

## NEUROFILAMENT DEPHOSPHORYLATION AND MICROGLIAL ACTIVATION FOLLOWING TRAUMATIC BRAIN INJURY IN THE IMMATURE RAT

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### **ABSTRACT**

Traumatic brain injury (TBI) is a leading cause of death and disability in infants and children under 4 years of age. Two major pathological mechanisms for disability following TBI are traumatic axonal injury (TAI) and inflammation. To examine the relationship between TAI and inflammation following TBI, immunohistochemical techniques were used to determine regional and temporal patterns of neurofilament dephosphorylation (marking TAI) and microglial activation (marking inflammation). The controlled cortical impact injury model was utilized to induce a contusive lateral injury in the left hemisphere. The presence of nonphosphorylated neurofilaments and active microglia were then assessed in the corpus callosum, cingulum, and lateral white matter tracts of 17-day-old rats that were euthanized at 1, 3, or 7 days postinjury. There was consistent evidence of both neurofilament dephosphorylation and microglial activation in each region at each of the time points following injury, as well as regionally and temporally similar levels of staining density. Morphological changes suggestive of axonal degeneration were also present. Nonphosphorylated neurofilament staining revealed that axonal swellings present at 1 day postinjury did not persist at 3 days or 7 days, terminal bulbs present at 3 days did not persist at 7 days, and the staining took on a punctate appearance that predominated at 3 days and 7 days. Microglia staining revealed an increase in microglial activation from 1 day to 3 days and a slight decrease thereafter. These data have revealed a possible relationship between TAI and inflammation following TBI and suggest that microglial activation might be a response not only to injury but also to axonal degeneration induced by neurofilament dephosphorylation.

### **INTRODUCTION**

Traumatic brain injury (TBI) is a leading cause of death and disability among infants and children under 4 years of age, with the pediatric population exhibiting higher morbidity, mortality, and TBI-related hospitalization rates compared to older children and adults (Cattalani et al., 1998; Langlois et al., 2005; Levin et al., 1992). Traumatic axonal injury (TAI) is regarded as the pathophysiologic hallmark of pediatric TBI. Following injury, a limited number of injured axons undergo primary axotomy because of the immediate mechanical damage, while a majority of injured axons deteriorate more slowly via secondary axotomy, the result of progressive structural damage from pathologic biochemical reactions that take place in the neurons (Maxwell et al., 1997; Povlishock and Katz, 2005; Gennarelli, 1996). Traumatic axonal injury is the major pathological mechanism for disability following TBI (Büki and Povlishock, 2006; Ross et al., 1994; Adelson et al., 2001) and an area in which therapeutic intervention could prove beneficial. Another consequence of TBI is inflammation, a condition detrimental to neuronal health (Vilhardt, 2005). Microglial cells are the resident immune cells of the brain and become activated by noxious stimuli, including brain injury. Microglial cells secrete pro-inflammatory substances when in the active state. In fact, sustained microglial activation has been associated with both initiation and perpetuation of chronic inflammation, which may lead to neuronal dysfunction and cell death (Vilhardt, 2005).

One means of identifying TAI is by detecting nonphosphorylated neurofilament. Neurofilaments are heteropolymeric components of the neuron cytoskeleton that consist of a 68 kDa light neurofilament subunit (NF-L) backbone with either 160 kDa medium (NF-M) or 200 kDa heavy subunit (NF-H) side-arms (Julien and Mushynski, 1998). Injury triggers calcium influx into the neuron through an as yet undefined mechanism (Wolf et al., 2001). Calcium influx has been reported to cause a cascade of events resulting in TAI. For example, elevated intracellular calcium levels activate calcineurin, a calcium-dependent phosphatase that dephosphorylates neurofilament side-arms (Büki and Povlishock, 2006). Alteration of the phosphorylation state of neurofilaments presumably affects neuronal structure and contributes to axonal injury. Neurofilament dephosphorylation is a marker of TAI and may be detected reliably using immunohistochemical techniques (Ross et al., 1994; Povlishock, 1993). Neuronal inflammation, on the other hand, may be detected through visualization of microglial cells in the active state. When in the so called resting state, these cells are surrounded by spiky protrusions or ramified processes. Upon activation, changes in microglial function – most notably induction of inflammation – are accompanied by a morphological transformation as the cells assume an ameboid appearance (Vilhardt, 2005). Hence, ameboid microglia mark inflammation.

Because inflammation and TAI are two main pathological mechanisms responsible for disability following TBI (Ashwal et al., 2006; Vilhardt, 2005), the focus of this study was to determine the relationship between TAI and inflammation following TBI. While it is well established that TAI and inflammation each follow TBI (Huh et al., 2008; Ma et al., 2009; Raghavendra Rao et al., 2000), it is unclear whether TAI itself can induce inflammation following TBI. To begin to address this issue, immunohistochemical techniques were used to determine regional and temporal patterns of neurofilament dephosphorylation (marking TAI) and microglial activation (marking inflammation) following TBI. Seventeen day-old (PND17) rats were subjected to controlled impact on the intact skull to replicate a clinically relevant model of pediatric closed head injury (Huh and Raghupathi, 2007). PND17 rats were used because they are neurologically equivalent to a toddler (Huh et al., 2008). Neurofilament dephosphorylation and inflammation were assessed in the cingulum, corpus callosum, and lateral white matter tracts at 1, 3, or 7 days postinjury. Consistent evidence of both neurofilament dephosphorylation and microglial activation in each region at each of the time points following injury, as well as regionally and temporally similar levels of staining density, were found. These findings suggest a possible relationship between TAI and inflammation following TBI.

## **METHODS**

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### **Brain Injury**

All procedures in this study were approved by the Institutional Animal Care and Use Committee of Drexel University College of Medicine and were in compliance with PHS guidelines for the Care and Use of Laboratory Animals. Brain injuries were induced using an electronically driven controlled cortical impact (eCCI) device (Custom Design and Fabrication, Richmond, VA), a modification of the pneumatic CCI (Dixon et al., 1991). The 5 mm diameter metal indenter was convex and driven with a velocity of 5 m/s with a dwell time of 100 msec. Seventeen-day-old male and female Sprague–Dawley rat pups (Charles River Laboratories, Wilmington, MA; 33±4g (mean±SD) body weight) were anesthetized with 5% isoflurane using a nose cone. When adequately anesthetized (loss of a tail-pinch reflex), a midline incision was made to expose the skull. The periosteum was reflected and the animal was placed in a standard mouse restrainer (Braintree Scientific, Braintree, MA). The head was supported by a soft foam pad to make it level with the body. The restrainer was positioned under the CCI device, the nose cone was removed, and the zero-point for the indenter was positioned on the skull for a contusive lateral injury. At about 45 s after removal of anesthesia (by which time the animals began to exhibit a pain reflex, as evidenced by a toe pinch and retraction of the limb), rats were subjected to an impact, as the indenter traveled a distance of 4 mm into the skull. Sham animals were prepared in an identical fashion through the step of positioning the zero-point for the indenter on the skull. The total time from initiation of anesthesia to removal of the nose cone prior to zeroing the impactor tip to the surface of the skull was 4 min. Once the animals regained a normal breathing pattern, they were reanesthetized, the scalp was sutured shut, and the pups were returned to the dam. Animals were placed on a heating pad (37 C) to

maintain body temperature throughout the procedures and during recovery. Animals were euthanized at 1, 3, and 7 days postinjury ( $N = 4$  injured and 1 sham per time point,  $N = 15$  animals total).

### **Tissue Preparation**

Sham and brain-injured animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and euthanized by transcardial perfusion with 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde, first in the skull for 24 h, and then for an additional 24 h outside the cranial cavity, cryoprotected in 30% sucrose, and frozen in liquid isopentane at  $-35^{\circ}\text{C}$ . Twelve sets of coronal sections (40  $\mu\text{m}$  thick) were taken every 0.5 mm from 1 mm anterior to bregma and 5.6 mm posterior to bregma; each set contained approximately 10 sections.

### **Immunohistochemistry**

To determine the extent of neurofilament dephosphorylation caused by TBI, a mouse antibody that binds to nonphosphorylated NF-M and NF-H was used (SMI-32; Sternberger Monoclonals Inc., Baltimore, MD, 1:1,000). Sections were first rinsed in Tris-Buffered Saline (TBS) 3 times for 5 minutes each (3x5 min) at room temperature, then blocked in 5% normal horse serum (NHS) with TBS-T (TBS with 0.1% Triton X-100) for 60 min at room temperature with gentle agitation. Sections were then incubated in mouse monoclonal anti-SMI-32 primary antibody (1:5000, diluted in 5% NHS/TBS-T solution) overnight at  $4^{\circ}\text{C}$ , again with gentle agitation. The following day, sections were washed 3x5 min in TBS, then incubated in donkey antimouse IgG (diluted 1:1000 in 5% NHS/TBS-T solution, Jackson ImmunoResearch) for 2 hours at room temperature with agitation. Sections were then incubated with hydrogen peroxide: methanol: TBS (1:3:6, v:v:v) for 15 min at room temperature with agitation. Following this incubation, sections were washed 4x10 min in TBS. During the washing period, AB solution was prepared using an ABC Elite kit (PK1600, Vector Laboratories, Burlingame, CA); 50  $\mu\text{L}$  solution A was first mixed with 2.4 mL of Tris Buffered Solution/Bovine Serum Albumin (TBS/BSA) and 50  $\mu\text{L}$  of solution B was added. After 30 min, 5 ml TBS/BSA was added, and the solution was mixed for an additional 30 min. Sections were then incubated in the AB solution for 1 hour at room temperature with gentle agitation. Next, sections were washed 4x5 min in TBS and incubated with Diacetylbenzidine (DAB) substrate solution for 7 min. A kit from Vector Laboratories (SK4100) was used to prepare the DAB substrate solution, comprised of 5 ml ddH<sub>2</sub>O, 2 drops of buffer stock solution, 4 drops of DAB stock solution, and 2 drops of hydrogen peroxide stock solution, added in order and mixed well between each addition by vortexing. Sections were then briefly rinsed 2x with ddH<sub>2</sub>O, then 1x with TBS-T. Lastly, sections were mounted on gelatin-coated glass slides and coverslipped with Hardset Vectashield (Vector Laboratories). As a negative control, 1 section from each animal was incubated with secondary antibody and the ABC reagent prior to exposure to DAB (i.e., no primary antibody).

To assess microglial activation a rabbit polyclonal antibody that binds to Iba, a 17-kDa EF hand protein that is specifically expressed in microglial cells and is upregulated during activation of these cells, was used (Iba-1 antibody; Wako Pure Chemical Industries, Ltd., Osaka, Japan, 1:20,000). Sections were rinsed 3x5 min in TBS, then incubated with hydrogen peroxide: methanol: TBS (1:3:6) for 15 min. Sections were then washed 4x10 min in TBS before being blocked in 5% NHS/TBS-T for 60 min. All previous incubations/washes were performed at room temperature with gentle agitation. Following the blocking procedure, sections were incubated in rabbit polyclonal Iba1 primary antibody (diluted 1:20,000 in 5% NHS/TBS-T solution) overnight at  $4^{\circ}\text{C}$  with agitation. The following day, sections were rinsed 3x5 min in TBS, then incubated for 2 hours in secondary antibody (biotinylated donkey antirabbit IgG H+L chains, diluted 1:500 in 5% NHS/TBS-T solution, Jackson ImmunoResearch). Sections were then washed 3x5 min in TBS and incubated in the AB solution (see above) for 1 hour. After this incubation, sections were washed 3x5 min in TBS, exposed to Diaminobenzidine (DAB) substrate solution for 5 minutes, briefly rinsed 3x with ddH<sub>2</sub>O, then 1x with TBS-T, mounted on gelatin-coated glass slides, and coverslipped with Hardset Vectashield (Vector Laboratories). As a negative control, 1 section from each animal was incubated with secondary antibody and the ABC reagent prior to exposure to DAB (i.e., no primary antibody).

### **Qualitative Assessment of Regional and Temporal Patterns of TAI and Inflammation**

Subcortical white matter tracts (regions that are composed predominantly of axons) including the lateral white matter, cingulum, and corpus callosum, were examined in this study for signs of injury-induced axonal degeneration and inflammation. Images of sections were captured at 10X and/or 40X magnification using an Eclipse E400 microscope (Nikon Corporation, Tokyo, Japan) and digitized as TIFF images using Nikon ACT-1 software version 2.62 (Nikon). Qualitative assessments were then made concerning the presence and levels of TAI and inflammation in each subcortical white matter region at each time point.

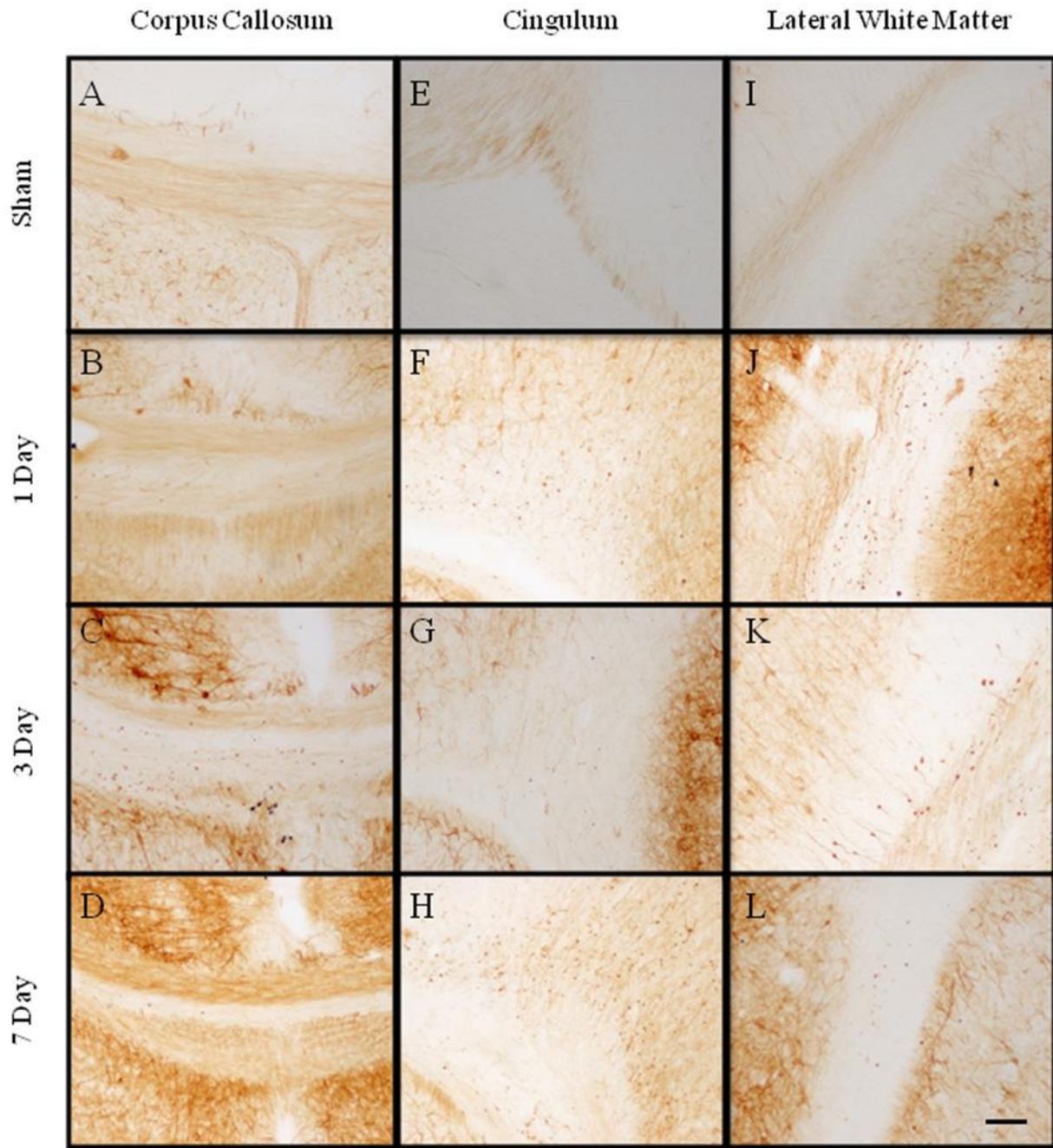
### **RESULTS**

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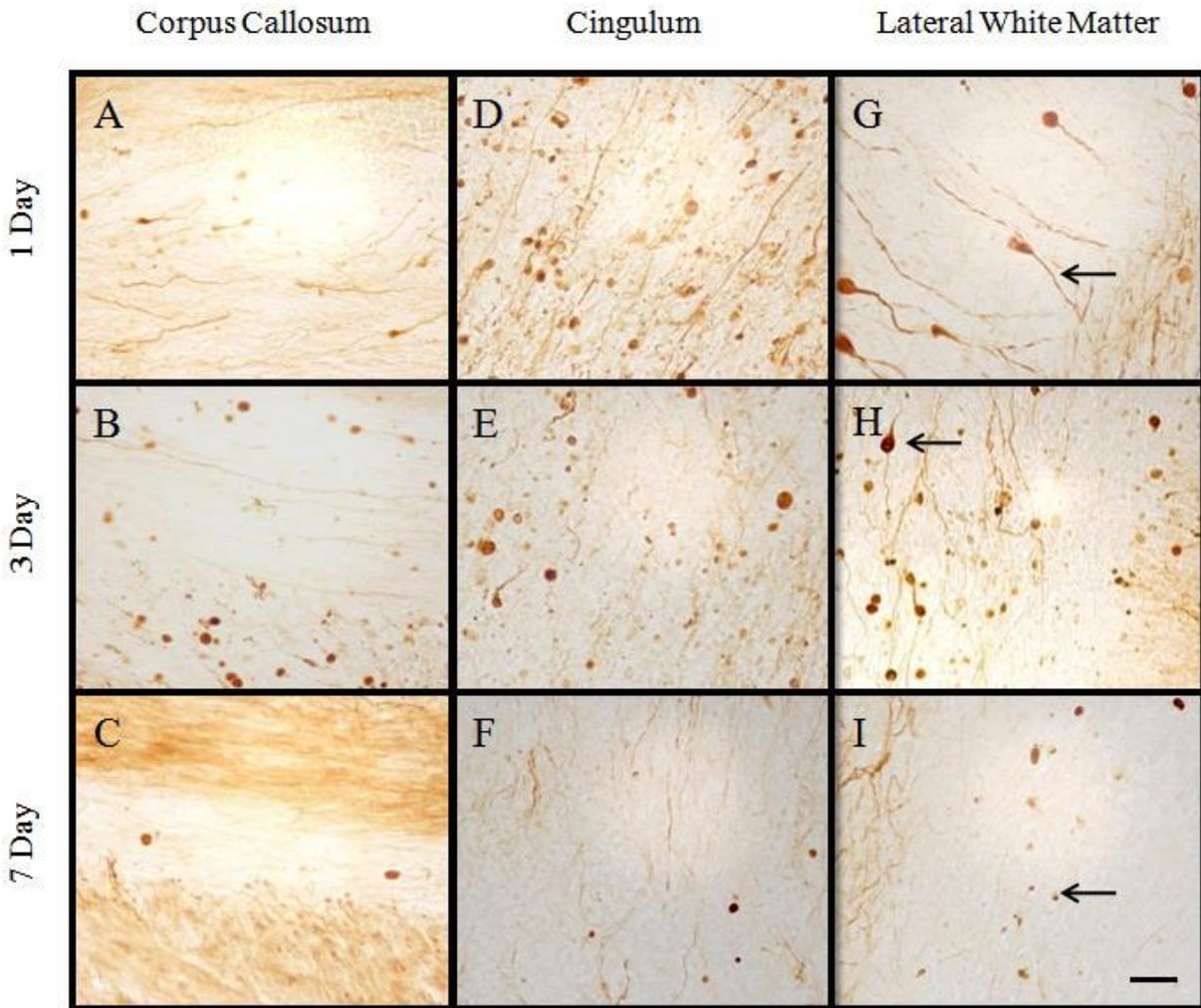
Nonphosphorylated neurofilament, a marker of traumatic axonal injury (TAI), was observed in each white matter region (corpus callosum, cingulum, and lateral white matter) in the injured hemisphere (Fig. 1). There was no evidence of nonphosphorylated neurofilament in sham animals (Fig. 1A, E, I). At 1 day postinjury, SMI-32 immunoreactivity was observed in each region (Fig. 1B, F, J), mainly presenting as swollen axons (Fig. 2 A, D, G). By 3 days post-injury, an overall peak in SMI-32 staining density was observed (Fig. 1C, G, K), with SMI-32 immunoreactivity still present in each region. At this time, terminal bulbs were also present (Fig. 2 B, E, H). At 7 days postinjury, overall SMI-32 staining density was decreased relative to day 3 (Fig. 1D, H, L) and was observed predominantly as punctate immunoreactivity (Fig. 2C, F, I), indicative of axonal degeneration.

Microglial activation, as assessed through visualization of Iba-1 immunoreactivity as a marker of inflammation, was also observed in each white matter region of interest (Fig. 3), most abundantly in the injured hemisphere. There was no evidence of microglial activation in sham animals, as indicated by the ramified morphology of microglial cells (Fig. 3A, E, I). At 1 day postinjury, microglial cells appeared less ramified and more amoeboid in each region (Fig. 3B, F, J) compared to inactive microglia. Similar to SMI-32 immunoreactivity, an overall peak in microglial activation occurred at day 3 postinjury; at this time point, microglia appeared most amoeboid-like, indicative of the active state (Fig. 3C, G, K). Subsequently, there was an overall decrease in microglial activation by day 7, with more microglia appearing ramified, compared to day 3 (Fig. 3D, H, L). Nevertheless, microglial activation remained heightened because the presence of amoeboid microglia persisted at this time.

(Fig. 3D, H, L).

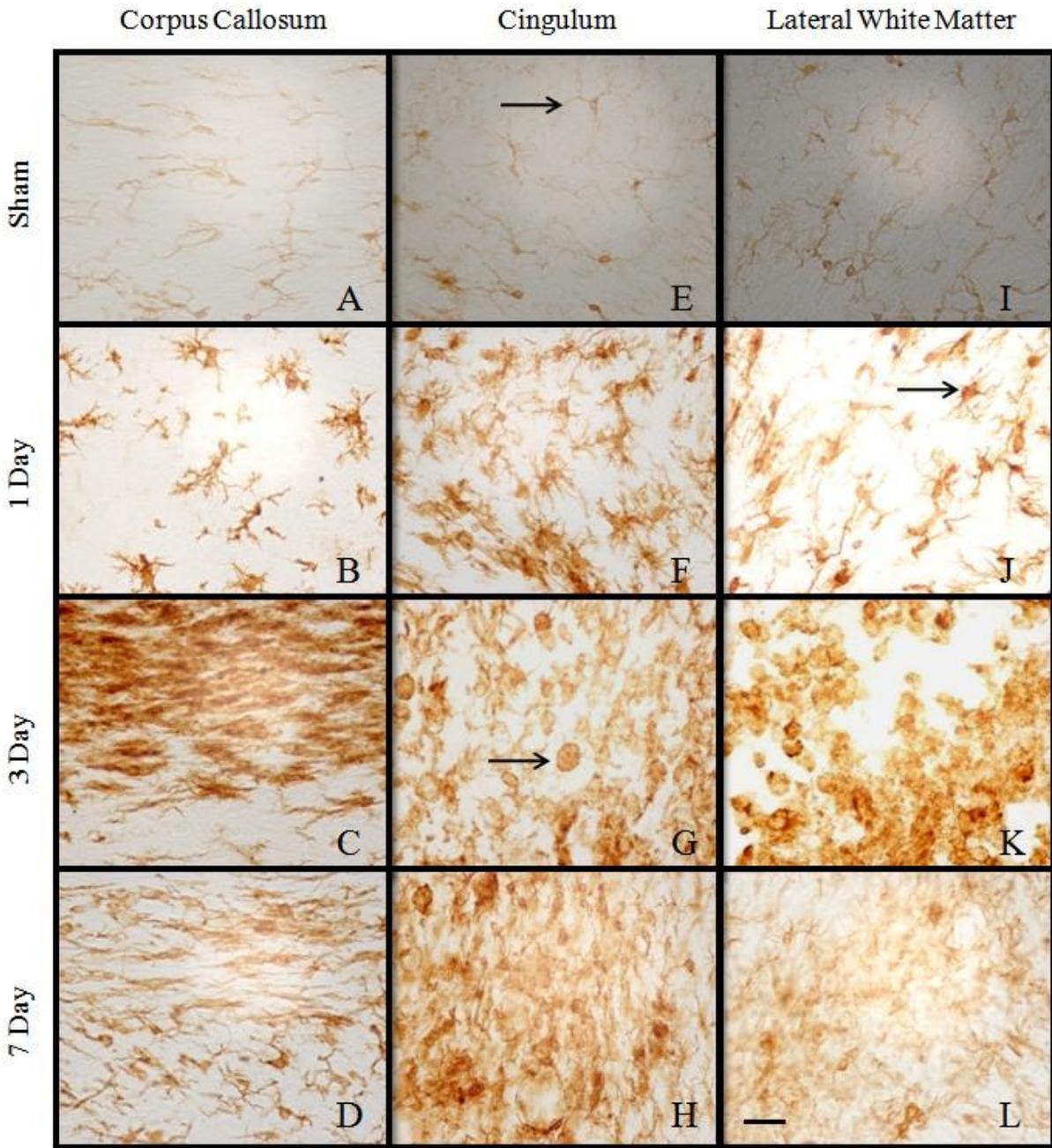


**Figure 1.** SMI-32 immunoreactivity following contusive lateral brain injury in immature rats. Representative photomicrographs of SMI-32-labeled axons in sham animals (A, E, I), and at 1 day (B, F, J), 3 days (C, G, K), and 7 days (D, H, L) postinjury in the corpus callosum (A-D), cingulum (E-H), and lateral white matter tracts (I-L). Scale bar = 100  $\mu$ m for all panels.



**Figure 2.** Morphological appearance of injured axons exhibiting SMI-32 immunoreactivity. Representative photomicrographs of the corpus callosum (A-C), cingulum (D-F) and lateral white matter (G-I) at 1 day, 3 days, and 7 days postinjury. SMI-32-labeled injured axons appeared mainly as axonal swellings at 1 day (G, arrow), as terminal bulbs at 3 days (H, arrow), and exhibited a punctate appearance at both 3 days and 7 days (I, arrow) after injury. Scale bar = 25  $\mu$ m for all panels.

Taken together, evidence for both TAI and inflammation was observed following traumatic brain injury in each region examined and at each time point. In addition, morphological changes occurred over time with respect to SMI-32-labeled injured axons, indicating progressive axonal degeneration. Morphological changes were also evident through time in the microglial cell population, as heightened microglial activation persisted until 7 days postinjury.



**Figure 3.** Iba-1 immunoreactivity following contusive lateral brain injury in immature rats. Representative photomicrographs of Iba-1-labeled axons in sham animals (A, E, I), and at 1 day (B, F, J), 3 days (C, G, K), and 7 days (D, H, L) postinjury in the corpus callosum (A-D), cingulum (E-H), and lateral white matter tracts (I-L). Note the typical ramified form of an inactive microglial cell (E, arrow), compared to the ameboid-like appearance of an active microglial cell (G, arrow). A microglial cell with an intermediate morphology is shown in panel J (arrow). Scale bar = 25  $\mu$ m for all panels.

## DISCUSSION

The present study revealed a temporal and spatial progression of neurofilament dephosphorylation and microglial activation marking traumatic axonal injury (TAI) and inflammation, respectively, following traumatic brain injury (TBI) in the immature rat. Both TAI and inflammation were observed in the

corpus callosum, cingulum, and lateral white matter at 1, 3, and 7 days postinjury. The consistent observation of neurofilament dephosphorylation and microglial activation at the same place and time, along with persisting microglial activation at 7 days in treated but not control animals, provides evidence for a relationship between TAI and inflammation. The SMI-32 immunoreactivity used to detect nonphosphorylated neurofilament exhibited a characteristic temporal pattern in each region. At 1 day postinjury, the staining appeared to define axonal swellings. The SMI-32 staining intensity peaked at 3 days after injury and was also seen in terminal bulbs at this time point. Staining density then decreased by 7 days postinjury, and was punctate in appearance, suggesting axonal degeneration (DiLeonardi et al., 2009). This observation was consistent with previous studies and suggests that during the post-traumatic response cellular fragments pinched off the axon downstream of the swelling (Coleman, 2005).

Microglial activation, a marker of inflammation, was also observed in each region of interest and at each time point. Microglial activation had begun by 1 day postinjury in each subcortical white matter tract, and at 3 days this activation had peaked. At 7 days postinjury, microglial activation had noticeably decreased, however, the presence of amoeboid microglial cells persisted. Microglial cells, which are the resident immune cells in the central nervous system, become activated by noxious stimuli, including brain injury. The activation process entails acquisition of phagocytic functions as well as the induction of inflammation (Lucas et al., 2006). Prolonged microglial activation, however, may cause chronic inflammation, leading to neuronal death and dysfunction (Vilhardt, 2005). Although we have shown a temporal correlation between microglial activation and neurofilament dephosphorylation, whether microglial activation is a specific response to the injury itself, to subsequent progressive axonal degeneration resulting from neurofilament dephosphorylation, or to both, remains to be seen.

TAI involves axonal deterioration, leaving behind cellular debris. Evidence for axonal degeneration in many subcortical white matter tracts was observed in this study via SMI-32 immunoreactivity. We found that injured axons first swell and over time become terminal bulbs. We speculate that the punctate staining we observed a week after injury represents cellular fragments that have pinched off the axon downstream of the initial swelling leaving free cellular debris for microglial to eliminate via phagocytosis. A process similar to this has already been described (Coleman, 2005). Thus, it is possible that microglial activation is not only a response to the immediate mechanical injury, but also to axonal degeneration following neurofilament dephosphorylation. Presumably, the initial damage and subsequent presence of cellular debris resulted in microglial activation and the consequent sustained phagocytic function required to clear this debris.

The present study provides testable hypotheses for future studies. While TAI and inflammation were both observed in each region and at each time point examined, this does not prove a causal relationship between neurofilament dephosphorylation-induced TAI and microglial activation. To address this issue, an antagonist to calcineurin (a calcium-activated neurofilament phosphatase) could be administered to the animal prior to and/or after injury. If microglial activation is a direct response to neurofilament dephosphorylation-induced TAI, then preventing neurofilament dephosphorylation following TBI should result in a significant depression of microglial activation compared to that observed in the present study. Furthermore, data from the present study must be carefully quantified for future comparisons of TAI levels and microglial activation. Understanding the pathophysiology of TBI is a crucial step in developing effective therapeutic interventions. If neurofilament dephosphorylation can be blocked, both TAI and inflammation may be attenuated, which may then lead to improved outcomes following traumatic brain injury.

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