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Neurologic impairment following closed head injury predicts post-traumatic neurogenesis

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Abstract

In the mammalian hippocampus, neurogenesis persists into adulthood, and increased generation of newborn neurons could be of clinical benefit following concussive head injuries. Post-traumatic neurogenesis has been well documented using “open” traumatic brain injury (TBI) models in rodents; however, human TBI most commonly involves closed head injury. Here we used a closed head injury (CHI) model to examine post-traumatic hippocampal neurogenesis in mice. All mice were subjected to the same CHI protocol, and a gross-motor based injury severity score was used to characterize neurologic impairment one hour after the injury. When analyzed 2 weeks later, post-traumatic neurogenesis was significantly increased only in mice with a high degree of transient neurologic impairment immediately after injury. This increase was associated with an early increase in c-fos activity, and subsequent reactive astrogliosis and microglial activation in the dentate gyrus. Our results demonstrate that the initial degree of neurologic impairment after closed head injury predicts the induction of secondary physiologic and pathophysiologic processes, and that animals with severe neurologic impairment early after injury manifest an increase in post-traumatic neurogenesis in the absence of gross anatomic pathology.

Keywords

closed head injury; traumatic brain injury; adult neurogenesis; hippocampus; neurologic severity score

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Introduction

The generation of newborn hippocampal neurons persists throughout life in mammals, including humans (for review, see (Zhao et al., 2008), and experiments in rodents strongly suggest that these new cells contribute to cognitive function (Dupret et al., 2008; Sahay et al., 2011; Shors et al., 2001). Traumatic brain injury (TBI) enhances hippocampal neurogenesis (Richardson et al., 2007), and this enhancement may contribute to the restoration of cognitive function (Blaiss et al., 2011; Kleindienst et al., 2004; Lu et al., 2005; Lu et al., 2003). Post-traumatic neurogenesis has been well documented in open head injury models involving controlled cortical impact (Dash et al., 2001; Kernie et al., 2001; Lu et al., 2003) or lateral fluid percussion (Chirumamilla et al., 2002; Kleindienst et al., 2004; Rice et al., 2003; Sun et al., 2005) in which impacts are made onto exposed dura through a craniotomy. Most cases of human TBI, however, involve a closed head injury (CHI; Centers for Disease Control and Prevention (CDC), 2012). Surprisingly, increased hippocampal neurogenesis has not been demonstrated following experimental CHI, despite increases in the generation of new glial cells (Bye et al., 2011; Carthew et al., 2012; Ng et al., 2012).

Given the variability of CHI models (Xiong et al., 2013), it remains possible that the inability to detect an increase in post-traumatic neurogenesis was secondary to non-uniform injury in experimental animals. Humans have highly variable clinical presentations after closed head injury (Moser and Schatz, 2002; Saatman et al., 2008), making experimental closed head injury models both mechanistically and clinically relevant despite their variability. Initial neurologic impairments are used to score TBI severity in humans (CDC, 2012; Murray et al., 1999; Sherer et al., 2008; Teasdale and Jennett, 1974) and ultimately help to predict neurologic outcome (Narayan et al., 1981; Pal et al., 1989). Thus, to account for the variability inherent in experimental closed head injury, we studied CHI in mice, and categorized each animal's neurogenic and glial response as a function of their neurologic status 1-hour after injury.

Material and Methods

Animals

All procedures were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were in compliance with approved IACUC protocols at Oregon Health & Science University. Subjects were three-month-old male and female C57BL/6J (wild-type) mice as well as proopiomelanocortin-enhanced green fluorescent protein (POMC-EGFP) transgenic mice, in which newborn neurons transiently express EGFP, maximal at approximately two weeks post-mitosis (Overstreet et al., 2004). In POMC-EGFP mice, the sham group included 5 males and 6 females; and the CHI group included 11 males and 13 females. In wild-type mice, the sham group included 6 males and 5 females; and the CHI group included 6 males and 7 females. Separate cohorts of wild-type mice were used to assess c-fos protein 3 hours after injury and triphenyl tetrazolium chloride (TTC) or Fluoro-Jade C staining 1 week after injury, and included 18 sham and 22 CHI treated mice. A few mice died immediately (within minutes) after CHI, and were excluded from the study.

Closed head injury

We used a closed head injury protocol (Flierl et al., 2009) to induce CHI. Mice were anesthetized using spontaneously inhaled isoflurane (2%) and mounted on a stereotaxic apparatus. A scalp incision was made along the midline, and the target area (1 mm left of the midline; 2 mm posterior to bregma) was marked. The head was then immobilized on a metal platform and a guided free-falling metal rod (310 g, 3 mm diameter silicone tip) was dropped on the target region from a height of 2.0 cm (all female mice) or 2.4 cm (all male mice). These sex-based drop heights were established in pilot studies to induce a transient neurologic impairment after injury with minimal mortality. Mice were weighed prior to sham or CHI treatment and there was no difference between groups (in grams, males: sham = 26.9 ± 0.8 , CHI = 26.3 ± 0.6 ; females: sham = 20.5 ± 0.8 , CHI = 21.3 ± 0.5). Following injury, the scalp was sutured and mice recovered in a warm padded chamber. Sham mice received the same treatment (anesthetic, scalp incision/closure, marking, head immobilization on platform), with the exception of the weight drop. One hour after injury, mice were assessed for gross sensorimotor and locomotor deficits using an abbreviated 8-point neurologic severity score (NSS) (identical to the published scale (Flierl et al., 2009) but omitting the 2 and 3 cm beam walk). The NSS assessed exploratory behavior, gait, motor coordination and startle response. Each mouse was individually coded and the experimenters were blinded for subsequent analyses. Mice were sacrificed two weeks after sham or CHI treatment to determine potential differences in hippocampal neurogenesis and glial activation, 3 hours after CHI to assess c-fos gene activation early after injury, or 1 week after injury to detect cell death with the neurodegeneration marker Fluoro-Jade C or macroscopic tissue damage using the vital dye TTC.

BrdU injections

Bromodeoxyuridine (BrdU) was used to examine the effect of CHI on neurogenesis in wild-type mice. BrdU (Sigma-Aldrich, St. Louis, MO) was dissolved in warm sterile saline (10 mg/ml) and injected at 300 mg/kg i.p. twice a day (4 hour interval between doses) for 7 days starting 24 hours after injury. This dose of BrdU was chosen to saturate mitotic cell labeling as determined previously (Cameron and McKay, 2001). These mice were sacrificed 2-weeks after injury, such that BrdU-labeled cells sampled the same population of newborn neurons as those labeled in POMC-EGFP mice (Overstreet-Wadiche et al., 2006).

Immunohistochemistry

Mice were terminally anesthetized according to IACUC-approved protocols, transcardially perfused with 4% paraformaldehyde in Phosphate-Buffered Saline (PBS), and post-fixed overnight. Coronal sections (150 μ m thick) through the hippocampus were prepared from each mouse and permeabilized in 0.4% Triton in PBS (PBST) for 45 min. Sections were then blocked for 30 min with 10% horse serum in PBST and incubated overnight (4°C) with primary antibody in 1.5% horse serum/PBST. The primary antibodies were as follows: anti-GFP (Alexa Fluor 488 conjugated; 1:400, Invitrogen); anti-BrdU (1:500, Abcam); anti-doublecortin (1:400, Millipore); anti-glial fibrillary acidic protein (GFAP; 1:20,000, Dako); anti-Mac-2 (1:400, Cedarlane Labs); and anti-c-fos (1:300, Santa Cruz). Sections incubated with anti-BrdU were first incubated in 2N hydrochloric acid in potassium PBST for 30 min

(37°C), washed twice and blocked with horse serum as described above. The samples that required secondary antibodies were washed in PBST (2×10 min) the following day and incubated with either goat anti-rabbit (1:400, Alexa Fluor 568, Invitrogen); goat anti-rat (1:400, Rhodamine Red, Jackson Labs); or goat anti-guinea pig (1:400, Alexa Fluor 488, Invitrogen) for 2-hours at room temperature. The sections were then washed in PBST (2×10 min) and mounted with Dapi Fluoromount-G (SouthernBiotech).

Slides were coded, and three alternate 150 μ m thick coronal slices were taken from each animal, starting 300 μ m from the anterior tip of the dorsal hippocampus of each mouse. This region was chosen as it was directly below the impact site and easily located between animals. Slices were imaged with a Zeiss LSM780 confocal microscope using a 10 $\times$ 0.45NA or 20 $\times$ 0.8NA lens and subsequently quantified using ImageJ software by an investigator blinded to experimental condition. For POMC-EGFP animals, all GFP positive cells in a 10 μ m Z-stack through a 100 μ m segment from the middle of the ipsilateral and contralateral suprapyramidal blade of the dentate gyrus granule cell layer (GCL), including the subgranular zone (SGZ), were counted in three separate slices for each animal. These same slices were subsequently blindly re-imaged at lower power (10 $\times$) using single confocal sections in order to assess neurogenesis across the entire span of both blades (supra and infrapyramidal) and the crest of the dorsal dentate gyrus as well as the dentate gyrus of the ventral (temporal) hippocampus, and normalized to GCL cross-sectional area. In wild-type mice, BrdU positive cells were quantified in a similar fashion, although using a 20 μ m thick Z-stack through the entire dentate SGZ and GCL and normalized to GCL volume.

To assess cell migration, the distance from the center of each cell body to the SGZ/hilar border was measured in the middle section of the suprapyramidal blade of the dentate gyrus. Some injured animals had more cells, and thus more observations per animal. Thus, to give each animal equal weight in a distribution of the granule cell migration distances, the migration distance of 40 randomly chosen cells (randomized using Microsoft Excel) was chosen per animal for each condition, resulting in 200-280 cells for each experimental group/sex/laterality to represent the migration distribution for that particular condition.

Subsets of CHI and sham mice were randomly chosen pre-hoc to undergo staining for additional markers, in order to provide adequate samples for each assay given the limited amount of tissue available from each mouse and to allow for littermate controls. All samples selected for an assay were included in the analysis without exception, and all tissue was stained side-by-side and imaged/analyzed by an experimenter blind to experimental condition, using the same acquisition settings. For quantification of c-fos and GFAP immunoreactivity, the mean intensity of all pixels in a 5 μ m Z-stack of the dentate gyrus GCL was obtained using ImageJ. For quantification of Mac-2 positive cells, the number of Mac-2 positive cells in a 10 μ m Z-stack of the dentate GCL was blindly counted, and normalized to GCL area in samples from wild-type mice. Cortical thickness and hippocampal volumes were quantified using low power images in which each structure was traced in 3 contiguous slices immediately below the CHI impact site using ImageJ. POMC-EGFP mice were used for the GFAP staining and anatomical measurements, and wild-type mice were used for Mac-2 and cFos analyses. TTC staining was performed on acutely prepared live brain slices (Schnell et al., 2012) from wild-type animals 1 week after CHI or

sham, and subsequently incubated in 2% TTC in PBS at 37°C for 20 minutes, fixed in PBS + 4%PFA, and imaged in accordance with established protocols (Glover et al., 2012). Fluoro-Jade C staining was performed as previously described on wild-type mice (Schmued et al., 2005) with minor modifications. Three contiguous perfusion-fixed slices taken from directly below the site of injury were dried, ethanol rehydrated, exposed to 0.06% potassium permanganate for 15 min and incubated in 0.001% Fluoro-Jade C (Histo-Chem Inc.) for 30 minutes. Slices were cleared in xylene and mounted with DPX.

Statistics

For all statistical analyses, data were first assessed for normality and homogeneity of variance to determine use of parametric or non-parametric tests as indicated in the Results section. Data are expressed as means $\pm$ SEM. Statistical analyses were conducted using SPSS Statistics (IBM, Armonk, NY) and group comparisons were considered significant at $p < 0.05$. All figures were generated using Prism Software (GraphPad Software, La Jolla CA).

Results

Neuroseverity scores and CHI-induced neurogenesis in POMC-EGFP transgenic mice

POMC-EGFP mice showed substantial variation in NSS following an identical CHI protocol, ranging from 0 (no deficits) to 8 (maximum deficit). Sham mice were unimpaired after sham surgery (NSS = 0 ± 0 , $n = 11$). Regardless of the 1-hour NSS, however, impairments after CHI improved within 48 hours and were almost completely resolved at 14 days (NSS in a randomly selected subset of 10 CHI mice: 1-hour, 4.9 ± 0.72 ; 48-hours 2.4 ± 0.62 ; 7-days 1.2 ± 0.39 ; 14-days 0.1 ± 0.1 ; main effect of time, $F_{3, 27} = 25.92$; $p < 0.001$, repeated measures ANOVA), as has been described previously for this model (Flierl et al., 2009).

To determine whether CHI increased hippocampal neurogenesis in POMC-EGFP mice, we counted newborn neurons within the GCL two weeks after injury (sham, $n = 11$; CHI, $n = 24$), after the majority of post-neurogenesis apoptosis had occurred (Sierra et al., 2010). POMC-EGFP mice transiently express GFP in recently born granule cells (Overstreet-Wadiche et al., 2006). In these mice, CHI increased the density of newborn granule cells in a manner predicted by the degree of neurologic impairment immediately after injury (R square = 0.21; $p < 0.01$, linear regression analysis, Fig. 1A-C). Following criteria that define the severity of injury according to the NSS at 1 hour after injury (Beni-Adani et al., 2001), we analyzed the neurogenic response of sham (no CHI) mice, mice after CHI with a resulting mild impairment (NSS < 5 ; $n = 10$) and mice after CHI with a resulting moderate to severe impairment (NSS ≥ 5 ; $n = 14$), henceforth referred to as the “severe” group. A two-way ANOVA with sex and impairment group as between subject factors determined that there was no significant sex by impairment group interaction ($p = 0.6$) on the number of GFP positive cells within the ipsilateral hemisphere; therefore, males and females were combined. There was however a main effect of impairment group ($F_{2, 29} = 5.46$; $p < 0.01$): the severely impaired group had a 50% increase in the number of newborn neurons in the ipsilateral dentate gyrus compared to sham or the mildly impaired group, $p < 0.01$ and 0.05

respectively, Fisher's LSD, Fig. 1D). There was no significant difference between sham mice and the CHI mice with a mild impairment ($p > 0.5$) in the ipsilateral hemisphere. A similar two-way ANOVA determined no effect of impairment group ($p > 0.7$) or a significant sex by impairment group interaction ($p > 0.4$) in the contralateral hemisphere. Variability in neurogenesis appeared to be biological rather than sampling error, because the variability between animals in each group was greater than that between slices from within each animal (data not shown).

To determine whether the effect of severe CHI impairment on neurogenesis was limited to the middle of the suprapyramidal blade as imaged above, we quantified the number of GFP positive cells in the entire span of the suprapyramidal and infrapyramidal blades as well as in the crest of both the dorsal and ventral ipsilateral dentate gyrus. Using this broader sampling method, we observed the same main effect of impairment group on post-traumatic neurogenesis (two-way ANOVA, $F_{2,29} = 6.64$, $p < 0.01$): the severely impaired group had more GFP positive newborn neurons ($1,270 \pm 90$ cells/mm²) compared to sham (780 ± 100 cells/mm²) or the mildly impaired group (920 ± 90 cells/mm²; $p < 0.01$; $p < 0.05$, respectively, Fisher's LSD). This effect was again independent of sex (sex by impairment group interaction $p > 0.3$). Additionally, there was no significant interaction between impairment group and dentate subregions (repeated measures ANOVA with the different blades and crest as within subject factors assessed in the ipsilateral dorsal and ventral dentate gyrus), indicating that the effect of CHI on neurogenesis is uniform throughout the entire ipsilateral dentate gyrus.

Neuroseverity scores and CHI-induced neurogenesis in wild-type mice

Neuropathology can alter neuronal gene expression, and mature dentate granule cells can adopt an “immature” phenotype in certain disease models (Ohira et al., 2013). To confirm that this result was not due to a CHI-induced alteration of the timing of GFP expression in the POMC-GFP mouse, we assessed incorporation of the mitotic marker BrdU into proliferating hippocampal cells after injury in wild-type mice. Numerous BrdU⁺ cells were noted throughout the dentate gyrus in the CHI animals, but we restricted our quantification to the GCL, as most of the BrdU⁺ cells in the molecular layer and dentate hilus co-labeled with glial cell markers (data not shown). Consistent with the results from POMC-EGFP mice, the 1-hour post-injury NSS predicted the degree of cell proliferation in the GCL (R square = 0.39; $p < 0.01$, linear regression analysis). Compared to sham mice, proliferation within the GCL more than doubled in the severely impaired group (Figure 1E-F; $p < 0.01$, Fisher's LSD; BrdU⁺ cell density in the GCL: sham, $49,500 \pm 6,200$ cells/mm³, $n = 11$; mildly impaired group, $63,300 \pm 11,100$, $n = 4$; severely impaired group, $104,600 \pm 18,800$, $n = 9$, main effect of injury group, $F_{2,21} = 5.2$; $p < 0.05$, one-way ANOVA). There was no significant difference between sham and the mildly impaired group ($p = 0.3$). The percentage of BrdU⁺ cells in the GCL that was positive for the immature neuronal marker doublecortin (DCX) was similar between the severely impaired group and the sham group (sham group = $91.1 \pm 1.9\%$, $n = 3$; severe group = $86.2 \pm 3.1\%$, $n = 5$; $p = 0.3$, Student's t -test; Figure 1G-H), indicating that the increased BrdU labeling in the GCL of the severely impaired group resulted from an increase in the number of newborn neurons.

Migration of newborn neurons following CHI

During granule cell maturation, neurons migrate away from the SGZ as they integrate into the hippocampal circuitry (Cameron et al., 1993) and insults such as seizures can accelerate the migration and dispersion of newborn hippocampal cells (Jessberger et al., 2007; Overstreet-Wadiche et al., 2006; Parent et al., 1997). To determine whether CHI also enhances the dispersion of newborn cells, we measured the migration of newborn neurons away from the SGZ for each POMC-EGFP mouse. Although increased cell migration was observed in some mice with a severe impairment (for example, see Fig. 1B), overall mean migration distances were not significantly different from sham mice (mean distance from SGZ in $\mu\text{m} \pm \text{SEM}$: sham 15.3 ± 0.7 , $n = 11$, mildly impaired 16.2 ± 0.8 , $n = 10$, severely impaired 16.9 ± 1.0 , $n = 14$; $p = 0.9$, Kruskal-Wallis test). When we split the data by sex, the mean migration distances in injured animals were also not significantly different from shams (males: sham $18.3 \pm 1.2 \mu\text{m}$, $n = 5$, mildly impaired $20.8 \pm 1.4 \mu\text{m}$, $n = 5$, severely impaired $14.9 \pm 1.0 \mu\text{m}$, $n = 6$; females: sham $11.5 \pm 0.7 \mu\text{m}$, $n = 6$, mildly impaired $11.5 \pm 0.8 \mu\text{m}$, $n = 5$, severely impaired $18.9 \pm 1.7 \mu\text{m}$, $n = 8$; $p > 0.05$ for all groups vs. sham, Kruskal-Wallis test). However, the severely impaired female mice had a significantly different distribution of migration distances than sham females ($p < 0.05$, Kolmogorov-Smirnov test), but this did not reach statistical significance in males ($p > 0.1$). It is unclear whether this sex difference was related to underlying differences in the biological response to injury or in the induction of the injury itself. Analysis of BrdU+ cells similarly demonstrated no significant difference in the mean distance of newborn cells from the SGZ between sham and severely impaired CHI mice in either sex (mean migration distance from SGZ in $\mu\text{m} \pm \text{SEM}$: sham males 12.5 ± 1.3 , $n = 6$, severely impaired males 16.7 ± 2.7 , $n = 4$; sham females 11.7 ± 1.0 , $n = 5$, severely impaired females 15.7 ± 2.3 , $n = 5$; $p > 0.1$ each, Mann-Whitney test). An analysis of the distribution of migration distances of the severely impaired mice now demonstrated that significant differences in both sexes between sham and severely impaired mice (males, $p < 0.05$; females, $p < 0.01$; Kolmogorov-Smirnov test).

Gross neuropathology and c-fos activity following CHI

Brains did not show gross anatomical damage following CHI, as assessed by quantification of cortical thickness below the impact site, as well as hippocampal cross-sectional area ipsilateral to the injury site (cortical thickness ipsilateral to injury, $n = 5$ per group: sham $1.04 \pm 0.08 \text{ mm}$, mildly impaired $1.13 \pm 0.04 \text{ mm}$, severely impaired $0.94 \pm 0.05 \text{ mm}$, $p = 0.2$; hippocampal cross-sectional area ipsilateral to the injury: sham $2.9 \pm 0.09 \text{ mm}^2$, mildly impaired $2.7 \pm 0.2 \text{ mm}^2$, severely impaired $2.7 \pm 0.2 \text{ mm}^2$, $p = 0.9$). Vital dye staining (TTC) in acutely prepared live slices performed one week after injury in a separate cohort of animals also failed to demonstrate differences between sham and CHI brains (sham $n = 7$, CHI $n = 8$; data not shown), suggesting that there was no overt pathological damage after CHI. Finally, we performed Fluoro-Jade-C staining 7 days after CHI to detect degenerating neurons, and found no positive cells in the hippocampus of either mild or severely impaired animals after CHI at this timepoint (sham $n = 4$, CHI $n = 6$, data not shown), but with some rare Fluoro-Jade-C positive cells noted in the ipsilateral cortex and thalamus of severely impaired animals.

We also examined other factors that could affect the neurogenic response. To determine whether CHI was associated with increased neuronal activity in the dentate gyrus near the time of injury, we stained perfusion-fixed slices for the activity-dependent gene c-fos (Barone et al., 1993) three hours after CHI. There was a main effect of impairment group on c-fos immunoreactivity in the GCL of the ipsilateral dentate gyrus ($F_{2,12} = 7.54$; $p < 0.01$, one-way ANOVA). Mice with a severe impairment ($n = 5$) demonstrated greater c-fos staining than sham mice ($n = 7$) or mice with a mild impairment ($n = 3$) ($p < 0.01$, and 0.05 , respectively, Fisher's LSD, Fig. 2A,D). In contrast, mice with a mild impairment were similar to sham mice. Interestingly, increased c-fos activity was not observed in the contralateral dentate gyrus (data not shown). Thus, both the anatomic location and magnitude of c-fos activation were associated with a subsequent increase in hippocampal neurogenesis.

Glial activation following CHI

Experimental TBI is also associated with reactive astrogliosis (Kernie et al., 2001) and activation of microglia (Albert-Weissenberger et al., 2012). We stained slices 2 weeks after CHI for GFAP to identify astrocytes and Mac-2 to identify activated microglia. There was a main effect of impairment group on GFAP immunoreactivity ($F_{2,19} = 4.63$; $p < 0.05$, one-way ANOVA). Compared to sham mice ($n = 8$), mice with a severe impairment ($n = 7$), but not mice with a mild impairment ($n = 7$) had increased GFAP staining in the GCL of the dentate gyrus ($p < 0.05$; Fisher's LSD; Fig. 2B,E). The GCL in the severely impaired group also contained an increased number of GFAP-positive linearly oriented fibers, indicative of radial glia (neuronal precursors; Fig. 2B). Similarly, there was an increase in Mac-2 positive cell density within the GCL of the dentate gyrus of the severely impaired group ($n = 5$) compared to sham mice ($n = 5$; $p < 0.05$, Kruskal-Wallis, Fig. 2C, F). The number of Mac-2 positive cells observed in the mildly impaired group ($n = 3$) was not significantly different from the sham group ($p = 0.12$; Fig. 2F), although this lack of significance might partially reflect the low n in the mildly impaired group.

Discussion

We observed an increase in hippocampal neurogenesis following experimental closed head injury using a clinically relevant TBI model. Not unlike the human response to closed head injury, CHI in mice produced a variable degree of neurologic impairment. Importantly, the degree of neurologic impairment 1 hour after the injury predicted the post-traumatic neurogenic response as assessed 2 weeks later. Consistent with our data, impairments early after TBI are also associated with other biological outcome and injury measures, including brain edema (Beni-Adani et al., 2001; Shapira et al., 1988), brain damage as assessed by MRI (Tsenter et al., 2008), and long-term cognitive impairments (Sanders et al., 1999). Accordingly, glial activation, as quantified by GFAP and Mac-2 immunoreactivity, increased in mice with a higher initial NSS, suggesting that these mice had sustained higher degrees of neural injury. It is not clear whether the differences between the mild and severely impaired CHI groups resulted from uncontrolled differences in the actual initial impact, or to biological differences in secondary responses.

In contrast, earlier studies did not observe significant changes in CHI-induced hippocampal neurogenesis, as assessed by quantification of cells labeled with neuronal markers or morphology (Bye et al., 2011; Carthew et al., 2012; Ng et al., 2012). One explanation for this discrepancy may be that these studies included all animals after CHI, regardless of the degree of neurologic impairment. Consistent with this idea, combining all our injured animals into a single group reduced the magnitude of the increase in neurogenesis and decreased its statistical significance despite a larger *n* (ipsilateral newborn granule cell densities: sham = 6.1 ± 1.0 , *n* = 11; all CHI = 8.6 ± 0.7 , *n* = 24; *p* = 0.04), because the mice with a mild impairment had similar rates of neurogenesis as sham animals. One prior study attempted to circumvent the variability of the CHI model by only including animals that sustained a skull fracture (Ng et al., 2012), however skull fracture did not clearly predict neurologic impairment in our experiments (data not shown). Other potential predictors, such as the weight of mice prior to injury and the incidence and duration of apnea immediately after the closed head injury, also did not predict neurologic impairment or the induction of neurogenesis in our experiments (data not shown).

In addition to enhanced neurogenesis, we also observed that more severe neurologic impairment was associated with increased c-fos induction early after the injury. Further studies are needed to determine whether synaptic activity generated during or immediately after the injury are causally related to the subsequent increase in neurogenesis. Enhanced c-fos activity has also been noted after seizures (Barone et al., 1993; Dragunow and Robertson, 1987; Overstreet-Wadiche et al., 2006), which also increase hippocampal neurogenesis (Bengzon et al., 1997; Parent et al., 1997). We did not observe seizure activity in animals after CHI; however we did not monitor the animals continuously throughout the three-hour period after injury, nor did we perform EEG recordings to identify subclinical seizure activity. Thus it remains possible that severely injured mice had undetected seizure activity that may have enhanced neurogenesis. Future work will be necessary to determine whether increased neurogenesis after CHI is dependent on neuronal activity including subclinical seizure activity, or whether other mechanisms such as glial activation (Chirumamilla et al., 2002; Yang et al., 2010) or neuronal degeneration drive the increase in neurogenesis. Although our assays of gross neuropathology failed to demonstrate any substantial differences between groups, we limited our analyses to later timepoints, and would have failed to detect changes that had occurred at an earlier timepoint. We presume that there is some functional or anatomic neuropathology underlying the initial neurologic impairment, which subsequently drives the glial reaction and post-traumatic neurogenesis. Additional work could help to define these processes. Additionally, because both our BrdU protocol and the POMC-EGFP mice sampled cells born over the course of the entire first post-CHI week, future studies are needed to determine whether the increased neurogenesis results primarily from enhanced proliferation, survival, or both. Furthermore, later timepoints after injury are necessary to determine the long-term survival of CHI-induced newborn neurons, as not all cells that have survived for 14 days post-mitosis reach maturity (Kempermann et al., 2003; Sierra et al., 2010).

The experimental NSS provides an early assessment of injury impairment in a manner similar to the clinical grading scales for human TBI (Saatman et al., 2008). As progress is made in non-invasively quantifying neurogenesis in humans (Couillard-Despres and Aigner,

2011; Ho et al., 2013), we may soon be able to study neurogenesis after human TBI and ultimately increase our understanding of its relationship to injury mechanisms and subsequent recovery.

Conclusions

Closed head injury induces post-traumatic hippocampal neurogenesis, and the magnitude of this induction is related to the severity of the initial neurologic impairment. The close association between initial impairment and subsequent neurogenesis has important experimental implications for the study of TBI, as it provides quick and simple criteria that may reduce variability in the CHI model.

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Highlights

- Closed head injury (CHI) in mice causes variable degrees of neurologic impairment
- Hippocampal neurogenesis increases in mice with a severe but not a mild impairment
- Severe impairment is also associated with increased c-fos and glial staining
- Neurologic impairment after CHI predicts histologic changes including neurogenesis

Figure 1.

Hippocampal neurogenesis following closed head injury (CHI) in mice. (A) POMC-EGFP mice express GFP in newborn hippocampal granule cell neurons, and all experimental mice were subjected to the same standard CHI protocol. Images of newborn dentate granule cells are shown from control (Sham), CHI with a resulting mild impairment (Mild; one-hour NSS < 5) and CHI with a resulting severe impairment (Severe; one-hour NSS ≥ 5). Scale = 200 µm. (B) Higher power magnification of the dentate gyrus in sham, mildly impaired post-CHI, and severely impaired post-CHI animals (white lines bracket the granular layer). Scale = 100 µm. (C) Neurologic impairment one hour after injury predicts the degree of hippocampal neurogenesis two weeks later (R square = 0.21; $p < 0.01$, linear regression analysis). All sham mice had an NSS = 0. (D) Severely impaired mice demonstrated a significant increase in the number of newborn granule cells compared to mice in the sham or mildly impaired groups (** $p < 0.01$, * $p < 0.05$, severely impaired versus sham and mildly impaired groups respectively). (E, F) CHI increases granule cell layer mitosis in mice with a severe impairment. (E) Representative sections from sham (left) and severely impaired (right) ipsilateral dentate gyrus in mice administered BrdU for 7 days following CHI. Scale = 100 µm. (F) Summary data of BrdU+ cell density within the granule cell layer in sham, mildly impaired CHI, and severely impaired CHI mice. (** $p < 0.01$, ipsilateral sham vs. severely impaired mice). (G, H) Doublecortin immunohistochemistry demonstrates that

BrdU-labeled cells in the granule cell layer are immature neurons. (G) Representative images from a severely impaired mouse after CHI demonstrating co-localization of BrdU with DCX in orthogonal views. Scale = 10 μ m. (H) Summary data showing no change in co-localization after CHI ($p > 0.3$).

Figure 2.

Neurologic impairment after closed head injury predicts glial marker staining and correlates with c-fos activation. (A, B, C) Representative images of c-fos (A), GFAP (B), and Mac-2 (C) staining in the ipsilateral dentate gyrus of sham and CHI treated mice. Scale bar = 50 μ m (A, B) and 25 μ m (C). (D, E, F) Quantification of immunoreactivity for c-fos protein (D, $**p < 0.01$, $*p < 0.05$, severely impaired group versus sham and mildly impaired groups); GFAP (E, $*p < 0.05$, severely impaired group versus sham and mildly impaired groups); and the number of Mac-2 positive activated microglia (F, $*p < 0.05$, severe group versus sham mice) within the GCL of the ipsilateral dentate gyrus.