PERSPECTIVE

Linking axon transport to regeneration using *in vitro* laser axotomy

Spinal cord injury has devastating consequences because adult central nervous system (CNS) neurons do not regenerate their axons after injury. Two key reasons for axon regeneration failure are extrinsic inhibitory factors and a low intrinsic capacity for axon regrowth. Research has therefore focused on overcoming extrinsic growth inhibition, and enhancing intrinsic regeneration capacity. Both of these issues will need to be addressed to enable optimal repair of the injured spinal cord.

To re-establish motor function after spinal cord injury, descending corticospinal axons need to regenerate over long distances and past the site of injury before making meaningful connections (Tedeschi and Bradke, 2017). Current approaches to overcome inhibitory molecules stimulate sprouting and plasticity leading to some recovery of function, but do not enable long-range axon regrowth. Approaches to enhance the neurons intrinsic capacity for regeneration also stimulate short-range growth leading to limited functional recovery, however there are currently no interventions that stimulate the regeneration of descending motor axons over long distances through the adult spinal cord. Long-range regeneration is possible through the spinal cord, as has been recently demonstrated for sensory neurons regenerating their axons from the periphery towards the brain (Cheah et al., 2016). This was made possible by providing the dorsal root ganglia (DRG) with an activated integrin, which allows axon growth over the extracellular matrix (ECM) molecule tenascin-C (which is upregulated in the spinal cord after injury). Integrins are cell surface receptors for ECM molecules that mediate axon growth during CNS development and adult peripheral nervous system (PNS) regeneration after injury. Integrin α9β1 is one of the receptors for tenascin-C and had been shown to promote axon growth and regeneration. Expression of α9 integrin together with its activator kindlin-1 endows sensory axons with the ability to ignore inactivation by injury-induced molecules leading to vigorous effects on regeneration and functional recovery (Cheah et al., 2016). This method works for ascending sensory axons because PNS neurons efficiently transport integrins into their axons, allowing them to drive regeneration from the axon surface. The approach could be used to drive long-range regeneration of descending motor axons in the corticospinal tract (CST), however integrins are not transported into these axons. AAV mediated delivery of α9 integrin into CST neurons allows transport of integrins into dendrites but not into axons (Andrews et al., 2016). Endogenous integrins are similarly not transported into adult CNS axons but instead confined to dendrites. Examining the mechanisms controlling axonal integrin transport could identify ways of directing integrins into CNS axons. This would mean that the integrin method which drives long-range sensory regeneration could be applied to CST motor neurons. It might also help us to understand whether the CNS blockade of integrin axon transport contributes to regenerative failure.

Integrins are transported into PNS axons in recycling endosomes controlled by two traffic-regulating small GTPases, Rab11 and ADP-ribosylation factor 6 (ARF6). Rab11 is necessary for targeting integrins to the axonal growth cone surface, whilst ARF6 controls the direction of axonal integrin transport. Active ARF6 stimulates retrograde transport, whereas inactive ARF6 allows anterograde transport. In PNS axons, integrins are efficiently transported and move bidirectionally, but in mature CNS axons integrins are removed by predominant retrograde transport controlled by ARF6 activation and the axon initial segment (AIS) (Eva and Fawcett, 2014; Nieuwenhuis et al., 2018).

This perspective article describes two recent papers that established roles for Rab11 and ARF6 in the regulation of regenerative capacity (Eva et al., 2017; Koseki et al., 2017). We reasoned that studying ARF6 and Rab11 might identify targets for increasing integrin transport into CNS axons, whilst ascertaining whether there are roles for these trafficking regulators in determining the regenerative capacity of mature CNS axons. In order to reduce the reliance on animal models to investigate CNS regeneration, we developed an *in vitro* model for axonal regeneration using primary cortical neurons and laser axotomy (see **Figure 1**). We have used this model to determine roles for Rab11, ARF6 and the ARF6 activator EFA6 in the intrinsic regulation of CNS regeneration. We review here that these molecules can be targeted to increase the transport of integrins in recycling endosomes into CNS axons, leading to an increase in regenerative capacity.

Neurons of the CNS can regenerate their axons during development, but fail to do so with maturity. In order to model this intrinsic loss of regenerative ability in a manipulable *in vitro* culture system, the study by Koseki et al. (2017) used embryonic day 18 rat cortical neurons, and grew these to maturity (up to 24 days *in vitro* (DIV)) on glass-bottomed imaging dishes with astrocyte feeder coverslips overlaying the cultures. In this way, neurons could be separated from glial cells but still receive their trophic support. Cultures of this type have been used before to investigate maturation-related changes, but had not been used to investigate axon regeneration. Neuronal maturity was tracked by measuring electrical activity and changes in gene expression at 4-, 8-, 16- and 24-DIV, confirming that electrical activity increased in line with maturity, whilst RNA sequencing showed progressive changes in many genes towards expression patterns typical of mature neurons. Ingenuity pathway analysis showed increasing expression of molecules involved in synapse formation and function, and decreasing expression of genes involved in neuronal development. After confirming the maturation state of the cultured neurons, their response to a laser injury was quantified at increasing developmental time points, recording whether the axon regenerated after a laser cut, as well as measuring a number of other indices of regeneration. As expected, the axonal regenerative capacity of CNS neurons declined in line with maturity. To confirm that these changes were intrinsic to neurons, the authors measured axon regeneration of young neurons (4 DIV) plated on mature cultures (25 DIV). Neurons grown in a 25 DIV environment regenerated their axons as well as neurons plated on poly-d-lysine, indicating that the environment was not inhibitory to regeneration. In summary, Koseki et al. (2017) achieved a model to study the intrinsic axonal regeneration capacity of cultured neurons (**Figure 1**).

The paper by Koseki et al. (2017) also examined the integrin transporter Rab11 in the context of axonal regeneration by utilizing the above *in vitro* model, hypothesising that part of the decline in regenerative capacity might be due to the polarised distribution that membrane proteins adopt as neurons mature (when post-synaptic molecules are targeted to dendrites, and molecules required for synaptic transmission are targeted to axons). Rab11 has a somatodendritic distribution in adult brain *in vivo*, and this was found to be the same *in vitro*. Importantly,

Rab11 was present in equal amounts in axons and dendrites at 4 DIV, but by 16 DIV Rab11 was as almost exclusively in the cell body and dendrites, with only very low levels present in axons. Given its role in axon growth during development, and in transporting regenerative molecules, this deficit of axonal Rab11 in adult CNS axons might contribute to their weak regenerative capacity. Overexpressed Rab11 is also preferentially distributed to dendrites, however overexpression forces some Rab11 into axons. This led to an increase in axonal integrins, and an increase in regenerative capacity after laser axotomy *in vitro*, suggesting a role for Rab11 and integrins in enabling CNS axon regeneration. Overall, this study demonstrates that cortical neurons can be used to investigate the intrinsic decline in axon regeneration ability that occurs with maturation in the CNS, and that part of the reason for the decline is the selective targeting of integrins in Rab11 endosomes away from axons towards the somatodendritic domain (Koseki et al., 2017).

Eva et al. (2017) continued to use the *in vitro* model to investigate a role for ARF6 in the intrinsic regulation of regenerative capacity, firstly confirming that active ARF6 removes integrins from CNS axons as they mature. Our previous studies had found that ARF6 activation and the AIS were separately involved in directing integrins away from axons by retrograde transport (Franssen et al., 2015), but it was not known whether the two mechanisms might be linked. We discovered that there is an ARF6 activator, EFA6, which is strongly enriched in the AIS as cortical neurons mature. EFA6 expression increases in line with development, being absent at 4 DIV but strongly enriched in the AIS at 14- and 21-DIV. As active ARF6 stimulates retrograde integrin transport, we measured axonal ARF activation state using a GST tagged probe that binds only to active ARF. ARF protein activation was not restricted to the AIS, but instead a strong signal was observed throughout axons. Importantly, this signal was not evident at 4 DIV, when EFA6 is not enriched in the AIS and integrins and Rab11 are transported into axons. Silencing EFA6 with shRNA lead to a reduction in axonal ARF activation, indicating that EFA6 is responsible for maintaining ARF6 activation in mature CNS axons. This is required to stimulate retrograde removal of integrins, because silencing EFA6 caused a substantial increase in axonal integrins. Importantly, silencing EFA6 also led to an increase in Rab11 endosomes in axons, because Rab11 and ARF6 cooperate to control the direction of endosomal transport. EFA6 therefore functions to remove axon growth machinery (integrins in Rab11 endosomes) from CNS axons as they mature. This contributes to the axon's weak capacity for regeneration, because silencing EFA6 led to a substantial increase in axonal regeneration after laser axotomy (Eva et al., 2017). It is likely that this increase in regeneration is due to a number of molecules in the axon, in addition to integrins and Rab11, because Rab11 regulates the transport of a variety molecules, many of which have the potential to facilitate axon growth. Rab11 is known to be required for developmental axon growth, because removing Rab11 from the growth cone using optogenetics leads to growth cone collapse (van Bergeijk et al., 2015). The study by Eva et al. (2017) suggests that ARF6 is an intrinsic regulator of axonal regeneration, and that ARF6 activation negatively regulates regenerative capacity. This concept is supported by experiments using adult DRG neurons (PNS, sensory neurons) and laser axotomy. These neurons rapidly regenerate their axons after a laser injury, and are therefore a good model of neurons with a superior regenerative ability. Because EFA6 opposes regeneration in CNS neurons, we expected that it might be absent (or present at low levels) in regenerative PNS neurons. We were surprised to find that EFA6 is actually expressed at high levels in adult DRG neurons, but further investigation revealed that its activity is counterbalanced by an ARF6 inactivator, ACAP1, which is not present in CNS neurons. Overexpressing EFA6 led to a strong reduction in regeneration after laser injury, whilst expressing the ARF6 activation-incompetent EFA6 (EFA6 E242K) only weakly inhibited regeneration. This suggests that EFA6 opposes regeneration, functioning mostly through activation of ARF6. ARF6 activation state is therefore an intrinsic determinant of the axon's regenerative capacity. Overall, our two recent papers suggest that ARF6 and Rab11 are intrinsic regulators of regenerative capacity, and that a supply of growth promoting machinery in recycling endosomes is an important pre-requisite for re-establishing a growth cone which can drive robust axon growth (**Figure 2**).

A role for ARF6 in the regulation of regenerative ability is in keeping with a well-known regeneration pathway, phosphatase and tensin homolog deleted on chromosome ten (PTEN)/ Phosphoinositide 3-kinase (PI3K) (Liu et al., 2010). PTEN and pI3K counteract each other to regulate the phosphoinositides PIP_2 and PIP_3, with PI3K generating PIP_3 from PIP_2 and PTEN converting PIP_3 back to PIP_2. ARF6 has a large family of activators and inactivators, and the majority these molecules are regulated (directly or indirectly) downstream of PIP_2 or PIP_3 (Randazzo et al., 2001). This includes the ARF6 activator EFA6, which is strongly activated in the presence of PIP_2 (Macia et al., 2008). As deletion of PTEN (which leads to enhanced regeneration) results in less PIP_2, this could consequently lead to lowered EFA6 activity and increased axonal transport and regeneration. It will be important to see whether the PI3K pathway functions to promote regeneration through the axonal mobilisation of endosomes transporting growth promoting machinery. It seems that this is a crucial factor determining whether a damaged axon can reconstruct a functional growth cone to drive axon regeneration after injury. Future work will determine whether our trafficking interventions, which lead to increased axonal integrin and Rab11 expression, can be used together with integrin overexpression to enable long-range regeneration of CST axons, as has previously been achieved for sensory neurons (Cheah et al., 2016).

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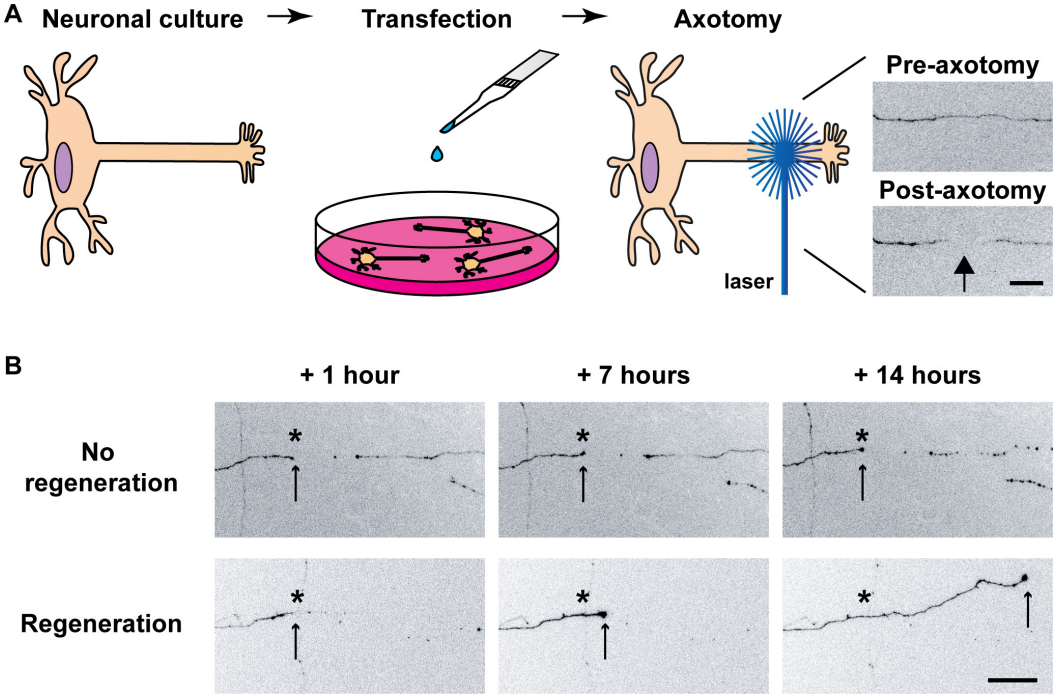

Figure 1 Cell culture model to study the intrinsic axonal regeneration response.

(A) A three-step set-up to investigate axonal regeneration in cultured neurons. In this example, primary cortical neurons were plated on a glass-bottomed imaging dish and were transfected with various DNA plasmids. The axons of the transfected neurons underwent laser axotomy and were video recorded for 14 hours. The arrow shows the site of injury. (B) Representative images of a non-regenerating axon (upper panels) and a regenerating axon (lower panels) from cortical neurons that were cultured for two weeks. The asterisk indicates the location of the proximal stump after one-hour post-axotomy, while the arrow marks the growth cone at indicated time-point. There is no difference in distal degeneration rate when comparing between regenerating and non-regenerating axons. Scale bars: 50 µm.

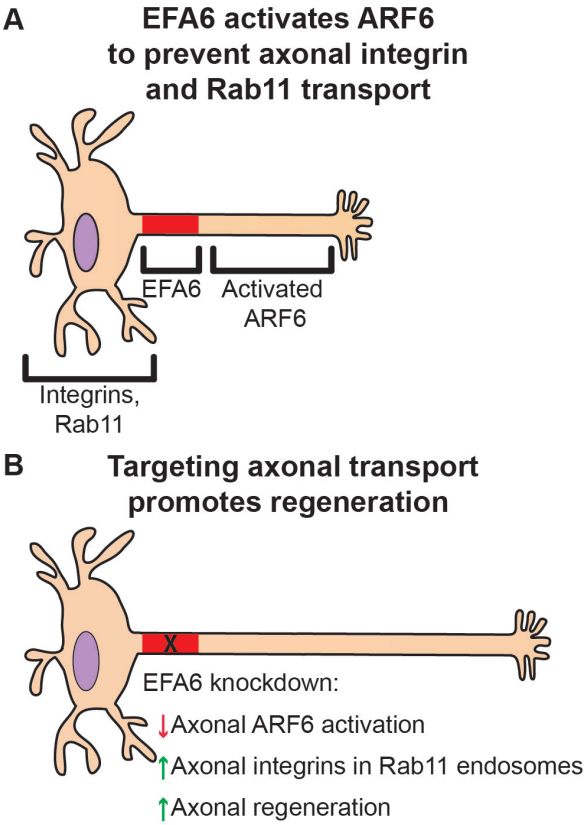

Figure 2 Enhancing axonal regeneration of central nervous system neurons by targeting selective transport.
(A) Endogenous distribution of trafficking-associated molecules and integrins in 14 days *in vitro* (DIV) primary cortical neurons. (B) Summary of the trafficking intervention by Koseki et al. (2017) and Eva et al. (2017) that promotes axonal regeneration in central nervous system neurons *in vitro*.

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