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Mild Traumatic Brain Injury Leads to Decreased Inhibition and a Differential Response of Calretinin Positive Interneurons in the Injured Cortex

Mariana Brizuela,* Catherine A. Blizzard,* Jyoti A. Chuckowree, Kimberley A. Pitman, Kaylene M. Young, and Tracey Dickson

Abstract

It is clear that even mild forms of traumatic brain injury (TBI) can have lasting cognitive effects; however, the specific cellular changes responsible for the functional deficits remain poorly understood. Previous studies suggest that not all neurons respond in the same way and that changes to neuronal architecture may be subtype specific. The current study aimed to characterize the response of interneurons to TBI. To model TBI *in vitro*, the neurites of primary cortical neurons were transected at 15 days *in vitro*. In response, calretinin$^+$ interneurons underwent significant neurite remodeling around the injury site. By examining the response of pyramidal neurons, GAD67-GFP$^+$ interneurons, and calretinin$^+$ interneurons to the injury, we found that this response was specific to the calretinin$^+$ cells. To determine whether calretinin$^+$ interneurons respond in this way to a clinically relevant *in vivo* model of mild diffuse and focal injury, we subjected mice to the lateral fluid percussion injury model. We found that calretinin$^+$ interneuron density was unaltered by this mild injury, but consistent with our *in vitro* data, these neurons underwent morphological alterations in their dendrites. These alterations evolved over a 28-day period, and calretinin$^+$ interneurons in the injured mice had a reduction in mean dendrite length and reduced number of secondary dendrites than those in the sham-injured controls by 7 days post-injury. Further, these structural alterations were accompanied by a reduction in the frequency of miniature inhibitory post-synaptic currents in layer V pyramidal neurons. These data suggest that even a mild TBI can lead to an overall change in the excitatory/inhibitory balance of the cortex that may play an important role in the longer-term behavioral pathology associated with mild TBI.

Keywords: calretinin; cortex; GAD67; interneuron; mild traumatic brain injury; mouse

Introduction

The incidence of traumatic brain injury (TBI) is rising worldwide.[1–3] Seemingly mild injuries account for approximately 80 percent of the total number of brain injuries that occur globally, and up to 25% can have persistent cognitive, behavioral and emotional consequences, with a small proportion developing post-traumatic epilepsy.[4–9] The specific cellular changes that underlie the functional alterations resulting from a mild injury remain poorly understood and elucidating these mechanisms is a fundamental priority in neurotrauma research.

Accumulating evidence indicates that the brain can undergo substantial reorganization after injury. Plastic changes after injury can include alterations in synaptic strength, remodeling of axonal and dendritic arbors, and neurogenesis.[10–16] The degree of plasticity occurring after injury has been classically linked to age, mode of injury, and/or the extent and region of injury.[17–19] More recent studies suggest that post-injury plasticity also can be dependent on the subpopulation of neurons affected, specifically in terms of dendritic remodeling, implying that a particular response to injury can be intrinsic to a specific neuronal subtype.[15,20] These results indicate that a broader understanding of the response of different subpopulations of neurons to structural injury and that the possible mechanisms underlying these responses is needed.

Inhibitory interneurons play a crucial role in neuronal plasticity during development, as increases in cortical inhibition regulate the onset and closure of critical periods.[21–26] However, recent studies have indicated that changes in the inhibitory circuitry also are involved in regulating plasticity in adulthood. Interestingly, in contrast to excitatory pyramidal neurons, inhibitory interneurons seem to be highly plastic and exhibit a range of dendritic structural modifications when imaged over time in the primary visual cortex, as well as other cortical regions.[24,27,28] Further, it has been suggested that morphological changes to inhibitory cell axons and

Menzies Institute for Medical Research, University of Tasmania, Tasmania, Australia.
*These authors contributed equally.

dendrites occur after sensory deprivation and can precede and drive the changes observed in the excitatory circuitry.[29–32] Despite this key evidence indicating that interneurons and inhibition may play an important role in the adult cortex, the response of these cells to cortical trauma remains relatively underexplored. However, studies examining a discreet focal injury to the somatosensory cortex indicate that subpopulations of interneurons, specifically the calretinin$^+$ interneurons, have an underappreciated potential for plasticity and remodeling following trauma.[20]

Calretinin$^+$ interneurons represent approximately 10–30% of the total interneuron population in the cortex.[33] Even though this is a relatively small fraction of the inhibitory neurons, their unique connectivity and placement in the circuitry affords them significant control over inhibitory regulation as a whole.[34] They have been described as being at the top of the interneuron hierarchy due to the fact that they preferentially regulate the activity of other interneuron populations and specifically target dendritic inhibitory interneurons to synchronize their activity.[35,36] This synchronization of dendritic inhibitory cells is a crucial process to provide effective inhibitory control of excitatory pyramidal cells.[37] As a result, calretinin$^+$ interneurons influence the whole cortical inhibitory circuit and may play an important role in the sequelae of pathogenic events that follow mild brain injury.[38] To determine whether this subpopulation of interneurons plays a role in the adverse outcomes that can manifest following a mild TBI, this study employed *in vitro* and *in vivo* models of brain injury. Post-injury plasticity was explored in terms of morphological changes to the dendritic arbor and the downstream consequences to the output of the cortical inhibitory network.

Methods

All experimental procedures involving animals were approved by the Animal Ethics Committee at the University of Tasmania and were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2013). Thy1-YFP transgenic mice (B6; CBA-Tg Thy1-YFP GJrs / GfngJ) were obtained from the Jackson Laboratory. GAD67-GFP mice were developed by Prof. Nobuaki Tamamaki (Kyoto University) and were provided by Prof. John Bekkers (Australian National University). GAD67-GFP mice are maintained on a C57BL6/J background and express green fluorescent protein (GFP) in neurons expressing GAD67, one of the two genes that encode isoforms of the GABA-synthesizing enzyme glutamic acid decarboxylase.[39,40] Animals were bred at the University of Tasmania and were housed in individually ventilated cages maintained at ~20°C on a 12-h light/dark cycle, with access to food and water *ad libitum*.

Cell culture and axonal transection in vitro

Primary dissociated cortical neuron cultures were prepared from mouse embryos as previously described.[41] Briefly, Thy1-YFP and GAD67-GFP transgenic mice were time-mated, the pregnant females killed by CO_2 exposure at 15.5 days of gestation, and the embryos removed and genotyped by exposure to a fluorescent light source. Neocortical hemispheres were dissected into 5 mL of Hanks Buffered Salt Solution (Thermo Fisher Scientific). Cortical tissue was dissociated by enzymatic digestion (0.025% w/v Trypsin, 5 min, 37°C), which was halted by the addition of 1 mL of pre-warmed medium (Neurobasal supplemented with 2% v/v B27, 10% v/v fetal calf serum, 0.5mM L-glutamine, 25μM glutamate and 1% streptomycin penicillin; Thermo Fisher Scientific). Tissue was dissociated with gentle trituration in 500 μL of pre-warmed medium. Cell viability and concentration was assessed using trypan blue vital dye exclusion. 3.5×10^4 cells/mm^2 were plated per 19 mm diameter glass 0 (Marienfeld). Each cover-slip was pre-coated with 0.001% Poly-L-lysine (Sigma-Aldrich) in 0.01M phosphate buffered saline (PBS; pH 7.0). Cultures were maintained in a 37°C humidified atmosphere of 5% CO_2 for up to 15 days. At 1 day *in vitro* (DIV), the medium was removed and replaced with serum-free growth medium (Neurobasal, 2% v/v B-27 supplement, 0.5 mM L-glutamine, and 1% streptomycin penicillin; Thermo Fisher Scientific), after which time ~50% of the medium was replaced every 3–4 days.

Neuronal transection was carried out using a Barkan goniotomy knife (Kaisers) on cortical cultures at 15 DIV.[41] A single injury extending the full diameter of the cover-slip was made, ensuring complete axonal transection and producing a cell free lesion of ~50–150 μm wide. Cells were fixed for 30 min with 4% (w/v) paraformaldehyde (PFA) in 0.01M PBS at 0, 4, and 24 h post-injury for immunocytochemistry.

In vivo fluid percussion injury model

Adult male GAD67-GFP mice were exposed to mild TBI at 8–10 weeks of age in the form of a lateral fluid percussion injury (FPI). Animals were anesthetized with isoflurane and using a trephine, a 3 mm diameter craniectomy was performed 2.0 mm posterior and 2.5 mm lateral to bregma on the right hand side of the skull over the somatosensory cortex and a needle hub (3 mm inner diameter) was secured above the skull opening. One to 2 h later, post-craniectomy animals were re-anaesthetized with isoflurane. Once surgical anesthesia was achieved the isoflurane was withdrawn, the hub was filled with saline and mice were connected to the FPI device. Once a normal breathing pattern resumed but before sensation was restored, an injury was induced by releasing the pendulum of the FPI device onto the fluid-filled piston to induce a brief fluid pressure pulse upon the intact dura (1.4 ± 0.10 atmospheres). The time taken for the mouse to regain its righting reflex was recorded. After injury the hub was removed and the animal was re-anesthetized with isoflurane and the incision sutured. Sham-injured animals received the same surgeries without receiving the injury.

To identify cells generated in the post-injury period, the thymidine analogue 5-ethynyl-2′-deoxyuridine (EdU, 0.2mg/mL; Invitrogen), was administered *ad libitum* via the drinking water, as previously described.[42] EdU is incorporated into the DNA of dividing cells during S phase of the cell cycle. Mice were sacrificed at 1 day, 1 week, and 4 weeks post-injury. Mice ($n = 6$ FPI and $n = 6$ sham injured per time-point) were terminally anesthetized (sodium pentobarbitone, 140 mg/kg, intraperitoneally) and transcardially perfused with 4% PFA in PBS. Brains were post-fixed in 4% PFA-PBS overnight and stored at 4°C in PBS containing 0.1% w/v sodium azide (Sigma Aldrich).

For sectioning, brains were embedded in 5% (w/v) molecular grade agarose (Bioline) dissolved in PBS. Using a Leica VT1000S vibratome (Biosystems Australia Pty Ltd, VIC Australia), 50-μm coronal sections were produced and collected as floating sections into 24 well plates (Corning Life Sciences) containing PBS sodium azide.

Immunolabelling and EdU detection

Somatosensory cortices containing the injury site were identified by referring to anatomical landmarks, such as the appearance of the lateral ventricles, the third ventricle, and the corpus callosum. Sections/cover-slips were incubated for 24 h at room temperature with primary antibodies that included rat anti-GFP to detect YFP or GFP (1:3000; Nacalai tesque), rabbit anti-calretinin (1:1000; Swant), and rabbit anti-activated caspase 3 (1:200; Millipore) diluted in PBS containing 0.3% triton x-100. Sections/cover-slips were washed thrice in PBS before the application of isotype- and species-specific secondary antibodies. Secondary antibodies (Alexa Fluor 594- and AlexaFluor 488-conjugated goat anti-rat and goat

anti-rabbit IgG H+L; Invitrogen) were diluted 1:1000 in PBS and applied to sections/cover-slips for 2 h at room temperature. Sections/cover-slips were washed thrice with PBS before mounting in fluorescent mounting medium (Thermo Fisher Scientific). For EdU labeling after the final immunohistochemistry washes, EdU was visualized using the AlexaFluor-647 Click-iT EdU kit (Life Technologies) as previously described.[43] Coronal cortical sections from sham and FPI GAD-GFP$^+$ mice were immunostained with anti-calretinin antibody to detect calretinin$^+$ interneurons. Somata and processes of GAD positive cells labeled for calretinin were identified as calretinin$^+$.

Neuronal quantification

To perform cell counts from uninjured cultures, four images were taken from distinct regions of each cover-slip. Each time-point (0, 4, and 24 h) was examined in four replicate cover-slips across three independent experiments. For cell counts of injured cultures and remodeling analysis, images were taken across the length of the scratch injury at specific distances from the injury site (0–500 μm, 500–1000 μm, and more than 1000 μm from the injury). Approximately 10 images were taken from each of four replicate cover-slips across three independent experiments. To further characterize the populations of Thy1-YFP$^+$ neurons, calretinin$^+$ interneurons and GAD67-GFP$^+$ interneurons after injury, the cell tracing software Neurolucida was utilized. Each neuron was further assessed with Neurolucida Explorer to determine the dendritic angle by performing the segment dendrite analysis (Supp. Fig. 2). For caspase immunolabeling, a minimum of four images were taken from two cover-slips that contained the injury site at every time-point analyzed (0, 4, and 24 h post-injury).

Quantification of the total number of interneurons in tissue sections from GAD67-GFP mice was based on the endogenous expression of GFP enhanced by the use of an anti-GFP antibody. Quantification of the subpopulation of calretinin$^+$ interneurons was based on their expression of this calcium binding protein. Interneuron numbers was quantified within the injury site in six age-matched sham-injured ($n=6$) and FPI ($n=6$) mice per time-point (1, 7, and 28 days post-injury). Images were collected using an UltraVIEW spinning disk confocal microscope running Volocity Software (PerkinElmer) equipped with a Plan Apo 60x/1.20 water objective (Nikon) configured to capture 30 μm Z-stacks (slices 1 μm apart). The supragranular and infragranular lamina were defined as two adjacent fields containing cortical layers I-IV and V-VI, respectively, and demarcated as extending from the pial surface to the gray–white matter border. To perform unbiased and inclusive quantitative analysis, ImageJ freeware was utilized for automation of cell counts. Neuronal morphology was characterized using Neurolucida software.

Electrophysiological characterization of neurons ex vivo

Thy1-YFPH or GAD67-GFP male mice received either an FPI or a sham injury at 8 weeks of age. Following cervical dislocation, mice were decapitated and their brains dissected into ice-cold sucrose solution containing: 75 mM sucrose, 87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 7 mM $MgCl_2$, and 0.95 mM $CaCl_2$. Acute coronal slices (300 μm) were prepared using a Leica VT1200s vibratome, and incubated at 31.7°C for ≥45 min in artificial cerebral spinal fluid (ACSF) containing 119 mM NaCl, 1.6 mM KCl, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 1.4 mM $MgCl_2$, 2.4 mM $CaCl_2$, and 11 mM glucose (300 ± 5 mosm/kg). During recordings, slices were perfused with ACSF containing 500 nM tetrototoxin and 1mM kynurenic acid, saturated with 95% O_2/5% CO_2 (all Sigma Aldrich). Whole–cell recordings were obtained from layer V pyramidal cells within the injury site that were identified by the shape of the cell soma and presence of a long apical dendrite. Neurobiotin (1.5 mg/mL) was included in the intracellular solution and following electrophysiological analysis slices were immersion fixed in 4% PFA-PBS for histological processing.

Recording electrodes were prepared from glass capillaries and had a resistance of 3–5 MΩ when filled with an internal solution containing 120 mM cs-methanesulfonate, 20 mM HEPES, 0.4 mM EGTA, 5 mM TEA, 2 mM $MgCl_2$, 2.5 mM MgATP, and 0.3 mM GTP, and set to a pH of 7.2–7.3 and an osmolarity of 280 ± 5 mOsm/kg. Recordings were collected using a HEKA patch clamp EPC800 amplifier, with a holding potential of 0 mV. Recordings were made using a gap-free protocol for 3 min and 10 sec, sampled at 50 kHz and filtered at 1 kHz using PClamp10 software (Molecular Devices). Data were not included in the analysis if the access resistance was ≥20 MΩ, or if the access resistance changed by ≥10% during the course of the recording. There was no significance difference in access resistance between the sham and FPI groups ($p>0.05$). Data collection was carried out only if the recordings were stable for more than 3 minutes. Mini inhibitory post-synaptic currents (mIPSCs) were defined as having an amplitude ≥8 pA, and were analyzed using the MiniAnalysis60 program (Synaptosoft, Decateur). Post-recording, the patch electrode was removed from the neuron, which was later identified by the detection of Neurobiotin with an Alexa Fluor 594 conjugated anti-streptavidin antibody (Molecular Probes; 0.1% triton-X-diluent detergent), to confirm that recorded cells were indeed layer V pyramidal neurons in the injury site.

Statistical analysis

All data was analyzed using a two-way analysis of variance followed by Bonferroni post hoc tests (GraphPad Prism, version 6.0) for group and regional comparisons. Average values were expressed as means ± standard error of the mean. For electrophysiological comparisons, two-tailed t-tests were performed. A p value <0.05 was considered significant.

Results

Calretinin$^+$ interneurons remodel their neurites after injury in vitro

Neurons derived from Thy1-YFP transgenic mice were grown for 15 DIV before being subjected to neuronal transection and fixed at 0, 4, and 24 h post-injury. Immunolabeling with antibodies to detect the calcium binding protein calretinin and YFP identified calretinin$^+$ interneurons and pyramidal neurons, respectively. Neurites labeled with calretinin or YFP were classified as projecting "away" from the injury site if they formed an angle of 0° to 180° with the injury site or "towards" the injury site if they formed an angle of 180° to 360° with the injury site. Neurites were examined at increasing distances from the injury site, and at different time-points post-injury (Fig. 1 A–D). The orientation of individual neurites for each neuron subtype was determined and the percentage of neurites that were projecting away or towards the injury site was calculated.

At 4 h post-injury, YFP$^+$ neurons located within 500 μm of the injury site did not appear to alter the orientation of their neurites relative to uninjured controls (Fig. 1F). However, the orientation of calretinin$^+$ neurites was altered, with the proportion of neurites projecting away from the injury being significantly increased (Fig. 1F). When we performed the same analysis at 0 and 24 h post-injury, we did not detect any significant change in the orientation of YFP$^+$ or calretinin+ neurites on injured relative to control cover-slips (Fig. 1E, 1G). Additionally, neurite orientation was unaltered at distances great than 500 μm from the injury site (Supplementary Fig. 1; see online supplementary material at www.liebertpub.com).

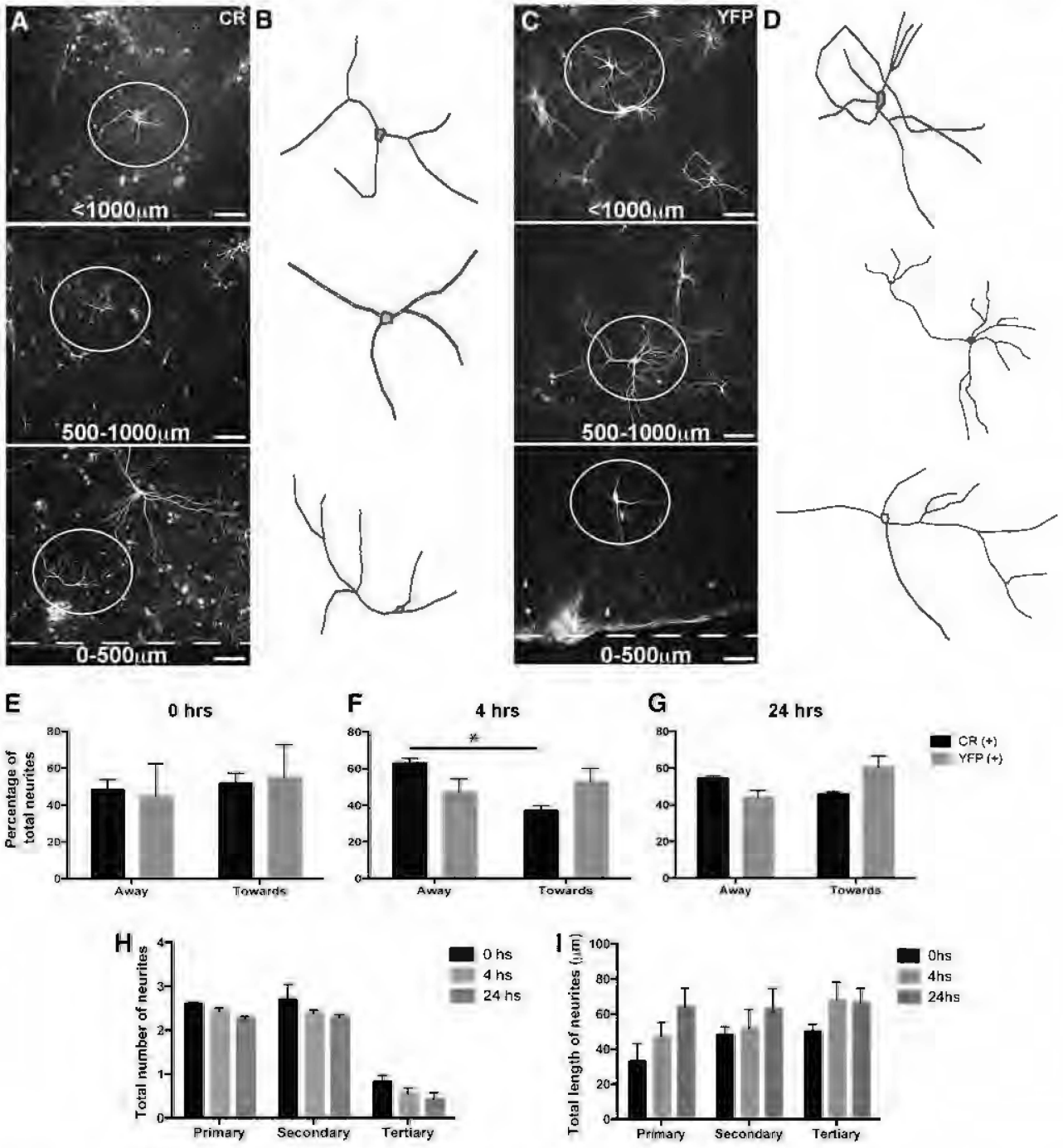

FIG. 1. Post-injury neurite remodeling of calretinin+ interneurons at 15 days *in vitro* (A–D) Representative images (A and C) and Neurolucida tracings (B and D) of calretinin+ interneurons (A,B) and YFP pyramidal neurons (C,D) at 4 h post-injury at increasing distances from the injury site. (E–G) The percentage of calretinin+ or YFP+ neurites that were directed away or towards the injury within 0–500 μm of the injury site at 0 h (E), 4 h (F), and 24 h (G). At 4 h, there was a significant difference between the percentage of neuritis directed away and toward the injury site. (H) There was no difference in the total number of neurites at 0, 4, and 24 h. (I) There was no difference in the total length of neurites at 0, 4, and 24 h. Two-way analysis of variance followed by Bonferroni test. Data are presented as mean ± standard error of the mean. Scale bar = 50 μm, Dotted line indicates edge of injury site. CR, calretinin.

Quantification of the total number and length of primary, secondary, and tertiary neurites within 500 μm of the injury site (Fig. 1H), confirmed that the change in orientation of calretinin$^+$ neurites at 4 h post-injury did not result from a change in the total number or length of neurites at any time-point investigated.

To determine whether remodeling neurites away from an injury site is a plasticity response common to all interneurons or is a response unique to calretinin$^+$ interneurons, cultures derived from GAD67-GFP mice were grown to relative maturity *in vitro* (15 DIV) and subjected to a transection or sham injury. Cultures were

fixed at 4 h post-injury and processed to detect GFP and calretinin. Interneurons that were either GAD67-GFP$^+$ alone or calretinin$^+$/GAD67-GFP$^+$ were analyzed as previously described to determine their capacity for neurite remodeling after injury. Only calretinin$^+$/GAD67-GFP$^+$ interneurons remodeled their neurites away from the injury site (Fig. 2A, 2B; $p=0.005$). By contrast, the neurite trajectories of GAD67-GFP$^+$ interneurons that were not calretinin$^+$ were not significantly changed in the injury relative to control conditions (Fig. 2B).

To investigate neuronal viability after injury, we quantified the proportion of calretinin$^+$ interneurons relative to the total number of GAD67-GFP$^+$ interneurons in control and injured cultures. The proportion of calretinin$^+$ interneurons increased relative to the total number of GAD67-GFP$^+$ interneurons between 9 to 12 DIV, increasing from ~15% at 9 DIV to ~30% at 12 DIV. However, by 15 DIV the proportion of GAD67-GFP$^+$ interneurons that expressed calretinin stabilized at ~27%. When the proportion GAD67-GFP$^+$ interneurons that expressed calretinin was examined within 500 μm of the injury site at 0, 4, and 24 h post-injury, there was no significant difference between injured and control cultures (Fig. 3A, 3B), grossly indicating that this injury was not affecting interneuron number.

To further investigate the viability of calretinin$^+$ neurons "close" to the injury site, immunocytochemistry to detect activated caspase 3 was performed. Caspase 3 is a protease involved in initiating the neuronal cell death cascade and is therefore an important marker of cells entering the apoptotic-signaling pathway.[44] GAD67-GFP$^+$ interneurons in control or injured cultures did not express caspase 3, indicating that these interneurons were not undergoing programmed cell death (Fig. 3C, 3D). These results indicate that there was no significant death of interneurons in response to the transection injury.

Mild TBI induces cell proliferation but not neurogenesis

To investigate the possibility that calretinin$^+$ interneurons were differentially vulnerable to injury *in vivo*, we used a fluid percussion device to deliver a unilateral mild injury over the somatosensory

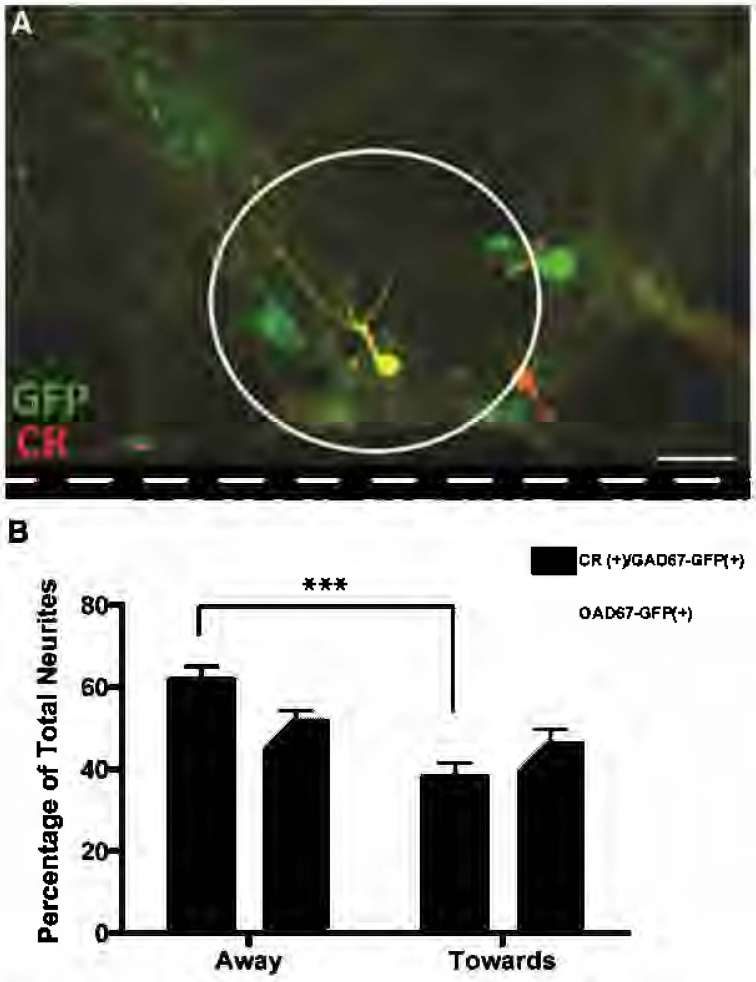

FIG. 2. Post-injury neurite remodeling is specific to the calretinin+ subpopulation. (**A**) Representative image of calretinin+ (red) and GAD67-GFP+ (green) interneuron at 4 h post-injury at 0–500 μm from the injury site. (**B**) The percentage of GAD67-GFP+ or calretinin+/ GAD67-GFP+ labeled neurites that orientated away or towards the injury at 0–500 μm from the injury site. There was a significant difference between the proportion of calretinin+/GAD67-GFP+ interneurons directed away and toward the injury site at 4 h post-injury (**B**; $p=0.005$). There was no significant difference in the GAD67-GFP+ neurites at 4 h post-injury. Scale bar = 40 μm. Dotted line indicates edge of injury site. CR, calretinin. Color image is available online at www.liebertpub.com/neu

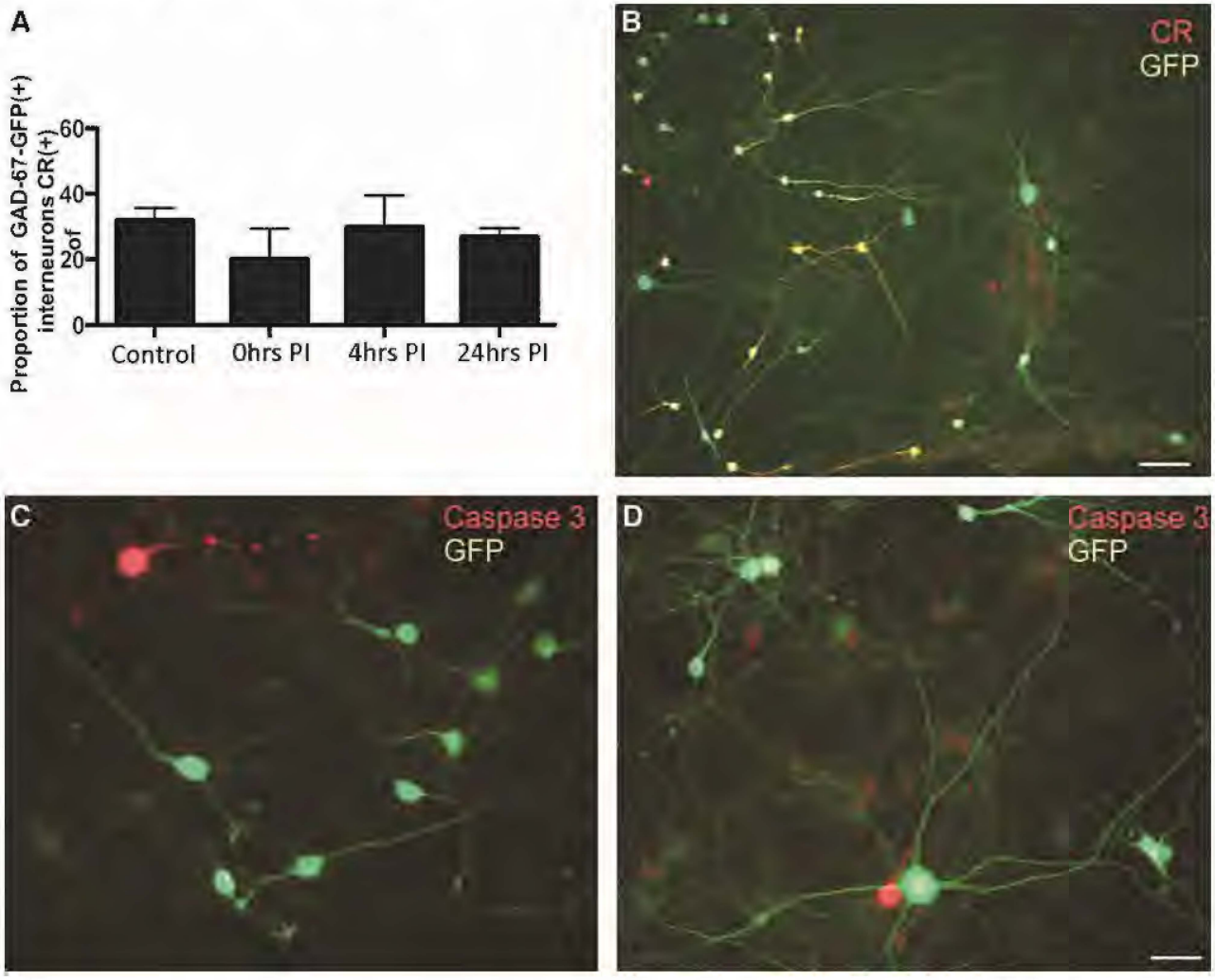

FIG. 3. Calretinin+ interneuron viability over 24 h post neurite injury. **(A)** There were no significant differences found in the proportion of calretinin+/GAD67-GFP+ interneurons from the total number of GAD67-GFP+ interneurons at any of the post-injury time-points analyzed, compared with control cultures. **(B)** Representative image of calretinin+/GAD67-GFP+ interneurons at 4 h post-injury. **(C, D)** Representative images of immunolabeling with anti-activated caspase 3 antibody and anti-GFP in control (C) and injured (D) cortical cultures at 4 h post-injury. Scale bar (B) = 50 μm, (C) and (D) = 30 μm. CR, calretinin. Color image is available online at www.liebertpub.com/neu

cortex of GAD67-GFP mice. We confirmed that our protocol delivered a mild injury, as the mice showed a short post-injury righting time (between 2–4 min) and did not experience mortality after injury (Table 1).[45] The mild FPI induced a transient behavioral suppression of the righting reflex in all injured animals of 2.60 ± 0.58 min, which was significantly longer than sham-injured animals (0.15 ± 0.10 min; $p < 0.001$). There were no significant differences in age or weight between the sham-injured and FPI mice at any time post-injury. This mild unilateral fluid percussion injury did not result in contusion, intraparenchymal hemorrhage, or notable cortical loss (Fig. 4A). In conclusion, the parameters measured were consistent with the delivery of a mild injury to the cortex.[45]

TABLE 1. PARAMETERS MEASURED IN THE GAD67-GFP MICE EXPOSED TO LATERAL FPI

	1 day PI		*7 days PI*		*28 days PI*	
	Sham n=6	*FPI* n=6	*Sham* n=6	*FPI* n=6	*Sham* n=6	*FPI* n=6
Age (weeks)	11.3 ± 0.88	9.6 ± 0.71	11.2 ± 0.48	11.3 ± 0.56	12.7 ± 0.21	12.5 ± 0.48
Weight (g)	28.6 ± 0.9	27.3 ± 1.4	29.2 ± 0.6	29.9 ± 0.9	26.0 ± 0.78	26.2 ± 1.1
FPI (atm)	NA	1.44 ± 0.10	NA	1.37 ± 0.12	NA	1.39 ± 0.15
Righting time (min)	0.2 ± 0.1	2.16 ± 0.53**	0.1 ± 0.1	2.67 ± 0.61**	0.1 ± 0.1	2.04 ± 0.30**

FPI, fluid percussion injury; PI, post-injury.

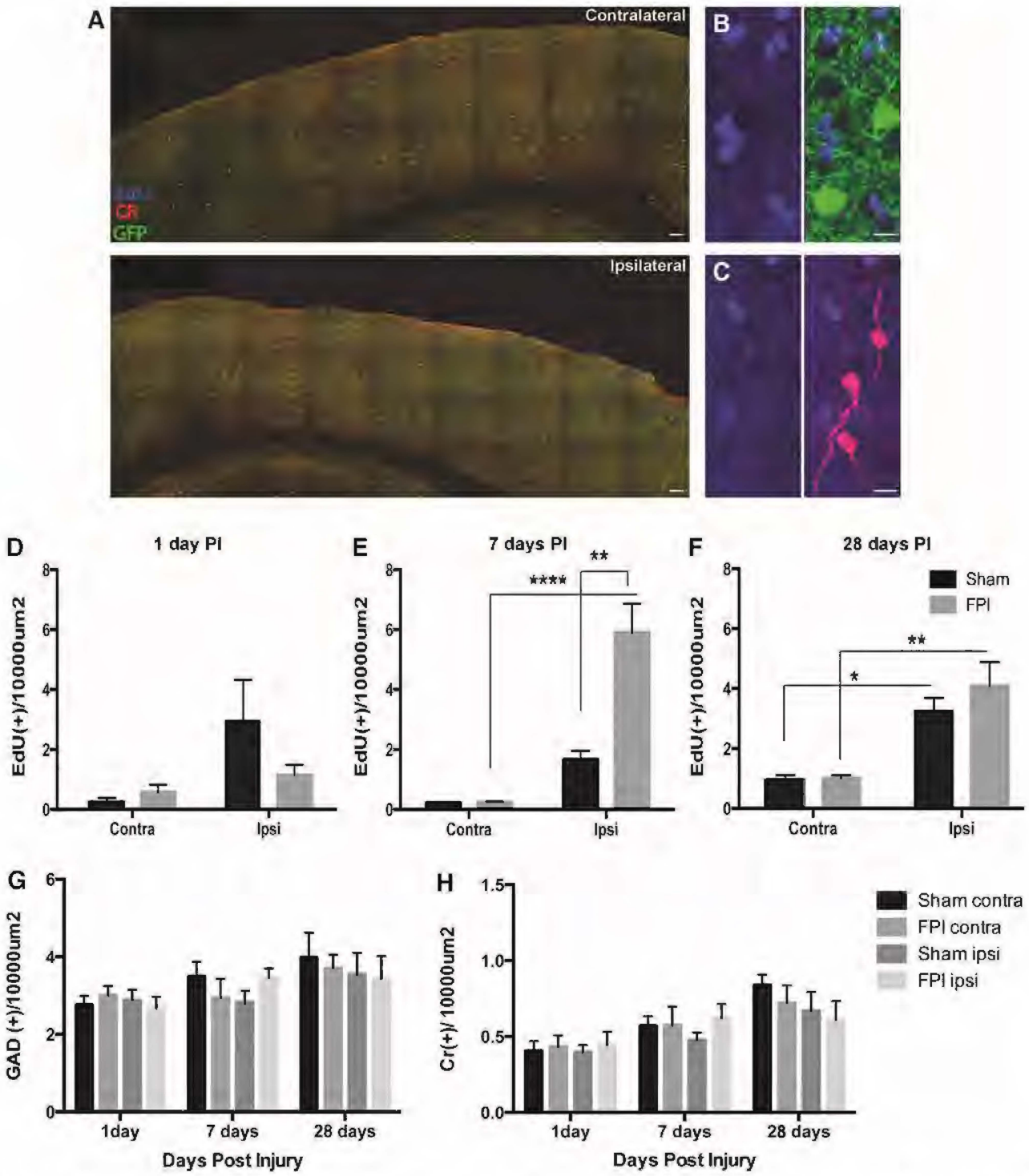

FIG. 4. Analysis of newly produced cells after mild TBI indicate no interneuron neurogenesis. In order to visualize newly produced cells GAD67-GFP mice received EdU via the drinking water after injury for 1 day, 7 days or 28 days to label dividing cells. **(A)** Immunolabeling for calretinin, EdU and GFP contralateral and ipsilateral to the injury site. Mild lateral fluid percussion injury did not result in contusion, intraparenchymal hemorrhage or cortical loss. **(B, C)** Representative images of the ipsilateral GAD67-GFP cortex after mild FPI injury immunolabeled for EdU^+ (B, blue) and $GAD67\text{-}GFP^+$ (B, green) and labelled for EdU^+ (C, blue) and $calretinin^+$ (C, red) cells. **(D)** There was no significant increase of EdU^+ cells at 1 day post-injury. **(E)** There was a significant increase in the number of EdU^+ cells at 7 days post-injury in the ipsilateral cortex of FPI injured mice compared to the contralateral side and the ipsilateral cortex of Sham mice (E, $p<0.0001$, $p<0.001$). **(F)** At 28 days post-injury there was a significant increase in the number of EdU^+ cells in the ipsilateral cortex of Sham and FPI mice in respect to the contralateral cortex ($p<0.05$, $p<0.01$ respectively). **(G)** There were no significant differences found in the total number of $GAD67\text{-}GFP^+$ interneurons found between the Sham and FPI injured mice at different time points post-injury, ipsilateral or contralateral to the injury site. **(H)** There were no significant differences found in the total number of $calretinin^+$ interneurons found between the Sham and FPI injured mice at different time points post-injury, ipsilateral or contralateral to the injury site. Data are presented as mean ± SEM. One-way ANOVA, followed by Bonferroni test. Scale A = 100μm; B and C = 30μm. Color image is available online at www.liebertpub.com/neu

To investigate a potential induction of neurogenesis following the mild injury, EdU was administered via the drinking water to sham-injured and FPI mice for 1, 7, or 28 days post-injury. Coronal brain sections were processed to detect GFP, calretinin, and EdU, and no newly generated GAD67-GFP$^+$ interneurons or calretinin$^+$ interneurons were detected in the cortex ipsilateral or contralateral to the injury site of FPI or sham-injured mice (Fig. 4B, 4C). The number of EdU$^+$ cells was quantified in the ipsilateral and contralateral cortex of sham-injured and FPI mice (Fig. 4D–F), and there was no significant change in the number of EdU-labeled cells present in the somatosensory cortex at 1 day post-injury ipsilateral or contralateral to the injury site when comparing sham-injured or FPI mice (Fig. 4D). However, the number of EdU$^+$ cells was significantly elevated in the ipsilateral cortex of FPI mice relative to sham-injured mice, and relative to the contralateral cortex 7 days (Fig. 4E) and 28 days post-injury (Fig. 4F). These data indicate that a mild FPI triggers a proliferative response, presumably from local glial populations, in the weeks to months following the impact. Of particular importance for this study, a mild FPI did not trigger interneuron addition to the injury site. There were no newly generated GFP$^+$ interneurons or CR$^+$ interneurons detected in the injured cortex ipsilateral or contralateral to the injury site in the FPI or sham animals at any of the post-injury time-points analyzed (0 GFP$^+$ EdU$^+$ cells from a minimum of 200 GFP$^+$ cells per section per animal and 0 CR$^+$ EdU$^+$ cells from a minimum of 25 CR$^+$ cells per section per animal).

To determine whether mild TBI had an effect on interneuron viability, the total number of GFP$^+$ interneurons was examined in the cortex of GAD67-GFP FPI and sham-injured mice at 1, 7, and 28 days post-injury (Fig. 4G). Our mild FPI did not affect the total number of interneurons present in the ipsilateral or contralateral somatosensory cortex at 1, 7, or 28 days post-injury (Fig. 4G; $p=0.85$). Further, as subpopulations of interneurons populate specific cortical layers, we examined the total number of interneurons in different layers of the cortex, and found that the injury did not influence the number of interneurons present in the supragranular (layers I–IV; $p=0.77$) or infragranular layers (layers V, VI; $p=0.66$) (Supplementary Fig. 1). These results suggest that interneuron number is not altered *in vivo* by a mild TBI.

To build on our earlier results indicating that calretinin$^+$ interneurons had a cell type specific response to injury *in vitro*, we quantified the number of calretinin$^+$ interneurons in the cortex of GAD67-GFP sham-injured and FPI mice at 1, 7, and 28 days post-injury (Fig. 4H). Quantification of the total number of calretinin$^+$ interneurons demonstrated that mild injury had no effect on the total number of calretinin$^+$ interneurons ipsilateral or contralateral to the injury site at any of the post-injury time-points analyzed, compared with sham-injured mice (Fig. 4H; $p=0.32$). There also was no significant change in the number of calretinin$^+$ interneurons in the supragranular (layers I–IV; $p=0.55$) or infragranular layers (layers V, VI; $p=0.50$) of the cortex at any of the post-injury time-points examined (data not shown). These data indicate that mild TBI does not lead to a loss of calretinin$^+$ interneurons or a change in their cortical distribution.

Mild TBI induces morphological changes in calretinin$^+$ interneurons

Calretinin$^+$ interneurons responded to *in vitro* transection injury by rearranging their dendritic arbors away from the injury site (Fig. 1). To determine whether calretinin$^+$ interneurons respond to a mild diffuse and focal injury *in vivo* in a similar fashion, we analyzed the dendritic arbor of calretinin$^+$ interneurons ipsilateral and contralateral to the injury site at 7 and 28 days post-injury, comparing sham-injured and FPI mice (Fig. 5A, 5B).

By 7 days post-injury, the total length of the dendritic arbor of calretinin$^+$ interneurons located ipsilateral to the injury, was reduced in the somatosensory cortex of GAD-67-GFP$^+$ FPI mice relative to sham-injured controls (Fig. 5C; 64 neurons analyzed per mouse, $n=6$ mice per group, two-way analysis of variance [ANOVA]; $p=0.004$). By 28 days post-injury, there was no longer a significant difference in the total length of the dendritic arbor of calretinin$^+$ interneurons in the ipsilateral cortex of sham-injured and FPI mice (Fig. 5D, 5C). Additionally, no difference was detected when comparing the total arbor length of calretinin$^+$ interneurons on the contralateral side of sham-injured or FPI mice at 7 or 28 days post-injury (Fig 5C, 5D; two-way ANOVA; $p=0.22$).

We next compared the number of dendritic branches elaborated by calretinin$^+$ interneurons located ipsilateral to the injury site, in GAD-67-GFP$^+$ sham-injured and FPI mice at different time-points post-injury. This analysis indicated that calretinin$^+$ interneurons respond to a mild TBI by decreasing their dendritic arbor complexity over the first 7 days post-injury (Fig. 5E). The reduction in dendritic complexity detected at 7 days post-injury was the result of calretinin$^+$ interneurons located ipsilateral to the FPI losing their secondary dendrites. However, this was a transient plasticity response, with the neurons elaborating new secondary dendrites by 28 days post-injury (Fig 5F; two-way ANOVA; $p=0.09$). There were no differences found in soma size or the length of individual dendritic branches for calretinin$^+$ interneurons in the injured cortex, compared with the sham-injured cortex, at any of the time-points analyzed (Supplementary Fig. 1).

Reduced synaptic inhibition in layer V pyramidal neurons after mild TBI

To determine whether the injury-induced alterations in the dendritic arbor of calretinin$^+$ interneurons altered inhibitory signaling in the cortex, we performed whole–cell patch clamp analysis of layer V pyramidal neurons in acute slices from Thy1-YFP-H sham-injured and FPI mice. Upon patching, each YFP$^+$ pyramidal neuron was filled with neurobiotin, which allowed us to histologically confirm its location in layer V of the injured somatosensory cortex (Fig. 6A, 6B). There was no difference in the capacitance of pyramidal neurons in the cortex of FPI and sham-injured mice (sham 58.5pF ±9.2pF, FPI 48.1pF ±3.5pF; $p=0.268$, two-tailed *t*-test). The average amplitude (Fig 6C, 6D; sham 12.7 pA ±0.8 pA, FPI 13.5 pA ±0.7 pA; $p=0.491$, two-tailed *t*-test), rise time (Fig. 6F; sham 4.33 msec ±0.64 msec, FPI 4.78 msec ±0.71 msec, $p=0.67$, two-tailed *t*-test) and decay time (Fig. 6G; sham 7.38 msec ±0.95 msec, FPI 13.32 msec ±2.74 msec, $p=0.14$, two-tailed *t*-test) of miniature inhibitory post-synaptic currents (mIPSCs) recorded from layer V pyramidal neurons located in the cortex of sham-injured and FPI mice at 7 days post-injury was equivalent, indicating that there were no changes to post-synaptic $GABA_A$ receptors. The mean rising time for sham and FPI was 4.41 msec but most neurons had a mIPSC rising time between 2–4 msec ($n=15$; sham and FPI), fewer neurons had a slower rising time greater than 4 msec ($n=4$; sham and FPI)). The neurons with a rise time of greater than 4 msec had no difference in capacitance or access resistance when separated from the total population and hence did not stratify any criteria for exclusion. The fast mIPSCs could represent currents originating from synapses on the soma and proximal dendrites, whereas the slow mIPSCs could be generated in synapses located

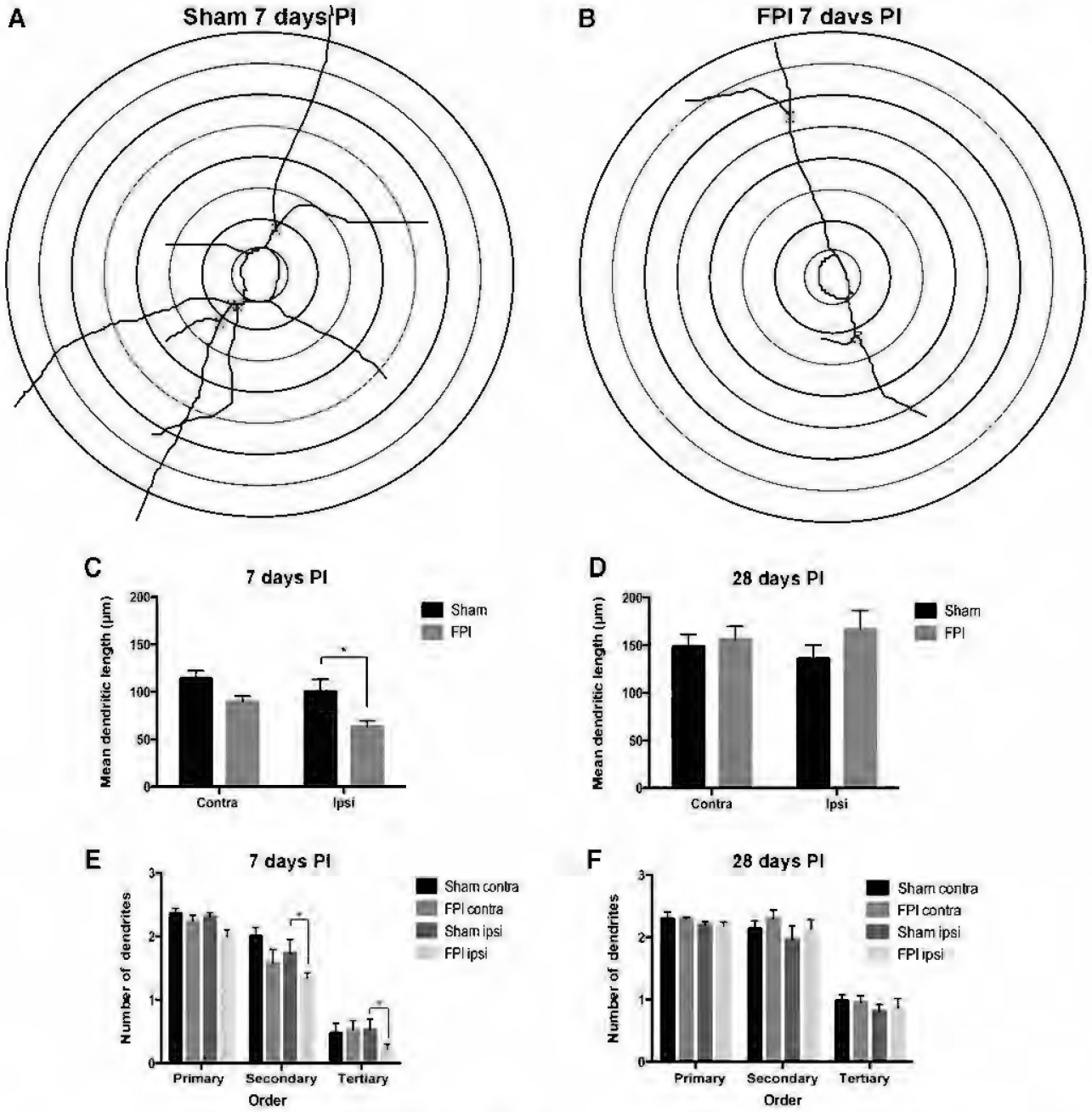

FIG. 5. Calretinin interneurons dendritic arbor complexity analysis at 7 and 28 days after mild TBI. Calretinin+ interneurons were traced using Neurolucida software in order to investigate their dendritic morphology at 7 and 28 days post-injury. (A, B) Representative images of calretinin+ interneurons in the ipsilateral cortex of fluid percussion injury (FPI; A) and sham (B) mice at 7 days post-injury. Each concentric circle represents 10 μm from the cell soma. (C) There was significant decrease in total dendritic length in calretinin+ interneurons found in the ipsilateral side in FPI-injured mice, compared with sham mice at 7 days post-injury ($p < 0.05$). (D) There was no significant decrease in total dendritic length in calretinin+ interneurons found in the ipsilateral side in FPI injured mice, compared with sham mice at 28 days post-injury ($p < 0.05$). (E, F) The number of primary, secondary and tertiary dendrites was evaluated at 7 (E) and 28 days post-injury (F). (F) There was a significant decrease in the number of secondary and tertiary order processes in calretinin+ interneurons found in the ipsilateral side of FPI-injured mice, compared with the ipsilateral side of sham animals (D; $p < 0.05$). (C–F) Data presented as mean ± standard error of the mean. Two-way analysis of variance, followed by Bonferroni test.

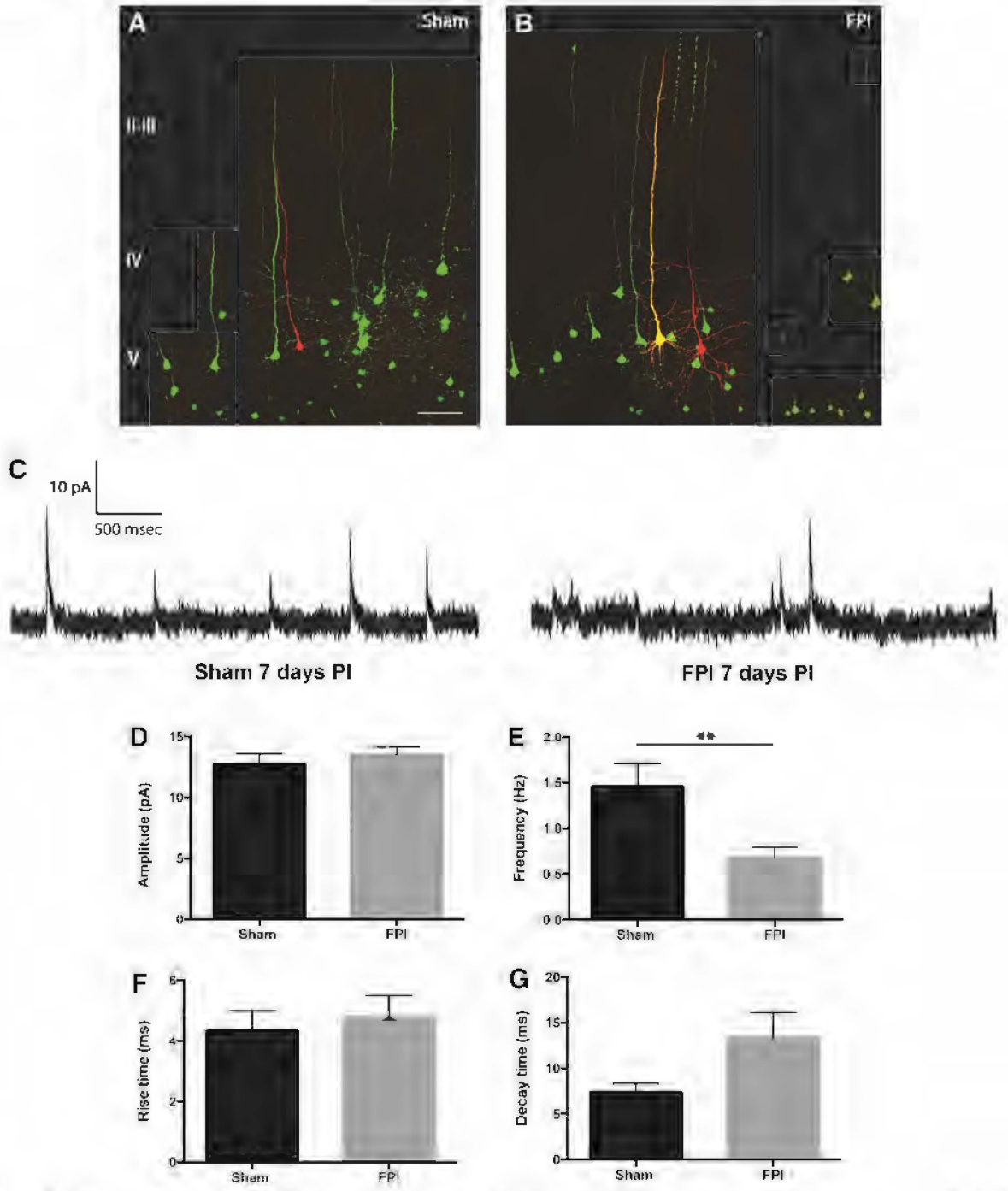

FIG. 6. Analysis of synaptic inhibition in layer V pyramidal neurons after mild traumatic brain injury. Whole-cell patch clamp recordings from layer V pyramidal neurons from sham and FPI-injured mice were taken. Representative images of layer V Neurobiotin filled neurons after whole cell patch clamping in sham (**A**) and fluid percussion injury (FPI) injured (**B**) cortical slices. Examples of mini inhibitory post-synaptic currents (mIPSCs) recorded in pyramidal neurons from sham and FPI-injured mice (**C**). There were no significant differences found in mIPSCs amplitude (sham 12.74 ± 0.68 pA; FPI 13.50 ± 0.67 pA) (**D**), rise time (sham 4.34 ± 0.64 msec; FPI 4.78 ± 0.71 msec; **F**) or decay time (sham 7.38 ± 0.95 msec; FPI 13.32 ± 2.74 msec) (**G**) in layer V pyramidal neurons at 7 days post-injury between sham and FPI mice. There was a significant decrease in mIPSCs frequency (sham [$n = 8$ cells from five animals] 1.46 ± 0.25 Hz, FPI [$n = 12$ cells from eight animals] 0.68 ± 0.12 Hz; $p < 0.01$). Paired t-test. Scale bar = 100 μm. Color image is available online at www.liebertpub.com/neu

more distally on the dendrites. However, there was a significant decrease in the frequency of mIPSCs from 1.46 ± 0.26 Hz to 0.68 ± 0.12 Hz (Fig. 6E; $p < 0.0061$, two-tailed t-test), indicating that the pyramidal neurons in the somatosensory cortex of injured mice received significantly fewer inhibitory synaptic inputs compared to sham-injured controls.

Discussion

A majority of cases of TBI are mild injuries that involve only brief loss of consciousness.[46,47] Although the deficits produced by mild TBI are frequently subtle, memory, affective, and executive dysfunction can nevertheless emerge and cause substantial impairment and life disruption.[48,49] These impairments arise despite the absence of clear structural damage, suggesting the resultant deficits are not a direct result of cortical loss and that adaptive and maladaptive plasticity alterations are at play.[50] It is well established that after a traumatic injury to the brain, neurons can respond and attempt regeneration through the spontaneous rearrangement of the affected network in the form of compensatory morphological or structural plasticity; however, the role that neuronal plasticity plays in mild forms of trauma is not clear.[20,51] The current study utilized two contrasting models of injury (*in vivo* mild diffuse injury vs. *in vitro* single complete neurite transection in relatively mature neurons) to investigate the response of interneurons, focusing on calretinin$^+$ interneurons, to injury. Mild injuries *in vivo* were confirmed by the lack of clear structural damage, absence of post-injury mortality, and short time required for mice to regain their righting reflex post-TBI.[45,52] Our investigations demonstrated that following mild injury, calretinin$^+$ interneurons have the capacity for neurite plasticity around the injury site and that this plasticity corresponded to decreased inhibitory input in pyramidal neurons. Further, our investigations indicate that the response of the calretinin$^+$ interneurons is replicated in different models of injury, suggesting this response is a conserved mechanism of these cells and may be generalizable to the array of varying forms of trauma that can occur. Understanding the aberrant alterations in excitability in the brain following trauma will aid in unraveling why even mild forms of injury can have such lasting effects cognitively.

Lateral FPI is a well-characterized model of brain injury that produces both focal and diffuse effects, including the generation of new cells.[53–58] Therefore, cell proliferation in the cortex was evaluated as the presence of newly generated EdU$^+$ cells following mild FPI. It was determined that mild FPI induces cell proliferation within the injury site but not in other regions of the cortex, including the contralateral hemisphere. Several studies have observed neurogenesis in rodents in response to moderate-to-severe traumatic brain injury.[59–63] However, most studies have indicated that neurogenesis does not lead to neuronal replacement in the cortex.[20,64] Since the focus of the current study is the inhibitory network, the presence of newly generated cortical interneurons was evaluated. There was no induction of interneuron neurogenesis after injury indicated by the lack of either GFP$^+$ or calretinin$^+$ neurons positive for EdU in the cortex.

Several studies have reported a deficit in GABAergic activity and loss of GABAergic interneurons in the hippocampus following various types of injury.[65–68] There also is evidence that severe injuries induce changes in the total number of interneurons and in specific populations of interneurons in the cortex.[69] However, alterations in the cortical inhibitory circuit after mild injury have not been previously evaluated. The mild injury employed here does not induce overall changes in the total number of interneurons or number of interneurons that reside in different layers of the cortex shortly after injury or as a delayed mechanism at later post-injury time-points. Moreover, there was no change in the number of calretinin$^+$ interneurons at any of the post-injury time-points analyzed. However, we cannot discount the other populations of interneurons were differentially altered. Our investigations did reveal significant changes in calretinin$^+$ neurite plasticity and potential changes in GABAergic activity.

Previous studies have indicated that cortical GABAergic interneurons have the capacity to remodel their dendritic arbors under physiologically normal and pathological conditions.[20,24,27,28] There is evidence indicating that interneurons are more plastic than pyramidal neurons. It has been reported that interneurons exhibit dynamic arbor rearrangements in the adult visual cortex while pyramidal neurons remain stable.[27,28] Moreover, our previous experiments using a focal stab injury to the somatosensory cortex have found differences in post-injury structural plasticity between calretinin$^+$ interneurons and pyramidal neurons.[20] To first probe the cell type specific potential for neuroplasticity, an *in vitro* model of axonal injury was utilized. Using neurons derived from the transgenic mouse strains Thy1-YFP and GAD67-GFP that express fluorescent proteins in cortical pyramidal and inhibitory neurons, respectively. Analysis of post-injury neurite angle indicated that calretinin$^+$ interneurons reorientate their processes towards the intact neuropil, while there were no changes found in pyramidal neurons. The direct mechanisms for this cell type specific plasticity are unclear. After injury there is an important release of neurotransmitters, neurotrophic factors, and calcium.[70,71] Given that remodeling was found to be restricted to an area close to the injury site and at an early post-injury time-point, it is plausible that the release of these diffusible factors around an injury site might have differential effects on specific cell types mediating neurite remodeling on calretinin$^+$ interneurons. Further, it is important ot note that the *in vivo* environment affords a unique post-injury environment that is not completely replicated *in vitro*. These results support the hypothesis that there are intrinsic differences in the neuronal response to injury between different neuronal subtypes and indicate that the remodeling of interneurons may play an important role after injury.

Using an *in vivo* model of injury, our studies demonstrate that sub-population specific morphological changes of calretinin$^+$ interneurons occur transiently in response to mild injury. At 7 days post-injury, calretinin$^+$ interneurons in the injury site had fewer secondary and tertiary processes when compared to the contralateral cortex and sham animals. While the changes in dendrite complexity are small, given the calretinin interneuron's unique connectivity and placement in the circuitry that enables significant control over inhibitory regulation as a whole, relatively small changes have the potential to elicit functionally significant effects. There was no significant reduction in the mean number of secondary order dendrites between the contralateral and ipsilateral sides after FPI. This could be due to the potential wide-reaching effects of the injury and provides evidence as to why the contralateral side is not an appropriate control. The changes in dendritic arbor complexity were time dependent and were restored to sham levels by 28 days post-injury. This transient response, observed both *in vitro* and *in vivo*, is indicative of a response of these interneurons to potential changes in either the extracellular environment or changes in intrinsic network connections occurring as a result of the injury; however, these mechanisms were not the focus of this current study. The vulnerability of calretinin$^+$ interneurons, in the form of loss and reorganization, has been described in animal

models of ischemia and epilepsy.[37,38] Previous studies indicate that in the naïve brain dendritic branch tip remodeling of interneurons in the adult visual cortex is mainly contained within a superficial strip of layer II/III of the cortex.[27] Moreover, it has been reported that dendritic remodeling of GABAergic interneurons is not determined by genetic lineage but rather is a general feature of all interneuron subtypes imposed only by cortical laminar circuitry.[24,27,28] Although these previous elegant studies have indicated that there are no subtype specific differences in the capacity of structural remodeling in interneurons, results in our studies point to a cell type specific effect rather than laminar specific.

Functional studies were carried out to determine if the morphological changes found in the subpopulation of calretinin$^+$ interneurons after mild injury corresponded to functional alterations to the whole inhibitory circuit. Recordings of the inhibitory activity or inhibitory afferents onto pyramidal neurons were used as an indication of the overall inhibitory network activity after injury.[69,72] To investigate this, mIPSCs were recorded from layer V pyramidal neurons within the injury site. mIPSCs are spontaneously occurring events that reflect spontaneous non-action potential–dependent release of single vesicles of GABA from individual pre-synaptic terminals of GABAergic interneurons.[73] Results indicated that at 7 days post-injury the average amplitude of the mIPSCs was equivalent between sham and FPI-injured mice. The equivalent mIPSCs amplitude found between groups suggests that the expression of $GABA_A$ receptors in layer V pyramidal neurons after injury was likely unaffected. Mild TBI was associated with a significant decrease in mIPSCs frequency. The observed decrease in mIPSCs frequency indicates that the number of inhibitory connections onto layer V pyramidal neurons in the injured cortex was potentially decreased at 7 days post-injury. Given that there were differences found between the sham and FPI groups in mIPSCs frequency but not mIPSCs amplitude, these results suggest that changes in inhibitory synapses onto layer V pyramidal neurons are predominantly pre-synaptic rather than post-synaptic, reflecting changes in the inhibitory network. Moreover, there were no changes in mIPSCs kinetics (rise and decay time) indicating no changes in $GABA_A$ receptor-subunit composition in the post-synaptic neuron, further pointing to pre-synaptic involvement.[74,75] Similar results have been reported for granule cells and pyramidal neurons in the hippocampus after both mild and severe brain injury at similar post-injury time-points.[65,66,68] Either a decrease in the number or efficacy of inhibitory synaptic afferents onto the excitatory cells or a decrease in neurotransmitter release probability from these afferents, or a combination of both mechanisms could produce a decrease in mIPSC frequency. The synchronization of dendritic inhibitory cells is a crucial process to provide effective inhibitory control of excitatory synaptic input of pyramidal cell dendrites.[37] As a result, calretinin$^+$ interneurons have a far-reaching and wide influence on the whole cortical inhibitory circuit.[38] It is therefore hypothesized, while not directly tested, that the post-injury dendritic remodeling of calretinin$^+$ interneurons, in terms of loss of secondary and tertiary processes, could result in less effective synchronization of dendritic inhibitory interneuron firing, leading to less effective inhibition of pyramidal neuron firing and ultimately resulting in a pathologic shift toward excess excitability.

The current studies demonstrate that calretinin$^+$ interneurons undergo a cell type–specific response to injury both *in vitro* and *in vivo* resulting in significant dendritic remodeling. Importantly, these studies indicate that the structural alterations to calretinin$^+$ interneurons observed correlate to functional changes in inhibitory input that may contribute to the cognitive impairment that can manifest following even seemingly mild forms of brain trauma. Targeting these inhibitory neurons after injury could potentially improve functional deficits found as a result of mild TBI.

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Author Disclosure Statement

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Address correspondence to:
Tracey Dickson, BscHons PhD
Menzies Institute for Medical Research
University of Tasmania
Private Bag 23
Hobart 7000, Tasmania, Australia

E-mail: Tracey.Dickson@utas.edu.au