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Does time heal all wounds? Experimental diffuse traumatic brain injury results in persisting histopathology in the thalamus

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Abstract

Background—Thalamic dysfunction has been implicated in overall chronic neurological dysfunction after traumatic brain injury (TBI), however little is known about the underlying histopathology. In experimental diffuse TBI (dTBI), we hypothesize that persisting histopathological changes in the ventral posteromedial (VPM) nucleus of the thalamus is indicative of progressive circuit reorganization. Since circuit reorganization in the VPM impacts the whisker sensory system, the histopathology could explain the development of hypersensitivity to whisker stimulation by 28 days post-injury; similar to light and sound hypersensitivity in human TBI survivors.

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Methods—Adult, male Sprague-Dawley rats underwent craniotomy and midline fluid percussion injury (FPI) (moderate severity; 1.8–2.0 atm) or sham surgery. At 1d, 7d, and 28 days post-FPI (d FPI) separate experiments confirmed the cytoarchitecture (Giemsa stain) and evaluated neuropathology (silver stain), activated astrocytes (GFAP), neuron morphology (Golgi stain) and microglial morphology (Iba-1) in the VPM.

Results—Cytoarchitecture was unchanged throughout the time course, similar to previously published data; however, neuropathology and astrocyte activation were significantly increased at 7d and 28d and activated microglia were present at all time points. Neuron morphology was dynamic over the time course with decreased dendritic complexity (fewer branch points; decreased length of processes) at 7d FPI and return to sham values by 28d FPI.

Conclusions—These data indicate that dTBI results in persisting thalamic histopathology out to a chronic time point. While these changes can be indicative of either adaptive (recovery) or maladaptive (neurological dysfunction) circuit reorganization, they also provide a potential mechanism by which maladaptive circuit reorganization could contribute to the development of chronic neurological dysfunction. Understanding the processes that mediate circuit reorganization is critical to the development of future therapies for TBI patients.

Keywords

Traumatic brain injury; post-concussive symptoms; thalamus; whisker-circuit; somatosensory

1.0 Introduction

Recently, the athletic and military communities have highlighted the long-lasting impact of mild traumatic brain injuries (TBI) ¹. According to Centers for Disease Control, 2.5 million TBIs occur in the United States annually and are a major source of death and permanent disability². Seventy-five percent of the TBIs suffered can be categorized as mild diffuse axonal injuries or concussions ^{3,4}. These injuries result from shearing forces leading to axotomy, vascular permeability and inflammation that progress towards neural circuit disconnection and dysfunction ^{5–8}. The consequences of the mechanical damage include both degenerative and regenerative neuronal processes that alter circuit structure and consequently its function ^{9,10}. Regenerative efforts can overcome injury-induced damage to some extent, however, uncoordinated regenerative responses likely lead to maladaptive circuit reorganization underlying clinical morbidity ¹¹.

While most who suffer from mild TBI recover without residual complications, an estimated 15–20% of patients have post-concussive symptoms (PCS) lasting at least 1-month post-injury or longer. Alternatively, symptoms may not develop until weeks after the injury and then can persist for months to years ^{12–15}. The true incidence of PCS is unknown, as many mild TBI patients do not seek care for their injury, and therefore would not identify the TBI event as the etiology of their symptoms^{1,3}. PCS can broadly include symptoms of cognition (fatigue, foggy, memory, concentration, cognitive slowing), emotion (depression, irritability, anxiety) and sensation (headaches, visual disturbances, dizziness, photo/phonophobia, nausea, vomiting, balance disturbances, numbness/tingling, sleep disturbances) ^{15–19}.

The range of PCS reflects the diffuse nature of the injury and its impact on multiple brain regions and circuits^{20–22}. The thalamus plays an important role as a relay for multiple cortical pathways and TBI-induced thalamic damage has been associated with post-concussive headache, hyperesthesia, fatigue and cognitive deficits^{23–30}. Specialized imaging studies, which may be more sensitive to pathophysiological changes after mTBI, such as diffusion-weighted imaging, diffusion tensor imaging, functional magnetic resonance imaging and spectroscopy, have demonstrated changes in thalamic metabolism, decreased tissue volume, perfusion alterations, microstructural injury, and changes in connectivity after TBI^{4,25,31–33}. Thalamic susceptibility to damage after mTBI is thought to be due to high sheer forces upon the axons of the neural circuitry that relay through this structure³⁴. While it is postulated that these structural changes contribute to the persistence in PCS, no translational behavioral or clinical diagnostic imaging experiments have evaluated the impact of diffuse axonal injury on the development of PCS or potential changes in thalamic histology over an extended time course^{35,36}.

A few experimental models of CNS injury have implicated thalamic pathology and dysfunction in the development of post-injury behavioral deficits. In a series of experiments, spinal cord injury has shown hyperesthesia associated with increased spontaneous and sensory-evoked activity, reactive microglia and activated astrocytes in the thalamus^{37–40}. In this model, the chronic inflammatory response to the spinal cord injury was speculated to be associated with ongoing neurodegeneration in the thalamus as indicated by a significant loss of thalamic neurons at 10 weeks post-injury³⁹. Evidence from rodent models of central pain syndrome after stroke have also suggested that the etiology of central pain is secondary to thalamic hyperactivity and altered connectivity⁴¹.

Similarly, 3–4 weeks after a mild-moderate midline fluid percussion injury (FPI), rodents develop a late-onset gain-of-function hypersensitivity to whisker stimulation, which persists to at least 56 days post-FPI^{42,43}. This hypersensitivity to whisker stimulation is mediated through a glutamatergic circuit in rodents that connects the somatosensory ventral posteromedial (VPM) thalamic nucleus to the barrel fields of the primary somatosensory cortex (S1BF) and corresponds with the onset of hypersensitive presynaptic glutamate release, increased regional activation in response to whisker stimulation and increased microglial activation at 7 and 28 d FPI in the VPM and S1BF^{42–53}. Sensory hypersensitivity in this model is similar to photophobia and phonophobia experienced by human TBI survivors^{54–57}.

The emerging diffuse traumatic brain injury (dTBI)-induced late-onset sensory hypersensitivity is indicative of altered whisker circuit connectivity, however little is known about the underlying histopathology in the VPM after dTBI^{42–44,58–60}. Lifshitz et al. (2007) and Povlishock et al. (1992) found that FPI results in axotomy and neuronal atrophy, but no overt cell death in the VPM^{49,58}. This is then followed by an expanded cFos staining, indicating broader distribution of whisker stimulation-evoked neuronal activation at 4–6 weeks post-injury⁶¹. However, it is unknown if these plastic events are a result of adaptive circuit reorganization (recovery) or maladaptive circuit reorganization (morbidity).

Readily available and rapid forms of imaging most commonly used in clinical facilities, such as computer tomography and magnetic resonance imaging, often do not demonstrate evidence of injury with mild TBI nor can they distinguish microscopic pathology⁶². In this study, we test the hypothesis that experimental dTBI results in persisting histopathological changes in VPM. After experimental dTBI using midline fluid percussion injury (mFPI), we will confirm consistent cytoarchitecture (Giemsa) and evaluate neuropathology (silver stain), astrocytic activation (GFAP), neuron morphology (Golgi stain) and microglial morphology (Iba-1) at 1d, 7d and 28d days post-FPI.

2.0 Methods

2.1 Diffuse brain injury: Midline fluid percussion injury

Male Sprague-Dawley rats (300–350 g; Harlan, IN) were allowed access to food and water *ad libitum*, with all experiments and animal care conducted in accordance with National Institutes of Health and Institutional Animal Care and Use Committee approved protocol. Female rats were not used due to the variation in estrus cycle that may confound data. Rats were subjected to midline FPI as previously described resulting in bilateral diffuse axonal injury without overt tissue destruction^{42,63–68}. Briefly, rats were anesthetized with 5% isoflurane, transferred to a stereotaxic frame (Kopf Instrument, Tujunga, CA), and maintained at 2% isoflurane via a nose cone for the duration of the surgery. During surgery, body temperature was maintained at 37°C with an isothermal heating pad (Braintree Scientific). A midline scalp incision exposed the skull and a 4.8-mm circular craniotomy was performed (centered on the sagittal suture midway between bregma and lambda) without disrupting the underlying dura or superior sagittal sinus. An injury cap was fabricated from the female portion of a Leur-Loc needle hub. A skull screw was secured in a 1-mm hand-drilled hole into the right frontal bone. The injury hub was affixed over the craniotomy using cyanoacrylate gel and methylmethacrylate (Hygenic Corp., Akron, OH) was applied around the injury hub and screw. The incision was sutured closed over the hub and rats were then returned to a warmed holding cage and monitored until ambulation returned.

For the injury induction, animals were re-anesthetized with 5% isoflurane approximately 60–90 min after the conclusion of the surgical procedure. The female end of the injury hub assembly flushed with normal saline and attached to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). Upon the return of the pedal withdrawal reflex, an injury of moderate severity (1.8–2.0 atm) was administered by releasing the pendulum onto the fluid-filled piston. Sham animals were connected to the FPI device, but the pendulum was not released. After injury or sham procedure, the injury hub assembly was removed *en bloc*, the craniotomy was inspected and the incision was sutured. Animals were monitored for changes in respiration, the fencing response and the return of the righting reflex⁶⁶. A mild-moderate severity brain injury was determined by a righting reflex recovery time of 5–10 mins. Sham animals recovered from anesthesia within 15 seconds. Wounds were covered with topical lidocaine and bacitracin antimicrobial creams. After recovery of the righting reflex, animals were placed in a warmed holding cage before being returned to their home cage.

2.2 Histological and Immunohistochemical Tissue Preparation

At 1, 7, or 28 days post-injury (d FPI) or sham-injury rats were over-dosed with sodium pentobarbital (200 mg/kg, i.p.), decapitated, and the brain tissue rapidly removed and then prepared in one of four ways. For Giemsa staining, the brains were removed and blocked in the coronal plane (12mm extent in the rostral-caudal plane) and paraffin processed⁴⁹. For Aminocupric silver staining, rats were transcardially perfused with phosphate buffered saline (PBS), followed by a fixative solution containing 4% paraformaldehyde. Brains were then shipped to Neuroscience Associates Inc. (Knoxville, TN). Data has been previously published for other brain regions^{51,69–71}. For GFAP immunohistochemistry, rats were perfused with 4% paraformaldehyde after a PBS flush, then cryoprotected in graded sucrose solutions (15%, 30%) and frozen in optimal cutting temperature compound (Fisher Scientific, #14-373-65). Data have been previously published for other brain regions^{70,71}. Brains were stored at -80°C until cryosectioned at 20 μm . Lastly, brain tissue for Golgi analysis was collected fresh according to the instructions provided by the manufacturer and placed in solutions provided in the FD Rapid GolgiStain Kit (FD NeuroTechnologies, #PK401). The brain was rinsed with ddH₂O, grossly dissected into 3 pieces (anterior to bregma, posterior to bregma, and cerebellum).

Images of all staining were captured on a Zeiss Imager A2 with attached digital camera with the exception of the Golgi stained tissue. Quantified images were taken from either hemisphere, based on the quality of the section mounted (no folds or tearing). All quantified measurements were made in comparison to sham, where images were also taken from both hemispheres. At least one uninjured sham animal was prepared for each time point. Details for each stain from previously published methods are highlighted below.

Giemsa Stain—Sections were cut 30 μm in the coronal plane and mounted on 2% gelatin-subbed slides. Slides were heated, deparaffinized, rehydrated, stained with 10% Giemsa stain (EM Sciences, #26156-01) at 60°C , differentiated with 1% acetic acid, dehydrated and cover-slipped with DPX mounting medium (Fisher, #NC9092474).

Aminocupric Silver Staining and Analysis—Argyrophilic reaction was examined using the de Olmos aminocupric silver histochemical technique as previously described^{65,72,73}. Sham ($n = 3$) or brain-injured rats ($n = 3$ per time point) were embedded into a single gelatin block (Multiblock Technology; Neuroscience Associates), sectioned at 40 μm and stained with the de Olmos aminocupric silver technique according to proprietary protocols (Neuroscience Associates), and cover-slipped. Quantification was measured in the VPM by calculating the percentage of area stained black using ImageJ software⁷⁴. The VPM was identified in coronal sections based on topographical localization to the hippocampus and medullary lamina (AP: -3.0 – -4.0 ; ML: ± 3 ; DV: -6.0 – -7.0). Eight images were analyzed per animal, with the average pixel density (per animal) compared to sham using a one-way ANOVA with Tukey's post-hoc comparison. Data are shown as mean $\pm$ SEM, significance indicates a $p < 0.5$. The same set of silver stained brains have also been used to assess neuropathology in the principal trigeminal nuclei (PrV) of the brainstem, S1BF of the cortex, basolateral amygdala and substantia nigra^{51,69,71,75}.

GFAP Staining and Analysis—Coronal brain tissue sections were immunostained for Glial Fibrillary Acidic Protein (GFAP) (rabbit anti-GFAP, 1:1000; Dako, Carpinteria, CA, catalog# Z0334) using a biotinylated horse anti-rabbit IgG secondary (biotinylated horse anti-rabbit, 1:250; Vector Laboratories, Burlingame, CA; Catalog# BA-1100). Sections were then washed and placed in ABC elite (Vector) and developed with nickel enhanced diaminobenzidine (Vector). Sections were then dehydrated in ethanol, cleared in Citrisolv and cover slipped with DPX mounting media. Four brains were analyzed per time point in comparison to sham, with a total of six sections per brain being analyzed per time point. Images were taken with a Zeiss ImagerA2 microscope, with an AxioCam mRc5 camera using Zeiss microscope software Zen 2011.

Changes in GFAP staining over time post-injury were quantified in the VPM using Pixel Density Analysis, identical to densitometric analysis in the silver stained tissue^{70,71,74}. For each rat (n=4–6/time point; at least 1 sham at each time point), 4–8 representative images from the VPM were collected at 20x magnification and analyzed using Image J software, identical to previously published methods^{70,71}. Pixel density following mFPI was compared to sham. Data were analyzed by one-way ANOVA with Tukey's post-hoc comparison. Data are shown as mean $\pm$ SEM, significance indicates a $p < 0.05$.

Golgi Stained Neurons and Analysis—Changes in VPM neuron morphology of Golgi stained tissue were assessed over time post-injury using Neurolucida software (MicroBrightField, Inc., Colchester, VT)^{70,71}. Neurons were traced by an investigator blind to injury status. All neurons chosen for reconstruction had an intact soma and absence of unnatural truncations or ends. We defined the natural end as the termination of a completely tapered dendrite which can be identified by an end swelling, spine or spine cluster or gradual narrowing. All neurons and soma were traced at 40x magnification, tracing the boundaries of each cell soma, following the predicted contour of the cell soma and then all dendrites emerging from said soma to their full extent. Neurons were traced in three-dimensions (x, y, z planes).

Reconstructions were analyzed using NeuroExplorer software (MicroBrightField, Inc., Colchester, VT) to determine soma area, branch points, ends, dendrite length, surface area, and construct a Sholl analysis^{70,76}. The same characteristics were taken from the sham animals that served as the control population. Ten neurons were traced per animal and four animals were used for sham and each time point, resulting in a total of 160 traced neurons for 3D reconstruction.

The calculated areas for each neuronal structure including the soma, branch points, ends, dendrite length, and surface area for each animal were averaged over the time point and then compared and statistically analyzed by one-way ANOVA with a Tukey post-hoc comparisons compared groups to identify significant differences at $p < 0.05$. Sholl analysis was performed to evaluate the distribution of dendritic processes. A series of concentric spheres with increasing radius (10 μm) centered on the soma was used to assess the number of process crossings as a function of distance and space using NeuroExplorer software. Image z-stacks (1 μm increments) were created from the top-most process to the bottom-most process. The 3D reconstructions from Neurolucida software were inverted and

converted to black and white in Photoshop (Adobe CS6) prior to analysis. Sholl analysis was analyzed by two-way ANOVA with a Tukey's post-hoc test. Data are shown as mean $\pm$ SEM, significance indicates a $p < 0.05$.

Iba-1 staining—Brains ($n=3$ /time point) were embedded into a single gelatin block (Multiblock_ Technology; Neuroscience Associates). Cryosections containing all study brains were mounted and immunostained for ionized calcium binding adaptor molecule 1 (Iba-1), to identify all microglia. The immunostained slides were imaged using a Zeiss Imager A2 microscope with AxioCam MRc5 digital camera. Images were rotated 90 degrees to the left. These sections were obtained from the same brains processed for aminocupric silver staining above. Data from these Iba-1 stained sections have been published for this and other brain regions^{52,75,77,78}.

3.0 Results

3.1 Cytoarchitecture is preserved after dTBI

Previous studies using the same injury method have demonstrated acute membrane perturbation, perisomatic axotomy and protease activation across the thalamocortical whisker circuit^{49,79–81}. Despite this damage, this injury model continues to produce no overt hemorrhage, edema, contusion or cavitation at 1d, 7d and 28d FPI in the VPM of the thalamus (Figure 1).

Giemsa staining was used to confirm the cytoarchitecture of sham and injured brains in this study. Giemsa staining is a solution of methylene blue and eosin. The methylene blue component stains nuclear elements and the eosin non-specifically binds cytoplasmic and connective tissue components. Gross histopathology was unremarkable between sham and injured brains at a macroscopic level similar to previously published using stereological quantification (Figure 1)⁴⁹. The representative images are indicative of preserved cytoarchitecture across all time points.

3.2 Neuropathology identified by argyrophilic reaction increases in the first week without resolution by 28 days post-FPI

Silver staining is a marker of neuropathology and the stain is thought to permeate the membranes of injured neurons. While uninjured animals display virtually no silver staining, brain-injured animals demonstrated staining at all time points (Figure 2A). Pixel density, as defined by the area stained black, of sham animals was $1.45\% \pm 0.50$. Pixel density of injured animals at 1, 7, 28 d FPI were $8.96\% \pm 3.97$, $20.72\% \pm 2.51$, and $11.33\% \pm 0.79$, respectively. Pixel density analysis with one-way ANOVA with Tukey's post-hoc of silver staining identified increased pixel density at 7d and 28d FPI ($F(3, 8)=22.05$, $p=0.0003$) compared to the absence of staining in uninjured sham brain (Figure 2B).

3.3 Astrocyte activation indicated via density of Glial fibrillary acid protein (GFAP) staining in the VPM gradually increased over 28 days post-FPI

Glial fibrillary acidic protein (GFAP) is an astrocytic marker indicating activation. When an astrocyte is activated, GFAP levels increase and the astrocytic processes become thicker,

resulting in a greater level of staining. We assessed GFAP staining, defined as the percentage of area stained black, at 1, 7 and 28 days post-FPI (n=3–5) in comparison to sham (representative images in Figure 3A). Pixel analysis was used to quantify levels of GFAP staining. The average density of black pixilation in shams was $5.4\% \pm 1.50$. After injury, pixilation increased to $8.85\% \pm 2.13$ at 1d FPI, $15.42\% \pm 1.69$ at 7d FPI and 13.83 ± 0.84 at 28d FPI; a 3-fold increase compared to sham (Figure 3B). A one-way ANOVA revealed a significant increase in GFAP staining (pixilation) as a function of time post-injury ($F(3,12)=23.79$, $p<0.0001$). Post-hoc analysis indicated a significant increase in GFAP staining at 7 and 28d FPI in comparison to sham and 1d FPI. These data provide evidence of persistent astrocyte activation without resolution by 28d FPI.

3.4 Three-dimensional reconstruction of Golgi stained neurons indicate dynamic morphology after dTBI

Golgi stains both the neuronal cell body and its processes (Figure 4A). From this, neuron morphology was quantified using Neurolucida software for 3D reconstruction of Golgi stained neurons in the VPM (Figure 4B). The 3D reconstructions were used for quantification of total number of processes, branch points, total number of process ends, mean process length, and soma area (Figure 4C–4F). A Sholl analysis was used to assess changes in complexity of process structure over time. The average process quantity was increased to 8.665 ± 0.481 at 28d FPI in comparison to 6.669 ± 0.137 at 7d FPI; however, 1d and 7d FPI measures were similar to sham, implying that results at 28d FPI are changes in arbor complexity rather than loss of whole processes ($F(3, 12)=4.02$; $p=0.034$; Figure 4C). The number of branch points were significantly decreased to 16.08 ± 2.74 at 7d FPI, returning to levels similar to sham by 28d FPI (45.54 ± 1.83 ; $F(3, 12)=7.79$; $p=0.004$; Figure 4D). The number of process ends significantly changed over time ($F(3, 12)=9.08$; $p=0.002$). Post-hoc analysis revealed significantly decreased ends at 7d FPI 26.19 ± 4.07 in comparison to sham 52.88 ± 10.02 , regaining ends by 28d FPI (67.73 ± 4.07); reinforcing the trend seen with branch point numbers (Figure 4E). Mean process length was significantly decreased at 7d FPI (87.95 ± 5.96) in comparison to sham (185.60 ± 20.03), and significantly increased at 28d FPI (171.80 ± 21.41) in comparison to 7d FPI; supporting the interpretation of neuron recovery ($F(3, 12)=6.85$; $p=0.006$; Figure 4F). Soma area was significantly increased at 7d FPI (838.70 ± 76.45) compared to sham (604.8 ± 33.43) and 1d FPI (574.30 ± 27.73 ; $F(3, 12)=5.72$; $p=0.011$; Figure 4G). Sholl analysis revealed fewer intersections at 7d FPI in comparison to sham between 50 and 70 μm from the soma, as well as significant differences between 28d and 7d FPI between 140 μm and 170 μm from the soma (Figure 4H). Only sham and 28d FPI rats have any interactions beyond 110 μm ($F(25,78)=29.93$; $p<0.0001$). Taken together, the neuronal morphology primarily showed injury-induced reductions in dendritic arbors at 7d FPI, with measures returning to uninjured sham values by 28d post-FPI, despite the ongoing neuropathology. For some outcome measures, upto four-fold changes in morphology were measured between 7d and 28d FPI.

3.5 Evidence of early and prolonged activation of microglia through 28d FPI

Microglia are specialized glial cells that are the primary immune defense in the CNS and can play a role in synaptic plasticity. When microglia are exposed to degenerating or foreign materials, pro-inflammatory and/or anti-inflammatory factors, they undergo a rapid change

in morphology, indicative of their function. In sham animals, there is an even distribution of ramified microglia typical of microglial surveillance in uninjured tissue, characterized by elongated processes with multiple fine branches (Figure 5). At 1 DPI, the even distribution of microglia is disrupted, processes are shorter and there are fewer fine branches in comparison to sham, indicative of activated microglia. There are also the first indications of microglia with an elongated cell body and polarized processes, referred to as ‘rod’ microglia. At 7d FPI, the distribution of microglia is still uneven, with several activated microglia and increased evidence of rod microglia. At 28d FPI, the distribution of microglia is becoming more evenly distributed, however activated microglia, bushy or hyper-ramified microglia (multiple short branches, see top right corner of insert) and rod microglia are still present. The representative images are indicative of early activation of microglia that is unresolved by 28d FPI.

4.0 Discussion

These data support that experimental midline FPI, a model of mild diffuse TBI, results in persisting histopathology in the thalamus that is chronic in nature to at least 28d FPI, which has similarly been demonstrated in previous studies in other brain regions using this model^{49,65}. Despite the preservation of the tissue cytoarchitecture post-injury as demonstrated by the Giemsa staining, activated microglia at all time points and pixel density of silver and GFAP staining at 7d and 28d FPI remain significantly elevated indicating that post-injury processes remain unresolved. Altered neuronal morphology was demonstrated at 7d FPI with decreased mean process length and number of branch points. Sham morphology returns by 28d FPI which coincides with the development of increased glutamate signaling and whisker hypersensitivity as previously described⁴⁴. Taken together, these data support that chronic histopathology likely results in altered circuitry and may precipitate whisker hypersensitivity in rodents after TBI^{15,82}.

While neuronal death and neuronal counts were not quantified in this set of Giemsa stained tissue, the staining was similar to previously published work from our lab that carried out bilateral systematic-random quantification of neuronal number and neuronal density using design-based stereology. Those experiments indicated that the density of neurons was decreased at 7 days by ~16%, however the total estimated number of neurons were not significantly different⁴⁹. It is plausible that the glial response to injury could contribute to the changes in neuron density measured in the previous study, however, improved methods of glial quantification would be necessary to support this statement.

Neuropathology in the VPM was increased at 7 and 28d FPI, which likely results from neuronal damage as a result of primary and secondary traumatic injury processes in the trigeminothalamic and corticothalamic pathways. Previously, we have published neuropathological findings from the principal trigeminal nuclei (PrV) in the brainstem and S1BF relays of the whisker circuit, from the same set of silver stained brains processed through Neuroscience Associates^{51,69}. Miremami et. al. (2014) reported increased neuronal damage in the PrV at 7d and 28d FPI⁶⁹. In the S1BF, Lifshitz et al. (2012) reported significantly increased neuronal pathology that progressed deeper into the cortex over time; beginning at 1d and still present at 28d FPI⁶⁵. The argyrophilic reaction is preferential to

damaged neurons due to increased membrane permeability. It is also possible that neuropathology observed in the VPM is exacerbated as a result of disrupted local afferent and efferent signaling stability and prolonged inflammatory response from glial cells^{52,39,73,83}. Regardless, reactive compensatory processes for the presence of neuronal pathology would be expected to account for the loss of synapses and maintenance of circuit function.

In the presence of neuropathology, activated astrocytes can contribute to ongoing degenerative, protective, and regenerative processes^{84–86}. After CNS insult, astrocytes can be activated as a result of compromised blood brain barrier (BBB), disruption of extracellular homeostasis, abnormal calcium signaling cascades, atypical neurotransmission, the presence of inflammatory mediators, oxidative stress, ischemia, and metabolic toxins^{87–89}. GFAP, a cytoskeletal filament, is upregulated in activated astrocytes and is the most commonly used indicator of activated astrocytes in the literature^{90,91}. Activated astrocytes in the absence of a destructive lesion (glial scar) can function to protect neurons from injurious substances, aid in the repair of synapses, promote axonal outgrowth and protect the BBB⁸⁸. Persistent activation of astrocytes at 28d FPI supports unresolved disruption in normal neuronal homeostasis, likely relevant to the unresolved neuropathology. Trophic factors secreted by activate astrocytes could promote neuronal growth and synaptic connectivity leading to either adaptive or maladaptive circuit reorganization. A longer time course would be required to further define the beneficial or detrimental relevance of persisting activation of astrocytes after dTBI in the thalamus, especially in regards to its contribution to synapse formation, ongoing post-traumatic circuit reorganization, and the development of chronic morbidity^{92–97}.

Neuropathology in the presence of a preserved cytoarchitecture in the VPM suggests that this model does not result in overt cell death, but rather substantial structural changes to neurons that could impact circuit function^{49,58}. In this study, we have identified changes in neuron morphology in the VPM over time post-injury with respect to the presence of circuit-wide histopathology. The initial decrease in neuronal complexity over the first week after injury, signified by the decrease in branch points, branch ends and mean process length, suggests an initial injury effect and is paralleled by peak levels of silver staining and activation of astrocytes at 7d FPI. This period is then speculated to be followed by regenerative and reparative processes resulting in increased neuronal complexity measured at 28d FPI, similar to sham, noted by the increase in process quantity, branch points, branch ends and mean process length.

However, values returning to sham levels at 28d FPI however do not necessarily imply that pre-injury functionality or connectivity has been restored (e.g. adaptive circuit reorganization). To the contrary, the lack of significant differences quantitative values of neuronal complexity between 28d FPI and sham animals supports the possibility that maladaptive circuit reorganization has occurred as these data coincide with the onset of behavioral morbidity, changes in presynaptic glutamate release and broadened distribution of cFos activated cells after whisker stimulation, indicating that while the length and branch points returned to sham levels, connectivity and function significantly changed^{42,44,61}. This regenerative phase is concomitant with astrocytosis. Trophic factors secreted by activated

astrocytes, therefore, may play a role in adaptive or maladaptive circuit reorganization after dTBI or other neuronal insult processes^{98–100}.

An initial increase in the average neuron soma area at 7d FPI then returns to sham size by 28d FPI, similar to previous findings in the hippocampus at 28 days post-focal injury in juvenile rats⁷⁶. An increase in average soma area is an indication of increased metabolic or transcriptional demands of neurons preparing to undergo growth, perhaps as compensation for the damaged or dysfunctional neurons^{101,102}. Carmichael et. al (2006) found similar findings in stroke when there was initial spine turnover followed by a period of regeneration, indicated by increased axonal growth and plasticity¹⁰³. More studies are necessary to confirm that increased soma area directly corresponds with predictable growth after injury.

The increase in soma area at 7d FPI that returns to sham size by 28d FPI in this study contradicts previously published data from our lab supporting a shift of the injured neurons toward smaller nuclear volumes in the ventral basal complex (which houses the VPM) at 1, 7 and 28 days after mFPI⁴⁹. Neuronal nuclear volumes are indicative of neuronal somatic volume in neurodegenerative diseases^{104–106}. The methods for assessing soma size were different between studies, with tracing of the cell soma at the largest diameter within the plane was used in the present experiments and a vertical nucleator was used in the previous study. However, in the previous study, the vertical nucleator was used on approximately 284 neurons per animal, providing greater power with this sample size in comparison to the sample size in the present study (10 neurons per animal). The difference in these data sets may also result from bias due to our neuron selection criterion. Many of the neurons in the VPM that stained for Golgi were observed to be grouped together with multiple processes intertwining with adjacent neurons to form fiber bundles. Therefore, it cannot be ruled out that our neuron selection process, requiring isolated neurons, resulted in choosing neurons that had processes damaged during injury, increasing the probability of the neuron being isolated. This bias, if legitimate, would make it tempting to speculate that the Golgi method and selection criterion had a higher probability for identifying injured neurons surviving 1-week post-injury and subsequent hypertrophy of the soma indicating growth, which could contribute to circuit reorganization.

The dynamic morphological changes in the VPM after injury could also be the result of deafferentation of afferent and efferent signaling from the PrV and S1BF, resulting in reorganization of neurons within the circuit to compensate for these changes. It is also possible that localized depolarizations, secondary to ionic membrane imbalances and excitatory neurotransmission after TBI promote neuroplasticity, similar to synaptic pruning in viable surviving neurons after an ischemic event^{107,108,109}.

There are few studies of reconstructed neurons in the VPM of rats. Golgi stained VPM neurons identified in these experiments were chosen based on location and not further classified into subtypes. These neurons contained approximately 4–9 primary dendrites with arbors that less frequently extended beyond 180 μm from the soma as previously observed by Chiaia et al. (1991)¹¹⁰.

Activated microglia are evident at each time point post-FPI, in agreement with what we have previously published⁵². Microglia can play a significant role in synapse formation and removal by secretion of synaptogenic molecules and pruning of ineffectual synapses in the ramified and activated states. In addition to activated microglia, rod microglia were observed at each time point, with the most observed at 7d FPI. These rod microglia have been demonstrated to parallel neuronal elements and are reported to be in highest numbers in the S1BF at time points prior to the evolution of aberrant behaviors, suggesting these microglia may play a role in synaptic plasticity and circuit reorganization^{52,92–97,111,112}. Additional studies are required to address the role of microglia in synaptic pruning after diffuse TBI.

Persisting pathology and changes in neuron morphology in this dTBI model parallel functional and behavioral changes. Previous studies indicate increased basal and potassium-evoked pre-synaptic glutamate release in the VPM in comparison to sham and 7d FPI rats, without evidence of changes in glutamate uptake rates⁴⁴. The amount of evoked glutamate release positively correlated with the severity of whisker hypersensitivity at 28d FPI, supporting a role for abnormal glutamate neurotransmission in the onset of whisker hypersensitivity after injury^{42,44}. Abnormal glutamate neurotransmission, concomitant with changes in neuron morphology, provide a mechanism by which greater areas of activation within the VPM and S1BF are induced by whisker stimulation at 28d FPI⁵³. Taken together with the persisting neuronal pathology and activation of astrocytes and microglia, these structural and functional changes in the VPM over time could potentially have significant implications for neuronal function, circuit reorganization and the development of late-onset hypersensitivity to whisker stimulation in our animal model^{42–44,58–60}.

Conclusion

The current communication builds on our previous studies describing the consequences of diffuse TBI on the whisker somatosensory circuit. The pathology resulting from moderate severity diffuse TBI is not resolved by 28 days post-FPI, indicating that the aftermath of trauma, rather than the traumatic event itself, promotes persisting and potentially late-onset morbidity. Additionally, persisting activated astrocytes and microglia may be a source of molecules that support morphological changes in neurons and could contribute to ongoing adaptive and maladaptive circuit reorganization. Clinically, as imaging techniques advance and are readily available, identification of the equivalent to these histological findings could aid clinicians in diagnosing mild TBI and predicting its long-term functional consequences. Understanding the mechanism by which circuit reorganization results from the pathophysiology of dTBI is critical to the development of prophylactic therapies as a novel means of mitigating the wounds that may not have necessarily healed by time alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

dTBI	Diffuse traumatic brain injury
FPI	Fluid percussion injury
GFAP	Glial fibrillary acid protein
mTBI	Mild traumatic brain injury
PBS	Phosphate buffered saline
PCS	Post-concussive symptoms
S1BF	Primary somatosensory barrel field of the cortex
TBI	Traumatic brain injury
VPM	Ventral posterior medial nuclei of the thalamus

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Highlights

- Experimental diffuse TBI causes thalamic neuron pathology and astrocyte activation that persists up to 1 month
- Onset of neuron pathology and glial activation occurs with dynamic changes in neuron morphology
- Histopathological changes in the presence of preserved cytoarchitecture support circuit reorganization
- Histopathological changes coincide with previously published changes in circuit function and behavior
- TBI-induced persisting histopathology indicates prolonged reparative processes rather than rapid recovery

Fig 1.

Giemsa stained VPM tissue confirms cytoarchitectural preservation over the entire post-injury time course in terms of cellular size and relative density. $n=2-3/\text{group/time point}$; scale bars=100 μm Giemsa staining is a marker which stains DNA and so is used to show changes in cytoarchitecture, over the time course it is seen that there is no significant changes at any point.

Fig 2.

Neuronal pathology develops over time and persists to at least 28 day post injury. (A) Representative images of VPM silver staining shows marked neuropathology at 7 and 28 days post-injury compared to the complete absence of staining in uninjured sham brain. Scale bars show 100µm for 20x. (B) Quantification of silver staining via pixel density analysis reveals significantly increased staining at 7 and 28 days post-injury in comparison to sham animals ($p < 0.001$). Significance is represented by *, where $p < 0.05$ compared to shams. Bar graphs represent the mean $\pm$ SEM. * $P < 0.05$ via one way ANOVA with a Tukey's post-hoc.

Fig 3.

Astrocyte activation in the VPM increases after FPI. (A) GFAP stained VPM tissue shows the increase in rate of astrocytic staining and difference between baseline and increased staining around astrocytes. Representative images at 20x images magnification. Arrows show examples of increased GFAP staining around astrocytes and arrowheads show baseline levels of GFAP staining around astrocytes. Scale bar=100 μ m. (B) Pixel analysis reveals a significant increase in GFAP staining over time, where staining at 7d FPI and 28d FPI are greater than sham. Data were analyzed by one-way ANOVA with Tukey's post-hoc comparison. Significance is represented by *, where $p < 0.05$. Bar graphs represent the mean $\pm$ SEM (n=3–5/group).

Fig 4.

Golgi-stained neurons in the VPM display altered morphologies over the post-injury time course. (A) Representative neurons are displayed at 40x magnification. (B) 2D reconstructions representing neurons in panel A. Scale bar=50 μm. (C–H) Quantification on 3D reconstructed neurons. (C) Neuronal process quantity was significantly greater at 28d FPI in comparison to 7d FPI, but not sham (* $p < 0.05$). (D) The number of branch points are significantly fewer at 7d FPI in comparison to sham, with a significant increase between 7d FPI and 28d FPI (* $p < 0.05$). (E) The number of end points is significantly lower at 7d FPI in comparison to sham. By 28d FPI, end points are similar to sham, but significantly greater than 1d FPI and 28d FPI (* $p < 0.05$). (F) Mean process length is significantly decreased at 7d FPI in comparison to sham, with a significant increase between 7d FPI and 28d FPI (* $p < 0.05$). (G) The mean soma area is significantly increased at 7d FPI in comparison to sham and 1d FPI (* $p < 0.05$). (H) Sholl analysis reveals decreased complexity at 7d FPI in comparison to sham with recovery by 28d FPI in comparison to 7d FPI. * $p < 0.05$ in comparison to sham, $^+p < 0.05$ in comparison to 7d FPI via two-way ANOVA. Bar graphs represent the mean $\pm$ SEM ($n = 4$ per group, 10 neurons per animal).

Fig 5.

Evidence of early and prolonged activation of microglia through 28d FPI. Representative images at 20x and 40X (insert) of Iba-1 stained coronal sections of the VPM demonstrates morphology consistent with activated microglia at 1, 7, and 28 days post-injury in comparison to sham. Note the decrease in process length and complexity in activated microglia as well as rod microglia (arrowheads) across the time course. Scale bar at 20X = 100 μ m; at 40x= 20 μ m.