PERSPECTIVE

# Physical interactions between activated microglia and injured axons: do all contacts lead to phagocytosis?

Axonal injury is a pathological hallmark of both head injury and inflammatory-mediated neurological disorders, including multiple sclerosis (Schirmer et al., 2013). Such axonal disruptions and/or disconnections typically result in proximal axonal segments that remain in continuity with the neuronal soma while losing contact with their distal targets. These disconnected axonal segments contribute to loss of signal transduction and overall circuit disruption, via subsequent deafferentation. Due to the disruption of anterograde transport in this cohort of axons, immunolabeling of the normally transported protein, amyloid precursor protein (APP), in post-mortem brain tissue is the most commonly used method for the visualization of the swollen end of proximal axonal segments. While proximal axonal segments remain connected to the neuronal cell body, axonal segments distal to the point of injury progress to anterograde Wallerian degeneration, forming myelin and axonal debris that negatively affect the surrounding tissue (Vargas and Barres, 2007; Mietto et al., 2015).

Wallerian debris signals the resident microglia within the central nervous system (CNS) to activate and become phagocytic. Phagocytosis of this Wallerian debris by microglia, and later by peripheral monocytes, in instances of blood-brain barrier breakdown, has been well characterized and is regarded as one of the main beneficial effects of acute neuroinflammation in both the healthy and injured brain. Specifically, mutations of the Mecp2 or Trem2 genes in microglia, resulting in reduced or absent phagocytic function, are correlated to the negative outcomes of Rett syndrome or Alzheimer's respectively (Chen and Trapp, 2015; Mietto et al., 2015). Microglia, however, have multiple roles, apart from phagocytosis. Within the healthy brain surveying microglia have highly ramified morphologies and are dynamic cells that, as the name suggests, survey the immediate environment and form brief, but frequent contacts with axonal segments (Kumar and Loane, 2012; Eyo and Wu, 2013). Virtually nothing is known regarding the molecular mechanisms mediating microglial process interactions with axons, however, microglial signaling molecules, such as fractalkine, DAP12 and complement proteins, are vital for the proper formation and maintenance of neuronal circuits. Further, the absence of microglia in the normal brain results in a host of negative effects, including extensive developmental defects and loss of neuronal electrophysiological adaptation to inflammatory signals (Eyo and Wu, 2013).

Upon activation, microglia undergo distinct morphological changes, transforming from highly ramified phenotypes to microglia, with truncated processes, larger cell bodies, and less complex process networks or amoeboid morphologies. As noted previously, phagocytosis by morphologically simple or amoeboid phagocytic microglia is vital for clearance of debris from degenerating neurons and distal axonal segments (Chen and Trapp, 2015; Mietto et al., 2015). These phagocytic activities have been the primary focus of axon/microglial associations following injury. In contrast, knowledge regarding interactions between activated microglia and the disconnected swellings of proximal axonal segments has been extremely limited. Accordingly, our recent study began to explore this interaction (Lafrenaye et al., 2015).

Using an adapted central fluid percussion injury model of mild traumatic brain injury we evaluated the extent of axonal injury and microglial activation in the micro pig 6 hours following injury. This mild central fluid percussion injury model has been well characterized and is routinely used to generate diffuse axonal injury in rodents; however, to our knowledge this was the first recorded use of this mild injury paradigm within the adult micro pig. Due to the similarity in neuroanatomy and systemic immune response between pigs and humans, this could constitute a highly clinically relevant model system for the study of axonal injury in an experimental setting. Systemic physiological readings of blood pressure, heart rate, temperature, hemoglobin oxygen saturation and blood gasses were rigorously monitored and maintained within normal ranges to ensure that brain pathology was not complicated by systemic physiological abnormality (Lafrenaye et al., 2015). Mild diffuse central fluid percussion brain injury did not produce gross pathology, such as contusion or hematoma formation. Injured axons, however, were found diffusely scattered throughout the thalamic domain (Lafrenaye et al., 2015). These same thalamic sites also demonstrated robust microglial activation, determined by both expression of ionized calcium-binding adapter molecule 1 (Iba-1) and morphological characteristics consistent with microglial activation (Kumar and Loane, 2012).

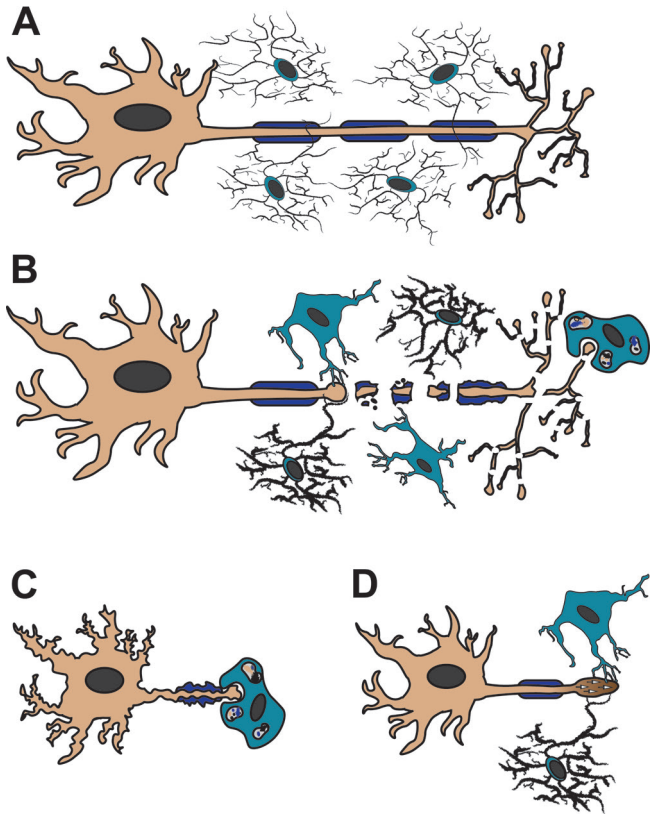


**Figure 1 Acute microglial process convergence on proximal swellings of injured axons could indicate either pre-phagocytic or neurotropic microglial response.**
(A) In the uninjured micro pig, thalamus ramified processes of surveying microglia were found in contact with normal myelinated axons. (B) Acutely (6 hours) following injury, processes of activated microglia converge onto the proximal swelling of injured axons. (C) This interaction could be a prequel to phagocytic engulfment of the proximal axonal segment, following retrograde degeneration, by phagocytic microglia. (D) Alternatively, acute microglial process convergence on the proximal axonal swelling could promote axon regeneration.

Due to this close mapping of axonal injury and microglial activation acutely post-injury, the number of surveying microglial contacts on normal myelinated axons within the thalamus of sham-injured micro pigs or the number of activated microglial contacts on the disconnected swelling of the proximal segment of injured myelinated axons was assessed. Analysis was done on 3D reconstructed confocal images triple-labeled for Iba-1, to identify microglia, myelin basic protein, to identify myelinated axons, and APP, to identify the tip of the proximal axonal segment (Lafrenaye et al., 2015). Immunolabeling of APP was used to identify this discrete region of the injured proximal axonal segment, as it is at the tip of the proximal axonal segment where potential axon regeneration has been observed previously (Schirmer et al., 2013). As illustrated in **Figure 1**, we found that while surveying microglia only made occasional contacts with normal myelinated fibers in the sham-injured micro pig (**Figure 1A**), processes of activated microglia converged on the proximal swelling of disconnected axons 6 hours following injury (Lafrenaye et al., 2015). Microglia demonstrating process convergence on damaged proximal axonal swellings did not contain any signs of engulfed debris, which is the hallmark of active phagocytosis.

It is possible, due to the single acute time point assessed in this study, that the observed acute microglial process convergence could represent a pre-phagocytic contact on injured axons undergoing early stages of retrograde degeneration (**Figure 1C**). As mentioned previously, phagocytic microglia have been well documented to engulf debris from damaged axons and neurons undergoing cell death in the healthy brain as well as following injury (Trapp et al., 1998; Oehmichen et al., 1999; Mietto et al., 2015). Indeed scattered phagocytic microglia were observed at 6 hours post-injury in our micro pig model, specifically in areas with demonstrable degeneration (as illustrated in **Figure 1B**; Lafrenaye et al., 2015). The lack of engulfed debris within the microglial processes that contact the proximal swelling of injured axons, however, indicates that this population of microglia is not phagocytic (Lafrenaye et al., 2015). Further, both retrograde axonal degeneration and the majority of anterograde Wallerian degeneration progress days following injury, with phagocytic microglial activation in the CNS occurring after axonal degeneration (Vargas and Barres, 2007; Mietto et al., 2015). The physical interactions between processes of activated microglia and proximal swellings of damaged axons, however, were observed very acutely at 6 hours post-injury suggesting that this interaction is not phagocytic in nature due to the later timing of phagocytosis previously observed following CNS injury. While additional studies exploring these interactions more chronically post-injury would be required to reach any conclusions regarding the ultimate nature of the microglia involved, it appears less likely that this acute association between microglia and injured axons is a precursor to phagocytic engulfment.

Alternatively, the observed acute microglial process convergence may represent a non-phagocytic association, as depicted in **Figure 1D**. Microglia are highly dynamic even in their ramified, surveying state, and have been shown to contact axons in response to molecular signals as well as neuronal electrophysiological activity in both the healthy and injured brain (Kumar and Loane, 2012; Eyo and Wu, 2013). Processes of surveying and activated microglia converge on the soma of hyperactive neurons via a pathway involving glutamate, NMDA receptors and the purinergic receptor, P2Y12, in epileptic animals. This somatic microglial process convergence is associated with decreased seizure activity (Eyo et al., 2014). Activated microglial processes have also been shown to rapidly converge on neurons following laser ablation via an ATP regulated pathway (Kumar and Loane, 2012). These microglial responses utilize two distinct and independent signaling pathways to manifest process convergence (Eyo et al., 2014), indicating that process convergence is associated with a variety of microglial functions and can be stimulated by multiple of mechanisms.

Activated microglia are traditionally differentiated into classically activated M1, inflammatory microglia, or alternatively activated/ anti-inflammatory M2 microglial subtypes. While controversial, mounting evidence suggests that the alternatively activated, M2, microglia play a neuroprotective role (Kumar and Loane, 2012; Chen and Trapp, 2015). Following CNS injury activated M2 microglia have been shown to produce insulin-like growth factors that promote neurogenesis and help to suppress pro-inflammatory cytokines (Kumar and Loane, 2012; Chen and Trapp, 2015), possibly promoting axon regrowth (**Figure 1D**). The association of microglial process convergence with a particular microglial subtype is currently unknown, however, the possibility that microglial process convergence on injured axons is potentially linked to the M2 phenotype warrants further investigation.

A recent study found that there is also a subtype of surveying microglia that preferentially interacts with the initial segment of axons in the non-injured rodent brain (Baalman et al., 2015). The interaction between these microglial processes and axon initial segments appears to be unaffected by brain injury and does not result in phagocytosis. The proportion of this AXIS (axon-initial segment associated) type of microglia is, however, variable among brain regions, with a reduced number of AXIS microglia in the thalamus as compared to the cortex (Baalman et al., 2015). Additionally, another group has been exploring a morphologically unique subtype of activated microglia. This group found that multiple activated rod-shaped microglia, in close proximity to each other, form "trains" that run along axons in the rodent cortex weeks following diffuse brain injury (Ziebell et al., 2012). These non-phagocytic rod microglia have also been shown to align with and wrap around apical dendrites of non-degenerating pyramidal neurons in cases of neurosyphilis (Graeber, 2010; Chen and Trapp, 2015). The studies of both the AXIS and rod microglia suggest that different brain regions and microglia subtypes could manifest different non-phagocytic axon/microglial associations following injury. Based on the non-rod-shaped morphology of the microglia assessed in our recent study, as well as their localization within the thalamic domain where AXIS microglia are sparse, it appears that the microglia investigated in the current study were neither AXIS nor rod microglia, leaving the subtype of the activated microglia assessed in our study undetermined.

Additionally, while not an inflammatory-mediated injury, as in multiple sclerosis, neuroinflammation and axonal injury are present weeks and even years following traumatic brain injury in the human population with a suggestion that these two processes are directly associated. In multiple sclerosis, microglial processes have been found in direct contact with swellings of injured axons in areas around active lesions in the human population (Trapp et al., 1998). Additionally, it has recently been theorized that the higher level of microglial activation following multiple sclerosis could be linked to a greater amount of axon regeneration observed in multiple sclerosis tissue as compared to tissue from people who suffered traumatic brain injury (Schirmer et al., 2013).

Ultimately, while the study of non-phagocytic axon-microglial interactions following injury is still in its infancy and therefore the information presented here is primarily speculative, the possibilities bear significant clinical relevance. Our recently reported work demonstrated that activated microglia contact

injured axons directly within 6 hours of injury. Due to recent technological advancements in the imaging of activated microglia via positron emission tomography (PET) scanning (Folkersma et al., 2011) this association could be exploited as a surrogate marker of axonal injury in the human population, that would not rely on post-mortem brain samples for assessment. Knowing the extent and localization of microglial activation, and thus axonal injury, in the living patient could give clinicians unique insight into brain pathology in different disease states that could previously only be speculated. Information regarding the amount and location of axonal injury via the surrogate marker of microglial activation could also be used to direct therapeutic interventions acutely in the course of a disease or following an injury. Additionally, based on the hypothesis of Schirmer et al. (2013) that increased neuroinflammation could be enhancing neuroregeneration, combined with the current finding of microglial process convergence on injured axons (Lafrenaye et al., 2015), the possibility that acute microglial activation is beneficial not only as a mechanism of clearing away damaging Wallerian degeneration *via* phagocytosis, but also as a means of enhancing regeneration through physical contact, could drastically alter current perceptions of neuroinflammation and precipitate the development of new therapeutics for the treatment of axonal injury.

*The work at the center of the current manuscript was performed as a component of the Operation Brain Trauma Therapy consortium, which is supported by U.S. Army grants W81XWH-10-1-0623 and WH81XWH-14-2-0018. Microscopy was performed at the VCU Department of Anatomy and Neurobiology Microscopy Facility, supported, in part, with funding from NIH-NINDS Center core grant 5P30NS047463.*

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***orcid:** 0000-0001-5986-2228 (Audrey D. Lafrenaye)*
***doi:** 10.4103/1673-5374.180726* ***http**://www.nrronline.org/*

***How to cite this article:** Lafrenaye AD (2016) Physical interactions between activated microglia and injured axons: do all contacts lead to phagocytosis? Neural Regen Res 11(4):538-540.*

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