Binge ethanol in adulthood exacerbates negative outcomes following juvenile traumatic brain injury

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Abstract
Traumatic brain injuries (TBI) are a major public health problem with enormous costs in terms of health care dollars, lost productivity, and reduced quality of life. Alcohol is bidirectionally linked to TBI as many TBI patients are intoxicated at the time of their injury and we recently reported that, in accordance with human epidemiological data, animals injured during juvenile development self-administered significantly more alcohol as adults than did sham injured mice. There are also clinical data that drinking after TBI significantly reduces the efficacy of rehabilitation and leads to poorer long-term outcomes. In order to determine whether juvenile traumatic brain injury also increased the vulnerability of the brain to the toxic effects of high dose alcohol, mice were injured at 21 days of age and then seven weeks later treated daily with binge-like levels of alcohol 5 g/kg (by oral gavage) for ten days. Binge-like alcohol produced a greater degree of neuronal damage and neuroinflammation in mice that sustained a TBI. Further, mice that sustained a juvenile TBI exhibited mild learning and memory impairments in adulthood following binge alcohol and express a significant increase in hippocampal ectopic localization of newborn neurons. Taken together, these data provide strong evidence that a mild brain injury occurring early in life renders the brain highly vulnerable to the consequences of binge-like alcohol consumption.

1. Introduction
Traumatic brain injuries (TBI) are a major public health problem with millions of injuries and tens of thousands of deaths occurring annually in the US alone (Langlois et al., 2006). Further, the long-term health consequences and economic costs in terms of health spending and loss of productivity are staggering (Coronado et al., 2012).

Alcohol use is tightly linked to traumatic brain injuries as alcohol intoxication is linked to, by some estimates, more than half of all traumatic brain injuries (Tagliaferri et al., 2006). Much of the research into this relationship therefore has focused on the role of alcohol as a risk factor for TBI, and intoxication at injury as a variable in functional outcomes and recovery (Berry et al., 2011; Chen et al., 2012; Opreanu et al., 2010; Pandit et al., 2014). However, there is also some experimental and clinical evidence that TBI itself could serve as an independent risk factor for substance abuse issues, particularly when injuries occur early in development (Bjork and Grant, 2009; Corrigan et al., 2013; Weil et al., 2016a). Most epidemiological studies have reported that although there is a population of patients that abstain from alcohol after TBI there is also a substantial number that resume drinking in the months after injury (Bom bardier et al., 2003; Kreutzer and Harris, 1990; Ponsford et al., 2007). There is some limited, though controversial, evidence that intoxication at the time of TBI can be neuroprotective (Berry et al., 2011; Chen et al., 2012; Opreanu et al., 2010; Pandit et al., 2014), but alcohol abuse following TBI is clearly problematic as it produces significantly poorer outcomes including: impairing the efficacy of rehabilitation programs; reducing vocational opportunities; and substantially increasing the risk for psychiatric disorders, post-traumatic seizures, and additional injuries (Corrigan, 1995; Corrigan et al., 2014; Jorge et al., 2005; Vaaramo et al., 2014a,b).

Recently, we reported that female, but not male, mice that received a single mild traumatic brain injury at 21 days of age self-administered significantly more alcohol in adulthood than did sham-injured mice or those injured in adulthood (Weil et al., 2016b). These data revealed that injuries occurring at a critical...
developmental time point in pre-adolescent mice resulted in long-lasting consequences that contributed to voluntary alcohol abuse in adulthood.

Although it is clear that drinking after traumatic brain injury reduces the effectiveness of rehabilitation therapy and produces poorer outcomes, the precise mechanisms that link drinking to impaired neuronal recovery and function in this clinical population are not fully understood. However, there is mounting evidence from both the experimental and clinical literature that heavy or binge-like drinking, even in the absence of other insults, induces neuroinflammation and degeneration (Alfonso-Loeches et al., 2010; Crews et al., 2011; Hayes et al., 2013; Qin and Crews, 2012; Vetreno and Crews, 2012). Further, TBI itself induces long lasting impairments in neuronal structure and function and also induces neuroinflammatory responses. Additionally, inflammatory events early in life appear to prime the neuroimmune system to exhibit greater inflammatory responses to other stimuli, such as high-dose alcohol, later in life (Bilbo et al., 2010). Thus, heavy drinking by TBI survivors may impair functional outcomes, at least in part, by inducing neuroinflammatory responses and further impairing already dysfunctional neuronal circuits.

Our previous study using the self-administration paradigm allowed us to investigate injury-induced alterations in alcohol reward processing, but it did not allow us to investigate the functional consequences of alcohol consumption after injury because sham-injured mice did not voluntarily self-administer high doses of ethanol (Weil et al., 2016a). Thus, in order to determine whether heavy drinking after TBI exacerbates the behavioral, neuropathological, and inflammatory consequences of juvenile injuries in female mice, we administered ten daily binge-like doses of ethanol via oral gavage to adult females that had sustained a TBI or sham injury in early life. The goals of this study were to 1) determine whether juvenile TBI increased the functional deficits and tissue damage induced by binge-like levels of alcohol and 2) to begin to uncover the mechanisms that link TBI, binge-like alcohol, and tissue damage. We hypothesized that binge-like exposure to alcohol would exacerbate tissue damage and functional deficits in previously injured female mice in part by inducing neuroinflammation and impairing neuroplasticity.

2. Methods

2.1. Animals

All procedures were conducted on female Swiss-Webster mice derived from breeders purchased from Charles River (Wilmington, MA) and bred at the Ohio State University. Pups were weaned at 21 days of age into a standard mouse cage (32 × 16 × 12 cm) with ad libitum access to food and filtered tap water. All animals were housed in a 14:10 light-dark cycle. All procedures were approved by the OSU Institutional Animal Care and Use Committee, and were conducted in accordance with the National Institute of Health guidelines.

2.2. Traumatic brain injury

On the day of weaning, 21 day-old female mice sustained a mild closed-head injury or a sham injury as described previously (Weil et al., 2016b). All mice were anesthetized with inhaled isoflurane, secured in a stereotaxic frame, and a round 2 mm impactor (Impact One device, Leica Biosystems, Richmond IL) was placed on the surface of the exposed skull (−1 mm AP, −1 mm ML). TBI mice underwent an impact at 3 mm/s (dwell time 30 ms) to a depth of 1 mm, while sham mice were exposed to an equivalent amount of anesthesia in the absence of impact. The skin was closed with nylon suture and mice were returned to their home cages.

2.3. Alcohol administration

Beginning seven weeks following TBI or sham injury, all mice were administered 5 g/kg of a 31.5% solution of ethanol or water control (dosing based on (Bertola et al., 2013)). Ethanol or water was administered via daily oral gavage for 10 consecutive days. Mice were weighed daily to account for body mass fluctuations in the dosing. This paradigm resulted in 4 groups (sham/water, TBI/water, sham/ethanol, TBI/ethanol), which were divided into 3 separate cohorts. One cohort was used for assessment of pathophysiology only (n = 8–10 per group), one for microglial extraction (n = 6–8 per group), and another cohort underwent behavioral testing followed by histological assessment (n = 9–12 per group). See Fig. 1 for experimental timeline.

2.4. Blood ethanol concentration

Blood ethanol concentration (BEC) was assessed via a colorimetric 96-well plate assay (modified from Prencipe et al., 1987). Blood was drawn from the retro-orbital sinus 50 min after the final oral gavage. Blood samples were centrifuged and serum stored at −80 °C until use. A BEC assay buffer (100 mM KH2PO4; 100 mM K2HPO4; 0.7 mM 4-aminoantipyrine; 1.7 mM chromotropic acid disodium salt; 50 mg/l EDTA; 50 mM/l triton X-100) was used to dilute ethanol standards and serum and all wells were assessed in duplicate. A reaction mixture of alcohol oxidase (Sigma A2404) and peroxidase from horseradish (Sigma P8375) diluted in BEC assay buffer was added to all wells and the plate was read at 600 nm.

2.5. Histology

Axonal damage was assessed via silver staining using the NeuroSilver kit (FD Neurotechnologies, PK301) per manufacturer’s instructions. Mice were overdosed with sodium pentobarbital (200 mg/kg) and transcardially perfused with 4% paraformaldehyde. Following an overnight postfix and cryopreservation, brain sections throughout the forebrain were sliced on a cryostat, (40 µm) and collected into a 24-well culture plate for free-floating immunohistochemistry and silver staining. For immunohistochemistry, tissue was washed with 0.1 M phosphate-buffered saline, quenched in hydrogen peroxide, and incubated in primary antibody overnight (rabbit anti-Iba1, Wako 019-19741; goat anti-doublecortin, Santa Cruz sc-8006). Biotinylated secondary antibodies (goat anti-rabbit and donkey anti-goat, Vector Laboratories BA-1000 and BA-5000) were applied the following day, and staining was visualized using the avidin biotin-diaminobenzidine staining method (Vector Laboratories PK-6100 and SK-4100).

2.6. Histological analysis

Axonal degeneration (silver staining) was assessed qualitatively as previously reported (Weil et al., 2014). Briefly, representative tissue throughout the entire forebrain were assessed and scored on a 4-point scale (0 = few axonal profiles, 3 = dense axonal degeneration throughout white matter tracts bilaterally).

Microglial cells (Iba1-positive staining) were assessed in the prefrontal cortex. Morphological analysis was conducted using Neurolucida software (MicroBrightfield). Briefly, 2–3 cells per hemisphere from each brain were selected using strict criteria as previously reported (Karelina et al., 2016) and traced for subsequent sholl analysis. Using NeuroExplorer (MicroBrightfield) concentric sholl rings spaced 5 µm apart were centered on the cell body, and the number of intersections, total process length, and cell body surface area were obtained for each cell. Total microglial...
numbers were counted in a 0.26 mm² region of interest using ImageJ software (Abramoff et al., 2004) and converted to cell number/mm².

Doublecortin-positive neurons were counted in the hippocampal dentate gyrus, sections spanning approximately −1.94 mm through −2.18 mm from bregma. Total cell counts were collected in the granule cell layer in both the top and bottom blades of the dentate, as well as the molecular layer and dentate hilus.

2.7. Microglial isolation

Microglia were extracted from bilateral frontal lobes of sham and injured mice 24 h following their final oral gavage. Fresh brains were collected, olfactory bulbs were removed and tissue was dissected (approximately bregma 0.56 mm through 3.56 mm) and homogenized in Dulbecco’s phosphate buffer solution (DPBS). The homogenate was centrifuged and the pellet resuspended in 70% isotonic Percoll (GE Healthcare Life Sciences #17-0891-01). A discontinuous Percoll gradient was created by layering 50% and 35% Percoll and topping off with DPBS. The gradient was then centrifuged and the microglia-containing layer in the 50%/70% interface was collected and washed.

2.8. qPCR

Extracted microglia were lysed and cDNA synthesized using the SuperScript III CellsDirect cDNA synthesis kit (Invitrogen #11739010 and #18080200) according to the manufacturer’s protocol. A TaqMan 18S ribosomal RNA primer/probe set (Applied Biosystems #4319413E) was used as an internal control. Primer/probe sets for the target genes were also purchased at Applied Biosystems (TLR4: Mm00445273_m1; IL1β: Mm00434228_m1) and amplification was performed on an ABI 7500 Sequencing Fast System using the TaqMan Fast Advanced Master Mix (#4444963). The universal two-step RT-PCR cycling conditions used were: 50°C for 2 min, 95°C for 3 s and 60°C for 30 s. Gene expression was analyzed relative to a standard curve and normalized to the sham/water group.

2.9. Passive avoidance

Learning and memory was evaluated via the passive avoidance task. The task consisted of a 2-chambered testing environment (one chamber was illuminated and the other was dark) with a grid floor and guillotine door separating the chambers. On training day, mice were placed individually into the illuminated chamber for 1 min; the door was then lifted and remained open until the mouse stepped into the dark chamber. Once the mouse entered the dark chamber, the door closed, a brief shock (1 mA, 2 s) was delivered to the grid floor, and the mouse was immediately replaced into its home cage. Twenty-four hours following the training, each mouse was again individually placed into the illuminated chamber for 1 min. Once the guillotine door was open, latency to enter the dark chamber was recorded (with an upper limit of 300 s). No shock was administered on testing day.

2.10. Statistical analysis

Qualitative analysis of silver staining was assessed via the non-parametric Mann-Whitney U and Kruskall-Wallis tests. Significant overall Kruskall-Wallis results were followed up by pairwise comparisons. Microglial sholl analysis was conducted using a repeated measures ANOVA. All histology analyses (cell counts, process length, cell body area), behavioral data, and gene expression data were assessed via a 2-way ANOVA (injury × ethanol administration). All statistics were analyzed using the IBM SPSS V.23 software. All significant overall ANOVA results were followed by the Least Significant Differences posthoc test. Data were considered significant for p-values <0.05.

3. Results

3.1. Binge-like alcohol administration exacerbates TBI-associated pathology

Blood ethanol concentrations were assessed on the final day of oral gavage (90 min following the gavage) and indicate a high level of ethanol in the blood (~0.4 g/dL) which did not differ between sham and TBI mice (Fig. 2A). In order to determine the pathological impact of high-dose alcohol exposure, we examined whether binge-like alcohol levels further exacerbate axonal degeneration in previously injured mice (Fig. 2B–C). As expected, TBI sustained in pre-adolescence led to a significant degree of axonal degeneration (U = 14.0, p < 0.05). However, a 10-day period of binge-like alcohol administration in adulthood significantly exacerbated axon damage in mice that had sustained a TBI as juveniles (H₁ = 20.009, p < 0.05). The overall significant Kruskall-Wallis test was followed up by pairwise comparisons which indicated significant differences between the sham/water and TBI/water groups, as well as a significant increase in degeneration in the TBI/ethanol mice compared to TBI/water (all p < 0.05).

We further examined microglial morphology as a measure of neuroinflammation. Brain tissue from all four groups was processed for Iba1 immunohistochemistry. Sholl analysis of
representative microglia in the prefrontal cortex revealed a significant effect of injury history on microglial morphology (F(1.141) = 194.942, p < 0.01) such that mice that had sustained a TBI exhibited microglia with fewer shell ring intersections (reduced process complexity), indicative of an activated state (Fig. 3A). TBI also significantly reduced overall microglial process length compared to sham injury (F(3.145) = 3.346, p < 0.05; Fig. 3C). An analysis of the total number of microglia in the prefrontal cortex revealed main effects of both injury (F(1.29) = 6.294, p < 0.05) and ethanol treatment (F(1.29) = 80.959, p < 0.01), as well as an interaction (F(1.29) = 8.382, p < 0.01) such that the ethanol-administered TBI mice exhibited the greatest number of cortical microglia (Fig. 3D). Finally, a measure of microglial cell body area revealed a main effect of injury (F(1.27) = 8.879, p < 0.001), and an interaction (F(1.27) = 5.427, p < 0.05) such that ethanol significantly increased cell body area in TBI mice compared to all other groups (Fig. 3E).

3.2. Microglia from injured mice treated with alcohol show increased pro-inflammatory gene expression

Given that both TBI and alcohol resulted in activated microglia, and the increased total number of microglia in the TBI/ethanol group, we examined the microglia directly in order to determine whether the activational state corresponds to increased neuroinflammation. Both Toll-like receptor 4 (F(3.16) = 3.516, p < 0.05; Fig. 4A) and interleukin-1 beta (F(3.19) = 3.311, p < 0.05) gene expression were significantly increased in the TBI/ethanol group relative to all other groups assessed.

3.3. High doses of alcohol impair learning and memory in previously injured mice

Learning and memory was assessed via the passive avoidance test in all four groups beginning 24 h after the final ethanol (or water) administration day. This test takes advantage of the fear-motivated tendency of mice to avoid brightly illuminated areas. Latency to enter the dark chamber on the training day was recorded and indicates no significant difference between groups (Fig. 5A). However, analysis of the latency to enter the dark chamber (in which each mouse had previously endured a painful shock) revealed an interaction of injury history and ethanol treatment (F(1.34) = 5.247, p < 0.05). These data indicate that the TBI/ethanol group exhibited a significantly shorter latency to enter the shock-associated chamber compared to the TBI/water group. Importantly, this difference is not likely to be a result of increased anxiety in the TBI/ethanol group as all mice had similar latencies to escape the light chamber on the training day.

3.4. Binge alcohol and TBI interact to induce ectopic expression of doublecortin-positive cells in the hippocampus

Given the learning/memory impairment observed in the TBI/ethanol mice, we conducted an immunohistochemical analysis of doublecortin in the hippocampus. Doublecortin (DCX) is transiently expressed in progenitor cells committed to the neuronal lineage and serves as an indirect marker of hippocampal neurogenesis. DCX is expressed for approximately 2–3 weeks in newborn neurons before it is replaced by mature neuronal markers (Brown et al., 2003), thus brain tissue fixed 13 days after the start of the ethanol administration paradigm is likely to contain a substantial subset of neurons developing during our experimental manipulation. Here, we assessed DCX-positive cells in the dentate gyrus (Fig. 6). The total number of DCX-positive cells in the dentate gyrus granule cell layer did not differ significantly by injury or ethanol manipulations. However, we identified a significant increase in the ectopic expression of DCX-positive neurons in ethanol-administered TBI mice. Specifically, the TBI/ethanol group had significantly more DCX-positive neurons in the dentate gyrus (F(1.33) = 7.129, p < 0.05) than any other group. Moreover, among mice that sustained a TBI, binge ethanol significantly increased DCX expression in the molecular layer (F(1.4) = 2.420, p < 0.03).

4. Discussion

Binge-like levels of alcohol produce significant neuropathology, neuroinflammation, and functional deficits in mice that had experienced a traumatic brain injury during juvenile development. Specifically, alcohol administration led to greater axonal degeneration and microglial reactivity in mice that had been previously injured. Further, microglia isolated from previously injured animals exhibited significantly increased gene expression for both TLR4 and IL-1β following alcohol administration. The combination of binge-like levels of alcohol and TBI also impaired normal neurogenesis, resulting in increased ectopic expression of DCX-positive cells in the hippocampus. Finally, the combination of high-dose alcohol and juvenile TBI produced impairments in learning and memory as assessed in the passive avoidance paradigm. Taken together, these data indicate that a history of traumatic brain injury significantly alters the neural and functional responses to binge-like levels of alcohol and that the deleterious consequences of high levels of alcohol in human populations may be related to inflammatory responses and impairments in neural plasticity.

Imaging and post-mortem studies have revealed pronounced neuroinflammation and degeneration, particularly of white matter, in the brains of alcoholics. Specifically, there is evidence of ongoing inflammation, microglial activation, and cortical and subcortical atrophy (Harper et al., 2003; He and Crews, 2008; Ikegami et al., 2003). The neurotoxicity of alcohol varies across the lifespan, but is most prominent in cases of long term heavy drinking. Similarly, axonal degeneration is a key pathological marker of TBI, however this pathology resolves over time following a mild injury. Here...
we report that chronic exposure to binge-like levels of alcohol contribute to sustained axonal damage even when the drinking occurs many weeks after the actual injury. Importantly, the binge-like alcohol administration paradigm used in this study was not sufficient to induce axonal degeneration in mice that had not experienced a prior TBI; rather TBI renders axons vulnerable to the toxic effects of high dose alcohol.

Mild traumatic brain injury, such as that used in the current study, is characterized by a significant but transient inflammatory response and a number of cognitive and emotional disruptions that occur in the acute phase following a TBI typically improve as the pathophysiology resolves. However, even mild neuroinflammation, particularly when it occurs during development, has been shown to prime or sensitize the neuroimmune system to later inflammatory events. For instance, neonatal *E. coli* infection enhances the cognitive deficits and microglial responses to lipopolysaccharide-induced inflammation later in life (Bilbo et al., 2008, 2010). Further, TBI itself can act as a priming stimulus as microglial reactivity and behavioral depression induced by experimental inflammation is enhanced in mice with a history of mild traumatic brain injury (Penn et al., 2014). A primary goal of the work presented here was to determine whether an early life brain injury could similarly potentiate the consequences of repeated high dose alcohol exposure in adulthood.

![Figure 3](image_url)

**Fig. 3.** Binge alcohol and TBI alter microglial morphology. A) Sholl analysis in the prefrontal cortex reveals that both alcohol and TBI reduce microglial process complexity, indicative of an activated state, as seen in B) representative images, scale bar = 50 µm. C) TBI also significantly reduced process length. D) The total number of microglia were significantly increased in ethanol-treated mice that sustained a juvenile TBI. E) The cell body area of cortical microglia were also significantly increased in ethanol-treated TBI mice. An asterisk (*) indicates statistically significant differences between the indicated groups (p < 0.05).

![Figure 4](image_url)

**Fig. 4.** TBI and alcohol administration increase proinflammatory gene expression. Relative mRNA gene expression is shown relative to the sham/water group and indicates that A) TLR4 and B) IL-1β is exacerbated by alcohol in mice that underwent juvenile TBI. An asterisk (*) indicates statistically significant differences between the indicated groups (p < 0.05).
Fig. 5. Learning and memory are mildly impaired in alcohol-treated mice that sustained a juvenile TBI. A) All mice exhibit a similar latency to enter the dark side of the passive avoidance chamber during training. B) However, the latency to re-enter the dark, shock-associated, side 24 h later was considerably longer, but was significantly reduced in alcohol-treated mice that sustained a juvenile TBI. An asterisk (*) indicates statistically significant differences between the indicated groups (p < 0.05).

Fig. 6. Binge alcohol increases the number of ectopic doublecortin-positive cells in the hippocampus of mice that sustained a juvenile TBI. A) While the number of total DCX-positive cells did not differ in the granule cell layer, high doses of alcohol significantly increased the number of ectopic newborn neurons in the B) hilus and C) molecular cell layers of the hippocampus (white arrows). D) Representative images of the dentate gyrus, scale bar = 100 μm. An asterisk (*) indicates statistically significant differences between the indicated groups (p < 0.05). ML = molecular layer, GCL = granule cell layer.
Neuroinflammatory responses are bidirectionally linked to alcohol abuse as neuroinflammation can increase drinking behavior (Crews et al., 2011). Conversely, chronic exposure to high levels of alcohol is neuroinflammatory as alcohol can activate glial cells and promote the expression of cytokine and chemokine molecules (He and Crews, 2008). Although the mechanisms of alcohol-induced neuroinflammation are incompletely understood, alcohol does appear to signal in part through toll-like receptors (Alfonso-Loeches et al., 2010; Fernandez-Lizarbe et al., 2009). For instance, the long term behavioral and neurochemical effects of adolescent alcohol exposure require TLR4 (Montesinos et al., 2016).

Microglia isolated from the injured frontal lobes following the binge-like administration of alcohol exhibited marked upregulation of mRNAs encoding the genes toll-like receptor 4 and IL-1β. Critically, this increase in expression of TLR4 and IL-1β only occurred in mice that had been previously injured and treated with binge-like levels of ethanol in adulthood. We have hypothesized that TBI sets up a vicious cycle wherein TBI during development primes inflammatory responses to exhibit exaggerated inflammatory responses to alcohol, which in turn promotes increased drinking (Weil et al., 2016a). As an extension of this theory, the neurotoxic effects of juvenile TBI could also be exacerbated by high doses of alcohol. The up-regulation of both TLR4 and IL-1β only occurred in mice that had been injured and exposed to high levels of alcohol would tend to support this hypothesis. It remains unspecified why we did not detect changes in gene expression in microglia from sham-injured mice although this largely agrees with our histological evidence including reduced microglial numbers, activated morphology and axonal degeneration.

In the current study, we report exacerbation of the TBI-induced damage to white matter tracts by binge-like levels of alcohol in accordance with increased numbers and activation state of microglia as well as exaggerated inflammatory gene expression profiles. Given that much of the tissue damage induced by chronic exposure to high levels of alcohol appears to require microglial-mediated inflammatory responses (Bojadzieva and Sarkar, 2010; Chastain and Sarkar, 2014; Yang et al., 2014), we conclude that a transient period of TBI-induced inflammation in juvenile development is sufficient to significantly exacerbate the neurotoxic effects of binge alcohol in adulthood.

Interestingly we report a differential effect of TBI and alcohol exposure on microglial morphology, counts and gene expression profiles. Specifically, both TBI and alcohol exposure reduced the length of microglial processes as assessed by Sholl analysis but only microglia from mice that were exposed to alcohol and were previously injured were more numerous, had larger cell bodies and upregulated TLR4 and IL-1β. That microglial morphology does not fully track gene expression profiles is consistent with previous reports (McClain et al., 2011) and suggests that both alcohol exposure and TBI are independently associated with changes in microglial physiology and morphology but that can induce a fuller activation when combined.

Moreover, exposure to binge-like levels of alcohol greatly increased the presence of immature doublecortin-positive neurons in the non-neurogenic molecular layer and hilus of the dentate gyrus but did so only in mice that had experienced TBI. Importantly, the exposure to binge-like alcohol in the current study occurred more than six weeks after the TBI, thus the DCX-positive cells assessed at this time point were those cells that were born around the time of alcohol exposure rather than at the time of injury. Although the combination of injury and alcohol-exposure increased the number of hilar DCX-positive cells, the total number was relatively small. Further there were no differences in the number or localization of DCX-positive cells induced by injury alone indicating that any acute effects of TBI on this cell population had resolved by adulthood.

CNS trauma often leads to an increase in the proliferation rate of neuronal precursors, and this phenomenon has been explored as a potential replacement source for cells lost to trauma (Dash et al., 2001; Kernie and Parent, 2010). However, this strategy is complicated by reactive and aberrant neurogenesis that can occur after injury, seizure, or following withdrawal from heavy drinking which can render the brain more vulnerable to cognitive dysfunction, excitotoxicity, and additional seizures (Parent et al., 2006). Ectopic granule cells that have been identified in the brains of epileptic animals and humans exhibit abnormal morphology, connectivity and electrophysiological properties that contribute to hyperexcitability (Parent et al., 2006; Scharfman et al., 2000). Additionally, alcohol (and alcohol withdrawal) even in the absence of trauma modulates neurotransmission and generally biases towards excitatory neurotransmission via changes in neuronal and glial neurotransmitter metabolism, transport, and receptor expression (Adermark and Bowers, in Press; Dodd et al., 2000). Clinically, TBI patients at high risk for post-traumatic epilepsy are advised to abstain from alcohol as drinking is positively correlated with the occurrence of seizures (Centers for Disease Control and Prevention; Corrigan, 1995). The data collected here suggest that an increased propensity for alcohol-induced ectopic neurogenesis in the previously injured brain may at least in part, underlie this relationship. Further, we cannot rule out the possibility that alcohol or alcohol-withdrawal induced seizures may also have occurred and underlies the ectopic neurogenesis rather than the other way around.

The behavioral results reported here, namely relatively subtle deficits in simple associative learning and memory in the passive avoidance paradigm, were conducted on animals experiencing alcohol withdrawal (retention trials occurred approximately 48 h after their final dose of alcohol). Importantly, although there were statistical differences between alcohol-and water-treated injured mice there were not statistical differences between the sham injured and TBI groups. In any case, we elected to test animals during withdrawal because we wanted to avoid any direct effects of intoxication on behavioral outcomes. Additionally, it will be important in future investigations to determine whether the mild impairments in learning and memory persist or improve beyond the acute phase of alcohol withdrawal. Given the enhanced axonal degeneration some degree of long-term functional deficit is likely and may require more extensive behavioral characterization. In any case, these data provide further compelling evidence that binge-like alcohol consumption negatively impacts functional outcomes in animals that were previously injured. Importantly, over the 10-day treatment paradigm, mice were administered a single daily high dose of alcohol every 24 h, thus in effect these animals experienced repeated cycles of intoxication and withdrawal over a short period of time. In addition, although we did not assess the estrous cycle, the prolonged alcohol exposure paradigm did presumably span at least two cycles. Finally, the paradigm we utilized produced high (but similar between sham-injured and injured mice) blood alcohol concentrations. Such a high dose of alcohol was utilized in order to evaluate whether neurotoxic doses would produce worse outcomes in the previously injured brain, however, it remains unclear whether a longer exposure to more moderate levels of alcohol would also be deleterious.

5. Conclusions

The human epidemiological literature has clearly demonstrated that 1) the TBI patient population consists of a high proportion of heavy and binge drinkers, 2) some percentage of TBI patients continue to drink at high levels after TBI, and critically 3) that drinking
after TBI impairs functional and occupational recovery and puts the patient at risk for serious complications including additional brain injuries and seizures. Here we begin to demonstrate the underlying neurobiological mechanisms that underlie this phenomenon including alcohol-induced axonal degeneration, ectopic neurogenesis, and enhanced neuroinflammation induced by alcohol in previously injured mice. This vulnerability to alcohol-induced histopathology and functional impairment occurred despite a long delay (more than 6 weeks) between the injury and the beginning of binge-alcohol administration. These data reinforce the importance of targeting substance abuse in this population and are of even more importance given our previous observation that TBI can also increase alcohol self-administration and reward.

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