Developmental Neurobiology

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

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1 **Title:** The virtuous cycle of axon growth: Axonal transport of growth-promoting machinery

2 as an intrinsic determinant of axon regeneration

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4 **Short title:** Axon transport governs axon regeneration

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6 **Authors:** Veselina Petrova and Richard Eva.

7 John Van Geest Centre for Brain Repair, Department of Clinical Neurosciences, University

8 of Cambridge, Cambridge CB2 OPY, UK.

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10 **Corresponding Author:** Richard Eva, re263@cam.ac.uk

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59   **Abstract**

60   Injury to the brain and spinal cord has devastating consequences because adult central

61   nervous system (CNS) axons fail to regenerate. Injury to the peripheral nervous system

62   (PNS) has a better prognosis, because adult PNS neurons support robust axon regeneration

63   over long distances. CNS axons have some regenerative capacity during development, but

64   this is lost with maturity. Two reasons for the failure of CNS regeneration are extrinsic

65   inhibitory molecules, and a weak intrinsic capacity for growth. Extrinsic inhibitory molecules

66   have been well characterised, but less is known about the neuron-intrinsic mechanisms which

67   prevent axon re-growth. Key signalling pathways and genetic / epigenetic factors have been

68   identified which can enhance regenerative capacity, but the precise cellular mechanisms

69   mediating their actions have not been characterised. Recent studies suggest that an important

70   prerequisite for regeneration is an efficient supply of growth-promoting machinery to the

71   axon, however this appears to be lacking from non-regenerative axons in the adult CNS. In

72   the first part of this review, we summarise the evidence linking axon transport to axon

73   regeneration. We discuss the developmental decline in axon regeneration capacity in the

74   CNS, and comment on how this is paralleled by a similar decline in the selective axonal

75   transport of regeneration-associated receptors such as integrins and growth factor receptors.

76   In the second part, we discuss the mechanisms regulating selective polarised transport within

77   neurons, how these relate to the intrinsic control of axon regeneration, and whether they can

78   be targeted to enhance regenerative capacity.

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84 **Introduction**

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

86 Unlike axons of the peripheral nervous system (PNS) which readily regenerate after injury,

87 mammalian central nervous system (CNS) axons lose their regenerative capabilities with

88 maturity (Bradke and Marin, 2014; Nicholls and Saunders, 1996). Injury to the adult brain

89 and spinal cord can therefore have life-altering consequences. This problem was considered

90 unassailable until a series of seminal studies in the 1980s challenged the long-standing dogma

91 that CNS axons lack the capacity for repair. Aguayo and colleagues showed that injured adult

92 rat axons arising in the CNS can regenerate through a peripheral nerve graft where the

93 environment is more permissive to growth (Benfey and Aguayo, 1982; David and Aguayo,

94 1981; Richardson et al., 1980). These experiments were instrumental in demonstrating that

95 certain CNS neurons retain a limited amount of their intrinsic ability for regeneration, and

96 that failure of regeneration after injury could be attributed to the environment of the injured

97 brain or spinal cord. Since these studies, numerous inhibitory molecules have been identified

98 which oppose regeneration after a spinal cord injury, including the glial-derived chondroitin

99 sulphate proteoglycans (CSPGs) and myelin-associated inhibitors such as Nogo, myelin-

100 associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (Schwab

101 and Strittmatter, 2014; Silver et al., 2014; Yang et al., 2014; Yiu and He, 2006; Yu et al.,

102 2018). These discoveries focused research on overcoming the inhibitory environment after

103 injury, and led to the identification of interventions such as chondroitinase (Bradbury and

104 Carter, 2011) and anti-Nogo antibody treatment (Ineichen et al., 2017). The chondroitinase

105 approach, aimed at neutralising CSPGs is currently under intense investigation as a promoter

106 of CNS repair, whilst anti-Nogo is undergoing clinical trials aimed at enhancing recovery

107 after spinal cord injury. Both of these strategies lead to functional recovery through enhanced

108 plasticity and sprouting from spared axons, with modest effects on axon regeneration.

109 Regenerated axons extend over limited distances, forming new synapses with spared circuits

110 which connect beyond the lesion (Fawcett, 2015; Schwab and Strittmatter, 2014). Recent

111 work has confirmed that neutralizing the astrocytic scar does not enable robust regeneration,

112 whilst providing evidence that glial derived molecules can actually support regenerative

113 growth of ascending sensory fibres stimulated by growth factor treatment and a growth-

114 priming injury (Anderson et al., 2016). Without these growth-promoting stimuli, injured

115 axons can become ensnared by NG2 positive cells which cause dystrophic axons to form

116 synapses, further hindering attempts at regrowth (Filous et al., 2014).

117 Enabling long-range axon regrowth after injury to the spinal cord remains a challenging

118 objective, particularly with respect to corticospinal tract (CST) axons. These axons descend

119 from the cortex and are responsible for motor functions. CST axons have an outstandingly

120 weak intrinsic capacity for regeneration, even in a permissive environment (Richardson et al.,

121 1984). For this reason, there are concerted efforts to understand the mechanisms regulating

122 intrinsic growth capacity, in order to identify new strategies which might be used together

123 with extrinsic interventions to optimise regeneration.

124

125 *Targeting neuron intrinsic mechanisms to promote regeneration and repair*

126 Studies aimed at intrinsically increasing regenerative capacity have so far identified key

127 signalling pathways, transcription factors and epigenetic mechanisms that can be targeted to

128 increase regeneration (Dergham et al., 2002b; He and Jin, 2016; Hu and Selzer, 2017;

129 Leibinger et al., 2017; Lindner et al., 2013; Liu et al., 2011; Moore et al., 2009; Moore and

130 Goldberg, 2011; Muramatsu et al., 2009; Puttagunta et al., 2014; Qiu et al., 2002; Tedeschi

131 and Bradke, 2017). Whilst these have not yet led to clinical treatments, they have generated

132 new approaches that are potentially clinically translatable. A good example is the study of the

133 tumour suppressor PTEN. PTEN was identified ten years ago as an intrinsic inhibitor of axon

134 regeneration, functioning to oppose the actions of PI3 kinase (PI3K). Deleting PTEN leads to

135 robust retinal ganglion cell regeneration after optic nerve crush (Park et al., 2008), and can

136 enable CST axons to regenerate for short distances past a lesion site (Liu et al., 2010). Due to

137 its nature as a tumour suppressor, deletion of PTEN is not considered a clinically translatable

138 strategy. However, the PI3K pathway can be successfully targeted with a more translatable

139 approach that works by potentiating signalling downstream of the growth factor IGF1.

140 Combined viral delivery of IGF1 and the matrix molecule osteopontin was found to enhance

141 signalling through the PI3K pathway leading to robust axon regeneration after an optic nerve

142 crush (Duan et al., 2015). This discovery lead to subsequent experiments combining IGF1

143 with osteopontin as a treatment after a model of spinal cord injury (T10 hemi-section). This

144 strategy promoted profuse CST axonal sprouting and short-range regeneration leading to

145 recovery of hind limb function, demonstrating that intrinsic growth capacity can be targeted

146 in a translatable fashion to promote regeneration and recovery (Liu et al., 2017). However,

147 the combination did not enable long-range CST regrowth, highlighting the need for further

148 studies to understand the intrinsic regulation of axon regrowth ability. One particularly

149 evident obstruction is the change in gene expression that occurs with maturity, combined

150 with the lack of a cell body response after an axonal injury. Injury to axons in the PNS leads

151 to an upregulation of regeneration-associated genes (Hoffman, 2010; Neumann and Woolf,

152 1999), but this is not seen in the CNS (Plunet et al., 2002). Efforts have therefore been made

153 to enhance growth capacity via intervening with gene expression through transcription factor

154 manipulation. This has been done either by removing inhibitory transcription factors such as

155 KLF4 or overexpressing growth-promoting transcription factors such as KLF7 (Blackmore et

156 al., 2012; Moore et al., 2009; Wang et al., 2015). These approaches have yielded encouraging

157 results; however, they have not been able to promote the type of regeneration seen in the PNS

158 (Wang et al., 2017b). It is becoming apparent that this may be due to complex epigenetic

159   factors such as closed chromatin affecting transcription factor binding site availability

160   (Trakhtenberg and Goldberg, 2012; Venkatesh et al., 2016). In regenerative neurons, there

161   are mechanisms to enhance chromatin availability in response to an axonal injury, and these

162   can be targeted to facilitate CNS regeneration (Weng et al., 2017; Weng et al., 2018).

163

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

165   The studies described above have been instrumental in demonstrating that there are

166   developmental changes in gene expression and intracellular signalling in CNS neurons that

167   contribute to their feeble regenerative ability, and that these can be targeted to facilitate

168   regeneration. However, there remains a gap in our knowledge regarding the mechanisms

169   downstream of these events that lead to effects on axon growth. The mechanisms regulating

170   developmental axon growth are well-known and include cytoskeletal reorganisation, axon

171   transport, membrane addition, and insertion of guidance molecules onto the growth cone

172   surface (Allen and Chilton, 2009; Bradke et al., 2012; Hilton and Bradke, 2017; Quiroga et

173   al., 2018), but the extent to which these are involved in mediating the effects of the

174   interventions described above is not known. Cytoskeletal reorganisation is clearly an

175   important consideration as demonstrated by the stimulation of CNS regeneration by targeting

176   both the microtubule and actin cytoskeleton (Dergham et al., 2002a; Ruschel et al., 2015),

177   and the neutralisation of growth inhibition by targeting growth cone non-muscle myosin (Hur

178   et al., 2011).

179   Another critical factor mediating regenerative ability is an efficient axonal supply of the

180   machinery required for growth. It is becoming clear that there are developmental changes in

181   CNS neurons which limit the axonal availability of growth-promoting molecules such as the

182   integrin family of adhesion/guidance molecules and their transporters, Rab11 positive

183   endosomes (Andrews et al., 2016; Franssen et al., 2015). Integrins and Rab11 endosomes can

184   be manipulated into mature CNS axons *in vitro*, which enables axon regeneration after a laser

185   injury (Eva et al., 2017; Koseki et al., 2017), but it remains to be seen whether these

186   manipulations can used to promote long-range regeneration *in vivo*.

187   Intrinsic stimulation of integrin-driven long-range axon growth is possible through the spinal

188   cord, as has been demonstrated by recent studies in sensory (PNS) axons regenerating

189   towards the brain (Cheah et al., 2016). Viral transduction of a growth promoting integrin

190   (alpha9) together with its activator kindlin-1 into dorsal root ganglion (DRG) neurons

191   enabled them to regenerate their central axons (after a dorsal root crush) into the spinal cord

192   and over long distances through the spinal cord (from the level of forepaw to medulla). The

193   study demonstrated that long-range axon growth is possible through a normally inhibitory

194   environment, as a result of intrinsic manipulations. The strategy works because PNS axons

195   efficiently transport integrins into their distal axons. It could potentially be used to enable

196   injured CNS axons to regenerate over lengthy distances, except for the issue with axonal

197   localisation: integrins are not transported into adult CNS axons, being instead confined to cell

198   bodies and dendrites (Andrews et al., 2016). An important question is whether this absence of

199   integrin receptors reflects a general axonal deficit of growth-promoting molecules. Growth

200   factor receptors are a good example of this type of molecule, and there is evidence that these

201   are not present in abundant levels after CNS axons have matured. Both TrkB and the IGF

202   receptor are reportedly excluded from CST axons in the spinal cord (Hollis et al., 2009a;

203   Hollis et al., 2009c), and TrkB is similarly not detectable in rubrospinal axons whilst being

204   present in their cell bodies (Kwon et al., 2004). BDNF (the TrkB ligand) can also rescue

205   injured rubrospinal neurons from atrophy and prevent cell death, but only when applied at the

206   level of the soma, and not the axon (Kwon et al., 2002).

207   If growth factor receptors are not abundant in CNS axons, it may explain why previous

208   experiments aimed at facilitating CNS regeneration through growth factor stimulation have

209　not yielded strategies to promote long-range axon growth (Kordower and Tuszynski, 2008).

210　Integrins and growth factor receptors are transported efficiently into CNS axons during

211　developmental growth, being essential for the correct development of the nervous system

212　(Myers et al., 2011), so it is clear that there are developmental changes that occur that limit

213　their presence in adult axons.

214　Here we argue that distributing growth-enabling machinery to the axon is necessary to switch

215　on a pro-regenerative neuronal program for successful regeneration. We discuss the idea that

216　a developmental decline in axonal transport of growth-associated molecules contributes to the

217　intrinsic decline in regenerative ability observed in the CNS; that changes in axonal transport

218　and trafficking result in redistribution of growth molecules and receptors from axons to the

219　somatodendritic domain. We also consider the evidence that interventions that promote

220　regeneration (e.g. conditioning injury or permissive nerve grafts) also enhance axonal

221　transport. We discuss the axon transport of specific regeneration-associated molecules, and

222　the mechanisms that regulate a polarised distribution within neurons. Focusing on these

223　topics provides insight into mechanisms that can be targeted to facilitate the axon transport of

224　growth-promoting machinery and enable axon regeneration.

225

226　Developmental decline in axon regeneration capacity in the CNS

227　*In vivo studies*

228　Evidence from numerous animal models including C. elegans, rats, hamsters and opossums,

229　suggest that there is a decline in CNS regeneration capacity that begins after birth and

230　continues to dwindle (Kalil and Reh, 1979; Keifer and Kalil, 1991; Nicholls and Saunders,

231　1996; Wu et al., 2007). The studies discussed in the introduction demonstrate that this decline

232　is due to both the extrinsic environment, and cell intrinsic programmes. Even in cases where

233　regeneration does occur in the adult such as after peripheral nerve injury, the regenerative

234 ability of the peripheral neurons is reduced and delayed in older organisms (Verdu et al.,

235 2000). For example, developmental changes such as impaired clearance of cell debris and the

236 accumulation of obstacles in the endoneurial tubes of aged animals can affect not only the

237 speed but also the extent of motor neurons' axonal regeneration after peripheral nerve injury

238 (Kang and Lichtman, 2013). Importantly, the CNS decline in regenerative capacity appears to

239 continue further into adulthood, as demonstrated by a study into the effects of PTEN deletion

240 during ageing. PTEN deletion promotes axon regeneration in corticospinal tract (CST)

241 neurons after injury in young adult mice (Liu et al., 2010). Geoffroy and colleagues

242 examined the effects of ageing on the ability of PTEN deletion to induce regeneration of CST

243 axons. Ageing did not reduce the effects of PTEN deletion on the intrinsic ability of axons to

244 regenerate proximally to a spinal cord injury, but greatly reduced axon regeneration distal to

245 the injury, suggesting that long range regeneration becomes increasingly problematic with

246 maturity (Geoffroy et al., 2016).

247

248 *In vitro studies*

249 As it is difficult to separate the effects of the extrinsic environment from intrinsic factors *in*

250 *vivo*, studies have also investigated regenerative ability using CNS neurons cultured *in vitro*

251 (Bradke et al., 2012). A recent investigation by Koseki et al. (2017) used *in vitro* laser

252 axotomy to investigate the intrinsic changes regulating axon regeneration of embryonic

253 cortical neurons cultured to maturity. The study used E18 rat brain cortical neurons which

254 were cultured up to 24 days *in vitro* using astrocyte feeder layers to separate neurons from

255 glia. Development of neurons to a mature state was confirmed by assessing electrical activity

256 and analysing gene expression, and regenerative capacity was measured by recording the

257 axonal response to a laser injury. The study confirmed that the regenerative ability of cultured

258 cortical neurons negatively correlates their maturational state, with less than 10% of neurons

259   cultured for 24 days regenerating compared to 70% of neurons cultured for 4 days. This

260   effect was shown to be due to an intrinsic change by culturing young neurons on 24 day old

261   cultures. The young neurons retained their regenerative capacity despite the aged

262   environment, regenerating as well as neurons plated on poly-d lysine (Koseki et al., 2017).

263   Retinal ganglion cells show a similar age-dependent decline in axon growth capacity,

264   whether grown on neonatal or adult optic nerve sections , whilst DRG neurons from the PNS

265   retain their intrinsic ability to regrow on either of these substrates (Goldberg et al., 2002;

266   Shewan et al., 1995). Koseki et al continued by analysing gene expression by RNA

267   sequencing and found that that there are vast changes in gene expression as cortical neurons

268   mature in culture, with an increase in the expression of genes involved in electrical activity

269   and synapse formation and function, and a decrease in genes associated with growth and

270   development. They also investigated the axonal delivery of growth-promoting machinery,

271   finding that there is a developmental decline in the axonal transport of recycling endosomes,

272   and that restoring this transport leads to an increase in regeneration. These findings support

273   the notion that genetic factors are partly responsible for the change in regenerative capacity,

274   and that there are developmental changes in selective axonal transport that limit axon

275   regrowth.

276

277   **Developmental decline in axon transport**

278   *Early studies*

279   It has long been assumed that efficient axon transport is necessary for effective regeneration,

280   with many studies addressing this hypothesis. The foremost studies were aimed at

281   understanding whether axon transport rates varied in regenerative vs. non-regenerative axons,

282   and whether there is an increase in axon transport after a peripheral injury when neurons

283   mount a regenerative response. These studies used techniques such as radiolabelling to

284   measure the transport of peptides into axons *in vivo* comparing early development with later

285   stages, and finding that molecules such as the growth-associated GAP43 are rapidly

286   transported into the optic nerve of neonatal rabbits, but that transport rates declined rapidly

287   with development (Skene and Willard, 1981). Cytoskeletal transport declined similarly

288   (Hoffman et al., 1983), as revealed by examining neurofilament transport. A number of

289   studies also addressed whether the enhanced growth observed after a conditioning lesion is

290   associated with an increase in axon transport rates by measuring the synthesis and axonal

291   transport of cytoskeletal components, again using radio-labelling, and finding that enhanced

292   regeneration is associated with elevated axon transport of both microtubules and

293   neurofilaments (McQuarrie and Grafstein, 1982; McQuarrie and Jacob, 1991; Oblinger and

294   Lasek, 1988). Similar observations were also reported in later studies examining enhanced

295   regeneration of optic nerve axons into a peripheral nerve bridge (McKerracher et al., 1990).

296   Collectively, these early experiments suggested a deceleration in axonal transport during

297   maturation, and an increase in transport during enhanced regeneration (Fournier and

298   McKerracher, 1995; Hoffman, 2010; McQuarrie et al., 1989).

299

Live cell imaging to characterise axon transport

301   The majority of early studies focused on cytoskeletal material as an example of machinery

302   that is required for axon growth. Similar techniques were recently used to examine axon

303   transport more completely, by coupling radiolabelling with mass spectroscopy and analysing

304   the transport of various subcellular components in the central branch of DRG axons after a

305   conditioning injury to the peripheral branch (which results in enhanced regeneration). The

306   study found enhanced transport of both actin and microtubule cytoskeletal elements, as well

307   as cytosolic proteins including glycolytic enzymes and regenerative molecules such as the

308   14-3-3 proteins and the RhoA inhibitor RhoGDI, and membranous vesicles including

309 lysosomes, and synaptophysin and APP-containing vesicles. Increased levels of molecular

310 motors and of polyglutamylated tubulin were also observed (Mar et al., 2014). This study

311 demonstrates global changes in axon transport after a conditioning lesion. It also highlights

312 that techniques such as live cell imaging of fluorescently labelled proteins make it possible to

313 examine axon transport in a highly selective fashion, focusing on individual cellular

314 components.

315 These techniques were recently used to analyse age-dependant transport as well as

316 regeneration-dependent changes focusing on two specific cargoes, the axon survival factor

317 NMNAT2, and mitochondria. Although this study focused on a much later developmental

318 period (between 1.5 and 24 months), some specific changes in mammalian CNS and PNS

319 axonal transport with development were identified (Milde et al., 2015). Interestingly, aged

320 neurons in an ageing environment (in 24-month-old mice) were capable of supporting higher

321 levels of axonal transport as stimulated by a peripheral nerve crush – an increase in the

322 number of anterogradely moving particles containing NMNAT2 and mitochondria was

323 observed at rates similar to young mice, whilst retrograde transport was unaffected. This

324 observation suggests that enhanced anterograde transport contributes to axon regeneration

325 after peripheral nerve injury. The movement of mitochondria has long been associated with

326 axon growth (Morris and Hollenbeck, 1993). A number of recent studies have demonstrated

327 the importance of axonal mitochondria for the regeneration process, and this will be

328 discussed in detail later.

329

330 *Developmental decline in axon transport of growth machinery*

331 The studies described above suggest that mature CNS axons lack the correct tools for axonal

332 extension, and that PNS axons mount a regenerative response by enabling enhanced transport

333 leading to an increase in axonal growth machinery. However, they do not elucidate precisely

334 what the necessary machinery is, or to what extent it is missing from non-regenerative adult

335 CNS axons. Axon growth is driven by the growth cone and can be compared to the process of

336 cell migration, in that there are requirements for the interaction of the cell with its

337 environment through adhesion and guidance molecules which subsequently reorganise the

338 cytoskeleton and surface membrane in the direction of growth (Dequidt et al., 2007; Itofusa

339 and Kamiguchi, 2011; Robles and Gomez, 2006; Tojima et al., 2007). One class of molecules

340 that is important for this process is the integrin family of cell surface receptors for the

341 extracellular matrix. These are important for developmental CNS axon growth and PNS axon

342 regeneration but are excluded from CNS axons after development, both endogenously and

343 after viral transduction (Nieuwenhuis et al., 2018)(discussed in detail below). Because

344 integrins can promote PNS regeneration but are excluded from mature CNS axons, there have

345 been substantial efforts to understand the mechanisms regulating their transport, with a view

346 to manipulating this to enable regeneration. These studies initially examined axonal integrin

347 transport in regenerative PNS axons *in vitro*, focusing on the integrin alpha9 and its binding

348 partner beta1. Two small GTPases were found to be responsible for regulating integrin

349 transport into axons, the recycling endosome markers Rab11 and ARF6 (Eva et al., 2012b;

350 Eva et al., 2010; Nieuwenhuis and Eva, 2018). Rab11 governs integrin transport into axons in

351 recycling endosomes, and additionally controls integrin recycling onto the growth cone

352 surface through an interaction with its effector, Rab coupling protein (Rab11-FIP1). ARF6 is

353 also involved in integrin trafficking within the growth cone, but crucially plays an additional

354 role in the axon itself, regulating the direction of integrin transport. ARF6 activation state

355 governs the direction of axon transport such that active ARF6 favours retrograde transport

356 whilst inactive ARF6 enhances anterograde transport. This has important implications,

357 because there are stark differences in directional transport in PNS vs CNS neurons. In PNS

358 neurons there is a balance between anterograde and retrograde transport which results in an

359   integrin presence throughout the axon, often in immobile structures (Eva et al., 2012b). In

360   CNS neurons (rat brain cortical neurons) there is a developmental increase in retrograde

361   integrin transport which results in removal of integrins from mature axons in the CNS (Fig.

362   1), so that live cell imaging reveals there are very few immobile integrin structures in axons

363   (Franssen et al., 2015). Predominant retrograde movement has previously been associated

364   with a state of axon growth arrest, whilst a balance of anterograde and retrograde endosomal

365   movement is associated with axonal elongation (Hollenbeck, 1993; Hollenbeck and Bray,

366   1987).

367   With regard to regenerative ability, it may prove to be crucial that not only are integrins

368   sequestered from adult CNS axons and retained in the cell body and dendrites, but so are their

369   carriers – Rab11 endosomes. Rab11 is important for developmental axon growth but its

370   axonal presence is diminished with maturity, being difficult to detect in mature CNS axons,

371   instead localising principally in the somatodendritic domain (Koseki et al., 2017; Sheehan et

372   al., 1996). In adult neurons Rab11 is involved in the regulation of post-synaptic plasticity,

373   with roles in receptor recycling (AMPA and TrkB receptors), dendritic spine development,

374   and synapse structure (Correia et al., 2008; Esteves da Silva et al., 2015; Lazo et al., 2013;

375   Sui et al., 2015). Conversely, there is less evidence for a presynaptic role for Rab11 in

376   mammalian CNS neurons, although it has been observed at low levels in the synaptic vesicle

377   fraction of synaptosomes. These are enriched with a different set of Rab proteins that are

378   important for the correct cycling of synaptic vesicles (Rabs 3 and 27) (Binotti et al., 2016;

379   Pavlos et al., 2010).

380   It is likely then that the developmental decline in axonal Rab11 reflects a change in the

381   requirements for recycling within the axon. There is evidence that as axons lose the necessity

382   for growth and instead become geared for neurotransmission, there is a change in the type of

383   endosomal recycling machinery present, with a decline in receptor and membrane protein

384   recycling and a shift towards synaptic vesicle recycling (Bonanomi et al., 2008). Recent

385   evidence suggests that membrane proteins and synaptic vesicle proteins are also degraded

386   through separate pathways in axon terminals, in support of the hypothesis that the machinery

387   for synaptic vesicle turnover is distinct from that which is required for membrane protein

388   turnover (Jin et al., 2018b). Moreover, the development of synapses has recently been

389   associated with regenerative decline (Tedeschi et al., 2016). In summary, Rab11 is known to

390   transport many molecules that are important for axon growth (in addition to integrins) so it

391   may not be surprising that Rab11 is not needed in large amounts in the fully developed axon.

392   Integrins, Rab11 and other regenerative Rab11 cargo are discussed the next section.

393

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

395   *Integrins*

396   Integrins are a diverse family of cell surface receptors for the extracellular matrix (Hynes,

397   2002). They transduce signals from the extracellular environment that lead to the

398   reorganisation of the cytoskeleton and activation of numerous influential signalling pathways.

399   As they have no enzymatic activity, integrins rely on a vast array of interacting molecules to

400   mediate their actions. They are also regulated by signalling from the intracellular

401   environment (termed inside-out signalling), which modifies the activation state of the

402   receptors. Integrins have active and inactive conformations, and ultimately depend on

403   activation by molecules such as the kindlins and talin, which lead to changes in their

404   extracellular structure (Cheah and Andrews, 2018; Kim et al., 2011). Integrins function as

405   heterodimers composed of an alpha and a beta subunit, the combination of which governs

406   their specificity for their individual ligands, so that different heterodimers can bind with

407   differing affinities to molecules such as laminin, collagen, fibronectin, tenascin and

408   vitronectin (Barczyk et al., 2010; Hynes, 2002). They also exhibit some interaction with other

409   secreted signalling molecules including certain growth factors (LaFoya et al., 2018). There

410   are therefore a number of ways that the extracellular environment can modify cellular

411   behaviour mediated by integrin activation, so it is unsurprising that integrins are involved in a

412   vast array of biological processes in most cell types both during development and in

413   adulthood. Neurons are no exception to this; integrins have been implicated in many aspects

414   of neuronal function, including axonal growth and guidance during development (Myers et

415   al., 2011) as well as playing an influential role in the regulation of synaptic function during

416   adulthood (Park and Goda, 2016). Integrins are also involved in the PNS regenerative

417   response after injury with the axon transport of alpha5 integrin being enhanced on fibronectin

418   after a conditioning lesion, whilst the integrin alpha7 mediates enhanced regeneration on

419   laminin after a peripheral nerve crush (Eva et al., 2012a; Gardiner, 2011; Gardiner et al.,

420   2005; Gardiner et al., 2007; Nieuwenhuis et al., 2018). Integrins have long been proposed as

421   a means of promoting axon regrowth (Condic, 2001), and have recently been used to promote

422   sensory regeneration through the spinal cord after an injury to the central branch of DRG

423   axons. Viral introduction of alpha9 integrin (AAV injection into DRG cell bodies) allows

424   regenerating PNS axons to re-enter the spinal cord and synapse with their targets as well as

425   continuing to regenerate almost as far as the brain (reaching the medulla but stopping short of

426   the cuneate nucleus) (Cheah et al., 2016). Because integrins are inactivated by inhibitory

427   molecules (Hu and Strittmatter, 2008; Tan et al., 2011), this sensory axonal repair strategy

428   relies on co-expression of the integrin activator kindlin-1. The observed integrin-dependent

429   regeneration is also only possible because PNS axons support integrin transport (Andrews et

430   al., 2016). The integrin alpha9 was selected for these experiments because it has two key

431   features – it binds to tenascin-C and promotes axon extension when bound to this ligand

432   (Andrews et al., 2009). Tenascin-C is an extracellular matrix glycoprotein that is normally

433   inhibitory to axon growth in the adult CNS and is strongly upregulated after CNS injury in

434 the brain, spinal cord and optic nerve (Andrews et al., 2009; Gervasi et al., 2008; Reinhard et

435 al., 2017; Tang et al., 2003; Zhang et al., 1997). Alpha9 integrin therefore mediates its

436 regenerative actions by localising to the axon surface to stimulate axon growth over a

437 molecule which normally inhibits axon growth. Integrin alpha9 is not normally expressed in

438 the adult nervous system, so exogenous viral introduction is necessary to observe its effects.

439 Other endogenous integrins are important for PNS regeneration, but these do not allow PNS

440 axons to regenerate into the environment of the spinal cord, despite being efficiently

441 transported into their axons (Gardiner, 2011). Integrins are therefore potent mediators of the

442 PNS regenerative response and could potentially be used to enable CNS regeneration after

443 injury. Alpha9 integrin particularly could be able to promote the regeneration of descending

444 corticofugal axons when virally introduced with kindlin-1, however the developmental

445 change in the subcellular distribution of integrins in neurons in the CNS means that they are

446 no longer present in axons, but instead selectively targeted to the somatodendritic domain

447 (Andrews et al., 2016; Bi et al., 2001; Franssen et al., 2015). This has been observed for

448 pyramidal neurons in the cortex, hippocampal neurons in the CA1 and CA3 regions,

449 cerebellar Purkinje neurons, and granule neurons in the dentate gyrus (Bi et al., 2001; Chan et

450 al., 2003; Einheber et al., 1996; Rodriguez et al., 2000). Virally transduced exogenous

451 integrins are also restricted from entry to the axons of mature cortical neurons *in vivo*, whilst

452 they are transported into the axons of young CNS neurons. However, there does appear to be

453 a role for integrins in retinal ganglion cell (RGC) neurons in the optic nerve, but it is not clear

454 if they are present in the axons of all the various subtypes of neurons within the retina

455 (Andrews et al., 2016; Vecino et al., 2015). *In vitro* studies have revealed a similar picture,

456 with alpha and beta integrins detectable in the axons and growth cones of adult DRG

457 neurons, but not in the axons of mature cortical neurons. Integrins are detectable in the axons

458 of E18 cortical neurons cultured for 4-7 days, but after this period they become difficult to

459   detect so that by 10-14 days *in vitro* endogenous integrins are almost completely absent from

460   axons (Bi et al., 2001; Franssen et al., 2015; Koseki et al., 2017). This developmental decline

461   in CNS integrin axon transport contributes to the decline in regenerative ability, because

462   restoring integrin transport leads to a restoration of regeneration after a laser injury to the

463   axons of cortical neurons maturing *in vitro* (Eva et al., 2017). Importantly, restoring integrin

464   transport also leads to an increase in axonal Rab11, suggesting that enabling integrin

465   transport may also facilitate the transport of additional machinery which is transported along

466   with integrins in Rab11 endosomes.

467

468   *Rab11 and recycling endosomes*

469   Rab11 and ARF6 are small GTPases that regulate recycling endosome trafficking and

470   function as the central regulators of axonal integrin transport, with emerging roles in the

471   intrinsic regulation of axon regeneration. Axonal Rab11 declines with development, whilst

472   axonal ARF6 activity is raised (Eva et al., 2012b; Eva et al., 2010; Eva et al., 2017; Franssen

473   et al., 2015; Sheehan et al., 1996).

474   Small GTPases are molecular switches that cycle between an active GTP bound state and an

475   inactive GDP bound state. Their activation state governs the molecules that they interact

476   with, so that some proteins will interact whilst GTP-bound, and others only whilst they are

477   bound to GDP. They possess intrinsic GTPase activity, so that bound GTP will be catalysed

478   to GDP. The rate at which this occurs is regulated by molecules known as GAPs (GTPase

479   activating proteins) whereas activation (to a GTP-bound state) is regulated by GEFs (Guanine

480   nucleotide exchange factors). Intrinsic GTPase activity varies between small GTPases, with

481   ARF6 in particular having very little intrinsic GTPase activity (Campa and Randazzo, 2008;

482   Gillingham and Munro, 2007). ARF6 is therefore entirely reliant on its GAPs and GEFs for

483   regulation of its activation state, and a large number of these molecules have been identified,

484   some of which are implicated in the regulation of axon growth and regeneration (Eva et al.,

485   2012b; Eva et al., 2017; Hernandez-Deviez et al., 2004).

486   Rab11 is a regulator of recycling endosomes. These are endosomes that return membrane

487   proteins to the cell surface after they have been internalised. Rab11 was originally identified

488   as regulating a long loop of receptor recycling via an organelle close to the nucleus (the peri-

489   nuclear recycling centre) (Ullrich et al., 1996). Through this trafficking pathway, membrane

490   proteins can be internalised from one part of the surface membrane and recycled to another.

491   Subsequently it emerged that Rab11 is additionally involved in a more localised, rapid

492   recycling which occurs when there is high-capacity membrane turnover, such as occurs at the

493   leading edge of migrating cells (Howes et al., 2010). It is also involved in the process of

494   membrane protein exocytosis (Welz et al., 2014).

495   ARF6 is also a regulator of recycling endosome traffic but is additionally involved in a range

496   of mechanisms that are central to cellular function. These include the regulation of the actin

497   cytoskeleton through key regulators such as Rac and Cdc42, and the control of

498   phosphoinositide signalling. ARF6 activates PIP5 kinase, an enzyme which is responsible for

499   generating phosphatidylinositol 4,5-bisphosphate (PIP2) (Gillingham and Munro, 2007). This

500   molecule is converted by PI3 kinase to make phosphatidylinositol 3,4,5-triphosphate (PIP3),

501   which can in turn be metabolised by PTEN back to PIP2 (Vanhaesebroeck et al., 2012). This

502   is a key step in an extremely influential signalling pathway which can have wide-ranging

503   consequences in cell functions such as transcription, translation, epigenetics, cytoskeletal

504   regulation, trafficking and transport, neurotransmission, apoptosis, cell growth, proliferation,

505   and survival. The majority of ARF6 GEFs and GAPs are either directly regulated by PIP2 or

506   PIP3 or regulated by phosphorylation from key kinases in the PI3 kinase pathway (Hawkins

507   et al., 2006; Randazzo et al., 2001). ARF6 is therefore both a trafficking and signalling

508   molecule closely involved with pathways that are central to controlling cell function.

509 Both ARF6 and Rab11 were first implicated as regulators of integrin function in non-

510 neuronal cells, being important for recycling of integrins during cell migration and invasion

511 of cancer cells. Recycling of integrins is central to their function in mediating cell migration,

512 being required for the correct turn-over of focal adhesion complexes (Caswell and Norman,

513 2008; Caswell et al., 2008; Caswell and Norman, 2006; Dai et al., 2004; Dunphy et al., 2006;

514 Jones et al., 2006; Pellinen and Ivaska, 2006; Powelka et al., 2004; Roberts et al., 2001; Vitali

515 et al., 2017). Subsequent studies found that Rab11 is required for targeting integrins to axons

516 in DRG neurons in culture, as well as being important for recycling integrins locally within

517 the growth cone (Eva et al., 2012b; Eva et al., 2010). Recycling is necessary within the

518 growth cone to control directional changes (Tojima et al., 2007; Tojima et al., 2010). More

519 recently, there have been a number of studies that confirmed the importance of Rab11 for

520 correct growth cone function, with its targeted removal from developing growth cones

521 leading to growth cone collapse, whilst increased growth cone targeting leads to an increase

522 in axon growth (van Bergeijk et al., 2015). Optogenetic disruption of growth cone Rab11 also

523 leads to a reduction in growth cone area, similar to the decreased growth cone area observed

524 when Rab11 is silenced (Eva et al., 2010; Nguyen et al., 2016). *In vivo* studies have

525 confirmed that Rab11 is required at the growth cone for the correct guidance of axons

526 crossing the midline of the spinal cord during development of the nervous system (Alther et

527 al., 2016).

528 ARF6 similarly regulates integrins in both the axon and at the growth cone, but crucially it

529 also regulates the direction of integrin transport within axons, with active ARF6 triggering

530 retrograde transport whilst inactive ARF6 allows anterograde transport (Eva et al., 2012b;

531 Franssen et al., 2015). Inactivating ARF6 enables both integrin and Rab11 transport leading

532 to enhanced axon growth and regeneration after laser axotomy (Eva et al., 2012b; Eva et al.,

533 2017; Hernandez-Deviez et al., 2004). This may be due to an increased integrin presence, but

534   may equally be due to an increase in Rab11 itself, as this has also been shown to increase

535   regeneration when its axonal localisation is increased by overexpression (Koseki et al., 2017).

536   There may also be a role for other Rab proteins in this process, as recycling endosomes are

537   also regulated by Rabs 8, 10 and 35 which are all involved in the regulation of neurite

538   outgrowth in PC12 cells and axon growth in cortical and hippocampal neurons *in vitro*

539   (Chevallier et al., 2009; Furusawa et al., 2017; Homma and Fukuda, 2016; Huber et al., 1995;

540   Kobayashi and Fukuda, 2012; Villarroel-Campos et al., 2016; Wang et al., 2011). It may also

541   be that Rab11 enables regenerative axon growth by providing other growth-related molecules

542   to the site of injury, because a number of growth factor receptors and the regenerative

543   reggie/flotillin proteins are also associated with trafficking via Rab11 (Bodrikov et al., 2017;

544   Hulsbusch et al., 2015; Koch et al., 2013). These are discussed below.

545

546   *IGF-1 and TrkB receptors*

547   With well-defined roles in regulating axon growth, it is understandable that research has

548   focused on promoting axon regeneration through growth factor / growth factor receptor

549   manipulation. Two prominent growth factor receptors that have been investigated in this

550   respect are tropomyosin-related kinase B (TrkB) and insulin-growth factor receptor 1 (IGFR-

551   1), which are activated by the growth factors BDNF and IGF respectively (Duan et al., 2015;

552   Hollis et al., 2009c; Liu et al., 2017; Lu and Tuszynski, 2008). IGFR-1 has a vital function in

553   neuronal survival and glial progenitor protection against glutamate toxicity after injury. In

554   order to perform these functions, IGFR-1 undergoes dynamic recycling and internalisation

555   upon ligand stimulation which is essential for sustained downstream signalling. Furthermore,

556   the IGF-1 receptor colocalises with Rab11-positive endosomes and with the transferrin

557   receptor identifying the IGF-1 receptor as one of the receptors packaged in recycling

558   endosomes (Romanelli et al., 2007). As Rab11-positive endosomes are excluded from the

559   mature axons of CNS neurons, the low availability of IGFR-1 in the axon after injury might

560   be a further reason for poor CNS axonal properties. The IGF-1 receptor has been implicated

561   in the specification and initial axon growth in hippocampal neurons acting through the PI3K

562   pathway, in the absence of TrkA or TrkB activation (Dupraz et al., 2009; Nieto Guil et al.,

563   2017; Sosa et al., 2006). This activation is preceded by targeted accumulation of IGFR-1 in

564   the developing axon. IGF-1 has also been shown to markedly enhance axon outgrowth in

565   young cultures of pure corticospinal motor neurons through the PI3K and the ERK/MAPK

566   pathways, an effect which is separate from its effects on neuronal survival (Ozdinler and

567   Macklis, 2006). IGF-1 receptor signalling was also shown to be necessary for proper axonal

568   outgrowth of retinal ganglion cells *in vitro* (Dupraz et al., 2013) and of corticospinal motor

569   neurons *in vivo* (Ozdinler and Macklis, 2006). Surprisingly, application of IGF-1 to injured

570   corticospinal tract neurons resulted in their improved survival but not regeneration. The

571   authors speculated that the effects of the IGFR-1 activation on survival and growth are

572   developmentally distinct and the reduced availability of the receptor in axons compared to the

573   soma can explain the inability of overexpressed IGF-1 to promote regeneration (Hollis et al.,

574   2009c). In a different study, the activation of the IGF-1 receptor by the application of IGF-1

575   and sensitisation with osteopontin resulted in robust sprouting and partial recovery of

576   function in two different models of corticospinal tracts injury (Liu et al., 2017). This effect

577   was attributed to osteopontin's ability to interact with integrins or other surface molecules to

578   cause IGFR-1 clustering, and most likely occurs at the site of the cell body plasma

579   membrane. Taking into account the versatile functions of the IGFR-1 receptor in neuronal

580   survival, growth and regeneration, its proper transport and trafficking is essential for optimal

581   function in development and regeneration. The investigation of osteopontin as a growth

582   stimulator arose from studies in retinal ganglion neurons. These have as small subpopulation

583   of cells that regenerate better than their counterparts. These are known as αRGCs and were

584 found to express higher levels of osteopontin. Experiments in the optic nerve determined that

585 osteopontin facilitates robust regeneration in combination with either IGF, or BDNF, the

586 TrkB receptor ligand (Duan et al., 2015).

587 TrkB is similar to the IGF receptor in that it too is involved in developmental axon growth

588 (Gates et al., 2000), has been targeted to promote CNS regeneration (Hollis et al., 2009b;

589 Kwon et al., 2004; Plunet et al., 2002), and is transported in Rab11 endosomes. The recycling

590 of TrkB receptor through Rab11-positive recycling endosomes regulates its neuronal

591 localisation, and in mature neurons is involved in post-synaptic receptor recycling in

592 dendrites (Huang et al., 2013; Lazo et al., 2013; Sui et al., 2015). This is in contrast to its

593 developmental enrichment at the growth cone of CNS neurons, from where it stimulates axon

594 growth. Importantly, BDNF signalling at the growth cone stimulates anterograde transport of

595 its receptor, TrkB (Cheng et al., 2011). This autocrine feedforward mechanism demonstrates

596 that signalling from the distal axon can stimulate growth-promoting mechanisms, and is

597 similar to the transport of TrkA in PNS neurons, which also transport TrkA in Rab11

598 endosomes (Ascano et al., 2009). Despite its role in axon growth promotion during

599 development, TrkB is unable to stimulate regeneration of injured CST axons in adults, and

600 this appears to be as a result of its somatodendritic localisation. Adult corticospinal neurons

601 show abundant TrkB receptor distribution in their soma and dendrites, but not in their axons

602 which correlated with their inability to regenerate after a subcortical lesion (Lu et al., 2001).

603 Similarly, rubrospinal axons do not appear to support TrkB transport, although application of

604 BDNF to their cell bodies can promote their survival after injury and encourage regeneration

605 into a peripheral nerve graft (Kwon et al., 2002; Kwon et al., 2004; Plunet et al., 2002). In

606 contrast, motor neurons which expressed TrkB throughout their axons were able to re-grow

607 past the injury site suggesting that the presence of the receptor in the axon plays a key role

608 after injury in order for the axon to initiate a growth program (Lu et al., 2001). TrkB agonists

609 can also promote structural and functional repair of cut peripheral nerves (English et al.,

610 2013). Given that integrins, the IGF receptor and TrkB all traffic via Rab11 and all adopt a

611 somatodendritic localisation, it seems inconsistent that overexpressed TrkB localises to CST

612 axons, and can improve regeneration when stimulated by BDNF after a subcortical lesion

613 (Hollis et al., 2009b). However, it is important to note that regeneration was only found

614 subcortically, and that the TrkB receptor was not transported to more distal sites, such as the

615 spinal cord. The presence of TrkB in the axon is not completely unexpected, because there is

616 evidence that TrkB has a presynaptic role in regulating neurotransmission (Xu et al., 2000).

617 This may be as a result of trafficking under the control of different endosomal regulators, as

618 has been reported in hippocampal axons (Arimura et al., 2009), however an axonal presence

619 of TrkB does not appear to be at adequate levels to support robust regeneration. This seems to

620 be the case for many growth factor receptors, as stimulation with NGF, BDNF, NT-3, NT-4,

621 GDNF, has limited effects on CST regeneration (Kordower and Tuszynski, 2008; Thoenen

622 and Sendtner, 2002)

623

624 *Reggie/flotillin proteins*

625 Reggie and flotillin are the same proteins with different names; reggie 1 and 2 are flotillin 2

626 and 1, respectively. The two proteins localise to lipid-rich microdomains in the surface

627 membrane, (often referred to as lipid rafts) as well as to other endosomal membranes. They

628 have two names because they were identified simultaneously in two separate labs. The

629 Stuermer lab identified two proteins from larval goldfish using an antibody against Thy-1 to

630 isolate microdomain enriched molecules, and subsequently named them the reggie proteins

631 because they are upregulated by retinal ganglion cells during axon regeneration in the fish

632 visual system (Schulte et al., 1997). At the same time, the Lodish lab identified flotillin-1

633 from a screen to identify novel components of caveolae (small invaginations of the plasma

634 membrane involved in clathrin independent endocytosis and signal transduction), using the

635 term flotillin because they found the protein resided in a detergent resistant buoyant (floating)

636 membrane fraction in the brain. Importantly, the Lodish lab noted the absence of caveolae in

637 the brain, highlighting a potential role for the protein outside of caveolae (Bickel et al., 1997).

638 There is now a large literature regarding the cell biological role of the reggie/flotillin

639 molecules, mostly regarding their roles in caveolae and clathrin-independent endocytosis

640 (using the flotillin name), and there are detailed reviews around this (Babuke and Tikkanen,

641 2007; Bodin et al., 2014; Hansen and Nichols, 2009). Here we mention the neuronal

642 functions of reggie/flotillin in axon regeneration, their association with Rab11 endosomes,

643 and their function in synapse regulation.

644 A role for reggie/flotillin in the regulation of axon growth and regeneration was first

645 suggested by experiments to silence their expression in the zebrafish retina. Depletion led to a

646 substantial decrease in the number of regenerating axons. Experiments in hippocampal

647 neurons found the presence of the reggie/flotillin along axons and at the growth cones of

648 neurons developing *in vitro*, and silencing with siRNA led to a similar reduction in axon

649 length (Munderloh et al., 2009). This resulted in experiments aimed at increasing RGC

650 regeneration after an experimental optic nerve crush in rats. Reggie 1 was transduced into

651 RGCs by intravitreal AAV injection two weeks before an optic nerve crush, and regeneration

652 was assessed four weeks later. Overexpression of reggie-1 increased the number of axons

653 crossing the lesion site by 3-5 times, and regenerating axons were found up to 5mm beyond

654 the injury site, confirming a role for the reggie/flotillin molecules in facilitating axon

655 regeneration (Koch et al., 2013). As neurons lack caveolae, it is unlikely that these effects are

656 mediated by membrane internalisation/endocytic functions but may have more to do with

657 trafficking or targeting of molecules onto the cell surface. T-cells also lack caveolae, and in

658 these cells, reggie/flotillin mediates the targeting of the T-cell receptor from an intracellular

659   compartment to a region on the cell surface known as the T-cell cap (Langhorst et al., 2006).

660   The identity of the intracellular compartment is debated, with some studies finding

661   reggie/flotilin on early endosomes, being involved in endocytosis (Glebov et al., 2006),

662   however in neurons, reggie/flotillin are also found enriched in recycling endosomes (as

663   marked by Rab11). Amongst the molecules implicated in mediating trafficking together with

664   reggie/flotillin, are the GTPase TC10 and the secretory exocyst component exo70. Crucially,

665   this complex (TC10 and exo70) mediates growth cone membrane addition and axon growth

666   downstream of the IGF-1 receptor and PI3 kinase (Dupraz et al., 2009). TC10 is found on

667   Rab11 positive endosomes and stimulates neurite outgrowth by stimulating exocytic fusion of

668   Rab11 endosomes with the plasma membrane (Fujita et al., 2013). Rab11 was subsequently

669   shown to interact with reggie/flotillin at the recycling endosome, functioning to return

670   cadherins and the transferrin receptor to the plasma membrane (Solis et al., 2013).

671   Reggie/flotillin also regulate EGF receptor trafficking (Solis et al., 2012), and together with

672   Rab11 control integrins and focal adhesion recycling (Hulsbusch et al., 2015).

673   In mature neurons, reggie/flotillin were recently shown to function with Rab11 in the

674   regulation of synapse development and post-synaptic trafficking, being involved in the

675   trafficking of cadherins, glutamate receptors and PSD95 into dendritic spines (Bodrikov et

676   al., 2017). Other studies have implicated reggie/flotillin in the regulation of synapses, being

677   involved in strengthening glutamatergic synapses *in vitro* (Swanwick et al., 2010), whilst

678   protein levels are altered *in vivo* when somatosensory cortical synapses are modulated by

679   sensory deprivation (Butko et al., 2013). These studies imply a dendritic localisation for

680   reggie/flotillin in mature CNS, however their localisation to mature axons has not been fully

681   investigated. It is clear that the reggie/flotillin molecules regulate developmental axon growth

682   and function together with regenerative molecules such as integrins and Rab11 but localise to

683   dendrites after development. It will be important to determine if they are absent/diminished in

684   mature CNS axons. It is likely that interventions that promote *in vitro* regeneration via

685   enhanced integrin/Rab11 axon transport may also be functioning by facilitating the transport

686   of other regenerative molecules such as reggie/flotillin, which are also present on recycling

687   endosomes.

688

689   Other organelles/complexes and axon regeneration

690   *Mitochondria*

691   Mitochondrial axonal transport and morphology are altered with cortical neuron maturation.

692   During development mitochondria are highly mobile and move bidirectionally along the axon

693   to meet the high energy demands of the developing cells while also acquiring shortened

694   morphology to aid this dynamic movement. This situation changes with development, so that

695   in mature neurons, mitochondria elongate and become less dynamic to serve the updated

696   functions of the cell (Chang and Reynolds, 2006). Using live imaging of mitochondrial

697   transport, a subsequent study revealed one reason why mitochondria are less mobile in

698   mature neurons is that they are being anchored to the axon by syntaphilin (Kang et al., 2008).

699   These observations have recently been confirmed *in vitro* and *in vivo*. Immature neurons *in*

700   *vitro* (3-7DIV) exhibit very mobile mitochondria whereas in more mature neurons (10+ DIV)

701   the mitochondria tend to be less mobile with up to 95% of all axonal mitochondria being

702   stationary by 28DIV (Lewis et al., 2016). Interestingly, the number of mobile vs. stationary

703   lysosomes did not seem to change, so this mitochondrial reduced mobility with maturation

704   seems to be specific which could be explained by the localisation of mitochondria to pre-

705   synaptic terminals with the development of synapses. The authors also examined the

706   transport of mitochondria using two-photon imaging and showed that more than 90% of

707   mitochondria in the distal axons of layer 2/3 cortical neurons are actually stationary. This

708   lack of mobility corresponds with a decline in regenerative capacity and can be targeted to

709   facilitate regeneration, either by interfering with the docking of mitochondria by syntaphilin

710   or by overexpressing the mitochondrial motor adaptor Miro-1. These interventions lead to

711   increased axonal mitochondrial dynamics, and enhanced regeneration of mature cortical

712   neurons *in vitro*. Axons from syntaphilin knockout mice also exhibit enhanced mitochondrial

713   motility and a rescue of energy deficits after *in vitro* axotomy, and enhanced regeneration

714   after a sciatic nerve crush injury. This study suggests that unmet energy demands after axonal

715   injury is one reason why mature axons are poor regenerators (Zhou et al., 2016). Enhancing

716   mitochondrial motility can also stimulate regeneration of injured CNS axons as demonstrated

717   by a recent study focusing on the mitochondria-associated molecule Armcx1. This molecule

718   is upregulated when regeneration is stimulated in the optic nerve by interventions such as

719   PTEN deletion. Overexpression of Armcx1 stimulates mitochondrial motility, *in vitro* CNS

720   axon growth, and axon regeneration after an optic nerve crush. It also enhances optic nerve

721   regeneration when overexpressed in mice with genetic PTEN deletion (Cartoni et al., 2016).

722

723   *Proteasome*

724   The ubiquitin-proteasome plays an important role in regulating the concentration of

725   individual proteins within the cell, by clearing excessive or unwanted proteins. It is also

726   responsible for clearing damaged or mis-folded proteins (Korhonen and Lindholm, 2004).

727   Proteasome function ensures that the correct amount of protein is present in specific

728   subcellular regions at any given time. Proteasome transport into axons is dependent upon

729   association with membranous vesicles, which are transported by kinesin and dynein motors

730   (Otero et al., 2014). In the PNS, there appears to be predominant anterograde transport of the

731   proteasome because ligation of the sciatic nerve leads to the accumulation of proteasomal

732   subunits on the proximal side of the ligation (indicating the blockade of proteasomes moving

733   away from the cell body). Proteasomal subunits also accumulate on the distal side of the

734   lesion, indicating retrograde transport also occurs, however this is not as profound as is seen

735   on the proximal side (Otero et al., 2014). Importantly, this anterograde transport appears to be

736   enhanced after a growth-stimulating pre-conditioning injury (Verma et al., 2005), suggesting

737   that PNS axons increase the anterograde transport of proteasomal components as part of their

738   response to an injury. Proteasomal activity appears to be an important part of the PNS

739   regeneration process, because inhibition of the proteasome by lactacystin leads to a reduction

740   in the percentage of axons that can reform a growth cone after in vitro axotomy (Verma et al.,

741   2005). CNS neurons also transport proteasomal subunits into their axons, both early on in

742   development (Hsu et al., 2015), and also at a more mature developmental stage (Otero et al.,

743   2014), although it is not clear whether the proportion of anterograde or retrograde transport

744   changes with development. The study by Hsu et al suggests that there is predominant

745   retrograde transport, at least at an early developmental stage (embryonic day 18 cortical

746   neurons cultured for 3 days), whilst the study by Otero et al suggest that later in development

747   (10 days *in vitro*) the majority of CNS proteasomal transport (80%) is random and diffuse,

748   with only small amounts of clearly anterograde or retrograde transport. This difference may

749   be to do with maturation, but could also be down to experimental conditions, Hsu et al

750   imaging proteasomal transport by use of a fluorescent dye (MV151), and Otero et al imaging

751   YFP tagged proteasomal subunits. Whatever the case, these studies did not find that the

752   predominant anterograde proteasomal transport observed in the PNS is also present in the

753   CNS. Instead, there may be an increase in retrograde proteasomal transport which increases

754   with axon length (Hsu et al., 2015). Given that a dynamic balance between local protein

755   synthesis and protein degradation is important for PNS axon regeneration after injury (Gumy

756   et al., 2010), it may be that efficient axonal proteasome transport is required for a properly

757   functioning growth cone which can drive regenerative axon growth.

758

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;

784   Kulkarni and Maday, 2018; Nikoletopoulou and Tavernarakis, 2018; Stavoe and Holzbaur,

785   2018). Here we discuss the involvement of autophagy in the process of axon growth and

786   regeneration, the presence of autophagy specifically within the axon, how autophagic

787   mechanisms may vary between neuronal types of differing regenerative ability, and the

788   regulation/interaction of Rab11 and recycling endosomes with the autophagosome. We also

789   comment on a role for non-acidic lysosomes at the growth cone.

790   Currently the majority of evidence suggests that autophagy is required for axon growth and

791   regeneration. Whilst one study reports that inhibition of autophagy by silencing of ATG7

792   leads to increased axon extension (Ban et al., 2013), the majority of studies suggest that

793   autophagy is required for axon growth. The autophagy genes ULK1 and 2 are required for

794   normal axon extension in the developing mouse brain (Wang et al., 2017a), and inhibition of

795   autophagy opposes axon growth and survival of DRG neurons in culture, suggesting a

796   positive role for autophagy during regenerative axon growth (Clarke and Mearow, 2016).

797   Autophagy also seems to be beneficial for CNS axon regeneration, with a recent study

798   demonstrating that stimulation of autophagy can promote axon growth over inhibitory

799   molecules *in vitro*, whilst activation of autophagy *in vivo* (by delivery of of Tat-beclin) leads

800   to enhanced regeneration of monoaminergic neurons after a spinal cord injury (He et al.,

801   2016). This study used EM to demonstrate that beclin-induced autophagosomes were present

802   specifically within axons. If autophagy is required within the axon in order to regenerate a

803   growth cone, it is possible that there may be differences in the transport of autophagy

804   machinery between regenerative and non-regenerative axons. It is important to note that

805   monoaminergic neurons have a better regenerative ability than corticospinal tract axons, and

806   the induction of autophagy by Tat-beclin does not stimulate CST regeneration (He et al.,

807   2016). This may indicate that the necessary growth-promoting autophagy machinery is not

808   present in these axons.

809   Autophagy, and the transport of autophagosomes, has been examined in both CNS and PNS

810   axons and whilst there is clear evidence for the existence of autophagy in both neuronal

811   types, there may also be differences. Axonal autophagosome biogenesis was first studied in

812   adult mouse DRG neurons (Maday et al., 2012), by imaging GFP-LC3 (an autophagosome

813   marker). Autophagosomes were observed developing in the distal part of axons, however in

814   this location they remained negative for lysosomal markers. Instead autophagosomes became

815   positive for lysosomal markers as they were retrogradely transported towards the cell body.

816   This suggests that mature autophagy (degradation) does not occur in the distal axon but is

817   targeted to the cell body (Maday et al., 2012). Despite this, there is apparent contact of

818   autophagosomes with lysosomes within the distal part of the axon, indicating that there may

819   be some fusion events that may not be degradative. It is not clear if the contents of distal axon

820   autophagosomes can escape autophagy by being passed to lysosomes. However, there is

821   evidence that lysosomes may be required at the growth cone to enable the trafficking of cargo

822   out of the autophagosome because decreasing the amount of lysosomes at the growth cone

823   leads to enlargement of autophagosomes (Farias et al., 2017). There is also evidence that

824   growth cone lysosomes may not be degradative as they are not acidified in the same way as

825   the rest of the cell (Farias et al., 2017; Overly and Hollenbeck, 1996). Lysosomes supply

826   integrins to enable invasive and migratory behaviour in non-neuronal cells (Dozynkiewicz et

827   al., 2012; Rainero and Norman, 2013) and can undergo exocytosis to contribute to axon

828   growth (Arantes and Andrews, 2006). It is therefore possible that there is a form of recycling

829   that occurs at the growth cone via autophagosome/endosome interaction. We speculate that

830   this may be required to enable regeneration when an axon needs to alter its machinery to

831   switch from a state of neurotransmission to a state of axon growth. The process of autophagy

832   could engulf unwanted machinery, which in turn could either be degraded or recycled

833   through lysosomes or other endosomes.

834   The above study by Maday et al demonstrated that axonal autophagosomes are

835   predominantly transported retrogradely towards the cell body in DRG neurons. Subsequent

836   work confirmed that a similar phenomenon occurs in CNS neurons, through the study of

837   E15.5 mouse neurons in vitro (Maday and Holzbaur, 2014; 2016).   These investigations

838   found that axonal autophagy was not induced in response to nutrient deprivation (as occurs in

839   other cell types), but rather that autophagosome generation appears to be a homeostatic

840   process. They also showed that autophagosome biogenesis is four times slower in the distal

841   axons of hippocampal neurons compared to DRG neurons. The studies also demonstrated that

842   the axonal ER is a source of membrane for the axonal autophagosome and not the plasma

843   membrane, but the authors did not examine a potential role for Rab11. Rab11 positive

844   recycling endosomes are a source for autophagosomal membrane during starvation induced

845   autophagy in non-neuronal cells (Lamb et al., 2016; Longatti et al., 2012; Longatti and

846   Tooze, 2012). Given that Rab11 endosomes are transported away from CNS axons as they

847   mature (Koseki et al., 2017) it is possible that this may limit the type of autophagy that exists

848   within the axon. The question remains as to whether starvation or stress induced autophagy is

849   a mechanism that needs to be activated in order to stimulate the process of regeneration

850   within the axon. It is also not known if there are developmental changes in the rate or type of

851   autophagy that occurs within axons (which may contribute to regeneratve ability) or whether

852   the type of autophagy varies between neurons of different regenerative abilities. Perhaps

853   recycling autophagy (as opposed to degradative) is more suited to axon regeneration? There

854   is strong evidence for the involvement of Rab11 in both the generation of the autophagosome

855   (as mentioned above) and also in mediating traffic away from autophagosomes towards

856   multivesicular bodies and exocytosis (Chen et al., 2017; Fader et al., 2008; Fader et al.,

857   2009). Given the difference in Rab11 transport between non-regenerative and regenerative

858   axons, it may be that there are differences in the way autophagy functions in the axons of

859 various neuronal types, that might contribute to their differential regenerative ability. Much

860 work is needed to determine whether this is the case.

861

862 **Mechanisms regulating polarised transport**

863 As described above, the capacity for axon regeneration varies between neuronal types and

864 with development. Adult PNS axons are considered good regenerators, whilst most CNS

865 axons lose their ability to regenerate with maturity. The ability to transport regenerative

866 molecules into axons also varies. For example, PNS axons continue to support integrin

867 transport into adulthood whilst transport declines with maturity in CNS axons (Fig. 1). This

868 also appears to be the case for other growth promoting receptors, which are either absent or

869 expressed at low levels in most mature CNS axons. To understand the mechanism behind

870 these differences, it is necessary to examine the regulation of polarised transport in neurons,

871 particularly focusing on membrane proteins. The axon growth promoting receptors and

872 guidance molecules that mediate regeneration such as TrkB, the IGF1 receptor and integrins

873 are all cell surface membrane proteins. This class of molecule is subject to tightly controlled

874 trafficking processes in all cells. As an extreme example of polarised cells, neurons have

875 intricate mechanisms for maintaining the correct distribution of membrane proteins to

876 specific neuronal compartments. This ensures that post synaptic receptors and associated

877 machinery are targeted to dendrites, whilst the machinery for synaptic vesicle cycling and

878 neurotransmission are directed to presynaptic sites within axons. In this section, we describe

879 the mechanisms that are known to regulate polarised distribution in neurons, focusing on

880 their relevance to regulating regenerative capacity. We have described above how a

881 conditioning injury that promotes regeneration can also facilitate axon transport. This

882 response relies partly on the retrograde injury signal, which transmits signals from the axon

883 to the cell body via retrograde axonal transport. We will not be focusing on this retrograde

884 response (which has been reviewed in detail: (Abe and Cavalli, 2008; Rishal and Fainzilber,

885 2014; Tasdemir-Yilmaz and Segal, 2016), but rather the mechanisms involved in facilitating

886 or preventing anterograde delivery, and how these might relate to axon regeneration.

887

888 *Neuronal membrane transport*

889 All membrane proteins are synthesised in the ER membrane, and spend their lives restricted

890 to a membranous environment. After leaving the ER, membrane proteins pass through the

891 Golgi membrane before being transported to the cell surface in endosomes. At the cell

892 surface, membrane proteins can be internalised (again into endosomes) and are subject to a

893 variety of regulatory mechanisms which can decide their fate – recycling to the plasma

894 membrane, redirecting to a different part of the cell, clustering into signalling platforms,

895 degrading, or even priming for extracellular cues (Yap and Winckler, 2012). Membrane

896 protein trafficking is therefore subject to precise and complex regulation by numerous

897 processes including cytoskeletal elements, motor proteins, adaptor molecules, protein

898 scaffolds, signalling molecules (including kinesins and phosphatases), and small GTPases

899 such as the Rab and ARF families. Investigations into neuronal polarised membrane transport

900 has focused on all of these mechanisms, and each of them plays a role in regulating transport

901 into either dendrites or axons.

902

903 *Microtubules and associated motors*

904 The principle regulator of polarised transport in neurons is arguably the cytoskeleton, which

905 defines axons and dendrites by virtue of the orientation of microtubules. Axonal microtubules

906 are unipolar, with the plus end facing into the axon, whilst dendrites have microtubules

907 aligned both into and away from the dendrite (Baas et al., 1988; Tas et al., 2017; Yau et al.,

908 2016). As the majority of membrane protein transport occurs in endosomes transported on

909 microtubules by kinesin or dynein/dynactin motor proteins, the unipolar nature of axonal

910 microtubules ensures that only kinesin motor proteins can drive anterograde axonal transport,

911 and that molecules transported principally by dynein are enriched in dendrites. Proteins

912 intended for axonal transport will also enter dendrites, but overall their distribution is biased

913 towards axons (Nakata and Hirokawa, 2003). Microtubule orientation alone is not sufficient

914 to determine whether a protein can enter the axon, and so further control is exerted by the

915 wide variety of the kinesin family. Neurons express as many as 20 types of kinesin that will

916 transport cargo towards the plus end (Silverman et al., 2010), but only some of these

917 specifically target to axons, whilst others target to both dendrites and axons. Interestingly, no

918 kinesins have been found to be targeted only to dendrites (Huang and Banker, 2012). This

919 suggests that dynein-dependant transport is an important determinant in dendritic targeting,

920 and this has been shown to be the case, because linking axon specific cargo to dynein motors

921 results in their dendritic delivery (Kapitein et al., 2010). The specificity of individual kinesins

922 for axons may contribute to the developmental decline in regenerative ability, in that the

923 expression of certain kinesins changes with development. Kinesin KIF4A is involved in the

924 axonal delivery of integrins during development, but it is downregulated postnatally at a time

925 when integrins are excluded from axons (Heintz et al., 2014). However, whilst re-expressing

926 KIF4A in mature cortical neurons *in vitro* leads to its presence in axons, it does not facilitate

927 integrin axonal transport. Integrins can be manipulated into axons at this time (through other

928 trafficking interventions), suggesting that other kinesins are also capable of transporting

929 integrins into axons.

930 The targeting of specific kinesins to the axon is thought to be partly as a result of specific

931 modifications to the microtubules, including acetylation, glutamylation and detyrosination

932 which regulate axonal kinesin transport (Hammond et al., 2010; Kaul et al., 2014; Konishi

933 and Setou, 2009). Importantly, interventions targeting these modifications such as low dose

934   taxol can permit axonally excluded growth promoters such as integrins to enter the axon

935   (Franssen et al., 2015), however this can also cause axonal proteins to accumulate in

936   dendrites (Hammond et al., 2010). Interestingly, low dose taxol treatment only permits

937   integrins transport into the proximal part of the axon, suggesting microtubule modifications

938   are particularly critical in this area. The early part of the axon is an area of intense study

939   regarding polarised transport. This region of the axon includes two important regions

940   implicated in the regulation of polarised transport, the axon initial segment (AIS) and before

941   it, the pre-axonal exclusion zone (PAEZ).

942

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

944   The AIS is in the very proximal part of the axon. It is primarily responsible for the

945   propagation of the action potential, being enriched in the ion channels necessary for this

946   function. It is also enriched in cytoskeletal elements such as AnkyrinG, actin, and beta IV

947   spectrin, and microtubule associated proteins such as EB1 and EB3 (Leterrier et al., 2011;

948   Zhang and Rasband, 2016). Importantly, the AIS develops at a time when CNS axons lose

949   their regenerative ability, and is strongly associated with polarised membrane transport and

950   axon dendrite identity (Rasband, 2010). Ankyrin G is considered to be the orchestrator of the

951   AIS because its depletion causes demolition of the entire structure. Depleting Ankyrin G

952   leads to a loss of axonal identity, with the proximal axon exhibiting dendritic molecules such

953   as post-synaptic receptors and taking on dendritic features such as spines (Sobotzik et al.,

954   2009). A number of mechanisms have been proposed to explain how the AIS might regulate

955   polarised transport, in addition to the post-translational modifications of microtubules

956   (Hammond et al., 2010; Konishi and Setou, 2009; Tapia et al., 2013). The actin cytoskeleton

957   is proposed to regulate transport from within the AIS by acting as a dense barrier to diffusion

958   (Song et al., 2009) or by diverting myosin motors back to the cell body (Lewis et al., 2009).

959   This idea is currently debated because other studies have not found a dense actin mesh within

960   the AIS (Farias et al., 2015), but rather the usual actin and spectrin rings that are present

961   throughout the axon (Leterrier et al., 2015; Xu et al., 2013) as well as actin patches (Jones et

962   al., 2014). Interfering with actin through latrunculin treatment leads to a small increase in

963   integrin transport into the proximal part of mature CNS axons (Franssen et al., 2015), but

964   does not facilitate long range axonal transport.

965   Another AIS-related mechanism functions to regulate dynein dependant retrieval of dendritic

966   cargo from within the AIS. The dynein regulator Ndel1 is attached to the AIS through

967   binding to Ankyrin G and functions through its binding partner Lis1 to activate dynein

968   leading to increased retrograde transport of molecules not intended for axons, such as the

969   transferrin receptor. Silencing Ndel1 leads to anterograde transport of the transferrin receptor

970   (Kuijpers et al., 2016). Importantly, Ndel1 is localised to the initial part of axons from an

971   early developmental stage, at a time when integrins are still present throughout the axon,

972   suggesting it is not the central regulator of integrin dendritic localisation. Retrograde

973   transport of early endosomes marked by Rab5 is also regulated from this part of the axon

974   through the Rab5 interactor FHF (Guo et al., 2016). Crucially, there is also retrograde

975   transport of dendritic vesicles away from the base of the axon even before the AIS develops,

976   from a region termed the pre-axonal exclusion zone (PAEZ) (Farias et al., 2015). This area

977   has been implicated in the dendritic targeting of glutamate receptor AMPA-GluR1, the Golgi

978   protein GM130, and the ER protein CLIMP-63, and the endoplasmic reticulum, indicating

979   that the region is critical to maintaining a wide range of cell machinery away from axons

980   (Britt et al., 2016; Gumy and Hoogenraad, 2018). This early developmental axonal exclusion

981   is in keeping with a previous study which demonstrated that dendritic proteins become

982   polarised even before the axon is specified (Petersen et al., 2014). Interestingly, the PAEZ

983   overlaps with a region within the AIS which labels strongly for TRIM46. This is a

984   microtubule organising molecule which is present before the AIS is fully developed, which is

985   responsible for arranging microtubules in their polarised fashion. Depletion of TRIM 46 leads

986   to mixed microtubule orientation and decreased transport of axonal cargo (van Beuningen et

987   al., 2015). Another level of selectivity is achieved within this region by the microtubule

988   associated protein, MAP2. Whilst classically considered to be a dendritic marker, MAP2

989   strongly labels the earliest section of the axon, and its localisation there depends on TRIM46.

990   Depletion of MAP2 leads to altered cargo transport, with some dendritic molecules being

991   transported into axons, and some axonal molecules appearing in the somatodendritc domain.

992   MAP2 functions in this location to regulate kinesin activity, specifically inhibiting KIF5

993   motor activity, so that cargo that is transported by KIF5 requires an additional motor such as

994   KIF1 (which is not affected by MAP2) in order to enter the axon (Gumy et al., 2017).

995   Crucially, MAP2 is present in the early part of the axon in non-regenerative and regenerative

996   neurons being observed in both mature CNS neurons that do not support integrin transport, as

997   well as PNS neurons that do. MAP2 therefore permits anterograde transport of the kinesins

998   that carry integrin endosomes, but in CNS axons there are additional signalling and

999   trafficking mechanisms that are upregulated with development that result in an increased

1000   affinity of integrin containing endosomes for the dynein/dynactin complex, and subsequent

1001   retrograde removal from axons. Recent studies have found that the principle regulators of this

1002   process are ARF6 and its activator EFA6, which is enriched in the initial part of the axon as

1003   cortical neurons mature (Eva et al., 2017).

1004

1005   *Axon initial segment, ARF6 and the JIP family of proteins*

1006   The ARF6 GEF EFA6 is upregulated in the brain along with development, playing a role in

1007   the development and maintenance of dendrites and spines (Choi et al., 2006; Raemaekers et

1008   al., 2012). It was recently discovered to have an additional axonal role, colocalising with

1009   neurofascin in the early part of the AIS, and functioning to activate ARF6 throughout the

1010   axon stimulate retrograde integrin transport (Eva et al., 2017). This may occur through the

1011   priming of another ARF6 GEF, ARNO, which is distributed along the whole length of the

1012   axon. EFA6 is known to function together with ARNO to sustain ARNO and ARF6 activity

1013   (Padovani et al., 2014). This appears to happen in axons, because silencing EFA6 leads to a

1014   striking decrease in axonal ARF6 activation. Importantly, silencing EFA6 leads to an

1015   increase in the anterograde axonal transport of integrin and Rab11 endosomes and an increase

1016   in regeneration after a laser injury. Interfering with ARNO also leads to an increase in axonal

1017   integrin transport in cortical neurons, as does overexpression of the ARF GAP ACAP1

1018   (Franssen et al., 2015). These molecules were first shown to regulate directional axon

1019   transport in PNS axons (which allow bi-directional integrin transport) (Eva et al., 2012b).

1020   Elevating ARF6 activity in adult DRG neurons *in vitro* leads to an increase in retrograde

1021   integrin transport and a decrease in regeneration after a laser injury. DRG neurons enable

1022   integrin transport through the expression of the GAP ACAP1, which is not present in the

1023   CNS. ACAP1 localises throughout DRG axons, and is strongly enriched in the growth cone

1024   (Eva et al., 2017). In addition to its ARF6 GAP activity ACAP1 also targets integrins to the

1025   surface membrane (Li et al., 2005). How does ARF6 regulate the direction of integrin

1026   transport in axons? The ability of ARF6 to control directional transport was first

1027   demonstrated in dividing non-neuronal cells, and relies on the interaction of ARF6 with the

1028   scaffold molecules JIP3 and 4. Active ARF6 increases the affinity of these JIPs for the

1029   dynein/dynactin complex whilst inactive ARF6 increases their affinity for kinesin motors

1030   (Montagnac et al., 2009). The JIP family of molecules therefore appear to exert a level of

1031   selectivity on axonal cargo and have the ability to exert this at many levels. As scaffolds, the

1032   JIPs function to link motor proteins to their cargo. The interaction between specific kinesins

1033   and the various JIPs defines one level of selectivity, while another is governed by the cargo

1034   that can or cannot interact with the JIPs. A good example of this is the difference between

1035   integrins and the amyloid precursor protein (APP). Integrins are transported away from

1036   mature CNS axons, whilst APP is bidirectionally transported. This may be as a result of their

1037   different interactions with the JIPs. There are four JIP family members. JIP 1 and 2 are

1038   similar but diverge from JIP 3 and 4 which are often classed together. APP interacts directly

1039   with JIP 1 and 2, and does not interact with JIP 3 and 4. ARF6 activation does not affect JIP1

1040   or 2 interactions with kinsesin or dynein, but only JIP3 and 4 (Koushika, 2008). Consistent

1041   with this, altering ARF6 activation does not alter APP axonal transport (Eva et al., 2017).

1042   APP directional transport is regulated through a different signalling mechanism. When JIP1

1043   is directly phosphorylated at a JNK phosphorylation site, S421, anterograde transport is

1044   stimulated, whilst dephosphorylation favours retrograde transport (Fu and Holzbaur, 2013).

1045   Importantly, the interactions between JIP/motor/cargo can occur in complexes with other

1046   proteins, meaning that their functions can impact on the directional transport of numerous

1047   proteins. The interaction between ARF6, JIP3 and 4 and motor proteins occurs in a complex

1048   that also involves Rab11 (Montagnac et al., 2009), meaning that ARF6 activation can also

1049   control the direction of transport of Rab11 and associated endosomes (Eva et al., 2017). This

1050   begins to explain how trafficking to specific endosomes might contribute to the targeting of

1051   membrane proteins to a specific part of the cell, with different endocytic regulatory or

1052   adaptor molecules adding another level of complexity.

1053

1054   *Endocytic transport, adaptors and sorting motifs*

1055   A number of endosome associated molecules have been implicated in polarised transport in

1056   neurons, including the EHD1/4 proteins and NEEP21/P19 (Nsg1 and 2), which regulate the

1057   trafficking of the adhesion molecule L1/NgCAM through early endosomes towards the axon

1058   (Lasiecka et al., 2010; Yap et al., 2008) via a transcytotic mechanism. Transcytosis

1059 (internalisation from the somatodendritic surface to the axonal surface) occurs in DRG

1060 neurons for integrins and the TrkA receptor via Rab11 endosomes (Ascano et al., 2009; Eva

1061 et al., 2010). Transport of the TrkA receptor is additionally regulated in a complex feed

1062 forward fashion which enables transcytosis to supply new receptors to the axon in response to

1063 retrograde signalling from within the axon. Anterograde transport is increased when

1064 retrograde signalling endosomes recycle to the somatic cell surface and transactivate resident

1065 receptors, causing them to be internalised. These are then transported to the ER, where they

1066 can be dephosphorylated by the phosphatase PTP1B before being anterogradely transported

1067 into the axon (Yamashita et al., 2017).

1068 The adapter molecules that regulate endocytosis are also important for regulating polarised

1069 distribution within neurons. These include the clathrin adaptors AP1-AP5 which exist as

1070 heterotetrameric complexes and the monomeric GGA adaptors (Golgi-localising, Gamma-

1071 adaptin ear homology, ARF-binding proteins) (Robinson, 2004). The clathrin adaptors

1072 interact with specific motifs that have been implicated in polarised transport in neurons and

1073 non-neuronal cells, including the AP1 adaptor which controls the dendritic distribution of the

1074 transferrin receptor through an interaction between the YXXØ motif in the cytoplasmic tail of

1075 transferrin and the μ1A subunit of AP1 (Farias et al., 2012). However, the presence of this

1076 motif does not guarantee targeting a protein away from the axon. Integrin subunits α 3, 4, 5,

1077 7, and 9 contain a YXXΦ motif, but preventing AP1 from a potential interaction with the

1078 motif by expression of a dominant negative mutant does not result in targeting of integrins

1079 into the axon, as was found for the transferrin receptor (Franssen et al., 2015).

1080

1081 **Summary: A virtuous cycle of axon growth and regeneration**

1082 Since the Aguayo experiments of the 1980s kick-started an era of research into CNS axon

1083 regeneration, there has been a broad-ranging, sustained effort to understand the mechanisms

1084 preventing regeneration. Understanding of intrinsic factors has increased dramatically, and it

1085 is apparent that there are three key elements which prevent regeneration: epigenetics,

1086 signalling pathways, and axon transport. Developmental gene changes maintain adult CNS

1087 neurons in a low state of growth (Venkatesh et al., 2016), compounded by underactive

1088 growth promoting signalling pathways (Liu et al., 2010), and a low abundance of growth

1089 machinery within the axon (Andrews et al., 2016; Eva et al., 2017; Hollis et al., 2009a; b).

1090 These areas represent targets for simulating regeneration and raise the tantalising prospect of

1091 an intervention that could function by affecting all three processes. One can envisage a

1092 strategy that targets epigenetic changes could lead to the expression of genes which enhance

1093 signalling through growth promoting pathways, as well as increased axonal transport of

1094 regenerative machinery such as integrins or growth factor receptors. Once in the axons,

1095 growth-promoting receptors could be activated by their ligands, leading to retrograde

1096 signalling via endosomes, amplification of growth promoting signals, and somatic effects on

1097 transcription, translation and axonal transport. In theory, this cycle of events could be

1098 targeted at any point to stimulate a "virtuous cycle of axon growth" (Fig. 2). A good example

1099 is intervening at the level of axon transport of integrins and growth factor receptors.

1100 Increasing the levels of these molecules on the surface of the axon could lead to increased

1101 signalling through known regenerative pathways, such as the PI3 kinase pathway. This would

1102 elevate growth promoting signals locally, but these are additionally capable of signalling to

1103 the cell body via retrograde signalling endosomes, which can lead to effects on transcription

1104 and translation (Tasdemir-Yilmaz and Segal, 2016). Signalling through PI3 kinase can lead to

1105 epigenetic changes which can activate a growth program (Spangle et al., 2017), and can also

1106 lead to increased axonal transport of PI3 kinase coupled receptors, such as TrkB (Cheng et

1107 al., 2011). Initiating the axonal transport of growth-enabling molecules can therefore have

1108 wide-ranging effects throughout the cell. So far, *in vitro* experiments have demonstrated that

1109 CNS regeneration can be stimulated through the axonal mobilisation of growth machinery

1110 (Eva et al., 2017; Koseki et al., 2017). Much work is needed to see if this approach can be

1111 used to stimulate regeneration *in vivo* after a brain or spinal cord injury.

1112

1113 **Figure Legends**

1114 **Figure 1. Integrin transport in regenerative vs non-regenerative axons.**

1115 Integrins and Rab11 are bidirectionally transported in regenerative adult PNS axons but are

1116 removed from non-regenerative CNS axons by predominant retrograde transport after

1117 development.

1118

1119 **Figure 2. A virtuous cycle of axon growth and regeneration.**

1120 Adult CNS axons are weak regenerators because of gene suppression by epigenetic factors

1121 and a lack of growth promoting machinery in the axon. The figure illustrates a cycle of events

1122 which can enable regeneration. Intervening at any point can feed forward to stimulate the

1123 subcellular changes that can drive axon growth. For example, increasing the transport of

1124 growth promoting receptors in recycling endosomes facilitates growth cone development and

1125 axon growth. Activated growth cone receptors signal retrogradely to the cell body.

1126 Signalling downstream of growth factors (eg through PI3K) can lead to changes in gene

1127 expression and altered protein translation. Retrograde signals from growth factor receptors

1128 can also stimulate anterograde transport in an autocrine fashion.

1129

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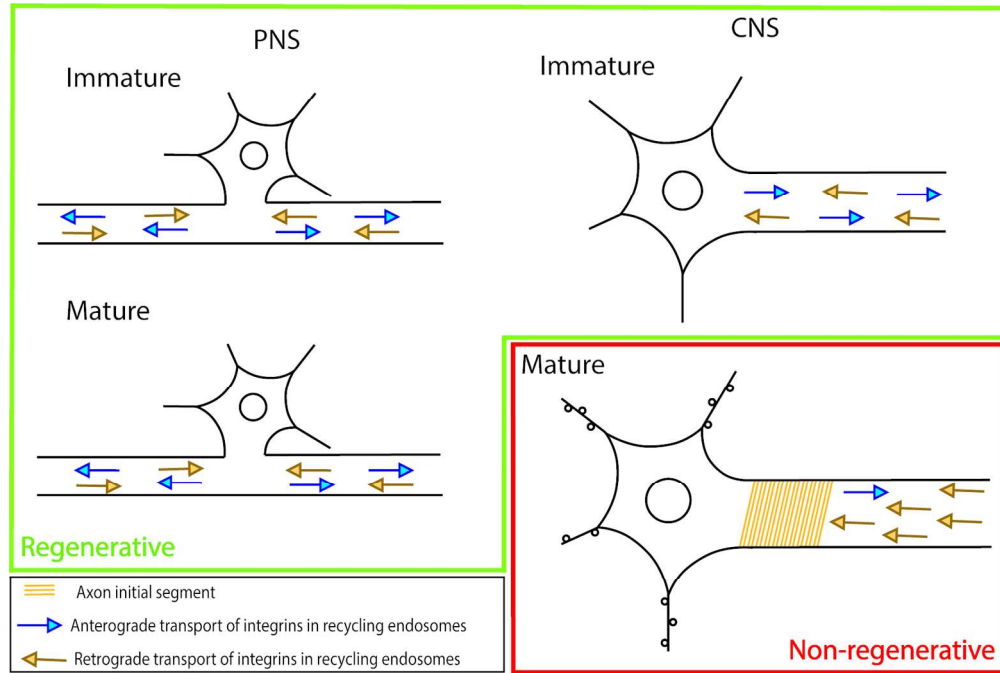1887   214(1):103-119.

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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.
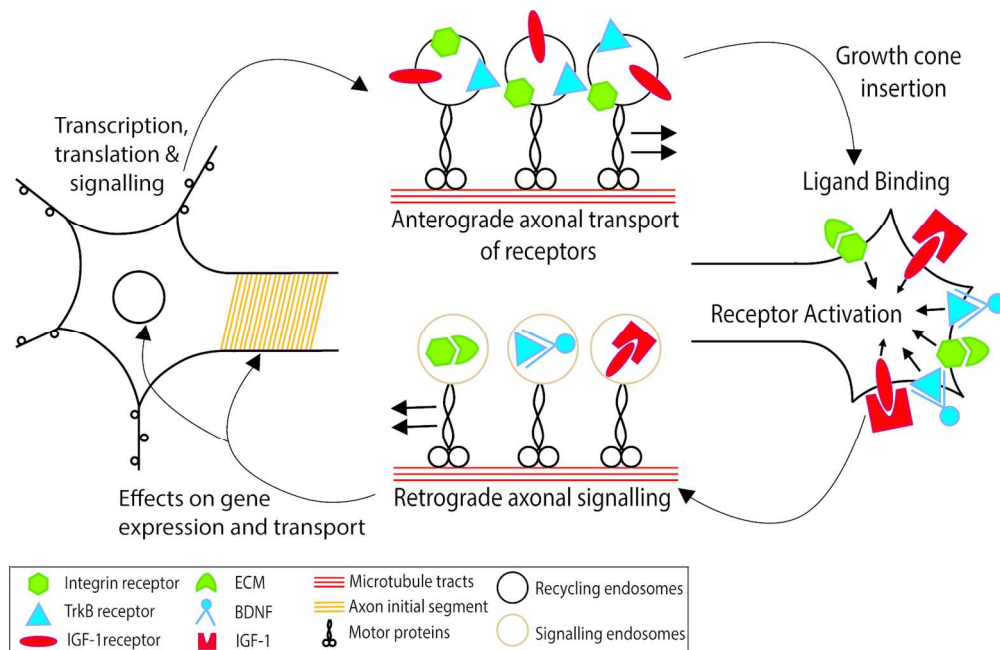
150x100mm (300 x 300 DPI)

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)