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Recapitulate development to promote axonal regeneration: good or bad approach?

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In the past decade there has been an explosion in our understanding, at the molecular level, of why axons in the adult, mammalian central nervous system (CNS) do not spontaneously regenerate while their younger counterparts do. Now a number of inhibitors of axonal regeneration have been described, some of the receptors they interact with to transduce the inhibitory signal are known, as are some of the steps in the signal transduction pathway that is responsible for inhibition. In addition, developmental changes in the environment and in the neurons themselves are also now better understood. This knowledge in turn reveals novel, putative sites for drug development and therapeutic intervention after injury to the brain and spinal cord. The challenge now is to determine which of these putative treatments are the most effective and if they would be better applied in combination rather than alone. In this review I will summarize what we have learnt about these molecules and how they signal. Importantly, I will also describe approches that have been shown to block inhibitors and encourage regeneration *in vivo*. I will also speculate on what the differences are between the neonatal and adult CNS that allow the former to regenerate and the latter not to.

Keywords: regeneration; myelin; MAG; Nogo; p75; cAMP

1. REGENERATION IN THE ADULT CENTRAL NERVOUS SYSTEM

Whether it is damage to the spinal cord after injury or axonal loss due to disease, the adult mammalian central nervous system (CNS), quite simply, does not spontaneously regenerate. In sharp contrast, it appears that neonatal CNS axons do indeed regenerate after being severed. This occurs for only a limited time during development, and the precise age at which this ability is lost depends on the species, which for rat, at least, is by the end of the first post-natal week (Bregman & Goldberger 1982; Kunkel-Bagden *et al.* 1992). What causes this loss of ability to spontaneously regenerate with development? We know from the work of Aguayo and colleagues in the early 1980s that it is not because adult CNS neurons have lost the intrinsic ability to extend axons at all, because if they are presented with a known, favourable environment, they will regenerate, albeit significantly more slowly than their younger counterparts (Richardson *et al.* 1980; David & Aguayo 1981; Goldberg *et al.* 2002). This suggests that something in the adult CNS environment, and perhaps also the response of the adult CNS to injury/disease, changes with development. That is in fact what happens—after injury the adult CNS is inhibitory for axonal regeneration. This inhibitory environment comprises the formation of a glial scar, a response to injury not observed in young animals, and the presence of inhibitory molecules present in both the scar itself and also in myelin debris. Surprisingly, the neuron's response to this environment also changes. That is to say, young neurons can quite happily extend axons through/on these inhibitors (Cai *et al.* 2001). In this review I will focus on these two developmental events: the change in the CNS environment and the switch in the neuron's response to that environment. I will describe what is known regarding the molecules involved, how their inhibition can be overcome to encourage regeneration and what is required to have full functional recovery after, for example, spinal cord injury.

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One contribution of 13 to a Theme Issue 'The regenerating brain'.

2. THE DEVELOPMENTAL CHANGE IN THE CENTRAL NERVOUS SYSTEM ENVIRONMENT

(a) *The glial scar*

The major component of the glial scar is astrocytes that have undergone reactive gliosis. In general, this occurs after injury to the brain or spinal cord when astrocytes change their morphology by enlarging and by putting out processes that interdigitate, forming a physical barrier to axonal regeneration. This change in morphology is marked by an upregulation in intermediate filaments and, indeed, increased expression of one such filament, glial fibrillary acidic protein, is used as the hallmark of a reactive astrocyte (Eng 1985). In addition, these reactive astrocytes increase expression of extracellular matrix components, most notably chondroitin sulphate proteoglycans (CSPGs), which are very inhibitory for axonal regeneration (Rudge & Silver 1990; Snow *et al.* 1990; McKeon *et al.* 1991). The formation of the glial scar is triggered by the invasion of non-CNS factors as a result of the disruption of the blood–brain barrier (BBB; Fitch *et al.* 1999; Preston *et al.* 2001). Following from this, the severity of the lesion dictates the severity of scar

formation. For example, relatively minor lesions, in which the BBB is minimally disrupted, result in reactive gliosis only very close to the lesion site (Preston *et al.* 2001). For lesions where there is greater disruption of the BBB but in which the meninges are left intact, the reactive gliosis is much greater, with a much stronger and more widespread upregulation of inhibitory proteoglycans. In addition, a cavity now forms at the lesion centre. Finally, in those severe lesions in which the meninges are disrupted, not only is there a strong reactive gliosis and cavitation, but also fibroblasts invade the lesion core and induce the astrocytic expression of particular guidance cues that are repulsive during development, such as Slit and ephrin-B2 as well as the ephrin receptors EphB2 and EphA4 (Turnley & Bartlett 1998; Bundesen *et al.* 2003; Hagino *et al.* 2003; Goldshmit *et al.* 2004). The fibroblasts themselves express the repulsive guidance cue, Sema3 and axons attempting to regenerate express the Sema3 receptor, neuropilin, and are strongly repelled by the lesion core (Pasterkamp *et al.* 1999; De Winter *et al.* 2002; Jin *et al.* 2002). So to encourage axons to grow through the glial scar, they must not only overcome inhibitory proteoglycans, which are expressed at all lesions, but also, depending on the severity of the lesion, ephrin-B2, Slit and Sema3.

Recently, an interesting study showed considerable regeneration of corticospinal and rubrospinal axons after a hemisection lesion in the EphA4-deficient mouse (Goldshmit *et al.* 2004). Interestingly, there was also attenuation of reactive gliosis as marked by less hypertrophy of astrocytes and less production of CSPGs. Although EphA4 has been shown to be a receptor for ephrin B3, Eph–ephrin interactions have been shown to signal in both directions. It is conceivable then, that for inhibition of growth, EphA4 is the ligand and the signal is transduced via ephrinB3 on the axon, while on astrocytes EphA4 is the receptor and transduces the signal. The question now to be addressed is whether or not blocking Eph4A with antibodies or peptides has a similar effect on regeneration *in vivo* in wild-type mice as that recorded in the knockout mouse.

The CSPGs in the glial scar include brevican, phosphocan, aggrecan, neurocan and NG2, which has also been shown to be expressed by oligodendrocyte progenitor cells (Henderson *et al.* 1981; Dou & Levine 1994; Jones *et al.* 2002). Proteoglycans consist of a protein core linked to a sulphated glycosaminoglycan (GAG) chain by four sugar moieties. They differ not only in the protein core but also in length, number and pattern of sulphate of the side chains (Margolis & Margolis 1993). The GAG portion of the molecules was shown to be the inhibitory portion, because if it is removed from reactive astrocytes in culture with the enzyme chondroitinase, they were no longer inhibitory for regeneration. Importantly, application of the same enzyme *in vivo* after lesion resulted in considerable axonal regeneration in the spinal cord and some functional recovery (Bradbury *et al.* 2002). Interestingly, although the neuronal receptors for CSPGs are not yet known and little is known regarding their signalling pathway, they have been shown to activate the small GTPase, Rho, and this activation is necessary for their inhibitory effects (Borisoff *et al.* 2003; Monnier *et al.* 2003). Myelin inhibitors also signal through Rho, which points to a common target whereby inhibition by both CSPGs and myelin inhibitors can be blocked simultaneously. Another approach that has been shown to block the inhibition by both CSPGs and myelin is to elevate neuronal cAMP (Chierzi *et al.* 2005). Manipulations of the signalling cascades as a possible therapy are discussed in more detail below.

Unlike the mature CNS, young CNS axons will spontaneously regenerate after injury (Ferretti *et al.* 2003). The stage of development when the switch from being able to regenerate to not being able to do so depends on the species and on the particular neural tract, but is in all cases neonatal. For example, in the chick spinal cord, the ability to regenerate is lost between E11 and E15, whereas for the rat it is in the first post-natal week (Bregman & Goldberger 1982; Shimizu *et al.* 1990; Kunkel-Bagden *et al.* 1992; Hasan *et al.* 1993). Although no real scar forms after injury at the ages when spontaneous regeneration does occur, astrocytes still undergo a reaction to injury (McKeon *et al.* 1991). In sharp contrast to the adult, however, reactive astrocytes from these very young animals are very permissive for axonal growth. While it is not known what the precise molecules that promote growth are, it has been shown that these young reactive astrocytes do not express inhibitory CSPGs. The reason for this difference in reactive gliosis in young and old astrocytes is not known, but it has been suggested that not only is there less haemorrhage in young animals after injury but also the immune cells and the factors they secrete may be different (Ferretti *et al.* 2003; Silver & Miller 2004).

(b) *Inhibition of regeneration by myelin*

In both the CNS and peripheral nervous system (PNS), myelination is one of the last events to occur during development (Morell 1984). The growth cone of the nascent axon has either reached its target, or at least is well past the region of the nerve tract that undergoes myelination. Hence, when first growing towards their targets, young axons never encounter myelin, and, consequently, the inhibitors therein. Intact myelin is of course beneficial as it insulates the axon and allows salutatory conduction to take place. After injury or disease, when axons are severed, myelin is inevitably disrupted and rather than being tightly wrapped around the axon, myelin membrane fragments are strewn throughout the injury site. The newly formed growth cone of the transected axon now comes into contact with this myelin debris, growth stops and the axon will most likely retract (Ramon y Cajal 1928). This is the scenario in the CNS, but in the PNS the picture is quite different as the reaction to injury is very different. In the CNS, Wallerian degeneration (degeneration of the distal axon and clearing of debris, including myelin) is very slow, taking months or years, and it is questionable if it is ever complete. In addition, a glial scar forms. In contrast, Wallerian degeneration is very rapid in the PNS, and although there are inhibitors in PNS myelin, they are cleared very rapidly, along with the distal axon. No glial scar forms in the PNS; instead,

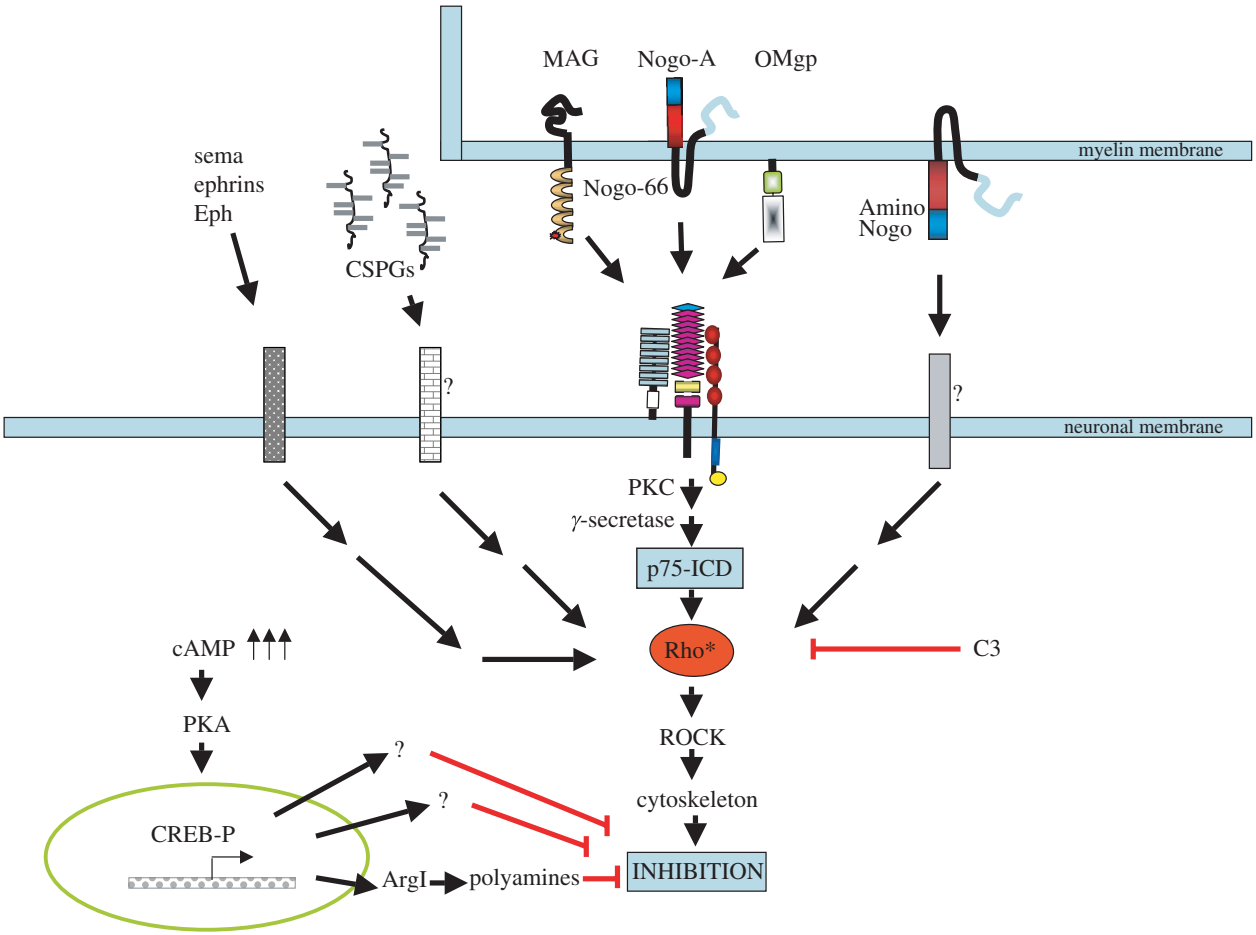

Figure 1. Inhibitors of axonal regeneration, their receptors and how they signal. Three myelin inhibitors, MAG, Nogo-66 and OMgp, all interact with the NgR/Lingo/p75 (TROY) receptor complex. The receptors for Amino-Nogo and CSPGs are not known. All the inhibitors exert inhibition by activating Rho. Inactivation of Rho or elevation of cAMP each overcome all the inhibitors simultaneously. The cAMP effect is CREB- and transcription-dependent. ArgI is up-regulated, resulting in an increase in polyamine synthesis. Polyamines can block inhibition.

Schwann cells de-differentiate and become permissive for axonal regeneration, before going on to re-myelinate the regenerated axon. Regeneration and the environment of the adult PNS is more reminiscent of axonal growth during development in either the CNS or PNS (Scherer & Salzer 2001).

(i) *The inhibitors*

To date three myelin-associated proteins have been clearly demonstrated to be inhibitory for regeneration (figure 1). These are the three very different proteins: Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (Filbin 2003).

Nogo

Nogo was identified as an antigen of the IN-1 monoclonal antibody, which had been shown 10 years prior to the cloning of Nogo to encourage axonal regeneration both on myelin in culture and in the CNS *in vivo* (Caroni & Schwab 1988; Schnell & Schwab 1990; Bregman *et al.* 1995). Nogo is a member of the reticulon family of proteins, which, except for Nogo, are found only in the endoplasmic reticulum (Chen *et al.* 2000; GrandPre *et al.* 2000; Prinjha *et al.* 2000). Nogo exists in three isoforms, A, B and C; of these Nogo-A is enriched in oligodendrocytes, Nogo-B in neurons and Nogo-C is found largely outside the nervous system (GrandPre *et al.* 2000; Huber *et al.* 2002; Wang *et al.* 2002*c*). Nogo-A is reported to have at least two inhibitory domains, a 66-amino acid sequence, referred to as Nogo-66, which is common to all three isoforms, and a sequence found in the amino terminus which is unique to Nogo-A and is termed Amino-Nogo (Oertle *et al.* 2003). Although it carries no signal sequence, Nogo-A has been localized to both the inner- and the outermost wrap of the myelin sheath (GrandPre *et al.* 2000; Huber *et al.* 2002; Wang *et al.* 2002*c*). At least two topologies have been proposed for Nogo-A: one in which both Amino-Nogo and Nogo-66 are extracellular and the other in which only Nogo-66 is exposed at the surface (Oertle *et al.* 2003).

Myelin-associated glycoprotein

Unlike Nogo, MAG was identified about 20 years before it was shown to be inhibitory for axonal growth (Quarles 1983; McKerracher *et al.* 1994; Mukhopadhyay *et al.* 1994). It is a member of the immunoglobulin (Ig)-superfamily, containing five Ig-like domains in its extracellular sequences. The two MAG isoforms, large (L) and small (S), differ only in their cytoplasmic sequences (Lai *et al.* 1987; Salzer *et al.* 1987). MAG is a sialic acid binding protein, and is a member of the

Siglec (sialic acid binding lectins) family of proteins (Siglec 4; Kelm *et al.* 1994; Crocker *et al.* 1998). It binds specifically to the gangliosides GT1b and GD1a. The sialic acid binding site for MAG has been mapped to Arg118 in the first Ig-domain (Tang *et al.* 1997). However, it would appear that sialic acid binding alone is not sufficient to effect inhibition by MAG, because a truncated form of the protein, consisting of only MAG Ig-domains 1–3, can bind neurons in a sialic acid-dependent manner but does not inhibit neurite outgrowth. This suggests that the inhibitory domain is carried by, or at least requires the presence of, MAG Ig-domains 4 and 5 (Tang *et al.* 1997). In the PNS, MAG represents about 0.1% of the total myelin protein, and is found in both the inner and outer loops of the sheath, as well as in regions of uncompacted myelin at the nodes of Ranvier. In the CNS, MAG is more than 10-fold more abundant, and is found only at the inner loop and in the uncompacted loops at the nodes (Trapp 1988, 1990).

Oligodendrocyte myelin glycoprotein

Like MAG, OMgp was first described many years before it was shown to be inhibitory (Mikol & Stefansson 1988; Kottis *et al.* 2002; Wang *et al.* 2002*b*). It is a glycosyl phosphatidyl inositol (GPI)-linked protein and carries a conserved leucine-rich repeat (LRR) and a C-terminal LRR. It is a relatively minor component in both CNS and PNS myelin, and may indeed be expressed in more abundance by other non-myelinating cells (Huang *et al.* 2005). Recently, OMgp expression was described in cells that have processes that extend to the node of Ranvier. There is evidence to suggest that these OMgp-containing processes prevent aberrant sprouting from the node in the intact nervous system (Huang *et al.* 2005). OMgp is believed to be localized in myelin to uncompacted regions.

Development guidance cues as inhibitors of regeneration in the adult

Recently, the inhibitory guidance cue, ephrin-B3 has been shown to be expressed by adult oligodendrocytes and to associate with myelin when extracted. There is a modest improvement in neurite growth on myelin when ephrin-B3 is blocked (Benson *et al.* 2005). Two other repulsive guidance cues of the semaphorin family, Sema5A and Sema4D, have been shown to be expressed by mature oligodendrocytes *in vivo* (Moreau-Fauvarque *et al.* 2003; Goldberg *et al.* 2004). The contribution these inhibitors make to inhibition by myelin has not yet been reported. Netrin is another guidance cue that is expressed by mature oligodendrocytes (Manitt *et al.* 2001). Netrin is bifunctional and can function as either an attractive or a repulsive cue to axon growth (Colamarino & Tessier-Lavigne 1995). It is not known if netrin contributes to the effect of myelin on axon growth, either positively or negatively.

(ii) *The receptors*

The first receptor for any of the myelin-associated inhibitors to be described was for Nogo-66. It was identified as a GPI-linked protein and was termed Nogo-66 receptor (NgR). It is predicted to contain eight LRRs and a flanking LRR that is rich in cysteines (Fournier *et al.* 2001). The second inhibitory domain on Nogo-A, Amino-Nogo, does not interact with this receptor. Surprisingly, however, both MAG and OMgp were subsequently shown to interact with NgR to exert their inhibitory action (Domeniconi *et al.* 2002; Kottis *et al.* 2002; Liu *et al.* 2002; Wang *et al.* 2002*b*). This was unexpected because these three proteins bear no obvious sequence similarity or even domain similarity. However, when the crystal structure of NgR was solved it was apparent that there were a number of 'binding platforms' that were predicted to be able to bind a diverse group of ligands (Barton *et al.* 2003). There are two conflicting reports of regeneration in the NgR-deficit mouse—one report finds extensive regeneration and the other none (Kim *et al.* 2004; Zheng *et al.* 2005). It is possible that other receptors exist for Nogo-66, MAG and OMgp. Indeed, two other NgR isoforms have been described, one of which has been shown to specifically bind MAG (Barton *et al.* 2003; Venkatesh *et al.* 2005). The binding partner for Amino-Nogo has yet to be described.

Because NgR is a GPI-linked protein, it cannot transduce the inhibitory signal across the membrane; a transducing partner was needed in the receptor complex. Revelation of the transducing component in the receptor complex as the well-known neurotrophin receptor p75, was also a surprise (Wang *et al.* 2002*a*; Wong *et al.* 2002). However, even before NgR was identified, p75 was implicated in transducing the signal for MAG, through MAG's ability to bind specific gangliosides and so cluster and activate p75 (Yamashita *et al.* 2002). Although it has been shown that the ganglioside binding capability of MAG is not necessary for it to inhibit neurite outgrowth (Tang *et al.* 1997), it has been reported that if GT1b and GD1a are absent or blocked, MAG does not inhibit neurite outgrowth (Vinson *et al.* 2001; Vyas *et al.* 2002). This apparent discrepancy in findings may arise from differences in how the neurite outgrowth assay is conducted and how MAG behaves when expressed by live cells compared to when immobilized as a substrate. It is of note, however, that when gangliosides are clustered with antibodies, in the absence of MAG, inhibition occurs (Vinson *et al.* 2001; Yamashita *et al.* 2002). This demonstrates that gangliosides can activate the p75 pathway that signals inhibition. The question remains as to whether this ever occurs *in vivo*. It is, however, highly likely that gangliosides can augment the signalling of inhibitory molecules, regardless of whether or not they are necessary for the effect.

Although p75 was clearly a member of this receptor complex and could indeed transduce the signal, it is not expressed by all types of neurons; yet neurons that express no p75 are still inhibited by myelin. It was subsequently found that another member of the tumour necrosis factor receptor family, of which p75 is a member, was able to substitute for p75 in the receptor complex and transduce the inhibitory signal; this protein was a known protein termed TROY or TAJ (Park *et al.* 2005; Shao *et al.* 2005). Finally, a third component of the complex was identified as a protein called LINGO, which is believed to be involved in bringing ligand-bound NgR and p75 or TROY together (Mi *et al.* 2004).

The receptors for the axonal guidance molecules that are believed to contribute to inhibition of axonal growth in the adult CNS are the same as those that inhibit growth during development.

(iii) *Signalling by myelin-associated inhibitors*

The final changes that occur in response to a signal, regardless of whether that signal triggers axonal growth or inhibition of growth, are changes in the arrangement of the cytoskeleton. The ultimate goal is to characterize all the signal transduction steps arising from receptor–ligand interaction that culminate in a direct effect on the cytoskeleton. It therefore seems reasonable to suggest that eventually all inhibitors of regeneration must at some point in the signalling pathway converge, such that they exert the same changes on the dynamics of the cytoskeleton. One such convergence point is probably the activation of the small GTPase, Rho, as all the inhibitors described above, regardless of whether they are myelin-associated or guidance cues, and regardless of what receptor complex they initially activate, have been reported to activate Rho (Liu & Strittmatter 2001; Schmucker & Zipursky 2001; Niederost *et al*. 2002; Yamashita *et al*. 2002; Borisoff *et al*. 2003; Fournier *et al*. 2003; Monnier *et al*. 2003). Activated Rho changes the dynamics of the actin cytoskeleton by activating the Rho kinase, ROCK (Hall 1998). What have yet to be completely characterized are the steps before and after Rho/ROCK activation. Of the myelin-associated inhibitors, other than its ability to activate Rho, little else is known regarding the signalling initiated by Amino-Nogo. Considerably, more is known regarding signalling through the NgR receptor complex by the other myelin-associated inhibitors.

Recently, it has been shown that upon MAG binding to the NgR complex, p75 undergoes regulated intramembrane proteolysis (RIP; Domeniconi *et al*. 2005). It is believed that RIP begins when MAG binds by, first, the extracellular domain of p75 being released by cleavage close to the membrane by an α-secretase. This is necessary for the second step to occur, which is a protein kinase C (PKC)-dependent, γ-secretase cleavage within the membrane, RIP. This second cleavage in turn releases the C-terminus of p75 into the cytoplasm, which is necessary both to activate Rho and to effect inhibition of neurite outgrowth. It is not known if this p75 fragment then enters the nucleus to exert its effect, as do the cytoplasmic fragments of other proteins that have undergone RIP, such as Notch (De Strooper *et al*. 1999). Alternatively, the fragment could have a direct effect in the cytoplasm by altering the cytoskeleton. If any one of the players in the RIP of p75—α-secretase, PKC or γ-secretase—is blocked, inhibition by myelin is blocked. In addition, it is of note that if the cytoplasmic fragment of p75 is expressed in neurons, even in the absence of myelin-associated inhibitors, Rho is activated and neurite outgrowth is inhibited (Yamashita & Tohyama 2003; Domeniconi *et al*. 2005). Interestingly, it has been proposed that p75 in the receptor complex is associated with Rho bound to its inhibitor Rho-GDI. Upon interacting with myelin inhibitors, p75 is proposed to activate Rho by displacing Rho-GDI (Yamashita & Tohyama 2003). What is unclear, however, is whether p75 cleavage directs this proposed displacement.

More recently, a novel *trans*-activation of the epidermal growth factor receptor (EGFR) and its downstream effector, Erk, by myelin-associated inhibitors has been reported (Koprivica *et al*. 2005). If activation of the EGFR pathway is blocked, the myelin-associated inhibitors have no effect, but activation of the pathway by its own ligand, EGF, does not induce inhibition of neurite outgrowth. This demonstrates that although activation of the EGFR by myelin inhibitors is necessary to bring about inhibition of neurite growth, it is not sufficient; the implication is that in addition to the EGFR pathway, activation of the NgR receptor complex must activate a separate pathway, which is also required to effect inhibition. It is of note that CSPGs also *trans*-activate the EGFR pathway, which is also necessary for them to bring about inhibition (Koprivica *et al*. 2005).

(iv) *Overcoming myelin inhibitors to promote regeneration in vivo*

It may appear futile to try and encourage regeneration by blocking only the myelin-associated inhibitors without also blocking those in the glial scar. However, one study, a number of years ago, suggested that immediately after the injury the major impediments to regeneration are myelin-associated inhibitors (Huang *et al*. 1999). In that study, mice were immunized with myelin proteins prior to carrying out a spinal cord lesion. In the myelin-immunized mice, many axons regenerated and some appeared to be 'pinched', suggesting that the scar had indeed formed after the axons had re-grown. Therefore, there may be a window of opportunity, immediately after injury, when blocking myelin inhibitors may be sufficient to promote axon regeneration.

In general, there are two ways in which to overcome inhibitors in the CNS and encourage regeneration: either block the inhibitors or their receptors with antibodies or peptides, or change the neuron from within such that it no longer interprets the signal as inhibitory. Antibodies or peptides corresponding to Nogo and to the NgR have received the most attention in recent years and varying degrees of success have been reported (Bregman *et al*. 1995; Brosamle *et al*. 2000; Fournier *et al*. 2002; GrandPre *et al*. 2002; Li & Strittmatter 2003). However, it is difficult to reconcile these different studies, as success has been reported by one group with antibodies specifically to Amino-Nogo, while others have blocked only Nogo-66 or the NgR receptor. Hence, it is difficult to interpret the results when one study would argue that blocking only Amino-Nogo is sufficient while the other indicates that Nogo-66–NgR is more important. Conflicts are also reflected in the results from mice that are deficient in various combinations of Nogo isoforms or in NgR, as findings range from no improvement in regeneration, to limited regeneration, to considerable regeneration with functional recovery (Kim *et al*. 2003, 2004; Simonen *et al*. 2003; Zheng *et al*. 2003; Willis *et al*. 2005). These issues remain to be resolved. It is noteworthy that, as described above, substantial regeneration was reported

in the Eph4A-deficient mouse, which complicates the situation even further (Goldshmit *et al.* 2004).

Two strategies can also be taken to change the neuron from within: interfere with the cascade that signals inhibition or activate a parallel pathway that will overcome the inhibition signalling pathway. Both approaches have been taken with some success. As mentioned above, all signals from all the inhibitors described to date converge on Rho, an observation supported by the fact that there is considerable regeneration and functional recovery in animals treated with the Rho inhibitor C3 (Lehmann *et al.* 1999; Dergham *et al.* 2002; Winton *et al.* 2002). In addition, others have reported that a PKC inhibitor (Sivasankaran *et al.* 2004) or an inhibitor of Erk activation promotes regeneration of lesioned optic nerve (Koprivica *et al.* 2005). It is difficult to compare these strategies and determine which is more potent, as the nerve tracts lesioned are often different as are, in most cases, the types of lesion.

A number of groups have shown that activation of the cAMP signalling pathway in neurons not only blocks inhibition of neurite outgrowth by myelin-associated inhibitors, but also changes growth repulsion to attraction for some guidance cues, as well as CSPGs (Cai *et al.* 1999; Song *et al.* 1998; Chierzi *et al.* 2005). To activate this pathway, cAMP has been elevated in cultured neurons in a variety of ways: with membrane permeable, non-hydrolysable cAMP analogues; by inhibiting phosphodiesterases, the enzymes that degrade cAMP; and by prior exposure of neurons to neurotrophins, such nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Cai *et al.* 1999; Lu *et al.* 2004; Nikulina *et al.* 2004; Pearse *et al.* 2004). It is of note that if the neurons are exposed to the neurotrophin at the same time as the myelin inhibitor, the neurotrophin has no effect and inhibition occurs. It has been established that neurotrophins elevate cAMP by an Erk-dependent inhibition of phosphodiesterase, and signalling by myelin-associated inhibitors, by a mechanism not yet fully understood, blocks this event (Cai *et al.* 1999; Gao *et al.* 2003). However, if cAMP is elevated with neurotrophins and the cAMP pathway triggered before exposure to the inhibitors, the inhibitors have no effect. Regardless of how cAMP is elevated, transcription is triggered by activation of the transcription factor cAMP response element binding protein (CREB) and the ability of cAMP to overcome inhibition by myelin is both CREB- and transcription-dependent (Gao *et al.* 2004). At least one gene is known to be upregulated by cAMP that plays a role in this phenomenon (Cai *et al.* 2002). It is the enzyme arginase I (ArgI), which is key in the synthesis of polyamines, and either overexpression of ArgI in neurons or exogenous polyamines are able to allow growth in the presence of myelin-associated inhibitors in culture. The mechanism whereby polyamines overcome inhibition of regeneration remains to be determined. However, what is of particular importance is that cAMP overcomes inhibition through the initiation of a genetic programme, by affecting transcription in the nucleus. This presents the attractive possibility of treating neuronal cell bodies, which can be more accessible to manipulation, rather than the lesion site *per se* to promote axonal regeneration *in vivo*. Indeed, this has been done successfully with injection of dibutyryl cAMP directly into the dorsal root ganglion cell body, which is sufficient to promote regeneration of spinal dorsal column axons lesioned at the T7/T8 level (Neumann *et al.* 2002; Qiu *et al.* 2002).

3. THE NEURONAL RESPONSE TO THE ENVIRONMENT

As described previously, during development, myelin is not an impediment to axonal growth because it is not present. Myelination is one of the last events to occur in development. In rodents, it begins in the ventral roots and the ventral and lateral tracts of the spinal cord at about post-natal day 2. It then proceeds in a rostrocaudal direction with the first myelin appearing in the brain at post-natal day 10 (Morell 1984; Lazzarini 2003). Consistent with the lack of myelin inhibitors, coupled with the fact that no glial scar forms after injury, spontaneous regeneration occurs in neonatal animals after spinal cord injury. Indeed, it has been shown for some regions of the nervous system that the loss in ability to spontaneously regenerate coincides with the onset of myelination (Ferretti *et al.* 2003). However, surprisingly, even though there are no inhibitors around, if young neurons are removed and grown on myelin, they are not inhibited (Cai *et al.* 2001). Depending on the age and the type of neuron, with development comes a switch in response to myelin and they become strongly inhibited from extending processes. It is not known if there is a physiological reason why young neurons are not inhibited by myelin, despite the fact that they are unlikely to encounter it. Perhaps it is as a safeguard in case growth of some axons is delayed in reaching their targets and so they would then come in contact with myelin. Regardless of whether or not there is a physiological reason for young neurons being able to grow on myelin, it is important to know mechanistically how they can grow in the presence of these inhibitors. Two possibilities are that they do not express the receptors for myelin inhibitors or that they are intrinsically different from older neurons and respond to the inhibitors in a positive manner. The latter seems to be the most likely explanation. We have shown that if young neurons that grow very well on myelin and on individual myelin inhibitors such as MAG are treated with a protein kinase A inhibitor, neurite outgrowth is now inhibited (Cai *et al.* 2001). Similarly, the ability of neonatal spinal axons to spontaneously regenerate is blocked by a PKA inhibitor administered *in vivo*. Consistent with the suggestion that cAMP/PKA signalling plays some role in the developmental switch to myelin inhibitors, endogenous cAMP levels are high in young neurons and the levels fall quite sharply with age at a time when the switch to inhibition occurs. The indication that it is the intrinsic state of the neuron that changes raises two questions: as with artificially elevating cAMP in older neurons described above, what are the downstream effectors of the cAMP/PKA pathway that allow these young neurons to grow in an inhibitory environment and spontaneously regenerate and what triggers the

decrease in cAMP levels with development? For the downstream effectors, ArgI levels are also higher in young neurons than in older neurons, which is consistent with what is upregulated in older neurons when cAMP is elevated, suggesting that the mechanisms are the same. As to the trigger for the decrease in cAMP, it is tempting to speculate that myelination *per se* is the trigger, given that it has been shown that interaction of myelin with the neuron activates a pertussis toxin-sensitive Gi/Go protein, which could bring about a drop in cAMP (Cai *et al*. 1999). However, there are many events that occur during development that could affect the neuronal cAMP levels, such as a decline in neurotrophins and their receptors. The trigger(s) remains to be described.

4. CONCLUSIONS

Is the best approach to achieving successful regeneration in the adult CNS to try and recapitulate development? The answer is not clear. Obviously, it would be a tremendous advance if axons could be encouraged to grow as fast as their young counterparts and also not to be blocked from growing by all the inhibitors described previously. Perhaps inactivation of Rho and elevating cAMP or some of its downstream effectors are steps in the right direction. The timing of treatment then becomes crucial, in that the axons need to re-grow before the glial scar matures and physically locks them in. To overcome this restriction on timing of treatment, perhaps methods could be devised to try and prevent the scar from forming, such as manipulation of the immune response. However, this in itself may present its own set of problems, in that it appears that the main function of the scar is not to stop axons regenerating but to limit damage to healthy tissue by locking in the site of injury and the immune cell invasion (Bush *et al*. 1999; Faulkner *et al*. 2004). An attractive alternative would be to induce in old animals the reactive gliosis that occurs in young astrocytes, a daunting task given the complexity of the reaction. To date the most successful strategy to encourage axons to regenerate, which in some cases achieves functional recovery and attenuation of the glial scar, has been to use a combination of treatments: transplant cells that are permissive for growth at the lesion site, as well as elevate cAMP and neurotrophins (Lu *et al*. 2004; Nikulina *et al*. 2004; Pearse *et al*. 2004). To this combination, inactivation of Rho, inactivation of EGFR and, importantly, digestion of CSPGs with chondroitinase should be added and maybe then many more axons can be encouraged to grow long distances. If this is achieved, the next hurdles will be giving the axon direction such that it reaches its correct target, ensuring it makes a functional synapse and finally that it is remyelinated. This then would be a true recapitulation of development.

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