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Traumatic brain injury induces neuroinflammation and neuronal degeneration that is associated with escalated alcohol self-administration in rats

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Abstract

Background—Traumatic brain injury (TBI) affects millions of people each year and is characterized by direct tissue injury followed by a neuroinflammatory response. The post-TBI recovery period can be associated with a negative emotional state characterized by alterations in affective behaviors implicated in the development of Alcohol Use Disorder in humans. The aim of this study was to test the hypothesis that post-TBI neuroinflammation is associated with behavioral dysfunction, including escalated alcohol intake.

Methods—Adult male Wistar rats were trained to self-administer alcohol prior to counterbalanced assignment into naïve, craniotomy, and TBI groups by baseline drinking. TBI was produced by lateral fluid percussion (LFP; >2 ATM; 25 ms). Alcohol drinking and neurobehavioral function were measured at baseline and following TBI in all experimental groups. Markers of neuroinflammation (GFAP & ED1) and neurodegeneration (FJC) were determined by fluorescence histochemistry in brains excised at sacrifice 19 days post-TBI.

Results—The cumulative increase in alcohol intake over the 15 days post-TBI was greater in TBI animals compared to naïve controls. A higher rate of pre-injury alcohol intake was associated with a greater increase in post-injury alcohol intake in both TBI and craniotomy animals. Immediately following TBI, both TBI and craniotomy animals exhibited greater neurobehavioral dysfunction compared to naïve animals. GFAP, IBA-1, ED1, and FJC immunoreactivity at 19 days post-TBI was significantly higher in brains from TBI animals compared to both craniotomy and naïve animals.

Conclusions—These results show an association between post-TBI escalation of alcohol drinking and marked localized neuroinflammation at the site of injury. Moreover, these results

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highlight the relevance of baseline alcohol preference in determining post-TBI alcohol drinking. Further investigation to determine the contribution of neuroinflammation to increased alcohol drinking post-TBI is warranted.

Keywords

Traumatic brain injury; alcohol; self-administration; neuroinflammation; anxiety

Introduction

Traumatic brain injury (TBI) is an increasingly prevalent health problem. In the United States alone, approximately 3.5 million patients are hospitalized annually while an additional 50,000 die from TBI (Coronado et al., 2012). TBI can be categorized as mild, moderate, or severe, with the vast majority of TBIs falling under the mild category (mTBI). mTBI, also referred to as a concussion, is defined as a “brief change in mental state or consciousness” (Centers for Disease Control (CDC) 2003). TBI is frequent in motorists, athletes, and military personnel. In fact, TBI has been called the “signature injury” of the recent theaters of war in Iraq and Afghanistan (Reger et al., 2012). Approximately 175,000 athletes visit an emergency department every year due to TBI, mainly as a result of participation in contact sports such as football and boxing (CDC). Many symptoms of mTBI are behavioral rather than physical and therefore cases of mTBI frequently go unreported, leading some to label TBI a “silent epidemic” (Reger et al., 2012).

TBI is characterized by tissue injury produced by a mechanical insult and the subsequent neuroinflammatory response (Al Nimer et al., 2013; Kumar and Loane, 2012; Loane and Faden, 2010; McIntosh et al., 1996). The mechanical insult is followed by disruption of the blood-brain barrier, compromised tissue integrity, and vessel rupture at the site of injury; therefore resulting in inflammatory activation characterized by astrogliosis and microgliosis (Al Nimer et al., 2013; Kumar and Loane, 2012; Loane and Faden, 2010; McIntosh et al., 1996). Although this neuroinflammation is initially beneficial for tissue recovery, it can be harmful if it persists for an extended period after the injury, and this often occurs with TBI. Sustained neuroinflammation can last months to years, contributing to secondary injury, neuronal death, and negative affective states (Al Nimer et al., 2013; Kumar and Loane, 2012; Loane and Faden, 2010; McIntosh et al., 1996).

Alcohol intoxication increases the risk for TBI, and up to 50% of all TBIs occur under the influence of alcohol (CDC). Individuals with a history of abusing alcohol prior to sustaining a TBI are particularly susceptible to the tendency for escalated alcohol drinking post-TBI (Bombardier et al., 2003; Hall and Johnston, 1994; Horner et al., 2005; Kreutzer et al., 1996). Studies of active duty US Airmen have shown an increased hazard ratio for nondependent alcohol abuse in the first three months following TBI (Miller et al., 2013). Furthermore, TBI promotes negative affective symptoms including anxiety, depression, sleep disturbances, heightened stress and pain sensitivity, and impulse control deficits (Garden and Sullivan, 2010; Garden et al., 2010; Konrad et al., 2011; Levin et al., 2001; Moore et al., 2006; Rao et al., 2010; Vanderploeg et al., 2007). These behaviors, particularly heightened stress sensitivity (Breese et al., 2005; Koob, 2009; Pohorecky, 1991) and anxiety

and depression (Kushner et al., 1999; Möller et al., 1997; Primeaux et al., 2006; Spanagel et al., 1995; Stewart et al., 1993), are often correlated with escalated alcohol use in humans.

Pathology resulting from TBI is driven by unchecked neuroinflammation; interestingly, neuroinflammation alone (modeled by injection of an agent such as bacterial lipopolysaccharide (LPS)) has been shown to increase alcohol drinking in rodents (Osterndorff-Kahanek et al., 2013). The combined effects of intoxicating levels of alcohol intake and (LPS) injection result in similar enhancement of proinflammatory gene expression in the prefrontal cortex; these gene changes are associated with increased alcohol drinking (Osterndorff-Kahanek et al., 2013). In the postmortem human alcoholic brain, levels of innate immune signaling molecules including high mobility group box 1 (HMGB1) and toll-like receptors 2, 3, and 4 (TLR2, TLR3, TLR4) are significantly elevated compared to nonalcoholic brains (Crews et al., 2013). Taken together, these findings suggest an important causal relationship between neuroimmune activation and alcohol intake.

Neuroimmune activation has also been reported to increase alcohol drinking in mice (Blednov et al., 2011). We hypothesize that neuroinflammation resulting from TBI may contribute to behavioral sequelae including escalated alcohol drinking. Here we used a rodent model of mild TBI produced by lateral fluid percussion (LFP), one of the most extensively validated models of TBI, (Thompson et al., 2005) to examine whether TBI-induced neuroinflammation is associated with increased alcohol self-administration. In addition, we determined whether anxiety-like behavior, which has been proposed as an underlying mechanism for escalated alcohol drinking (Kushner et al., 1999; Möller et al., 1997; Primeaux et al., 2006; Spanagel et al., 1995; Stewart et al., 1993) is present following TBI and associated with neuroinflammation and increased alcohol self-administration.

Materials and Methods

Animals

Male Wistar rats weighing between 175 and 200 grams were purchased from Charles River Laboratories (Wilmington, Massachusetts) and pair-housed in a temperature- and humidity-controlled animal housing room with a 12 hour (h) light/dark cycle. The rats had *ad libitum* access to water and standard rat chow. All animal procedures and experiments were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center and were in accordance with the guidelines of the National Institutes of Health.

Operant self-administration

After one week of habituation, the animals were trained to self administer ethanol for four weeks as previously described (Roltsch et al., 2014). Rats were allowed to drink on Monday-Friday, six hours into the dark cycle, in limited access sessions of 30 minutes. Access was permitted in a two-lever contingency (water vs. alcohol) on a FR1 schedule, in which one press of a lever delivered 0.1 ml of water or 10% w/v alcohol. Blood alcohol levels (BALs) were measured at baseline to ensure animals were consuming alcohol. 500 µl of tail blood was collected via a small incision immediately following an operant drinking

session and that blood was analyzed using an analox machine according to manufacturer's instructions (Analox Instruments USA, Lunenburg MA).

Once the animals reached a consistent baseline drinking level, defined as three consecutive days during which the number of lever presses on the alcohol lever did not exceed $\pm 20\%$ variance, the animals were divided into experimental groups counterbalanced for baseline alcohol drinking levels: TBI (N=11), Craniotomy (N=20), and Naïve (N=12). Baseline responding was calculated as mean lever presses for the last five 30-minute operant sessions prior to surgery day.

Traumatic Brain Injury via Lateral Fluid Percussion

Average body weight prior to surgery was 486 ± 42 grams. Animals underwent craniotomy (-2mm bregma and -3mm lateral to midline; 2 ATM; 25 ms) prior to TBI by LFP (*Fluid Percussion Injury (FPI), Model 01-B. Custom Design and Fabrication, Virginia Commonwealth University*) as previously described (Teng and Molina, 2014). Animals in the craniotomy group were anesthetized and received craniotomy but were not subjected to TBI (surgical controls). The naïve animals did not receive any surgical manipulation or injury, but were trained to self-administer ethanol (drinking-only controls). Only animals with an injury of at least 2 ATM of pressure were included for analysis. Following TBI, several physiologic outcome measures were recorded. Apnea was measured in seconds before the first breath post-injury. Respiratory rate was measured as breaths per minute, one minute post-injury. Righting reflex was measured as the amount of time that an animal, placed on its side directly after injury, took to "right" itself onto all four paws—an analysis that relates to how long it takes humans to regain consciousness post-injury. Topical lidocaine was applied to the incision sites following surgery. Animals were allowed to recover in home cages for 48 hours with food and water *ad libitum* prior to being placed in operant chambers and being allowed to drink alcohol.

Neurological and Neurobehavioral Assessments

In order to measure the immediate effect of TBI on behavioral outcomes, neurological severity scores (NSS) and neurobehavioral scores (NBS) were obtained on each animal 24 hours pre-TBI (baseline), 24 hours post-TBI, 72 hours post-TBI, and 7 days post-TBI as described previously (Teng and Molina, 2014). Higher scores indicated greater impairment. NSS tests "motor, sensory, reflexes, beam walking, and beam balancing" on a scale of 0 to 25, while NBS evaluates "sensorimotor task, proprioception, exploratory behavior in home cage, and novel object recognition" on a scale of 0 to 12. (Teng and Molina, 2014). Animals were always tested for NBS and NSS on non-drinking days. Data are presented as change from baseline (each post-TBI time point minus baseline score). Seventeen days post-TBI, anxiety-like behavior was assessed using a light-dark box test (Onaivi and Martin, 1989). The animals had five minutes (300 seconds) to explore two chambers: one chamber was dark with black painted walls, and the other chamber was lit by a white light with white painted walls. The two boxes were separated by a guillotine door. A video camera mounted on the ceiling directly above the light-dark box wirelessly transmitted video to a computer in the lab and recorded each test. Behavior was later scored by an observer blind to the treatment. The amount of time each rat spent exploring the light and dark chamber was

quantified. Data are expressed as percent time in light box (seconds in light box divided by 300).

Post-TBI Operant Drinking

Following TBI, the animals' drinking behavior was monitored during 30-minute operant sessions on days 2, 4, 6, 8, 10, 13, and 15 post-injury. Responding on the alcohol lever was compared within subjects between groups (naïve vs. craniotomy vs. TBI), relative to baseline within subjects. Lever presses were used to calculate alcohol intake as grams of alcohol consumed per kilogram of body weight (g/kg).

Tissue Collection and Tissue Fixation

Data reported in this manuscript were obtained from three different cohorts of animals. Only animals from the third cohort were used for immunohistochemistry; the tissues generated from animals in cohorts 1 and 2 were stored for future mechanistic analyses. Animals from cohort 3 (N=22) were sacrificed 19 days post-TBI by decapitation under isoflurane anesthesia. Brains were excised, fresh frozen, and stored until analysis. A subset of brains from cohort 3 (N=9; 3 from each experimental group) were postfixed and sectioned for IHC. The selected animals had behavior that was representative of their experimental group. Brains were placed in 4% paraformaldehyde overnight followed by one night in 20% sucrose solution. Brains were then flash frozen in isopentane and stored at -80°C until sectioning.

Immunohistochemistry and immunofluorescence

Brains were sectioned in 40 micron slices in a cryostat at -20°C , then permeabilized with 0.3% Triton-X 100 in PBS for 30 minutes and blocked with blocking buffer (containing bovine serum albumin, normal donkey serum, Triton-X 100, and PBS) for one hour, both at room temperature.

Primary antibody incubation with GFAP (1:200; Abcam, Cambridge, England), ED1 (1:200; Abcam, Cambridge, England), and Iba-1 (1:500, Wako, Osaka, Japan) lasted 24 hours at 4 degrees Celsius in a humidification chamber. The next day, slides were washed in PBS for 15 minutes (3×5 minutes with fresh PBS). Secondary incubation (1:200; Alexa Fluor 488, Life Technologies, Carlsbad, CA) lasted two hours at room temperature in the dark. The slides were again washed for 15 minutes in PBS as described above. The slides were dried and coverslipped using mounting media with DAPI (ProLong Gold, Life Technologies Carlsbad, CA). Images were captured at 40x or 20x magnification using a three-millisecond exposure time on a Nikon Eclipse TE2000-U (Nikon, Tokyo, Japan). The imaging software was NIS Elements (Version 3.22.11, Nikon, Tokyo, Japan). IHC was quantified using ImageJ software at 40x magnification.

Fluoro Jade C staining was completed according to the manufacturer's instruction (Ready-to-Dilute (RTD) Fluoro-Jade C Staining Kit, Biosensis, California). Slides were incubated in sodium hydroxide for five minutes, then washed with 70% ETOH followed by distilled water. Slides were then incubated in potassium permanganate for 10 minutes. Next, slides were washed with distilled water and moved to low-light for staining with Fluoro-Jade C

and DAPI for 15 minutes. Still in low light, slides were rinsed with distilled water, and cleared by brief immersion in xylenes. Slides were then coverslipped using Permount, and visualized. Images were captured at 20x magnification using a three-millisecond exposure time on a Nikon Eclipse TE2000-U (Nikon, Tokyo, Japan). The imaging software was NIS Elements (Version 3.22.11, Nikon, Tokyo, Japan).

Three sections were used for analysis at the site of injury for each animal (Figure 5A). For each antibody, three 400 μ m diameter pictures were taken at the site of injury per animal (scale bars for reference); each image was quantified and the three quantification values per animal were averaged. Three animals were quantified per group and averaged together to obtain a single value for each group. Slide selection to determine the site of injury was made based on coordinates (-2 mm bregma) and then within each section the area to be quantified was determined with a standard size box used for each animal, always at the same location (site of injury). Quantification was completed using ImageJ software (NIH Public Domain). Briefly, color images were split into single color channels and each channel was converted to grayscale. The grayscale image was then converted to a binary image by adjusting the image threshold to highlight only the structures of interest (activated cells). Once the image was in binary, analysis was completed using the ImageJ command analyze $\rightarrow$ analyze particles. Output is given as percent positive staining over total image size.

Statistical analyses

Alcohol intake was analyzed using two-way repeated measures (RM) ANOVA with factors alcohol intake and time. Physiological outcomes of TBI (apnea and righting reflex), sum change in alcohol drinking, anxiety-like behavior, and IHC quantification were analyzed using one-way ANOVA at each time point with a Bonferroni post-hoc test. Correlations between baseline drinking and post-TBI drinking were analyzed using an R-square goodness of fit test. Where data was not normal due to uneven sample sizes, a Kruskal-Wallis non-parametric test was utilized. All values are mean $\pm$ SEM and significance was set at $P=0.05$.

Results

TBI Causes Apnea, Decreased Respiratory Rate, and Prolonged Righting Reflex

TBI animals displayed apnea following injury while craniotomy animals did not. TBI animals also displayed a significantly reduced respiratory rate following TBI compared to craniotomy animals ($P=0.02$), while the righting reflex was significantly extended in TBI animals compared to craniotomy controls ($P=0.04$) (Table 1).

TBI and Craniotomy Animals Displayed Behavioral Alterations Following TBI

NSS and NBS scores were analyzed using a two-way ANOVA with a Bonferroni post-test for group analysis. NSS scores, when comparing each group to naïve controls, had a significant interaction effect ($P=0.0001$, $F=17.61$), a significant treatment effect ($P=0.0001$, $F=187.6$), and a significant effect of time ($P=0.0001$, $F=67.20$). The Bonferroni post-test revealed both craniotomy and TBI groups to be significantly different than naïve controls at all three time points ($P=0.0001$). At 24 hours, TBI was also significantly different than craniotomy ($P=0.001$, $t=3.945$). The NBS test, when comparing all groups to naïve controls,

resulted in a significant interaction effect ($P=0.0014$, $F=4.698$), a significant treatment effect ($P=0.0001$, $F=252.6$), and a significant effect of time ($P=0.0001$, $F=14.93$). Using a Bonferroni post-test, we determined craniotomy was different from naïve controls at 24 hours ($P=0.0001$, $t=8.056$) and 72 hours ($P=0.0001$, $t=5.239$) but not at 7 days ($P>0.05$, $t=2.050$). TBI was different than naïve at 24 hours ($P=0.0001$, $t=12.86$), 72 hours ($P=0.0001$, $t=12.86$), and 7 days ($P=0.0001$, $t=9.596$). TBI was also different from craniotomy at 24 hours ($P=0.0001$, $t=6.708$), 72 hours ($P=0.0001$, $t=9.445$), and 7 days ($P=0.0001$, $t=8.855$).

Anxiety-like behavior was examined by a light-dark box test 17 days post-TBI in a subset of animals upon completion of a 15-day period of alcohol drinking assessments (Fig. 1C). TBI animals spent less time exploring the light box compared to naïve controls, but this difference failed to reach statistical significance ($P=0.09$; Kruskal-Wallis statistic = 4.657).

TBI Animals Increase Alcohol Intake Post-TBI

Mean pre-TBI baselines for alcohol intake (g/kg) did not differ among groups. To ensure animals were actually consuming alcohol during operant sessions, blood alcohol levels (BALs) were measured at baseline. BALs ranged from 57 mg/dl to 110 mg/dl and were consistent with amount of lever presses. When analyzing daily drinking over time using a two-way RM ANOVA (Fig. 2A), there was no treatment effect ($P=0.25$). However, there was a significant effect of time ($P=0.0005$) and a trend towards treatment x time interaction effect ($P=0.053$). Examining the sum change in alcohol intake over baseline during the 15 days post-TBI (Fig. 2B) with a one-way ANOVA revealed that TBI animals displayed a significantly greater escalation ($P=0.016$) of alcohol intake relative to naïve controls.

Baseline alcohol drinking was used to stratify animals into quartiles (Q1–Q4). Craniotomy animals and TBI animals with the highest pre-TBI baseline alcohol drinking (Q1) showed a significant increase using a Bonferroni post-hoc analysis in post-TBI alcohol intake ($P=0.006$) (Fig. 3). In contrast, only TBI animals in Q2 and Q3 showed a significant increase in alcohol drinking post-TBI ($P=0.04$). No Q4 animals showed an increase in alcohol drinking post-TBI.

Pre-TBI Alcohol Intake Positively Correlates with Post-TBI Alcohol Intake in Craniotomy and TBI animals

Baseline alcohol intake was not correlated with post-TBI alcohol intake ($P=0.50$) in naïve animals (Fig. 4A). In contrast, a positive correlation ($P=0.004$) was observed for these parameters in craniotomy animals (Fig. 4B). TBI animals exhibited the strongest positive correlation between baseline and post-TBI alcohol intake ($P=0.001$) (Fig. 4C). In both craniotomy and TBI groups, the animals that increased post-TBI intake by the greatest amount were those with the highest pre-TBI baseline alcohol intake.

TBI Produces Greater Neuroinflammation than Craniotomy

Astrocyte activation, measured with GFAP staining, was significantly greater ($P=0.027$) in TBI animals compared to all other groups (Fig. 5B; representative images Fig. 5 panels F, J, N). Microglial activation, measured with ED1 staining, was significantly greater ($P=0.04$) in TBI animals compared to all other groups (Fig. 5C; representative images Fig. 5 panels G, K,

O). Neuronal degradation, measured with FJC staining, was significantly greater ($P=0.033$) in TBI animals compared to all other groups (Fig. 5D; representative images Fig. 5 panels H, L, P). There was also significantly more positive iba-1 staining in TBI animals compared to craniotomy and naïve animals (Fig. 5E; representative images Fig 5 panels I, M, Q).

Discussion

In this study, we examined the impact of TBI on alcohol drinking in rats, and on neuroinflammation and neurodegeneration in alcohol-drinking rats. Our results revealed an association between mild TBI and increased alcohol intake. Moreover, we found that animals with high baseline alcohol intake were more likely to increase alcohol drinking post-craniotomy, and even more likely to increase alcohol drinking post-TBI. As shown in our results, TBI produced neuroinflammation characterized by astrocyte and microglial activation and neuronal degeneration, which persisted at least 19 days post-TBI at the site of injury.

Our results are consistent with previous clinical reports that have associated TBI with increased risk for alcohol abuse in humans (Horner et al., 2005; Kreutzer et al., 1996; Miller et al., 2013; Simpson and Tate, 2002). Reports in the literature suggest that pre-TBI alcohol use may increase risk for post-TBI alcohol abuse in humans (Bombardier et al., 2003; Hall and Johnston, 1994; Kreutzer et al., 1996; Ponsford et al., 2007). We found that TBI increased drinking in high and moderate baseline drinkers, while craniotomy increased drinking in high baseline drinkers only. Our results show that pre-TBI baseline drinking was directly correlated with post-TBI alcohol drinking; the correlation was strongest for animals that underwent TBI than for those that underwent craniotomy alone, whereas no relationship was noted between baseline alcohol intake and post-TBI alcohol drinking in naïve animals.

These results suggest that baseline alcohol intake can determine not only whether alcohol intake post-TBI will increase, but also the magnitude of that increase. Furthermore, these findings underline the importance of identifying the history and patterns of alcohol drinking in TBI victims in an effort to ensure implementation of appropriate interventions designed to ameliorate the risk for increased drinking during the post-TBI recovery period. We predict that alcohol consumption during the post-TBI period is likely to delay neurobehavioral recovery and resolution of neuroinflammation, and this prediction is the focus of current investigations in our laboratory.

We included a craniotomy-only group as a surgical control for TBI, as other researchers have done (Cole et al., 2011). Craniotomy itself has been shown to result in “quantifiable structural and functional damage to the underlying brain,” potentially resulting in behavioral and biochemical disruptions not seen in naïve non-surgical controls (Cole et al., 2011). Minor disruptions of the microvasculature and innervation connecting the rat brain to the scalp as a result of craniotomy have been proposed to produce secondary injury cascade similar to, but less severe than, that seen in TBI. Previously published studies have also reported craniotomy-induced brain damage, and have hypothesized different reasons for the observed effects (Crone and Olesen, 1982; Edvinsson and West, 1972; Navari et al., 1978; Olesen, 1987). For example, in 1972 Edvinsson et al. tested the hypothesis that the post-

craniotomy exposed skull may lose CO₂ to the environment, thus promoting local vasoconstriction and tissue and neuronal damage. Though no research has conclusively shown the mechanism of craniotomy-induced changes in behavioral and biochemical outcomes, we hypothesize that craniotomy may actually be a mild brain injury and that it could possibly account for the TBI-like behavior exhibited by these animals. The close similarity in behavioral patterns observed in TBI and craniotomy animals seen in our studies strongly suggests that craniotomy is not a clean control for the injury. Moreover, the similarities support the need for inclusion of naïve control animals in the experimental design in order to decompound the subtle differences between TBI and craniotomy.

Head injuries such as TBI can foster negative affect in humans, resulting in symptoms that include anxiety and depression (Garden and Sullivan, 2010; Garden et al., 2010; Konrad et al., 2011; Levin et al., 2001; Moore et al., 2006; Rao et al., 2010; Vanderploeg et al., 2007). TBI can lead to dysregulation of the hypothalamic-pituitary-adrenal axis and subsequent deficits in stress coping behavior, providing a potential mechanism for the development of this negative affect (Taylor 2013). This HPA axis dysregulation may be driven by damage to rich glucocorticoid receptor (GR) brain regions, impairing the GR-feedback termination of the HPA stress response, resulting in perpetual HPA activation and disruption of affective behaviors (Taylor 2013). Among these, anxiety and depression, are often correlated with escalated alcohol use (Kushner et al., 1999; Möller et al., 1997; Primeaux et al., 2006; Spanagel et al., 1995; Stewart et al., 1993). To examine anxiety-like behavior in alcohol drinking animals following TBI, we used the light-dark box test (Fig. 1C). Our results did not show statistically significant differences in anxiety-like behavior between TBI and naïve controls. However, we did observe a trend towards heightened anxiety in the TBI animals. Though not definitive, further studies are warranted to better dissect the potential role of anxiety-like behaviors in escalated alcohol drinking post-TBI. It is possible that the time point selected for determination of this parameter was not optimal. We speculate that testing anxiety-like behaviors at a time point closer to the time of injury might reveal robust, TBI-induced increases in anxiety-like behavior, and this effect may drive post-TBI escalation of alcohol intake. This requires further investigation. In addition, a limitation of this test was the inability to measure locomotor activity due to the fact that animals in the dark box were out of view of the overhead camera.

Neuroinflammation resulting from mild TBI may last several weeks after injury (Perez-Polo et al., 2013). We predict that alcohol use post-TBI increases neuroinflammation, which is therefore considered to possibly be a mechanism underlying the escalation in alcohol drinking. Neuroinflammation has been shown to increase alcohol self-administration in rodents (Blednov et al., 2011). Our results indirectly support a role of neuroinflammation in post-TBI alcohol drinking. We observed that TBI caused a 19-day period of neuroinflammation and neuronal degeneration at the site of injury. Because of these findings, we are currently investigating our prediction that greater neuroinflammation post-TBI may contribute to increased alcohol intake, and that alcohol drinking worsens TBI recovery relative to alcohol-naïve TBI animals. A limitation to this study is the small sample size of our IHC (n=3 for each experimental group), but we believe these results to be representative of all animals.

Based on our findings and the relevant literature, we propose a feed-forward interaction between TBI and alcohol drinking, in which TBI results in increased neuroinflammation (Al Nimer et al., 2013; Kumar and Loane, 2012; Loane and Faden, 2010; McIntosh et al., 1996), increased neuroinflammation promotes escalation of alcohol drinking (Blednov et al., 2011), and escalated alcohol drinking exacerbates neuroinflammation (Crews et al., 2013). The most important step in this cycle is the synergistic effect that alcohol and TBI can have on neuroinflammation. As mentioned previously, small amounts of neuroinflammation are necessary for recovery from injury, but alcohol-exacerbated neuroinflammation post-TBI may be the driving force for TBI-related pathologies including negative affect, continued alcohol drinking, and heightened risk for sustaining future injuries. In this report, we document the occurrence of persistent neuroinflammation for several weeks post-TBI, suggesting that the time period immediately following TBI may be an important therapeutic window during which TBI-related pathologies can be moderated.

In summary, alcohol self-administration increased post-TBI and the magnitude of escalation appeared to be related to pre-injury alcohol intake. Increased post-TBI alcohol drinking was associated with sustained neuroinflammation and neuronal degeneration at the site of injury. The mechanisms linking cortical neuroinflammation to the subcortical circuitry that drives excessive alcohol drinking remain to be determined (Moonat and Pandey, 2012; Zamora-Martinez and Edwards, 2014). Future studies will examine these mechanisms with the goal of identifying pharmacological targets to reduce escalation of alcohol drinking following TBI.

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Highlights

- TBI increased alcohol self-administration in rats
- Post-TBI escalated alcohol self-administration was higher in animals with high pre-TBI drinking
- TBI and craniotomy induced neurobehavioral dysfunction and a trend toward anxiety like behavior
- TBI resulted in neuroinflammation and neurodegeneration at 19 days post-injury

Figure 1.

Impairment following TBI as measured by neurological severity score (NSS) (A) and neurobehavioral score (NBS) (B); and the light-dark box test for anxiety-like behavior expressed as % time spent exploring the light box (C). Data are presented as mean $\pm$ SEM. Data was analyzed using a two-way ANOVA, with $n=12$ for naïve, $n=20$ for craniotomy and $n=11$ for TBI. $\#p<0.05$ compared to naïve controls, $\$p<0.05$ compared to craniotomy animals.

Figure 2.

Changes in post-TBI alcohol intake per drinking session in g/kg (A) and calculated as sum change for the entire 15 days post-TBI (B). Data are presented as mean $\pm$ SEM. Panel A was analyzed using a two-way ANOVA with repeated measures, while panel B was analyzed using a one way ANOVA, with $n=12$ for naïve, $n=20$ for craniotomy and $n=11$ for TBI. $\#p<0.05$ compared to naïve controls only.

Figure 3.

High (Q1), moderate (Q2 and Q3), and low (Q4) baseline drinkers for naïve, craniotomy, and TBI groups. Changes in post-TBI alcohol intake were calculated as a sum change from baseline (g/kg) for the entire 15 days post-TBI. Baseline values for each experimental group were as follows: TBI = 0.54 g/kg/30 min session. Craniotomy = 0.59 g/kg/30 min session. Naïve = 0.57 g/kg/30 min session. Data are presented as mean $\pm$ SEM. Data were analyzed using a one-way ANOVA, with $n=12$ for naïve, $n=20$ for craniotomy, $n=11$ for TBI. * $p<0.05$ difference from naïve group in the same quartile.

Figure 4.

Correlation between alcohol intake at baseline and post-TBI sum change in alcohol intake for naïve (A), craniotomy (B), and TBI (C) groups. All panels were analyzed using a goodness of fit test, with $n=12$ for naïve, $n=20$ for craniotomy, and $n=11$ for TBI. $P<0.05$ indicates a slope is significantly different from zero.

Figure 5.

Location of TBI and image analysis (A). Quantification of post-TBI neuroinflammation as measured by GFAP immunoreactivity (B) and ED1 immunoreactivity (C), neuronal degradation as measured by FJC immunoreactivity (D), and general microglial staining as measured by iba-1 (E). Data are presented as mean $\pm$ SEM. Data were analyzed using a one way ANOVA. # $p < 0.05$ compared to all other groups, with $n = 3$ for each experimental group. Representative immunohistochemistry images for GFAP (F, J, N), ED1 (G, K, O), FJC (H, L, P), and Iba-1 (I, M, Q). DAPI overlay is shown for each experimental group. Figures contain scale bars for size reference.

Table 1

Mean injury severity, mean apnea, mean respiratory rate, and mean righting reflex assessed immediately following TBI.

Group ID	Mean Injury Severity (atm)	Mean Apnea (s)	Mean Respiratory Rate (breaths/min)	Mean Righting Reflex (s)
Craniotomy	-	-	98 ± 5	382 ± 85
TBI	2.20 ± 0.01	13 ± 4	69 ± 3 [*]	864 ± 141 [*]

Data are presented as mean ± SEM, and analyzed using a paired t-test, with n = 20 in the craniotomy group and n = 11 in the TBI group.

* p<0.05 to craniotomy values.