Traumatic Brain Injury Severity Affects Neurogenesis in Adult Mouse Hippocampus

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Abstract

Traumatic brain injury (TBI) has been proven to enhance neural stem cell (NSC) proliferation in the hippocampal dentate gyrus. However, various groups have reported contradictory results on whether TBI increases neurogenesis, partially due to a wide range in the severities of injuries seen with different TBI models. To address whether the severity of TBI affects neurogenesis in the injured brain, we assessed neurogenesis in mouse brains receiving different severities of controlled cortical impact (CCI) with the same injury device. The mice were subjected to mild, moderate, or severe TBI by a CCI device. The effects of TBI severity on neurogenesis were evaluated at three stages: NSC proliferation, immature neurons, and newly-generated mature neurons. The results showed that mild TBI did not affect neurogenesis at any of the three stages. Moderate TBI promoted NSC proliferation without increasing neurogenesis. Severe TBI increased neurogenesis at all three stages. Our data suggest that the severity of injury affects adult neurogenesis in the hippocampus, and thus it may partially explain the inconsistent results of different groups regarding neurogenesis following TBI. Further understanding the mechanism of TBI-induced neurogenesis may provide a potential approach for using endogenous NSCs to protect against neuronal loss after trauma.

Key words: injury severity; neurogenesis; neural stem cell proliferation; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a tremendous public health issue in the United States.^{1–4} According to Centers for Disease Control and Prevention statistics, TBI affects 2.5 million people each year and costs 76.5 billion U.S. dollars for direct and indirect medical expenses.⁵ As well, about 7000 U.S. soldiers on active duty suffer TBI from exposure to blast-waves every year.⁶ Despite physical disabilities, TBI induces multiple neurological complications, such as memory and learning impairment, seizure, Alzheimer's disease, and Parkinson's disease.^{1–4,7,8} This wide range of effects results from complicated pathological changes made by TBI, including cell death and profound neural degeneration.^{9,10} Besides focal injury in the cortex, TBI also causes diffuse injury in other regions, among which the hippocampus is most vulnerable.^{11,12} However, there is no U.S. Food and Drug Administration–approved drug to treat or prevent cell death after TBI. Since the hippocampus is important for human memory and learning capacity, an approach to generate new neurons is urgently needed to repair the damage.

Adult neurogenesis in the hippocampus throughout an entire lifetime has been proven in rodents, primates, and humans.^{13–18} Neurogenesis derives from neural stem cells (NSCs) that reside in the subgranular zone (SGZ) of the hippocampal dentate gyrus (HDG). In physiological condition, NSCs maintain basal activity.

They activate, proliferate, and generate neural progenitors that differentiate into a neural lineage and migrate to the granule cell layer of the HDG, which eventually produces new granular neurons.^{19–21} NSC-derived adult neurogenesis provides a potential approach to compensate for the loss of neurons following TBI.

Studies have shown that TBI promotes NSC proliferation,^{22–34} while data for elucidating neurogenesis after TBI are contradictory. Increased,^{22,29,30} unchanged,^{23,32,34} and decreased²⁸ neurogenesis have all been reported post-TBI. However, these studies have been done in different TBI models: fluid percussion injury (FPI),^{23,27,29,30} controlled cortical injury (CCI),^{28,32} cortical contusion,²² and impact-acceleration.³⁴ Because different models represent different aspects of human pathological characteristics,³⁵ they may induce different severities of TBI, although most studies were conducted in moderate levels in specific models. We consider injury severity as the key variation among controversial reports. In this study, we investigated whether neurogenesis is affected differently by different TBI severities in a consistent system. To decipher this question, we used a CCI model to induce TBI ranging from mild to moderate-to-severe levels. Using 5-bromo-2'-deoxyuridine (BrdU) incorporation and immunostaining with specific cell-type markers, we tracked the fate of proliferated cells and detected the effects of injury severity on proliferation of NSCs, survival of immature neurons, and maturation of adult-born neurons in the HDG. Through our study, we may better understand the response of NSCs to TBI,

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thus providing insights into the therapeutic potential of manipulating endogenous neurogenesis to repair damage caused by TBI.

Methods

Animal care

Male C57 BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were kept in a 12/12-h light/dark cycle environment with access to food and water *ad libitum*. Nestin-enhanced green fluorescent protein (EGFP) mice were kindly provided by Dr. Enikolopov at Cold Spring Harbor Laboratories (New York) and described previously.³⁶ All procedures were conducted under protocols approved by Indiana University's Animal Care and Use Committee.

Controlled cortical impact traumatic brain injury

Male C57 BL/6 mice ($n = 56$) were subjected to mild, moderate, and severe CCI injury or sham surgery as previously described.^{32,37} Mice were used in experiments at the age of 9 weeks. Briefly, the mice were anesthetized with a solution of 2.5% Avertin (Sigma-Aldrich, St. Louis, MO) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). Using sterile procedures, the skin was retracted and a 4 mm craniotomy was performed at a point midway between the lambda and bregma sutures and laterally midway between the central suture and the temporalis muscle. The skullcap was carefully removed without disruption of the underlying dura. Prior to injury induction, the tip of the impactor was angled and kept perpendicular to the exposed cortical surface.

The mouse CCI model uses an electromagnetic impactor that allows alteration of injury severity by controlling contact velocity and the level of cortical deformation independently. In these experiments, the contact velocity was set at 3.5 m/sec and deformation depth was set at 0.2 mm, 1.0 mm, and 1.2 mm, respectively, for mild, moderate, and severe TBI. The injury site was allowed to dry prior to suturing the wound. During surgery and recovery, a heating pad was used to maintain the core body temperature of the animals at 36–37°C.

BrdU injection post-trauma

Mice were subjected to sham surgery or different severities of TBI as described above. To evaluate NSC proliferation, BrdU was administered at 44 h following TBI (five mice for each group, BrdU at 100 mg/kg in saline, intraperitoneally [i.p.]; Sigma-Aldrich; Fig. 4A). To quantify surviving proliferated cells and newly-generated mature neurons, BrdU administration was performed once per day consecutively for 7 days following TBI (four mice for each group, BrdU at 50 mg/kg in saline, i.p.; Fig. 3A).

Tissue processing

For NSC proliferation assessment, animals were sacrificed at 48 h after initial injury (Fig. 4A). For immature neuron number evaluation, mice were perfused at 2 weeks after surgery (Fig. 6A). To quantify surviving proliferated cells and newly-generated mature neurons, animals were sacrificed at 4 weeks after TBI (Fig. 3A). Briefly, the animals were deeply anesthetized and transcardially perfused with cold saline, followed by a fixative containing 4% paraformaldehyde (PFA) in PBS. The brains were collected and were post-fixed overnight in PFA followed by cryoprotection for 48 h in 30% sucrose. Serial 30 μ m thick coronal sections were cut using a cryostat (LeicaCM 1950; Buffalo Grove, IL), and stored at –20 °C. The sections were then processed for histological and/or immunohistochemical analysis.

Histology and immunohistochemistry

Nissl staining was performed to analyze histological changes. Briefly, sections were incubated in a solution of 0.1% cresyl violet

(Sigma-Aldrich) for 20 min. After a quick rinse in distilled water, the sections were then differentiated in 95% ethanol for 3 min and followed by dehydration in 100% ethanol for 5 min, twice. The sections were then cleared in xylene for 5 min twice, air-dried, and mounted with DPX (Sigma-Aldrich).

Sections were stained with Fluoro-Jade B (FJB) to detect dying cells. Briefly, sections were hydrated in distilled water for 5 min. The sections were then incubated in a solution of 0.06% potassium permanganate (Sigma-Aldrich) for 20 min at room temperature. After washing in distilled water for 5 min twice, the sections were incubated in a 0.0004% solution of FJB (Sigma-Aldrich) for 20 min, followed by washing in distilled water three times and incubation in a solution of 0.01% 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 10 min. Sections were then air-dried and mounted with DPX.

For immunostaining, free floating sections were washed in PBS three times at room temperature, incubated with blocking buffer (0.1% Triton X-100, 1% bovine serum albumin, 5% normal goat serum in PBS), and then incubated overnight with primary antibody at 4 °C. After washing in PBS three times, sections were incubated with secondary antibody for 2 h at room temperature, followed by DAPI treatment for 2 min. The sections were washed in PBS again three times and mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL). For BrdU incorporation, HCl pretreatment was performed prior to incubation in blocking buffer as follows: sections were incubated in 2N HCl for 1 h at room temperature, soaked in 0.1M (pH 8.4) borate buffer for 10 min, followed by PBS washing three times and then were processed according to standard protocol in blocking solution. The following primary antibodies and their final concentrations were used: anti-BrdU (1:200, rat; AbDSerotec, Raleigh, NC), anti-Sox2 (1:1000, rabbit; Abcam, Cambridge, CA), anti-GFP (1:1000, chicken; Abcam), anti-doublecortin (DCX; 1:500; guinea pig; Millipore, Darmstadt, Germany), anti-neuronal nuclei (NeuN; 1:500, mouse; Millipore), anti-glial fibrillary acid protein (GFAP; 1:1000, mouse; Sigma-Aldrich), anti-NG2 (1:200, rabbit; Millipore) and anti-CD11b (1:500, rabbit; Abcam). Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) secondary antibodies were applied in a dilution of 1:1000.

Cortical cavity volume measurement

Series of every sixth sections (30 μ m thickness, 180 μ m apart) from covered injured cortex were stained with cresyl violet to show the spare cortex. The boundary contours of the contralateral and ipsilateral spare cortex were drawn with a Zeiss microscope (Jena, Germany) attached to a Neurolucida system (Microbrightfield Inc., Colchester, VT). The contours enclosed volume was measured. The percent cortex of the cavity was calculated with the following formula: percentage of the cortical cavity = (contralateral cortex volume – ipsilateral spare cortex volume)/contralateral cortex volume $\times$ 100%.

Cell counting

Sections were simultaneously processed for immunostaining to detect target cells. An inverted fluorescent microscopy system (Zeiss Axiovert 200M) was used to analyze sections. For BrdU incorporated cell proliferation, series of every sixth section (30 μ m thickness, 180 μ m apart, 12 to 16 sections for each animal) from covered injured hippocampus were processed with antibody against BrdU. Cells were counted under the fluorescent microscope using the 40 $\times$ objective through the whole series of sections. The total cell number was determined through the profile count method. Briefly, every single BrdU-positive cell (even partial BrdU-positive nuclei at the borders of sections) was counted in multi-planes throughout the entire dentate gyrus in the 30 μ m section. For cell type specification, series of every sixth section (30 μ m thickness, 180 μ m apart) from covered injured hippocampus were processed with antibodies against BrdU and specific cell type marker. The double-labeled cell was counted using the profile count method as mentioned

FIG. 1. Traumatic brain injury (TBI) severities shown by Nissl staining. Brain tissues collected at 48 h after trTBI. **(A-D)** Nissl staining of serial coronal sections from sham (A), mild TBI (B), moderate TBI (C), and severe TBI (D) animals show the characteristic structures in different severities of TBI. **(E)** Quantification of cortical cavity volume in different severities. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. $n = 3$ for each group. n.s., not significant.

above and determined as follows. We used BrdU as an indicator. When the BrdU-positive cell showed in the frame, we switched to the channel matching the cell specific marker. If the target cell also had been marked, we considered it as a double-positive cell. For NSC proliferation evaluation, the entire SGZ was examined for the whole series of sections. To quantify surviving proliferated cells and distinguish newly-generated mature neurons, the entire dentate gyrus was examined through the whole series of sections. The total number of quantified cells was adjusted by correction.⁴⁸ The volume of granule cell layer or dentate gyrus area was measured by creating contours in Zeiss software (AxioVision v4.8). For NSC proliferation evaluation, cell density was calculated by dividing the total cell number by the volume of the granule cell layer. For surviving proliferated cells and newly-generated mature neurons assessment, cell density was calculated by dividing the total cell number by the volume of the dentate gyrus. Thus cell density was expressed as average number/mm³.

For quantifying immature neurons, three epicentral sections (30 μ m thickness, 180 μ m apart) were processed with antibody against DCX. The total cell number was determined through the profile count method as described above. The volume of granule

cell layer was measured in Zeiss software (AxioVision v4.8). Cell density was calculated by dividing the total cell number by the volume of the granule cell layer. DCX-positive cells were expressed as average number/mm³.

To assess proportions of mature neurons and reactive glia in surviving proliferated cells, epicentral sections of each animal were processed with antibodies against BrdU and GFAP, or NG2 or CD11b. BrdU positive cells and double positive cells were quantified as described above. The percentage of target cell type was then calculated. The proportion of each cell type in the whole BrdU positive population was then normalized to match 100% in total.

Microscopy

The inverted microscopy system (Zeiss Axiovert 200M) mentioned above was combined with an apotome and interfaced with a digital camera (Zeiss Axio Cam MRc5) controlled by a computer. Representative images were captured using the apotome in software (AxioVision v4.8), and stacked as a cut view image in the software. Then, the images were assembled and labeled in Photoshop 7.0 (Adobe System, San Jose, CA).

FIG. 2. Severe traumatic brain injury (TBI) mainly causes mature neuron death. Brain tissues collected at 24 h after TBI induction. (A–D) Fluoro-Jade B (FJB) staining shows dying cells in hippocampal dentate gyrus (HDG) in sham (A), mild (B), moderate (C), and severe TBI (D) animals. (E, F) Immunostaining with neuronal nuclei (NeuN; red) for mature neurons followed by FJB staining (green) for dying cells. (G, H) Immunostaining with doublecortin (DCX; red) for immature neurons followed by FJB staining (green) for dying cells. 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei shows HDG structure. (F, H) Enlarged images of E and G (indicated by white boxes) to show co-label of NeuN and FJB (F, indicated by white arrows) and co-label of DCX and FJB (H, indicated by white arrows). Color image is available online at www.liebertpub.com/neu

FIG. 3. Traumatic brain injury (TBI) severity affects surviving proliferated cells. (A) Schematic shows experimental strategy. (B-E) Immunostaining with 5-bromo-2'-deoxyuridine (BrdU; green) to identify surviving proliferated cells in the hippocampal dentate gyrus (HDG) in sham (B), mild TBI (C), moderate TBI (D), and severe TBI (E) mice. 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei shows HDG structure. (F) Quantification of BrdU-positive surviving proliferated cells in HDG in sham and different TBI severities. (G) Percentage of BrdU-positive surviving proliferated cells in individual HDG subregions, molecular layer (ML), granule cell layer (GCL), and hilus, in sham and different TBI severities. Pie chart sizes indicate ratio of total BrdU-positive cell number in different groups. (H-J) Quantification of BrdU-positive surviving proliferated cells in ML, GCL, and hilus respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 3$ for sham group, $n = 4$ for mild and moderate TBI groups, and $n = 5$ for severe TBI group. n.s., not significant. Color image is available online at www.liebertpub.com/neu

Statistical analysis

Cell quantification data were shown as average $\pm$ standard deviation and analyzed via One-way analysis of variance followed by *post hoc* analysis with Tukey's honest significance test using SPSS software IBM Corporation, Armonk, NY. Significance was set at $p < 0.05$.

Results

Assessing different severities of TBI induced by a CCI model

TBI is a complex disease ranging from mild to severe levels. To generate different injury severities in rodents, a CCI model was used as previously described^{32,37} and injury levels were controlled by deformation depths at 0.2 mm, 1 mm, and 1.2 mm, which correspond to mild, moderate, and severe TBI, respectively. Injury level was further confirmed by histological examination (Fig. 1). Consistent with our previous reports, animals with mild injury showed limited cortical disruptions without obvious cavity formation (lesion volume is $0.2\% \pm 5.4\%$; $p = 1.0$; Fig. 1B, 1E).³⁷ Moderate TBI introduced a cortical cavity (cavity volume is $14.3\% \pm 1.0\%$; $p = 0.001$; Fig. 1C, 1E) with an intact corpus callosum,³⁸ while severe TBI dramatically increased cavity volume to $26.2\% \pm 1.8\%$ ($p < 0.001$; Fig. 1D, 1E), further destroyed the corpus callosum, and even deformed the hippocampal structure.

At the cellular level, we evaluated cell death in the hippocampal dentate gyrus (HDG), where the NSCs reside, with FJB staining. FJB-positive cells were barely observed in the HDG of mouse brain that received sham (Fig. 2A) or mild CCI injury (Fig. 2B). FJB-positive

cells were easily detectable in the HDG of mouse brain that received moderate CCI injury (Fig. 2C), mainly at the inner 1/3 of the granular cell layer, where the immature neurons locate. We previously reported that most of the dead cells in the HDG following moderate CCI-injury are immature neurons.³⁸ As the injury severity increased to the severe level, the FJB-positive cells were not only located at the inner 1/3 of the granular cell layer, they also were observed in the outer granular cell layer and hilus (Fig. 2D), where mature granular neurons and mature interneurons locate. Further immunostaining confirmed that the majority of cells dying in severe TBI were co-stained with NeuN-positive mature neurons (Fig. 2E and 2F, indicated by white arrows) and a small proportion were co-labeled with DCX-positive immature neurons (Fig. 2G and 2H, indicated by white arrows).

Evaluating the effects of different severities of TBI on surviving proliferated cells in the hippocampal dentate gyrus

The effect of TBI on cumulative cell proliferation in the HDG during the first week post-trauma has been investigated widely in different models using BrdU incorporation.^{22–26,28–34} Whether the severity of TBI affects cell proliferation and the following survival is unclear. To address this question, we injected animals i.p. with BrdU (50 mg/kg) to label the proliferating cells once per day in the first week after surgery and collected tissues at 4 weeks following TBI (Fig. 3A). Series of every six sections (12 to 16 sections in total for each animal), which covered the distance of the whole hippocampus, were processed for BrdU staining.

BrdU-positive cells in the HDG were then quantified. Sham animals showed the baseline of surviving proliferated cells in HDG

FIG. 4. Traumatic brain injury (TBI) severity affects neural stem cell (NSC) proliferation. (A) Schematic shows experimental strategy. (B–E) Immunostaining with 5-bromo-2'-deoxyuridine (BrdU; green) to detect proliferating cells in sham (B), mild TBI (C), moderate TBI (D), and severe TBI (E) mice between 44–48 h after TBI induction. 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei shows hippocampal dentate gyrus (HDG) structure. (F–I) Double immunostaining with BrdU (green) and transcription factor Sox2 (red) to identify NSC proliferation in the subgranular zone (SGZ) in sham (F), mild TBI (G), moderate TBI (H), and severe TBI (I) mice. (J–M) Three-dimensional reconstruction of proliferating NSCs in F–I (indicated in white boxes) to show BrdU and Sox2 co-label. (N) Quantification of BrdU and Sox2 double-positive proliferating NSCs in SGZ in sham and different TBI severities. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5$ for each group. n.s., not significant. Color image is available online at www.liebertpub.com/neu

to be $969 \pm 175/\text{mm}^3$ (Fig. 3B, 3F); mild injury animals had a similar level at $1267 \pm 161/\text{mm}^3$ ($p=0.985$; Fig. 3C, 3F). In moderate injury animals, density of surviving proliferated cells was dramatically elevated to $3739 \pm 1250/\text{mm}^3$ ($p=0.032$; Fig. 3D, 3F). The number was further extended to $5406 \pm 1611/\text{mm}^3$ ($p=0.001$; Fig. 3E, 3F) in severely-injured animals. Collectively, moderate and severe, but not mild TBI, increased the number of surviving proliferated cells 4 weeks after injury.

Determining the effects of different severities of TBI on neural stem cell proliferation

Different types of cells proliferate in the HDG following TBI, including neural stem cells and reactive glia.^{22–33} Our previous study in moderate TBI showed that injury transiently enhanced NSC proliferation, peaking at 44–48 h after initial injury.³³ To assess NSC proliferation responding to different severities of TBI,

FIG. 5. Validation of neural stem cell (NSC) proliferation following traumatic brain injury (TBI) with nestin-enhanced green fluorescent protein (GFP) transgenic mice. Brain tissue collected at 48 h after moderate TBI in nestin-GFP mouse. (A–D) Immunostaining with green fluorescent protein (GFP), 5-bromo-2'-deoxyuridine (BrdU), and transcription factor Sox2 confirms that nearly all BrdU and Sox2 double-positive cells quantified in the subgranular zone (SGZ; indicated by white arrows) are GFP-positive neural stem cells. (E–H) Three-dimensional reconstruction of D (indicated in white box) shows co-label of Sox2 and GFP in SGZ. A proliferating neural stem cell in SGZ with Sox2-, GFP-, and BrdU-triple positive was indicated by a white arrowhead. A reactive glia in the hilus with only Sox2 positive was indicated by a white arrow. Color image is available online at www.liebertpub.com/neu

we pulse-labeled the proliferating cells with BrdU (100 mg/kg, i.p.) at 44 h and collected the brains at 48 h to quantify the proliferating cells between 44–48 h following surgery (Fig. 4A). In the HDG of sham animals, we observed a small number of cells undergoing proliferation, mainly in the SGZ, where the neural stem cells reside (Fig. 4B). Mild TBI animals showed a similar number and distribution of BrdU-positive cells in the HDG (Fig. 4C). Moderate and severe TBI animals showed an obvious increase of BrdU-positive cells and also a dispersed distribution into the molecular layer and hilus (Fig. 4D, 4E).

It is known that cell proliferation post-trauma also contains glial reactivation.³⁹ To further discern NSC proliferation, we performed double immunostaining with antibodies against BrdU and transcription factor Sox2, as Sox2 is required for NSC self-renewal and widely used as an NSC marker.^{40,41} Because NSCs in the HDG exclusively reside in the SGZ, we quantified proliferating NSCs based on their co-staining with Sox2 and specific location within the SGZ (Fig. 4F–4M). In sham animals, we observed the baseline of NSC proliferation to be $1038 \pm 135/\text{mm}^3$ (Fig. 4F, 4J, 4N), which represents normal adult neurogenesis under physiological conditions. In mild TBI animals, NSC proliferation was not altered, compared with sham animals ($1096 \pm 105/\text{mm}^3$; $p = 0.998$; Fig. 4G, 4K, 4N). After moderate TBI, NSC proliferation showed a dramatic 2.2-fold increase ($2332 \pm 540/\text{mm}^3$; $p = 0.001$; Fig. 4H, 4L, 4N). This result is consistent with our previous data.³² In severe TBI animals, NSC proliferation was further boosted by 3.2-fold, compared with sham animals ($3352 \pm 714/\text{mm}^3$; $p < 0.001$; Fig. 4I, 4M, 4N).

Since Sox2 also expressed in some of the reactive glia, in order to rule out the possible interference from the reactive glia adjacent to the SGZ, we took advantage of a nestin-EGFP transgenic mouse line, in which EGFP driven by nestin promoter is expressed in the NSCs but not in reactive glia.³⁶ With this unique mouse line, we validated that more than 83% of BrdU and Sox2 double-positive cells in the SGZ were GFP-positive NSCs in the HDG following a moderate level of CCI-injury (Fig. 5).

Assessing the effects of different severities of TBI on immature neuron number

Previously, we demonstrated that immature neurons are the most vulnerable cell type in the HDG following moderate TBI.³⁸ To assess the effects of TBI severity on immature neuron number, we collected brains at 2 weeks after TBI at different severities and counted DCX-positive immature neurons in the HDG (Fig. 6A). Two weeks is the critical time for a newly-born and immature neuron to survive and differentiate into a mature neuron.¹⁹ In all the animals, DCX-positive cells resided in the inner 1/3 of the granule cell layer (GCL) with neurites growing towards the molecular layer (ML; Fig. 6B–6E). We then quantified DCX-positive cell numbers in each group (Fig. 6F–6J). In animals receiving sham surgery, there were $23,432 \pm 3861/\text{mm}^3$ DCX-positive cells residing in the GCL (Fig. 6F, 6J). No significant alteration was seen in the number of DCX-positive cells in the HDG following mild TBI ($30,528 \pm 6495/\text{mm}^3$; $p = 0.738$; Fig. 6G, 6J) and moderate TBI ($31,640 \pm 5778/$

FIG. 6. Traumatic brain injury (TBI) severity affects immature neuron number. (A) Schematic shows experimental strategy. (B–I) Immunostaining with doublecortin (DCX) (red) to identify immature neurons in the hippocampal dentate gyrus (HDG) in sham (B, F), mild TBI (C, G), moderate TBI (D, H), and severe TBI (E, I) mice. 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei shows HDG structure. (F–I) Enlarged images of B–E (indicated in white boxes) to show DCX-positive immature neurons in granule cell layer (GCL) in higher magnification. (J) Quantification of DCX-positive immature neurons in sham and different TBI severities. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 4$ for sham and moderate TBI groups, $n = 5$ for mild TBI group, and $n = 7$ for severe TBI group. n.s., not significant. Color image is available online at www.liebertpub.com/neu

mm³; $p=0.681$, Fig. 6H, 6J). However, in severely injured animals, the number of DCX-positive cells was dramatically elevated by 2.2-fold to $51,586 \pm 15,246/\text{mm}^3$ ($p=0.003$; Fig. 6I, 6J). These results indicated that severe TBI, but not mild or moderate TBI, increases immature neuron number within the HDG at 2 weeks after injury.

Determining the effects of different severities of TBI on generation of new mature neurons

To assess number of newly-generated mature neurons following different severities of TBI, we combined BrdU staining with NeuN, a specific cell type marker for mature neurons, to detect adult-born mature neurons following injury (Fig. 7A). In sham HDG, we ob-

served BrdU-positive cells mainly located in the GCL, where newly-generated granule neurons are supposed to be (Fig. 7B). Mild TBI animals showed a similar pattern of BrdU-positive cells, mostly in the GCL (Fig. 7C). While in the HDG of moderate and severe TBI animals, a large proportion of BrdU-positive cells distributed in the ML and hilus, as well (Fig. 7D, 7E), conferring robust gliogenesis. We further quantified BrdU and NeuN double-positive cells in different groups (Fig. 7F-7N). In sham animals, $108 \pm 11/\text{mm}^3$ new neurons were generated in the HDG as the baseline (Fig. 7F, 7J, 7N). The number was increased in mild TBI ($145 \pm 66/\text{mm}^3$; $p=0.935$; Fig. 7G, 7K, 7N) and moderate TBI ($223 \pm 118/\text{mm}^3$; $p=0.316$; Fig. 7H, 7L, 7N) without a significant difference. However, severe TBI dramatically promoted newly-produced neurons by three-fold ($329 \pm 84/\text{mm}^3$; $p=0.016$; Fig. 7I,

FIG. 7. Traumatic brain injury (TBI) severity affects adult-born mature neurons in hippocampal dentate gyrus (HDG). (A) Schematic shows experimental strategy. (B-M) Double immunostaining with 5-bromo-2'-deoxyuridine (BrdU; green) and neuronal nuclei (NeuN; red) to identify newly-generated mature neurons in the hippocampal dentate gyrus (HDG) in sham (B, F, J), mild TBI (C, G, K), moderate TBI (D, H, L), and severe TBI (E, I, M) mice. (F-I) Enlarged images from B-E (indicated in white boxes) to show BrdU and NeuN double-positive cells in the granular cell layer (GCL). (J-M) Three-dimensional reconstruction of newly-generated mature neurons in F-I (indicated in white boxes) to show BrdU and NeuN co-label. (N) Quantification of BrdU and NeuN double-positive cells in HDG in sham and different TBI severities. (O) Percentage of BrdU and NeuN double-positive cells in individual HDG subregions, molecular layer (ML), GCL, and hilus, in sham and different TBI severities. Pie chart sizes indicate ratio of total BrdU and NeuN double-positive cell number in different groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n=3$ for sham group, $n=4$ for mild and moderate TBI groups, and $n=5$ for severe TBI group. n.s., not significant. Color image is available online at www.liebertpub.com/neu

7M, 7N). Regardless of injury groups, the vast majority of newly-generated mature neurons located in the GCL (more than 91%) with few in the ML and hilus, and there was no significant difference for the distribution among four groups (Fig. 7O). Taken together, generation of adult-born mature neurons was increased only in animals with severe TBI, but not in animals with mild or moderate TBI. This conclusion agrees with our previous report that demonstrated moderate CCI- injury does not increase neurogenesis.³²

Evaluating gliogenesis after different severities of TBI

Glial response to TBI is another source of cell proliferation besides NSC proliferation. As shown in Figure 3, the number of proliferated cells in the HDG increased as the severity of injury increased. Further, the distribution of the proliferated cells was different in the HDG following different severities of TBI. The proliferated cells mainly located to the SGZ at the HDG of mice that received sham surgery or mild TBI, while proliferated cells dispersed to other regions in the HDG of mice that received moderate or severe TBI. Since location of cells can partially indicate their identities, we first divided surviving proliferated cells into different subregions of the HDG (Fig. 3G-J). Compared with sham animals, mild TBI mice showed a similar distribution of BrdU-positive cells in the molecular layer (ML), the granule cell layer (GCL) and the hilus. In moderate and severe TBI mice, the distribution of BrdU-positive cells slightly shifted away from the GCL (43.5% in sham animals and 41.1% in mild TBI, compared with 31.5% in moderate and 33.0% in severe TBI animals in the GCL, Fig. 3G). When each subregion was examined separately, mild TBI did not change the number of BrdU-positive cells in any of the regions, while moderate and severe TBI increased the number in both the ML and the GCL, but not in the hilus. Specifically, moderate TBI resulted in 4.6-fold and 2.8-fold promotion of BrdU-positive cell number in the ML and GCL respectively, and severe TBI further elevated the number to 5.5-fold in the ML and 4.2-fold in the GCL (Fig. 3H-3J). As the majority of cells in the ML are glial cells, and neurons mainly reside in the GCL, adult-born mature neurons only account for a small proportion of those BrdU-positive cells, the rest of the BrdU labeled cells are likely reactive glia.

To further confirm glial cell identities, we double-stained BrdU-positive cells with different types of glial cell markers: GFAP for astrocytes (Fig. 8A-8C), NG2 for oligodendrocyte precursors (Fig. 8D-8F), or CD11b for microglia (Fig. 8G-8I). We quantified the proportion of each cell type in the BrdU-positive cell population in each subregion (Fig. 8J). Although no significant difference was detected in the cellular compositions in any of the subregions, some subtle changes were observed as follows. In the ML, the contribution of each cell type did not change much among different

groups (Fig. 8J, top panel). In the GCL, generation of mature neurons accounted for a similar proportion in the BrdU-positive cell population in all animals (52.9% in sham, 61.3% in mild, 52.3% in moderate, and 52.8% in severe TBI animals, Fig. 8J, middle panel), although the absolute number of new neurons increased in severe TBI. Whereas, an increase in proliferated NG2-positive cell proportion was observed in moderate and severe TBI (5.8% in sham, 3.8% in mild, 11.6% in moderate, and 14.1% in severe TBI, Fig. 8J, middle panel). In the hilus, an interesting elevation of proliferated CD11b-positive microglia percentage was noticed instead (0 in sham, 9.4% in mild, 26.0% in moderate and 26.3% in severe TBI; Fig. 8J, bottom panel).

Discussion

Neural stem cells support adult neurogenesis through a lifetime and enable the regenerative potential of the adult brain. NSC proliferation in the HDG following TBI brings about the possibility of injury repair by endogenous neurogenesis. However, whether endogenous NSC proliferation supports neurogenesis following injury is disputed. Current contradictory studies were conducted by different injury models and mostly in rodents. Comparing reported works, CCI injury applied in adult mice^{28,32} and cortical contusion injury induced in adult rats²² both recapture focal injury characteristics in human TBI, whereas impact acceleration model performed in adult rats³⁴ mainly mimics diffused axonal injury in TBI patients, and lateral FPI injury induced in adult rats^{23,29,30} exhibits mixed focal and diffuse injury in the clinical setting.³⁵ Since diffuse injury (i.e., FPI and impact acceleration) tends to affect a larger brain area than focal injury (i.e., CCI and cortical contusion model), diffuse models generally produce injury with greater severity regarding the whole-brain network. Overall, neural activity, metabolism level, and neurotrophic factors in the hippocampus may all be altered by injury model and resulting injury severity. Since all the signals mentioned above are essential for adult neurogenesis regulation,⁴² we consider injury severity as one of the most crucial variations in previous discrepancies. Besides injury severity, the time-point at which neurogenesis was examined is also critical, as TBI pathogenesis and adult neurogenesis are both dynamic processes. Current studies that evaluated neurogenesis at early as 2 days post-injury to as late as 10 weeks post-injury might reflect different single points on the dynamic curve. Here, we assessed neurogenesis after different severities of TBI in a consistent system at three crucial stages in order to attain a better profile for understanding how NSCs respond to injury.

By combining BrdU labeling with immunostaining of specific cell type markers, we evaluated the effects of TBI severity on NSC proliferation, immature neuron number, and maturation of adult-

FIG. 8. Gliogenesis accounts for the rest of surviving proliferated cells. Brain tissues were collected by the procedure shown in Figure 3; tissues from moderate traumatic brain injury (TBI) mice shown as example. (A-C) Double immunostaining with 5-bromo-2'-deoxyuridine (BrdU; green) and glial fibrillary acid protein (GFAP; red) to identify astrocytes in proliferated cell population in the ipsilateral side of injured brain. (D-F) Double immunostaining with BrdU (green) and NG2 (red) to identify the oligodendrocyte lineage in proliferated cell population in the ipsilateral side of injured brain. (G-I) Double immunostaining with BrdU (green) and CD11b (red) to identify microglia in proliferated cell population in the ipsilateral side of injured brain. 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei shows the hippocampal dentate gyrus (HDG) structure. (B, E, H) Enlarged images of A, D, G (indicated in white boxes) to show gliogenesis in higher magnification. (C, F, I) 3-dimensional reconstruction of proliferated glial cells in B, E, H (indicated in white boxes) to confirm their cell types. (J) Percentage of different cell types in BrdU-positive surviving proliferated cell population in individual HDG subregions, molecular layer (ML), granule cell layer (GCL), and hilus, in sham and different TBI severities. Pie chart sizes indicate ratios of total BrdU-positive cell number in different groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 3$ for sham group, $n = 4$ for mild and moderate TBI groups, and $n = 5$ for severe TBI group. n.s., not significant. Color image is available online at www.liebertpub.com/neu

born neurons. In our study, we observed that mild TBI barely causes alteration of NSC proliferation, immature neuron number, or generation of mature neurons, indicating low NSC or glial response. Previously, we reported that mild TBI causes limited cell death in the cortex but not in the HDG,³⁷ and this may explain why mild TBI does not trigger cell proliferation.

Moderate TBI leads to increased NSC proliferation followed by neither dramatic change in immature neuron number nor mature neuron production. We have reported that immature neurons are the most vulnerable cell type in the hippocampus, leading to a dramatic reduction in the number of immature neurons in the hippocampus at 24 h after injury.³⁸ Although moderate CCI injury promotes NSC proliferation, it may only make up for the loss of immature neurons at the early acute phase. A slight but not significant increase of immature neuron number shown in our results is likely compensating effect. Moreover, immature neurons have to integrate into local circuits and then develop into mature neurons. Since no pre-existing mature neuron dies after moderate TBI, unchanged newborn mature neuron may relate to immature neuron death resulting from failure to integrate into neural circuits.

In contrast, severe CCI injury induces a much more severe tissue lesion in the cortex and increased cell death, mainly mature neuron death, in the hippocampus. Severe CCI significantly promoted cell proliferation and mature neuron production, compared with moderate TBI, corresponding to the mature neuron death as injury severity increased. This result suggests that NSCs can sense the environmental changes, and respond to different intensities of stimuli.

We also detected subtle changes in gliogenesis in different subregions of the HDG. In the ML, cellular composition was comparable among all four groups. In the GCL, moderate and severe TBI caused an increased percentage of proliferated NG2 cells. In the hilus, the proliferated microglia proportion was elevated in all TBI groups instead. It is interesting to detect these trends, as glial cells play important roles in regulating neuronal activity and adult neurogenesis. Previously considered only as oligodendrocyte precursors, NG2 cells have currently been proven to show population heterogeneity. Some subsets of NG2 cells can respond to neuronal stimulation in the adult hippocampus by elevating intracellular calcium and are considered as modulators of synaptic activity.⁴³ NG2 cells observed in the GCL after injury predominantly located close to the inner or outer border of the GCL (data not shown), thus indicating their potential involvement in modulating neuronal activity in spared or newly-formed neural circuitry after injury. Microglia have been reported to play opposite roles in adult neurogenesis. Classic activation of microglia was reported to suppress neurogenesis by expressing pro-inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor α , while with alternative activation, microglia release anti-inflammatory cytokines (i.e., IL-4, IGF-1 and transforming growth factor β), and promote neurogenesis.⁴⁴ In our case, subtype identification of microglia was not performed, whereas the elevated microglia proportion in the hilus is likely one of the mechanisms regulating neurogenesis, although no clear clue refers whether they are supportive or detrimental to neurogenesis.

Taken together, our results demonstrate that TBI severity affects neurogenesis. Mild TBI does not affect neurogenesis. Moderate TBI promotes NSC proliferation without increasing neurogenesis. Severe TBI enhances NSC proliferation, immature neuron number, and mature neuron generation. Further investigation is needed to evaluate characteristics of newly-generated neurons, including their long-term survival, their integration into local circuits, and

their functional properties. Although behavioral assessments are not included in this study, other groups have demonstrated that learning and memory capacity recoveries, determined by Morris water maze test performances, are positively correlated with the level of neurogenesis following TBI.^{45–47}

By illustrating the phenomenon above, we may partially explain the dispute existing among different groups regarding neurogenesis following TBI. Moreover, this effect on neurogenesis indicates that in the adult brain, NSCs have the potential to make up for cell loss after trauma. By further investigating the mechanism that regulates this inner neuroplasticity, we may be able to provide insights into a potential therapeutic use of endogenous NSCs against cell loss post-trauma.

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