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Nogo presence is inversely associated with shifts in cortical microglial morphology following experimental diffuse brain injury

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Abstract

Diffuse traumatic brain injury (TBI) initiates secondary pathology, including inflammation and reduced myelination. Considering these injury-related pathologies, the many states of activated microglia as demonstrated by differing morphologies would form, migrate, and function in and through fields of growth-inhibitory myelin byproduct, specifically Nogo. Here we evaluate the relationship between inflammation and reduced myelin antigenicity in the wake of diffuse TBI and present the hypothesis that the Nogo-66 receptor antagonist peptide NEP(1-40) would reverse the injury-induced shift in distribution of microglia morphologies by limiting myelin-based inhibition. Adult male rats were subjected to midline fluid percussion sham or brain injury. At 2h, 6h, 1d, 2d, 7d, and 21d post-injury, immunohistochemical staining was analyzed in sensory cortex (S1BF) for myelin antigens (myelin basic protein; MBP and CNPase), microglia morphology (ionized calcium binding adapter protein; Iba1), Nogo receptor and Nogo. Pronounced reduction in myelin antigenicity was evident transiently at 1d post-injury, as evidenced by decreased MBP and CNPase staining, as well as loss of white matter organization, compared to sham and later injury time points. Concomitant with reduced myelin antigenicity, injury shifted microglia morphology from the predominantly ramified morphology observed in sham-injured cortex to hyper-ramified, activated, fully activated, or rod. Changes in microglial morphology were evident as early as 2h post-injury, and remained at least until day 21. Additional cohorts of uninjured and brain-injured animals received vehicle or drug (NEP(1-40), i.p., 15 min and 19 h post-injury) and brains were collected at 2h, 6h, 1d, 2d, or 7d post-injury. NEP(1-40) administration further shifted distributions of microglia away from an injury-induced activated morphology towards greater proportions of rod and macrophage-like morphologies compared to vehicle-treated. By 7d post-injury, no

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differences in the distributions of microglia were noted between vehicle and NEP(1-40). This study begins to link secondary pathologies of white matter damage and inflammation after diffuse TBI. In the injured brain, secondary pathologies co-occur and likely interact, with consequences for neuronal circuit disruption leading to neurological symptoms.

Keywords

Microglia; Nogo; traumatic brain injury; myelin; oligodendrocytes

Introduction

Worldwide, traumatic brain injury (TBI) is a leading cause of death and disability, despite dramatic improvements in critical care medicine. The initial insult activates complex cellular and biochemical pathways, which if not tightly controlled, can exacerbate the injury (Morganti-Kossmann, Rancan, Stahel, & Kossmann, 2002). These secondary insults include the disruption of the blood-brain barrier, edema, inflammation, neuropathology and the activation of glial cells, all of which contribute to changes in neurological function (Ziebell & Morganti-Kossmann, 2010). Functional recovery following injury to the adult CNS is often impeded by the failure of axons to regenerate after injury.

Part of the secondary injury cascade involves the loss of myelin from axons, both injured and uninjured. When myelin on axons is disrupted, myelin-associated neurite outgrowth inhibitor, Nogo-A, is released (Filbin, 2003). Nogo-A, plays a key role in inhibition of axonal regeneration following injury and ischemia in the central nervous system (David, Fry, & Lopez-Vales, 2008; Fry, Ho, & David, 2007; Satoh, Onoue, Arima, & Yamamura, 2005; Yan et al., 2012). The Nogo gene encodes three main protein isoforms: Nogo-A, Nogo-B and Nogo-C, of which Nogo-A is the most widely studied (Satoh et al., 2005). A 66 residue domain of Nogo-A expressed on the surface of oligodendrocytes binds to Nogo-66 receptor (NgR) on neurons causing growth cone collapse and arrest of neurite/axon outgrowth (GrandPré, Li, & Strittmatter, 2002). Recently, Nogo proteins have been found to regulate neuronal precursor migration, neurite growth, and branching in the developing nervous system (Schwab, 2010). Since cells beside neurons migrate, grow and branch in the nervous system, it is compelling to consider a relationship of cellular movement and morphological transitions to myelin debris.

In addition to neurons, NgR is reported to be expressed on microglia/macrophages in multiple sclerosis demyelinating lesions (Satoh et al., 2005). This implies that, as well as acting on neighboring NgR-expressing neurons and their axons, Nogo-A released from damaged oligodendrocytes interacts directly with NgR on the cell surface of reactive astrocytes and microglia. This may result in down-regulation of glial proliferation and cytokine production, as well as sequestration of Nogo-A (Satoh et al., 2005). Binding of Nogo-A to NgR on microglia, has been found to block microglial adhesion and to inhibit their migration *in vitro*. Nogo-A also impairs microglia polarization and membrane protrusion formation, contributing to a decreased capacity for cell mobility (Yan et al., 2012). Nogo-66 is also reported to play a key role in inhibition of neurite outgrowth in the

central nervous system by binding to NgR expressed on neurons. Moreover, it has been reported that NgR has a role in clearance of macrophages from injured peripheral nerve (Fry et al., 2007). These studies indicate that the Nogo/NgR relationship may modulate microglia function in secondary neuropathology.

Microglia have a plethora of functions within the CNS and are vital to the maintenance of homeostasis (Graeber, 2010; Hanisch, 2013; Ziebell, Adelson, & Lifshitz, 2015). Diffuse TBI activates microglia from their normal surveillance ramified morphology into one of four activated morphologies; 1) hyper-ramified, 2) reactive, 3) rod, 4) fully activated/macrophage-like. It has been suggested that function follows morphology. Recently, ramified microglia have been implicated in the homeostasis of normal synapse function (Tremblay, 2011; Tremblay & Majewska, 2011; Tremblay et al., 2011; Wake, Moorhouse, Miyamoto, & Nabekura, 2013). Whereas, hyper-ramified and activated microglia have been proposed to release cytokines and chemokines which drive secondary injury cascades (Ziebell & Morganti-Kossmann, 2010). The fully activated microglia/macrophage-like morphology is thought to scavenge debris and pathogens, but no role has been ascribed to rod microglia.

Our recent work has identified rod microglia within the primary sensory barrel fields (S1BF) of the cortex following diffuse brain injury in the rat. Rod microglia formation peaked at 7 days post-injury in S2BF (Ziebell, Taylor, Cao, Harrison, & Lifshitz, 2012). By this time, rod microglia abut one another to form trains which align in a trajectory parallel to axons and perpendicular to the dural surface (Ziebell et al., 2012). Necessarily, some of these axons will be myelinated, which creates a microenvironment for axonal, myelin, and microglial interactions. At these junctions, the possibility exists for neuronal:glial signaling to regulate functional states of these components. At present, the function of rod microglia is unknown, including their affinity for myelinated or unmyelinated axons, which may influence the microglia response to TBI. Since the myelin sheath is the immediate cellular component encountered by rod microglia when interacting with axons, it is plausible that rod microglia modulate myelin, or vice versa, in the injured brain. Indeed, we recently reported an apparent decrease in CNPase (myelin) staining in close proximity to rod microglia, indicating that these morphologically distinct microglia may act in preventing or exacerbating neuropathology (Ziebell et al., 2012).

Here, we investigate whether Nogo-A has a role in microglia reactivity, especially the rod morphology, following diffuse brain injury. Following TBI each morphology of microglia is present, however, the proportion of each morphology varies as a function of time. The relative proportions of microglia morphologies can represent the pathological state of the tissue. It stands to reason that directing microglia from an activated, pro-inflammatory state back towards a ramified surveillance morphology may improve outcomes post-injury. Therefore, we sought to determine whether population distributions of microglial morphology were responsive to the inhibition of Nogo-A function with the NgR antagonist NEP(1-40) (GrandPre et al., 2002; Wang et al., 2012). This would support the hypothesis that Nogo-A in the wake of TBI may contribute to directing microglial population responses.

Materials and Methods

Surgical Preparation and Diffuse Traumatic Brain Injury

A total of 74 male Sprague-Dawley rats (328–377 g) were used in this study. Rats received midline fluid percussion injury (mFPI) or sham-injury, as described elsewhere (Hosseini & Lifshitz, 2009; Lifshitz, Kelley, & Povlishock, 2007; Lifshitz & Lisembee, 2012). Briefly, rats were anesthetized with 5% isoflurane in 100% O₂ prior to the surgery and maintained at 2% isoflurane via nose cone. Rats were placed in a stereotaxic frame and a midline scalp incision was made to expose the skull. 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 hub was fabricated from the female portion of a Luer-Loc needle hub, which was cut, beveled, and scored to fit within the craniotomy. 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 methyl-methacrylate (Hygenic Corp., Akron, OH) was applied around the injury hub and screw. The incision was sutured at the anterior and posterior edges and topical Lidocaine ointment was applied. Animals were returned to a warmed holding cage and monitored until ambulatory.

For injury induction, animals were re-anesthetized with 5% isoflurane 60–90 min after surgery. The dura was inspected for patency and debris through the injury-hub assembly, which was then filled with physiological saline and attached to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). As the rats' reflexive responses returned, the injury (1.9–2.0 atm) was administered by releasing the pendulum onto the fluid-filled cylinder. Animals were monitored for the presence of a forearm fencing response as well as the return of the righting reflex as indicators of injury severity (Hosseini & Lifshitz, 2009). Sham animals were connected to the FPI device, but the pendulum was not released. The injury-hub assembly was removed *en bloc*, integrity of the dura was observed, and bleeding was controlled prior to the incision being stapled. Brain-injured animals had righting reflex recovery times of 5 to 10 minutes, and sham-injured animals recovered within 15 seconds. Surgical recovery was monitored post-operatively for three days, for which no overt differences (e.g. weight, movement, grooming) were observed in animals. Experiments were conducted in accordance with NIH guidelines and approved by the institutional animal care and use committee (IACUC) at St. Joseph's Hospital and Medical Center (Phoenix, AZ). Measures were taken to minimize pain and discomfort.

Tissue collection and processing for immunohistochemistry

Brain tissue was collected at 6 pre-determined time points with respect to brain injury (2h, 6h, 1d, 2d, 7d, and 21d; n=4 per time point for a total of 24 rats). Brain tissue was collected from uninjured sham rats at 7 days post-injury. Rats were overdosed with sodium pentobarbital (200 mg/kg i.p.) and transcardially perfused with PBS, followed by 4% paraformaldehyde in PBS. Following removal, the brains were cryoprotected in sucrose and frozen in O.C.T. (Optimal Cutting Temperature, Tissue Tek, Sakura) media. Coronal sections (20 µm) were cut on a cryostat and mounted on Superfrost Plus slides (Fisherbrand)

using PBS and dried before being stored at -80°C . See Figure 1A for a schematic timeline of injury and tissue collection schedule.

Nogo receptor antagonist, NEP(1-40), treatment

A total of 50 rats were used in this experiment; 5 rats were excluded from analysis due to post-surgery complications. There were 3 treatment groups: brain-injured treated with NEP(1-40), brain-injured treated with vehicle, and sham treated with NEP(1-40). Briefly, NEP(1-40) (Sigma-Aldrich; St. Louis, MO) was dissolved at $89\text{ }\mu\text{g/ml}$ in sterile vehicle solution consisting of 97.5% PBS and 2.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich). NEP(1-40) was administered at time points prior to microglial activation, because Nogo and peak rod microglial presentation were temporally delayed after brain injury. Animals received drug or vehicle at $89\text{ }\mu\text{g/kg}$ by intraperitoneal (i.p.) injections at 15 minutes and 19 hours following mFPI or sham-injury, with the dosage based on a similar study carried out by (Wang et al., 2012). See Figure 1B for a schematic timeline of injury, treatment, and tissue collection schedule. Brain tissue was collected at 2h, 6h, 1d, 2d, and 7d after brain injury ($n = 4$ per group for NEP(1-40); $n = 3$ per group vehicle; $n=4$ vehicle 7d), as described above. Analysis of sham animals injected with NEP(1-40) revealed no changes in microglia morphology with treatment over time, thus these animals were pooled to one group ($n=9$). Animals were randomly assigned to treatment or vehicle by an investigator who had not performed surgery or injury. Care was taken to ensure the righting reflex times, and thus injury severity, were equivalent between the treatment and vehicle groups.

Immunohistochemical staining for microglia and Nogo Receptor

Immunohistochemistry for microglia was performed using ionized calcium binding adaptor molecule 1 (Iba-1) antibody, consistent with previously described methods (Cao, Thomas, Ziebell, Pauly, & Lifshitz, 2012; Ziebell et al., 2012). In addition, staining for NgR was conducted on adjacent slides using the same protocol. Briefly, 6 sections per animal (3 sections at approximately Bregma -2.40mm and 3 sections at approximately Bregma -3.48mm) were thawed and dried in an oven at 60°C for 4 hours, following which they were rinsed three times in PBS. Sections were then placed in blocking solution consisting of 4% normal horse serum (NHS) and 0.4% Triton X-100 in PBS for 60 minutes. Sections were then incubated at 4°C overnight in 1% blocking solution containing either rabbit anti-Iba-1 primary antibody (Wako, cat #019-19741, 1:5000) or rabbit anti-Nogo receptor (Alomone Labs, cat #ANT-008, 1:500).

The following day, sections were rinsed three times in PBS, then incubated for 1 hour in 4% blocking solution containing horse anti-rabbit IgG biotinylated (Vector Laboratories, cat #BA-1100, 1:250). Slides were then rinsed three times in PBS and endogenous peroxidases were inhibited by placing slides in PBS containing 1% v/v H_2O_2 . Slides were then rinsed in PBS and incubated in Vectastain Elite ABC (Vector Laboratories, cat #PK-6100) for 30 minutes. Sections were developed with 3,3'-diaminobenzidine (DAB) solution (Vector laboratories, cat #SK-4100) after washing in PBS. Following DAB, slides were rinsed in tap water, counterstained with methyl green and dehydrated in 100% ethanol. Sections were cleared in Citrisolv (Fisher Scientific) and cover slipped with DePeX (Sigma-Aldrich).

Immunofluorescence labeling of myelin or Nogo with microglia

Double labeling immunofluorescence for Iba-1 and myelin associated molecules was performed consistent with previously described methods (Ziebell et al., 2012). Briefly, 3–6 sections per animal were thawed and dried in an oven at 60°C for 4 hours, following which they were rinsed three times in PBS. Sections were then incubated for 60 minutes in blocking solution consisting of 2% NHS, 2% normal donkey serum (NDS) and 0.4% Triton X-100 in PBS. Sections were then incubated at 4°C overnight in 1% blocking solution containing mouse anti-Iba-1/AIF (Millipore, cat #MABN92 1:1000) or rabbit anti-Iba-1 and one of the following: rabbit anti-CNPase (Cell Signaling, cat #5664 1:500), rabbit anti-Nogo (Abcam, cat #ab32298 1:2000) or mouse anti-myelin basic protein (MBP; Covance, cat #SMI-99P).

The next day, sections were rinsed three times in PBS containing 0.05% Tween-20 (PBST). They were then incubated for 1 hour in 4% blocking solution containing appropriate secondary antibodies at 1:250 including biotinylated horse anti-mouse (Vector Laboratories, cat #BA-2000), biotinylated horse anti-rabbit, donkey anti-mouse conjugated to AlexaFluor 488 (Jackson Laboratories, cat #715-545-150), or donkey anti-rabbit AlexaFluor 488 (Jackson Laboratories, cat #711-545-152). Sections were then rinsed three times in PBST and incubated for 1 hour in streptavidin bound AlexaFluor488 or AlexaFluor594 (Jackson Laboratories, cat #016-540-084 and cat #016-580-084, respectively, 1:1000) in PBS. Sections were then rinsed 3 times in PBST and dipped in Hoechst (Invitrogen, 1:1000) for 60 seconds, rinsed 3 more times in PBS then in ddH₂O and cover slipped with Fluoromount-G (Southern Biotech, cat #0100-01). To ensure reproducible results, parallel immunohistochemistry runs for MBP and CNPase were conducted simultaneously.

Semi-quantitative analysis of microglial proportions

Analysis was undertaken by an investigator that was blinded to treatment, injury status of each animal, or time post-injury. For semi-quantitative cell count analysis, photomicrographs were acquired from Iba-1 labelled sections. Two regions of interest (ROIs) were analyzed: 1) the S1BF due to its known association with neuropathology after midline fluid percussion injury (Cao et al., 2012; Lifshitz & Lisembee, 2012; Ziebell et al., 2012); and 2) the secondary somatosensory cortex (S2) as a control, since neuropathology is less evident this region (Cao et al., 2012). All photomicrographs were acquired on a Zeiss Imager A2 microscope with AxioCam MRc5 digital camera. The S1BF and S2 were imaged at 20× in the left and right hemisphere of two sections at approximately Bregma –2.40mm and Bregma –3.48mm. Images were acquired of the center of the ROIs, such that analysis did not include the border of other brain regions. Four images were obtained per ROI, per animal. Microglial cell counts were performed using Image J software (National Institute of Health, USA). A 1 cm² grid was applied to each image, and all microglia within the center four squares were counted using the cell counter tool, with a target count of 100 microglia per ROI per animal (distributed over the 16 squares in four images). For this communication microglia were categorized as one of four morphologies: ramified (small cell body, multiple fine processes), rod (polarized apical and basal processes), active (enlarged cell body, hyper-ramified), or amoeboid/macrophage (rounded, no processes) (Figure 2) (Bye et al., 2007; Kreutzberg, 1996; Taylor, Morganti-Kossmann, Lifshitz, & Ziebell, 2014; Ziebell et al.,

2012). The activated microglia category was inclusive of non-ramified, non-rod, and non-macrophage morphologies, as such these morphologies represented those that were shifted away from ramified, mostly towards de-ramified. However, we make no claims of subsets within the activated group.

Microglia proportions were calculated by counting each of the 4 morphologies in each photomicrograph. The number of each morphology was then summed across the 4 images. The percent of each morphology was then calculated for each animal before averaging each time point or treatment group. Proportions of microglia morphologies were collated for each group and graphs of mean proportions were created using Microsoft Excel (2007). For each morphology type in each group, the SEM was calculated using the microglia proportions in each group.

Statistical Analysis

Statistical analysis was conducted using two-tailed Fisher's exact tests. Each morphology was summed within a time point and/or treatment and compared to the total number of all remaining microglia morphologies. For Fisher's exact test analysis, GraphPad Prism version 6 for Windows (GraphPad Software, La Jolla, California, USA) was used. To meet the assumptions of the test, comparisons were not made when any cell in the contingency table had an expected value of fewer than 5 counts, indicated in tables 1–6. In order to account for multiple comparisons, adjusted significance levels of $P < 0.00056$ for the 90 comparisons made in the first cohort and $P < 0.001$ for the 50 comparisons made in the NEP(1-40) treated tissue were used.

Results

Microglial activation is a hallmark of brain injury; however, few investigations have distinguished the different microglia morphologies. In this study, we first analyzed the proportions of microglial morphologies across a time course following diffuse TBI. Using contemporary classifications that potentially relate to function, if not an indicator of neuropathology, microglia were identified as: ramified, small cell body with highly ramified, thin processes (Figure 2A); hyper-ramified, rounder cell body with bushy appearance of processes (Figure 2B); reactive, round cell body, reduced number of processes (Figure 2C); amoeboid microglia/macrophage, round cell body and no processes (Figure 2D); or rod microglia, highly polarized with reduced planar processes (Figure 2E). Due to the plethora of overlapping functions described for hyper-ramified and reactive morphologies, cell counts of these two morphologies were combined for this study as activated microglia.

A time-course of microglial activation was observed following TBI in comparison to sham for the S1BF and S2 regions (Figure 3A). In both the S1BF and S2 of sham-injured rats, microglia were predominantly ramified in morphology, with their processes forming a fine mosaic across cortical tissue. However, following injury the pattern of microglia activation differed in these two cortical regions. In the S1BF, as early as 2 hours post-injury, the population of ramified microglia had changed to predominantly rod and activated morphologies. By 1 day post-injury activated microglia started to become the predominant morphology which lasted through day 21. Despite this population shift towards activated

morphologies, there was still a robust proportion of rod microglia evident through all time points analyzed. Whereas in the S2, microglia populations tended to stay predominantly ramified with small proportions of activated and rod microglia evident in the first hours post-injury. By day 1 and 2 post-injury, a large proportion of ramified microglia had shifted to an activated state. By day 21 post-injury, microglia proportions had shifted back to a predominantly ramified state. These data indicate that microglia in the S1BF have a more robust shift in the proportions of each morphology than the S2.

Microglial proportions changed in the S1BF over time post-injury

In sham animals, ramified microglia were predominant, with only 8% activated microglia, with no rod or macrophage morphologies. Activated microglia increased in proportion post-injury, reaching a peak of 64% after 21 days in S1BF (Figure 3B). However, amoeboid microglia/macrophage presence reached a peak of 6% at 2 days post-injury (Figure 3B).

Rod microglia appeared as early as 2 hours post-injury in the S1BF, and were present through all post-injury time-points. As expected from prior work (Ziebell et al., 2012), rod microglia aligned perpendicular to the dural surface (Figure 3A). Rod microglia at 2 hours post-injury appeared to have fine apical and basal processes, and a thin cell body. By 6 hours post-injury, the rod microglia processes and cell bodies had become more linear. However, at days 1 and 2 post-injury, rod microglia had shorter and more highly ramified processes from both the polar and planar areas of the soma. At 7 days post-injury, rod microglia had the most robust response, not only being in the highest proportions, but also aligned perpendicular to the dural surface. At this time-point, some rods abutted one another to form trains that lay in the same trajectory, which mostly abated by 21 days post-injury.

The proportions of rod microglia, as a fraction of the population counted in the ROI in four nonconsecutive sections of cortex, changed over the post-injury time course (Figure 3B). At 2 hours post-injury, rod microglia peaked at 46% percent of the sampled population, decreasing to 43% by 6 hours post-injury. At 1–2 days post-injury, rod microglia only accounted for 16% of the sampled population. This increased to 33% by 7 days which subsequently decreased to 22% at 21 days post-injury. Therefore, the presentation of rod microglia was bi-phasic: elevated proportions at 2–6 hours and 7 days post-injury, with detectable, but lower proportions at 1, 2 and 21 days post-injury (Table 1; Figure 3C).

Ramified and activated microglia were the most prevalent microglia morphologies in the S2 post-injury

In the S2 cortex, Iba-1 immunohistochemistry revealed different proportions of microglial morphologies over the post-injury time-course than S1BF (Figure 3A). As in the S1BF, ramified microglia were the predominant morphology in sham-injured animals with a 5% proportion of activated microglia. An injury-induced increase in activated microglia was observed, which was most profound at days 1 through 7 post-injury. The proportion of activated microglia peaked at 52% by 2 days post-injury, when ramified microglia were at the lowest proportion of 42%. By day 21 post-injury, the proportion of ramified microglia had returned to over 90%. Amoeboid microglia/macrophages were present in small numbers, reaching only 1% of the total population from 1 to 2 days post-injury (Figure 3B).

Rod microglia were observed in the S2 post-injury, peaking at 6% of the total population by 1 day post-injury (Figure 3B). No significant differences in the proportions of rod microglia were observed in the S2 over the post-injury time course (Table 2; Figure 3C). These shifts in the proportions of microglia morphology support previous data published by our group (Lifshitz & Lisembee, 2012), which indicate this region undergoes minimal pathology following injury.

Reduced myelin antigenicity in the injured cortex through 1 day post-injury

Myelin that wraps axons would lie between the neuronal processes and adjacent rod microglia. As such, we examined the relationship between the presence of rod microglia and myelin-derived antigens in histological sections double-labeled with Iba1 and CNPase. Myelin antigenicity was identified as long, fine filamentous staining in the S1BF and S2 of uninjured sham animals (Figure 4).

After diffuse brain injury, characteristic myelin staining was altered in the cortex. Indicative of reduced antigenicity, CNPase staining was reduced, and long, fine filamentous staining was absent. This reduced quality of CNPase staining was observed through 1 day post-injury in the S1BF and S2 regions, indicating widespread reduction of myelin antigenicity, which mirrored neuropathology previously reported by silver staining (Lifshitz & Lisembee, 2012). By 2 days, intensity of CNPase staining had begun to return, with long, fine filaments becoming evident, suggesting return of myelin antigenicity. At 21 days post-injury staining appeared similar to sham, indicating subsequent restoration of myelin antigenicity (Figure 4). Similar myelin findings were observed between S1BF and S2, indicating that the proportions of activated microglia may not be associated with myelination, and vice versa. Adjacent sections were stained for CNPase and myelin basic protein (MBP), which corroborated the reduced myelin antigenicity observed with CNPase (Figure 5). These time-course data for myelin stain with respect to microglial activation indicate that the phenomenon of abnormal myelination is more widespread than the previously appreciated for the S1BF at day 7 post-injury (Ziebell et al., 2012).

In the S1BF, rod microglia aligned in the same trajectory as neuronal processes (perpendicular to the dural surface) regardless of myelination (Figure 5, insets). The present study investigated the relationship between rod microglia and myelin at multiple time points post-injury, whereas previously we had only reported on day 7. Contrary to previous findings, there was no evidence of reduced myelin antigenicity (CNPase or MBP) in close proximity to rod microglia (Figure 4 and 5). Moreover, there was no clear evidence of myelin ingestion by microglia in the present study.

Nogo and its receptor NgR were present in S1BF, but not S2, following injury

Myelin breakdown is associated with the release of neurite outgrowth inhibitor protein, Nogo. As disruption to normal myelin was evident throughout cortical regions post-injury, we next investigated the release of Nogo. Double-labelling immunofluorescence for Iba-1 and Nogo revealed low levels of Nogo staining in sham, which was as expected. These low levels of Nogo in the S1BF continues through to 2 hours post-injury. Subsequently, Nogo staining increased transiently in the S1BF from 6 hours to 2 days post-injury (arrows, Figure

6A). Peak Nogo accumulation occurred at 1 day post-injury in the S1BF, coinciding with the lower proportions of rod microglia relative to the early and delayed increase in rod microglia (Figure 6A). Few, if any, amoeboid microglia/macrophages were evident in this model of diffuse brain injury, and none co-localized with Nogo. Some activated microglia, however, co-labeled with Nogo in the S1BF at 1–2 days post-injury, suggesting that microglia with an activated morphology could phagocytose Nogo (Figure 6B). Additionally, when Nogo was present, rod microglia were at their lowest proportions and vice versa suggesting this morphology may be influenced by Nogo signaling.

Nogo was not evident in the S2 in sham-injured animals. As expected from the absence of rod microglia, minimal Nogo was found in the S2 after brain injury. Due to the low abundance Nogo and the lack of clustering of stained elements, the S2 region was determined to be devoid of staining (arrow, Figure 6A). Taken together, these data indicate that Nogo is present where neuropathology and microglial activation is high following diffuse TBI.

To further examine the role of Nogo signaling following diffuse TBI, Nogo-66 receptor (NgR) expression was examined via immunohistochemistry at a subset of time points. NgR was found when Nogo was not evident (2h and 7d post-injury) and at maximal expression (1d post-injury). In sham-injured rats, minimal NgR staining was observed, however by 2 hours post-injury punctate staining was seen in the cytoplasm and processes of cells with the size and shape of neurons in the S1BF (Figure 7). This staining was more intense at 1 day post-injury before declining in intensity at day 7. Similar to Nogo staining, NgR was negligible in the S2.

NEP(1-40) application shifted microglial populations in S1BF and S2

Since Nogo presence after brain injury was offset from peak rod microglia presentation, a NgR antagonist was administered to rats at time points prior to microglial activation in order to assess the role of Nogo in injury-induced activation of microglia, particularly rod microglia. NEP(1-40), an NgR antagonist, was delivered to animals at 15 minutes and 19 hours post-injury. These administration times were chosen to maximize NEP(1-40) efficacy: the injection at 15 minutes post-injury served to reduce NgR function immediately post-injury, whilst the injection at 19 hours post-injury allowed 5 hours for NEP(1-40) to take effect preceding the 1 day post-injury time point.

Just like with no treatment, vehicle treatment was associated with an injury-induced shift in population proportion of microglia from ramified to activated morphologies. Unlike no treatment (Figure 3B), which had a shift in proportions to rod microglia, DMSO vehicle treatment was associated with a shift to activated microglia (Figure 8A). This is not surprising, as microglial activation is exquisitely sensitive to any chemical disturbance. DMSO can cross the BBB and disturb the chemical balance, which has been shown as sufficient to activate microglia (Broadwell, Salcman, & Kaplan, 1982). The shift in proportions of microglia to an activated form rather than rod was still evident at 6 hours post-injury in vehicle group (Table 3 and 5; Figure 8A). At later time points (1 and 7 days), the proportion of rod microglia in the population was significant ($P < 0.0001$). At 2 days post-injury, the proportion of rod microglia was not significantly different from 1 day post-injury

($P=0.0510$; Table 5) or 7 days post-injury ($P=0.0067$; Table 5). For this study, the statistical results were in contrast to those for the untreated animals in the initial time course ($P<0.0001$; Table 1). Therefore, it is possible that the vehicle alone had an effect on rod microglia proportions post-injury.

Similar to vehicle treatment, NEP(1-40) treatment was associated with an injury-induced decrease in the proportion of ramified microglia post-injury (Figure 7). The proportion of ramified microglia was similar between vehicle and NEP(1-40) treated rats at 2 hours post-injury, however the proportions of activated and macrophage-like were increased (Table 4). Treatment with NEP(1-40) was associated with a bimodal increase in rod microglia proportions. Unlike vehicle treatment, the first significant increase in rod microglia proportions was at 6 hours post-injury (Table 6), indicating that the initial NEP(1-40) treatment altered the activation profile of microglia. In the NEP(1-40) treated S1BF, there was a significant increase in rod microglia proportions between 2 and 6 hours post-injury ($P<0.0001$), and between 6 hours and 1, 2, and 7 days post-injury ($P<0.0001$; Table 6). Also, between 1 and 2 days post-injury, there was a significant decrease in rod microglia proportions ($P=0.0026$; Table 6), contrary to untreated and vehicle treated animals ($P=0.3815$; Table 1; and $P=0.0510$; Table 5, respectively). There was a second increase in rod microglia proportions at day 7 compared to days 1 and 2 post-injury ($P<0.0001$).

Macrophage accumulation in outer cortical layers

A limitation of the current study was the use of DMSO in the vehicle. Compared to brain-injured untreated rats, the vehicle and NEP(1-40) treated rats had higher proportions of macrophages (Figure 3B compared to Figure 8). Due to the compromised blood-brain barrier (BBB) in the acute hours following diffuse brain injury, influx of macrophages was expected, however, the greater proportion observed in vehicle-treated rats was not. This increased proportion may have, in part, been due to DMSO. DMSO has been reported to disrupt the BBB at a dose of 1–4 g/kg (Broadwell et al., 1982) and it is plausible that the disturbed BBB was more sensitive to DMSO (~0.03 g/kg in this study) than previous reports. And then, the accumulation of macrophages at the outer cortical layers was even greater in NEP(1-40) treated, brain-injured rats, suggesting that NEP(1-40) had a combined effect on microglia (Figure 9).

Discussion

Diffuse brain injury results in neuropathology, which includes reduced myelin antigenicity and neuroinflammation (Gennarelli & Graham, 1998; Inglese et al., 2005; Ziebell & Morganti-Kossmann, 2010). In this study, we examined reduced myelin antigenicity, the consequent release of Nogo, and the relationship of these processes with microglial activation based on morphological states. Analysis focused on the S1BF, a region known to preferentially harbor neuropathology, and the S2, due to its more distal location from sites of principal neuropathology. The primary aim of the study was to determine if microglia of specific morphologies, specifically rod microglia, were associated with reduced myelin antigenicity. Moreover, we sought to determine whether blocking Nogo-A function with the NgR antagonist NEP(1-40) would alter the proportions of microglia morphology. No

specific microglial morphology was directly associated with the reduction of myelin antigenicity. However, the presence of Nogo was inverse to the presence of rod microglia, suggesting a role for the Nogo signaling cascade in rod microglia presentation.

In the current study, we found widespread reduction in myelin antigenicity throughout both the S1BF and S2 following diffuse brain injury, which peaked at 1 day post-injury. Previous reports have found that diffuse brain injury leads to widespread myelin loss and oligodendrocyte apoptosis (Flygt, Djupsjo, Lenne, & Marklund, 2013; Lotocki et al., 2011). Although immunohistochemistry is not routinely used as the sole marker for myelination, these results are consistent with previous findings in an experimental model of focal TBI; where diminished MBP isoforms were reported by western blot in the cortex as early as 2 hours post-injury. These isoforms reached the lowest levels at 2 days post-injury before recovering by 7 days (Liu et al., 2006). It has been hypothesized that microglia play a role in reduction of myelin antigenicity following injury, since active microglia/macrophages can exacerbate neurodegeneration by releasing reactive oxygen species and cytokines during phagocytosis of myelin (Williams, Ulvestad, Waage, Antel, & McLaurin, 1994). Further research suggests that activated microglia may also be essential for remyelination. For instance, activation of microglia by high doses of LPS does not elicit a neurotoxic response, despite the robust production of inflammatory molecules (Glezer, Simard, & Rivest, 2007). As microglial morphology has previously been shown to change following diffuse TBI (Cao et al., 2012; Taylor et al., 2014; Ziebell et al., 2012), we chose to investigate whether these morphological changes were associated with decreased myelin antigenicity. We hypothesized that rod microglia may be directly associated with decreased myelin antigenicity at 1 day post-injury, and the re-establishment of myelin thereafter, suggesting that microglial populations at that time have an immunoregulatory (M2) microglial phenotype (Miron et al., 2013). Despite our previous observations, the present results indicate that rod microglia had not relation with decreased myelin antigenicity. Changes in CNPase/MBP antigenicity were widespread, with reduced staining in the S1BF and to a lesser extent the S2. However, microscope fields of Nogo (a myelin byproduct) were seen only in the S1BF post-injury, suggesting a unique pathophysiological milieu. The S1BF has been reported to show selective neuropathology (silver staining) and microglial activation (Cao et al., 2012; Lifshitz & Lisembee, 2012; Ziebell et al., 2012). Taken together, activation of the Nogo/NgR pathway may be uniquely associated with substantial neuropathology and neuroinflammation, including activation of microglia, after diffuse TBI.

Activated microglia and macrophages are reported to reside in the injured brain for weeks, or even years post-injury (Cao et al., 2012; Johnson et al., 2013). The appearance of rod microglia following diffuse brain injury is a subject that has only recently been explored in greater detail (Ziebell et al., 2012); (Wierzba-Bobrowicz et al., 2002). Their orientation and close anatomical association with neurons suggest that rod microglia may be providing structural support for damaged neurons, simply migrating through cortical tissue, or serving as a trophic bridge. Alternately, involvement of all microglia in the process of synaptic stripping may merely be “guilt by association”, since it is a neuron autonomous event (Perry & O’Connor, 2010). Conversely, microglia have a neurotrophic role in promoting axonal sprouting and synaptic plasticity (Batchelor et al., 1999; Batchelor et al., 2002).

Here, rod microglia accumulate as soon as 2 hours post-injury, and follow a bimodal distribution across the post-injury time-course. The appearance of rod microglia as early as 2 hours post-injury in diffuse brain injury indicates that signaling for rod microglia formation must develop immediately following TBI. As a second increase in rod microglia occurs 7 days post-injury, waxing and waning signals are likely involved in the formation and remission of rod microglia. Since a bimodal mechanism for microglial activation has not been previously reported, the question remains as to whether the rod microglia at 2 hours are in a different activation state to the rod microglia at 7 days post-injury. The activation and deactivation signals may be autocrine or paracrine, as mediated by cytokines, chemokines, or other cell-cell signaling molecules. The current study limited its analysis of microglia to morphology alone, which does not take into consideration the potential for morphologically similar microglia expressing different markers. By deciphering the neuro-glial signals necessary for each microglial morphology, further understanding and pharmacological control over the injury-induced inflammatory processes become clearer.

The rod microglia morphology may be merely transient; Franz Nissl suggested they were an initial activated microglial state (Nissl, 1899). This may not be the case, since rod microglia persist in the brain for weeks after the initial insult (Ziebell et al., 2012). In addition, rod microglia reside alongside activated microglia in subacute sclerosing panencephalitis (SSPE), Alzheimer's and Wilson's disease, regardless of disease duration (Wierzbak-Bobrowicz et al., 2002). The evidence suggests that rod microglia are not merely a transitional state between ramified and active phenotypes, but a unique independent phenotype based on morphology.

Neuropathology would include damage to oligodendrocytes, myelin/axonal debris, and the release of Nogo. The Nogo observed in the S1BF at 1 day post-injury likely represents axonal and/or oligodendroglial damage, as evidenced by the reduction in CNPase and MBP antigenicity. This was confirmed by repeat staining of both antigens of interest simultaneously, with positive controls. Moreover, hematoxylin and eosin staining of this tissue showed preserved neuronal cell bodies suggesting tissue quality was not an issue. Unexpectedly, this decreased myelin antigenicity was only associated with the expression of Nogo in the S1BF. Future studies can explore double labelling immunofluorescence for Nogo and oligodendrocytes. Co-labelling of Iba-1 positive microglia for Nogo suggests that active microglia may phagocytose myelin debris containing Nogo in the S1BF in order to clear the region, hence providing a more permissible environment. The low incidence of microglia with ingested Nogo suggests a minor role in this capacity. Further, Nogo-A may strongly influence restoration of myelin antigenicity post-injury (Chong et al., 2012; Pernet, Joly, Christ, Dimou, & Schwab, 2008). When present in myelin/axon debris, Nogo-A also reduces the capacity for axon regrowth following injury. Treatment approaches to remove myelin and associated growth-inhibitory factors are proposed to facilitate axon regeneration (Tanaka, Ueno, & Yamashita, 2009), potentially via activated microglia.

In a model of middle cerebral artery occlusion (MCAO), inhibition of Nogo-A by intracerebroventricular infusion of NEP(1-40) decreased neuronal loss and cell death, as well as the proliferation of oligodendrocytes and astrocytes (Wang et al., 2012). Concomitant GAP-43 and MAP-2 labelling of processes suggested that Nogo inhibition aided in network

remodeling. Midline FPI is not associated with substantial cell death (Lifshitz et al., 2007), thus NEP(1-40) treatment was not assessed in conjunction with neuronal density or cell death. Nogo staining in the NEP(1-40) treated brains showed no reduction in Nogo (data not shown), as expected since NEP(1-40) is a competitive binding agent for NgR rather than a Nogo inhibitor. In this study, only the effects of NEP(1-40) on microglia proportions were analyzed to determine whether treatment shifted microglial proportions. Future analysis will examine the effects on other markers of neurodegeneration and neuropathology.

Neurological recovery following injury to the adult CNS is often incomplete, due in part to the failure of axons to regenerate. Blocking Nogo/NgR function can be neuroprotective as it allows for a more permissive environment for axonal regeneration. To this end, NgR1 knockout mice have enhanced recovery of cognitive function after TBI compared to wild-type mice, using a CCI model (Tong et al., 2013). Others have reported impaired functional recovery of NgR1 KO mice or mice treated with soluble NgR1 following CCI (Hanell et al., 2010). Moreover, aged Nogo-A/B mice subjected to CCI also displayed impaired functional recovery (Marklund et al., 2009). On the other hand, when Nogo-A was blocked with mAb 7B12 following lateral FPI in rats, the neutralizing antibody treatment improved cognitive (but not motor) function compared with brain-injured, vehicle-treated controls (Marklund et al., 2007). More research on the therapeutic efficacy of Nogo/NgR signaling in recovery is needed. Given the conflicting results with Nogo investigations in TBI (including our own), it remains unclear what role rod microglia may play in the pathophysiology of TBI. Additional studies are required to determine whether the changes in microglial activation profiles reported here are associated with improved functional outcome.

Recent evidence suggests that that NgR is expressed on non-neuronal cells including microglia. Nogo-A itself has repulsive or anti-adherent effects on microglia, thereby reducing migration (Yan et al., 2012). Indeed, immunohistochemical results suggest that an influx of macrophages at the edge of the S1BF with administration of NEP(1-40) (Figure 9). Nogo-A may therefore have an important role in reducing macrophage infiltration after TBI. However, blocking Nogo-A function did not have an effect on rod microglial formation, suggesting rod microglia are a morphology from endogenous brain microglia rather than infiltrating from the periphery. Myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) are other proteins involved in the same signaling cascade as Nogo. These proteins have also been shown to bind to NgR and inhibit neurite growth by activating RhoA and inhibiting Rac1 (Sato et al., 2005). It is therefore possible that these proteins compensated for the Nogo/NgR pathway upon NEP(1-40) administration. Further studies which examine changes in MAG and OMgp expression in the S1BF following mFPI, in relation to rod microglial distribution, are required to more fully elucidate this relationship.

Conclusions

Data from this and previous studies indicate that diffuse TBI is associated with decreased myelin antigenicity and inflammation. The proportions of microglial morphologies represent time dependent alterations in inflammation, which are necessary for targeted therapies.

Moreover, by-products of myelin influence microglial activation, such that a Nogo receptor antagonist NEP(1-40) treatment shifted microglial proportions post-injury.

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Highlights

- Diffuse traumatic brain injury is associated with inflammation and reduced myelin antigenicity
- Diffuse brain injury shifts the distribution of microglia morphologies
- NEP(1-40) treatment shifted microglia proportions away from ramified to fully activated/macrophage
- Myelin by-products resulting from brain injury impact microglia distributions

Figure 1. Schematic timeline of traumatic brain injury and tissue collection

Male rats were subjected to traumatic brain injury or sham injury. At 3h, 6h, 1d, 2d, 7d, or 21d post-injury brain tissue was collected and analyzed via immunohistochemistry (A). In a second experiment (B), male rats were subjected to TBI or sham injury and treated with NEP(1-40) or vehicle at 15 minutes and 19 hours post-injury. Brain tissue was collected at 3h, 6h, 1d, 2d, or 7d post-injury for analysis via immunohistochemistry.

Figure 2. Morphological classifications of microglial activation states following diffuse brain injury

Ramified microglia had fine processes and smaller cell bodies (A). Hyper-ramified microglia had an enlarged cell body with processes of thicker nature which had retracted from the surrounding microenvironment (B). Reactive microglia had enlarged cell bodies and a reduction in process number (C). Amoeboid microglia/macrophages had no processes (D), whereas rod microglia had polarized apical and basal processes (E). Hyper-ramified and reactive microglia are considered as activated for quantification.

observed in the S1BF at every post-injury time point. Rod microglia were detectable in the S2 (C).

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Figure 4. CNPase antigenicity was not associated with rod microglia

Representative 20× images of double labelling immunofluorescence for Iba-1 (microglia) and CNPase (myelin) show that decreased myelin antigenicity was most evident in the S1BF and S2 at 1 day post-injury, indicated by a reduced quality of CNPase staining. Staining quality for CNPase returned by 2 days post-injury, and appeared most similar to sham at 21 days post-injury. Decreased myelin antigenicity did not occur in close proximity to rod microglia, rather changes in CNPase antigenicity were widespread. All sections were stained in a single batch.

Figure 5. MBP antigenicity was not associated with rod microglia

Representative 20× images of double labelling immunofluorescence for Iba-1 (microglia) and MBP (myelin) show that decreased myelin antigenicity was most evident in the S1BF and S2 at 1 day post-injury, indicated by a reduced quality of MBP staining. Staining quality for MBP returned by 7 days post-injury. Insets (40×) show rod microglia aligning to myelinated axons at 2 hours and 7 days post-injury in the S1BF. Decreased myelin antigenicity did not occur in close proximity to rod microglia.

Figure 6. Nogo presence peaked in the S1BF at 1 day post-injury

Representative images of double labelling immunofluorescence for Iba-1 (microglia) and Nogo A/B revealed Nogo in low levels within sham-injured brain, but began to accumulate in the S1BF at 6 hours post-injury and remained until 2 days post-injury (arrows). This coincided with fewer rod microglia (A). Nogo was occasionally present in the S2 between 6 hours and 2 days post-injury (arrow) (A). Some activated microglia and macrophages co-labelled for Nogo in the S1BF, suggesting that these cells were clearing Nogo from the area by phagocytosis (B).

Figure 7. Nogo receptor was evident in the S1BF post-injury

Representative images of DAB labelling for Nogo receptor (NgR). NgR was observed as punctate staining within processes and cell bodies; these cells were most likely neurons. Maximal staining was observed at day 1 post-injury.

Figure 8. NEP(1-40) did not affect proportions of rod microglia in the S1BF post-injury
 Graphs show mean proportions of macrophages (blue), active (red), rod (purple) and ramified (green) microglia in vehicle treated and NEP(1-40) treated animals over the post-injury time course. In the S1BF, NEP(1-40) treated animals had significantly different proportions of rod microglia between 1 and 2 days post-injury ($p=0.0026$) and 2 and 7 days post-injury ($p<0.0001$) whereas vehicle treated did not ($p=0.0510$ and 0.0067 respectively). However, untreated animals in the original post-injury time course had significantly different proportions of rod microglia between 2 and 7 days post-injury, raising the question of whether the vehicle itself had an effect on rod microglia proportions.

Figure 9. NEP(1-40) administration appeared to increase number of amoeboid microglia/macrophages at 2 days post-injury

Representative images of Immunohistochemistry for Iba-1 (microglia) revealed infiltration of amoeboid microglia/macrophages (black arrow heads) at the edge of the S1BF at 2 days post-injury in NEP(1-40) treated brains. Although regions analysed at 20× in the inner S1BF in vehicle (Ai) and NEP(1-40) (Bi) treated brains revealed similar macrophage proportions, outer regions in vehicle (Aii) and NEP(1-40) (Bii) brains appeared to have different macrophage proportions. This qualitative evidence suggests that NEP(1-40) may promote infiltration of amoeboid microglia/macrophages at the edge of the S1BF.

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Fishers Exact test *P*-values for comparing the proportions of rod microglia versus non-rod microglia in the S1BF at each post-injury time point

Table 1

Significant results are indicated in bold font. There were 2 distinct populations of rod microglia; elevated proportions at 2 and 6 hours and 7 days post-injury which were significantly different to the detectable but lower proportions at 1, 2 and 21 days post-injury. Since the elevated group interweaved with the detectable group, a bimodal distribution of rod microglia proportions was indicated with peak proportions at 2 hours and 7 days post-injury. The sham group was not compared to any injury group since the expected number of rod microglia in sham animals was fewer than 5; hence the assumptions of the Chi-squared test were not met. Results were considered significant at $P < 0.00056$; n.t. = not tested.

	Sham	2h FPI	6h FPI	1d FPI	2d FPI	7d FPI	21d FPI
Sham	x	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
2h FPI		x	0.3406	<0.0001	<0.0001	0.0002	<0.0001
6h FPI			x	<0.0001	<0.0001	0.0121	<0.0001
1d FPI				x	1.0000	<0.0001	0.1279
2d FPI					x	<0.0001	0.0728
7d FPI						x	<0.0001
21d FPI							x

Significance: 6 groups being compared (2h, 6h, 1d, 2d, 7d, 21d); 4 morphologies per group = $15 \times 6 = 90$ comparisons. $0.05/90 = \mathbf{0.00056}$ (3 s.f.)

Fishers Exact test *P*-values for comparing the proportions of rod microglia versus non-rod microglia in the S2 at each post-injury time point

Table 2

Rod microglia were present in the S2, but not in meaningful proportions. There were no significant changes in rod microglia proportions over the post-injury time course. The sham and 21 days post-injury groups were not compared to other groups since the expected numbers of rod microglia in these groups were fewer than 5; hence the assumptions of the Chi-squared test were not met. Results were considered significant at $P < 0.00056$, n.t. = not tested.

	Sham	2h FPI	6h FPI	1d FPI	2d FPI	7d FPI	21d FPI
Sham	x	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
2h FPI		x	0.8125	0.3970	1.0000	0.0211	n.t.
6h FPI			x	0.6674	0.8052	0.0093	n.t.
1d FPI				x	0.3815	0.0012	n.t.
2d FPI					x	0.0370	n.t.
7d FPI						x	n.t.
21d FPI							x

Significance: 6 groups being compared (2h, 6h, 1d, 2d, 7d, 21d); 4 morphologies per group = $15 \times 6 = 90$ comparisons. $0.05/90 = \mathbf{0.00056}$ (3 s.f.)

Table 3
Fishers Exact test *P*-values for comparing the proportions of activated microglia versus non-activated microglia in the S1BF of vehicle treated animals

The proportions of activated microglia were similar at all time points post-injury. Results were considered significant at $P<0.00167$.

	Sham	2h FPI	6h FPI	1d FPI	2d FPI	7d FPI
Sham	x	n.t.	n.t.	n.t.	n.t.	n.t.
2h FPI		x	0.5843	0.7679	0.7823	0.0083
6h FPI			x	0.8493	0.7903	0.0337
1d FPI				x	0.0263	1.0000
2d FPI					x	0.0164
7d FPI						x

Significance: 5 groups being compared (2h, 6h, 1d, 2d, 7d); 4 morphologies per group = $5 \times 10 = 50$ comparisons. 0.05/50 = **0.001** (3 s.f.)

Table 4
Fishers Exact test *P*-values for comparing the proportions of activated microglia versus non-activated microglia in the S1BF of NEP (1-40) treated animals

Similarly to the vehicle treatment the proportion of activated microglia was similar at all time points post-injury, except for 2 hours compared to 2 days ($P=0.0007$). Results were considered significant at $P<0.00167$.

	Sham	2h FPI	6h FPI	1d FPI	2d FPI	7d FPI
Sham	x	x	n.t.	n.t.	n.t.	n.t.
2h FPI		x	0.3681	0.3152	0.0007	0.2224
6h FPI			x	0.8190	0.0018	0.6693
1d FPI				x	0.0128	0.9345
2d FPI					x	0.0151
7d FPI						x

Significance: 5 groups being compared (2h, 6h, 1d, 2d, 7d); 4 morphologies per group = $5 \times 10 = 50$ comparisons. $0.05/50 = \mathbf{0.001}$ (3 s.f.)

Table 5

Fishers Exact test *P*-values for comparing the proportions of rod microglia versus non-rod microglia in the S1BF of vehicle treated animals

There was a significant difference in rod microglia proportions between 1 day and 7 days post-injury, similar to untreated animals. Contrary to no treatment there was no significant difference in rod microglia proportions at any other time point tested. Results were considered significant at $P<0.00278$.

	Sham	2h FPI	6h FPI	1d FPI	2d FPI	7d FPI
Sham	x	n.t.	n.t.	n.t.	n.t.	n.t.
2h FPI		x	0.6599	0.0256	0.8196	0.0182
6h FPI			x	0.0063	0.4484	0.0486
1d FPI				x	0.0510	<0.0001
2d FPI					x	0.0067
7d FPI						x

Significance: 5 groups being compared (2h, 6h, 1d, 2d, 7d); 4 morphologies per group = $5 \times 10 = 50$ comparisons. 0.05/50 = **0.001** (3 s.f.)

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Table 6
Fishers Exact test *P*-values for comparing the proportions of rod microglia versus non-rod microglia in the S1BF of NEP(1-40) treated animals

There was a significant difference in rod microglia proportions between 2 hours and 6 hours post-injury ($P=0.0011$) as well as 2 days ($P<0.0001$). At 6 hours post-injury, rod microglia proportions were significantly different to day 1 ($P<0.0001$) and day 2 ($P<0.0001$) post-injury. Whereas day 7 was different to day 1 ($P<0.0001$) and day 2 ($P<0.0001$). There was also a significant decrease in rod microglia proportions between 1 and 2 days post-injury ($P=0.0026$). Results were considered significant at $P<0.00278$.

	Sham	2h FPI	6h FPI	1d FPI	2d FPI	7d FPI
Sham	x	n.t.	n.t.	n.t.	n.t.	n.t.
2h FPI		x	<0.0001	0.0003	<0.0001	0.7620
6h FPI			x	<0.0001	<0.0001	<0.0001
1d FPI				x	0.0026	<0.0001
2d FPI					x	<0.0001
7d FPI						x

Significance: 5 groups being compared (2h, 6h, 1d, 2d, 7d); 4 morphologies per group = $5 \times 10 = 50$ comparisons. 0.05/50 = **0.001** (3 s.f.)