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Serotonin axons in the neocortex of the adult female mouse regrow after traumatic brain injury

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Abstract

It is widely held that injured neurons in the central nervous system do not undergo axonal regrowth. However, there is mounting evidence that serotonin axons are a notable exception. Serotonin axons undergo long-distance regrowth in the neocortex after amphetamine lesion and, following a penetrating stab injury, they can regrow from cut ends to traverse the stab rift. Traumatic brain injury (TBI) is clinically prevalent and can lead to pathologies such as depression that are related to serotonergic dysfunction. Thus, whether serotonin axons can regrow after TBI is an important question. We used two models for TBI, a persistent open skull condition and controlled cortical impact, to evoke injury in adult female mouse neocortex and assessed serotonin axon density one week, one month, and three months after injury by serotonin transporter immunohistochemistry. We found that following both forms of TBI, serotonin axon density is decreased posterior but not anterior to the injury site when measured in layer 1 at one week post-surgery, and that serotonin axons are capable of regrowing into the distal zone to increase density by one month post-surgery. This pattern is consistent with the anterior-to-posterior course of serotonin axons in the neocortex. TBI in these models is associated with significant reactive astrogliosis both anterior and posterior to the impact, but the degree of reactive astrogliosis is not correlated with serotonin axon density when measured one week after TBI. Microglial density remains constant following both types of injuries, but microglial condensation was detected one week after controlled cortical impact.

Graphical Abstract

It is widely held that injured neurons in the central nervous system do not undergo axonal regrowth. However, there is mounting evidence that serotonin axons are an exception. We found

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TJK designed experiments, performed experiments, analyzed data and wrote the paper.

SED performed experiments. DJL designed experiments and wrote the paper.

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that following traumatic brain injury serotonin axons are damaged, but capable of regrowing in the adult female mouse neocortex.

Keywords

serotonin; regeneration; traumatic brain injury; controlled cortical impact

Introduction

Traumatic brain injury (TBI) is a physical injury caused by a blow against or jolt of the head, a penetrating injury to the head, or exposure to blast that results in the disruption of normal brain function. In the United States there are approximately 2.5 million emergency department visits involving TBI annually, a number that likely underestimates the true incidence (CDC, 2015). TBI comprises a range of injury types that have diverse outcomes (Faul and Coronado, 2015). TBI tends to damage tissues through biomechanical forces generated by rapid acceleration, deceleration, compression against the intact skull, and changes in intracranial pressure.

While the initial insults resulting in TBI can be thought of as a discrete event, TBI itself is a chronic disease process (Masel and DeWitt, 2010). Following rehabilitation after injury, patients with TBI are at greater risk of death than the general population (Harrison-Felix et al., 2004) and are also at an increased risk for a wide range of morbidities, including neurological disorders, neurodegenerative diseases, and psychiatric disease such as major depression (Masel and DeWitt, 2010). Major depression is the most commonly reported neurobehavioral sequela of TBI (Riggio and Wong, 2009) with TBI patients showing a 17% higher rate of depression than the general population (Jorge et al., 2004). The causal link between TBI and depression remains unclear, but may be a result of disruption of normal monoaminergic modulation of the prefrontal cortex (Kohler et al., 2016). Indeed, the first line of treatment for depression following TBI are the selective serotonin reuptake inhibitors, which target the serotonergic transporter to increase serotonin concentration in the central nervous system (Silver et al., 2009).

Serotonin is a monoamine neurotransmitter implicated in diverse functions including mood, sex drive, sleep and thermoregulation (Lucki, 1998; Muller and Jacobs, 2010). Nearly every structure in the brain receives innervation from serotonin axons. Serotonergic cell bodies in the rostral group of raphe nuclei send projections to the forebrain (Figure 1A; Tork, 1990); these axons follow a C-shaped projection through the lateral hypothalamus and basal forebrain in a dense path termed the medial forebrain bundle, and turn dorsally at the frontal cortex before coursing posteriorly to continue through the parietal, occipital, and temporal cortex.

It is widely held that injured axons in the central nervous system do not have the capacity to regenerate (Tuszynski and Steward, 2012). Regrowth into or through an injured area can take one of two distinct forms, which are not mutually exclusive; regrowth from a transected or injured axon itself, whether directly from the severed end or proximal to it, is considered regeneration, while growth from non-damaged axons to compensate for innervation loss from damaged axons is called sprouting (Tuszynski and Steward, 2012). While there have been reports of injury-evoked local sprouting in the neocortex, axon regeneration is rare (Canty et al., 2013).

One reason that regrowth is rare following injury is that damaged tissue is not a permissive environment for axonal growth due to extracellular expression of molecules acting as stop signals for axonal growth cones (Fournier et al., 2001; Bovolenta et al., 1993; Hagino et al., 2000). While limited rewiring of the central nervous system suggests that neurons can retain some intrinsic capacity to regrow axons, not all cell types appear to have the capacity to regrow, even in a permissive environment. Indeed, recent studies are beginning to converge on the conclusion that diminished intrinsic regenerative capacity in mature neurons is a major factor limiting regeneration (Sun and He, 2010; Zou et al., 2009; He and Jin, 2016).

However, following injury, serotonin axons can regrow in the central nervous system, both in the spinal cord (Lee et al., 2010; Alilain et al., 2011; Lee et al., 2013; Yoo et al., 2013; Kanno et al., 2014) and in the brain (O'Hearn et al., 1988; Molliver et al., 1990; Mamounas et al., 2000; Hawthorne et al., 2011; Jin et al., 2016). However, the injury models used in these experiments reflect unique insults. For example, amphetamine lesion (O'Hearn et al., 1988; Molliver et al., 1990; Mamounas et al., 2000) does not create a glial scar (Wilson and Molliver, 1994), while stab injury (Jin et al., 2016) is less common clinically and leaves a relatively narrow rift of damaged tissue to be crossed by regrowing axons. Therefore, whether serotonin axons are able to regrow following the more clinically relevant forms of TBI remains unknown. To address this question we analyzed serotonin axon density using immunohistochemistry following two forms of TBI in mice.

Materials & Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins School of Medicine, Baltimore, Maryland. Animals were group housed (4–5 per cage) on a 12/12 light-dark cycle with access to food and water *ad libitum*. Nesting material was provided and bedding was changed weekly.

Controlled cortical impact (CCI) and open skull injury protocols

10 week-old female C57BL/6 mice (Charles River) were induced with 3% isoflurane anesthesia in a closed plastic container and the skull was then shaved in preparation for surgery. Mice were then placed in the stereotaxic device (Stoelting) with 1.5% isoflurane delivered via a nose cone. The skin above the skull was cleaned with alcohol and iodine and a midline incision was made to expose the skull. The skin was retracted medially and held in place with a modified Colibri retractor (Fine Science Tools). Fascia was removed from the skull and a round craniotomy was made with a 2.7 mm trephine drill bit (Fine Science Tools) using an electric drill. The craniotomy location was over the left hemisphere of the brain; the medial aspect of the craniotomy bordered the sagittal suture and the antero-posterior aspect of the craniotomy was centered on the coronal suture. The skull over the craniotomy was carefully removed using a scalpel and tweezers, and the dura was inspected to ensure that it remained intact. An electromagnetic impactor with a 1.5 mm tip (Leica) was then mounted to the stereotaxic device and centered over the craniotomy site at a 10 degree angle, so that it was perpendicular to the brain surface under the craniotomy. The impactor was extended and the tip lowered until it made contact with the dura, as measured by the completion of an electrical circuit between the impactor and a wire clipped to the tail. It was then retracted and the impact depth was set to 0.75 mm past the measured surface of the dura. The electromagnetic impactor was then activated resulting in an impact with a final velocity of 4 m/s and a dwell time of 100 ms. These parameters were chosen from a wide range of empirically tested values to provide an injury that was consistent between animals, resulted in visible damage to neocortical layers 1–4, and largely spared neocortical tissue anterior and posterior to the injury site to allow for analysis of fixed tissue in those areas.

After impact, the retractor was removed and the skin was closed above the craniotomy using 9 mm wound clips (Fine Science Tools). Mice were given subcutaneous injections of Baytril (2.5 mg/kg) to prevent infection and Buprenex (0.01 mg/kg) for pain management. Mice were removed from the stereotaxic device and put under a heat lamp in a recovery cage on their backs. Righting time, defined as the amount of time it took for the mice to flip and place all four paws on the bedding, was measured in seconds (control: range 9–144, median 46; open skull: range 39–650, median 214; CCI: range 36–597, median 301). Mice were then returned to group housing for the duration of the post-surgery time point ($n = 10$ for each time point). Open skull injury mice received the same treatment, but the impactor was not fired ($n = 10$ for each time point). Control mice received the same treatment (shaving, incision, staples, isoflurane anesthesia, Baytril and Buprenex), but did not receive a craniotomy nor was the impactor fired ($n = 8$ for each time point). Animals that did not survive to their designated time point were excluded from the study and no other exclusion criteria were used. Animals were killed to allow for tissue processing either one week, one month, or three months after surgery.

Viral injection in SERT-Cre mice

Slc6a4-Cre BAC transgenic mice created by the GENSAT consortium were purchased from the Mutant Mouse Regional Resource Center at U.C. Davis (stock #031028-UCD; RRID: MMRRRC_017260-UCD). Serotonin axons were labeled by rostral raphe injection of AAV5-EF1a-DIO-hChR2(H134R)-EYFP-WPRE-pA. Adult mice (~3 months old) were

anesthetized with 1–2% isoflurane and placed in a stereotaxic device (Stoelting). A glass pipette with 40 Nm tip diameter was filled with mineral oil and connected to a Nanoject II device (Drummond Scientific Company). The tip of the glass pipette was then front-filled with ~1 NL of the virus solution. Injections were made 4.7 mm posterior from bregma, at a depth of 2.8–3.2 mm. A volume of 0.1–0.2 NL was delivered over a 10 min period. The pipettes were left in place for 15 min before they were withdrawn. The virus was developed in Karl Deisseroth's lab (Tsai et al., 2009) and purchased from the UNC Vector Core. Animals then received CCI or control treatment at ~16 months of age.

Tissue processing

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and perfused intracardially with ice-cold 0.01 M phosphate-buffer saline (PBS) followed by ice-cold 4% paraformaldehyde (PFA) in PBS. After perfusion, the brain was removed and placed in 4% PFA in PBS at least overnight at 4 °C. Two days prior to slicing, brains were cyroprotected in 15% sucrose in PBS overnight at 4 °C followed by 30% sucrose in PBS overnight at 4 °C. The left hemisphere of samples was then sectioned into 40 Nm thick slices on a freezing sliding microtome. The slices were stored in PBS at 4 °C.

Immunohistochemistry and image acquisition

Tissue from all three treatment groups at each time point was processed together. Sections were washed with 0.3% Triton X-100 in PBS and blocked in 5% normal goat serum and 0.3% Triton X-100 in PBS for 2 hours at room temperature. The sections were then incubated with primary antibody in the blocking buffer overnight at 4 °C. The primary antibodies used were guinea pig anti-SERT (Frontier Institute #HTT-GP-Af1400; RRID: AB_2571777), mouse anti-NeuN (Millipore #ABN60; RRID: AB_2298767), chicken anti-GFP (Aves Labs #GFP-1010, RRID: AB_2307313), rabbit anti-GFAP (Millipore #AB5804; RRID: AB_10062746), and rabbit anti-Iba1 (Wako #019-19741; RRID: AB_839504). For SERT/NeuN, GFAP/NeuN, and Iba1/NeuN double staining, the primary antibody dilutions were all 1:1000. For SERT/YFP/NeuN triple staining the dilutions were 1:1000, 1:5000, and 1:500, respectively. Slices were washed in PBS and incubated with secondary antibody diluted in blocking buffer at room temperature for 2 hours. The secondary antibodies used were FITC-labeled goat anti-guinea pig (1:1000, Thermo Fisher #A18776; RRID: AB_2535553), Alexa Fluor 594-labeled goat anti-mouse (1:500, Jackson ImmunoResearch Laboratories #115-586-146, RRID: AB_2338899), Alexa Fluor 488-labeled goat anti-rabbit (1:500 Jackson ImmunoResearch Laboratories #111-547-003; RRID: AB_2338058), Cy3-labeled goat anti-guinea pig (1:800, Jackson ImmunoResearch Laboratories #111-165-003; RRID: AB_2337423), Cy5-labeled goat anti-mouse (1:200, Jackson ImmunoResearch Laboratories #115-175-146; RRID: AB_2338713) and FITC-labeled goat anti-chicken (1:1000, Thermo Fisher #A16055; RRID: AB_2534728). The Alexa Fluor-labeled secondary antibodies were reconstituted with water and diluted 1:1 with glycerol. All other secondary antibodies were reconstituted with water only. The sections were washed again and mounted on slides for imaging. A single photon laser scanning confocal microscope was used to acquire all images (Zeiss LSM 510). Imaging of tissue that was processed together was performed with the same settings and laser power for each sample and treatment groups were interleaved throughout the imaging process.

Image analysis

Raw acquired images were exported from Zeiss LSM software in an uncompressed tagged image file format and stored for subsequent analysis using custom code written using the Anaconda Python distribution (Continuum Analytics). Z-stacks were maximally projected and the projection was used to set a threshold for detection of pixels positive for SERT or Iba1 signal. The threshold was calculated using an iterative algorithm for feature extraction (Ridler and Calvard, 1978). The threshold is set to 0 to begin and is incrementally increased until the following condition is true (a background pixel is any pixel at or below threshold; an object pixel is any pixel above the current threshold):

$$threshold > \frac{average\ background\ pixel\ value + average\ object\ pixel\ value}{2}$$

After the threshold has been calculated it is applied to an image such that every background pixel is set to 0 (black) and every object pixel is set to 255 (the highest value for an 8-bit image). This is an unbiased systematic contrast enhancement technique that allows for the subsequent calculation of pixels positive for a given signal.

For quantification of serotonin fiber density, the threshold was applied to each individual slice of the z-stack and the number of pixels above threshold in each slice was summed together and expressed as the percent pixels above threshold, where the total number of pixels was the two-dimensional maximally projected area in the region of interest (i.e., layer 1, 2/3, or 4 as drawn by hand, guided by the NeuN images). This approach was used to account for the fact that in a maximally projected image any area where serotonin fibers run in the z-direction would result in an underestimate of fiber density in the two-dimensional projection. The percent pixels above threshold measure was then normalized to the control condition within each time point to allow comparisons between time points (which did not undergo tissue processing, immunohistochemistry, and imaging together). For quantification of microglial density, a 10 Nm maximal projection was generated, the threshold was applied, and quantification was performed on the projected image.

Staining for reactive astrocytes with GFAP reveals non-specific fluorescence in deeper neocortical layers that interferes with density measurements, such as ours, that use automated thresholding. Therefore, for quantification of reactive astrocytes, a manual counting method was employed on 10 Nm maximally projected images. All GFAP-positive cells that matched the distinct morphology of reactive astrocytes within the layer of interest were counted. In layer 1, GFAP-positive cells that were part of the pia mater were excluded.

To quantify microglial morphology the same images used for density analysis were manually scored. For each microglial cell, the short and long axes of the soma were measured and the area of the microglia soma was approximated as an ellipsis. Microglia which lay against the edge of the imaging plane were excluded from analysis.

Statistics

Statistical significance was determined using a linear regression model followed by targeted t-tests adjusted for multiple comparisons. Data were divided based on imaging regions. For each data set, an ordinary least squares regression model was run, fitting the data to take into account the effect of treatment group, time, and the interaction between treatment group and time. If the linear model suggested an effect of the regressors for the group, one-tailed heteroscedastic t-tests were performed within a timepoint across groups and across timepoints within a group. The significance level of the t-tests results were adjusted for multiple comparisons using the Bonferroni correction. P-values presented in text are for t-tests except where otherwise noted. Data are presented as means $\pm$ the standard error of the mean.

Results

Young female adult mice (10 weeks old) were exposed to one of three treatments. The CCI group which received a craniotomy and an impact directly to the dura, the open skull injury group which received a craniotomy but no impact, and a control group that received neither a craniotomy nor impact. All groups received anesthesia, a scalp incision, wound clips, and post-operative drugs. Mice were killed at one week, one month, and three months post-surgery.

Double-label immunohistochemistry was performed on sagittal brain sections corresponding to the center of the craniotomy (lateral +1.32 mm) to visualize the extent of serotonin fiber damage and recovery following injury. Serotonin fibers were labeled with an antibody against the serotonin transporter (SERT), which is expressed throughout the axon of serotonergic cells. In order to be able to identify neocortical layers, neuronal cell bodies were visualized with an antibody against NeuN, which is expressed in most mature neuronal nuclei.

First, low magnification images were acquired to assess the extent of the injuries. One week post-surgery there was always a clear deformation of layers 1–4 of the motor cortex in the CCI group (Figure 1B). In the open skull injury condition there was occasional damage confined to layers 1 and 2/3 underneath the craniotomy site. There was never damage to the neocortex in the control group.

Next, single-photon confocal stacks were acquired with a 40X objective lens to visualize serotonin fibers at several anatomical locations. Since serotonin axons run in an anterior to posterior gradient in the neocortex (Figure 1A), we wanted to observe damage and recovery both anterior (proximal) to the injury, where we predicted there would be relatively little damage, and posterior (distal) to the injury, where we predicted there would be significant fiber loss due to axonal damage within the injury zone. The anterior and posterior areas were chosen to border either edge of the craniotomy location (anterior aspect: Bregma +1.34 mm; posterior aspect: Bregma –1.34 mm). By choosing constant imaging areas we minimized between-animal variation in serotonin fibers due to the antero-posterior density gradient. We were also interested in whether any differences in damage and recovery exist among

different cortical layers. To this end, we imaged regions including layer 1, layer 2/3, and layer 4, both anterior and posterior to injury.

Anterior to the injury site in layer 1 there was a small decrease in serotonin axon density in both the open skull injury ($78.5 \pm 8.9\%$, $n = 9$) and CCI conditions ($78.6 \pm 13.3\%$, $n = 7$) when measured one week post-surgery compared to control treatment ($100.0 \pm 5.4\%$, $n = 8$) (Figure 2). These decreases were not statistically significant (linear regression $p = 0.290$) (Figure 5). Layers 2/3 and 4 anterior to the injury site show a similar pattern of serotonin fiber loss as layer 1 when measured one week post-surgery. Neither the open skull injury group (layer 2/3: $87.5 \pm 4.5\%$, $n = 9$; layer 4: $87.4 \pm 4.7\%$, $n = 9$; linear regression $p = 0.337$), nor the CCI group (layer 2/3: $85.5 \pm 8.2\%$, $n = 7$; layer 4: $96.0 \pm 6.8\%$, $n = 7$; linear regression $p = 0.249$) showed significant serotonin axon loss compared to the control group (layer 2/3: $100.0 \pm 2.6\%$, layer 4: $100.0 \pm 5.5\%$, $n = 8$).

In contrast to the region anterior to the injury site, there was a significant decrease in serotonin axon density in layer 1 posterior to the injury site, when measured one week post-surgery (Figure 3). This decrease was present in both the open skull injury group ($54.8 \pm 6.8\%$, $n = 9$, $p = 0.001$) and CCI group ($45.7 \pm 9.6\%$, $n = 7$, $p = 0.004$) compared to the control group ($100.0 \pm 6.4\%$, $n = 8$). In layer 2/3 posterior to the injury site there is a significant decrease in serotonin axon density in the open skull injury group ($79.7 \pm 4.4\%$, $n = 9$, $p = 0.008$) compared to the control group ($100.0 \pm 5.9\%$, $n = 8$), but the decrease in the CCI group ($79.9 \pm 7.2\%$, $n = 7$, $p = 0.026$) is not statistically significant after correction for multiple comparisons. In layer 4 there were no significant differences one week post-surgery compared to the control group ($100.0 \pm 3.6\%$, $n = 8$) for either the open skull injury ($89.1 \pm 4.3\%$, $n = 9$) or CCI ($80.8 \pm 7.3\%$, $n = 7$) groups despite a trend toward decreasing innervation (linear regression $p = 0.099$).

These results show that serotonin axons are damaged following TBI and that serotonin axon density is decreased posterior but not anterior to the injury site, consistent with their anterior-to-posterior / proximal-to-distal direction. Given this finding, that serotonin axon density is decreased in layer 1, posterior to injury, one week post-surgery, we sought to determine whether serotonin axons are capable of regrowth following traumatic brain injury.

In layer 1, posterior to the injury site, there was an increase in serotonin axon density for the open skull injury condition at one month ($77.4 \pm 8.3\%$, $n = 8$, $p = 0.027$) and three months ($68.7 \pm 2.2\%$, $n = 9$, $p = 0.04$) relative to one week post-surgery, but these increases were not statistically significant after adjusting for multiple comparisons. However, there was significant recovery of serotonin axon density observed in the CCI group (Figures 4 and 5). Density at one month ($81.3 \pm 8.7\%$, $n = 6$, $p = 0.009$) and three months ($72.0 \pm 6.1\%$, $n = 6$, $p = 0.021$) post-surgery were both significantly greater than the density observed one week post-surgery. In layer 2/3 posterior to the injury site there was no significant difference in innervation between groups at when measured one month post-surgery after adjusting for multiple comparisons (control: $100.0 \pm 4.8\%$, $n = 8$; open skull injury: $85.0 \pm 5.2\%$, $n = 8$, $p = 0.026$; CCI: $94.5 \pm 6.9\%$, $n = 6$, $p = 0.267$). However, at three months post-surgery density for both the open skull injury ($74.1 \pm 6.5\%$, $n = 9$, $p = 0.004$) and CCI ($80.7 \pm 6.9\%$, $n = 8$, $p = 0.013$) groups were significantly lower than the control ($100.0 \pm 5.3\%$, $n = 8$) group.

Density in layer 4 was not statistically different at one week post-surgery and this remained constant at one month (control: $100.0 \pm 5.9\%$, $n = 8$; open skull injury: $93.2 \pm 7.1\%$, $n = 8$; CCI: $91.0 \pm 3.7\%$, $n = 6$) and three months (control: $100.0 \pm 4.2\%$, $n = 8$; open skull injury: $83.7 \pm 4.2\%$, $n = 9$; CCI: $90.8 \pm 6.0\%$, $n = 8$) post-surgery (linear regression $p = 0.099$). Taken together these results support the conclusion that TBI leads to serotonin axon damage in superficial layers posterior to the injury site and that, in layer 1, serotonin axons are able to regrow one month and three months following TBI.

One potential confound to this interpretation is that controlled cortical impact might result in a decrease in expression of SERT posterior to the injury site, rather than a true loss of axons. In this scenario, the decrease in serotonin density would be an artifact of this expression change and the perceived regrowth could be explained by the return of SERT expression in undamaged axons. To address this issue, we repeated the controlled cortical impact protocol on SERT-Cre mice that received injections of the virus AAV5-EF1a-DIO-hChR2(H134R)-EYFP-WPRE-pA (Tsai et al., 2009) in the dorsal raphe. This yields mice in which infected dorsal raphe serotonin neurons, and their neocortical axon projections, have surface membrane EYFP that is constitutively expressed, under the control of the viral EF1a promoter rather than the native SERT promoter. We were then able to perform triple label immunohistochemistry to identify EYFP positive serotonergic axons, SERT positive serotonergic axons, and NeuN to identify neocortical layers in a single mouse. To measure axon loss following injury, both the SERT and EYFP signals were processed using the iterative thresholding algorithm described above (Figure 6). Of the SERT positive axons, 26.9% were EYFP positive in the control. We found that following CCI the SERT signal was decreased to 45.5% of the control level and the EYFP signal was similarly reduced to 43.2% of the control level. This argues against the possibility that changes in SERT expression are being misinterpreted as serotonin fiber axon loss and regrowth.

One of the key features of CCI is that it models common aspects of TBI such as reactive gliosis secondary to injury. This is important as reactive gliosis is known to inhibit axonal growth in some cases (Yiu and He, 2006) and promote it in others (Lee et al., 2010). We therefore wished to examine the scope and dynamics of reactive astrocytosis following CCI and open skull injury and assess its relationship to the degree of serotonin fiber loss.

To this end, sagittal sections adjacent to those used for quantification of serotonin axon density were processed with double-label immunohistochemistry against NeuN to identify neocortical layers and against GFAP, which is expressed by reactive, but not resting, astrocytes (Eng and Ghirnikar, 1994). These images (Figure 7) clearly reveal profound reactive gliosis across layers 1, 2/3, and 4 anterior to the injury site, one week post-surgery for both the open skull injury (layer 1: 7.7 ± 0.3 cells/100 Nm^2 , $n = 4$, $p < 0.001$; layer 2/3: 6.3 ± 1.4 , $n = 4$, $p = 0.01$; layer 4: 3.9 ± 1.3 , $n = 4$) and CCI groups (layer 1: 7.5 ± 0.4 , $n = 4$, $p < 0.001$; layer 2/3: 9.1 ± 0.5 , $n = 4$, $p < 0.001$; layer 4: 6.3 ± 0.1 , $n = 4$, $p < 0.001$) compared to control tissue (layer 1: 1.7 ± 0.3 , $n = 4$; layer 2/3: 0.2 ± 0.1 , $n = 4$; layer 4: 0.2 ± 0.1 , $n = 4$; Figure 8).

The reactive astrogliosis posterior to the injury site looks similar to that anterior to the injury site, when measured one week after surgery. Both the open skull injury and CCI groups have

significant increases in reactive astrocyte density in layer 1 (open skull injury: 7.4 ± 0.8 cells/100 Nm^2 , $n = 4$, $p = 0.002$; CCI: 8.1 ± 0.8 , $n = 4$, $p = 0.001$) and layer 2/3 (open skull injury: 7.5 ± 1.7 , $n = 4$, $p = 0.013$; CCI: 8.5 ± 1.3 , $n = 4$, $p = 0.004$) compared to control levels (layer 1: 1.2 ± 0.2 , $n = 4$; layer 2/3: 0.5 ± 0.2 , $n = 4$). There is a trend toward increased reactive astrogliosis in layer 4 as well, but due to large variability this is not statistically significant after adjusting for multiple comparisons (control: 0.3 ± 0.2 , $n = 4$; open skull injury: 5.0 ± 1.6 , $n = 4$, $p = 0.027$; CCI: 5.1 ± 1.8 , $n = 4$, $p = 0.036$).

Three months after surgery, the number of reactive astrocytes anterior to the injury site decreases to near-control levels (Figures 7, 8). In the open skull injury group there was a significant decrease in the number of reactive astrocytes in layer 1 at three months (1.9 ± 0.5 cells/100 Nm^2 , $n = 4$, $p < 0.001$) compared to one week post-surgery. The same pattern was observed in the CCI group (2.8 ± 0.7 , $n = 4$, $p = 0.001$). Significant decreases were also observed in layer 2/3 for both types of injuries (open skull injury: 0.7 ± 0.1 , $n = 4$, $p = 0.013$; CCI: 1.9 ± 0.8 , $n = 4$, $p < 0.001$) and layer 4, but only for the CCI group (0.8 ± 0.1 , $n = 4$, $p < 0.001$) at three months compared to one week post-surgery. The only significant difference that remained between groups three months post-surgery was the number of reactive astrocytes in the CCI group (0.8 ± 0.1 , $n = 4$, $p = 0.002$) in layer 4 anterior to the injury site compared to the control group (0.08 ± 0.08 , $n = 4$). Quantification of reactive astrocytes anterior to the injury site at one month after injury reveals a partial reduction in layer 1, while reactive astrogliosis in layers 2/3 and 4 had already resolved and appear similar to control levels. In summary, there is a profound degree of reactive astrogliosis anterior to injury one week post-surgery, which begins to resolve at one month, and no longer persists at a statistically significant level at three months after injury.

There are no differences between treatment groups in any measured neocortical layer at three months post-surgery posterior to the injury site (Figure 8). In layer 1, reactive gliosis in both the open skull injury (1.7 ± 0.3 cells/100 Nm^2 , $n = 4$, $p = 0.002$) and CCI (3.0 ± 0.9 , $n = 4$, $p = 0.003$) groups is significantly lower than it was at one week post-surgery. The same holds true for layer 2/3 (open skull injury: 0.4 ± 0.04 , $n = 4$, $p = 0.013$; CCI: 1.4 ± 0.7 , $n = 4$, $p = 0.003$).

Next we asked whether the degree of reactive gliosis was correlated with the degree of serotonin axon loss following injury. To answer this, reactive gliosis and serotonin innervation data were combined for each animal and presented in a scatterplot (Figure 8C). We restricted this analysis to only include the CCI and open skull injury groups as measured one week after surgery. In this data subset there is no significant relationship (Pearson correlation $r = -0.25$, $p = 0.101$) between the two variables. Thus, even profound reactive gliosis is not a predictor of serotonin axon damage. Furthermore, when analysis is restricted to the layer 1 and the injury groups, astrogliosis still fails to predict the degree of serotonin axon loss (Pearson correlation $r = -0.19$, $p = 0.487$).

The response to traumatic brain injury also contains an inflammatory component. Microglia are an important inflammatory cell in the neocortex (Dheen et al., 2007) and have been shown to rapidly respond to brain injury (Davalos et al., 2005; Szalay et al., 2016). Therefore, we sought to determine whether there was a change in microglial density

following both forms of TBI as well as whether there was a relationship between microglial density and serotonin axon loss. To measure microglia we utilized double-label immunohistochemistry against Iba1, which is expressed only by microglia in the neocortex (Ito et al., 1998), and NeuN to identify neocortical layers on sagittal sections adjacent to those used for quantification of serotonin axon density. In order to capture all of the layers of interest, two dorso-ventral adjacent 10 Nm deep confocal z-stacks were captured, maximally projected, and stitched together. The Iba1 signal was contrast enhanced using the iterative threshold algorithm previously described to isolate microglia from background signal. Microglial density was calculated as the percent of total pixels in the layer of interest that were positive for Iba1 signal.

One week after surgery there was no significant difference in microglial density between treatment groups anterior to the injury site in layer 1 (control: $100.0 \pm 12.8\%$, $n = 4$; open skull injury: $88.4 \pm 7.1\%$, $n = 4$; CCI: $123.1 \pm 17.4\%$, $n = 4$; linear regression $p = 0.261$), layer 2/3 (control: $100.0 \pm 9.9\%$, $n = 4$; open skull injury: $96.1 \pm 4.3\%$, $n = 4$; CCI: $99.9 \pm 13.1\%$, $n = 4$; linear regression $p = 0.956$), or layer 4 (control: $100.0 \pm 10.9\%$, $n = 4$; open skull injury: $72.4 \pm 3.9\%$, $n = 4$; CCI: $87.1 \pm 11.7\%$, $n = 4$; linear regression $p = 0.261$). Furthermore, microglial density remained constant anterior to the injury site throughout the experimental time points (Figure 9). While there is a trend toward increased microglial density in layer 1 compared to control density at one week, one month, and three months post-surgery, there were no statistically significant differences between the groups (linear regression $p = 0.261$).

As with the anterior to injury region, there were no microglia density differences between treatment groups posterior to the injury site one week after surgery (Figure 9). Microglia density for the open skull injury (layer 1: $92.5 \pm 14.2\%$, $n = 4$; layer 2/3: $89.7 \pm 4.7\%$, $n = 4$; layer 4: $92.8 \pm 4.5\%$, $n = 4$) and CCI (layer 1: $111.4 \pm 24.6\%$, $n = 4$; layer 2/3: $103.4 \pm 8.3\%$, $n = 4$; layer 4: $94.5 \pm 7.1\%$, $n = 4$) groups was comparable to control levels (layer 1: 100.0 ± 16.0 , $n = 4$; layer 2/3: $100.0 \pm 11.0\%$, $n = 4$; layer 4: $100.0 \pm 11.3\%$, $n = 4$) in layers 1, 2/3, and 4. When we quantified microglial density posterior to the injury site at later time points (one month and three months post-surgery) we also did not observe any significant changes between groups or across time points (linear regression; layer 1: $p = 0.933$, layer 2/3: $p = 0.588$, layer 4: $p = 0.193$).

Microglial activation secondary to injury leads to morphological changes called condensation, including increased soma size and shortened processes (Cho et al., 2006). We therefore estimated the area of microglial somata (Figure 10). CCI resulted in an increase in soma size both anterior ($67.0 \pm 2.7 \text{ Nm}^2$, $n = 214$) and posterior (63.4 ± 2.4 , $n = 198$) to the injury region as compared to the control treatment (anterior to injury: 51.0 ± 1.7 , $n = 141$, $p < 0.001$; posterior to injury: 50.5 ± 1.9 , $n = 131$, $p < 0.001$) as well as the open skull injury (anterior to injury: 56.4 ± 2.0 , $n = 194$, $p < 0.001$; posterior to injury: 53.5 ± 2.1 , $n = 186$, $p = 0.001$) as measured one week after surgery. There were no significant differences between the control treatment and open skull injury at this time point. One month after surgery there was no longer a significant condensation of microglia in the CCI group anterior or posterior to injury. In contrast, microglial soma size anterior to injury in the CCI group (52.7 ± 1.5 , $n = 170$) were significantly smaller than in the control group (58.3 ± 2.0 , $n = 136$, $p = 0.012$).

At three months after surgery, the only difference between groups was posterior to injury, where the open skull injury (50.5 ± 1.3 , $n = 199$) had significantly smaller microglial soma size than the control group (58.0 ± 1.7 , $n = 165$, $p < 0.001$). There were no differences between the CCI and open skull injury groups at one month (anterior: $p = 0.087$, posterior: $p = 0.430$) or three months (anterior: $p = 0.047$, posterior: 0.041) post-surgery when adjusting for multiple comparisons.

Taken together, the immunohistochemical analysis of inflammatory markers shows that CCI produced significant activation of both astroglia and microglia when compared with control (as indexed by soma size but not density). This activation was evident both anterior and posterior to the injury site 1 week after injury and was mostly resolved by 3 months after injury. The open skull injury also produced significant, transient astroglial activation in both anterior and posterior locations, but did not result in significant microglial activation, indexed by either soma size or density when compared with controls at any time point. These differences in injury-evoked inflammation validate the approach of using two different TBI models.

Discussion

Here, we have used SERT immunohistochemistry to measure the density of serotonin axons in the neocortex of adult mice following two different forms of TBI: open skull injury and CCI, and have compared these treatments to a sham-surgery control. We find that both open skull injury and CCI produce damage to serotonin axons posterior but not anterior to the injury site when measured 1 week after injury and that this damage is most apparent in cortical layer 1 (Figures 2, 3, 5). The restriction of the damage to the posterior region is consistent with the anterior-to-posterior direction of the serotonin axons in this dorsal aspect of the neocortex (Figure 1A): it is likely that that CCI and open skull injury damaged serotonin axons underlying the craniotomy, thereby producing degeneration in the posterior (distal) but not the anterior (proximal) axons. Likewise, because both CCI and open skull injury appeared to spare deeper cortical layers under the craniotomy (Figure 1), it is not surprising that the largest damage in the posterior region was seen in layer 1 with smaller damage in layer 2/3 and no statistically significant damage in layer 4. While it is likely that other axons were damaged by these injuries as well, we focused our analysis on serotonin axons because of their relevance to the pathophysiology of TBI.

There are multiple potential mechanisms by which serotonin axons could have been damaged in the open skull injury group. In the present study the craniotomy in both the open skull injury and CCI groups was left open, covered only by skin closed with a wound clip, before mice were returned to group housing. One likely explanation is that behaviors such as social grooming or scratching at the wound site could result in ongoing damage to the underlying tissue. While there was little gross deformity to the brain tissue under the craniotomy in these animals (Figure 1), this does not preclude a more subtle insult from damaging serotonin axons. Another possibility is that temperature dysregulation as a result of the craniotomy, which can lower brain temperature as much as 10°C below core body temperature (Kalmbach and Waters, 2012) leads to damage of serotonin axons. In addition,

this finding reinforces the importance of considering the craniotomy itself as an aspect of the injury when interpreting results from CCI experiments.

When control treated mice (which did not receive craniotomies) were killed either one week, one month, or three months after no-surgery sham treatment, the density of neocortical serotonin axons was stable (Figure 5). This finding replicates previous reports using serotonin immunohistochemistry (O'Hearn et al., 1988; Molliver et al., 1990; Mamounas et al., 2000), SERT immunohistochemistry (Jin et al., 2016), or in vivo two-photon observation of serotonin axons in SERT-EGFP BAC transgenic mice (Jin et al., 2016). Importantly, when serotonin axon density was measured in layer 1 of the posterior region, significant recovery was seen at one month and three months after injury when compared with the one week time-point, indicating that serotonin axon regrowth occurred (Figures 4 and 5). This is consistent with previous reports of damage and regrowth of neocortical serotonin axons following thermal lesion (Hawthorne et al., 2011), amphetamine lesion (O'Hearn et al., 1988; Molliver et al., 1990; Mamounas et al., 2000; Jin et al., 2016) and a neocortical stab injury (Jin et al., 2016). Thus, it appears that serotonin axons have an unusual capacity for regrowth and that this is evident following four different forms of brain injury.

While there was significant regrowth of serotonin axons over the period from one week after CCI to one month after CCI, there was no further significant increase in serotonin axon density in the period between one month and three months after CCI (Figure 5). There are two potential explanations for this finding (that are not mutually exclusive). The first is that there is a physiological limit to the amount of regrowth that serotonin axons are capable of after injury and that this limit is reached by or soon after the one month time point. The second is that the plateau in regrowth between one month and three months reflects a dynamic equilibrium between two effects: serotonin axon regrowth and progressive atrophy of surviving or new serotonin axons. It has been shown that TBI is a progressive degenerative process, with neuronal death persisting at least one year following a traumatic brain injury in rat (Smith et al., 1997). Therefore it is possible that secondary effects of injury are masking continuing regrowth between one and three months after injury.

We believe that SERT immunohistochemistry is an appropriate indicator of serotonin axon density. However, it is possible that the apparent reduction in serotonin axon density measured in this manner could result from attenuation of SERT expression without the loss of serotonin axon structure. Indeed, it was recently reported that SERT expression decreases seven days after controlled cortical impact injury in mice as measured by mRNA expression via real-time PCR and protein levels via western blotting (Abe et al., 2016). In this way, serotonin axons might be intact but their levels of SERT expression would be below the detectable limits of immunohistochemistry. To address this concern, we obtained adult SERT-Cre BAC transgenic mice and injected them in the rostral raphe with AAV5-EF1a-DIO-hChR2(H134R)-EYFP-WPRE-pA. Thus, a subset of serotonergic neurons were infected and they expressed EYFP under the control of the strong viral EF1a promoter, not the SERT promoter. Importantly, CCI produced a strong reduction in the level of both EYFP and SERT immunofluorescence when measured one week later, posterior to the site of CCI injury (Figure 6). This argues against the hypothesis that apparent serotonin axon degradation was merely an artifact of reduced SERT expression and is consistent with a

previously published result in which similar EYFP-expressing serotonin axons in the neocortex were degenerated by amphetamine treatment (Jin et al., 2016)

Injured brain tissue typically undergoes an inflammatory response and this is often accompanied by activation of astroglia (Eng and Ghirnikar, 1994) and recruitment of microglia (Davalos et al., 2005; Loane and Kumar, 2016). We measured activated astrocytes and microglia with GFAP and Iba1 immunohistochemistry, respectively. Although microglial density was not significantly altered at any time point after either form of TBI, CCI resulted in increased microglial somatic area. These results were consistent in tissue both anterior and posterior to the injury site (Figures 9 and 10). We also observed strong activation of astrocytes throughout layers 1–4, as indexed by GFAP immunoreactivity, one week after both open skull injury and CCI (Figure 7 and 8). Importantly, these reactive astrocytes were found both anterior and posterior to the injury site. The density of activated astrocytes following both forms of TBI was reduced one month after injury and further reduced at three months after injury.

It is useful to note that the presence of the astrocyte response did not predict the level of serotonin axon loss measured one week after injury. While both anterior and posterior sites showed strong astrocyte activation, only the region posterior to the injury showed loss of serotonin axon density. Furthermore, when analyzed on an animal-by-animal basis, the degree of astrocyte activation was not correlated with the degree of serotonin axon loss in mice that received TBI treatment (Figure 8C). It is clear that in these TBI models, astrocyte activation is not sufficient to produce serotonin axon loss. Whether astrocyte activation is necessary but not sufficient or, alternatively, is entirely unrelated to serotonin axon loss cannot be determined by the present experiments.

There are several caveats and limitations to the present findings that should be stated. First, the parameters used to guide the impactor in the CCI protocol (depth: 0.75 mm past the measured surface of the dura; final velocity: 4 m/s; impactor diameter: 1.5 mm) were chosen to produce a delimited area of damage that, by visual inspection, did not involve the deeper layers of the neocortex or subcortical structures (Figure 1B). It is possible that a more severe injury that would sever serotonin axons in all cortical layers and the underlying white matter, and would produce a greater and more widespread loss of serotonin axons in the region posterior (distal) to the injury site. It is also possible that the degree and timing of regrowth following injury might be different with a more severe insult.

Second, the present results, which rely upon analysis of fixed tissue and hence between-animal comparisons, cannot resolve the cellular dynamic details of regrowth. For example, it is unclear whether the recovery of serotonin axon density seen one and three months after TBI results from regeneration (growth from damaged serotonin axons) or sprouting (growth originating from neighboring undamaged serotonin axons; definitions from Tuszynski and Steward, 2012) or both phenomena. If there is regeneration, it could involve growth from the severed ends of serotonin axons (as has been reported following neocortical stab injury; Jin et al., 2016) or growth originating at more proximal sites that eventually extend to innervate the region of interest distal to the injury site. These important distinctions could be revealed by *in vivo* two-photon imaging of serotonin axons.

Third, because the labeling of SERT positive axons is dense, the individual trajectories of regrowing serotonin axons cannot be resolved. For example, we cannot determine whether regrowing axons that contribute to recovery after TBI are coursing through the damaged tissue underlying the craniotomy or are routed around this zone. By comparison, following neocortical stab injury, which produces a narrow rift in tissue, some regenerating serotonin axons were observed to completely traverse the rift that was filled with GFAP-positive activated astrocytes (Jin et al., 2016). It has also been observed in raphe spinal serotonergic axons that regenerating axons can sometimes pass through GFAP-positive tissue bridges that form following complete spinal transection (Lee et al., 2010).

Fourth, while the degree of astrocyte activation measured one week after TBI does not predict the degree of serotonin axon loss at this same time point (Figure 8C), it remains unclear if this early astrocyte activation is correlated with the speed or extent of serotonin axon regrowth in the weeks and months to follow. This would require within-animal comparisons as could be accomplished with two color *in vivo* two-photon microscopy in the appropriate GFAP/SERT double transgenic mice.

Fifth, it remains to be seen whether the serotonin axons that regrow following TBI are functional. While technically difficult, it is possible to measure serotonin release in mouse neocortex using fast-scan cyclic voltammetry when evoked by electrical stimulation of the medial forebrain bundle (Wood and Hashemi, 2013). This approach was used to show that the serotonin axons which regenerated following an amphetamine lesion were in fact competent to release serotonin (Jin et al., 2016).

Finally, we do not know if all serotonin neurons that project to the neocortex have the same capacity to regrow. Results from two-photo *in vivo* imaging of serotonin fiber regeneration following amphetamine lesion showed that serotonin fibers do not regrow in the exact same locations as fibers used to be, suggesting that the original innervation pattern does not need to be replicated for functional recovery (Jin et al., 2016). Developmentally, all of the dorsal raphe neurons originate from rhombomere 1, while the median raphe contains neurons originating from rhombomeres 1, 2, and 3 (Jensen et al., 2008) and it has been recently shown that serotonin neurons have rhombomere lineage-specific gene expression patterns (Okaty et al., 2015). These gene expression patterns are likely to confer subtype-specific functions, which could involve aspects of intrinsic regenerative capacity. Labeling of different serotonin neuron populations with these intersectional genetic strategies would help determine whether certain groups of neurons are preferentially susceptible to damage and/or have a particular ability regrow thereafter.

The main findings herein, that serotonin axons are damaged by TBI and are able to regrow following injury provides a potentially important insight into the potential biological basis behind certain outcomes secondary to TBI, such as depression. Loss of serotonin axons reinforces the importance of treating TBI as a disease process rather than a distinct injury event; even if the immediate effects of an injury resolve, there is the potential for continued dysfunction of this major neuromodulatory system in the neocortex. In the future, it may be useful to develop therapies to increase the rate or degree of serotonin axon regrowth.

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Significance Statement

Traumatic brain injury (TBI) is a prevalent medical problem that can result in long-term neurological and psychiatric disease, including depression. Little is known about how the brain recovers from TBI. Here, we have produced TBI in adult mice and have shown that nerve fibers that use the mood-regulating neurotransmitter serotonin are damaged by this injury, but then regrow within weeks thereafter. Ultimately, understanding how serotonin-using nerve fibers regrow may help us develop therapies to boost recovery of function after TBI.

Figure 1. Serotonin axon innervation of the adult mouse brain includes axons that run in the anterior-to-posterior direction in the superficial layers of the dorsal neocortex

A) Serotonin cell bodies are located in a diverse set of nuclei. The axons of the rostral serotonergic nuclei, including the median raphe (MnR) and dorsal raphe (DR), project throughout the forebrain innervating regions including the neocortex, nucleus accumbens (ac), amygdala (amg), hypothalamus (hyp), olfactory bulb (ob), striatum (str), and thalamus (th). Serotonergic nuclei of the caudal group, including the medullary reticular nuclei (MrN), raphe magnus (RMg), raphe obscurus (ROb) and raphe pallidus (RPa) project to the cerebellum (cer), brainstem and spinal cord.

B) Low magnification exemplar images of sagittal sections reveal the extent of injury evoked by two forms of TBI in two exemplar mice per group. One week after surgery the damage inflicted by controlled cortical impact is apparent (bottom row). The open skull injury group (middle row) and control groups (top row) have largely intact neocortical regions.

Occasionally damage secondary to the open craniotomy can be observed in the open skull injury condition (middle row, right side, dorsal to the lateral ventricle). The white boxes show the regions for analysis of tissue flanking the injury site.

Figure 2. Exemplar images show serotonin axon innervation in the neocortex, anterior (proximal) to the craniotomy site, measured one week after two different forms of TBI or control treatment

Maximally projected 40x confocal z-stacks with thresholded serotonin transporter (SERT, green) signal and NeuN signal (red) to show neuronal nuclei and thereby identify cortical layer boundaries reveal a small decrease in the density of serotonin axon innervation in the open skull injury (middle) and CCI groups (right) compared to the control group (left). Damage due to impact can be appreciated in the CCI group by the deterioration of the NeuN signal. These, and all of the images that follow, are sagittal sections.

Figure 3.

Exemplar images show serotonin axon innervation in the neocortex, posterior (distal) to the craniotomy site, measured one week after two different forms of TBI or control treatment. Note that both the open skull injury and CCI groups show reduced serotonin fiber innervation and that this reduction is most apparent in layer 1.

Figure 4. Exemplar images show sustained recovery of serotonin axon density following CCI treatment in neocortical layer 1, posterior to the CCI injury site
Following profound serotonin axon loss one week after CCI injury, serotonin axon density recovered when measured one month afterwards, and this recovery was sustained for at least three months after injury.

Figure 5. Quantification of serotonin axon density after two forms of TBI or control treatment. Percent of SERT-positive pixels above threshold were calculated to quantify serotonin axon density. These group data were normalized to control levels for each layer and time point

A) Anterior (proximal) to the injury site. There was no statistically significant axon loss following treatment one week post-injury, and no further change was noted one month and three months post-injury.

B) Posterior (distal) to the injury site. There was statistically significant serotonin axon loss following two forms of TBI treatment one week after surgery and this was most clearly evident in layer 1. Significant regrowth was evident in the CCI group in layer 1 at one month and three months compared to one week after injury. Significance is denoted as follows: * indicates $p < 0.0167$ (Bonferroni corrected threshold: $0.05/3$), ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

Figure 6. CCI treatment produces loss of serotonin axons that express EYFP under the control of strong viral promoter, arguing against SERT protein down-regulation as an explanation for apparent serotonin axon loss

SERT-Cre mice were injected with AAV5-EF1a-DIO-hChR2(H134R)-EYFP-WPRE-pA to label infected serotonergic neurons in the rostral raphe with membrane-associated EYFP under the control of the EF1a promoter. EYFP immunofluorescence is rendered in green and SERT immunofluorescence in red. In the top panels, the EYFP-positive (green) axons derived from raphe cell bodies that were infected with the virus are shown in control tissue and one week post-surgery. The bottom panel shows the same images with SERT staining (red) overlapping the subpopulation of EYFP-positive axons. Note that CCI treatment reduced the density of both SERT-positive and SERT-EYFP double-positive axons when measured in layers 1 and 2/3 one week after injury.

Figure 7. Exemplar images show reactive astrogliosis accompanying two forms of TBI treatment Immunohistochemistry against GFAP reveals profound reactive astrogliosis across all neocortical layers in both open skull injury and CCI groups compared to control tissue when measured one week after injury. The degree of reactive astrogliosis is largely, but not completely, returned to control values when measured three months after injury. **A)** Anterior to the injury site. Note astroglial endfeet outlining penetrating arterioles in open skull injury and CCI images. **B)** Posterior to the injury site. Note that anterior and posterior sites showed similar reactive astrogliosis.

Figure 8. Quantification of reactive astrogliosis accompanying two forms of TBI treatment

A) Anterior to the injury site. The number of reactive astrocytes in both open skull injury and CCI groups is greatly increased one week following treatment. This increase, which spans cortical layers 1–6, is transient as there is a significant decrease in the number of reactive astrocytes when measured one month and three months after surgery.

B) Posterior to the injury site. There is a significant increase in reactive astrocyte density across all layers of neocortex when measured one week post-surgery in the open skull injury and CCI groups. The astrogliosis begins to significantly resolve at one month, and there is no difference between treatment groups in any layer at three months post-surgery.

Significance is denoted as follows: * indicates $p < 0.0167$ (Bonferroni corrected threshold: 0.05/3), ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

C) Scatterplot shows that there is no correlation between the degree of reactive astrogliosis and serotonin axon density measured one week after injury in the two TBI groups. Each plot point is from a single layer, anterior or posterior to the injury site.

Figure 9. Exemplar images of microglial density and morphology accompanying two forms of TBI treatment

Iba1 immunohistochemistry was used to visualize microglia one week post-surgery. There was no difference in microglial density accompanying two forms of TBI treatment, but CCI treatment resulted in increased microglial soma size.

Figure 10. Quantification of microglial morphology accompanying two forms of TBI treatment reveals changes in soma size

Microglia soma size, an index of microglial condensation response, is increased one week post-injury in the CCI group compared to open skull injury or control treatment. There is no longer a microglial condensation in the CCI group at one or three months post-surgery. However, one month post-surgery, soma size in the CCI group is significantly smaller than in controls anterior to injury. Three months post-surgery, soma size in the open skull injury group is significantly smaller than in the control treatment posterior to the injury site. Significance is denoted as follows: * indicates $p < \text{Bonferroni corrected threshold}$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$.