Dentate Gyrus Proliferative Responses After Traumatic Brain Injury and Binge Alcohol in Adult Rats

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ABSTRACT

BACKGROUND: Traumatic brain injury is a significant public health issue that results in serious disability in survivors. Traumatic brain injury patients are often intoxicated with alcohol when admitted to the hospital; however, it is not clear how acute intoxication affects recovery from a traumatic brain injury. Our group has previously shown that binge alcohol prior to traumatic brain injury resulted in long-term impairment in a fine sensorimotor task that was correlated with a decreased proliferative and neuroblast response from the subventricular zone. However, whether binge alcohol prior to traumatic brain injury affects the proliferative response in the hippocampal dentate gyrus is not yet known.

METHODS: Male rats underwent binge alcohol (3 g/kg/day) by gastric gavage for 3 days prior to traumatic brain injury. Cell proliferation was labeled by BrdU injections following traumatic brain injury. Stereological quantification and immunofluorescence confocal analysis of BrdU+ cells in the hippocampal dorsal dentate gyrus was performed at 24 hours, 1 week, and 6 weeks post traumatic brain injury.

RESULTS: We found that either traumatic brain injury alone or binge alcohol alone significantly increased dentate gyrus proliferation at 24 hours and 1 week. However, a combined binge alcohol and traumatic brain injury regimen resulted in decreased dentate gyrus proliferation at 24 hours post-traumatic brain injury. At the 6 week time point, binge alcohol overall reduced the number of BrdU+ cells. Furthermore, more BrdU+ cells were found in the dentate hilar region of traumatic brain injury compared to vehicle traumatic brain injury groups. The location and double-labeling of these mismigrated BrdU+ cells was consistent with hilar ectopic granule cells.

CONCLUSION: The results from this study showed that pre-traumatic brain injury binge alcohol impacts the injury-induced proliferative response in the dentate gyrus in the short-term and may affect the distribution of newly generated cells in the dentate gyrus in the long-term.

KEYWORDS: Binge alcohol, traumatic brain injury, proliferation, hippocampal dentate gyrus, hilar ectopic granule cells

Introduction

Traumatic brain injury (TBI) is a major health problem with as many as 2.8 million cases reported each year in the United States.1,2 Patients face a long road to recovery and many will live with permanent deficits in sensory and motor functions and with an increased risk for other psychiatric and neurological disorders.3-5 Furthermore, several clinical studies showed up to nearly half of TBI patients present to the emergency department with elevated blood alcohol levels above the legal limit.6-9 It is noted however, that these studies only examined a limited TBI population, and the level of alcohol intoxication at time of injury may change if different criteria were examined, including age, sex or cause of TBI. In the United States today, binge drinking is the most common mode of alcohol misuse.5

According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), the definition of binge drinking is typically 5 drinks for men and 4 drinks for women during a 2-hour session, resulting in a blood alcohol level greater than 80 mg/dL.10 In experimental studies, alcohol given acutely or in a binge-like paradigm resulted in worse cognitive performance, impairment in spatial processing and decreased recognition memory in rats.11-13 Furthermore, in a rat model of TBI and binge alcohol, our group has shown that skilled sensorimotor recovery was worse in alcohol-treated animals.14 This correlated well with clinical findings in a population of TBI patients who were intoxicated at the time of injury.15-18 Subsequently, we showed that binge alcohol decreased TBI-induced subventricular zone (SVZ) cell proliferation, attenuated SVZ neuronal differentiation, and decreased the number of newborn migratory neurons to the olfactory bulbs.19

Neurogenesis is thought to be a compensatory mechanism observed to be up-regulated in the hippocampal dentate...
gyrus (DG) and the SVZ after a TBI. These newly produced neurons have been associated with recovery of function following brain injuries. On the contrary, post-traumatic neurogenesis could be contributing to significant pathology following a CNS injury, such as producing an epileptic focus. The goal of this study was to determine how a focal TBI in the forelimb motor cortex affects proliferation in the DG and if a prior alcohol binge would change this result. We report that binge alcohol before a TBI led to a reduced cell proliferative response from the DG and increased ectopic proliferation in the hilar region of the DG at 6 weeks. Therefore, the aberrant cellular responses following binge alcohol could result in negative ramifications on long-term hippocampal functions.

**Materials and Methods**

**Animal subjects**

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Edward Hines Jr. Veterans Affairs Hospital (permit #H13-001). Brain tissue sections encompassing the dorsal dentate gyrus from rats used in our previous study were utilized in the current study. Therefore, all animal procedures, including alcohol administration, controlled cortical impact, measurement of blood alcohol level, bromodeoxyuridine (BrdU) administration, perfusion, tissue processing, and histology were performed identically as our previous study. Briefly, we used 2 month-old adult male Sprague Dawley rats (Envigo, Indianapolis, IN; see Table 1). Rats were housed in a fully accredited animal care facility with a 12-hour light/dark cycle and food and water was available ad libitum. Rats were number coded and randomized before all procedures and codes were revealed only after data analysis.

**Alcohol administration**

We administered alcohol to rats by gastric gavage for 3 consecutive days as described in our previous study, (3 g/kg/dose/day at 9 AM, i.g. using Everclear (Luxco, St. Louis, MO) diluted to 40% alcohol by volume (ABV) with distilled water). Control animals received an equal dose of distilled water by gastric gavage. We observed no adverse effects following the above procedure.

**TBI by controlled cortical impact (CCI)**

One hour following the last alcohol administration, CCI procedure was performed as described in our previous studies. Rats were anesthetized (5% isoflurane (75 mg/ml) in 100% oxygen at induction, then 2%-3% to maintain anesthesia), and placed in a stereotaxic frame, with a skin incision and trephination (5 mm diameter) performed directly above the right forelimb area of the sensorimotor cortex (1 mm anterior, 1.5 mm lateral from bregma) (see Figure 1). CCI parameters were as follow: 3 mm diameter, 2.5 m/s velocity, 2 mm depth, 250 ms dwell time (Impact One, MyNeurolab, St. Louis, MO). Sham animals underwent the same duration of anesthesia, and received identical skin incision and trephination procedure.

**Blood alcohol level (BAL) quantification**

BAL measurement was performed identically as reported in our past studies. Blood samples by tail venipuncture were separated by centrifugation to obtain serum then de-proteinized per the manufacturer’s instructions (Pointe Scientific, Canton MI, USA). The following assay parameters were used: sample/reagent ratio (1:201), 5 minutes incubation (30°C), reading (30°C, absorbance 340 nm). All measurements were done in duplicate and then averaged; unknown samples were run at the same time as known ethanol samples.

**Bromodeoxyuridine (BrdU) administration**

Preparation of BrdU solution (20 mg/ml in sterile saline) and injection schedule were performed identically to our previous study. All rats received BrdU at 100 mg/kg (i.p.) dose immediately following surgery (sham or CCI). Rats in the 1 and 6 week groups were given 1 BrdU injection (100 mg/kg, i.p.) daily and this continued until each animal received a total of 7 injections.

| GROUP | ALCOHOL | TBI | 24H | 1WK | 6WK |
|-------|---------|-----|-----|-----|-----|
| 1     | –       | –   | 6   | 6   | 6   |
| 2     | –       | +   | 6   | 7   | 7   |
| 3     | +       | –   | 6   | 6   | 6   |
| 4     | +       | +   | 6   | 7   | 7   |
| Subtotal |       |     | 24  | 26  | 26  |
| Total  |         |     | 76  |     |     |

Animal number (n) and groups for the 3 different time points examined (24 hours, 1 week, and 6 weeks, respectively). Animals were randomly assigned to each of the 4 experimental groups (1-4), either with alcohol or vehicle and TBI or sham surgery.
Perfusion, tissue processing, and histology

The following procedures were performed identically to our previous studies.14,19 Rats were overdosed (phenytoin/pentobarbital, 390 mg/kg, i.p.) and transcardially perfused (at 4°C, first with heparinized saline, then 4% paraformaldehyde (PFA)). Brains were processed as follow: post fixed (4°C in 4% PFA), cryoprotected (30% sucrose in PBS, pH 7.4), cryosectioned (coronal, 40 μm thickness), storage (−20°C in ethylene glycol), antigen retrieval (99°C–100°C, 10 mM sodium citrate, pH 6.0, 15 minutes). See Table 2 for primary and secondary antibodies used. All incubation times and washes were performed identically as described in our previous publication.19 Phosphate-buffered saline (PBS) (pH 7.4, 0.2% Tween-20, 5% normal goat serum) was used to dilute antibodies to the correct working concentrations (see Table 2). Primary antibodies were incubated overnight (4°C) and secondary antibodies were incubated at room temperature for 2 hours. Nickel-enhanced diaminobenzidine (DAB) staining was performed exactly as described by manufacturer’s instruction (Vector Laboratories). All sections were mounted on gelatin-subbed slides and coverslipped with either Fluoromount G or Permount mounting medias.

Stereology

All stereological counting procedures were performed similarly to previous published protocols from our lab.19,26 The following parameters were used: 6 sections/animal (dorsal dentate gyrus, 480 μm apart, between −2.0 mm posterior and −4.8 mm posterior to bregma).27 The granule cell layer (GCL) was outlined by tracing (2.5X objective), and the sampling grid size (64 × 150 μm) and the dissector window size (25 × 25 μm) were used. Counting was performed under high magnification (40X/0.75 NA objective, MBF Bioscience Leica DM400B microscope) but with upper and lower focal planes excluded to avoid oversampling. Cell counting and analysis were performed using StereoInvestigator software version 9.0.

Confocal imaging

To identify cellular fates of newborn cells, we examined tissue sections on a Leica SPE confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL). Using a 10x objective, we identified representative equivalent areas of the DG from a series of stained sections. We subsequently used a 63x/1.3 NA oil immersion objective to confirm double labeling of BrdU− cells. All image acquisition settings were kept constant and image stacks were imported into Leica Application Suite X and compressed to maximum intensity Z projections.

Analysis of BrdU+ dentate gyrus cell distribution

In order to determine the distribution of newborn cells within the hippocampal dentate gyrus (1 week and 6 week survival groups), we used two 40 μm sections per animal encompassing the middle of the dorsal dentate gyrus (−3.14 mm to −3.30 mm posterior to bregma). Outline tracings of the GCL were performed in StereoInvestigator software version 9.0 in a similar manner as the stereology protocol above (Figure 6). The
thickness of the contour encompassing the GCL was kept at approximately 60 μm and was further sub-divided into 2 zones: GCL1 was adjacent to the subgranular zone (SGZ) and GCL2 was adjacent to the molecular layer (ML). Furthermore, two 30 μm contours encompassing the hilar zone adjacent to the SGZ were constructed: Hilus1 was most adjacent to the SGZ and Hilus 2 was furthest away. This method was adapted from previous reports.28,29 The number of BrdU + cells was determined in each of these zones by manual counting on a MBF Bioscience Leica DM400B microscope under high magnification of 40X/0.75 NA objective.

**Statistical analysis**

All data analysis was performed using either Minitab version 17 (Minitab, Inc. State College, Pennsylvania, USA) or Graphpad Prism version 5.0 (GraphPad Software, San Diego California, USA). Statistical methods including data

**Table 2. Summary of antibodies used for immunofluorescence and immunohistochemistry.**

| PRIMARY ANTIBODIES | TARGET/ANTIGEN | ANTIGEN SPECIES | IMMUNOGEN | SOURCE | DILUTION |
|--------------------|----------------|-----------------|-----------|--------|----------|
| Rabbit anti-GFAP polyclonal | Astrocytes glial fibrillary acidic protein intermediate filament | Cow | Spinal cord GFAP | Dako Z0334 [RRID: AB_10013382] | 1:1000 |
| Rabbit anti-doublecortin (DCX) polyclonal | Neuroblast microtubule associated protein | Synthetic | peptide of human DCX | Cell Signaling 4604S [RRID: AB_1069771] | 1:500 |
| Rabbit anti-Iba1 polyclonal | Macrophage/ microgliia calcium binding protein | Synthetic | Peptide of C-terminus of Iba1 | Wako 019-19741 [RRID: AB_839503] | 1:5000 |
| Mouse IgG2a anti-BrdU monoclonal | 5-Bromo-2-deoxyuridine (BrdU) | Synthetic | 5-iodouridine covalently coupled to ovalbumin | Pierce MA3-071 [RRID: AB_10986341] | 1:1000–4000 |
| Mouse IgG1 anti-NeuN monoclonal | DNA-binding, neuron-specific protein NeuN | Mouse | Purified cell nuclei from mouse brain | Chemicon MAB377 [RRID: AB_2298772] | 1:1000 |
| Rabbit anti-S100B polyclonal | S100 calcium binding protein B | Synthetic | S100 beta fusion protein Ag7440 | Proteintech 15146-1-AP [RRID: AB_2254244] | 1:1000 |
| Rabbit anti-PROX1 polyclonal | Prospero homeobox 1 | Synthetic | PROX1 fusion protein Ag1543 | Proteintech 11067-2-AP [RRID: AB_2268604] | 1:500 |
| Rabbit anti-Calbindin-D-28K (EG-20) polyclonal | Vitamin D-dependent calcium-binding protein or cholecalcin | Synthetic | Rat Calbindin-D-28K C-terminal conjugated to KLH | Sigma C2724 [RRID: AB_258818] | 1:500 |

**Secondary antibodies**

| Goat anti-mouse (AlexaFluor 488) polyclonal | Mouse IgG | Mouse | Gamma immunoglobulins heavy and light chains | ThermoFisher A11001 [RRID: AB_10566289] | 1:500 |
| Goat anti-mouse IgG2a (AlexaFluor 488) polyclonal | Mouse IgG2a | Mouse | IgG2a | ThermoFisher A21131 [RRID: AB_141618] | 1:500 |
| Goat anti-mouse IgG2a (biotinylated) polyclonal | Mouse IgG, Fcγ subclass 2a specific | Mouse | IgG2a | Jackson Immunoresearch 115-065-206 [RRID: AB_2338572] | 1:500 |
| Goat Anti-Rabbit (AP-conjugated) polyclonal | Rabbit IgG | Rabbit | Gamma immunoglobulins heavy and light chains | Invitrogen 31340, RRID: AB_228339 | 1:500 |
| Goat anti-rabbit (AlexaFluor 568) polyclonal | Rabbit IgG | Rabbit | Gamma immunoglobulins heavy and light chains | ThermoFisher A11036 [RRID: AB_143011] | 1:500 |
transformations, ANOVA and WITH-IN group analysis were performed similarly to a previous study. All data transformations were performed using the Box-Cox procedure, and were necessary to satisfy the Normality-and-Constant-Variance requirements. The 24 hour data was square-root transformed, the 1 week data was natural log transformed, and the 6 week data was square-root transformed. All data was analyzed by 2-way ANOVA (F tests, general linear model). We looked for main effects and interactions between treatment and injury groups. Furthermore, we performed WITH-IN group analysis (regression analysis) to determine if certain group means were significantly different.

Results

Binge alcohol given before TBI results in high blood alcohol level (BAL), however, did not affect lesion size

As reported in our previous study, the alcohol gavage protocol resulted in 156.1 mg/dl ± 8.3 mg/dl mean BAL. Previous reports using the same rat strain resulted in similar BAL. Furthermore, binge alcohol did not significantly alter the lesion size as measured at any time point post-TBI as reported in our previous study.

TBI stimulated bilateral proliferation in the GCL of the dentate gyrus at 24 hours post TBI and binge alcohol decreased this response

After a TBI, cell proliferation in the dentate gyrus has been shown to markedly increase (see Ngwenya and Danzer for review). In our model of TBI, we also saw the dentate gyrus respond robustly through up-regulation of the number of proliferating cells in the GCL starting as early as 24 hours after injury (Figure 2A, A’-A’’). There was a statistically significant Injury (TBI) × Treatment (alcohol) interaction for ipsilesional \( F_{(1,24)} = 14.55; P < .005 \) and contralesional \( F_{(1,24)} = 26.89; P < .005 \) BrdU+ cell counts. Pre-planned multiple comparisons (WITH-IN group regression analysis) showed that the mean number of proliferating cells in the GCL of the Vehicle/TBI group was higher than that of the Vehicle/Sham group for both ipsilesional \( P < .005 \) and contralesional \( P < .005 \) hemispheres, indicating that TBI alone induced an approximately 2 fold increase in cell proliferation in the GCL. The mean BrdU+ cell count in the Alcohol/TBI group was not significantly different than that of the Vehicle/TBI group in the contralesional hemisphere \( F_{(1,24)} = 2.97; P = .099 \) but was very close to being significantly different in the ipsilesional hemisphere \( F_{(1,24)} = 3.92; P = .061 \) (Figure 2B, B’). However, the number of proliferating cells of the Alcohol/Sham group was 2 fold higher than that of the Vehicle/Sham group in the ipsilesional \( F_{(1,24)} = 5.12; P = .034 \) hemisphere (Figure 2B, B’), indicating that alcohol alone stimulated proliferation in the GCL at 1 week after the last binge.

Binge alcohol decreased BrdU+ cell number in the GCL of the dentate gyrus at 6 weeks post-TBI

We found that there was no significant main effect of Injury (TBI) or Alcohol treatment on the BrdU+ cell count of the contralesional GCL at 6 weeks post-TBI (Figure 3A). Multiple comparisons showed that the mean BrdU+ cell count was not significantly different among comparison groups. In the ipsilesional GCL, while there was no statistically significant effects of Injury or Injury x Treatment interaction, there was a significant overall difference between Vehicle and Alcohol groups \( F_{(1,27)} = 4.91; P = .036 \). This indicated that TBI had no effect on BrdU+ cell number in the GCL while binge alcohol still had a significant effect in decreasing the number of BrdU+ cells in the ipsilesional GCL at 6 weeks post-binge (Figure 3B-D).

Binge alcohol altered the distribution of BrdU+ cells in dentate gyrus at 1 week and 6 weeks post-TBI

In adult animals, DG granule cells are being continuously generated from neural precursor cells located in the SGZ, and these cells express markers as seen during development.
Figure 2. Binge alcohol and TBI alone and in combination altered BrdU+ cell number in the granule cell layer at 24 hours and 1 week post TBI. (A and B) plots of un-transformed BrdU+ cells count in the ipsi and contra-lesional GCL at 24 hours and 1 week post-TBI respectively: (A) at 24 hours after injury, binge alcohol significantly increased DG proliferation in sham groups and decreased proliferation in TBI groups bilaterally, (B) at 1 week after injury, binge alcohol and TBI alone significantly increased GCL proliferating cells. (A'-A''') and (B'-B'''') Representative immunohistochemical staining demonstrates labeling of BrdU+ cells in the ipsilesional DG at 24 hours and 1 week post TBI respectively. Yellow dash lines outline the SGZ; yellow arrows point to BrdU+ cells. Scale bar = 60 μm. Two-way ANOVA, post-hoc within-group analysis, * denotes P < .05, error bars = SEM.
Doublecortin (DCX) is a microtubule-associated protein and has become a reliable marker for neuronal differentiation. At 1 week post TBI, BrdU+/DCX+ double labeled cells were seen populating the GCL of the dentate gyrus (Figure 4B). Also at 1 week, many BrdU+ cells within the GCL were expressing Iba1, a microglial marker (Figure 4E). At this timepoint, we did not see GCL BrdU+ cells with mature neuronal markers such as NeuN or Calbindin (Figure 4C, D); however, we only qualitatively examined the Vehicle TBI group. In contrast, at 6 weeks post-TBI, a number of GCL BrdU+ cells expressed NeuN, Calbindin, and Prox1 (Figure 5A-C) and very few expressed Iba1 (Figure 5D); as before, we only qualitatively examined the Vehicle TBI group. Of particular importance is Prox1, a transcription factor expressed during DG granule cell development that persists in adult granule cells. At both 1 week and 6 week timepoints, we did not see many BrdU+ cells co-label with astrocyte markers (GFAP and S100B). Additionally, the distribution of BrdU+ cells in the GCL and

Figure 3. At 6 weeks after injury, binge alcohol had an overall effect on decreasing GCL BrdU+ cell number: (A) plot of un-transformed BrdU+ cell count in the ipsi and contra-lesional GCL at 6 weeks post TBI, (B and C) representative immunohistochemical staining demonstrates labeling of BrdU+ cells in the ipsilesional DG at 6 weeks post TBI, and (D) interaction plot of square-root transformed 6 week data showing overall binge alcohol effect. Yellow dash lines outline the SGZ; yellow arrows point to BrdU+ cells. Scale bar = 60 μm. Two-way ANOVA, post-hoc within-group analysis, * denotes P < .05, error bars = SEM.
Figure 4. Representative immunofluorescence staining of BrdU\(^+\) (red) cells with proliferative, neuronal and glial markers from the ipsilesional DG of a vehicle treated rat at 1 week post TBI. BrdU\(^+\) cells co-labeled with Ki67 a proliferative marker (A) and DCX, a marker for new neurons (B), while it did not co-labeled with markers of matured neurons such as NeuN (C) and calbindin (D). BrdU\(^+\) cells in the GCL expressed Iba1, a microglial marker (E), while no co-labeling with the astrocyte marker GFAP could be seen (F).

White arrowheads point to double-labeled cells, yellow dash lines outline the SGZ, scale bar in A = 20 \(\mu\)m, and in magnified panels = 10 \(\mu\)m. Abbreviations: GCL, granule cell layer; ML, molecular layer.
part of the dentate hilus were measured at both 1 week and 6 weeks post injury (Figure 6A–D). At 1 week post injury, we detected a significant main effect of TBI on the number of BrdU+ cells in the 2 most distant layers from the SGZ, the Hilus 2 (ipsilesional \( F_{(1,25)} = 10.27, P = .0037 \); contralesional \( F_{(1,25)} = 4.47, P = .0446 \)) and far GCL2 (ipsilesional \( F_{(1,25)} = 15.31, P = .0006 \); contralesional \( F_{(1,22)} = 5.71, P = .0247 \)) as defined in the methods section. At 6 weeks post TBI, there was a significant main effect of alcohol on the percent of BrdU+ cells (defined as BrdU+ cell number in each zone divided by total BrdU+ cell number in the DG) in the contralesional Hilus 2 (\( F_{(1,22)} = 6.67, P = .0170 \)). Additionally, there was a significant

Figure 5. Representative immunofluorescence of the ipsilesional DG from a vehicle treated rat showing BrdU+ cells within the GCL at 6 weeks post TBI expressing makers of matured neurons but very few expressed glial markers. BrdU+ cells (red nuclei) can be seen co-labeled with Prox1 (A), NeuN (B) and calbindin (C), which are markers of matured neurons in the GCL. Very few BrdU+ cells are seen co-labeled with the microglial marker Iba1 (D) and none can be seen co-labeled with the matured astrocyte marker S100B (E). White arrowheads point to double-labeled cells, yellow dash lines outline the SGZ, scale bar in A = 20μm, and in magnified panels = 10μm. Abbreviations: GCL, granule cell layer; ML, molecular layer.
main effect of alcohol on the number of BrdU+ cells in the ipsilesional Hilus1 ($F_{1,22} = 5.70, P = .026$). Furthermore, there was a significant main effect of TBI on the percent of BrdU+ cells in ipsilesional GCL2 ($F_{1,22} = 6.71, P = .0167$). Multiple comparisons showed that the percentage of BrdU+ cells was significantly higher in that of Alcohol TBI compared to Vehicle TBI groups in the contralesional Hilus 2. This also corresponds with a significant lower percentage of BrdU+ cells in Alcohol TBI compared to Vehicle TBI groups in the contralesional Hilus1. This indicates that in TBI groups at 6 week post TBI, binge alcohol shifts the distribution of newly generated cells to the more distal regions of the hilus, an area of ectopic granular cells as shown with BrdU+/Prox1+ double labeling in (Figure 6I-J), reported to be disruptive to hippocampal related tasks.40

**Discussion**

Our results show that a single episode of binge alcohol (3 consecutive daily doses) before a TBI resulted in a reduction in DG proliferation when measured at 24 hours post-injury. Although TBI did not have a significant effect on the number of proliferating cells in the DG when measured at 6 weeks post injury, binge alcohol overall significantly reduced the number of BrdU+ cells. Surprisingly, binge alcohol alone increased DG proliferation in the short-term when measured at 24 hours and 1 week after the last binge episode. Furthermore, binge alcohol prior to TBI led to an increase in the number of BrdU+ cells in the hilar region of the dentate gyrus. The staining pattern of these cells suggests they are mismigrated granule cells (hEGCs), which may play a role in increased susceptibility to seizures and negatively contribute to hippocampal function.

**TBI alone increased proliferation in the dentate GCL**

In our study, cell proliferation in the DG increased post-TBI when measured at 24 hours and 1 week post injury, as in agreement with past literature.31,42 Experimental manipulations that resulted in increased hippocampal neurogenesis such as exercise or environmental enrichment lead to improvements in learning, memory and other hippocampal-related tasks.38 Additionally, blocking hippocampal proliferation resulted in worse cognitive recovery.

**Figure 6.** Binge alcohol and TBI altered the distribution of newly generated cells within different layers of the dentate gyrus. (A-D) BrdU DAB IHC of the contralesional DG from the 6 week time point. Inserts in C and D are a magnified view of the boxed area, and red arrows point to BrdU+ cells in the Hilus 2 zone. (E-H) Plots of BrdU+ cell number and percent of total BrdU+ cells over 2 coronal brain sections per rat encompassing the dorsal hippocampus in different layers of the DG at 1 week and 6 weeks post-TBI. (I, J) Representative Prox1 (green) and BrdU (red) immunofluorescence of contralesional dentate gyrus at the 6 week time point. (I) schematic of hilar ectopic granule cell quantification method. The GCL was sub-divided into 2 zones, GCL1 and GCL2. The dentate hilar area adjacent to the GCL was also sub-divided into the Hilus 1 and Hilus 2. Each zone was approximately 30 μm in thickness. Immunolabeled sections were processed for DAB and BrdU+ cells in each zone were manually counted. Scale bars: A = 60 μm, insert=30 μm, l=30 μm, and (J) alcohol TBI group showed more Prox1+ cells in the ectopic hilar region. White arrowheads point to single-labeled hilar Prox1+ cells, and yellow arrows point to double-labeled cells (hilar subdivisions not shown). Two-way ANOVA, post-hoc Dunn’s multiple comparison, * denotes $P \leq .05$, Error bars = SEM.
post-TBI. Furthermore, the DG proliferative response to TBI in our study appeared to peak after 1 week and is similar to other studies. Interestingly, qualitative examination of BrdU staining of vehicle/sham groups (Figures 2A, 2B, 3B) indicated that in control animals, there was incorporation of additional new cells into the GCL during this time. This appears to be possible due to a several reasons such as the cumulative BrdU labeling strategy, the high sensitivity of the immunohistochemistry method and the heterogeneity of the proliferative pool of the DG. However, the cell proliferation effect of TBI at early time points did not extend to the 6 weeks post-injury time point. In fact, the mean BrdU+ cell count was lower in the vehicle TBI group as compared to vehicle sham group, bilaterally. This indicates that the elevated cell count as seen early on at 24 hours and 1 week did not persist, and these new cells could have either migrated away from the GCL or undergone cell death as seen in another study.

Additionally, we saw that many BrdU+ cells co-expressed the microglial marker Iba1 at 1 week post-TBI (Figure 4E), but not many of these double-labeled BrdU+/Iba1+ cells were seen in the dentate GCL at 6 weeks. Microglia are highly motile cells and therefore could have migrated away from the GCL during the time between 1 week and 6 weeks post-TBI. Microglia play a major role in the inflammatory response in the secondary phase of TBI, thought to be both beneficial and detrimental to CNS recovery. Pertinent to this study, neuroinflammation can stimulate neurogenesis yet, excessive microglial activation and inflammatory cytokines released by microglia such as IL-1β may cause the neurogenic microenvironment of the DG to be non-supportive for the survival and maturation of newborn cells.

Binge alcohol alone increased short-term proliferation in the dentate GCL but had the opposite effect in the long-term We found that binge alcohol alone elicited a bilateral proliferative response in the DG, as alcohol only treated animals exhibited up to a 4 fold increase in BrdU+ cells at 24 hours compared to controls. While certain proliferation is thought to be beneficial after brain injuries, stem cells within the dentate SGZ can only undergo a finite number of cell divisions, and the cell proliferation effect of TBI at early time points did not extend to the 6 weeks post-injury time point. In fact, the mean BrdU+ cell count was lower in the vehicle TBI group as compared to vehicle sham group, bilaterally. This indicates that the elevated cell count as seen early on at 24 hours and 1 week did not persist, and these new cells could have either migrated away from the GCL or undergone cell death as seen in another study.

Binge alcohol combined with TBI decreased TBI-induced proliferation in the dentate GCL We found that animals receiving binge alcohol had up to a 4 fold decrease in proliferating cells in the dentate GCL at 24 hours post-TBI. At later time points, the number of proliferating cells in the GCL of animals in the vehicle TBI and alcohol TBI groups were not significantly different. However, at the 1 week time point, the mean number of BrdU+ cells in the ipsilesional dentate GCL of animals in the alcohol TBI group was 40% lower than that of the animals in the vehicle TBI group. Our previous study also reported a similar significant effect of alcohol but in a different proliferative location, that is, the SVZ. At both the 24 hour and 1 week time points, alcohol significantly decreased the TBI induced SVZ cell proliferative response. In adult rats, the SVZ produces new neurons that migrate toward cortical lesion sites post-injury. Therefore, the negative effect of binge alcohol on SVZ neurogenesis could be the underlying cellular basis for the reduced recovery in skilled forelimb use as we have reported. In regards to the current study, proliferation may contribute to functional recovery post-TBI by incorporation of new neurons into areas of the hippocampus, and the reduction of the DG proliferative response associated with binge alcohol as shown here could worsen TBI associated cognitive deficits.

Binge alcohol combined with TBI altered the distribution of BrdU+ cells in the dentate gyrus We found that binge alcohol significantly altered the distribution of BrdU+ cells in the DG at 6 weeks after TBI. The shift in the distribution of newly generated cells (BrdU+) was consistent with mismigration of granule cells into the hilus, known as hilar ectopic granule cells (hEGCs), which rarely occur under normal circumstances. HEGCs express immunohistochemical markers that are found in granule cells such as the homeobox protein
and reduce co-morbidities following TBI complicated by alcoholic process could lead to better therapy to improve recovery, public health, a better understanding of the post-injury proliferation in the hilus DG area. These putative hEGCs are known to be detrimental firing activity than normal granule cells located in the GCL.40,65 This interesting property of hEGCs has been shown in a computational model to disrupt circuitry functions involved in pattern separation and completion,70 specific processes that underlie higher cognitive functions that depend on the neural circuitry between the DG and CA3.70 While our study quantified the number of BrdU+ cells within different layers in relation to the anatomical location of the SGZ, it is possible some of the cells counted could have non-neuronal identities. Therefore, it will be important in future studies exploring the relationships between binge alcohol, hEGCs and TBI to utilize double-labeled immunohistological methods.

In conclusion, our results show that TBI in the forelimb motor cortex robustly stimulated DG proliferation short-term in the adult male rat and that binge alcohol given prior to TBI dampened this reactive proliferative process. Furthermore, binge alcohol had a broad effect in reducing the number of newly generated cells in the DG at the 6 week time point, and led to an increased distribution of BrdU+ cells in the ectopic hilar DG area. These putative hEGCs are known to be detrimental to hippocampal dependent functions. Important to public health, a better understanding of the post-injury proliferative process could lead to better therapy to improve recovery, and reduce co-morbidities following TBI complicated by alcohol intoxication.

**Author Contributions**

Conceptualization: STT, ICV, S-YT, GLK; Methodology: STT, ICV, S-YT, GLK; Investigation: STT, S-YT, JYW, KH, NSA, JPG; Formal Analysis: STT, TEO; Writing – Original Draft: STT, GLK; Writing – Review & Editing: STT, GLK, S-YT, NSA; Visualization: STT; Supervision: GLK; Funding Acquisition: STT, GLK.

**Significance Statement**

Binge alcohol drinking is the most common mode of excessive alcohol consumption and patients presenting at the hospital for traumatic brain injury (TBI) are often under alcohol intoxication. This study provides evidence that a binge pattern of alcohol administration prior to TBI reduced injury-induced hippocampal proliferation in the short term, and suggests an increase in aberrant hilar ectopic granule cells in the long term. These changes in the proliferative capacity of the brain have important implications for recovery from TBI for many patients suffering with these injuries.

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