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PERSPECTIVE

Reassembly of the axon initial segment and nodes of Ranvier in regenerated axons of the central nervous system

Myelinated axons of the peripheral and central nervous system (PNS & CNS) are divided into molecularly distinct excitable domains, including the axon initial segment (AIS) and nodes of Ranvier. The AIS is composed of a dense network of cytoskeletal proteins, cell adhesion molecules, and voltage gated ion channels and is located at the proximal most region of the axon (Kole and Stuart, 2012). The primary functions of the AIS are to both maintain neuronal polarity by regulating vesicular traffic across the somatodendritic and axonal domains as well as initiate the action potential when sufficiently depolarized by integrated postsynaptic potentials (Zollinger et al., 2015). Nodes of Ranvier, which are structurally similar to the AIS, are found adjacent to myelin sheaths and are interspersed at regular intervals along the axon. Enriched with voltage gated ion channels, the primary function of the node is to regenerate the action potential as it propagates along the myelinated axon away from the soma (Rasband and Peles, 2016). Thus, AIS and nodes play critical roles in normal neural physiology and their specific disruption has been implicated as a common pathology in a wide spectrum of neurological disorders including stroke, epilepsy, and traumatic injury (Schafer et al., 2009; Baalman et al., 2013).

There is considerable interest in axon regeneration as a therapeutic tool for CNS injury and disease. Consequently, much effort has been invested in teasing out and understanding the various molecular mechanisms that are associated with axon degeneration and regeneration in the CNS. What is now understood is that axons of the CNS are mostly incapable of regenerating themselves after traumatic injury. The CNS responds to injury by differentially regulating a variety of genetic survival and growth programs that often result in neuronal death. As axons degenerate, unmetabolized growth inhibitory myelin debris and chondroitin sulfate proteoglycans released from the glial scar accumulate in the extracellular space hindering axon regrowth (Benowitz et al., 2016). Nevertheless, several genetic and pharmacological axon regeneration models have been developed which demonstrate robust axon regrowth past the lesion site and in some instances, reinnervation of downstream CNS targets. For example, Baldwin et al. (2015) demonstrated that intravitreal injection of β (1,3) glucan, a proinflammatory dectin-1 ligand, induces robust axon regeneration after optic nerve crush. Furthermore, de Lima et al. (2012) demonstrated that genetic deletion of phosphatase and tensin homolog (PTEN) in retinal ganglion cells (RGCs) coupled with intravitreal injection of zymosan, a proinflammatory yeast cell wall suspension, and 4-(chlorophenylthio) adenosine cyclic AMP (CPT-cAMP) in an optic nerve crush model significantly increases RGC survival, promotes axon regeneration into the dorsal lateral geniculate, and restores some basic forms of visual behavior.

These remarkable observations have set the stage for the development of future therapeutic strategies and provide a platform to answer deeper questions about CNS axon regeneration. In particular, a major unanswered question is whether regenerating CNS axons become remyelinated and reassemble their excitable domains. We recently answered this question by examining myelin, AIS and nodes in an optic nerve crush injury and regeneration model (Marin et al., 2016).

In our study, we first defined the time course of excitable domain disassembly after optic nerve crush. We found that loss of AIS and nodes occurs shortly after crush: within 12 hours of crush, regions within and immediately proximal and distal to the injury site were almost completely devoid of nodes. In the retina we observed a significant decrease in the length of RGC-AIS within 3 days, which was then followed by a significant decrease in AIS density by one week after crush injury. By 30 days after crush injury, AIS and nodes were almost completely gone in the retina and optic nerve. Previous studies show that loss of AIS and nodes is due to calcium-dependent calpain mediated proteolysis (Schafer et al., 2009).

Following our analysis of excitable domain disassembly after optic nerve crush, we next analyzed AIS and node reassembly in the regenerating retina and optic nerve of the $PTEN^{f/f}$+zymosan+CPT-cAMP regeneration mouse model. Briefly, RGCs were transduced using an adeno-associated virus (AAV) expressing Cre recombinase 2 weeks prior to optic nerve crush. Immediately after crush, a single bolus of zymosan and CPT-cAMP was injected intravitreally. For longer survival times, an additional bolus of zymosan and CPT-cAMP was given every 3 weeks. Axon regeneration, and AIS and node reassembly were assessed at 2, 6, and 12 weeks after crush. Remarkably, we found new nodes, paranodal junctions indicative of remyeliantion, and AIS in the regenerating optic nerve and retina 6 weeks after crush injury (**Figure 1**). Despite extensive axon regeneration, node reassembly was limited to the proximal optic nerve. Twelve weeks after nerve crush node, reassembly extended into areas distal to the crush site, indicating that node reassembly progresses in a proximal to distal direction down the optic nerve, and that remyelination and reassembly of excitable domains is a very protracted process (**Figure 2**).

We also determined if AIS were reassembled in the retina after axon regeneration. Flatmount retinas were immunostained for βIV spectrin and analyzed at 2, 6, and 12 weeks after optic nerve crush. We found that retinas were mostly devoid of AIS 2 weeks after crush. However, we found an increase in both AIS density and average AIS length 6 weeks after injury. Together, these results suggest that neuronal polarity is re-established and that the cytoskeletal and ion channel protein complexes necessary for action potential initiation and propagation are reassembled. However, further studies are required to determine whether RGCs with regenerating axons develop normal electrophysiological properties.

Although reassembly of nodes suggests that regenerating axons become remyelinated, it is difficult to demonstrate this fact using electron microscopy since myelin debris is not efficiently removed after CNS injury. Instead, we used immunostaining for caspr, a marker for the paranodal junction. Paranodal junctions form between myelinating glial cells and the axon and are an excellent surrogate marker for myelination. In parallel to these immunofluorescence studies, we also analyzed remyelination of regenerated axons defined by labeling with cholera toxin B conjugated to horseradish peroxidase (CTB-HRP) using electron microscopy (EM). Consistent with our studies using antibodies against the paranodal junction, we found myelinated, CTB-labeled axons. This EM analysis shows that regenerating axons may be remyelinated as far as 2.5 mm past the injury site. Together, these data demonstrate that regenerated axons can be remyelinated and that proper axo-glial interactions are reestablished.

Functional recovery is the ultimate goal of axon regeneration. Thus, any therapeutic approach aimed at functional recovery will require the re-establishment of nodes and AIS. Our results are the first to show that this is possible. As we continue searching for genetic and pharmacological mechanisms that bring about functional recovery in axon regeneration models, it is critical that we remain mindful of the physiology of these axons, especially those processes that underlie the electrogenicity of the neuron. Recent evidence suggests that neural activity drives mechanisms central to neuronal repair including axon outgrowth and remyelination (Stevens et al., 2002; Gibson et al., 2014; Lim et al., 2016). Thus, the reassembly of excitable domains in regenerating axons may underpin downstream repair processes. The discovery and use of optic nerve regeneration models provides new and unprecedented opportunities to begin

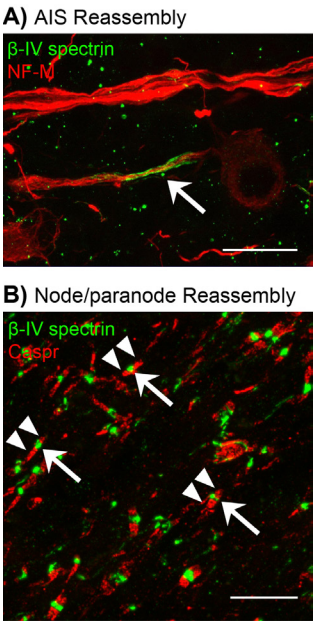

Figure 1 Reassembly of excitable domains in regenerated axons of the CNS.
(A) Reassembly of the axon initial segment (arrow) in retinal ganglion cells and (B) nodes of Ranvier (arrow) and paranodes (arrow head) in regenerated axons of the optic nerve after crush in the PTEN$^{f/f}$+zymosan+CPT-cAMP regeneration model. Scale bars: A, 20 μm, B, 5 μm. AIS: Axon initial segment; CNS: central nervous system; CPT-cAMP: 4-(chlorophenylthio) adenosine cyclic AMP; PTEN: phosphatase and tensin homolog.

answering critical questions about the viability and efficacy of strategies to promote regeneration of injured CNS axons.

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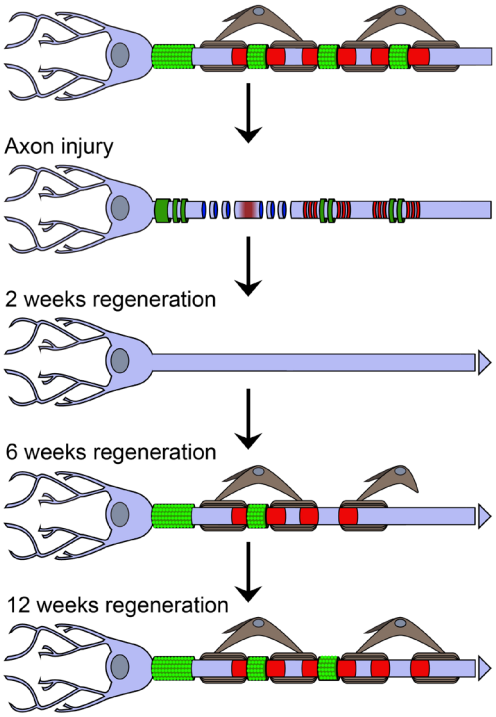

Figure 2 Disassembly and reassembly of excitable domains in regenerated axons of the optic nerve is a protracted process.
Following injury, axon fragmentation and excitable domain disassembly progresses from the injury site towards the proximal and distal ends of the axon. Two weeks after the onset of regeneration, axon outgrowth is apparent. Reassembly of excitable domains as well as paranodes (a surrogate marker for remyelination) are detectable 6 weeks after the onset of regeneration while node reassembly continues distally down the axon as time progresses.

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