Experimental Neurology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Brief Communication

Neurologic impairment following closed head injury predicts post-traumatic neurogenesis

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ARTICLE INFO

Article history:
Received 16 January 2014
Revised 14 May 2014
Accepted 16 May 2014
Available online xxxx

Keywords:
Closed head injury
Traumatic brain injury
Adult neurogenesis
Hippocampus
Neurologic severity score

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 1 h 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 astrocytosis 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.
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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., 2003, 2005). 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-h 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

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http://dx.doi.org/10.1016/j.expneurol.2014.05.016
0014-4886/Published by Elsevier Inc.

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 h 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 $= 26.9 \pm 0.8$, CHI $= 26.3 \pm 0.6$; females: sham $= 20.5 \pm 0.8$, CHI $= 21.3 \pm 0.5$). 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 h 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 h 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 μm 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-h 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 μm thick coronal slices were taken from each animal, starting 300 μm 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 μm Z-stack through a 100 μm 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 the GCL cross-sectional area. In wild-type mice, BrdU positive cells were quantified in a similar fashion, although using a 20 μm 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 μm 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 μm Z-stack of the dentate GCL was blindly counted, and normalized to the 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-GFP 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 37C for 20 min, 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)

Please cite this article as: Villasana, L.E., et al., Neurologic impairment following closed head injury predicts post-traumatic neurogenesis, Exp. Neurol. (2014), http://dx.doi.org/10.1016/j.expneurol.2014.05.016