Journal Club

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Epac2 Promotes Axonal Outgrowth and Attenuates the Glial Reaction in an *Ex Vivo* Model of Spinal Cord Injury

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Review of Guijarro-Belmar et al.

The failure of lesioned axons to regenerate in the mammalian CNS underlies some of the permanent functional deficits caused by ischemic stroke, traumatic brain injury, and spinal cord injury. Axonal regeneration is prevented by both extrinsic factors including inhibitory molecules found in the post-traumatic extracellular environment and intrinsic factors that involve the neuron's internal reaction to injury. One manipulation of an axotomized neuron's internal signaling that has led to an augmented regenerative response is the elevation of cAMP. cAMP levels were first manipulated to enhance the regeneration of a severed axon by Kilmer and Carlsen (1984) in the peripheral nervous system. Later, Song et al. (1998) demonstrated *in vitro* that a cAMP analog allowed axons to overcome the inhibitory effect of myelin-associated glycoprotein (MAG). Subsequent work was able to induce regeneration of primary afferent axons after a dorsal column lesion with the addition of a cell-permeable analog of cAMP *in vivo* (Neumann et al., 2002; Qiu et al., 2002). Eventually, cAMP elevation was demonstrated to improve functional outcomes after spinal cord injury in rats (Kajana and Goshgarian, 2009), although its most promising clinical applications have been as part of combination therapies (Pearse et al., 2004).

Given that cAMP is a second messenger involved in numerous signaling cascades, it is important to determine which downstream effectors underlie its ability to promote axon regeneration. The best studied effector of cAMP is protein kinase A (PKA). When PKA was inhibited, cultured axons' ability to overcome MAG inhibition was abolished indicating that targets of PKA promote axon growth (Qiu et al., 2002). Surprisingly, however, when PKA activity was blocked *in vivo* after a cervical spinal cord injury, functional recovery improved in rats (Wei et al., 2016). This suggests that cAMP activates separate pathways that produce beneficial or detrimental effects after spinal cord injury.

The exchange protein activated by cAMP (Epac) is a more recently established family of proteins targeted by cAMP, and they act as nucleotide exchange factors for the small GTPase Rap (de Rooij et al., 1998). Interestingly, activation of Epac1/2 has been shown to increase neurite outgrowth *in vitro* (Murray and Shewan, 2008). Guijarro-Belmar et al. (2019) published a study in *The Journal of Neuroscience* that examined the role of Epac2 in neurite outgrowth, growth cone guidance, and glial reactivity *in vitro* and *ex vivo*. Using both cortical and dorsal root ganglia neural cultures, the authors showed that an Epac2 agonist increased neurite outgrowth, induced attractive growth cone turning, and was sufficient to mitigate growth inhibition by chondroitin sulfate proteoglycans (CSPGs). Each of these results mirror the effects of cAMP: cAMP increases neurite outgrowth (Neumann et al., 2002), induces a growth cone turning response (Song et al., 1998), and is sufficient to overcome an inhibitory environment (MAG inhibition was tested in the cAMP studies, Cai et al., 2001; whereas CSPG inhibition was tested by Guijarro-Belmar et al., 2019). The resemblance between the effects of cAMP and Epac2 activation in axon regeneration experiments strengthens the claim that Epac2 is an important downstream mediator responsible for cAMP-induced axonal regeneration.

Guijarro-Belmar et al. (2019) next examined the effects of the Epac2 agonist in an organotypic spinal cord culture and injury model. Postnatal day 1–3 rat spinal cords were dissected, cultured as intact spinal cord slices, and then lesioned. To locally deliver the Epac2 agonist at the site of injury, the authors prepared a fluorenylmethyloxycarbonyl (Fmoc)-based hydrogel and incorporated the agonist in the hydrogel before treatment. Tissue treated with the Epac2 agonist delivered in the hydrogel exhibited significantly more axonal outgrowth into the lesioned area than tissue grown with the hydrogel alone.

Guijarro-Belmar et al. (2019) also examined the effects of Epac2 agonism on the glial reaction to injury. Treatment of dissociated microglia and astrocytes with lipopolysaccharide, a bacterial molecule, induced an inflammatory response, as indicated by an increase in the proportion of iNOS-positive microglia and GFAP inten-

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sity in astrocytes. The Epac2 agonist reduced these effects. Epac2 activation also reduced astrocyte reactivity (GFAP density) and microglial activation (perimeter of Iba1-positive cells) in the *ex vivo* spinal cord injury model. These findings are consistent with previous reports that Epac activation diminished the inflammatory response in microglia (Quan et al., 2013).

Finally, Guijarro-Belmar et al. (2019) assessed locomotor recovery after a spinal cord contusion in rats. Three groups were compared: a group that received the Epac2 agonist via an Fmoc-based hydrogel, a control group, and a sham surgery group. The Epac2 agonist was applied 3 weeks after injury, and by week 7 there was a significant improvement in open-field locomotion in the treatment group over the control group. However, the authors did not provide *in vivo* histological data showing the effects of Epac2 agonism on axonal regeneration, astroglial reactivity, or microglial activation. In addition, as the authors note, the control group received a spinal cord injury but did not receive the hydrogel as a vehicle control. Self-assembling hydrogels (Fmoc based hydrogels being one such subtype) alone can increase axonal regeneration in animal models of spinal cord injury (Cigognini et al., 2014). The absence of a vehicle control makes it impossible to fully parse out the effects of Epac2 agonism from the effects of providing a substrate for axons to adhere to *in vivo*.

The data presented by Guijarro-Belmar et al. (2019) provide important insight into the mechanisms underlying cAMP-triggered neurite growth and axonal regeneration. How Epac2 acts downstream to ultimately produce the observed neuronal growth behavior is still unresolved, but studies from other models may provide clues. Epac has been shown to activate CREB, which is sufficient to induce the regeneration of sensory axons after a spinal cord injury (Gao et al., 2004). CREB is a basic leucine zipper transcription factor that binds directly to DNA at highly conserved nucleotide sequences (CRE response elements) in promotor or enhancer regions to modulate downstream gene expression. Arginase 1 and BDNF are two genes upregulated by CREB (Gao et al., 2004; Shieh et al., 1998), and both could contribute to CREB's role in axon regeneration. Arginase-1 catalyzes the conversion of L-arginine to L-orinthine, which limits the availability of the substrate required to produces nitrous oxide, this ultimately shifts immune cells from a proinflammatory to pro-reparative phenotype (for review, see Yang and Ming, 2014). The CREB-mediated upregulation of arginase-1 might underlie the effect of Epac2 activation on the attenuation of activated microglia. BDNF and its role in axon regeneration and functional recovery following CNS damage has been the subject of extensive investigation. BDNF has been shown to promote neuronal survival, axonal regeneration, plasticity, and remyelination (for review, see Weishaupt et al., 2012). In addition to activating CREB, Epac activates the small GTPase Rap1, which subsequently leads to B-RAF signaling, that has also been shown to initiate a regenerative growth response in neurons (O'Donovan et al., 2014). B-RAF is a Raf kinase and component of the MAPK/ERK signaling pathway that has downstream roles promoting cell division, proliferation, and survival. Although the mechanisms surrounding B-RAF activation and axon regeneration are still the subject of debate, there is speculation that the activation of the ERK pathway promotes the stabilization of microtubules (O'Donovan et al., 2014) and this microtubule stabilization directly enhances the ability of axons to grow (Hellal et al., 2011). Further work is required to elucidate whether one, both, or neither of these downstream pathways are the mechanisms by which Epac activation leads to neuronal growth.

In summary, Guijarro-Belmar et al. (2019) show that Epac2 activation produces strikingly similar axonal regeneration as the administration of its upstream signaling molecule cAMP. Epac2 activation is sufficient to overcome CSPG inhibition and to induce axons to grow into the lesioned site of an organotypic model of spinal cord injury. Further work is needed to determine whether Epac2 activation alone is sufficient to improve function after spinal cord injury and to elucidate the downstream mechanisms that produce these neuronal growth responses.

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