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The virtuous cycle of axon growth: Axonal transport of growth-promoting machinery as an intrinsic determinant of axon regeneration

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Abstract

Injury to the brain and spinal cord has devastating consequences because adult central nervous system (CNS) axons fail to regenerate. Injury to the peripheral nervous system (PNS) has a better prognosis, because adult PNS neurons support robust axon regeneration over long distances. CNS axons have some regenerative capacity during development, but this is lost with maturity. Two reasons for the failure of CNS regeneration are extrinsic inhibitory molecules, and a weak intrinsic capacity for growth. Extrinsic inhibitory molecules have been well characterised, but less is known about the neuron-intrinsic mechanisms which prevent axon re-growth. Key signalling pathways and genetic / epigenetic factors have been identified which can enhance regenerative capacity, but the precise cellular mechanisms mediating their actions have not been characterised. Recent studies suggest that an important prerequisite for regeneration is an efficient supply of growth-promoting machinery to the axon, however this appears to be lacking from non-regenerative axons in the adult CNS. In the first part of this review, we summarise the evidence linking axon transport to axon regeneration. We discuss the developmental decline in axon regeneration capacity in the CNS, and comment on how this is paralleled by a similar decline in the selective axonal transport of regeneration-associated receptors such as integrins and growth factor receptors. In the second part, we discuss the mechanisms regulating selective polarised transport within neurons, how these relate to the intrinsic control of axon regeneration, and whether they can be targeted to enhance regenerative capacity.

Introduction

Long-range regeneration fails in the adult brain and spinal cord

Unlike axons of the peripheral nervous system (PNS) which readily regenerate after injury, mammalian central nervous system (CNS) axons lose their regenerative capabilities with maturity (Bradke and Marin, 2014; Nicholls and Saunders, 1996). Injury to the adult brain and spinal cord can therefore have life-altering consequences. This problem was considered unassailable until a series of seminal studies in the 1980s challenged the long-standing dogma that CNS axons lack the capacity for repair. Aguayo and colleagues showed that injured adult rat axons arising in the CNS can regenerate through a peripheral nerve graft where the environment is more permissive to growth (Benfey and Aguayo, 1982; David and Aguayo, 1981; Richardson et al., 1980). These experiments were instrumental in demonstrating that certain CNS neurons retain a limited amount of their intrinsic ability for regeneration, and that failure of regeneration after injury could be attributed to the environment of the injured brain or spinal cord. Since these studies, numerous inhibitory molecules have been identified which oppose regeneration after a spinal cord injury, including the glial-derived chondroitin sulphate proteoglycans (CSPGs) and myelin-associated inhibitors such as Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (Schwab and Strittmatter, 2014; Silver et al., 2014; Yang et al., 2014; Yiu and He, 2006; Yu et al., 2018). These discoveries focused research on overcoming the inhibitory environment after injury, and led to the identification of interventions such as chondroitinase (Bradbury and Carter, 2011) and anti-Nogo antibody treatment (Ineichen et al., 2017). The chondroitinase approach, aimed at neutralising CSPGs is currently under intense investigation as a promoter of CNS repair, whilst anti-Nogo is undergoing clinical trials aimed at enhancing recovery after spinal cord injury. Both of these strategies lead to functional recovery through enhanced plasticity and sprouting from spared axons, with modest effects on axon regeneration.

Regenerated axons extend over limited distances, forming new synapses with spared circuits which connect beyond the lesion (Fawcett, 2015; Schwab and Strittmatter, 2014). Recent work has confirmed that neutralizing the astrocytic scar does not enable robust regeneration, whilst providing evidence that glial derived molecules can actually support regenerative growth of ascending sensory fibres stimulated by growth factor treatment and a growth-priming injury (Anderson et al., 2016). Without these growth-promoting stimuli, injured axons can become ensnared by NG2 positive cells which cause dystrophic axons to form synapses, further hindering attempts at regrowth (Filous et al., 2014).

Enabling long-range axon regrowth after injury to the spinal cord remains a challenging objective, particularly with respect to corticospinal tract (CST) axons. These axons descend from the cortex and are responsible for motor functions. CST axons have an outstandingly weak intrinsic capacity for regeneration, even in a permissive environment (Richardson et al., 1984). For this reason, there are concerted efforts to understand the mechanisms regulating intrinsic growth capacity, in order to identify new strategies which might be used together with extrinsic interventions to optimise regeneration.

Targeting neuron intrinsic mechanisms to promote regeneration and repair

Studies aimed at intrinsically increasing regenerative capacity have so far identified key signalling pathways, transcription factors and epigenetic mechanisms that can be targeted to increase regeneration (Dergham et al., 2002b; He and Jin, 2016; Hu and Selzer, 2017; Leibinger et al., 2017; Lindner et al., 2013; Liu et al., 2011; Moore et al., 2009; Moore and Goldberg, 2011; Muramatsu et al., 2009; Puttagunta et al., 2014; Qiu et al., 2002; Tedeschi and Bradke, 2017). Whilst these have not yet led to clinical treatments, they have generated new approaches that are potentially clinically translatable. A good example is the study of the tumour suppressor PTEN. PTEN was identified ten years ago as an intrinsic inhibitor of axon

regeneration, functioning to oppose the actions of PI3 kinase (PI3K). Deleting PTEN leads to robust retinal ganglion cell regeneration after optic nerve crush (Park et al., 2008), and can enable CST axons to regenerate for short distances past a lesion site (Liu et al., 2010). Due to its nature as a tumour suppressor, deletion of PTEN is not considered a clinically translatable strategy. However, the PI3K pathway can be successfully targeted with a more translatable approach that works by potentiating signalling downstream of the growth factor IGF1. Combined viral delivery of IGF1 and the matrix molecule osteopontin was found to enhance signalling through the PI3K pathway leading to robust axon regeneration after an optic nerve crush (Duan et al., 2015). This discovery lead to subsequent experiments combining IGF1 with osteopontin as a treatment after a model of spinal cord injury (T10 hemi-section). This strategy promoted profuse CST axonal sprouting and short-range regeneration leading to recovery of hind limb function, demonstrating that intrinsic growth capacity can be targeted in a translatable fashion to promote regeneration and recovery (Liu et al., 2017). However, the combination did not enable long-range CST regrowth, highlighting the need for further studies to understand the intrinsic regulation of axon regrowth ability. One particularly evident obstruction is the change in gene expression that occurs with maturity, combined with the lack of a cell body response after an axonal injury. Injury to axons in the PNS leads to an upregulation of regeneration-associated genes (Hoffman, 2010; Neumann and Woolf, 1999), but this is not seen in the CNS (Plunet et al., 2002). Efforts have therefore been made to enhance growth capacity via intervening with gene expression through transcription factor manipulation. This has been done either by removing inhibitory transcription factors such as KLF4 or overexpressing growth-promoting transcription factors such as KLF7 (Blackmore et al., 2012; Moore et al., 2009; Wang et al., 2015). These approaches have yielded encouraging results; however, they have not been able to promote the type of regeneration seen in the PNS (Wang et al., 2017b). It is becoming apparent that this may be due to complex epigenetic

factors such as closed chromatin affecting transcription factor binding site availability (Trakhtenberg and Goldberg, 2012; Venkatesh et al., 2016). In regenerative neurons, there are mechanisms to enhance chromatin availability in response to an axonal injury, and these can be targeted to facilitate CNS regeneration (Weng et al., 2017; Weng et al., 2018).

Integrins drive long-range regeneration, but are absent from adult CNS axons

The studies described above have been instrumental in demonstrating that there are developmental changes in gene expression and intracellular signalling in CNS neurons that contribute to their feeble regenerative ability, and that these can be targeted to facilitate regeneration. However, there remains a gap in our knowledge regarding the mechanisms downstream of these events that lead to effects on axon growth. The mechanisms regulating developmental axon growth are well-known and include cytoskeletal reorganisation, axon transport, membrane addition, and insertion of guidance molecules onto the growth cone surface (Allen and Chilton, 2009; Bradke et al., 2012; Hilton and Bradke, 2017; Quiroga et al., 2018), but the extent to which these are involved in mediating the effects of the interventions described above is not known. Cytoskeletal reorganisation is clearly an important consideration as demonstrated by the stimulation of CNS regeneration by targeting both the microtubule and actin cytoskeleton (Dergham et al., 2002a; Ruschel et al., 2015), and the neutralisation of growth inhibition by targeting growth cone non-muscle myosin (Hur et al., 2011).

Another critical factor mediating regenerative ability is an efficient axonal supply of the machinery required for growth. It is becoming clear that there are developmental changes in CNS neurons which limit the axonal availability of growth-promoting molecules such as the integrin family of adhesion/guidance molecules and their transporters, Rab11 positive endosomes (Andrews et al., 2016; Franssen et al., 2015). Integrins and Rab11 endosomes can

be manipulated into mature CNS axons *in vitro*, which enables axon regeneration after a laser injury (Eva et al., 2017; Koseki et al., 2017), but it remains to be seen whether these manipulations can used to promote long-range regeneration *in vivo*.

Intrinsic stimulation of integrin-driven long-range axon growth is possible through the spinal cord, as has been demonstrated by recent studies in sensory (PNS) axons regenerating towards the brain (Cheah et al., 2016). Viral transduction of a growth promoting integrin (alpha9) together with its activator kindlin-1 into dorsal root ganglion (DRG) neurons enabled them to regenerate their central axons (after a dorsal root crush) into the spinal cord and over long distances through the spinal cord (from the level of forepaw to medulla). The study demonstrated that long-range axon growth is possible through a normally inhibitory environment, as a result of intrinsic manipulations. The strategy works because PNS axons efficiently transport integrins into their distal axons. It could potentially be used to enable injured CNS axons to regenerate over lengthy distances, except for the issue with axonal localisation: integrins are not transported into adult CNS axons, being instead confined to cell bodies and dendrites (Andrews et al., 2016). An important question is whether this absence of integrin receptors reflects a general axonal deficit of growth-promoting molecules. Growth factor receptors are a good example of this type of molecule, and there is evidence that these are not present in abundant levels after CNS axons have matured. Both TrkB and the IGF receptor are reportedly excluded from CST axons in the spinal cord (Hollis et al., 2009a; Hollis et al., 2009c), and TrkB is similarly not detectable in rubrospinal axons whilst being present in their cell bodies (Kwon et al., 2004). BDNF (the TrkB ligand) can also rescue injured rubrospinal neurons from atrophy and prevent cell death, but only when applied at the level of the soma, and not the axon (Kwon et al., 2002).

If growth factor receptors are not abundant in CNS axons, it may explain why previous experiments aimed at facilitating CNS regeneration through growth factor stimulation have

not yielded strategies to promote long-range axon growth (Kordower and Tuszynski, 2008). Integrins and growth factor receptors are transported efficiently into CNS axons during developmental growth, being essential for the correct development of the nervous system (Myers et al., 2011), so it is clear that there are developmental changes that occur that limit their presence in adult axons.

Here we argue that distributing growth-enabling machinery to the axon is necessary to switch on a pro-regenerative neuronal program for successful regeneration. We discuss the idea that a developmental decline in axonal transport of growth-associated molecules contributes to the intrinsic decline in regenerative ability observed in the CNS; that changes in axonal transport and trafficking result in redistribution of growth molecules and receptors from axons to the somatodendritic domain. We also consider the evidence that interventions that promote regeneration (e.g. conditioning injury or permissive nerve grafts) also enhance axonal transport. We discuss the axon transport of specific regeneration-associated molecules, and the mechanisms that regulate a polarised distribution within neurons. Focusing on these topics provides insight into mechanisms that can be targeted to facilitate the axon transport of growth-promoting machinery and enable axon regeneration.

Developmental decline in axon regeneration capacity in the CNS

In vivo studies

Evidence from numerous animal models including C. elegans, rats, hamsters and opossums, suggest that there is a decline in CNS regeneration capacity that begins after birth and continues to dwindle (Kalil and Reh, 1979; Keifer and Kalil, 1991; Nicholls and Saunders, 1996; Wu et al., 2007). The studies discussed in the introduction demonstrate that this decline is due to both the extrinsic environment, and cell intrinsic programmes. Even in cases where regeneration does occur in the adult such as after peripheral nerve injury, the regenerative

ability of the peripheral neurons is reduced and delayed in older organisms (Verdu et al., 2000). For example, developmental changes such as impaired clearance of cell debris and the accumulation of obstacles in the endoneurial tubes of aged animals can affect not only the speed but also the extent of motor neurons' axonal regeneration after peripheral nerve injury (Kang and Lichtman, 2013). Importantly, the CNS decline in regenerative capacity appears to continue further into adulthood, as demonstrated by a study into the effects of PTEN deletion during ageing. PTEN deletion promotes axon regeneration in corticospinal tract (CST) neurons after injury in young adult mice (Liu et al., 2010). Geoffroy and colleagues examined the effects of ageing on the ability of PTEN deletion to induce regeneration of CST axons. Ageing did not reduce the effects of PTEN deletion on the intrinsic ability of axons to regenerate proximally to a spinal cord injury, but greatly reduced axon regeneration distal to the injury, suggesting that long range regeneration becomes increasingly problematic with maturity (Geoffroy et al., 2016).

In vitro studies

As it is difficult to separate the effects of the extrinsic environment from intrinsic factors *in vivo*, studies have also investigated regenerative ability using CNS neurons cultured *in vitro* (Bradke et al., 2012). A recent investigation by Koseki et al. (2017) used *in vitro* laser axotomy to investigate the intrinsic changes regulating axon regeneration of embryonic cortical neurons cultured to maturity. The study used E18 rat brain cortical neurons which were cultured up to 24 days *in vitro* using astrocyte feeder layers to separate neurons from glia. Development of neurons to a mature state was confirmed by assessing electrical activity and analysing gene expression, and regenerative capacity was measured by recording the axonal response to a laser injury. The study confirmed that the regenerative ability of cultured cortical neurons negatively correlates their maturational state, with less than 10% of neurons

cultured for 24 days regenerating compared to 70% of neurons cultured for 4 days. This effect was shown to be due to an intrinsic change by culturing young neurons on 24 day old cultures. The young neurons retained their regenerative capacity despite the aged environment, regenerating as well as neurons plated on poly-d lysine (Koseki et al., 2017). Retinal ganglion cells show a similar age-dependent decline in axon growth capacity, whether grown on neonatal or adult optic nerve sections , whilst DRG neurons from the PNS retain their intrinsic ability to regrow on either of these substrates (Goldberg et al., 2002; Shewan et al., 1995). Koseki et al continued by analysing gene expression by RNA sequencing and found that that there are vast changes in gene expression as cortical neurons mature in culture, with an increase in the expression of genes involved in electrical activity and synapse formation and function, and a decrease in genes associated with growth and development. They also investigated the axonal delivery of growth-promoting machinery, finding that there is a developmental decline in the axonal transport of recycling endosomes, and that restoring this transport leads to an increase in regeneration. These findings support the notion that genetic factors are partly responsible for the change in regenerative capacity, and that there are developmental changes in selective axonal transport that limit axon regrowth.

Developmental decline in axon transport

Early studies

It has long been assumed that efficient axon transport is necessary for effective regeneration, with many studies addressing this hypothesis. The foremost studies were aimed at understanding whether axon transport rates varied in regenerative vs. non-regenerative axons, and whether there is an increase in axon transport after a peripheral injury when neurons mount a regenerative response. These studies used techniques such as radiolabelling to

measure the transport of peptides into axons *in vivo* comparing early development with later stages, and finding that molecules such as the growth-associated GAP43 are rapidly transported into the optic nerve of neonatal rabbits, but that transport rates declined rapidly with development (Skene and Willard, 1981). Cytoskeletal transport declined similarly (Hoffman et al., 1983), as revealed by examining neurofilament transport. A number of studies also addressed whether the enhanced growth observed after a conditioning lesion is associated with an increase in axon transport rates by measuring the synthesis and axonal transport of cytoskeletal components, again using radio-labelling, and finding that enhanced regeneration is associated with elevated axon transport of both microtubules and neurofilaments (McQuarrie and Grafstein, 1982; McQuarrie and Jacob, 1991; Oblinger and Lasek, 1988). Similar observations were also reported in later studies examining enhanced regeneration of optic nerve axons into a peripheral nerve bridge (McKerracher et al., 1990). Collectively, these early experiments suggested a deceleration in axonal transport during maturation, and an increase in transport during enhanced regeneration (Fournier and McKerracher, 1995; Hoffman, 2010; McQuarrie et al., 1989).

Live cell imaging to characterise axon transport

The majority of early studies focused on cytoskeletal material as an example of machinery that is required for axon growth. Similar techniques were recently used to examine axon transport more completely, by coupling radiolabelling with mass spectroscopy and analysing the transport of various subcellular components in the central branch of DRG axons after a conditioning injury to the peripheral branch (which results in enhanced regeneration). The study found enhanced transport of both actin and microtubule cytoskeletal elements, as well as cytosolic proteins including glycolytic enzymes and regenerative molecules such as the 14-3-3 proteins and the RhoA inhibitor RhoGDI, and membranous vesicles including

lysosomes, and synaptophysin and APP-containing vesicles. Increased levels of molecular motors and of polyglutamylated tubulin were also observed (Mar et al., 2014). This study demonstrates global changes in axon transport after a conditioning lesion. It also highlights that techniques such as live cell imaging of fluorescently labelled proteins make it possible to examine axon transport in a highly selective fashion, focusing on individual cellular components.

These techniques were recently used to analyse age-dependant transport as well as regeneration-dependent changes focusing on two specific cargoes, the axon survival factor NMNAT2, and mitochondria. Although this study focused on a much later developmental period (between 1.5 and 24 months), some specific changes in mammalian CNS and PNS axonal transport with development were identified (Milde et al., 2015). Interestingly, aged neurons in an ageing environment (in 24-month-old mice) were capable of supporting higher levels of axonal transport as stimulated by a peripheral nerve crush – an increase in the number of anterogradely moving particles containing NMNAT2 and mitochondria was observed at rates similar to young mice, whilst retrograde transport was unaffected. This observation suggests that enhanced anterograde transport contributes to axon regeneration after peripheral nerve injury. The movement of mitochondria has long been associated with axon growth (Morris and Hollenbeck, 1993). A number of recent studies have demonstrated the importance of axonal mitochondria for the regeneration process, and this will be discussed in detail later.

Developmental decline in axon transport of growth machinery

The studies described above suggest that mature CNS axons lack the correct tools for axonal extension, and that PNS axons mount a regenerative response by enabling enhanced transport leading to an increase in axonal growth machinery. However, they do not elucidate precisely

what the necessary machinery is, or to what extent it is missing from non-regenerative adult CNS axons. Axon growth is driven by the growth cone and can be compared to the process of cell migration, in that there are requirements for the interaction of the cell with its environment through adhesion and guidance molecules which subsequently reorganise the cytoskeleton and surface membrane in the direction of growth (Dequidt et al., 2007; Itofusa and Kamiguchi, 2011; Robles and Gomez, 2006; Tojima et al., 2007). One class of molecules that is important for this process is the integrin family of cell surface receptors for the extracellular matrix. These are important for developmental CNS axon growth and PNS axon regeneration but are excluded from CNS axons after development, both endogenously and after viral transduction (Nieuwenhuis et al., 2018)(discussed in detail below). Because integrins can promote PNS regeneration but are excluded from mature CNS axons, there have been substantial efforts to understand the mechanisms regulating their transport, with a view to manipulating this to enable regeneration. These studies initially examined axonal integrin transport in regenerative PNS axons *in vitro*, focusing on the integrin alpha9 and its binding partner beta1. Two small GTPases were found to be responsible for regulating integrin transport into axons, the recycling endosome markers Rab11 and ARF6 (Eva et al., 2012b; Eva et al., 2010; Nieuwenhuis and Eva, 2018). Rab11 governs integrin transport into axons in recycling endosomes, and additionally controls integrin recycling onto the growth cone surface through an interaction with its effector, Rab coupling protein (Rab11-FIP1). ARF6 is also involved in integrin trafficking within the growth cone, but crucially plays an additional role in the axon itself, regulating the direction of integrin transport. ARF6 activation state governs the direction of axon transport such that active ARF6 favours retrograde transport whilst inactive ARF6 enhances anterograde transport. This has important implications, because there are stark differences in directional transport in PNS vs CNS neurons. In PNS neurons there is a balance between anterograde and retrograde transport which results in an

integrin presence throughout the axon, often in immobile structures (Eva et al., 2012b). In CNS neurons (rat brain cortical neurons) there is a developmental increase in retrograde integrin transport which results in removal of integrins from mature axons in the CNS (Fig. 1), so that live cell imaging reveals there are very few immobile integrin structures in axons (Franssen et al., 2015). Predominant retrograde movement has previously been associated with a state of axon growth arrest, whilst a balance of anterograde and retrograde endosomal movement is associated with axonal elongation (Hollenbeck, 1993; Hollenbeck and Bray, 1987).

With regard to regenerative ability, it may prove to be crucial that not only are integrins sequestered from adult CNS axons and retained in the cell body and dendrites, but so are their carriers – Rab11 endosomes. Rab11 is important for developmental axon growth but its axonal presence is diminished with maturity, being difficult to detect in mature CNS axons, instead localising principally in the somatodendritic domain (Koseki et al., 2017; Sheehan et al., 1996). In adult neurons Rab11 is involved in the regulation of post-synaptic plasticity, with roles in receptor recycling (AMPA and TrkB receptors), dendritic spine development, and synapse structure (Correia et al., 2008; Esteves da Silva et al., 2015; Lazo et al., 2013; Sui et al., 2015). Conversely, there is less evidence for a presynaptic role for Rab11 in mammalian CNS neurons, although it has been observed at low levels in the synaptic vesicle fraction of synaptosomes. These are enriched with a different set of Rab proteins that are important for the correct cycling of synaptic vesicles (Rabs 3 and 27) (Binotti et al., 2016; Pavlos et al., 2010).

It is likely then that the developmental decline in axonal Rab11 reflects a change in the requirements for recycling within the axon. There is evidence that as axons lose the necessity for growth and instead become geared for neurotransmission, there is a change in the type of endosomal recycling machinery present, with a decline in receptor and membrane protein

recycling and a shift towards synaptic vesicle recycling (Bonanomi et al., 2008). Recent evidence suggests that membrane proteins and synaptic vesicle proteins are also degraded through separate pathways in axon terminals, in support of the hypothesis that the machinery for synaptic vesicle turnover is distinct from that which is required for membrane protein turnover (Jin et al., 2018b). Moreover, the development of synapses has recently been associated with regenerative decline (Tedeschi et al., 2016). In summary, Rab11 is known to transport many molecules that are important for axon growth (in addition to integrins) so it may not be surprising that Rab11 is not needed in large amounts in the fully developed axon. Integrins, Rab11 and other regenerative Rab11 cargo are discussed the next section.

Growth machinery at low levels in mature CNS axons (Rab11 and cargo)

Integrins

Integrins are a diverse family of cell surface receptors for the extracellular matrix (Hynes, 2002). They transduce signals from the extracellular environment that lead to the reorganisation of the cytoskeleton and activation of numerous influential signalling pathways. As they have no enzymatic activity, integrins rely on a vast array of interacting molecules to mediate their actions. They are also regulated by signalling from the intracellular environment (termed inside-out signalling), which modifies the activation state of the receptors. Integrins have active and inactive conformations, and ultimately depend on activation by molecules such as the kindlins and talin, which lead to changes in their extracellular structure (Cheah and Andrews, 2018; Kim et al., 2011). Integrins function as heterodimers composed of an alpha and a beta subunit, the combination of which governs their specificity for their individual ligands, so that different heterodimers can bind with differing affinities to molecules such as laminin, collagen, fibronectin, tenascin and vitronectin (Barczyk et al., 2010; Hynes, 2002). They also exhibit some interaction with other

secreted signalling molecules including certain growth factors (LaFoya et al., 2018). There are therefore a number of ways that the extracellular environment can modify cellular behaviour mediated by integrin activation, so it is unsurprising that integrins are involved in a vast array of biological processes in most cell types both during development and in adulthood. Neurons are no exception to this; integrins have been implicated in many aspects of neuronal function, including axonal growth and guidance during development (Myers et al., 2011) as well as playing an influential role in the regulation of synaptic function during adulthood (Park and Goda, 2016). Integrins are also involved in the PNS regenerative response after injury with the axon transport of alpha5 integrin being enhanced on fibronectin after a conditioning lesion, whilst the integrin alpha7 mediates enhanced regeneration on laminin after a peripheral nerve crush (Eva et al., 2012a; Gardiner, 2011; Gardiner et al., 2005; Gardiner et al., 2007; Nieuwenhuis et al., 2018). Integrins have long been proposed as a means of promoting axon regrowth (Condic, 2001), and have recently been used to promote sensory regeneration through the spinal cord after an injury to the central branch of DRG axons. Viral introduction of alpha9 integrin (AAV injection into DRG cell bodies) allows regenerating PNS axons to re-enter the spinal cord and synapse with their targets as well as continuing to regenerate almost as far as the brain (reaching the medulla but stopping short of the cuneate nucleus) (Cheah et al., 2016). Because integrins are inactivated by inhibitory molecules (Hu and Strittmatter, 2008; Tan et al., 2011), this sensory axonal repair strategy relies on co-expression of the integrin activator kindlin-1. The observed integrin-dependent regeneration is also only possible because PNS axons support integrin transport (Andrews et al., 2016). The integrin alpha9 was selected for these experiments because it has two key features – it binds to tenascin-C and promotes axon extension when bound to this ligand (Andrews et al., 2009). Tenascin-C is an extracellular matrix glycoprotein that is normally inhibitory to axon growth in the adult CNS and is strongly upregulated after CNS injury in

the brain, spinal cord and optic nerve (Andrews et al., 2009; Gervasi et al., 2008; Reinhard et al., 2017; Tang et al., 2003; Zhang et al., 1997). Alpha9 integrin therefore mediates its regenerative actions by localising to the axon surface to stimulate axon growth over a molecule which normally inhibits axon growth. Integrin alpha9 is not normally expressed in the adult nervous system, so exogenous viral introduction is necessary to observe its effects. Other endogenous integrins are important for PNS regeneration, but these do not allow PNS axons to regenerate into the environment of the spinal cord, despite being efficiently transported into their axons (Gardiner, 2011). Integrins are therefore potent mediators of the PNS regenerative response and could potentially be used to enable CNS regeneration after injury. Alpha9 integrin particularly could be able to promote the regeneration of descending corticofugal axons when virally introduced with kindlin-1, however the developmental change in the subcellular distribution of integrins in neurons in the CNS means that they are no longer present in axons, but instead selectively targeted to the somatodendritic domain (Andrews et al., 2016; Bi et al., 2001; Franssen et al., 2015). This has been observed for pyramidal neurons in the cortex, hippocampal neurons in the CA1 and CA3 regions, cerebellar Purkinje neurons, and granule neurons in the dentate gyrus (Bi et al., 2001; Chan et al., 2003; Einheber et al., 1996; Rodriguez et al., 2000). Virally transduced exogenous integrins are also restricted from entry to the axons of mature cortical neurons *in vivo*, whilst they are transported into the axons of young CNS neurons. However, there does appear to be a role for integrins in retinal ganglion cell (RGC) neurons in the optic nerve, but it is not clear if they are present in the axons of all the various subtypes of neurons within the retina (Andrews et al., 2016; Vecino et al., 2015). *In vitro* studies have revealed a similar picture, with alpha and beta integrins detectable in the axons and growth cones of adult DRG neurons, but not in the axons of mature cortical neurons. Integrins are detectable in the axons of E18 cortical neurons cultured for 4-7 days, but after this period they become difficult to

detect so that by 10-14 days *in vitro* endogenous integrins are almost completely absent from axons (Bi et al., 2001; Franssen et al., 2015; Koseki et al., 2017). This developmental decline in CNS integrin axon transport contributes to the decline in regenerative ability, because restoring integrin transport leads to a restoration of regeneration after a laser injury to the axons of cortical neurons maturing *in vitro* (Eva et al., 2017). Importantly, restoring integrin transport also leads to an increase in axonal Rab11, suggesting that enabling integrin transport may also facilitate the transport of additional machinery which is transported along with integrins in Rab11 endosomes.

Rab11 and recycling endosomes

Rab11 and ARF6 are small GTPases that regulate recycling endosome trafficking and function as the central regulators of axonal integrin transport, with emerging roles in the intrinsic regulation of axon regeneration. Axonal Rab11 declines with development, whilst axonal ARF6 activity is raised (Eva et al., 2012b; Eva et al., 2010; Eva et al., 2017; Franssen et al., 2015; Sheehan et al., 1996).

Small GTPases are molecular switches that cycle between an active GTP bound state and an inactive GDP bound state. Their activation state governs the molecules that they interact with, so that some proteins will interact whilst GTP-bound, and others only whilst they are bound to GDP. They possess intrinsic GTPase activity, so that bound GTP will be catalysed to GDP. The rate at which this occurs is regulated by molecules known as GAPs (GTPase activating proteins) whereas activation (to a GTP-bound state) is regulated by GEFs (Guanine nucleotide exchange factors). Intrinsic GTPase activity varies between small GTPases, with ARF6 in particular having very little intrinsic GTPase activity (Campa and Randazzo, 2008; Gillingham and Munro, 2007). ARF6 is therefore entirely reliant on its GAPs and GEFs for regulation of its activation state, and a large number of these molecules have been identified,

some of which are implicated in the regulation of axon growth and regeneration (Eva et al., 2012b; Eva et al., 2017; Hernandez-Deviez et al., 2004).

Rab11 is a regulator of recycling endosomes. These are endosomes that return membrane proteins to the cell surface after they have been internalised. Rab11 was originally identified as regulating a long loop of receptor recycling via an organelle close to the nucleus (the peri-nuclear recycling centre) (Ullrich et al., 1996). Through this trafficking pathway, membrane proteins can be internalised from one part of the surface membrane and recycled to another. Subsequently it emerged that Rab11 is additionally involved in a more localised, rapid recycling which occurs when there is high-capacity membrane turnover, such as occurs at the leading edge of migrating cells (Howes et al., 2010). It is also involved in the process of membrane protein exocytosis (Welz et al., 2014).

ARF6 is also a regulator of recycling endosome traffic but is additionally involved in a range of mechanisms that are central to cellular function. These include the regulation of the actin cytoskeleton through key regulators such as Rac and Cdc42, and the control of phosphoinositide signalling. ARF6 activates PIP5 kinase, an enzyme which is responsible for generating phosphatidylinositol 4,5-bisphosphate (PIP2) (Gillingham and Munro, 2007). This molecule is converted by PI3 kinase to make phosphatidylinositol 3,4,5-triphosphate (PIP3), which can in turn be metabolised by PTEN back to PIP2 (Vanhaesebroeck et al., 2012). This is a key step in an extremely influential signalling pathway which can have wide-ranging consequences in cell functions such as transcription, translation, epigenetics, cytoskeletal regulation, trafficking and transport, neurotransmission, apoptosis, cell growth, proliferation, and survival. The majority of ARF6 GEFs and GAPs are either directly regulated by PIP2 or PIP3 or regulated by phosphorylation from key kinases in the PI3 kinase pathway (Hawkins et al., 2006; Randazzo et al., 2001). ARF6 is therefore both a trafficking and signalling molecule closely involved with pathways that are central to controlling cell function.

Both ARF6 and Rab11 were first implicated as regulators of integrin function in non-neuronal cells, being important for recycling of integrins during cell migration and invasion of cancer cells. Recycling of integrins is central to their function in mediating cell migration, being required for the correct turn-over of focal adhesion complexes (Caswell and Norman, 2008; Caswell et al., 2008; Caswell and Norman, 2006; Dai et al., 2004; Dunphy et al., 2006; Jones et al., 2006; Pellinen and Ivaska, 2006; Powelka et al., 2004; Roberts et al., 2001; Vitali et al., 2017). Subsequent studies found that Rab11 is required for targeting integrins to axons in DRG neurons in culture, as well as being important for recycling integrins locally within the growth cone (Eva et al., 2012b; Eva et al., 2010). Recycling is necessary within the growth cone to control directional changes (Tojima et al., 2007; Tojima et al., 2010). More recently, there have been a number of studies that confirmed the importance of Rab11 for correct growth cone function, with its targeted removal from developing growth cones leading to growth cone collapse, whilst increased growth cone targeting leads to an increase in axon growth (van Bergeijk et al., 2015). Optogenetic disruption of growth cone Rab11 also leads to a reduction in growth cone area, similar to the decreased growth cone area observed when Rab11 is silenced (Eva et al., 2010; Nguyen et al., 2016). *In vivo* studies have confirmed that Rab11 is required at the growth cone for the correct guidance of axons crossing the midline of the spinal cord during development of the nervous system (Alther et al., 2016).

ARF6 similarly regulates integrins in both the axon and at the growth cone, but crucially it also regulates the direction of integrin transport within axons, with active ARF6 triggering retrograde transport whilst inactive ARF6 allows anterograde transport (Eva et al., 2012b; Franssen et al., 2015). Inactivating ARF6 enables both integrin and Rab11 transport leading to enhanced axon growth and regeneration after laser axotomy (Eva et al., 2012b; Eva et al., 2017; Hernandez-Deviez et al., 2004). This may be due to an increased integrin presence, but

may equally be due to an increase in Rab11 itself, as this has also been shown to increase regeneration when its axonal localisation is increased by overexpression (Koseki et al., 2017). There may also be a role for other Rab proteins in this process, as recycling endosomes are also regulated by Rabs 8, 10 and 35 which are all involved in the regulation of neurite outgrowth in PC12 cells and axon growth in cortical and hippocampal neurons *in vitro* (Chevallier et al., 2009; Furusawa et al., 2017; Homma and Fukuda, 2016; Huber et al., 1995; Kobayashi and Fukuda, 2012; Villarroel-Campos et al., 2016; Wang et al., 2011). It may also be that Rab11 enables regenerative axon growth by providing other growth-related molecules to the site of injury, because a number of growth factor receptors and the regenerative reggie/flotillin proteins are also associated with trafficking via Rab11 (Bodrikov et al., 2017; Hulsbusch et al., 2015; Koch et al., 2013). These are discussed below.

IGF-1 and TrkB receptors

With well-defined roles in regulating axon growth, it is understandable that research has focused on promoting axon regeneration through growth factor / growth factor receptor manipulation. Two prominent growth factor receptors that have been investigated in this respect are tropomyosin-related kinase B (TrkB) and insulin-growth factor receptor 1 (IGFR-1), which are activated by the growth factors BDNF and IGF respectively (Duan et al., 2015; Hollis et al., 2009c; Liu et al., 2017; Lu and Tuszynski, 2008). IGFR-1 has a vital function in neuronal survival and glial progenitor protection against glutamate toxicity after injury. In order to perform these functions, IGFR-1 undergoes dynamic recycling and internalisation upon ligand stimulation which is essential for sustained downstream signalling. Furthermore, the IGF-1 receptor colocalises with Rab11-positive endosomes and with the transferrin receptor identifying the IGF-1 receptor as one of the receptors packaged in recycling endosomes (Romanelli et al., 2007). As Rab11-positive endosomes are excluded from the

mature axons of CNS neurons, the low availability of IGFR-1 in the axon after injury might be a further reason for poor CNS axonal properties. The IGF-1 receptor has been implicated in the specification and initial axon growth in hippocampal neurons acting through the PI3K pathway, in the absence of TrkA or TrkB activation (Dupraz et al., 2009; Nieto Guil et al., 2017; Sosa et al., 2006). This activation is preceded by targeted accumulation of IGFR-1 in the developing axon. IGF-1 has also been shown to markedly enhance axon outgrowth in young cultures of pure corticospinal motor neurons through the PI3K and the ERK/MAPK pathways, an effect which is separate from its effects on neuronal survival (Ozdinler and Macklis, 2006). IGF-1 receptor signalling was also shown to be necessary for proper axonal outgrowth of retinal ganglion cells *in vitro* (Dupraz et al., 2013) and of corticospinal motor neurons *in vivo* (Ozdinler and Macklis, 2006). Surprisingly, application of IGF-1 to injured corticospinal tract neurons resulted in their improved survival but not regeneration. The authors speculated that the effects of the IGFR-1 activation on survival and growth are developmentally distinct and the reduced availability of the receptor in axons compared to the soma can explain the inability of overexpressed IGF-1 to promote regeneration (Hollis et al., 2009c). In a different study, the activation of the IGF-1 receptor by the application of IGF-1 and sensitisation with osteopontin resulted in robust sprouting and partial recovery of function in two different models of corticospinal tracts injury (Liu et al., 2017). This effect was attributed to osteopontin's ability to interact with integrins or other surface molecules to cause IGFR-1 clustering, and most likely occurs at the site of the cell body plasma membrane. Taking into account the versatile functions of the IGFR-1 receptor in neuronal survival, growth and regeneration, its proper transport and trafficking is essential for optimal function in development and regeneration. The investigation of osteopontin as a growth stimulator arose from studies in retinal ganglion neurons. These have as small subpopulation of cells that regenerate better than their counterparts. These are known as αRGCs and were

found to express higher levels of osteopontin. Experiments in the optic nerve determined that osteopontin facilitates robust regeneration in combination with either IGF, or BDNF, the TrkB receptor ligand (Duan et al., 2015).

TrkB is similar to the IGF receptor in that it too is involved in developmental axon growth (Gates et al., 2000), has been targeted to promote CNS regeneration (Hollis et al., 2009b; Kwon et al., 2004; Plunet et al., 2002), and is transported in Rab11 endosomes. The recycling of TrkB receptor through Rab11-positive recycling endosomes regulates its neuronal localisation, and in mature neurons is involved in post-synaptic receptor recycling in dendrites (Huang et al., 2013; Lazo et al., 2013; Sui et al., 2015). This is in contrast to its developmental enrichment at the growth cone of CNS neurons, from where it stimulates axon growth. Importantly, BDNF signalling at the growth cone stimulates anterograde transport of its receptor, TrkB (Cheng et al., 2011). This autocrine feedforward mechanism demonstrates that signalling from the distal axon can stimulate growth-promoting mechanisms, and is similar to the transport of TrkA in PNS neurons, which also transport TrkA in Rab11 endosomes (Ascano et al., 2009). Despite its role in axon growth promotion during development, TrkB is unable to stimulate regeneration of injured CST axons in adults, and this appears to be as a result of its somatodendritic localisation. Adult corticospinal neurons show abundant TrkB receptor distribution in their soma and dendrites, but not in their axons which correlated with their inability to regenerate after a subcortical lesion (Lu et al., 2001). Similarly, rubrospinal axons do not appear to support TrkB transport, although application of BDNF to their cell bodies can promote their survival after injury and encourage regeneration into a peripheral nerve graft (Kwon et al., 2002; Kwon et al., 2004; Plunet et al., 2002). In contrast, motor neurons which expressed TrkB throughout their axons were able to re-grow past the injury site suggesting that the presence of the receptor in the axon plays a key role after injury in order for the axon to initiate a growth program (Lu et al., 2001). TrkB agonists

can also promote structural and functional repair of cut peripheral nerves (English et al., 2013). Given that integrins, the IGF receptor and TrkB all traffic via Rab11 and all adopt a somatodendritic localisation, it seems inconsistent that overexpressed TrkB localises to CST axons, and can improve regeneration when stimulated by BDNF after a subcortical lesion (Hollis et al., 2009b). However, it is important to note that regeneration was only found subcortically, and that the TrkB receptor was not transported to more distal sites, such as the spinal cord. The presence of TrkB in the axon is not completely unexpected, because there is evidence that TrkB has a presynaptic role in regulating neurotransmission (Xu et al., 2000). This may be as a result of trafficking under the control of different endosomal regulators, as has been reported in hippocampal axons (Arimura et al., 2009), however an axonal presence of TrkB does not appear to be at adequate levels to support robust regeneration. This seems to be the case for many growth factor receptors, as stimulation with NGF, BDNF, NT-3, NT-4, GDNF, has limited effects on CST regeneration (Kordower and Tuszynski, 2008; Thoenen and Sendtner, 2002)

Reggie/flotillin proteins

Reggie and flotillin are the same proteins with different names; reggie 1 and 2 are flotillin 2 and 1, respectively. The two proteins localise to lipid-rich microdomains in the surface membrane, (often referred to as lipid rafts) as well as to other endosomal membranes. They have two names because they were identified simultaneously in two separate labs. The Stuermer lab identified two proteins from larval goldfish using an antibody against Thy-1 to isolate microdomain enriched molecules, and subsequently named them the reggie proteins because they are upregulated by retinal ganglion cells during axon regeneration in the fish visual system (Schulte et al., 1997). At the same time, the Lodish lab identified flotillin-1 from a screen to identify novel components of caveolae (small invaginations of the plasma

membrane involved in clathrin independent endocytosis and signal transduction), using the term flotillin because they found the protein resided in a detergent resistant buoyant (floating) membrane fraction in the brain. Importantly, the Lodish lab noted the absence of caveolae in the brain, highlighting a potential role for the protein outside of caveolae (Bickel et al., 1997). There is now a large literature regarding the cell biological role of the reggie/flotillin molecules, mostly regarding their roles in caveolae and clathrin-independent endocytosis (using the flotillin name), and there are detailed reviews around this (Babuke and Tikkanen, 2007; Bodin et al., 2014; Hansen and Nichols, 2009). Here we mention the neuronal functions of reggie/flotillin in axon regeneration, their association with Rab11 endosomes, and their function in synapse regulation.

A role for reggie/flotillin in the regulation of axon growth and regeneration was first suggested by experiments to silence their expression in the zebrafish retina. Depletion led to a substantial decrease in the number of regenerating axons. Experiments in hippocampal neurons found the presence of the reggie/flotillin along axons and at the growth cones of neurons developing *in vitro*, and silencing with siRNA led to a similar reduction in axon length (Munderloh et al., 2009). This resulted in experiments aimed at increasing RGC regeneration after an experimental optic nerve crush in rats. Reggie 1 was transduced into RGCs by intravitreal AAV injection two weeks before an optic nerve crush, and regeneration was assessed four weeks later. Overexpression of reggie-1 increased the number of axons crossing the lesion site by 3-5 times, and regenerating axons were found up to 5mm beyond the injury site, confirming a role for the reggie/flotillin molecules in facilitating axon regeneration (Koch et al., 2013). As neurons lack caveolae, it is unlikely that these effects are mediated by membrane internalisation/endocytic functions but may have more to do with trafficking or targeting of molecules onto the cell surface. T-cells also lack caveolae, and in these cells, reggie/flotillin mediates the targeting of the T-cell receptor from an intracellular

compartment to a region on the cell surface known as the T-cell cap (Langhorst et al., 2006). The identity of the intracellular compartment is debated, with some studies finding reggie/flotilin on early endosomes, being involved in endocytosis (Glebov et al., 2006), however in neurons, reggie/flotillin are also found enriched in recycling endosomes (as marked by Rab11). Amongst the molecules implicated in mediating trafficking together with reggie/flotillin, are the GTPase TC10 and the secretory exocyst component exo70. Crucially, this complex (TC10 and exo70) mediates growth cone membrane addition and axon growth downstream of the IGF-1 receptor and PI3 kinase (Dupraz et al., 2009). TC10 is found on Rab11 positive endosomes and stimulates neurite outgrowth by stimulating exocytic fusion of Rab11 endosomes with the plasma membrane (Fujita et al., 2013). Rab11 was subsequently shown to interact with reggie/flotillin at the recycling endosome, functioning to return cadherins and the transferrin receptor to the plasma membrane (Solis et al., 2013). Reggie/flotillin also regulate EGF receptor trafficking (Solis et al., 2012), and together with Rab11 control integrins and focal adhesion recycling (Hulsbusch et al., 2015).

In mature neurons, reggie/flotillin were recently shown to function with Rab11 in the regulation of synapse development and post-synaptic trafficking, being involved in the trafficking of cadherins, glutamate receptors and PSD95 into dendritic spines (Bodrikov et al., 2017). Other studies have implicated reggie/flotillin in the regulation of synapses, being involved in strengthening glutamatergic synapses *in vitro* (Swanwick et al., 2010), whilst protein levels are altered *in vivo* when somatosensory cortical synapses are modulated by sensory deprivation (Butko et al., 2013). These studies imply a dendritic localisation for reggie/flotillin in mature CNS, however their localisation to mature axons has not been fully investigated. It is clear that the reggie/flotillin molecules regulate developmental axon growth and function together with regenerative molecules such as integrins and Rab11 but localise to dendrites after development. It will be important to determine if they are absent/diminished in

mature CNS axons. It is likely that interventions that promote *in vitro* regeneration via enhanced integrin/Rab11 axon transport may also be functioning by facilitating the transport of other regenerative molecules such as reggie/flotillin, which are also present on recycling endosomes.

Other organelles/complexes and axon regeneration

Mitochondria

Mitochondrial axonal transport and morphology are altered with cortical neuron maturation. During development mitochondria are highly mobile and move bidirectionally along the axon to meet the high energy demands of the developing cells while also acquiring shortened morphology to aid this dynamic movement. This situation changes with development, so that in mature neurons, mitochondria elongate and become less dynamic to serve the updated functions of the cell (Chang and Reynolds, 2006). Using live imaging of mitochondrial transport, a subsequent study revealed one reason why mitochondria are less mobile in mature neurons is that they are being anchored to the axon by syntaphilin (Kang et al., 2008). These observations have recently been confirmed *in vitro* and *in vivo*. Immature neurons *in vitro* (3-7DIV) exhibit very mobile mitochondria whereas in more mature neurons (10+ DIV) the mitochondria tend to be less mobile with up to 95% of all axonal mitochondria being stationary by 28DIV (Lewis et al., 2016). Interestingly, the number of mobile vs. stationary lysosomes did not seem to change, so this mitochondrial reduced mobility with maturation seems to be specific which could be explained by the localisation of mitochondria to pre-synaptic terminals with the development of synapses. The authors also examined the transport of mitochondria using two-photon imaging and showed that more than 90% of mitochondria in the distal axons of layer 2/3 cortical neurons are actually stationary. This lack of mobility corresponds with a decline in regenerative capacity and can be targeted to

facilitate regeneration, either by interfering with the docking of mitochondria by syntaphilin or by overexpressing the mitochondrial motor adaptor Miro-1. These interventions lead to increased axonal mitochondrial dynamics, and enhanced regeneration of mature cortical neurons *in vitro*. Axons from syntaphilin knockout mice also exhibit enhanced mitochondrial motility and a rescue of energy deficits after *in vitro* axotomy, and enhanced regeneration after a sciatic nerve crush injury. This study suggests that unmet energy demands after axonal injury is one reason why mature axons are poor regenerators (Zhou et al., 2016). Enhancing mitochondrial motility can also stimulate regeneration of injured CNS axons as demonstrated by a recent study focusing on the mitochondria-associated molecule Armcx1. This molecule is upregulated when regeneration is stimulated in the optic nerve by interventions such as PTEN deletion. Overexpression of Armcx1 stimulates mitochondrial motility, *in vitro* CNS axon growth, and axon regeneration after an optic nerve crush. It also enhances optic nerve regeneration when overexpressed in mice with genetic PTEN deletion (Cartoni et al., 2016).

Proteasome

The ubiquitin-proteasome plays an important role in regulating the concentration of individual proteins within the cell, by clearing excessive or unwanted proteins. It is also responsible for clearing damaged or mis-folded proteins (Korhonen and Lindholm, 2004). Proteasome function ensures that the correct amount of protein is present in specific subcellular regions at any given time. Proteasome transport into axons is dependent upon association with membranous vesicles, which are transported by kinesin and dynein motors (Otero et al., 2014). In the PNS, there appears to be predominant anterograde transport of the proteasome because ligation of the sciatic nerve leads to the accumulation of proteasomal subunits on the proximal side of the ligation (indicating the blockade of proteasomes moving away from the cell body). Proteasomal subunits also accumulate on the distal side of the

lesion, indicating retrograde transport also occurs, however this is not as profound as is seen on the proximal side (Otero et al., 2014). Importantly, this anterograde transport appears to be enhanced after a growth-stimulating pre-conditioning injury (Verma et al., 2005), suggesting that PNS axons increase the anterograde transport of proteasomal components as part of their response to an injury. Proteasomal activity appears to be an important part of the PNS regeneration process, because inhibition of the proteasome by lactacystin leads to a reduction in the percentage of axons that can reform a growth cone after in vitro axotomy (Verma et al., 2005). CNS neurons also transport proteasomal subunits into their axons, both early on in development (Hsu et al., 2015), and also at a more mature developmental stage (Otero et al., 2014), although it is not clear whether the proportion of anterograde or retrograde transport changes with development. The study by Hsu et al suggests that there is predominant retrograde transport, at least at an early developmental stage (embryonic day 18 cortical neurons cultured for 3 days), whilst the study by Otero et al suggest that later in development (10 days *in vitro*) the majority of CNS proteasomal transport (80%) is random and diffuse, with only small amounts of clearly anterograde or retrograde transport. This difference may be to do with maturation, but could also be down to experimental conditions, Hsu et al imaging proteasomal transport by use of a fluorescent dye (MV151), and Otero et al imaging YFP tagged proteasomal subunits. Whatever the case, these studies did not find that the predominant anterograde proteasomal transport observed in the PNS is also present in the CNS. Instead, there may be an increase in retrograde proteasomal transport which increases with axon length (Hsu et al., 2015). Given that a dynamic balance between local protein synthesis and protein degradation is important for PNS axon regeneration after injury (Gumy et al., 2010), it may be that efficient axonal proteasome transport is required for a properly functioning growth cone which can drive regenerative axon growth.

Autophagosomes

In its simplest form, autophagy is a highly regulated process through which cellular components can be degraded and recycled for reuse within the cell. For example, unwanted or damaged proteins can be recycled as amino acids to feed protein translation. Autophagy can occur in a steady fashion, or in response to stress or starvation. The normal autophagy process involves the formation of the double membrane bound autophagosome, which envelopes isolated cellular constituents. These eventually fuse with lysosomes to form autolysosomes, which degrade proteins via their acidic environment (Bento et al., 2016; Glick et al., 2010; Kaur and Debnath, 2015). However, there is also significant crosstalk between autophagosomes and endosomes (Davis et al., 2017; Kim et al., 2012; Szatmari et al., 2014), particularly at the level of the late endosome (Hyttinen et al., 2013; Lamb et al., 2013), resulting in a multi-vesicular endo/autophag-osome termed the amphisome (Patel et al., 2013; Sanchez-Wandelmer and Reggiori, 2013). Amphisomes can fuse with the surface membrane causing the release of sequestered proteins to the extracelluar environment as well as adding membrane to the cell surface (Claude-Taupin et al., 2017). Addtionally, lysosomes can also function as exocytic vesicles (Arantes and Andrews, 2006; Naegeli et al., 2017; Padamsey et al., 2017) in addition to being organelles of degradation, even supplying integrins for directed migration (Rainero and Norman, 2013). Traffic through the autophagosome-lysosome pathway is therefore extremely complicated and not necessarily a means of degradation.

Neuronal autophagy is well studied (Jin et al., 2018a), largely because its misregulation is implicated in degenerative diseases such as Alzheimer's, Parkinson's and Huntingdon's disease as well as amyotrophic lateral sclerosis (ALS) / motor neuron disease (Dikic and Elazar, 2018). There are a number of excellent recent reviews which comprehensively discuss the function and regulation of autophagy within neurons (Kulkarni et al., 2018;

Kulkarni and Maday, 2018; Nikoletopoulou and Tavernarakis, 2018; Stavoe and Holzbaur, 2018). Here we discuss the involvement of autophagy in the process of axon growth and regeneration, the presence of autophagy specifically within the axon, how autophagic mechanisms may vary between neuronal types of differing regenerative ability, and the regulation/interaction of Rab11 and recycling endosomes with the autophagosome. We also comment on a role for non-acidic lysosomes at the growth cone.

Currently the majority of evidence suggests that autophagy is required for axon growth and regeneration. Whilst one study reports that inhibition of autophagy by silencing of ATG7 leads to increased axon extension (Ban et al., 2013), the majority of studies suggest that autophagy is required for axon growth. The autophagy genes ULK1 and 2 are required for normal axon extension in the developing mouse brain (Wang et al., 2017a), and inhibition of autophagy opposes axon growth and survival of DRG neurons in culture, suggesting a positive role for autophagy during regenerative axon growth (Clarke and Mearow, 2016). Autophagy also seems to be beneficial for CNS axon regeneration, with a recent study demonstrating that stimulation of autophagy can promote axon growth over inhibitory molecules *in vitro*, whilst activation of autophagy *in vivo* (by delivery of of Tat-beclin) leads to enhanced regeneration of monoaminergic neurons after a spinal cord injury (He et al., 2016). This study used EM to demonstrate that beclin-induced autophagosomes were present specifically within axons. If autophagy is required within the axon in order to regenerate a growth cone, it is possible that there may be differences in the transport of autophagy machinery between regenerative and non-regenerative axons. It is important to note that monoaminergic neurons have a better regenerative ability than corticospinal tract axons, and the induction of autophagy by Tat-beclin does not stimulate CST regeneration (He et al., 2016). This may indicate that the necessary growth-promoting autophagy machinery is not present in these axons.

Autophagy, and the transport of autophagosomes, has been examined in both CNS and PNS axons and whilst there is clear evidence for the existence of autophagy in both neuronal types, there may also be differences. Axonal autophagosome biogenesis was first studied in adult mouse DRG neurons (Maday et al., 2012), by imaging GFP-LC3 (an autophagosome marker). Autophagosomes were observed developing in the distal part of axons, however in this location they remained negative for lysosomal markers. Instead autophagosomes became positive for lysosomal markers as they were retrogradely transported towards the cell body. This suggests that mature autophagy (degradation) does not occur in the distal axon but is targeted to the cell body (Maday et al., 2012). Despite this, there is apparent contact of autophagosomes with lysosomes within the distal part of the axon, indicating that there may be some fusion events that may not be degradative. It is not clear if the contents of distal axon autophagosomes can escape autophagy by being passed to lysosomes. However, there is evidence that lysosomes may be required at the growth cone to enable the trafficking of cargo out of the autophagosome because decreasing the amount of lysosomes at the growth cone leads to enlargement of autophagosomes (Farias et al., 2017). There is also evidence that growth cone lysosomes may not be degradative as they are not acidified in the same way as the rest of the cell (Farias et al., 2017; Overly and Hollenbeck, 1996). Lysosomes supply integrins to enable invasive and migratory behaviour in non-neuronal cells (Dozynkiewicz et al., 2012; Rainero and Norman, 2013) and can undergo exocytosis to contribute to axon growth (Arantes and Andrews, 2006). It is therefore possible that there is a form of recycling that occurs at the growth cone via autophagosome/endosome interaction. We speculate that this may be required to enable regeneration when an axon needs to alter its machinery to switch from a state of neurotransmission to a state of axon growth. The process of autophagy could engulf unwanted machinery, which in turn could either be degraded or recycled through lysosomes or other endosomes.

The above study by Maday et al demonstrated that axonal autophagosomes are predominantly transported retrogradely towards the cell body in DRG neurons. Subsequent work confirmed that a similar phenomenon occurs in CNS neurons, through the study of E15.5 mouse neurons in vitro (Maday and Holzbaur, 2014; 2016). These investigations found that axonal autophagy was not induced in response to nutrient deprivation (as occurs in other cell types), but rather that autophagosome generation appears to be a homeostatic process. They also showed that autophagosome biogenesis is four times slower in the distal axons of hippocampal neurons compared to DRG neurons. The studies also demonstrated that the axonal ER is a source of membrane for the axonal autophagosome and not the plasma membrane, but the authors did not examine a potential role for Rab11. Rab11 positive recycling endosomes are a source for autophagosomal membrane during starvation induced autophagy in non-neuronal cells (Lamb et al., 2016; Longatti et al., 2012; Longatti and Tooze, 2012). Given that Rab11 endosomes are transported away from CNS axons as they mature (Koseki et al., 2017) it is possible that this may limit the type of autophagy that exists within the axon. The question remains as to whether starvation or stress induced autophagy is a mechanism that needs to be activated in order to stimulate the process of regeneration within the axon. It is also not known if there are developmental changes in the rate or type of autophagy that occurs within axons (which may contribute to regeneratve ability) or whether the type of autophagy varies between neurons of different regenerative abilities. Perhaps recycling autophagy (as opposed to degradative) is more suited to axon regeneration? There is strong evidence for the involvement of Rab11 in both the generation of the autophagosome (as mentioned above) and also in mediating traffic away from autophagosomes towards multivesicular bodies and exocytosis (Chen et al., 2017; Fader et al., 2008; Fader et al., 2009). Given the difference in Rab11 transport between non-regenerative and regenerative axons, it may be that there are differences in the way autophagy functions in the axons of

various neuronal types, that might contribute to their differential regenerative ability. Much work is needed to determine whether this is the case.

Mechanisms regulating polarised transport

As described above, the capacity for axon regeneration varies between neuronal types and with development. Adult PNS axons are considered good regenerators, whilst most CNS axons lose their ability to regenerate with maturity. The ability to transport regenerative molecules into axons also varies. For example, PNS axons continue to support integrin transport into adulthood whilst transport declines with maturity in CNS axons (Fig. 1). This also appears to be the case for other growth promoting receptors, which are either absent or expressed at low levels in most mature CNS axons. To understand the mechanism behind these differences, it is necessary to examine the regulation of polarised transport in neurons, particularly focusing on membrane proteins. The axon growth promoting receptors and guidance molecules that mediate regeneration such as TrkB, the IGF1 receptor and integrins are all cell surface membrane proteins. This class of molecule is subject to tightly controlled trafficking processes in all cells. As an extreme example of polarised cells, neurons have intricate mechanisms for maintaining the correct distribution of membrane proteins to specific neuronal compartments. This ensures that post synaptic receptors and associated machinery are targeted to dendrites, whilst the machinery for synaptic vesicle cycling and neurotransmission are directed to presynaptic sites within axons. In this section, we describe the mechanisms that are known to regulate polarised distribution in neurons, focusing on their relevance to regulating regenerative capacity. We have described above how a conditioning injury that promotes regeneration can also facilitate axon transport. This response relies partly on the retrograde injury signal, which transmits signals from the axon to the cell body via retrograde axonal transport. We will not be focusing on this retrograde

response (which has been reviewed in detail: (Abe and Cavalli, 2008; Rishal and Fainzilber, 2014; Tasdemir-Yilmaz and Segal, 2016), but rather the mechanisms involved in facilitating or preventing anterograde delivery, and how these might relate to axon regeneration.

Neuronal membrane transport

All membrane proteins are synthesised in the ER membrane, and spend their lives restricted to a membranous environment. After leaving the ER, membrane proteins pass through the Golgi membrane before being transported to the cell surface in endosomes. At the cell surface, membrane proteins can be internalised (again into endosomes) and are subject to a variety of regulatory mechanisms which can decide their fate – recycling to the plasma membrane, redirecting to a different part of the cell, clustering into signalling platforms, degrading, or even priming for extracellular cues (Yap and Winckler, 2012). Membrane protein trafficking is therefore subject to precise and complex regulation by numerous processes including cytoskeletal elements, motor proteins, adaptor molecules, protein scaffolds, signalling molecules (including kinesins and phosphatases), and small GTPases such as the Rab and ARF families. Investigations into neuronal polarised membrane transport has focused on all of these mechanisms, and each of them plays a role in regulating transport into either dendrites or axons.

Microtubules and associated motors

The principle regulator of polarised transport in neurons is arguably the cytoskeleton, which defines axons and dendrites by virtue of the orientation of microtubules. Axonal microtubules are unipolar, with the plus end facing into the axon, whilst dendrites have microtubules aligned both into and away from the dendrite (Baas et al., 1988; Tas et al., 2017; Yau et al., 2016). As the majority of membrane protein transport occurs in endosomes transported on

microtubules by kinesin or dynein/dynactin motor proteins, the unipolar nature of axonal microtubules ensures that only kinesin motor proteins can drive anterograde axonal transport, and that molecules transported principally by dynein are enriched in dendrites. Proteins intended for axonal transport will also enter dendrites, but overall their distribution is biased towards axons (Nakata and Hirokawa, 2003). Microtubule orientation alone is not sufficient to determine whether a protein can enter the axon, and so further control is exerted by the wide variety of the kinesin family. Neurons express as many as 20 types of kinesin that will transport cargo towards the plus end (Silverman et al., 2010), but only some of these specifically target to axons, whilst others target to both dendrites and axons. Interestingly, no kinesins have been found to be targeted only to dendrites (Huang and Banker, 2012). This suggests that dynein-dependant transport is an important determinant in dendritic targeting, and this has been shown to be the case, because linking axon specific cargo to dynein motors results in their dendritic delivery (Kapitein et al., 2010). The specificity of individual kinesins for axons may contribute to the developmental decline in regenerative ability, in that the expression of certain kinesins changes with development. Kinesin KIF4A is involved in the axonal delivery of integrins during development, but it is downregulated postnatally at a time when integrins are excluded from axons (Heintz et al., 2014). However, whilst re-expressing KIF4A in mature cortical neurons *in vitro* leads to its presence in axons, it does not facilitate integrin axonal transport. Integrins can be manipulated into axons at this time (through other trafficking interventions), suggesting that other kinesins are also capable of transporting integrins into axons.

The targeting of specific kinesins to the axon is thought to be partly as a result of specific modifications to the microtubules, including acetylation, glutamylation and detyrosination which regulate axonal kinesin transport (Hammond et al., 2010; Kaul et al., 2014; Konishi and Setou, 2009). Importantly, interventions targeting these modifications such as low dose

taxol can permit axonally excluded growth promoters such as integrins to enter the axon (Franssen et al., 2015), however this can also cause axonal proteins to accumulate in dendrites (Hammond et al., 2010). Interestingly, low dose taxol treatment only permits integrins transport into the proximal part of the axon, suggesting microtubule modifications are particularly critical in this area. The early part of the axon is an area of intense study regarding polarised transport. This region of the axon includes two important regions implicated in the regulation of polarised transport, the axon initial segment (AIS) and before it, the pre-axonal exclusion zone (PAEZ).

The axon initial segment and the regulation of axonal traffic and transport

The AIS is in the very proximal part of the axon. It is primarily responsible for the propagation of the action potential, being enriched in the ion channels necessary for this function. It is also enriched in cytoskeletal elements such as AnkyrinG, actin, and beta IV spectrin, and microtubule associated proteins such as EB1 and EB3 (Leterrier et al., 2011; Zhang and Rasband, 2016). Importantly, the AIS develops at a time when CNS axons lose their regenerative ability, and is strongly associated with polarised membrane transport and axon dendrite identity (Rasband, 2010). Ankyrin G is considered to be the orchestrator of the AIS because its depletion causes demolition of the entire structure. Depleting Ankyrin G leads to a loss of axonal identity, with the proximal axon exhibiting dendritic molecules such as post-synaptic receptors and taking on dendritic features such as spines (Sobotzik et al., 2009). A number of mechanisms have been proposed to explain how the AIS might regulate polarised transport, in addition to the post-translational modifications of microtubules (Hammond et al., 2010; Konishi and Setou, 2009; Tapia et al., 2013). The actin cytoskeleton is proposed to regulate transport from within the AIS by acting as a dense barrier to diffusion (Song et al., 2009) or by diverting myosin motors back to the cell body (Lewis et al., 2009).

This idea is currently debated because other studies have not found a dense actin mesh within the AIS (Farias et al., 2015), but rather the usual actin and spectrin rings that are present throughout the axon (Leterrier et al., 2015; Xu et al., 2013) as well as actin patches (Jones et al., 2014). Interfering with actin through latrunculin treatment leads to a small increase in integrin transport into the proximal part of mature CNS axons (Franssen et al., 2015), but does not facilitate long range axonal transport.

Another AIS-related mechanism functions to regulate dynein dependant retrieval of dendritic cargo from within the AIS. The dynein regulator Ndel1 is attached to the AIS through binding to Ankyrin G and functions through its binding partner Lis1 to activate dynein leading to increased retrograde transport of molecules not intended for axons, such as the transferrin receptor. Silencing Ndel1 leads to anterograde transport of the transferrin receptor (Kuijpers et al., 2016). Importantly, Ndel1 is localised to the initial part of axons from an early developmental stage, at a time when integrins are still present throughout the axon, suggesting it is not the central regulator of integrin dendritic localisation. Retrograde transport of early endosomes marked by Rab5 is also regulated from this part of the axon through the Rab5 interactor FHF (Guo et al., 2016). Crucially, there is also retrograde transport of dendritic vesicles away from the base of the axon even before the AIS develops, from a region termed the pre-axonal exclusion zone (PAEZ) (Farias et al., 2015). This area has been implicated in the dendritic targeting of glutamate receptor AMPA-GluR1, the Golgi protein GM130, and the ER protein CLIMP-63, and the endoplasmic reticulum, indicating that the region is critical to maintaining a wide range of cell machinery away from axons (Britt et al., 2016; Gumy and Hoogenraad, 2018). This early developmental axonal exclusion is in keeping with a previous study which demonstrated that dendritic proteins become polarised even before the axon is specified (Petersen et al., 2014). Interestingly, the PAEZ overlaps with a region within the AIS which labels strongly for TRIM46. This is a

microtubule organising molecule which is present before the AIS is fully developed, which is responsible for arranging microtubules in their polarised fashion. Depletion of TRIM 46 leads to mixed microtubule orientation and decreased transport of axonal cargo (van Beuningen et al., 2015). Another level of selectivity is achieved within this region by the microtubule associated protein, MAP2. Whilst classically considered to be a dendritic marker, MAP2 strongly labels the earliest section of the axon, and its localisation there depends on TRIM46. Depletion of MAP2 leads to altered cargo transport, with some dendritic molecules being transported into axons, and some axonal molecules appearing in the somatodendritc domain. MAP2 functions in this location to regulate kinesin activity, specifically inhibiting KIF5 motor activity, so that cargo that is transported by KIF5 requires an additional motor such as KIF1 (which is not affected by MAP2) in order to enter the axon (Gumy et al., 2017).

Crucially, MAP2 is present in the early part of the axon in non-regenerative and regenerative neurons being observed in both mature CNS neurons that do not support integrin transport, as well as PNS neurons that do. MAP2 therefore permits anterograde transport of the kinesins that carry integrin endosomes, but in CNS axons there are additional signalling and trafficking mechanisms that are upregulated with development that result in an increased affinity of integrin containing endosomes for the dynein/dynactin complex, and subsequent retrograde removal from axons. Recent studies have found that the principle regulators of this process are ARF6 and its activator EFA6, which is enriched in the initial part of the axon as cortical neurons mature (Eva et al., 2017).

Axon initial segment, ARF6 and the JIP family of proteins

The ARF6 GEF EFA6 is upregulated in the brain along with development, playing a role in the development and maintenance of dendrites and spines (Choi et al., 2006; Raemaekers et al., 2012). It was recently discovered to have an additional axonal role, colocalising with

neurofascin in the early part of the AIS, and functioning to activate ARF6 throughout the axon stimulate retrograde integrin transport (Eva et al., 2017). This may occur through the priming of another ARF6 GEF, ARNO, which is distributed along the whole length of the axon. EFA6 is known to function together with ARNO to sustain ARNO and ARF6 activity (Padovani et al., 2014). This appears to happen in axons, because silencing EFA6 leads to a striking decrease in axonal ARF6 activation. Importantly, silencing EFA6 leads to an increase in the anterograde axonal transport of integrin and Rab11 endosomes and an increase in regeneration after a laser injury. Interfering with ARNO also leads to an increase in axonal integrin transport in cortical neurons, as does overexpression of the ARF GAP ACAP1 (Franssen et al., 2015). These molecules were first shown to regulate directional axon transport in PNS axons (which allow bi-directional integrin transport) (Eva et al., 2012b). Elevating ARF6 activity in adult DRG neurons *in vitro* leads to an increase in retrograde integrin transport and a decrease in regeneration after a laser injury. DRG neurons enable integrin transport through the expression of the GAP ACAP1, which is not present in the CNS. ACAP1 localises throughout DRG axons, and is strongly enriched in the growth cone (Eva et al., 2017). In addition to its ARF6 GAP activity ACAP1 also targets integrins to the surface membrane (Li et al., 2005). How does ARF6 regulate the direction of integrin transport in axons? The ability of ARF6 to control directional transport was first demonstrated in dividing non-neuronal cells, and relies on the interaction of ARF6 with the scaffold molecules JIP3 and 4. Active ARF6 increases the affinity of these JIPs for the dynein/dynactin complex whilst inactive ARF6 increases their affinity for kinesin motors (Montagnac et al., 2009). The JIP family of molecules therefore appear to exert a level of selectivity on axonal cargo and have the ability to exert this at many levels. As scaffolds, the JIPs function to link motor proteins to their cargo. The interaction between specific kinesins and the various JIPs defines one level of selectivity, while another is governed by the cargo

that can or cannot interact with the JIPs. A good example of this is the difference between integrins and the amyloid precursor protein (APP). Integrins are transported away from mature CNS axons, whilst APP is bidirectionally transported. This may be as a result of their different interactions with the JIPs. There are four JIP family members. JIP 1 and 2 are similar but diverge from JIP 3 and 4 which are often classed together. APP interacts directly with JIP 1 and 2, and does not interact with JIP 3 and 4. ARF6 activation does not affect JIP1 or 2 interactions with kinsesin or dynein, but only JIP3 and 4 (Koushika, 2008). Consistent with this, altering ARF6 activation does not alter APP axonal transport (Eva et al., 2017). APP directional transport is regulated through a different signalling mechanism. When JIP1 is directly phosphorylated at a JNK phosphorylation site, S421, anterograde transport is stimulated, whilst dephosphorylation favours retrograde transport (Fu and Holzbaur, 2013). Importantly, the interactions between JIP/motor/cargo can occur in complexes with other proteins, meaning that their functions can impact on the directional transport of numerous proteins. The interaction between ARF6, JIP3 and 4 and motor proteins occurs in a complex that also involves Rab11 (Montagnac et al., 2009), meaning that ARF6 activation can also control the direction of transport of Rab11 and associated endosomes (Eva et al., 2017). This begins to explain how trafficking to specific endosomes might contribute to the targeting of membrane proteins to a specific part of the cell, with different endocytic regulatory or adaptor molecules adding another level of complexity.

Endocytic transport, adaptors and sorting motifs

A number of endosome associated molecules have been implicated in polarised transport in neurons, including the EHD1/4 proteins and NEEP21/P19 (Nsg1 and 2), which regulate the trafficking of the adhesion molecule L1/NgCAM through early endosomes towards the axon (Lasiecka et al., 2010; Yap et al., 2008) via a transcytotic mechanism. Transcytosis

(internalisation from the somatodendritic surface to the axonal surface) occurs in DRG neurons for integrins and the TrkA receptor via Rab11 endosomes (Ascano et al., 2009; Eva et al., 2010). Transport of the TrkA receptor is additionally regulated in a complex feed forward fashion which enables transcytosis to supply new receptors to the axon in response to retrograde signalling from within the axon. Anterograde transport is increased when retrograde signalling endosomes recycle to the somatic cell surface and transactivate resident receptors, causing them to be internalised. These are then transported to the ER, where they can be dephosphorylated by the phosphatase PTP1B before being anterogradely transported into the axon (Yamashita et al., 2017).

The adapter molecules that regulate endocytosis are also important for regulating polarised distribution within neurons. These include the clathrin adaptors AP1-AP5 which exist as heterotetrameric complexes and the monomeric GGA adaptors (Golgi-localising, Gamma-adaptin ear homology, ARF-binding proteins) (Robinson, 2004). The clathrin adaptors interact with specific motifs that have been implicated in polarised transport in neurons and non-neuronal cells, including the AP1 adaptor which controls the dendritic distribution of the transferrin receptor through an interaction between the YXXØ motif in the cytoplasmic tail of transferrin and the µ1A subunit of AP1 (Farias et al., 2012). However, the presence of this motif does not guarantee targeting a protein away from the axon. Integrin subunits α 3, 4, 5, 7, and 9 contain a YXXΦ motif, but preventing AP1 from a potential interaction with the motif by expression of a dominant negative mutant does not result in targeting of integrins into the axon, as was found for the transferrin receptor (Franssen et al., 2015).

Summary: A virtuous cycle of axon growth and regeneration

Since the Aguayo experiments of the 1980s kick-started an era of research into CNS axon regeneration, there has been a broad-ranging, sustained effort to understand the mechanisms

preventing regeneration. Understanding of intrinsic factors has increased dramatically, and it is apparent that there are three key elements which prevent regeneration: epigenetics, signalling pathways, and axon transport. Developmental gene changes maintain adult CNS neurons in a low state of growth (Venkatesh et al., 2016), compounded by underactive growth promoting signalling pathways (Liu et al., 2010), and a low abundance of growth machinery within the axon (Andrews et al., 2016; Eva et al., 2017; Hollis et al., 2009a; b). These areas represent targets for simulating regeneration and raise the tantalising prospect of an intervention that could function by affecting all three processes. One can envisage a strategy that targets epigenetic changes could lead to the expression of genes which enhance signalling through growth promoting pathways, as well as increased axonal transport of regenerative machinery such as integrins or growth factor receptors. Once in the axons, growth-promoting receptors could be activated by their ligands, leading to retrograde signalling via endosomes, amplification of growth promoting signals, and somatic effects on transcription, translation and axonal transport. In theory, this cycle of events could be targeted at any point to stimulate a "virtuous cycle of axon growth" (Fig. 2). A good example is intervening at the level of axon transport of integrins and growth factor receptors. Increasing the levels of these molecules on the surface of the axon could lead to increased signalling through known regenerative pathways, such as the PI3 kinase pathway. This would elevate growth promoting signals locally, but these are additionally capable of signalling to the cell body via retrograde signalling endosomes, which can lead to effects on transcription and translation (Tasdemir-Yilmaz and Segal, 2016). Signalling through PI3 kinase can lead to epigenetic changes which can activate a growth program (Spangle et al., 2017), and can also lead to increased axonal transport of PI3 kinase coupled receptors, such as TrkB (Cheng et al., 2011). Initiating the axonal transport of growth-enabling molecules can therefore have wide-ranging effects throughout the cell. So far, *in vitro* experiments have demonstrated that

CNS regeneration can be stimulated through the axonal mobilisation of growth machinery (Eva et al., 2017; Koseki et al., 2017). Much work is needed to see if this approach can be used to stimulate regeneration *in vivo* after a brain or spinal cord injury.

Figure Legends

Figure 1. Integrin transport in regenerative vs non-regenerative axons.

Integrins and Rab11 are bidirectionally transported in regenerative adult PNS axons but are removed from non-regenerative CNS axons by predominant retrograde transport after development.

Figure 2. A virtuous cycle of axon growth and regeneration.

Adult CNS axons are weak regenerators because of gene suppression by epigenetic factors and a lack of growth promoting machinery in the axon. The figure illustrates a cycle of events which can enable regeneration. Intervening at any point can feed forward to stimulate the subcellular changes that can drive axon growth. For example, increasing the transport of growth promoting receptors in recycling endosomes facilitates growth cone development and axon growth. Activated growth cone receptors signal retrogradely to the cell body. Signalling downstream of growth factors (eg through PI3K) can lead to changes in gene expression and altered protein translation. Retrograde signals from growth factor receptors can also stimulate anterograde transport in an autocrine fashion.

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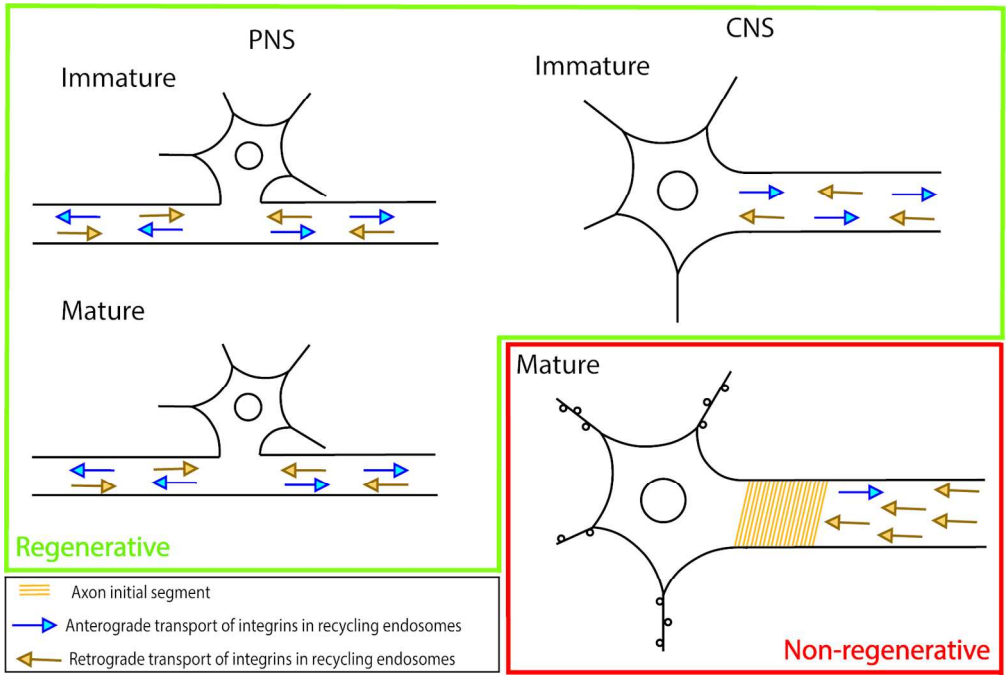

Figure 1. Integrin transport in regenerative vs non-regenerative axons.
Integrins and Rab11 are bidirectionally transported in regenerative adult PNS axons but are removed from non-regenerative CNS axons by predominant retrograde transport after development.

150x100mm (300 x 300 DPI)

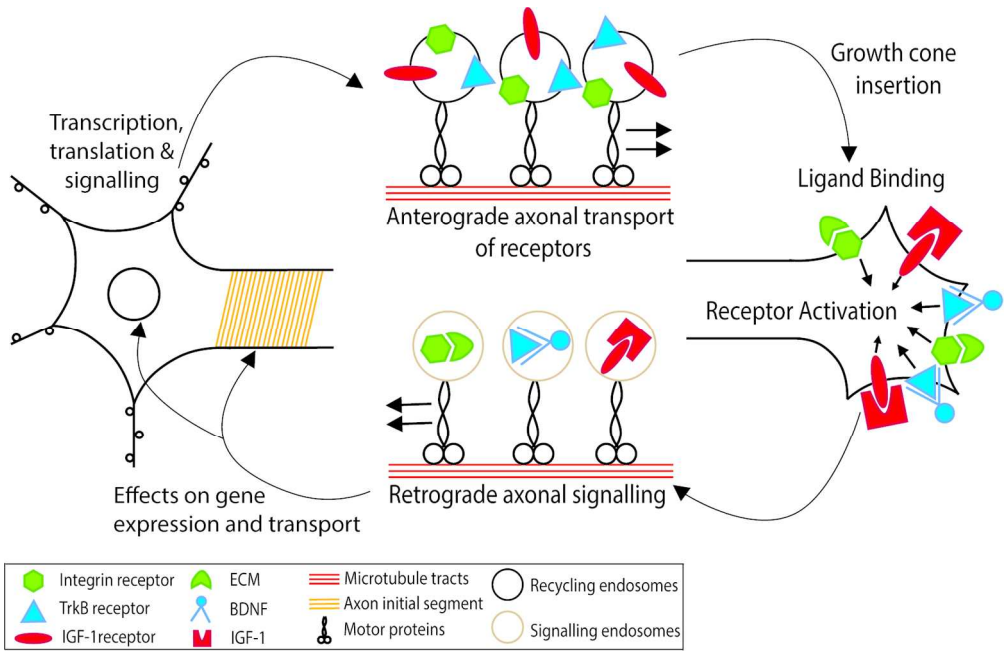

Figure 2. A virtuous cycle of axon growth and regeneration.
Adult CNS axons are weak regenerators because of gene suppression by epigenetic factors and a lack of growth promoting machinery in the axon. The figure illustrates a cycle of events which can enable regeneration. Intervening at any point can feed forward to stimulate the subcellular changes that can drive axon growth. For example, increasing the transport of growth promoting receptors in recycling endosomes facilitates growth cone development and axon growth. Activated growth cone receptors signal retrogradely to the cell body. Signalling downstream of growth factors (eg through PI3K) can lead to changes in gene expression and altered protein translation. Retrograde signals from growth factor receptors can also stimulate anterograde transport in an autocrine fashion.

149x96mm (300 x 300 DPI)