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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 2 h, 6 h, 1 d, 2 d, 7 d, and 21 d 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 1 d 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 2 h 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 2 h, 6 h, 1 d, 2 d, or 7 d post-injury. NEP(1–40) administration further shifted distributions of microglia away from an injury-induced activated morphology toward greater proportions of rod and macrophage-like morphologies compared to vehicle-treated. By 7 d post-injury, no 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.** 

**Key words: 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 et al., 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 and 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 (Satoh et al., 2005; Fry et al., 2007; David et al., 2008; 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 (GrandPre et al., 2002). Recently, Nogo proteins have been found to regulate neuronal precursor migration, neurite growth, and branching

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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 et al., 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 and Majewska, 2011; Tremblay et al., 2011; Wake et al., 2013). Whereas, hyper-ramified and activated microglia have been proposed to release cytokines and chemokines which drive secondary injury cascades (Ziebell and 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 et al., 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.

## EXPERIMENTAL PROCEDURES

### 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 (Lifshitz et al., 2007; Hosseini and Lifshitz, 2009; Lifshitz and Lisembee, 2012). Briefly, rats were anesthetized with 5% isoflurane in 100% $O^2$ 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