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Commentary

Awakening the stalled axon — Surprises in CSPG gradients

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ABSTRACT

The remarkably poor regeneration of axons seen after injury of the brain and spinal cord can result in permanent loss of neural function. This failure of meaningful regeneration has been attributed to both a low intrinsic growth potential of CNS neurons and extrinsic factors that actively block axon growth in the adult CNS. Injury exacerbates this situation by increasing the expression of and exposure to proteins that actively block axonal growth in the CNS. Much experimental efforts have been aimed at overcoming the extrinsic growth inhibitory environment of the injured brain and spinal cord. A recent publication in *Experimental Neurology* from Kuboyama and colleagues shows that activation of protein kinase A signaling is responsible for the stalling of axon growth in gradients of CNS inhibitory molecules. This observation is unexpected given the role of cAMP signaling in supporting intrinsic growth mechanisms, emphasizing the need to consider spatial and temporal aspects of intracellular signaling in future strategies for neural repair.

The lack of meaningful axon regrowth after injury in the central nervous system (CNS) has been a source of disappointment and frustration. Proteins expressed and/or exposed after injury of the brain and spinal cord actively inhibit the regrowth of axons (Busch and Silver, 2007). This is not the case in the peripheral nervous system (PNS) where injured axons form a growth cone and spontaneously regrow. Although nerve regeneration in the PNS oftentimes does not bring a full return to function, PNS axons regenerate remarkably better than do those in the brain and spinal cord. Even when injured CNS axons start to extend and form a terminal 'growth cone' that is thought necessary for elongation, they most often retract and halt the progression upon encountering the inhibitory proteins that concentrate around the CNS injury site (Bradke et al., 2012; Silver and Miller, 2004). Transforming these stalled axons into progressive growth cones that could ignore the growth inhibitory molecules is an appealing strategy for neural repair.

The failure of CNS axon regeneration is perhaps most readily apparent in the injured spinal cord, where the injury site can be anatomically defined along the length of the spinal cord with intact proximal and deafferented distal segments. Long distance regeneration of axons in both ascending and descending white matter tracts is needed to restore spinal function, and the retracted ends of injured axons can be seen near the injury site in the spinal cord where inhibitory molecules concentrate (Cote et al., 2011). These retracted ends of axons have variably been referred to as 'dystrophic growth cones', 'retraction bulbs' and 'endballs'. Here, we will use the term 'dystrophic endballs' in keeping with the current publication from Kuboyama et al. (2013) and as originally described by Cajal for the injured CNS (Cajal, 1928). Much effort has focused on reviving these retracted ends of axons into active growth cones so that the axon can regrow. Work reported by Kuboyama et al. (2013) in *Experimental Neurology* brings a surprising observation that needs to be considered in future therapeutic strategies to coax these injured axons to regenerate. Indeed, these authors show that the terminal axons respond differently to non-permissive chondroitin sulfate proteoglycans (CSPGs) when presented as a uniform concentration as compared with a gradient of increasing concentrations. These gradients of CSPGs more closely mimic the in vivo glial scar. Moreover, blocking protein kinase A (PKA) activity was sufficient to overcome growth inhibitory effects of the CSPG molecule aggrecan.

At first glance, this is a surprising result given previous studies pointing to cAMP signaling as increasing intrinsic neuronal growth programs (see Hannila and Filbin, 2008 and references therein). As indicated above, the neurons of the mature CNS are thought to have an overall lower growth potential than those in the mature PNS. Consistent with this, ascending sensory axons, that include some of those from sensory ganglia that lie in the PNS, have proven easier to coax into regeneration than descending motor axons whose cell bodies reside in the CNS (Hoffman, 2010). Despite the higher intrinsic growth potential of the PNS sensory neurons in the dorsal root ganglion (DRG), the CNS environment also inhibits growth from these PNS neurons (Cai et al., 1999). Thus, the extrinsic inhibitory factors in CNS also impinge upon the neuron's intrinsic capacity to grow.

Combining approaches that target both intrinsic growth mechanisms and extrinsic growth inhibitory molecules has garnered much attention for CNS regeneration, and these have success at supporting

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regeneration in the injured spinal cord (McCall et al., 2012). The complexity of promising combinatorial approaches for CNS regeneration may help to explain why single agents that have robustly supported in vitro axon growth have often not proven effective for in vivo CNS axon regeneration. Still, there is much to be learned from in vitro approaches as the present publication from Kuboyama et al. (2013) illustrates.

CSPGs and axon regeneration

Degenerated myelin, oligodendrocytes and activated astrocytes form a growth barrier termed as the 'glial scar' in the injured CNS (Silver and Miller, 2004). This 'scar' is composed of extracellular matrix (ECM) proteins including the CSPGs produced by reactive astrocytes and chemorepulsive molecules such as semaphorins and Nogo secreted by reactive astrocytes and other cells in the injured CNS white matter (Schwab et al., 2006). Degenerating myelin exposes growth inhibitory molecules including myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (Yiu and He, 2006). Together, these present formidable chemical and physical barriers that ultimately stall the growth cone and forward movement of the axon.

With spinal cord and other CNS injuries, there is an upregulation of several CSPGs. These glycoproteins are defined by their protein core and glycosaminoglycan (GAG) polysaccharide side chains (Jones et al., 2003; Morgenstern et al., 2002). The GAGs are thought to be responsible for mediating the inhibitory effects from CSPGs, and the bacterial enzyme chondroitinase ABC (ChABC) that removes these side chains is effective at inactivating the CSPGs, both in vitro and in vivo (Bradbury et al., 2002). Thus, ChABC represents a potential therapeutic strategy for spinal cord injury, but advancing this as a biologic agent for neural repair also brings the significant obstacle for targeted delivery of a protein to the CNS. Hence, there is a great need to develop small molecule antagonists for these and other inhibitory molecules that could be utilized with less invasive approaches than ChABC.

In vitro studies with cultured neurons have proven extremely useful for understanding the mechanisms of action of the non-permissive substrates from the injured CNS. For example, neurons plated onto substrates coated with CSPGs show extremely attenuated axonal outgrowth (Dou and Levine, 1994; Friedlander et al., 1994; Yamada et al., 1997). This effect extends to PNS sensory neurons that are argued to have a higher intrinsic growth potential than CNS neurons. Indeed, axons from cultured PNS neurons growing on a permissive substrate like laminin will actively avoid CSPGs when presented (Hynds and Snow, 1999); if the axon cannot avoid the CSPGs, its growth stalls unless provided some means to overcome the growth inhibition.

The stalled axon

After axotomy, the cut end of an axon either forms a new growth cone or retracts into a bulbous structure (i.e., 'retraction bulb') depending on the intrinsic growth status of the neuron and the permissiveness of the environment (Bradke et al., 2012). Tom et al. (2004) showed that axons stall while growing up gradients of CSPG forming bulbous structures referred to as dystrophic endballs. With this gradient approach, neurons are plated on laminin in the center of a coverslip with their axons exposed to increasing CSPG concentration (i.e., a laminin → aggrecan gradient) as they extend towards the periphery of the coverslip. In contrast to the turning that axons growing on permissive substrates show when they encounter a border of inhibitory substrate, growth cones stall in these aggrecan gradients as they reach higher concentrations of aggrecan rather than turning back towards the laminin-rich environment (Tom et al., 2004). Tom et al. (2004) showed that the stalled growth cone or terminal axon is not stationary but is rather quite dynamic, forming peripheral veils and recycling membrane through increased endocytosis at the leading edge with retrograde transport to the rear end of growth cones. Although these dystrophic endballs do not make any net forward movement, the dynamic features suggested the possibility that this lack of regenerative growth might be reversed with simple interventions (Tom et al., 2004).

In the present work from Kuboyama et al. (2013), the dystrophic endball that stalls in the aggrecan gradients can be morphologically distinguished from terminal axons on uniform concentrations of aggrecan and from those growing on permissive substrates (see Fig. 3A in Kuboyama et al., 2013). Inhibition of Rho-kinase or increase in intracellular cAMP levels has been shown to counteract myelin and Semaphorin-induced growth cone collapse (Gao et al., 2003; Yukawa et al., 2005). However, these and other manipulations that have previously been shown to support growth on non-permissive substrates did not restore locomotion in the dystrophic endball in the CSPG gradients (see Fig. 1 in Kuboyama et al., 2013). Despite the dynamic behavior at the periphery, the dystrophic endballs formed on the CSPG gradients have dispersed integrin localization and reduced filopodia formation (Tom et al., 2004). This raised the possibility that a defect in integrin-mediated adhesion could account for the stalling of axons in the face of increasing CSPG concentrations. The study from Kuboyama et al. (2013) provides a direct intracellular link between CSPGs and integrin signaling.

CSPG signaling

Growth inhibitory signaling of CSPGs is initiated by binding to cell surface receptors. Several of the inhibitory substrates of the CNS, including CSPGs, MAG, OMgp, and Nogo share components of their receptors for generating intracellular signals (Mi et al., 2004; Park et al., 2005; Shao et al., 2005; Wang et al., 2002). These molecules all bind to the Nogo66 receptors (NgR1), though CSPGs can also bind to NgR3 (Dickendesher et al., 2012). The signaling cascades from these receptors converge to activate the small GTPase RhoA (see Sharma et al., 2012 and references within). CSPGs have also been shown to bind to a related receptor phosphatase, leukocyte common antigen-related phosphatase (LAR) that can activate RhoA and inactivate Akt to inhibit axon growth (Fisher et al., 2011). The sulfate side chains of CSPG have been shown to associate with an immunoglobulin-like domain on transmembrane protein tyrosine phosphatase (PTPσ) (Shen et al., 2009). PTPσ was earlier linked to axon growth, since PTPσ$^{-/-}$ neurons show enhanced regeneration after sciatic, facial, and optic nerve injuries (Fry et al., 2010; McLean et al., 2002; Sapieha et al., 2005; Thompson et al., 2003). PTPσ is thought to signal through mechanisms similar to LAR in the growth cones (Sharma et al., 2012). Interestingly, integrin β1 can also mediate MAG-induced growth cone turning independent of NgR1 (Goh et al., 2008), which indicates a possibility that multiple receptors, including the subcellular structures/complexes responsible for adhesion to extracellular matrix (ECM), may be utilized for conveying signals from these negative cues.

The CSPGs can trigger several signal transduction pathways that have the ability to modulate axon growth and growth cone dynamics (see Sharma et al., 2012 and references within). Modification of the cytoskeleton is a key mediator of the CSPG's growth regulating activities. CSPG exposure can reorganize the cytoskeleton of the distal axon to block growth cone motility through activation of RhoA and modulating the activity of the collapsin response mediator proteins (CRMP2 and CRMP4). During ephrin A5-induced growth cone collapse, Rho kinase phosphorylates CRMP2 reducing its tubulin binding capability and thus preventing microtubule assembly (Arimura et al., 2005). CRMP-4 is an F-actin bundling protein, and its interaction with Rho is required for axon growth inhibition by myelin and aggrecan (Alabed et al., 2007; Arimura et al., 2005). CSPG's effects on microtubules have been demonstrated in neuronal-like PC12 cells, where neurites show reduced rates of microtubule polymerization upon contacting CSPG borders, with an increase in microtubules showing reverse polarity (Kelly et al., 2010). Post-translational modifications of microtubules may also be altered by CNS inhibitory molecules. Histone deacetylatase 6 (HDAC6)