| | | | | | | | | | |
| Sham | 1 | 12 | 55.30 | ± | 11.95 | 12 | 4.29 | ± | 0.28 |
| | 2 | 12 | 39.53 | ± | 9.98 | 12 | 4.28 | ± | 0.40 |
| | 3 | 10 | 33.26 | ± | 16.88 | 10 | 4.36 | ± | 0.44 |
| | 4 | 11 | 20.53 | ± | 8.53 | 11 | 4.30 | ± | 0.40 |
| TBI | 1 | 11 | 61.18 | ± | 12.02 | 11 | 3.59 | ± | 0.46 |
| | 2 | 12 | 33.27 | ± | 11.78 | 12 | 3.23 | ± | 0.77 |
| | 3 | 11 | 7.67 | ± | 1.53 | 11 | 3.00 | ± | 0.57 |
| | 4 | 12 | 7.64 | ± | 2.74 | 12 | 2.34 | ± | 0.43 |
| | | | | | | | | | |
| Sham | 1 | 11 | 84.87 | ± | 11.37 | 11 | 7.01 | ± | 0.47 |
| | 2 | 11 | 43.35 | ± | 14.67 | 11 | 5.70 | ± | 0.63 |
| | 3 | 11 | 39.74 | ± | 14.90 | 11 | 6.00 | ± | 0.55 |
| | 4 | 12 | 57.70 | ± | 17.51 | 12 | 6.42 | ± | 0.46 |
| | 5 | 12 | 47.29 | ± | 20.08 | 12 | 5.53 | ± | 0.44 |
| | 6 | 12 | 19.16 | ± | 9.84 | 12 | 4.12 | ± | 0.44 |
| TBI | 1 | 5 | 113.50 | ± | 31.58 | 5 | 6.56 | ± | 0.71 |
| | 2 | 8 | 62.79 | ± | 20.64 | 8 | 5.60 | ± | 0.55 |
| | 3 | 11 | 56.48 | ± | 14.62 | 11 | 5.88 | ± | 0.64 |
| | 4 | 9 | 68.97 | ± | 18.85 | 9 | 6.15 | ± | 0.62 |
| | 5 | 9 | 52.51 | ± | 12.40 | 9 | 4.46 | ± | 0.32 |
| | 6 | 12 | 15.38 | ± | 6.76 | 12 | 3.85 | ± | 0.28 |
| | | | | | | | | | |
| Sham | 1 | 11 | 96.91 | ± | 14.13 | 11 | 7.03 | ± | 0.42 |
| | 2 | 12 | 59.43 | ± | 10.85 | 12 | 6.78 | ± | 0.50 |
| | 3 | 12 | 56.60 | ± | 13.69 | 12 | 6.71 | ± | 0.35 |
| | 4 | 11 | 44.25 | ± | 12.51 | 11 | 6.05 | ± | 0.31 |
| | 5 | 12 | 71.74 | ± | 15.90 | 12 | 5.52 | ± | 0.36 |
| | 6 | 11 | 20.39 | ± | 7.84 | 11 | 4.50 | ± | 0.31 |
| | 7 | 12 | 46.50 | ± | 11.76 | 12 | 4.87 | ± | 0.23 |
| | 8 | 12 | 19.55 | ± | 6.24 | 12 | 4.01 | ± | 0.22 |
| TBI | 1 | 13 | 81.40 | ± | 17.16 | 13 | 5.97 | ± | 0.48 |
| | 2 | 11 | 42.36 | ± | 11.39 | 11 | 4.91 | ± | 0.58 |
| | 3 | 11 | 22.00 | ± | 7.35 | 11 | 4.54 | ± | 0.34 |
| | 4 | 10 | 23.06 | ± | 9.27 | 10 | 4.91 | ± | 0.49 |
| | 5 | 12 | 22.47 | ± | 6.65 | 12 | 4.56 | ± | 0.49 |
| | 6 | 12 | 15.33 | ± | 5.85 | 12 | 4.63 | ± | 0.54 |
| | 7 | 11 | 20.87 | ± | 11.42 | 11 | 4.31 | ± | 0.58 |
| | 8 | 11 | 18.55 | ± | 7.34 | 11 | 3.85 | ± | 0.42 |

BEC, blood ethanol concentration.

**Discussion**

The DID two-bottle choice paradigm was successfully employed as data demonstrated that all groups consistently consumed ethanol volumes per body weight that produced physiologically relevant BEC levels. The drinking levels are corroborated by the BEC collected on Day 4 each week. Both ethanol intake levels and BEC levels are consistent with what other groups using this drinking paradigm have previously published (Crabbe et al., 2009; Lee, Coehlo, Solton, & Szumlinski, 2017; Marianno, Abrahao, & Camarini, 2017; J. S. Rhodes et al., 2007; Justin S. Rhodes et al., 2005). Daily drinking stabilized after 4 weeks of ethanol exposure, with few groups exhibiting a change in ethanol intake between days 1&2 and days 3&4 from week 5 onward. The preference data showed a consistent preference for ethanol over water across 2, 4, 6, and 8 Week drinking cohorts. While there was initially more variability in daily preference scores, this also stabilized over time.

Whereas the DID paradigm was effective in producing reliable drinking data at similar levels observed by other researchers (Crabbe et al., 2009; Lee, Coehlo, Solton, & Szumlinski, 2017; Marianno, Abrahao, & Camarini, 2017; J. S. Rhodes et al., 2007; Justin S. Rhodes et al., 2005), there was no evidence for elevated voluntary ethanol consumption or an escalated drinking pattern following this model of repetitive mTBI when compared to sham. In fact, for the 4 and 8 week cohorts, rmTBI mice consumed less ethanol than Sham mice. In the 4 week cohort, the decrease in g/kg volumes were accompanied by decreased ethanol preference; however, this effect on preference was not repeated in the 8 week cohort. While escalated drinking was not present following TBI, a temporary decrease in voluntary consumption is similar to what is reported in some clinical populations following injury prior to possible escalations in drinking (J. Corrigan, Rust, & Lamb-Hart, 1995).

The decreases in ethanol consumption observed in this study differ from other animal models of TBI that observed a bimodal change in ethanol consumption producing high low

ethanol drinkers following blast induced TBI compared to sham (Mayeux, Teng, Katz, Gilpin, & Molina, 2015). However, Mayeux and colleagues (2015) used an outbred rat model which differs considerably from the inbred mouse strain, C57BL/6, used in this study. The difference in outcomes highlights two important choices of methods. First, Sprague Dawley rats are generally considered a low ethanol preferring model, whereas the C57BL/6J mice normally drink moderately high volumes of ethanol without training or forced consumption. Additionally, genetic variability may play an important role as only approximately 20% of patients in clinical populations are reported to excessively drink following TBI without previous history of AUD (Bombardier et al., 2003; Dikmen et al., 1995; Horner et al., 2005). The C57BL/6J mouse strain is genetically homogenous without the genetic variability that may contribute to the bimodal distribution of ethanol consumption producing high and low drinkers after TBI. Another study looking at an outbred mouse strain, Swiss Webster, found increased ethanol consumption in adulthood after a single TBI incurred during adolescence in female mice (Weil et al., 2015). It is possible that increased consumption may be seen because of genetic markers present in the outbred strain not found in the inbred C57BL/6J mice. -

**Conclusions**

Clinical populations sometimes show a decrease in drinking for up to two months post-injury, and then others report increases in problem or excessive drinking without previous history following TBI (Bombardier et al., 2003; Dikmen et al., 1995; Horner et al., 2005). It is possible that our C57BL/6J mouse model does not have a natural genetic variant that contributes to excessive drinking observed in a portion of the population following TBI. However, while the proposed hypothesis was not supported, because genes are commonly manipulated in C57Bl/6J mice using knock-out, knock-in, and transgenic models, C57Bl/6J mice may serve

as an excellent tool to explore the influences of specific genes on the vulnerability to rmTBI-induced increases in ethanol intake.

## EXPERIMENT 2: EFFECT OF REPETITIVE MILD TRAUMATIC BRAIN INJURY AND VOLUNTARY DRINKING ON NEURONAL CYTOKINES

Over 1.5 million patients suffer a traumatic brain injury (TBI) annually (Faul M, Xu L, Wald MM, 2010; Niska et al., 2010), TBI's represent a major health problem in America. In fact, TBI represents a leading cause of disability and death for otherwise healthy adolescents and adults (Holm et al., 2005; National Center for Injury Prevention and Control, 2003), and is estimated to have an economic impact of nearly $40 billion each year for those requiring hospitalization (Silver et al., 2001). Remarkably, the majority of TBI cases are closed head injuries (i.e. concussions) that are classified as 'mild' (mTBI) and often do not require hospitalization. Such injuries are not generally visualized standard MRI and CT scans; however, advanced techniques such as functional (fMRI) and diffusion tensor imaging (DTI) have recently had greater success identifying damage (Delouche et al., 2015; Honce et al., 2016). Typically, mTBIs are identified by reported symptoms such as transient confusion, disorientation, or impaired consciousness; dysfunction of memory around the time of injury; and/or loss of consciousness lasting less than 30 minutes (National Center for Injury Prevention and Control, 2003).

The physical tissue damage that occurs from a TBI is referred to as the primary injury or phase; this is followed by a number of secondary mechanisms that can also have deleterious consequences. These delayed effects can occur in the minutes and hours following an injury or after weeks or months after the physical trauma (Morganti-Kossmann et al., 2005; Schmidt, Heyde, Ertel, & Stahel, 2005). Inflammatory responses are among the secondary effects that have received much attention in hope of developing new treatments. Neuroinflammation is the

result of a number of mechanisms including the upregulation of various pro-inflammatory cytokines and chemokines (Ghirnikar, Lee, & Eng, 1998; Ziebell & Morganti-Kossmann, 2010). Ghirnikar and colleagues (1998) review early studies of neurological insult that describe the involvement of several cytokines and chemokines including interleukin-1 (IL-1), IL-6, IL-8/macrophage inflammatory protein-2 in the rodent (MIP-2), IL-10, tumor necrotic factor (TNF-α), Fas ligand (FasL), and monocyte chemoattractant protein (MCP-1). Neural cytokines and chemokines are involved with immune responses such as the regulation and migration of leukocytes, but they are also known to influence neuronal growth, communication, repair and survival (Ziebell & Morganti-Kossmann, 2010). While some of these molecules appear to have very clear neurotoxic effects and others appear to be neuroprotective, it has also been observed that the combined neuroinflammatory response is acutely harmful, but beneficial in the long term recovery from TBI (Finnie, 2013; Ziebell & Morganti-Kossmann, 2010). For example, IL-1 and TNF-α are both considered neurotoxic based on their ability to exacerbate TBI and extend recovery times, and they can even work together to initiate inflammatory processes, disrupt the BBB, and recruit leukocytes (Chao, Hu, Ehrlich, & Peterson, 1995). Alternatively, IL-6 and IL-10 are generally considered neuroprotective as evidence suggests that IL-6 can reduce apoptotic cell death and promote neuroregenerative factors, and IL-10 can reduce TNF-α induced inflammation (Kremlev & Palmer, 2005; Penkowa et al., 2003). IL-6 and IL-1 have also been shown to be significantly upregulated in patients with the most severe and even fatal TBI as presumably they are recruited to sites of injury to mediate tissue damage (Ferreira et al., 2014; Schneider Soares et al., 2012). Finally, the chemokines, IL-8/MIP-2 and MCP-1 are implicated in the recruitment of leukocytes contributing to neuroinflammation; however, IL-8 is also able to stimulate the production of nerve growth factor and therefore may also possess neurotropic benefits (Whalen et al., 2000; Xu et al., 2004).

Alcohol is a well-known neurotoxic substance causing predictable neurodegeneration especially following chronic, heavy and binge use, and a number of mechanisms for these neurodegenerative effects have been examined including neuroinflammatory processes (Collins, Corso, & Neafsey, 1996; Crews, 1999; Crews et al., 2006; Obernier, Bouldin, & Crews, 2002; Schmued & Hopkins, 2000). While neuroinflammation is only one of many ways alcohol can be detrimental to brain health, research demonstrating increased levels of cytokines in human alcoholics' brains postmortem corroborate this hypothesis (He & Crews, 2008). Ethanol can disrupt the immune response by decreasing levels of systemic cytokines that are crucial in fighting infection (Pruett, Zheng, Fan, Matthews, & Schwab, 2004), while also inducing pro-inflammatory systemic cytokines such as TNF-α that can also impact the brain (Crews et al., 2006). Furthermore, a study administering chronic ethanol to mice was able to confirm elevated pro-inflammatory cytokines, TNFα, IL-1β, IL-6, and MCP-1, contributed to the increased inflammation observed in the brain (Qin & Crews, 2012). There is still some ambiguity regarding the relationship between pro-inflammatory cytokine microglia activation after alcohol exposure and neurodegeneration, One study suggests a causal relationship (Qin et al., 2008; Qin & Crews, 2012), while another using a shorter ethanol exposure period observed neurodegeneration but only partial microglia activation (Marshall et al., 2013). It is then possible that microglia activation is a secondary mechanism following damage caused by ethanol or the neurodegeneration observed follow shorter bouts of ethanol exposure may be elicited by mechanisms other than neuroinflammatory responses.

While both TBI and alcohol consumption can have a detrimental impact on brain health, the effects of alcohol on TBI outcome are more complex and not well understood. Although the increased risk of sustaining a TBI when intoxicated is well established (Chen, Yi, Yoon, & Dong, 2012), observational data regarding beneficial or detrimental effects of alcohol consumed prior to TBI is less clear. Some researchers report no relationship to outcome (Chen et al., 2012),

while others report a reduction in mortality for intoxicated patients with moderate to severe injuries (Brennan, Bernard, Cameron, Rosenfeld, & Mitra, 2015). It has been suggested that a confound exists in severity ratings for TBI intoxicated patients as the CNS depressant nature of alcohol would artificially lower the Glasgow rating scale used to assess the severity of injury in many medical settings potentially leading to decreases in mortality (O'Phelan, McArthur, Chang, Green, & Hovda, 2008). One study using an animal model of alcohol consumption prior to TBI, confirms previous reports of alcohol's ability to suppress pro-inflammatory cytokines, but also showed an increased risk of mortality for intoxicated TBI subjects which was hypothesized to be a result of increased susceptibility to infection (Greiffenstein, Mathis, Stouwe, & Molina, 2007). This is in contrast to a study showing acute alcohol intoxication at the time of TBI delayed the resolution of neuroinflammatory cytokines (IL-1, IL-6, TNF-α, MCP-1) during TBI recovery (S X Teng & Molina, 2014). Alcohol consumption during the recovery period following a TBI appears to have predominately deleterious effects, but the mechanisms are still unknown (J. D. Corrigan, 1995; J. D. Corrigan et al., 2013). A recent study using ethanol vapor chamber exposure following mTBI showed increased astrocyte reactivity as well as microglia activation that correlated positively with greater deficits on neurological assessments of sensory, motor, reflexes, and balance skills (Sophie X. Teng et al., 2015).

**Hypotheses**

The proposed experiment investigated the combined effects of voluntary ethanol consumption following repetitive mTBI on the expression of the neuronal cytokine TFN-α as it is independently increased after TBI and alcohol consumption. It was hypothesized that increases in TNF-α expression would be greater following rmTBI compared to sham groups, but that ethanol consumption would lead to increased levels in both groups compared to controls. Length of the voluntary consumption period was hypothesized to positively correlate with

greater cytokine levels, therefore the cohort drinking for 8 weeks was expected to have the highest levels of TNF-α (He & Crews, 2008; Marshall et al., 2013; Ziebell & Morganti-Kossmann, 2010).

## Methods

Brain tissue was collected from mice in Experiment 1 (n = 80, 10 samples per experimental condition) and an additional set of brains were extracted from a group of non-manipulated mice (n =6) to determine TNF-α expression in drinking and non-drinking mice following rmTBI using real-time qPCR (Albert-Weißenberger et al., 2012; Yang et al., 2015).

### Real-time Polymerase Chain Reaction Assay

**RNA Isolation.** Frozen brains were brought to room temperature; TRIzol (Invitrogen, Carlsbad, CA) was added and tissue was homogenized until no solid pieces were visible. Homogenized samples were incubated at room temperature for 5 minutes to allow for complete dissociation of the nucleoprotein complex. Chloroform was added to each sample, thoroughly mixed, and allowed to incubate another 2-3 minutes. The samples were then centrifuged at 12,000g for 15 minutes at 4℃. The upper aqueous phase was removed and placed into a new collection tube. RNA purification occurred according to manufacturer protocol provided with PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). An aliquot (5ul) of RNA was diluted in 495ul of DEPC-treated water in order to measure RNA concentration and purity. Each sample was assessed with UV spectroscopy utilizing the absorbance measured at 260 and 280 nm (GENESYS Spectrometer, Thermo Scientific). The remainder of the RNA sample was stored at -80℃ until further use.

**cDNA Synthesis.** The cDNA synthesis reaction was prepared according to manufacturer provided protocol included in the iScript Advanced cDNA Synthesis Kit for RT-

qPCR (BioRad Laboratories, Hercules, CA). Briefly, a working solution was made using the provided 5x iScript Advanced Reaction mix, iScript Reverse Transcriptase, and water. The sample RNA template added to each well with the working solution. The samples went through reverse transcription by being held at 46℃ for 20min and then 1min at 95℃ to inactivate the reverse transcriptase. Samples were stored undiluted at -20℃ if not used for qPCR immediately.

**qPCR.** Quantitative PCR was conducted via manufacturer provided protocol for Sso Advanced Universal SYBR Green Supermix (BioRad Laboratories, Hercules, CA). Briefly, a working solution made using the provided SsoAdvanced Universal SYBR Green Supermix (2x), forward and reverse primers specific to the house gene (18S) or target gene (TNF-α), and nuclease-free H20. The samples were added to the plate in duplicate. Samples were then placed in iCycler MyIQ Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA) and heated to protocol specified temperatures for 35 cycles.

**Analyses**

All analyses were conducted with SPSS 25.0 (IBM). A p-value less than 0.05 was set to evaluate significance unless otherwise indicated. Outliers were identified and removed following Tukey's method creating boxplots and removing data points falling outside the ±1.5 interquartile range. Results of analyses that are not mentioned specifically are non-significant.

Relative quantification of gene expression of TNF-α was calculated as fold change using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak & Schmittgen, 2001) comparing changes in house gene (18S) to target gene in controls and experimental groups. If the unknown from the experimental group exhibits a greater fold change relative to the housekeeping gene than the unknown for the control group, the conclusion is that the sample from the experimental group began with a larger quantity of DNA than did the sample from the control group. This would explain the difference in fold-change between the groups.

In order to test the hypothesis that increases in the gene expression fold change of pro-inflammatory cytokine, TNF-α, would be greater following rmTBI compared to sham groups, and that ethanol consumption would exacerbate the presence of these markers compared to controls, a one-way ANOVA, was conducted for each of the drinking cohorts (2, 4, 6, and 8 Weeks).

**Results**

For each cohort, a one-way ANOVA was conducted to compare gene expression fold change in TNF- α expression; results are displayed in Table 1 and Figure 1. This revealed a significant effect in the 2 Week cohort, $F(2, 20) = 4.489$, $p < .05$. Post-hoc analyses using Bonferroni correction indicated a greater fold change in the Sham group, ($M= 2.669$) compared to the Control group ($M = 1.020$); this suggests there were similar amounts of DNA in the control condition relative the housekeeping gene, but the Sham group showed more than a 2.5x increase in relative quantity of DNA. In the 4 Week cohort, there was also a significant effect, $F(2, 15) = 5.811$, $p < .05$. Post-hoc analyses using Bonferroni correction also showed a larger fold change in Sham ($M= 2.182$) compared to Control ($M = 1.134$). There was also a significant effect in the 6 Week cohort, $F(2, 18) = 10.317$, $p < .05$. Post-hoc analyses using Bonferroni correction found a greater fold change from in the Sham group ($M= 2.523$) compared to Control ($M = .735$) and rmTBI ($M = .894$) groups. There were no significant differences in TNF- α fold change gene expression in the 8 Week cohort.

In summary, there is insufficient evidence to support a compounded elevation in TNF- α expression due to the combined effects of rmTBI and ethanol consumption. Instead, in the 2, 4, and 6 Week cohorts, fold change in TNF- α expression was elevated in Sham mice compared to controls presumably due to ethanol consumption. Both rmTBI and Sham groups in the 8 Week

cohort showed a return to baseline levels. Possible explanations of these phenomena are included in the discussion.

**Table 1.** TNF- α Fold Change Expression Means by Cohort

| | 2 Week | | | 4 Week | | | 6 Week | | | 8 Week | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Condition | M | SD | N | M | SD | N | M | SD | N | M | SD | N |
| Control | 1.02 | .23 | 4 | 1.13 | .57 | 5 | .74 | .28 | 4 | .77 | .10 | 4 |
| Sham | 2.67 | 1.24 | 10 | 2.18* | .34 | 6 | 2.52* | .97 | 7 | .59 | .48 | 8 |
| rmTBI | 1.97 | .70 | 9 | 1.45 | .62 | 7 | .89** | .79 | 10 | .54 | .30 | 6 |

*denotes significant difference from Control at the 0.05 level. ** denotes significant difference between Sham and rmTBI at the 0.05 level

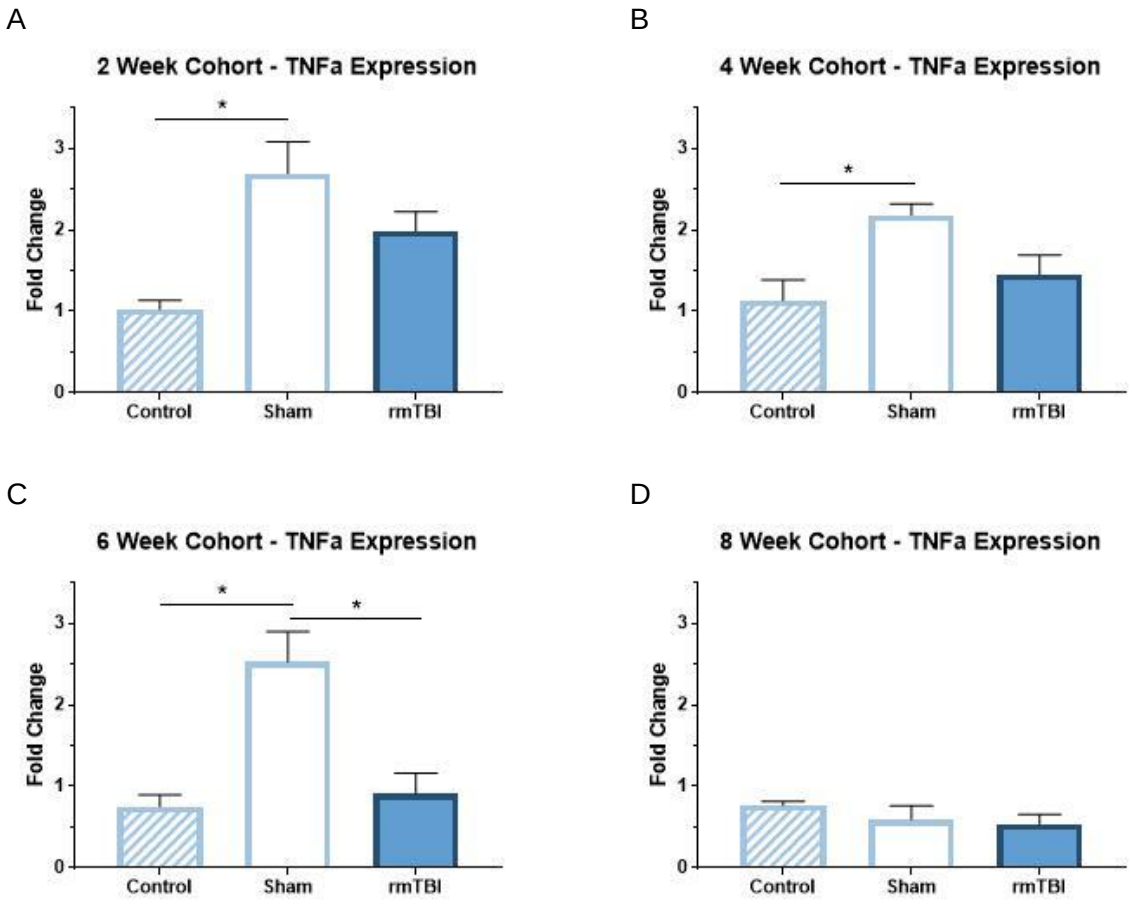



**Figure 1.** TNF- α Fold Change Expression Means by Cohort.
A) 2 Week Cohort, B) 4 Week Cohort, C) 6 Week Cohort, D) 8 Week Cohort

**Discussion**

Expression of TNF- α observed in the Sham groups compared to Controls in the 2, 4, and 6 Week cohorts were elevated likely due to ethanol consumption as predicted. Yet despite the rmTBI cohorts also voluntarily consuming ethanol, there was not a significant difference from Controls in any of the cohorts. This was somewhat surprising given other researchers accounts of increased TNF- α expression following TBI. In fact, due to both TBI and ethanol consumption independently increasing TNF- α expression, it was expected that there would be elevated levels in the rmTBI Drinking groups compared to Sham. Expression of TNF- α in the rmTBI groups were slightly elevated from controls in the 2 and 4 Week cohorts, but in the 6 Week group, the levels have returned to control levels. All TNF- α expression returned to control levels in the 8 Week cohort despite continued ethanol consumption.

Other researchers have shown suppression of pro-inflammatory cytokines when alcohol was administered prior to injury, so it is possible that the observed findings suggest ethanol attenuated the expression of TNF- α in the TBI group (Greiffenstein et al., 2007). However, others show an exacerbated response for neuroinflammatory cytokines (IL-1, IL-6, TNF-α, MCP-1) when alcohol was present at the time of TBI (S X Teng & Molina, 2014). Cytokines are often released in a cascade, some showing pro-inflammatory effects, others anti-inflammatory effects, and some have more diverse functions (Tisoncik et al., 2012). It is entirely possible that a more holistic approach would yield a better understanding of the recruitment and resolution of the cytokines in response to rmTBI, alcohol, and the combination. While it is beyond the scope of the current study, it is possible that the brain's response to TBI included anti-inflammatory cytokines also, that contributed to a prolonged reduction of TNF- α expression despite continued ethanol consumption in the TBI group.

## Conclusions

The current literature regarding the combined effects of rmTBI and voluntary ethanol consumption on neuroinflammatory effects is inconsistent in that some evidence suggests alcohol suppresses neuroinflammatory cytokine production and other research finds support for the prolonged elevation of cytokine levels. Given the varied effects of cytokines and the intricate cascades recruiting them to sites of neural insult, it is likely that single assessment approaches like the one used here are somewhat limited in the greater understanding of recovery from TBI. Future studies should consider examining other pro-inflammatory cytokines like those that have been previously observed after TBI or alcohol consumption such as IL-1β, IL-6, IL-8/MIP, IL-10, and MCP-1 simultaneously in each sample. Likewise, it would be useful to investigate microglia and neuron specific recruitment of the pro-inflammatory cytokines to better understand the specific targets of the pro-inflammatory cytokines.

## EXPERIMENT 3: COMBINED EFFECTS OF REPETITIVE MILD TRAUMATIC BRAIN INJURY AND CHRONIC ALCOHOL CONSUMPTION ON COGNITION AND BEHAVIOR

Each year an estimated 1.5 million Americans seek medical attention for a traumatic brain injury (TBI) while many more injuries go unreported altogether (Faul M, Xu L, Wald MM, 2010; Niska et al., 2010). Even still, the economic impact to treat TBI is nearly $40 billion dollars annually (Silver et al., 2001). The majority of these injuries are considered mild and are difficult to observe using MRI or CT scans that are the current standard for brain imaging (Delouche et al., 2015). Despite the relatively minor physical damage to brain tissue, symptoms include headache, dizziness, impaired memory, confusion, irritability, fatigue, and poor concentration (Alexander, 1995; Karr, Areshenkoff, & Garcia-Barrera, 2014; National Center for Injury Prevention and Control, 2003).

Many studies and meta-analyses show mTBI patients generally show full cognitive, behavioral, and cerebral glucose metabolic recovery by 3 months post-injury (Belanger, Curtiss, Demery, Lebowitz, & Vanderploeg, 2005; Karr et al., 2014; Weil, Gaier, & Karelina, 2014). However, 10-15% of patients with mTBI do not show recovery and continue to present with headache, neck pain, or dizziness, characteristics of symptomatic persistent post-concussive syndrome (PPCS) which is a more permanent form of post-concussive syndrome (PCS) (Alexander, 1995). Cognitive symptoms of PPCS are similar to initial cognitive complaints and include problems with attention, impaired memory, and other reduced executive functions (Alexander, 1995; Ashman et al., 2006; Belanger et al., 2005; Harmon et al., 2013; Holm et al., 2005; Vagnozzi et al., 2010). Recovery can be prolonged by additional disturbances to the brain during the initial recovery period of mTBI (e.g. a second mTBI, extreme physical exertion;

Ashman et al., 2006; Goodman et al., 2013; Harmon et al., 2013; Prins et al., 2011; Solomon & Zuckerman, 2015; Weil et al., 2014).

Alcohol use can also greatly impact an individual's recovery from TBI and lead to greater risk of incurring additional TBIs (Corrigan et al., 2013). Increased drinking following a TBI can be predicted by history of use prior to the injury (Bombardier et al., 2003; Dikmen et al., 1995; Horner et al., 2005; Rogers & Read, 2007), but can also occur in patients who had never had substance abuse disorder in either civilian (J. D. Corrigan, 1995; Hibbard et al., 1998; Massagli et al., 2004; Silver et al., 2001) or military populations (Adams et al., 2013; Herrold A. et al., 2014; Miller & Baktash, 2013; Tanielian, T., Jaycox, 2008). Regardless of previous drinking history, heavy alcohol consumption during recovery of TBI can lead to delayed recovery and detrimental outcomes in terms of health, interpersonal relationships, and psychological well-being (J. D. Corrigan, 1995). It is not surprising that patients can experience substance use disorders following TBI, as there is an increase in a number of other psychological disorders including depression/mood disorders, post-traumatic stress disorder, and anxiety disorders (Hibbard et al., 1998; Jorge et al., 2005; Silver et al., 2001).

Alcohol use disorders can be devastating to a person's health, finances, and interpersonal relationships and can co-occur with other mental health disorders independently of TBI (Center for Behavioral Health Statistics and Quality, 2015). Since heavy drinking following a TBI can increase recovery times, potentially due to additional damage via neuroinflammation or other mechanisms (Crews et al., 2006; Ghirnikar et al., 1998; He & Crews, 2008), it is important to determine how drinking influences cognitive and behavioral performance in those with TBI. Animal models of repetitive mTBI have already demonstrated poorer cognitive and behavioral outcomes after multiple mTBIs (Prins et al., 2011; Yang et al., 2015). However, researchers have found mixed results on cognitive performance when ethanol was administered prior to or following acute and repetitive mTBI in rodent models. For example, one group of researchers

found no effects on neurological or behavioral outcomes when ethanol was administered prior to acute mTBI (S X Teng & Molina, 2014), and others showed delayed recovery of sensorimotor function (Vaagenes et al., 2015). Others have found that ethanol administered chronically after mTBI, produced mild neurological deficits, impaired memory on a novel object recognition task, and decreased locomotor activity (Mayeux et al., 2015; Sophie X. Teng et al., 2015). This study sought to contribute to the growing body of literature by investigating the effects of voluntary alcohol consumption following repetitive mTBI in rodent models on cognitive and behavioral performance using the open field exploration test, novelty preference, novel object recognition, and the serial spatial discrimination reversal learning task (SSDRL, a measure of behavioral flexibility).

## Hypotheses

Previous literature has demonstrated deficits in behavioral flexibility after high dose ethanol administration in mice (Badanich, Becker, & Woodward, 2011) and various cognitive/behavioral deficits following mTBI have been reported (Prins et al., 2011; Yang et al., 2015), therefore it was hypothesized that all would mice show deficits on the behavioral flexibility task due to ethanol consumption, but those injured would have greater deficits including worse performance on the novel object recognition task.

## Methods

### Voluntary Ethanol Consumption

The same model of repetitive mTBI and ethanol voluntary consumption described in Experiment 1 was used again. A total of 216 (8-12 weeks old) male C57BL/6J mice (Envigo Laboratories, Indianapolis, IN, USA) were tested (n = 12 mice per condition, rmTBI or sham)

after 0, 2, 4, 6, and 8 weeks of ethanol exposure or no ethanol exposure. Two days following the last ethanol exposure, mice were subjected to a battery of cognitive-behavioral tasks over six days to determine how excessive drinking after a rmTBI effects cognitive performance. All behavior was recorded and analyzed using Any-maze video tracking software (Stoelting Co., IL, USA).

**Cognitive and Behavioral Measures**

Cognitive and behavioral tasks include the behavioral flexibility task, SSDRL, open field test (OF), novelty preference, and novel object recognition (NOR). The SSDRL task is a general measure of global cognitive function and behavioral flexibility. It requires the mice to learn a strategy using spatial cues, recognize when this strategy no longer works, and switch to a new strategy. The OF test provided a measure of general locomotor activity after habituating to a new environment. Novel object recognition served as a measure of novelty preference and working memory function; for a detailed outline of the schedule see Table 1.

| **Table 1.** Experiment 3 Cognitive Assessment Schedule | |
|---|---|
| Day 1 | *SSDRL*: Pretrial training (10 trials) |
| Day 2 | *SSDRL*: Test Day (30 trials) |
| Day 3 | *SSDRL*: Test Day (30 trials) |
| Day 4 | *SSDRL*: Test day (30 trials) |
| Day 5 | *Open Field*: Open field habituation/behavioral reaction to the novel environment for 3 min in a normal open field (Trial 1). 30min Delay. *Open Field*: 3 min open field exposure test (Trial 2) to test locomotor activity. |
| Day 6 | *Novelty Preference, and Novel Object Recognition*: One 3 min open field exposure test (Trial 3) with a novel object in the center to measure novelty preference. After a 30min delay, a final 3 min trial in the open field area with the familiar object and a new object to gage object recognition (Trial 4). |

**Serial Spatial Discrimination Reversal Learning.** Using a partially submerged T-Maze (overall upper arm length 24in, arm width 3-3.5 in, base leg length 15in, wall height 15in), mice were required to learn to escape from the water (24-26°C, dyed opaque with non-toxic, water-

soluble white paint) using a platform at one of the arms. The first day of this task there was a pre-trial training session to habituate the mice to the task. The arms of the T-Maze are blocked in a pseudorandom order ensuring the mice have learned to escape from both sides of the maze at least five times prior to testing. During the subsequent three days of testing, mice learned to escape by remembering the location of the escape platform to be on the left or right arm of the maze. Once a rule has been sufficiently learned (criterion of six correct choices with no errors), the rule was reversed so that the platform was on the other side of the maze. The mice completed 30 trials per day and were kept in a warmed environment (approximately 31°C) between trials (1 to 2 inter-trial minute intervals) to prevent hypothermia.

All animals started testing on their non-preferred arm. This was determined by allowing the animal to choose freely between the two arms on the first test trial and placing the escape platform in the opposite arm. Errors included entering the incorrect arm, re-entry to the starting stem, and backtracking through the correct arm without escaping. Because the criterion to reverse the contingencies was six correct trials, if an animal completed two consecutive trials during the final trials of the day, an additional four trials were added in an attempt to allow the animal to reach the criterion. If the mouse made an error during one of the extra trials, testing ended for the day.

**Open Field with Novelty Task and Recognition Task.** These tasks assessed locomotor activity (distance traveled, time spent in the center), preference for novelty, and working memory (via recognition of familiar object and time spent with novel object).

On day 5, mice freely explored the OF arena for 3 min to habituate to the environment; approximately 30 minutes later an additional 3 min session took place to monitor the animals locomotor activity. On day 6, a 3 min exploration included a novel object. After a 30min delay, a second object was also added to the arena and the mouse was allowed another 3 min to explore. All objects used were no more than twice the size of the mice and did not resemble

living stimuli (e.g. no animal shapes or eyes). The arena and all objects were cleaned with 40% ethanol wipes to sanitize and deodorize between animals.

**Analyses**

All analyses were conducted with SPSS 25.0 (IBM). A p-value of less than 0.05 was set to evaluate significance unless otherwise indicated. Outliers were identified and removed following Tukey's method creating boxplots and removing data points falling outside the ±1.5 interquartile range. Results of analyses that are not mentioned specifically are non-significant.

In order to test the prediction that mice with rmTBI would perform more poorly on the SSDRL compared to sham mice, an ANOVA was used to compare performance scores of rmTBI and sham groups for each cohort (0, 2, 4, 6, or 8 weeks). Poorer performance on this task was determined by an increased number of trials to reach criterion, increased number of errors, and fewer successful reversals of strategy.

In order to test the impact of injury on locomotor activity in the open field paradigm, the average distance traveled in Trial 2 of the open field series was determined using Any-maze video tracking software. An ANOVA was used to compare performance scores of rmTBI and sham groups for each cohort (0, 2, 4, 6, or 8 weeks). In order to test the prediction that memory performance during the novel object recognition task would be impaired due to rmTBI, an ANOVA was conducted examining the relative time spent with the novel object compared to the familiar object for each cohort.

## Results

### Voluntary Consumption

A 2 x 2 Procedure (rmTBI, Sham) x Week (1, 2) mixed ANOVA was conducted to determine whether there were differences in average daily consumption of ethanol in the 2 Week cohort. Results shown in Figure 1, indicated a significant main effect of Week, $F(1, 38) = 6.422$, $p < .05$, and Bonferroni corrected pairwise comparisons showed mice drank less ethanol in Week 2 (M= 1.45g/kg) compared to Week 1 (M = 2.00g/kg). A 2 x 2, Procedure (rmTBI, Sham) x Week (1, 2) mixed ANOVA was used to examine differences in daily ethanol preference. Results revealed a significant main effect of Procedure, $F(1, 38) = 5.072$, $p < .05$, and Bonferroni adjusted pairwise comparison showed rmTBI to cause a decreased ethanol preference in rmTBI mice (M = 75.1%) compared to Sham mice (M = 84.3%).

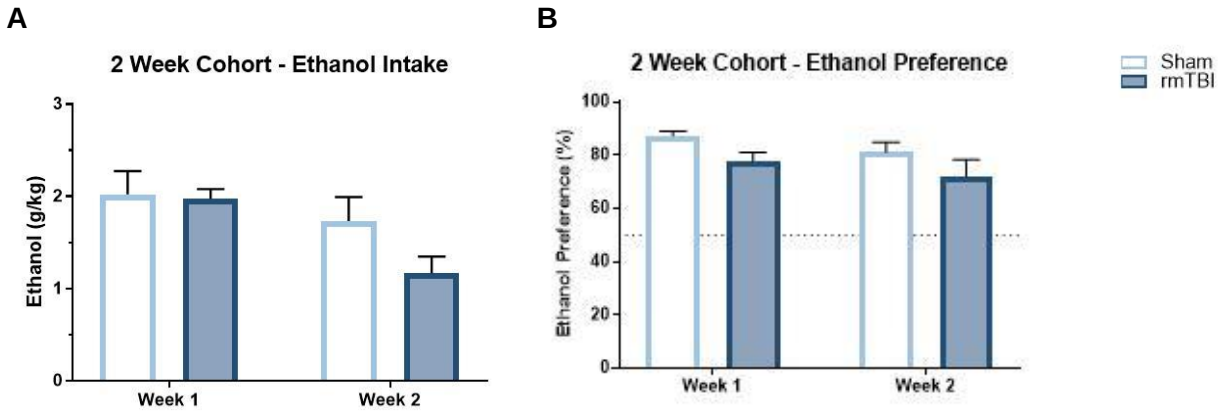



**Figure 1.** Experiment 3 Ethanol Intake – 2 Week Cohort
A) Average daily voluntary ethanol consumption and B) ethanol preference in the 2 Week Cohort.

In the 4 Week cohort, a 2 x 4 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4) mixed ANOVA was used to determine differences in average daily consumption of ethanol (g/kg). Results, as shown in Figure 2, revealed significant main effects of both Week, $F(3, 78) = 6.47$, p

< .05 and Procedure, F(1, 78) = 11.45, p < .05. Pairwise comparison using Bonferroni corrections indicated mice consumed less ethanol in Week 2 (M = 1.50g/kg) and Week 3 (M = 1.36g/kg) compared to Week 1 (M= 2.16g/kg), and recovered to initial levels in Week 4 (M = 1.91g/kg). Across time, rmTBI mice consumed less ethanol (M = 1.49g/kg) compared to Sham (M = 1.98g/kg). In order to evaluate the decreased ethanol consumption observed in the 4 Week cohort, a 2 x 4 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4) mixed ANOVA was used to examine total fluid (ethanol and water) intake (ml). Results showed a decrease in the total fluid consumed that corresponds with the decrease in rmTBI specific ethanol (g/kg) consumption during Week 2, F(3, 78) = 4.363, p < .05 suggesting the decrease in ethanol consumption coincides with a total decrease in fluid intake by rmTBI mice during Week 2.

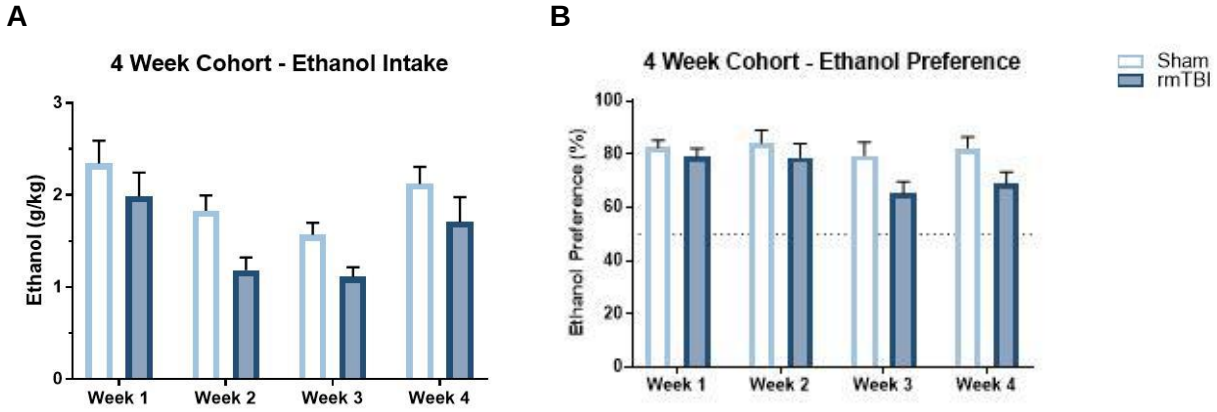



**Figure 2.** Experiment 3 Ethanol Intake – 4 Week Cohort
A) Average daily voluntary ethanol consumption and B) ethanol preference in the 4 Week Cohort.

Additionally, to examine differences in average ethanol preference a 2 x 4 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4) mixed ANOVA was conducted and revealed a significant main effect of TBI, F(1, 78) = 8.890, p < .05. Bonferroni adjusted pairwise comparison showed a decrease in ethanol preference for rmTBI mice (M = 73.1%) compared to Sham (M = 82.2%).

A 2 x 6 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4, 5, 6) mixed ANOVA examining average ethanol consumption and another comparing average ethanol preference in the 6 Week cohort did not reveal any differences across weeks or between the TBI and Sham conditions; results illustrated in Figure 3.

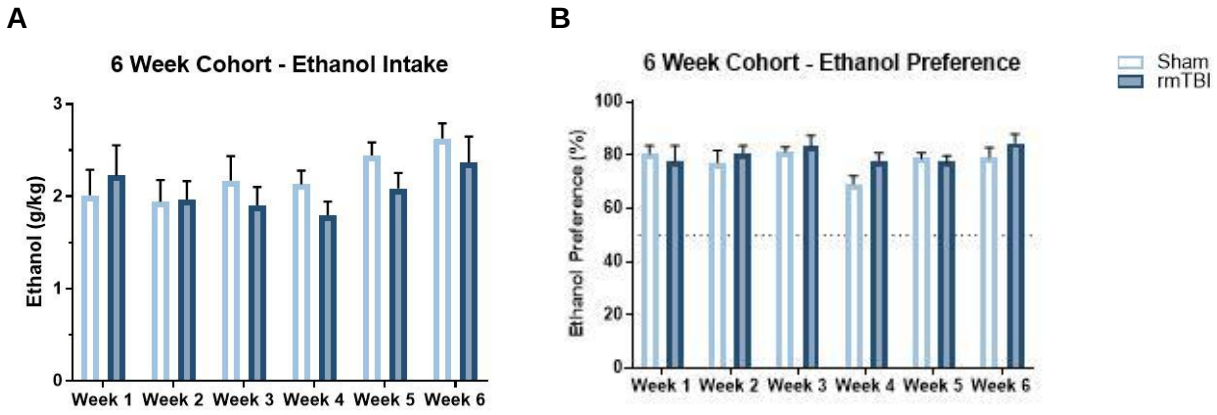



**Figure 3.** Experiment 3 Ethanol Intake – 6 Week Cohort
A) Average daily voluntary ethanol consumption and B) ethanol preference in the 6 Week Cohort.

In the Week 8 cohort, 2 x 8 Procedure (rmTBI, Sham) x Week (1, 2, 3, 4, 5, 6, 7, 8) mixed ANOVA comparing average ethanol consumption (g/kg) did not show any significant differences, but when an additional analysis was conducted examining differences in ethanol preference, a main effect of Procedure was found, $F(1, 160) = 28.432$, $p < .05$. As shown in Figure 4, pairwise comparison using Bonferroni corrections revealed rmTBI mice had a greater preference for ethanol (M = 87.2%) compared to Sham (M = 76.4%).

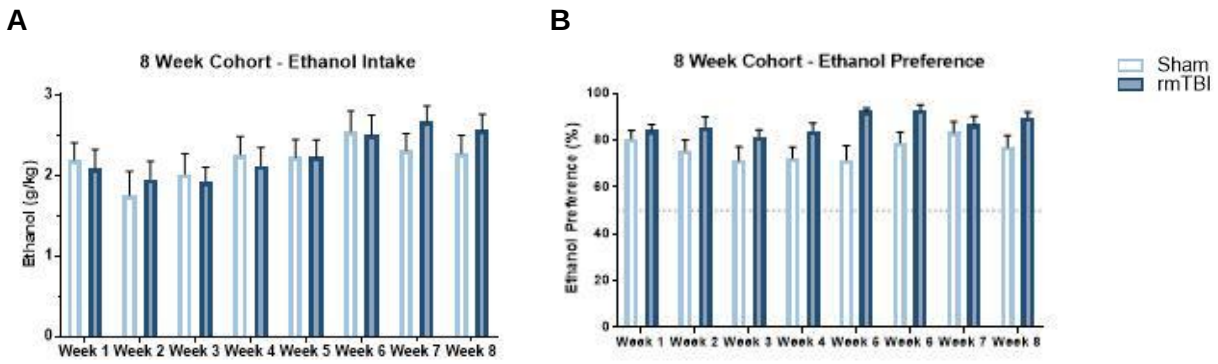



**Figure 4.** Experiment 3 Ethanol Intake – 8 Week Cohort
A) Average daily voluntary ethanol consumption and B) ethanol preference in the 8 Week Cohort.

Although in the 8 Week cohort rmTBI mice exhibited a unique increase in ethanol preference, the effects of rmTBI on voluntary ethanol consumption in the 2, 4 and 6 weeks cohorts were similar to what was observed in Experiment 1. Again, there was insufficient evidence to support the hypothesis that rmTBI leads to increased ethanol drinking and preference, and instead, the data indicate rmTBI may decrease ethanol consumption during weeks 2 and 3 specifically as observed in the 4 Week cohort.

### Cognitive and Behavioral Testing

To make the results of the SSDRL task more tenable, in each cohort, the group mean for the Sham, No Ethanol group during the simple discrimination task was set to 100% baseline for both “Trials to Criterion” and “Errors to Criterion” measures. The percent difference from baseline was analyzed to determine effects of the Task (Simple Discrimination or Reversal Learning), TBI, and Ethanol Exposure via repeated measures ANOVA for each cohort.

Measures of locomotor activity were analyzed for differences in distance traveled (See Table 2), and the percent time spent in the center of the OF arena for TBI and Ethanol Exposure for each cohort (0 Week, 2 Week, 4 Week, 6 Week, 8 Week). Additionally, novel object

recognition was measured by comparing the percentage of time spent with a novel object or familiar object by TBI and Ethanol Exposure for each cohort (0 Week, 2 Week, 4 Week, 6 Week, 8 Week).

**Table 2.** Distance Travelled (m)

| | 0 Week | | | 2 Week | | | 4 Week | | | 6 Week | | | 8 Week | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Condition | M | SD | N | M | SD | N | M | SD | N | M | SD | N | M | SD | N |
| Sham, No Drinking | 5.6 | 1.6 | 12 | 6.8 | 1.6 | 12 | 5.7 | 1.7 | 12 | 6.8 | 1.9 | 12 | 6.5 | 2.2 | 12 |
| Sham, Drinking | -- | -- | -- | 6.4 | 1.6 | 12 | 7.2 | 1.5 | 12 | 6.4 | 2.3 | 12 | 4.8 | 2.7 | 11 |
| TBI, No Drinking | 5.8 | 2.0 | 12 | 6.9 | 2.4 | 12 | 7.2 | 1.9 | 12 | 7.7 | 1.7 | 12 | 5.5 | 1.4 | 12 |
| TBI, Drinking | -- | -- | -- | 6.8 | 2.3 | 11 | 7.1 | 1.7 | 11 | 6.5 | 1.8 | 11 | 6.1 | 1.7 | 12 |

Locomotor activity – distance tables

**0 Week Cohort.** The 0 Week cohort was tested following the rmTBI or sham procedure period and therefore were not exposed to ethanol. A repeated measures ANOVA was used to determine differences in the percentage of trials to criterion and revealed a significant main effect of Task, $F(1, 18) = 18.396$, $p < .05$. As shown in Figure 5, mice displayed an increased percentage of trials necessary to meet learning criteria during the Reversal task (180.2%) compared to the Simple Discrimination trial (M = 104.4%). A repeated measures ANOVA analysis to compare the percentage of errors relative to the Sham, No Ethanol group found a similar result with a main effect of Task $F(1, 18) = 40.018$, $p < .05$. Adjusted pairwise comparisons also showed mice had an increased percentage of errors in Reversal (M = 446.7%) compared to Simple Discrimination (M = 104.4%). These findings verify the difficulty of reversal learning in both Sham and rmTBI mice and indicate that rmTBI by itself did not significantly alter behavioral flexibility 3 days following the final rmTBI exposure. No differences were observed for distance traveled, time spent in the center, or time spent with the novel object.

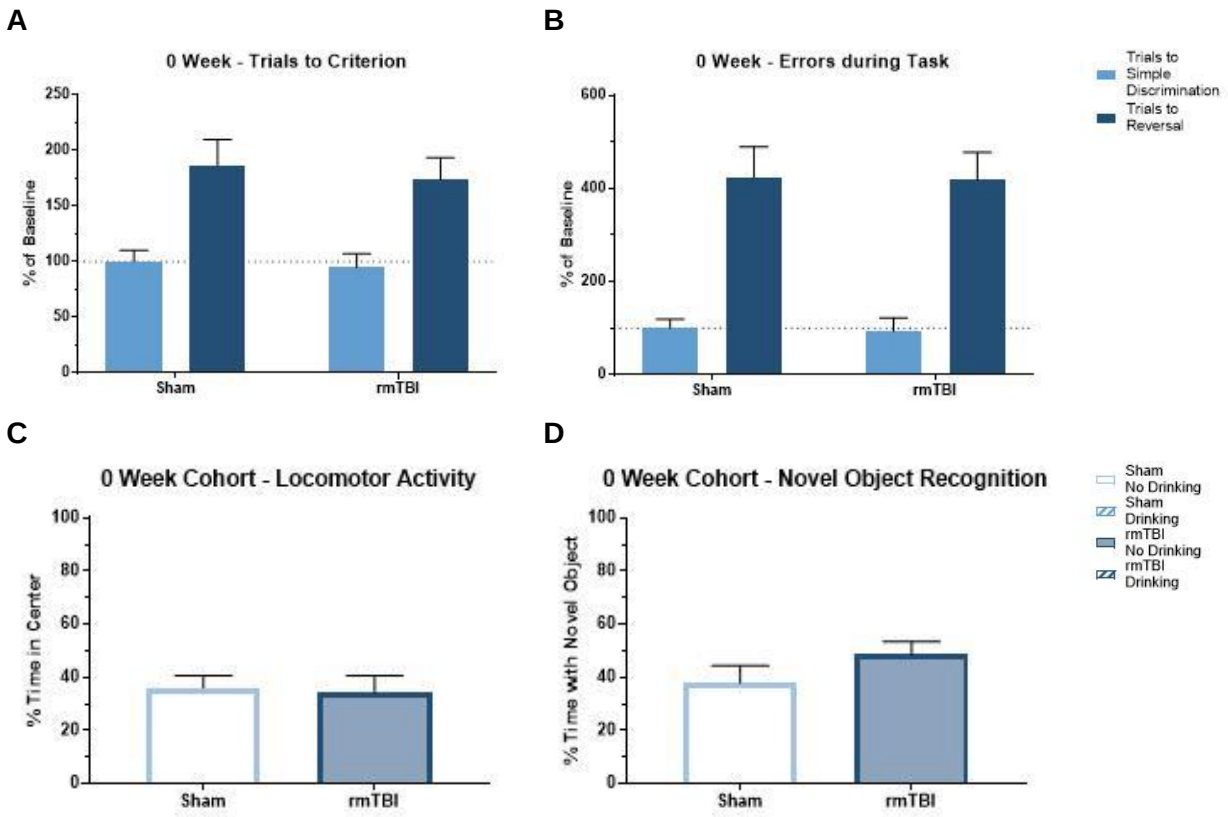



**Figure 5.** Cognition and Behavior for 0 Week Cohort
Panel A) Percentage of trials to criterion B) Percentage of errors during criterion, C) Percent time spent in center during locomotor activity trial, D) Percent time spent with novel object vs familiar object

**2 Week Cohort.** Results are illustrated in Figure 6. To determine differences in the percentage of trials to criterion in the 2 Week cohort, a repeated measures ANOVA was conducted. Results indicated a significant main effect of Task, $F(1, 38) = 16.027$, $p < .05$; however, this effect was qualified by a significant three-way interaction of Task x Procedure x Ethanol Exposure, $F(1, 38) = 7.628$, $p < .05$. Bonferroni adjusted pairwise comparisons revealed that mice in the rmTBI, No Ethanol group displayed an increase in the percentage of trials to criterion from the Simple Discrimination task (M = 69.4%) to the Reversal task (M = 168.6%). Additionally, mice in the rmTBI, Ethanol group required a greater percentage of trials to reach criterion during Simple Discrimination (M = 134.4%) than the rmTBI, No Ethanol group

(M = 69.4.4%). Finally, mice in the rmTBI, No Ethanol group displayed an increase in percent trials to Reversal (M = 168.6%) compared to the Sham, No Ethanol group (M = 90.1%).

To determine whether there were differences in the percentage of errors made, repeated measures ANOVA was conducted. Results revealed a significant main effect of Task, $F(1, 38) = 36.904$, $p < .05$; however, this effect was qualified by a significant three-way interaction of Task x Procedure x Ethanol Exposure, $F (1, 38) = 7.303$, $p < .05$. Corrected pairwise comparisons showed an increase in percent errors for the Sham, Ethanol group from simple discrimination (M = 79.8%) to reversal (M = 183.5%), as well as for the TBI, No Ethanol group from simple discrimination (M = 49.2%) to reversal (M = 309.7%). Additionally, mice in the rmTBI, Ethanol group committed a larger percentage of errors before reaching criterion during simple discrimination (M = 155.7%) compared to the rmTBI, No Ethanol (M = 49.2.0%). Finally, mice in the rmTBI, No Ethanol group showed an increase in percent errors during reversal (M = 309.7%) compared to the Sham, No Ethanol group (M = 170.9%). No differences were observed for distance traveled, time spent in the center, or time spent with the novel object. These findings suggest that 2 weeks following the final rmTBI exposure, mice in the rmTBI group exhibit some increased difficulty in both learning a simple discrimination and behavioral flexibility and that 2 weeks of ethanol exposure may exacerbate difficulties in learning a simple discrimination 2 weeks following rmTBI.

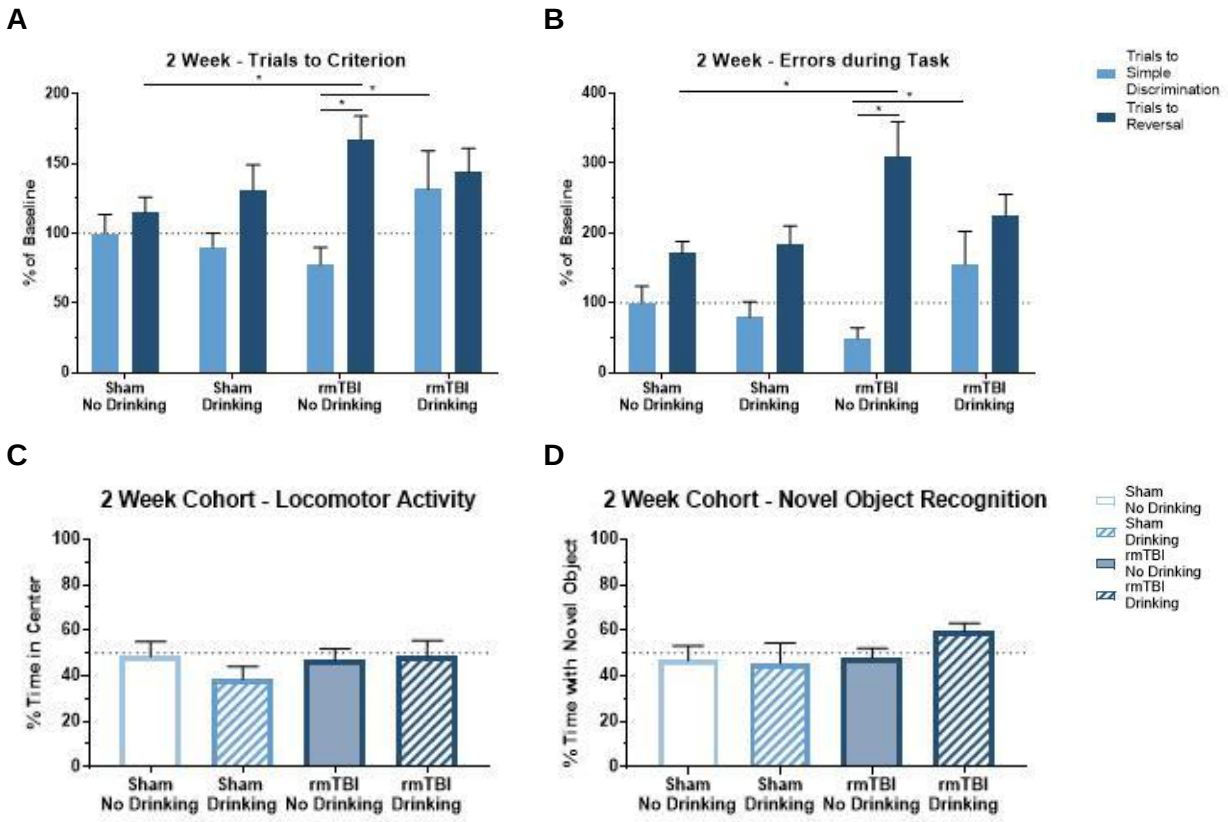



**Figure 6.** Cognition and Behavior for 2 Week Cohort
Panel A) Percentage of trials to criterion B) Percentage of errors during criterion, C) Percent time spent in center during locomotor activity trial, D) Percent time spent with novel object vs familiar object

**4 Week Cohort.** To determine differences in the percentage of trials to criterion in the 4 Week cohort, a repeated measures ANOVA was conducted and revealed a significant main effect of Task $F(1, 38) = 10.026$, $p < .05$. As illustrated in Figure 7, corrected pairwise comparisons showed mice required an increase in the percentage of trials to complete the Reversal task (122.2%) compared to the Simple Discrimination task (M = 93.3%). The repeated measures ANOVA used to compare the percentage of errors produce a similar finding with a main effect of Task $F(1, 38) = 35.468$, $p < .05$. Adjusted pairwise comparisons again showed an increased difficulty (more errors) to complete the Reversal task (M = 207.4%) compared to the Simple Discrimination task (M = 87.7%). Therefore, 4 weeks following rmTBI or a sham

procedure, mice given water or exposed to ethanol exhibited typical difficulties with behavioral flexibility.

To examine differences in novelty preference in the novel object recognition task, a 2 x 2, Procedure (rmTBI, Sham) x Ethanol Exposure (Drinking, No Drinking) ANOVA comparing percent time spent with a novel object vs a familiar object was conducted and revealed a main effect of Procedure, $F(1 , 43) = 4.138$, $p < .05$, as well as, a main effect for Ethanol Exposure, $F(1 , 43) = 10.519$, $p < .05$. Bonferroni corrected pairwise comparisons indicated that rmTBI mice spent a greater percentage of time with the novel object (M = 65.2%) than Sham mice (M = 52.2%). Pairwise comparisons for Ethanol Exposure indicated the mice that drank ethanol generally spent a greater percentage of time with the novel object (M = 69.1%) compared to those that did not drink ethanol (M =48.3%. These findings indicate that novelty preference is elevated 4 weeks following the final rmTBI procedure and that 4 weeks of ethanol exposure produces similar effects, but do not support the hypothesis that ethanol exposure exacerbates that effects of rmTBI on novelty preference.

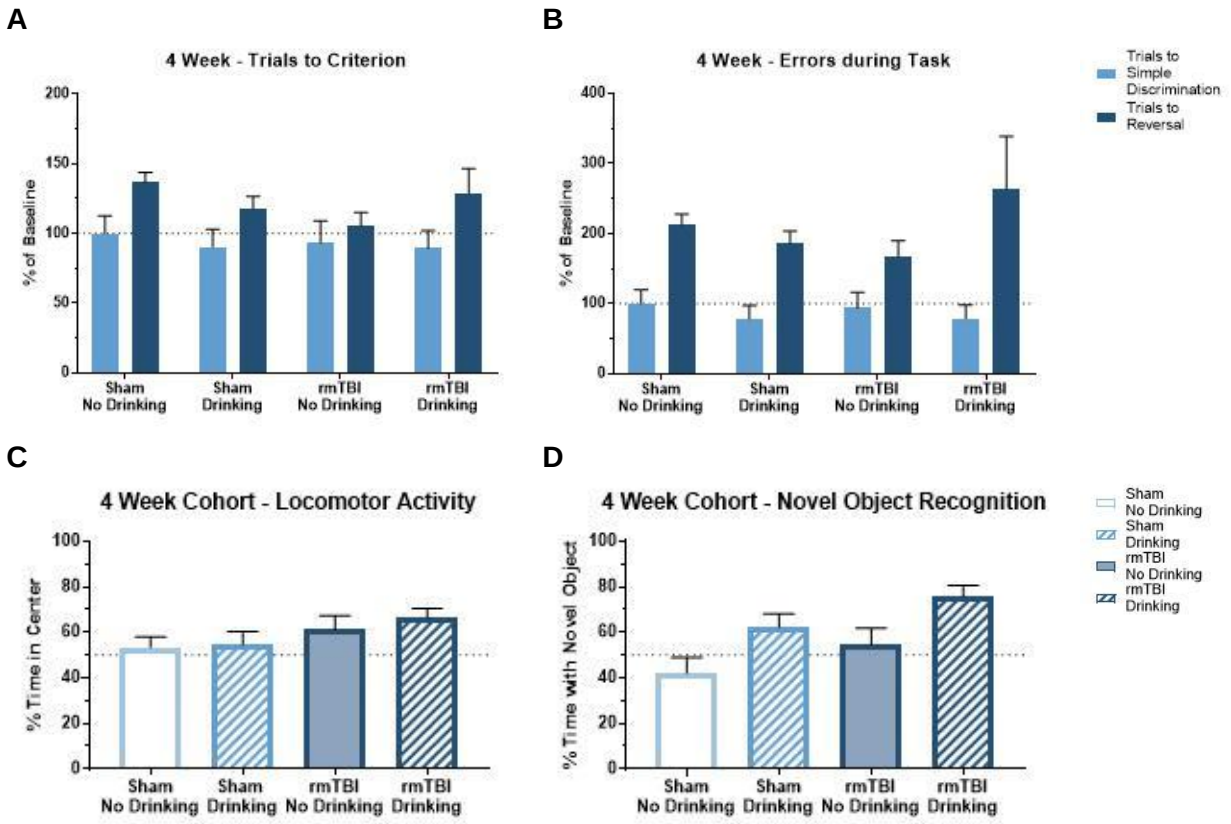



**Figure 7.** Cognition and Behavior for 4 Week Cohort
Panel A) Percentage of trials to criterion B) Percentage of errors during criterion, C) Percent time spent in center during locomotor activity trial, D) Percent time spent with novel object vs familiar object

**6 Week Cohort.** To determine differences in the percentage of trials to criterion in the 6 Week cohort, a repeated measures ANOVA was conducted and revealed a significant main effect of Task, $F(1, 36) = 11.476$, $p < .05$, and a significant Task by Procedure interaction, $F(1, 35) = 4.978$, $p < .05$; however, these effects were qualified by a significant three-way interaction of Task x Procedure x Ethanol Exposure, $F (1, 36) = 6.516$, $p < .05$. As illustrated in Figure 8, Bonferroni adjusted pairwise comparisons indicated that mice in the Sham, No Ethanol group showed an increased percentage of trials required to complete the Reversal task (M = 163.4%) compared to the Simple Discrimination task (M = 100.0%). The same effect was noted for the rmTBI, Ethanol mice when comparing Simple Discrimination (M = 78.8%) to Reversal (M = 117.9%). Additionally, mice in the rmTBI, No Ethanol showed increased difficulty completing the

Simple Discrimination task (M = 133.4%) compared to the rmTBI, Ethanol group (M = 78.8%). Finally, mice in the Sham, No Ethanol group showed increased difficulty with behavioral flexibility when comparing the percentage of trials to criterion for the Reversal task (M = 163.4%) to the rmTBI, No Ethanol group (M = 114.2%).

To examine differences in the percentage of errors committed during a task, a repeated measures ANOVA was conducted and revealed a significant main effect of Task, $F(1, 36) = 56.961$, $p < .05$, and a significant Task by Procedure interaction, $F(1, 35) = 7.102$, $p < .05$; however, these effects were qualified by a significant three-way interaction of Task x Procedure x Ethanol Exposure, $F (1, 36) = 6.041$, $p < .05$. Corrected pairwise comparisons showed mice in the Sham, No Ethanol group committed more errors during the Reversal task (M = 339.1%) compared to Simple Discrimination (M = 100.0%). This was also true for the Sham, Ethanol group when comparing percentage of errors in Reversal (M = 262.9%) and Simple Discrimination (M = 131.4%), as well as for mice in the rmTBI, Ethanol group, showing an increase in percentage of errors during Reversal (M = 223.0%) when compared to Simple Discrimination (M = 99.1%). Although rmTBI, No Ethanol mice did not exhibit a greater percentage of errors during reversal when compared to simple discrimination, these mice produced a higher percentage of errors (M = 185.7%) while completing the Simple Discrimination task than either the Sham, No Ethanol group (M = 100.0%) or the TBI, Ethanol group (M = 99.1%), indicating cognitive impairment. Interestingly, the rmTBI, No Ethanol group produced a lower percentage of errors during Reversal (M = 239.0%) when compared to the Sham, No Ethanol group (M = 339.1%), but this was accompanied by an impairment of simple discrimination suggesting increased difficulty with learning a simple discrimination in the rmTBI, No Ethanol group. No differences were observed for distance traveled, time spent in the center or time spent with the novel object.

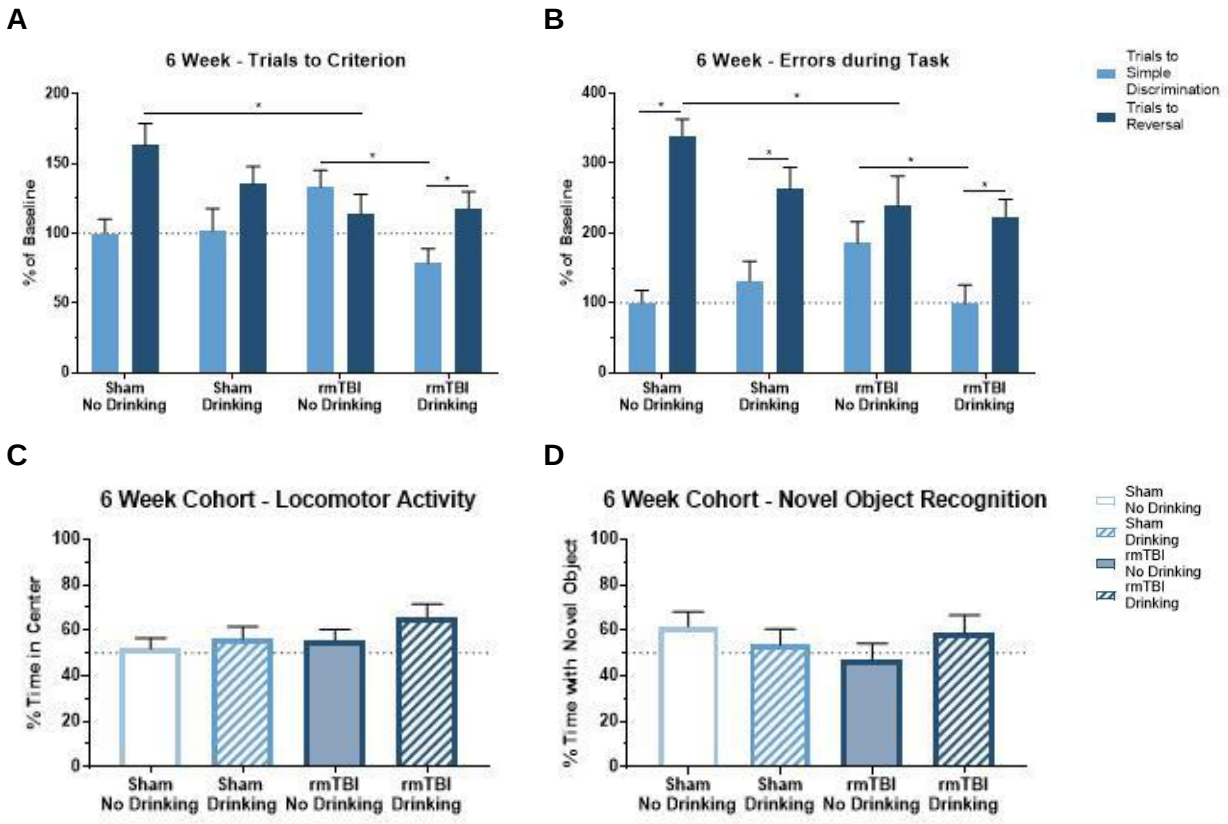



**Figure 8.** Cognition and Behavior for 6 Week Cohort
Panel A) Percentage of trials to criterion B) Percentage of errors during criterion, C) Percent time spent in center during locomotor activity trial, D) Percent time spent with novel object vs familiar object

**8 Week Cohort.** To determine differences in the percentage of trials to criterion in the 8 Week cohort, a repeated measures ANOVA was used and found a significant main effect of Task, $F(1, 35) = 12.891$, $p < .05$. As shown in Figure 9, Bonferroni adjusted pairwise comparisons confirmed mice required an increased percentage of trials to complete the Reversal task (167.3%) compared to the Simple Discrimination task (M = 110.4%).

To examine differences in the percentage of errors committed during a task, a repeated measures ANOVA was conducted and showed a significant main effect of Task, $F(1, 35) = 37.484$, $p < .05$; however, this effect was qualified by a significant three-way interaction between Task, rmTBI, and Ethanol Exposure, $F(1, 38) = 6.267$, $p < .05$. Corrected pairwise comparisons show mice in the Sham, No Ethanol group committed more errors in the Reversal task (M =

395.8%) compared to the Simple Discrimination task (M = 100.0%). Likewise, the Sham, Ethanol mice showed an increase in percentage of errors during the Reversal task (M = 276.3%) compared to the Simple Discrimination task (M = 147.0%) and the TBI, Ethanol group displayed a similar increased percentage of errors committed during Reversal (M = 371.4%) compared to Simple Discrimination (M = 138.7%). Mice in the rmTBI, No Ethanol group did not exhibit a greater percentage of errors during reversal than during simple discrimination which is suggestive of difficulty with learning the simple discrimination task. However, the rmTBI, No Ethanol group also produced a smaller percentage of errors (M = 199.3%) during reversal when compared to the Sham, No Ethanol group (M = 395.8%) or the TBI, Ethanol group (M = 371.4%), suggesting less difficulty with behavioral flexibility than the other groups.

To examine differences in novelty preference in the novel object recognition task, a 2 x 2, Procedure (rmTBI, Sham) x Ethanol Exposure (Drinking, No Drinking) ANOVA comparing percent time spent with a novel object vs a familiar object was conducted and revealed a main effect of Ethanol Exposure, $F(1, 42) = 6.995$, $p < .05$. Bonferroni correct pairwise comparisons showed Ethanol exposed mice spent more time with the novel object (M = 72.9%) compared to the No Ethanol group (M =54.9%).

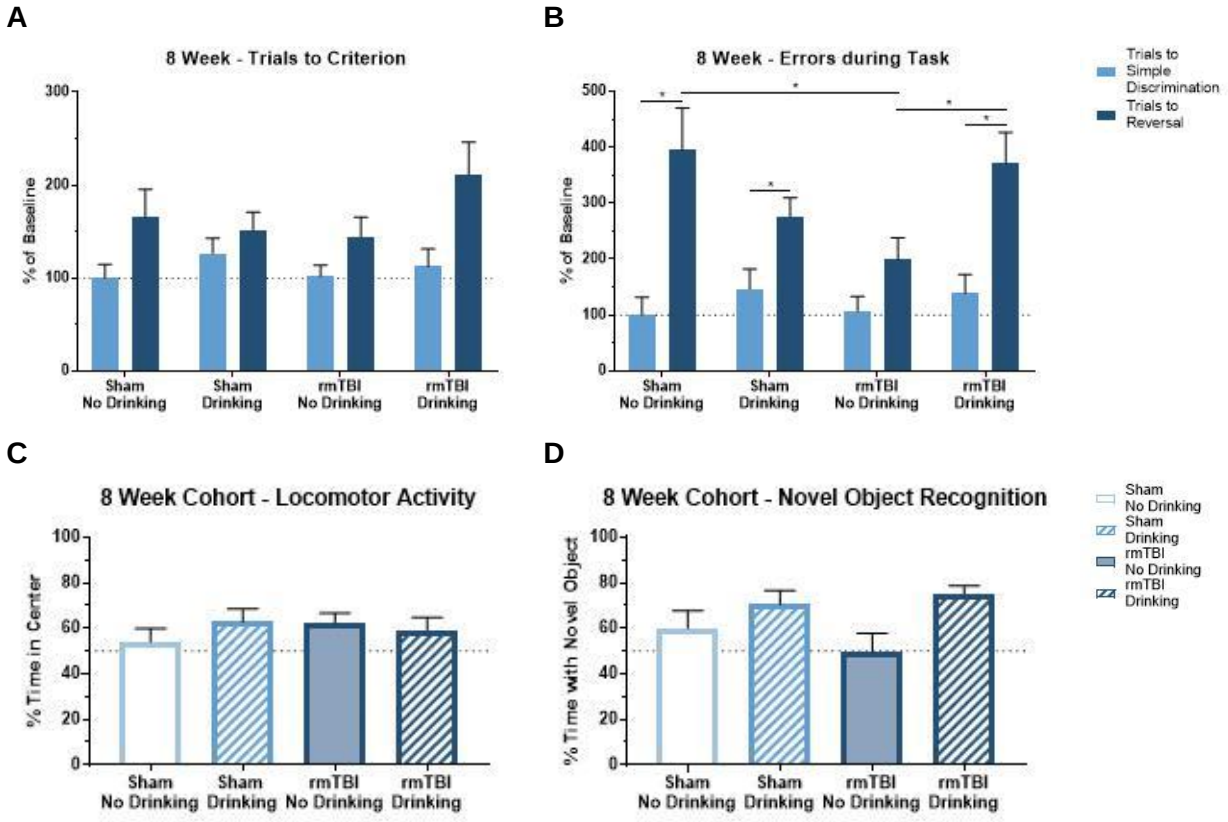



**Figure 9.** Cognition and Behavior for 8 Week Cohort
Panel A) Percentage of trials to criterion B) Percentage of errors during criterion, C) Percent time spent in center during locomotor activity trial, D) Percent time spent with novel object vs familiar object

In summary, the SSDRL task provided a valid measure of behavioral flexibility by demonstrating a general capacity for learning a simple discrimination task and increased difficulty with reversal learning. Inconsistent patterns of cognitive deficits were observed in the 2, 6 and 8 Week cohorts. This suggests nature of cognitive dysfunction related to rmTBI and voluntary ethanol exposure is perhaps transient and difficult to elicit consistently in animal models. The specific cognitive deficits observed came in the form of general learning dysfunction as indicated by increased difficulty learning the initial simple discrimination task, and deficits in behavioral flexibility assessed with performance on the reversal learning task. Ethanol exposure, rmTBI, or the combination did not consistently produce deficits in general learning or behavioral flexibility, rather the observed effects varied by cohort. For example in the 2 Week

cohort the rmTBI, Drinking group experienced general difficulty learning and the rmTBI, No Drinking group only displayed a strong deficit in behavioral flexibility. However, in the 6 Week cohort, the rmTBI, No Drinking group displayed general learning deficits and the rmTBI, Drinking group showed specific deficits in behavioral flexibility. In the 8 Week cohort, deficits in cognition can only be observed with the more sensitive measure of errors during each task, and suggest a more lasting rmTBI dependent effect for the rmTBI, Drinking group.

In general, the lack of evidence for differences between groups on the measures of distance traveled and time spent in center suggest there may not be lasting locomotor effects that bias the novel object recognition task or create notable anxiety dependent effects that would inhibit the mice from exploring the objects when placed in the center. The exception is for the 0 Week cohort tested following the rmTBI procedures, as this group did have notably lower percentages of time spent in center suggesting a possible increase in anxiety that also subsequently impacted the times spent with the novel objects. These animals had fewer interactions with humans as the nature of their assigned cohort (0 Week ethanol exposure) dictated a less total time in the colony. For the remaining cohorts, the novel object recognition task performance provided some support for the hypothesis predicting ethanol dependent increases in novelty preference. Specifically, increases in novelty preference due to ethanol consumption were observed in the 4 and 8 Week cohorts, and an additional influence of rmTBI on increased novelty seeking was present in the 4 Week cohort.

**Discussion**

The voluntary ethanol consumption and ethanol preference outcomes in this study generally support the transient effect of decreased intake and preference following repetitive mTBI first observed in Experiment 1. The ethanol consumption volumes also confirm a decrease in weekly variation as drinking levels stabilize over time.

The primary focus of this study was to investigate the cognitive and behavioral outcomes following the repetitive TBI or sham procedure combined with the voluntary consumption or waiting period. To do this, the SSDRL task was used to assess an animal's ability to learn a rule in a T-Maze swimming task, and then measure behavioral flexibility when that rule was changed unexpectedly. This was followed by an open field series investigating anxiety effects (decreased time spent in the center), and novelty preference as observed in a novel recognition paradigm.

There were no significant differences in distance traveled or time spent in the center for any of the cohorts, but a general increase in distance traveled and time spent in the center was observed as the cohorts' drinking or resting period increased. These effects may be due to increased handling and human exposure over time.

When there were no effects of TBI or Ethanol Exposure for the SSDRL paradigm as in the 0 Week and 4 Week cohorts, there was still increased difficulty observed when the mice were trying to reverse the previously learned simple discrimination task. The increased number of trials to criterion and errors during reversal learning help validate the assumption that behavioral flexibility is more challenging than simple discrimination learning.

More interestingly, in the 2 Week cohort, an increased difficulty with behavioral flexibility was observed in the TBI, No Ethanol group suggesting impairment due to TBI. However, a more non-specific difficulty with learning was noted in the TBI, Ethanol group as shown by deficits when learning both the simple discrimination and reversal tasks. This was supported by both the number of trials to reach criterion and the number of errors committed during each task. In the Week 6 cohort, non-specific deficits were observed in the TBI, No Ethanol group as evidenced by the increased trials to criterion and errors committed during both simple discrimination and reversal learning tasks. This general cognitive dysfunction was not repeated in the 8 Week cohort, but there was evidence for increased difficulty with behavioral flexibility in the rmTBI, Ethanol group when the number of errors during each trial were measured.

There appeared to be an increase in novelty preference for the Drinking group, and specifically the rmTBI, Drinking groups. These effects were statistically significant in the 4 and 8 Week cohorts, and this pattern persisted for the 2 and 6 Week cohorts as well. This increased novelty preference in drinking groups is not surprising, but it is interesting that it was intensified by TBI. This increase in novelty preference may be indicative of increased novelty seeking that can lead to drug and alcohol abuse or other risky behaviors in human populations.

When taken together, the data from this study do not suggest any major cognitive deficits due to repetitive mTBI, but the SSDRL task may have detected some transient deficits in non-specific learning and behavioral flexibility. This would parallel the cognitive dysfunction that is reported following rmTBI in human populations as the symptoms of confusion and disorientation are not typically apparent at all times and often show spontaneous recovery after a couple months. Furthermore, it is possible that the moderate doses of ethanol consumed in this study were not enough to exacerbate the deficits in behavioral flexibility that have been previously observed in ethanol dependent mouse models (Badanich et al., 2011) thus producing inconsistent effects of ethanol exposure.

While other researchers have been able to produce consistent cognitive deficits, important differences between the models or assessments used could be responsible. For example, the number of injuries and how the injuries are incurred could lead to differences in cognitive outcomes (Mouzon et al., 2018; Prins et al., 2011; Yang et al., 2015). It is also important to consider the type of task being measured as the diffuse nature of rmTBI vs local TBI would be important in distinguishing specific deficits, so measures of spatial memory may not produce the same deficits as a simple discrimination or reversal learning task. Similarly, in mouse models, the most noticeable protracted cognitive deficits are not produced at the moderate levels of ethanol consumption observed in this study but are more likely to be associated with dependence or binge ethanol models (Fulton T Crews et al., 2004). Combined

effects of ethanol and TBI on cognitive deficits are not consistently achieved, which impresses upon the importance for continued research (S X Teng & Molina, 2014; Sophie X. Teng et al., 2015).

**Conclusions**

The novel nature of research focusing on combined outcomes of TBI and ethanol consumption leaves plenty of opportunities for future research to expand the current findings. Cognitive and behavioral measures can be sensitive to damage to specific brain regions, and in the case of rmTBI, the damage is often non-specific. This means a number of paradigms will need to be characterized with this or similar models to better understand the memory concerns, confusion, and concentration difficulties often reported following a concussion, especially when combined with ethanol consumption during recovery. While using a model allowing voluntary consumption of ethanol is useful for translational studies, it may produce too much variability to isolate specific deficits while trying to fully characterize the deficits observed after repetitive mTBI and ethanol use. Future studies should consider the combined effects of repetitive mTBI and acute binge drinking or chronic ethanol dependence on cognitive and behavioral deficits.

## DISCUSSION

Experiment 1 of this series characterized ethanol consumption and preference after rmTBI or Sham procedures using a mouse model. The study suggested rmTBI could cause a temporary decrease in ethanol consumption, but intake values would return to Sham mice drinking levels over time. Experiment 2 investigated the gene expression of TNF-α following rmTBI and voluntary ethanol consumption. Interestingly, Sham, Drinking mice showed elevated TNF-α expression while the rmTBI, Drinking expression was attenuated in comparison. Experiment 3 examined the combined effects of rmTBI and voluntary ethanol consumption on cognition and behavior. Results indicated a transient impact of rmTBI and ethanol on learning a simple discrimination task and on behavioral flexibility, as well as a slight increase in novelty preference due to ethanol consumption and rmTBI independently.

### Experiment 1: Effects of Repetitive Traumatic Brain Injury on Voluntary Ethanol Consumption

In the first experiment of the series, mice drank ethanol (g/kg) and produced BECs at levels that would be expected with the DID paradigm (Crabbe et al., 2009; Lee et al., 2017; Marianno et al., 2017; J. S. Rhodes et al., 2007; Justin S. Rhodes et al., 2005). These results provide support for the use of this drinking model to elicit moderate voluntary ethanol consumption and high ethanol preference following rmTBI. The results also indicated a decreased ethanol consumption around the 3 and 4 week marks, similar to the spontaneous decrease in alcohol drinking observed in human populations following TBI (Ponsford et al., 2007). However, following this rmTBI-dependent decrease in ethanol consumption, mice

returned to previous drinking levels and did not show an increase in ethanol intake or ethanol preference due to rmTBI as predicted. While the goal to model excessive drinking following injury was not specifically met, this paradigm is still a useful control for future studies as the C57BL/6J is a commonly used base for creating specific genetic mutation models. While using this model has its advantages, using the C57BL/6J also inherently introduced some confounds that could be parsed out with additional studies.

The purpose of the DID paradigm is to produce relatively high levels of ethanol consumption and the chosen mouse strain, C57BL/6J, is considered a high ethanol preferring strain. Together, ethanol consumption (g/kg) and ethanol preference data showed all mice displayed an overwhelming preference for ethanol, and this may be indicative of a ceiling effect on voluntary ethanol consumption. This is important to note as it suggests that the rmTBI mice may indeed be excessively drinking post-injury, yet group differences are unable to be detected as the Sham mice are also excessive ethanol drinkers.

The DID paradigm only allows for short access to ethanol 4 of 7 days in the week to ensure the mice ramp up their drinking when ethanol is presented. It may be possible to discern more subtle differences due to rmTBI using a lower ethanol preferring rodent strain or a different drinking model (e.g. continuous access, operant self-administration, or sucrose vs quinine paradigm; for a review, see Leeman et al., 2011. Each of these drinking paradigms would offer specific advantages and disadvantages. For example, continuous access models would remove the urgency for the mice to drink during a specific window of time, thereby potentially leading to differences in total consumption or consumption periods if used with a continuous monitoring system. A disadvantage of the continuous access paradigm is the likelihood that the mice may drink more overall, but less in a given time point making it difficult to ensure that the mice drink to levels that produce physiologically relevant BECs. The use of an operant self-administration paradigm would specifically be useful if interested in how much effort a mouse would put forth in

order to drink which is important to assess motivational and reinforcing properties of ethanol. Because one of the proposed explanations of the increased ethanol consumption observed in portions of the clinical populations is related to problems with disinhibition, this paradigm could be useful to investigate ethanol consumption with progressive schedules. However, the operant paradigm requires significant training and typically makes it more difficult examine ethanol consumption following injury in a naïve model as some of the training would need to occur prior to or during the rmTBI portion of the experiment. Finally, a paradigm adding sucrose or quinine (to make the solution bitter) would be useful to compare and contrast motivational and aversive factors related to voluntary consumption patterns. These altered ethanol solutions could be used with any of the described drinking paradigms to further understand reported consumption and preference data.

An additional limitation of the study stems from the genetically homogenous nature of the C57BL/6J mouse strain. Because these mice exhibit minimal natural genetic variation, they are limited in the reproduction of effects observed in clinical populations that are likely at least partially due to genetic factors. Specifically, while millions of individuals struggle with alcohol abuse, only approximately 20% of TBI patients exhibit excessive drinking without a previous history of alcohol use disorder (Bombardier et al., 2003; Dikmen et al., 1995; Horner et al., 2005). This implies that there may be specific mechanisms such as genetic factors that increase vulnerability to excessive drinking post-injury in populations not previously engaged in problematic drinking behaviors. This mouse model is not ideal to specifically examine the possibility of genetic mechanism as the C57BL/6J mouse strain may lack the genetic characteristics necessary to model the increased drinking after TBI. However, this strain is ideal for modifying specific genes through knock-in, knock-out, and transgenic mutations to investigate genetic targets that may be responsible for this behavior.

The current mouse model represents a good control for future studies to examine specific factors related to changes in voluntary ethanol consumption following rmTBI. Changes to the drinking paradigm or the mouse model may help elucidate specific effects related to motivational factors or genetic mechanisms. Additionally, social or psychological factors may be responsible for the increased drinking post-injury, so future studies should consider the impact of stress (e.g. acute, chronic, and traumatic) and social factors like isolation on changes in drinking after TBI. These possible mediating factors could be explored with the current model or following modifications to the drinking paradigm or mouse model.

**Experiment 2: Effect of Repetitive Mild Traumatic Brain Injury and Voluntary Drinking on Neuronal Cytokines**

In the second experiment, it was expected that the combination of rmTBI and ethanol consumption would together compound the independent increase in TNF-α expression observed in ethanol consumption and TBI models. However, TNF-α expression was only notably elevated in the Sham, Drinking group presumably due to ethanol intake. Conversely, TNF-α expression was attenuated in the rmTBI, Drinking group relative to the Sham, Drinking group. These results suggest a more complex relationship between ethanol consumption and rmTBI mechanisms of inflammation.

It should be noted that the Sham and rmTBI procedures both involved exposure to 2-4% isoflurane to anesthetize the mice prior to the mTBI or to serve as the Sham procedure. Studies using mouse models to investigate clinically relevant durations of isoflurane of approximately 2hrs (1.4%) did find elevated levels of TNF-α expression due to this prolonged exposure, but the authors mention the considerably shorter exposure of 5-10s prior to euthanasia did not elicit the same TNF-α response (Wu et al., 2012). The mice in this study were exposed for a few minutes during each of the 4 rmTBI or Sham procedures, and prior to euthanasia, so while it is

possible that the reoccurring exposure to isoflurane contributed to the elevated TNF-α expression observed in this study, it is probably not a major contributing factor to the observed changes in TNF-α expression.

Understanding the possible explanations for the attenuated TNF-α expression in the rmTBI groups relative to the Sham groups for 2, 4, and 6 Week cohorts require a deeper consideration for the complicated cytokine cascade resulting from neuronal insult. The recruitment of cytokines can occur through high-mobility group protein box 1 (HMGB1) mediated effects on the family of toll-like receptors (TLR) or the receptor for advanced glycation end products (RAGE) pathways, each with unique intracellular mechanisms that lead to nuclear factor kappa B (NFκB)-dependent altered gene expression of cytokines (Crews, F. T., Sarkar, D. K., Qin, L., Zou, J., Boyadjieva, N., & Vetreno, 2015). In acute doses, ethanol has been reported to suppress some of the TLRs and thereby attenuate pro-inflammatory cytokines including TNF-α (Goodman et al., 2013; Pruett et al., 2004). Conversely, chronic ethanol exposure is most commonly associated with increases in pro-inflammatory cytokines (Fulton T. Crews, Qin, Sheedy, Vetreno, & Zou, 2013; Qin et al., 2008; Zou & Crews, 2014). To further complicate this response to neural insult via ethanol or injury, the recruitment of cytokines can independently occur at neurons, microglia, and astrocytes and may be mediated by different factors (Crews, F. T., Sarkar, D. K., Qin, L., Zou, J., Boyadjieva, N., & Vetreno, 2015).

Studies showing chronic ethanol consumption increased pro-inflammatory cytokines are in direct contrast to the attenuation of TNF-α expression observed in the rmTBI, Drinking mice. Many of these studies including the current study focus on the pro-inflammatory cytokines like TNF-α and IL1-β as researchers are interested in the mechanisms that mediate neuronal damage associated with ethanol exposure, however, other anti-inflammatory cytokines may play important roles especially in more complicated paradigms of neuronal insult. The first step to understanding the observed results would be to confirm ethanol consumption as the mediating

factor of the attenuated TNF-α expression in the current study by including rmTBI and Sham, Non-Drinking groups for comparison. Additional targets such as the anti-inflammatory cytokine IL-10, and other inhibitors of NF-κB could be assessed as well (Qin et al., 2008). It is possible that the elevated expression of IL-10 and IL-6 following TBI could continue to be neuroprotective against ethanol-induced upregulation of TNF-α and other pro-inflammatory cytokines when drinking is initiated following injury (Ziebell & Morganti-Kossmann, 2010).

While the 2, 4, and 6 Week cohorts all showed similar patterns of elevated TNF-α expression in the Sham, Drinking groups, the 8 Week cohort showed a return to control levels of TNF-α expression for rmTBI and Sham, Drinking groups. After 8 Weeks of drinking it is possible diminished TNF-α expression could be due to a form of tolerance to prolonged TNF-α elevation (Fraker, Stovroff, & Merino, 1988; Huber, Bikker, Welz, Christmann, & Brand, 2017). Recent work has indicated possible mechanisms for TNF-α tolerance that could be explored in future studies (Gunther et al., 2014).

The limitations of the current study include the solitary examination of TNF-α gene expression and lack of non-drinking groups for comparison. Future studies should consider exploring multiple cytokines simultaneously and provide additional groups for comparison, but should also consider histological measures of microglia or astrocyte reactivity, investigation of receptor density for the target cytokines, and observation of upstream targets such as HMGB1, TRL, and RAGE. This additional information would provide a better understanding of the neuronal response to combined insults of rmTBI and ethanol consumption as a whole.

**Experiment 3: Combined Effects of Repetitive Mild Traumatic Brain Injury and Chronic Alcohol Consumption on Cognition and Behavior**

The voluntary ethanol consumption data repeated in this experiment provided overall a good replication of the data obtained in Experiment 1. Key differences included increased

consumption in Experiment 1, 6 and 8 Week cohorts relative to most cohorts in Experiment 1 and Experiment 3 and increased ethanol preference specific to rmTBI in the last weeks of Experiment 3, 8 Week cohort compared to the other cohorts in Experiment 3. It is not clear how to account for the elevated ethanol intake in Experiment 1, 6 and 8 Week cohorts relative to most other groups in both Experiment 1 and Experiment 3, but it might be related to some of the variability and inconsistencies in other measures including BEC and related outcomes. The typical average daily consumption in most cohorts ranges from 1.5 – 2.5g/kg whereas the 6 and 8 Week cohorts are consistently closer to 3g/kg. The rmTBI specific increase in ethanol preference during the final weeks of Experiment 3, 8 Week cohort is less clear and possibly a matter of individual differences as this pattern was not evident in the first experiment but still within a similar range as the initial 8 Week cohort.

While the repetition of the voluntary ethanol consumption experiment was inherently useful to provide support for the initial results, the primary focus of Experiment 3 was to assess the cognitive and behavioral outcomes following the combination of rmTBI and ethanol consumption. As a whole, the SSDRL task provided a valid measure of behavioral flexibility by demonstrating a general capacity for learning a simple discrimination task and increased difficulty with reversal learning. The group dependent effects were interesting, but not entirely clear as the cognitive deficits observed were often transient between cohorts. For example, the observation of cognitive deficits in the 2 Week cohort suggested the rmTBI, Drinking group experienced general difficulty learning the simple discrimination task in addition to difficulties with reversal learning, whereas the rmTBI, No Drinking group displayed a strong deficit in behavioral flexibility only. This is in contrast to the lack of group differences in the 4 Week cohort, and contrary to the observation in the 6 Week cohort showing general learning deficits in the rmTBI, No Drinking group and behavioral flexibility specific deficits in the rmTBI, Drinking group. In the 8 Week cohort, deficits in cognition could only be observed with the more sensitive

measure of errors during each task, and suggest the more resistant rmTBI dependent effect is in the rmTBI, Drinking group.

The most obvious explanation for the inconsistent rmTBI dependent deficits is that concussive deficits are often transient in nature, and therefore are only sometimes made worse with the addition of ethanol consumption. However, this explanation cannot be empirically supported in this study as measures of neural damage are not available to correlate with the changes in cognition. The diffuse nature of mTBI makes a physical assessment of the injury more difficult, but measures of astrocyte reactivity or neuronal degeneration would be good measures to consider. Future studies should also consider using DTI imaging to measure the integrity of white matter tracts that are important to executive functions like reversal learning and working memory integral to success in the SSDRL task used in this study.

The behavioral data focusing on novelty preference measured in the novel object recognition task alluded to potentially interesting increases in novelty seeking due to ethanol consumption and rmTBI together. The objects used in this study were plastic, 3D shapes and may not have been different enough to elicit a greater response to novelty. Future studies should consider altering shape more dramatically, as well as, altering the material of the object to ensure a strong distinction between objects. Since a 30min inter-trial interval was used in the novel object portion of the open field series, deficits in short-term memory were not apparent. Future studies should consider 24hr inter-trial intervals to assess long-term memory as well.

The mild nature of the TBIs and the moderate levels of ethanol consumed in this paradigm lend to a translational model of rmTBI and ethanol intake, but also lead to increased variability in cognitive and behavioral changes. Additional work should consider the administration of ethanol at low, moderate, and high doses following rmTBI to better control ethanol mediated effects. Lastly, this study also only addresses cognition and behavior post-injury in ethanol naïve mice, and in humans, it is more likely that alcohol exposure starts prior to

injury. Future studies should also consider pre-treatment of ethanol and possibly even the use of ethanol-dependent mice with the rmTBI paradigm to assess changes in cognition and behavior.

**Conclusion**

The overall impact of this series of studies primarily resides in the novel contribution to the literature regarding rmTBI and voluntary ethanol consumption. While Experiment 1 was not able to model the excessive drinking observed in a portion of the clinical population following TBI, it provides a foundation for future investigations to uncover the mechanism underlying this problematic behavior. Experiment 2 examined TNF-α expression, a possible shared mechanism of neuronal damage of both rmTBI and ethanol consumption, and uncovered a curious attenuation of TNF-α expression when rmTBI was followed by voluntary ethanol consumption despite observing the expected increase in TNF-α expression due to ethanol consumption in Sham mice. The results of Experiment 3 investigating the combined effects of rmTBI and ethanol consumption demonstrated transient cognitive deficits and behavioral changes in novelty preference. The nature of mild TBI and moderate ethanol consumption make investigation of the combined and independent outcomes more difficult, but the widespread impact of both emphasize the need for translational models such as the one employed in these studies.

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## APPENDIX

USF UNIVERSITY OF SOUTH FLORIDA

**RESEARCH INTEGRITY AND COMPLIANCE**
**INSTITUTIONAL ANIMAL CARE & USE COMMITTEE**

**MEMORANDUM**

| | |
|---|---|
| TO: | Mark Kindy, |
| FROM: | Farah Moulvi, MSPH, IACUC Coordinator<br>Institutional Animal Care & Use Committee<br>Research Integrity & Compliance |
| DATE: | 7/28/2016 |
| PROJECT TITLE: | Traumatic Brain Injury in the mouse |
| FUNDING SOURCE: | USF department, institute, center, etc. |
| IACUC PROTOCOL #: | R IS00002500 |
| PROTOCOL STATUS: | **APPROVED** |

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC **APPROVED** your request to use the following animals in your **protocol for a one-year period beginning 7/28/2016:**

| | |
|---|---|
| Mouse: C57BL/6 (3 weeks to 24 months, 15 gm to 40 gm, M & F) | 9000 |
| Mouse: RAGE KO mice (3 weeks to 24 months, 15 gm to 40 gm, M & F) | 3000 |
| Mouse: Cathepsin B deficient mice (3 weeks to 24 months, 15 gm to 40 gm, M & F) | 3000 |

Please take note of the following:

• **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.