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Review

Setting the stage for functional repair of spinal cord injuries: a cast of thousands

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Here we review mechanisms and molecules that necessitate protection and oppose axonal growth in the injured spinal cord, representing not only a cast of villains but also a company of therapeutic targets, many of which have yet to be fully exploited. We next discuss recent progress in the fields of bridging, overcoming conduction block and rehabilitation after spinal cord injury (SCI), where several treatments in each category have entered the spotlight, and some are being tested clinically. Finally, studies that combine treatments targeting different aspects of SCI are reviewed. Although experiments applying some treatments in combination have been completed, auditions for each part in the much-sought combination therapy are ongoing, and performers must demonstrate robust anatomical regeneration and/or significant return of function in animal models before being considered for a lead role.
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Introduction

For clinician and scientist alike, the injured spinal cord is a formidable scene: a large cast of endogenous cells and molecules act in concert to prevent or restrict functional connectivity. The players and their roles in limiting spinal cord repair continue to be elucidated, and each discovery unveils the character of a molecular antagonist that hinders return of function after spinal cord injury (SCI). As the villains reveal themselves, our repertoire of possible targets for intervention grows, and potential treatments fall into one or more of five categories: (1) *protection*, to prevent death of neural cells undamaged by the initial injury; (2) *stimulating axonal growth*, either by enhancing the intrinsic regenerative capacity of spinal and supraspinal neurons or by blocking or removing endogenous inhibitors to repair; (3) *bridging*, to provide a permissive substrate for elongating axons and to replace lost tissue; (4) *enhancing axonal transmission*, to alleviate conduction block in spared or regenerated axons and (5) *rehabilitation*, to enhance functional plasticity within surviving circuits and consolidate anatomical repair.

As the cast of villains in SCI is vast and collaborative, so too must be the chorus of heroes that rise to meet them. It is widely acknowledged that a combination of treatments will be required to address the complex issues of SCI;[1] however, designing such a treatment strategy is incredibly daunting. The first step is to identify the most powerful and/or versatile performers to fill the roles of protection, stimulating growth, bridging, conduction and rehabilitation: each treatment must audition in isolation before it can take the stage as part of a combination therapy. The second step is equally ambitious: once the protagonists of SCI are identified, they must appear *in the appropriate spatial and temporal combination*. Like a performance, the development of a reliable therapy for SCI must be directed with intimate knowledge of each character, leading to the perfect balance among the players.

Neuroprotection in the setting of SCI

Although traumatic SCI is often the result of a single mechanical insult, the functional outcome is determined by a cascade of behind-the-scenes events described collectively as 'secondary injury'. Displacement or penetration of the spinal cord both inflicts direct tissue damage (ie primary injury) and initiates destructive processes that expand the injury site. This phenomenon of secondary injury, which extends for days after the initial trauma, presents the first opportunity for intervention after SCI. Events that fall under the

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umbrella of secondary injury include vascular changes (edema, ischemia, and hypoxia), excitotoxic biochemical events (formation of free radicals and nitric oxide, protease release), and cellular responses (invasion by immune cells, activation of glial cells, and death of neurons and glia) (reviewed in Hausmann[2]). How significantly each component of secondary injury contributes to loss of function remains unclear: what is evident is that the resulting pathology might be ameliorated by eliminating one or more of these players in the early stages of SCI.

Clinical trials of neuroprotective agents, delivered acutely following SCI, have been carried out with equivocal success. The two most widely discussed are the National Acute Spinal Cord Injury Study (NASCIS) trial of high-dose systemic methylprednisolone (MP)[3] and the Sygen® trial of GM-1 gangliosides.[4] GM-1 gangliosides appeared to enhance the rate of recovery from SCI, but did not significantly improve the ultimate functional outcome, whereas MP was reported to improve functional outcome when administered within 8 h of SCI.[5] Although MP has since been widely used as a neuroprotective therapy, several more recent studies have reported equivocal benefits and concerns about the potential toxicity of the required high doses and observed medical complications (reviewed by Kwon *et al*[6]). Administration of this steroid is no longer a standard treatment, nor even a guideline for treatment, of acute SCI in Canada[7] and many other countries have adopted similar policies.

In the midst of controversy surrounding the use of currently available pharmacologic interventions, the search for an effective neuroprotective agent is ongoing in animal models of SCI. Neuroprotective strategies have been reviewed at length previously.[8,9] Here we focus on recent work from our group and elsewhere dealing with some of the neuronal consequences of secondary injury, including axonal retraction/dieback and neuronal atrophy/death.

One potential mechanism for secondary neuronal injury which is undergoing thorough investigation in a number of laboratories involves endogenous (microglial) or invading (hematogenous) phagocytic cells. While the potential therapeutic use of previously myelin-activated macrophages has received much attention of late (reviewed in;[10] see 'Myelin and myelin signaling', below), several lines of evidence demonstrate that the microglial/macrophage response to SCI is responsible for demyelination, oligodendrocyte death, and damage to intact axons.[11–14] The nontraumatic injection of the micgroglia/macrophage activator zymosan into the brain and spinal cord results in cavitation, demyelination, and axonal injury.[15,16] The molecules released by mature phagocytes, including superoxide, hydrogen peroxide, hypochlorous acid, as well as neurotoxic chemokines and cytokines may be partially responsible for axonal damage and retraction, and neuronal death.[17]

The second-generation tetracycline derivative, minocycline, has been shown to be an antiinflammatory agent. Following SCI, systemic minocycline administration has also been documented to inhibit microglial activation, to promote oligodendrocyte survival, to reduce lesion-induced cavity formation, and to prevent the retraction or dieback of injured dorsal column, rubrospinal, and corticospinal axons.[18–20] The axonal protection afforded by this treatment may be of interest, since the rats also exhibit improved functional recovery,[18,19,21] although the restricted subtotal nature of the dorsal column lesion would permit neural plasticity within preserved pathways to contribute to recovery. The mechanism by which sensory dorsal column axons dieback following injury may involve macrophage-mediated degeneration of proximal myelin sheaths, since in myelin-deficient rats macrophage/microglial activation with zymosan does not induce axonal retraction.[20] Interestingly, suppression of p75, a putative signaling receptor for myelin inhibition, also reduced axonal retraction/dieback from the lesion site in p75 knockout mice ($p75^{-/-}$).[22] This effect of p75 deletion was evident in pictures of the lesion site, although the authors of the study did not discuss reduced retraction/dieback as a result of this study.

The loss of neurons and glia at the site of SCI is well-documented. However, at more remote distances, the extent of neuronal death following SCI has probably been overestimated. Even 1 year after a cervical SCI, the cell bodies of severed descending rubrospinal axons, which were previously thought to have perished, regained normal neuronal morphology when BDNF was administered in the vicinity of the red nucleus.[23] Rather than being generated *de novo*, these rubrospinal neurons had atrophied to the point where they were easily missed during previous histological screens, leading to an underestimation of their numbers and resulting in the premature declaration of their death. Recently, adeno-associated viral (AAV) vector-mediated BDNF gene transfer into the red nucleus similarly counteracted atrophy of chronically lesioned rubrospinal neurons.[24] It is not known to what extent severe atrophy (rather than neuronal loss) is a general response of CNS neurons to chronic injury, but corticospinal neurons also exhibit atrophy after spinal axotomy.[25] If SCI-induced atrophy is common among CNS neurons, it may mean that rather than attempting to replace neurons through transplantation of embryonic tissue or multipotent cells,[26] therapies directed at reviving these 'hibernating' neurons may be more appropriate. It has been observed that the direct CNS application of neurotrophic factors following SCI can prevent the atrophy of rubrospinal,[27,28] corticospinal,[25,29] and ascending propriospinal neurons,[30] as well as stimulate the expression of axonal growth-associated genes. Many of the same factors can also prevent corticospinal and ascending sensory axonal dieback.[31]

Stimulating axonal growth in the injured spinal cord

At the site of SCI, axons attempting to regrow and/or re-establish functional connections get a chilly recep-

tion, not only because of necrotic and apopotic forms of secondary cell death and the activation of immune cells (discussed in the previous section above), but also because a troupe of inhibitory molecules confront growth cones at and around the site of SCI. These molecules can be broadly classified into two groups: myelin-associated molecules produced by oligodendrocytes, and extracellular matrix (ECM) molecules, many of which are produced by astrocytes in response to injury. Nerve grafting experiments and other manipulations (discussed in the section on Bridging the site of SCI below) indicate that damaged CNS axons can regenerate when their local environment is replaced with a substrate that is less inhibitory (or more permissive). Even so, it has been demonstrated repeatedly that the capacity for axonal regeneration is hindered by an impoverished neuronal growth program being generated by severed neurons. Cajal was perhaps the first to recognize that '…for these [growth cones] to become consolidated and to progress, the action of the trophic center or nerve cell is indispensable from every point of view.'[32] In this section, we will consider both the molecular antagonists to regeneration after SCI, and the protagonistic manipulations that might coax CNS axons into a state of growth in their native environment, through direct targeting of the responsible inhibitory factors within CNS tissue, the axonal responses to those inhibitors, or through enhancing the response of the cell body to injury.

Myelin and myelin signaling: an inhibitory chorus line

In the normal CNS, myelin plays a critical role in solidifying connections established in development. After SCI, myelin-derived molecules take the stage in a new light, as critical inhibitors of axonal growth/regeneration. The role of myelin in suppressing plasticity after SCI was first illustrated in landmark experiments employing the Nogo-blocking antibody (IN-1):[33,34] antagonizing Nogo, the now notorious inhibitory constituent of central myelin, permitted extensive growth of cut corticospinal axons in young rats. In fact, Nogo – now known to exist in three isoforms[35,36] – is but one of a number of myelin-derived inhibitory proteins. Others identified thus far include myelin-associated glycoprotein (MAG)[37,38] and oligodendrocyte myelin glycoprotein (OMgp).[39]

The role of MAG as an impediment to axonal regeneration is exemplified by *in vitro* studies in which its immunodepletion from myelin extracts attenuated inhibitory activity.[37] It has since been shown that elevating cAMP can stimulate adult neurons to extend axons on MAG substrates,[40] an effect related to the growth-enhancing effects of conditioning lesions and neurotrophic factor application (see below). Studies in MAG knockout mice have been equivocal, however, since myelin preparations from these mice are still effective in blocking regeneration, and since severed optic nerve and corticospinal tract (CST) axons regenerate no better in MAG knockouts than in wild-type mice.[41] Recent characterization of plasticity following SCI in three lines of mice lacking one or more Nogo molecules has been similarly perplexing. While corticospinal axon sprouting following spinal hemisection was evident and apparently robust in some mice,[42,43] almost no augmentation of regeneration or sprouting was seen in others.[44] Immunizing mice with MAG-plus-NogoA enhances regeneration and/or sprouting of corticospinal axons following spinal hemisection; however, myelin immunization has a greater overall effect than MAG/NogoA immunization.[45,46] Manipulations of OMgp have yet to be carried out, but one might hypothesize that, like the focused targeting of MAG or Nogo alone, specifically reducing OMgp activity would have less effect on axonal regeneration than targeting multiple myelin-derived inhibitors.

Fortuitously, recent data indicate that this trio of myelin-derived inhibitors – NogoA, MAG and OMgp – signal through a common receptor (the Nogo receptor, NgR).[47–50] As an apparent point of convergence in inhibitory signaling, NgR has taken the stage as a target of strategies aimed at increasing plasticity in the injured spinal cord. A competitive antagonist of NgR, NEP1-40, blocks the inhibitory action of myelin *in vitro*[51] and enhances both growth of supraspinal axons and functional recovery when administered intrathecally or systemically after thoracic hemisection.[51,52] Another antagonist, NgREcto, is a soluble, truncated form of NgR, which has been shown to alleviate inhibition of both Nogo and myelin *in vitro* (Fournier *et al*, 2002). Recent work from our group examines the effects of NgR activity using a different form of soluble NgR *in vivo*;[53] in these experiments, we found that NgR antagonism augmented rhizotomy-induced sprouting of both descending monoaminergic axons and peptidgeric primary afferents in the cervical dorsal horn. The discovery of an anti-NgR monoclonal antibody, capable of inhibiting binding of Nogo, MAG, and OMgp, and of blocking inhibition of myelin *in vitro*, has now been reported.[54]

Downstream of NgR are several other molecules that might also serve as targets for intervention after SCI. The Nogo receptor is thought to transmit myelin-inhibitory signaling through its interaction with the low-affinity neurotrophin receptor p75.[55,56] Recently, LINGO-1, a neural transmembrane protein, has been revealed as an essential component of the functional NgR/p75 complex.[57] Ligand-bound NgR associates with p75 and LINGO-1 to activate the GTPase RhoA, which transduces myelin-derived inhibition to the cytoskeleton.[58–60] Since p75 binds neurotrophins, its contribution to axonal growth is complex (see below), whereas the activation of RhoA leads to the recruitment of Rho kinase and subsequent remodeling of the actin cytoskeleton to inhibit regeneration. Targeting the Rho pathway with the bacterial enzyme C3 transferase or with specific inhibitors of Rho kinase has led to reports of both corticospinal regeneration and functional recovery after rat SCI.[61,62] Interestingly, it seems that astrocyte or meningeal cell-derived ECM molecules may

also signal through the Rho pathway,[63–65] indicating a convergence of multiple inhibitory signaling pathways, as well as suggesting that multiple tiers of inhibition can be overcome by focusing on a single intracellular pathway (for a review, see Kwon *et al*[66]).

It is likely that other myelin-associated inhibitors remain to be elucidated. A broader, approach, then, is to temporarily and focally remove myelin and/or myelin debris from the site of SCI. We have used a complement-fixing antibody to galactocerebroside (GalC), coadministered intraspinally with serum complement, to achieve transient focal demyelination at the site of SCI.[67–69] Within the CNS, GalC is restricted to the cell membrane of oligodendrocytes and myelin; specifically, it is present in the outer layer of the myelin membrane. Binding of the GalC antibody triggers activation of the complement cascade, which results in disruption of myelin membranes and recruitment of endogenous microglia or invading macrophages to phagocytically clear CNS myelin within the injured region. Using this approach, the attack on myelin is volume- and concentration-dependent, can be readily tracked with noninvasive magnetic resonance imaging, ceases when the infusion is stopped, and is reversible, as remyelination occurs when treatment is withdrawn. Myelin disruption with the GalC antibody permits regeneration of brainstem-spinal axons when administered after acute hemisection in the adult rat.[68] More recently, we have found that immunological myelin disruption stimulates both regeneration of supraspinal axons and improved recovery of locomotion after severe thoracic contusion injuries (where controls are permanently paralyzed), even when treatment is delayed for 1 or 2 months after injury (JDS, unpublished observations).

It has been suggested that another method of reducing myelin-derived inhibition at the site of SCI is to transplant autologous macrophages at the lesion site to phagocytically clear myelin debris. In rat, macrophages activated by prior exposure to degenerating peripheral nerve segments stimulated both regeneration and functional recovery when grafted at the site of complete thoracic transection;[70] more recently, macrophages coincubated with skin improved functional outcome when grafted at the site of severe thoracic contusion.[71] Activated autologous macrophages have entered clinical trials as ProCord, a therapy administered by ProNeuron, based in Israel. Although patients have already received ProCord, no published reports of their progress are available. As all patients received ProCord within 14 days of SCI, the effect of stimulated macrophage transplantation on their clinical outcome may prove difficult to interpret: in spite of this potential limitation, a Phase II multicenter trial has been initiated (http://www.proneuron.com/ClinicalStudies/index.html). Despite their foray into clinical testing, macrophage transplantation remains a controversial therapy for SCI, largely because the activation of macrophages after SCI may also have other actions within the CNS, which have yet to be characterized.[72]

Astrocytes and the glial scar: another inhibitory ensemble
An excellent setting in which to study the astroglial contributions to inhibition of regeneration is that of the dorsal root entry zone (DREZ), the point at which sensory axons enter the spinal cord. First, dorsal root injury does not compromise the blood–brain barrier, and spinal cyst cavity formation does not occur; second, astrocytes of the glia limitans form a precisely demarcated border between peripheral and central nervous tissue; and third, astrocytic and degenerative changes (microglial activation and myelin breakdown) are spatially and temporally separated from one another. Astrogliosis begins within days of a dorsal rhizotomy, whereas frank Wallerian degeneration does not begin until 1 week later.[73,74] It is clear that activated astrocytes, whether part of a scar following SCI or as a component of the reactive DREZ following rhizotomy, deter regeneration. Following SCI, axons retract from the lesion site and are surrounded by CNS myelin. They approach, but do not come into contact with the newly formed glia limitans at the innermost margin of the glial scar. This was demonstrated in studies by Silver and colleagues,[75,76] in which eGFP-expressing axons from grafted sensory neurons elongated within the degenerating dorsal columns, but halted within a halo of chondroitin sulfate proteoglycan (CSPG) immunoreactivity surrounding a small, previously generated, wound. Sensory axons injured within the dorsal root also regenerate up to the DREZ. Once there, their growth is arrested by the astrocytes of the glia limitans, against which they often form terminal-like axon structures,[77] or from which they turn back, and are redirected peripherally.[78]

Based on observations such as these, it has been hypothesized that there exists an astrocytic 'physiological stop signal' at the DREZ which is responsible for the axonal arrest at the DREZ.[79] While the nature of the signal(s) remains unknown, a number of ECM proteins, induced by dorsal rhizotomy, are well-situated to prohibit penetration by sensory axons of CNS tissue, including CSPGs, the CSPG NG2 made by oligodendrocyte precursors, as well as Tenascin-C and Tenascin-R.[78] The astrocytic character of the inhibition strongly suggests secreted factors, and these are most likely to be components of the ECM laid down as a result of astrocyte activation. A recent DNA microarray carried out in our group to identify rapidly upregulated (3 days post-rhizotomy) astrocytic mRNAs has revealed a large number of candidate stop signals;[80] here we review recent data on a selection of these molecules.

Chondroitin sulfate proteoglycans A number of CSPGs are upregulated soon after dorsal rhizotomy, and are also candidate inhibitors of axonal regeneration at the site of SCI (reviewed in Morgenstern *et al*[81] and Rhodes and Fawcett[82]). These include neurocan and versican, both of which are made by reactive astrocytes and inhibitory to neurite outgrowth.[83–86] While the growth-inhibitory effects of versican are well established,[87] its

pattern of expression following SCI is controversial,[84,86] and thus its role in the failure of regeneration remains unclear. Neurocan is more consistently upregulated after SCI, and thus this CSPG is a better candidate to mediate scar-related inhibition. While more slowly upregulated (only by 6 days post-rhizotomy), chondroitin synthase is likely to also play an important role in glial scar formation since it is involved in CSPG synthesis.[88] The CSPG NG2 has been implicated in regenerative failure, based on its localization in the CNS part of the DREZ;[78] in fact, its upregulation occurs too slowly (after 1 week) to compellingly implicate it in the rapid conversion of the DREZ from permissive to inhibitory (within 3 days) following rhizotomy.

Inhibitory effects of CSPG accumulation after SCI have been partially alleviated by enzymatic degradation. Chondroitinase ABC (ChABC), a bacterial enzyme that cleaves glycosaminoglycan (GAG) side chains from the protein core to attenuate CSPG inhibitory activity,[89–91] promotes functional recovery when administered acutely following lesions of the cervical dorsal spinal cord in adult rats.[92] Recently, ChABC was applied after thoracic hemisection, at the distal end of a Schwann cell-seeded polyacrylonitrile/polyvinylchloride (PAN/PVC) polymer channel, where it was reported to enhance axonal growth across the caudal graft–host interface.[93] Although proven to increase plasticity in both spinal cord and brain,[94–97] ChABC does not completely remove GAG sidechains, and the 'carbohydrate-stubbed' protein core that persists after ChABC degradation of CSPGs does exert some inhibitory influence on growth of spinal axons.[98] A DNA enzyme against the GAG-chain initiating enzyme, xylosyltransferase-1 (XT-1), may remove GAG chains more completely, and was recently applied to spinal stab lesions in adult rat:[99] in this study, enzymatic treatment diminished GAG chain presence around the lesion and facilitated growth of GFP-positive axons from grafted sensory neurons beyond the lesion site.

Heparan sulfate proteoglycans (HSPGs) Syndecan-1 is a member of the HSPG family identified in our microarray data. Four different syndecan genes have been identified, and all are upregulated with a rapid time course following brain injury.[100] During development, the HSPGs are differentially expressed in lateral and medial astrocytes, with higher expression toward the midline thought to inhibit crossing of developing axonal projections.[101–103] While the more medial astrocytes tend to inhibit axonal outgrowth, their treatment with heparatinase caused them to be more permissive to axonal elongation and rendered their surfaces (as measured with atomic force microscopy) more like the growth-promoting lateral astrocytes.[104] A role for HSPGs in preventing inappropriate axon crossing at the optic chiasm, and in retaining retinal axons in the frog tectum has also been demonstrated.[105]

HSPGs also may act to increase inhibition by promoting astrocyte reactivity through an interaction with fibroblast growth factor (FGF) receptors:[106] HSPGs increase the affinity of FGFs for their receptors. FGFs (particularly FGF-2) increase astrogliosis *in vitro*[107] and *in vivo*,[108] and they also increase the expression of syndecan-1 in oligodendrocytes and astrocytes.[109] To date, few HSPGs have been identified at the DREZ, with the exception of glycipan, which is expressed in the E13-E16 DREZ,[110] but not in the adult.[111] The rapid upregulation of syndecan-1 revealed by the DNA microarray data is suggestive of a role for HSPGs in mediating inhibition at the DREZ and thus probably also in the damaged spinal cord, although HSPGs have not yet been targeted in animals models of SCI.

Keratan sulfate proteoglycans (KSPGs) Lumican is one of the few cloned KSPGs.[112] The induction of lumican in our DNA array data was surprising, given that its expression to date has been confined to cartilage and cornea.[113] It is also noteworthy that lumican has been shown, using the Affymetrix DNA microarray, to be massively upregulated by pyramidotomy.[114] This could mean either that lumican is *de novo* upregulated in reactive astrocytes or other CNS glia after injury, or equally plausibly, that there is sufficient sequence homology between lumican and other yet-to-be-cloned KSPGs to generate a false-positive result. In either case, the potential role of KSPGs in mediating axonal inhibition warrants discussion. Of the KSPGs so far identified in nervous tissue, claustrin,[113] and ABAKAN[115] are the best candidates for inhibiting axonal growth between CNS compartments. Claustrin was originally identified as a midline barrier molecule,[116,117] and the pattern of ABAKAN expression (separating thalamic nuclei, barrel fields and in astrocytes surrounding cortical stab lesions) is highly suggestive of a strong role in boundary formation.[115,118,119] A recent report of KSPG induction following SCI has indicated that astrocytes are not robust producers of some KSPGs;[120] however, the 5D4 antibody used has been reported to react with molecules other than claustrin and ABAKAN,[121] and so these remain potential candidates for regeneration failure in the CNS.

ECM modifying proteins A number of molecules involved in ECM synthesis and stabilization are also implicated by microarray data. Lysyl oxidase, while probably not astrocyte expressed, is rapidly upregulated following CNS trauma,[122] and is known to catalyze the cross linking of ECM proteins.[123,124] Osteopontin (secreted phosphoprotein 1) is expressed by neurons, macrophages/microglia, and astrocytes following stroke[125,126] and ventral root avulsion injury.[127] Its role in glial scar formation is suggested by its adhesive nature, as well as by its ability to promote astrocyte migration.[128] Thrombomodulin is upregulated in activated astrocytes and may act to stabilize a glial scar following an initial astrocytic proliferative response

induced by rhizotomy or direct CNS trauma.[129] Two inhibitors of matrix metalloproteinases (MMPs) were also upregulated: tissue inhibitor of matrix metalloproteinase 1 (TIMP1) and alpha-2-macroglobulin (A2M). Both TIMP1 and A2M counteract the ECM-degrading properties of MMPs, leading to ECM reinforcement;[130,131] however, A2M has also been shown to have both astrocyte proliferation and neurite outgrowth-promoting properties.[132]

In contrast to the stabilizing effects of lysyl oxidase, osteopontin, thrombomodulin, and MMP inhibitors, a number of astrocytic proteins were upregulated which are known to have antiscarring properties. These include decorin, which when delivered to the site of a cortical stab wound, inhibits glial scar formation,[133] and SC1, an antiadhesive glycoprotein synthesized and secreted by astrocytes.[134,135] It is likely that both stabilizing and destabilizing processes are operational in the first days following injury as the glial scar takes shape.

The cell body response and neurotrophic factors: upstaging intrinsic inhibition

Perhaps the most convincing studies demonstrating the requirement of an appropriate cell body response for axonal regeneration were carried out by Richardson and Issa,[136] in which a prior lesion of the peripherally projecting axon of a dorsal root ganglion (DRG) neuron enhanced penetration into the CNS of a grafted centrally projecting branch. These experiments have since been repeated.[137,138] Of the various effects of such 'conditioning' lesions, two of the most important appear to be the upregulation of regeneration-associated genes (RAGs) such as GAP-43 and CAP-23 (reviewed in Bulsara *et al*[139]), and the upregulation of cAMP (reviewed in Qiu *et al*[140]). While the expression of GAP-43 does not necessarily imply that a neuron is regenerating,[141] it does correlate well with the regenerative potential of a neuron or neuronal population. Even though GAP-43 is not upregulated following a dorsal rhizotomy or dorsal column crush lesion, a prior peripheral nerve injury both increases GAP-43[142] and improves the sprouting and/or regenerative response to a subsequent central lesion.[137]

Neurotrophic factors are also well-recognized for their ability to induce RAG expression and to promote the elongation of axons. Following a dorsal rhizotomy, neurotrophin-3 (NT-3) application promotes functional centripetal regeneration as well as GAP-43 upregulation.[74,143,144] Following dorsolateral funiculus lesions of the spinal cord, brain-derived neurotrophic factor application at the level of the red nucleus increases GAP-43 expression and improves regeneration of rubrospinal axons into a peripheral nerve graft.[27]

Like RAG expression, cAMP also appears to play an important role in regenerative potential. Cai *et al*[40] demonstrated that if sensory or cerebellar neurons were pretreated with neurotrophic factors, there was a resulting increase in intracellular cAMP, which allowed axons to elongate on inhibitory substrates. Since then, the same group has shown that by augmenting intracellular cAMP in DRG neurons *in vivo*, central sprouting/regeneration is enhanced following dorsal column lesions.[145] Intriguingly, the downstream effectors appear to be polyamines synthesized in part by Arginase 1.[140] Both Arginase 1 and cAMP levels are high in young (neonatal) DRG neurons, correlating well with their ability to elongate in the presence of MAG.[146,147] Rolipram, a compound previously tested in clinical trials for treatment of depression,[148] is a phosphodiesterase inhibitor, and thus causes cAMP to accumulate by preventing its enzymatic degradation. Rolipram has recently been administered systemically after SCI in rats, with encouraging results;[149,150] the central role of Rolipram in recent reports of combination approaches stimulating regeneration and functional recovery are discussed below (see 'Casting the combination').

Most studies examining the growth-enhancing effects of neurotrophic factors applied to the site of SCI have tested neurotrophins administered in conjunction with other manipulations. For example, nerve growth factor (NGF) can enhance axonal growth through fetal spinal cord transplants or peripheral nerve grafts.[151–153] Additionally, BDNF and NT-3 enhanced growth of supraspinal and propriospinal axons into Schwann cell-seeded guidance channels *in vivo*,[154] and boosted growth of descending axons into fetal transplants.[155] Recently, NT-3 was reported to promote regeneration of dorsal column sensory axons into and beyond a graft of bone marrow stromal cells, but only when these neurons were preconditioned by cAMP injection into the DRG.[156] Fewer studies have shown that treatment with neurotrophic factors on their own can induce axonal regeneration within the damaged spinal cord; however, intrathecally delivered NT-3 can induce regeneration of injured dorsal column axons.[157] Neurotrophins have also been delivered to the site of SCI via genetically modified cells, which could conceivably play dual roles as both suppliers of trophic molecules and substrates for axonal growth. Fibroblasts, SCs, neural multipotent cells and olfactory ensheathing cells (OECs) have been modified to express various neurotrophins by *ex vivo* gene transfer; results of these important experiments have been reviewed recently.[158]

Since DRG neurons have been the best-characterized population in terms of responsiveness to neurotrophic factors, we will use them as a primary example. Significant inroads have been made into promoting reentry of sensory axons into the adult spinal cord following dorsal root injury with these molecules (reviewed in Ramer *et al*[159]). Intrathecal NGF promoted the ingrowth of small-calibre axons that express CGRP, but not large-calibre axons that express NF200. By contrast, NT-3 promoted ingrowth of NF200-expressing axons, but not axons that express the $P2X_3$ receptor (ie glial cell line-derived neurotrophic factor (GDNF)-sensitive axons).[160] GDNF promoted the regeneration of large- and small-caliber axons, in line with the expression patterns of the requisite receptors.[161,162]

Encouragingly, regrowth was accompanied by synaptic reconnection, again in a selective fashion, such that NGF and GDNF treatments resulted in reactivation of dorsal horn neurons by slowly conducting (smaller diameter) sensory axons, and NT-3 and GDNF treatments facilitated the recovery of postsynaptic potentials that are evoked by rapidly conducting (larger diameter) sensory fibers. NGF- and GDNF-treated animals regained the ability to sense pain, indicating that appropriate spinal circuits were being activated by sensory input. NT-3 promoted the appropriate recovery of proprioception,[144] in-line with the distribution of trkC on proprioceptive sensory neurons. While much remains unanswered regarding the responsible mechanisms, effects other than those directly on neurons (such as nonspecific effects on glial cells) can largely be excluded as fundamental due to the specificity of the regenerative response and functional target reconnection.

Despite the robust regeneration obtained using neurotrophic factors in animal models, the use of neurotrophic molecules as potential therapeutic agents is not without some pitfalls. The most obvious example here is NGF, which while promoting the robust regeneration of small-diameter peptidergic afferents into the spinal cord following rhizotomy also produces severe hyperalgesia.[163] One of the apparent issues is that NGF-treated axons far overshoot their targets[164,165] to terminate deep in the dorsal horn. Tang *et al*[166] have taken advantage of a developmental mechanism of axon repulsion in the spinal grey matter by transfecting ventral cells with semaphorin 3A. This acts to prevent nociceptive axons from penetrating too deeply into the cord, and to partially inhibit the development of abnormal pain.[166] Direct spinal cord trauma leads to the NGF-mediated sprouting of CGRP-positive fibers, which may be partly responsible for the development of autonomic dysreflexia (AD), a severe and life-threatening condition characterized by large and inappropriate elevations in blood pressure:[167,168] in this situation, sequestering NGF improves outcomes in rats and mice. By refining our ability to manipulate guidance cues, it may eventually be possible to extract from NGF its desired effects. For now, however, it remains an impractical member of the neurotrophin family for clinical administration after SCI.

p75: a wolf in sheep's clothing or a sheep in wolf's clothing?

Despite the well-characterized interactions of trks with p75 in neurotrophin signaling[169] it remains unclear how p75 contributes to (or subtracts from) neurite outgrowth. *In vitro* studies using ciliary neurons have indicated that p75 activation by neurotrophins can increase trk-mediated neurite outgrowth: it has been suggested that by constitutively activating RhoA (a small GTPase involved in the control of actin filament assembly and focal adhesions), p75 impedes neurite elongation in the absence of neurotrophin. With the addition of ligand (such as NGF), RhoA activation is inhibited, promoting growth cone motility and neurite outgrowth.[58] On the other hand, several studies have pointed to a negative role of p75 in neurotrophin signaling and neurite outgrowth: activating p75 in PC-12 cells with BDNF inhibited trkA activation through the ceramide pathway;[170] in sensory neuronal compartmented cultures, binding of BDNF or a p75-specific antibody inhibited NGF-mediated trkA phosphorylation and slowed neurite outgrowth;[171] and *in vitro* and *in vivo*, BDNF-mediated p75 activation inhibited sympathetic neurite outgrowth and target innervation.[172] Additionally, in $p75^{-/-}$ mice *in vivo*, some brain structures are normally hyperinnervated,[173] and regeneration following peripheral nerve injury is more robust in p75 knockouts than in wild-type mice.[174,175] *In vivo*, $p75^{-/-}$ sympathetic axons invade injured DRGs (where glial expression of NGF and NT-3 are increased[176]) more profusely than wild-type axons following a spinal nerve lesion.[177] Other startling *in vivo* data argue strongly for a negative contribution of p75 to axonal growth in the CNS: in mice which overexpress NGF in CNS astrocytes, there is a massive developmental invasion of CNS white matter tracts by sympathetic axons, and this invasion was greatly augmented when the NGF overexpressors were crossed with p75 knockout mice.[178]

More recently, it has been suggested that p75 mediates inhibitory signaling of myelin-derived inhibitory proteins (see above).[58–60] Upon binding to the common receptor for these proteins (the Nogo receptor), p75 is activated, leading to RhoA activation and inhibition of actin polymerization at the growth cone. On balance, these findings indicate a clear rationale for blocking p75 function in order to extend neurotrophin-mediated regeneration into or within the CNS following injury. Indeed, our group has recently found that central regeneration following dorsal rhizotomy is enhanced in $p75^{-/-}$ mice, as is intraspinal sprouting of descending monoaminergic (serotonergic, noradrenergic, dopaminergic) fibers.[179] A recent publication showed that p75 antagonism did not promote the regeneration of injured dorsal column ascending (sensory) axons, but it noticeably prevented axonal retraction from the lesion site.[22] It is interesting to note that ascending dorsal column axons also appear to be refractory to anti-NogoA treatment,[180] indicating that the selection of a neuronal population for the study of regeneration is of critical importance in the development of potential therapies. It also argues for the investigation of multiple CNS pathways and at least the confirmation of any preclinical finding in another population, especially if the intervention is conceived as having a potential global therapeutic benefit for stimulating functional regeneration in patients.

A role for spared axons in functional recovery

Most SCIs are incomplete, and repair strategies should include the optimization of spared systems. In the absence of regenerative therapy, rodents exhibit func-

tional recovery of complex motor tasks after SCI, and detailed analysis is often required to reveal deficits.[181,182] Axonal sprouting was inferred by Liu and Chambers,[183] and has since been demonstrated repeatedly.[114,184–189] This response of undamaged axons has been credited not only with producing motor improvements (reviewed in Muir and Steeves[190] Dunlop and Steeves,[191] and 'Rehearsing roles', below), but also with the instigation of chronic pain and AD.[192]

The ability of intact CNS axons to sprout remains largely unknown, but appears to depend on phenotype and the degree of cord trauma. NGF-sensitive axons sprout more than GDNF-sensitive axons following partial cord deafferentation.[193] Intact descending systems (serotonergic and noradrenergic) respond differently to dorsal rhizotomy.[194] Intrinsic dorsal spinal cord neurons will sprout into dorsal roots, but only when there has been (at least) some mild direct trauma to the spinal cord, as occurs following dorsal root implantation or insertion of a fine needle into the cord, but not after dorsal root injury alone.[195,196] That mild trauma to the cord promotes sprouting of intact axons suggests the involvement of neurotrophic factors and cytokines produced as a result of inflammation. There have been a limited number of demonstrations of neurotrophic factor-promoted sprouting of intact spinal axons. While NGF has been shown to promote intraspinal sprouting of nociceptive primary afferents,[164,165] the usefulness of NGF in SCI is severely limited by its painful side effects (discussed above).[163] Exogenous NT-3 promotes sprouting of intact CST axons developmentally[197] and following rubrospinal tract ablation in adults.[184] Like neurotrophic factors, neutralizing inhibitory myelin and ECM molecules can promote sprouting of some intact systems: blocking Nogo-A promotes sprouting of adult CST axons,[198] and digestion of glial scar-associated CSPGs promotes behavioral recovery following dorsal column injury in the absence of robust regeneration of injured axons.[92]

Bridging the site of SCI

Neuroprotective treatments might soon increase the continuity across the lesion site (see 'Neuroprotection in the setting of SCI' above), but this advancement may not eliminate the need for bridging transplants. In addition to providing a permissive substrate for axonal elongation, the ideal bridge may confer continued growth support, remodel the lesion site to allow axons to pass through, lend protection from inhibitory molecules, and/or even stimulate remyelination. Here, we review recent data on axonal growth and behavioural recovery stimulated by cellular bridges tested in rats and humans. The use of cellular transplants to deliver neurotrophic factors (see 'Stimulating axonal growth in the injured spinal cord'), to remove inhibitory debris at the lesion site (see 'Stimulating axonal growth in the injured spinal cord'), or to stimulate remyelination (see 'Overcoming conduction block') is discussed elsewhere.

Classical experiments established that injured spinal axons can grow into a graft of peripheral nerve after complete transection of the spinal cord:[32,199] these seminal works initiated the search for the most suitable biological bridging agent. Peripheral nerves are still used experimentally to examine axonal growth in response to other manipulations, and can support some return of function after complete thoracic transection (in rat) with concomitant administration of growth factors.[200–202] Peripheral nerves have been implanted at the site of SCI in patients in Taiwan, China, Peru, and Brazil and a report of such surgery recently became available.[203] In this case, a patient with chronic paraplegia resulting from thoracic SCI was treated 4 years following injury with autologous sural nerve grafts and acidic FGF. In the 2 years following surgery, the patient exhibited motor and sensory improvements, indicated by the American Spinal Injury Association (ASIA) rating score (which changed from ASIA C to ASIA D) and pin-prick tests. However, data from a small study involving eight patients with functionally complete SCI who received similar treatment, presented at the International Clinical Trials Workshop on SCI earlier this year, was less encouraging.[204] Autografts of peripheral nerve have also been used to bypass the lesion site: sural nerves grafted between ventral spinal cord above a thoracic lesion and ventral roots below restored some voluntary movement in one person with chronic SCI.[205] Today, nerve bridges have largely given way to the development of cellular transplants for a number of reasons: single cells can be better-defined than the assortment of cells present in a whole nerve, and can be characterized *in vitro*; cells can be injected as a suspension, to fill all aspects of a cyst cavity at a lesion site, whereas implantation of a nerve segment may require resection; and finally, cells can be genetically modified to produce and release factors at the lesion site (see 'Stimulating axonal growth in the injured spinal cord' above).

In the spotlight: Schwann cells and OECs

Schwann cells (SCs) were the first to be cast as bridging agents for SCI. The rationale for injecting SCs into the injured spinal cord is clear, as SCs play a critical role in establishing the permissive nature for axonal regrowth within the peripheral nerve environment: when mitosis of host SCs is inhibited after sciatic nerve injury, axonal growth into acellular autographs is slowed.[206,207] After peripheral nerve injury, SCs act as ushers for growing axons by proliferating, decreasing their expression of various myelin proteins, and increasing the expression of neurotrophic factors and cell adhesion molecules.[208,209] Finally, Schwann cells can be obtained from adult peripheral nerve and rapidly expanded for autologous transplantation: human SCs have been successfully isolated, examined *in vitro*, and implanted into the injured rat spinal cord.[210–212]

Schwann cell transplantation at the site of SCI in the adult rat enjoys a prolific history and has been reviewed previously.[213] Many experiments have tested a SC-filled

PAN/PVC polymer channel, grafted at the site of acute thoracic transection in the adult rat.[154,211,212,214–219] Without genetic modification or addition of neurotrophic factors, SC-filled grafts fuse with the cut stumps of the spinal cord, support ingrowth of propriospinal, sensory, and brainstem-spinal axons, and may underlie modest recovery of function, such as rhythmic stepping.[213] However, supraspinal axons that enter SC bridges typically do not exit caudally to re-enter the spinal cord. The inability of descending axons to re-enter host tissue after complete transection may result from myelin inihibition and/or an accumulation of CSPGs at the distal graft-host interface,[220] an obstacle which might be overcome with concomitant application of anti-inhibitory compounds and/or neurotrophic factors[93] (see 'Stimulating axonal growth in the injured spinal cord' above).

In the early 1990s, another candidate for cellular bridging took the stage. OECs are glia that support growth of olfactory neurons – a routinely replenished population of neurons – from PNS-to-CNS throughout adult life. The rationale for implanting OECs into the injured spinal cord is less obvious, since when the axons of olfactory neurons are injured, they do not regrow their axons like peripheral nerves, but rather rapidly degenerate and die.[221–223] New neurons reconstitute the injured peripheral olfactory system,[224] but do not restore original connectivity, and this is reinforced clinically: permanent anosmia or dysosmia are relatively common after blunt head injury, when movement of the brain relative to the skull shears olfactory axons at the cribriform plate.[225] The rationale for using OECs, then, is not that they support axonal regeneration *in situ*, but that they exist in a unique capacity to permit axonal growth across a PNS–CNS interface in the adult.

Since olfactory axons grow across a PNS–CNS interface, OECs (from the rat olfactory bulb) were first injected into the rat spinal cord after dorsal root injury, to examine their ability to bridge the DREZ.[226] In this study, afferent ingrowth was reported to extend into the dorsal horn, in the areas of appropriate targets, and subsequent electrophysiological experiments suggested that these axons achieved functional reconnection.[227–229] However, recent data from our group and others indicate that OEC transplantation does not support regeneration of sensory axons across the injured DREZ.[230–232] Using OECs from the GFP mouse, we have observed that OEC injection into the spinal cord mechanically disrupts the DREZ, permitting sensory axon ingrowth but not functional regeneration involving the re-acquisition of appropriate targets on the other side of the injured DREZ.[232]

After SCI, OECs were also applied to remodel a PNS–CNS interface, namely, that established by grafting Schwann cells into CNS tissue. When OECs were injected into the proximal and distal stumps of transected spinal cord spanning a SC bridge, regenerating axons crossed the graft–host interfaces and re-entered the spinal cord.[233] Other experiments indicated that OECs alone were sufficient to support both axonal growth and functional recovery.[234–238] These data have generated sufficient enthusiasm in the SCI community to instigate several clinical trials testing OECs worldwide. The first report of a large trial in China was recently published.[239] Although OEC transplantation was reported to generally improve both sensory and motor function, clinical follow-up was limited, confounding interpretation of these results. As well, OECs in this trial were harvested and expanded from the fetal olfactory bulb, an ethically problematic cell source. In Lisbon, Carlos Lima has grafted pieces of autologous olfactory mucosa at the site of SCI in 20 patients, without intervening *in vitro* manipulation or isolation of OECs; some return of both sensory and motor function has been reported after these surgeries.[204] Recently, OECs have been successfully isolated and expanded from the adult human olfactory mucosa, accessible by nasal biopsy, potentially permitting autologous transplantation:[240] these cells have entered a small Phase I trial in Brisbane, Australia.

As OECs are being clinically tested, work in animal models continues, both to identify potential mechanisms of OEC-mediated recovery and to optimize methods of OEC delivery. Recent data suggest that the mechanism of functional return may be more complex than regrowth of lesioned axons. In fact, OEC transplantation protects spinal tissue from secondary damage and prevents cavitation,[241–246] enhances vascularization of the lesion site[235,246] and promotes branching of neighboring axons spared by the primary injury,[247] all of which might subserve improved functional outcome. While earlier transplant experiments sought to isolate OECs without other cellular components of the olfactory nerve, recent work suggests that the recovery of function is enhanced by including other cell types, such as olfactory nerve fibroblasts (ONFs), in the graft.[248] Grafts of OECs and ONFs were recently shown to reconstitute ipsilateral breathing rhythm in rats with cervical hemisections,[249] although functional recovery after spinal hemisection must be interpreted with caution, as it has been well-documented that both hemisected people and animals experience substantial and spontaneous recovery via preserved (undamaged) projections. Finally, the timing of OEC transplantation may be important. Recent data indicates that OEC transplantation has functional benefits even when delayed (for 1 week to 2 months after injury in the rat)[250,251] and one study suggests that delayed transplantation is actually more beneficial than acute delivery.[244] To address some of these important issues, and to more confidently predict the effects of OEC transplantation in humans, OEC transplantation is being investigated in non-human primates with complete spinal cord transection (A. Ramon-Cueto, pers. comm.).

SCs and OECs: a duel or a duet?

Despite the relatively rapid progression from animal models to clinical trials, OECs have not clearly out-

performed SCs as cellular bridging agents. The two cell types are comparable in some respects, but not in others.[252] Like SCs, OECs express adhesion molecules and neurotrophic factors and can support growth by many types of neurons.[152] Unlike SCs, OECs do not proliferate or migrate in response to injury of their associated axons.[253] Both types of cells have been genetically modified prior to transplantation to deliver growth factors at the lesion site.[245,254,255] OECs have been reported to integrate more successfully with astrocytes,[256] and to induce less glial scarring[257] than SCs, but may themselves express inhibitory molecules (such as Nogo or proteoglycans, discussed in 'Stimulating axonal growth in the injured spinal cord' above) after transplantation.[258] Few experiments have directly compared SC- and OEC-transplantation in the same injury. When SCs and OECs were injected (separately and together) into acute thoracic contusion injuries in rat, both cells induced tissue sparing and angiogenesis at the lesion; however, functional outcome was only improved in animals that received SCs.[243] SCs were also recently reported to support regeneration of rat retinal ganglion axons, while OECs applied in the same manner did not.[259] In a recent combination study, SCs bridged a thoracic contusion site to support growth and remyelination in rolipram and cAMP-treated animals (see 'Casting the combination', below).[260]

On a final note, OECs and SCs may share the stage: recent work from our group demonstrates that OEC transplantation enhances invasion of host SCs into the injured spinal cord.[246] This finding is especially significant in light of recent data indicating that myelin within OEC grafts is SC-, not OEC-derived[261] (discussed in the subsequent section below). Whether the observed axonal growth and/or functional recovery after SCI can be attributed to the presence of grafted OECs or might also be due to infiltrating host SCs remains to be seen. SC infiltration/penetration of the CNS has been linked to regeneration of spinal[262,263] and vagal afferents.[264]

In the wings: fetal tissue, multipotent/progenitor cells and synthetic implants

Spinal transection in the vertebrate embryo, prior to the onset of myelination, results in functional regeneration and repair.[265–268] Akin to the use of peripheral nerve grafts, grafts of fetal brain and spinal cord have been applied to the site of SCI to provide axons in the adult spinal cord with the permissive environment of the embryo[269–271] (reviewed in Anderson *et al*[272] and Lakatos and Franklin[273]). In addition to acting as a permissive substrate for elongating host axons, grafted embryonic neurons can both receive synaptic input from and extend axons to host spinal neurons, thereby acting as a relay across the injured spinal cord.[274] Fetal tissue can be used to replace some of the neurons lost in SCI: for example, pieces of ventral embryonic spinal cord can give rise to neurons that reinnervate muscular targets and reverse muscle atrophy in rat.[275] Analagous to the peripheral nerve environment, axons grow readily into grafts of fetal tissue, but emerge distally in a more limited fashion, a limitation that may be overcome by concomitant administration of neurotrophic factors.[155,276] Transplantation of fetal spinal tissue has been tested clinically in the United States in patients with progressive post-traumatic syringomyelia, and preliminary reports suggest that this treatment is safe,[277,278] although complete reports have not been published. Since the use of fetal tissue entails ethical challenges, it may need to be proven superior to other biological bridges in order to assume the role of a preferred clinical bridging intervention.

Recently, various populations of multipotent and progenitor cells have gained increasing attention as potential grafting agents for SCI. Spinal and supraspinal axons grow into grafts of neural multipotent cells, indicating that they do function as a permissive bridge,[279–281] but the possibility that multipotent/progenitor cells might differentiate into spinal neurons to replace lost spinal circuitry is perhaps more intriguing. Both embryonic multipotent cells and neural progenitor cells can differentiate into neurons and extend axons into host tissue,[26,282] raising the possibility that multipotent cell grafts could also function as a relay center within the injured spinal cord. To date, however, neuronal differentiation of multipotent cells grafted at the site of SCI has been limited, and the vast majority of grafted toti- or mulitpotent progenitors become glia.[283,284] Interestingly, functional recovery after thoracic hemisection was improved when neural multipotent cells were delivered in a degradable polymer scaffold, despite the fact that grafted cells did not differentiate into neurons.[285] In this study, improved functional outcome was associated with tissue sparing and reduced glial scarring, suggesting that multipotent cells may exert protective effects. Improved functional recovery from multipotent-cell-treated SCI associated with oligodendrocyte differentiation and remyelination of host axons is discussed as a potential remyelination strategy (in next section).

Multipotent cell-induced recovery in animal models has prompted clinical testing in Russia, where 15 patients received injections of fetal brain and hemapoietic (liver) cells; 11 patients received concomitant grafts of OECs and fetal spinal cord.[286] In this study, six patients that exhibited complete sensory and motor paralysis prior to transplantation regained the ability to walk with or without assistance. However, five patients in this group received transplants within 4 months of SCI, and four received OECs and fetal spinal cord in addition to multipotent cells, so the results of this study are difficult to interpret. While no complications related to multipotent cell transplantation have been reported, the incomplete understanding of multipotent cell regulation, proliferation, and differentiation both *in situ* and upon transplantation may have important implications for imminent clinical translation of this technology.[287,288]

Despite its promise in animal models, cellular transplantation presents several challenges for clinical translation. Timing of transplantation, cell source, and

cellular maintenance/manipulation are important inherent variables that may preclude comparison of results from clinical trials at different centers. Additionally, immunological compounds may be associated with graft rejection. Synthetic implants under development offer the attractive potential of being identical and reproducible in composition, immunologically inert, and even absorbable. If cellular transplantation after SCI does become a clinical reality, synthetic implants may be used to contain/deliver the cells at the injury site and/or to guide axons growing through these biosynthetic grafts.[213] Recently, biomaterials have been designed to deliver neurotrophic factors, and may provide an attractive alternative to the intrathecal pump, as delivery is reproducible and immobilized concentration gradients can be created.[289–291] In two recent experiments, synthetic implants were applied to the site of complete thoracic transection in rat; in these studies, BDNF-filled foam[292] and synthetic hydrogel guidance channels[293] integrated with host tissue and supported axonal ingrowth, although axons did not regenerate beyond the implant. While we want to stress the potential importance of synthetic implants in stimulating regeneration and recovery after SCI, we are constrained by space, and direct the interested reader to reviews of developments in this field.[294–296]

Overcoming conduction block

Although the CNS has the capacity to replace oligodendrocytes lost in spinal trauma, and remyelination of intact demyelinated axons can be spontaneous,[297,298] remyelination in the wake of SCI is not robust, and the resulting conduction block of action potentials due to persistent demyelination likely contributes to loss of function in SCI.[299] To supplement intrinsic CNS remyelination, myelinogenic and progenitor cells have been grafted at the site of SCI in rats, and such cells have been tested in both focal demyelinating lesions (inducing oligodendrocyte death without axonal trauma) and traumatic lesions (resulting in axon damage). Remyelination may differ among intact *versus* regenerating axons; nonetheless, models of focal deymelination have provided important information regarding the extent and mechanisms of spinal remyelination stimulated by cell transplants. Here, we review recent data in this field, and also consider another approach to alleviating conduction block via systemic drug treatment, which is currently being tested in a clinical trial.

Stage left: Schwann cells and OECs

In addition to the fame they have acquired in supporting axonal elongation (see 'Bridging the site of SCI'), SCs and OECs have also been acclaimed for their ability to remyelinate axons after SCI. Endogenous SCs can invade spinal lesions and form functional myelin,[300,301] providing the impetus for transplantation of SCs (and by association, OECs) to alleviate SCI-induced conduction block. Experiments reporting SC- and OEC-provoked remyelination after spinal insult have been reviewed recently;[302–304] therefore, we limit this discussion to recent data that implicates SCs as a source of peripherally derived myelin after both SC- and OEC-transplantation into spinal lesions.

OEC myelination was first reported *in vitro*. When rat OECs were cocultured with rat DRG neurons or olfactory receptor neurons, myelin sheaths formed that were indistinguishable from those formed by SCs under similar conditions.[305,306] In a recent study, however, OECs cultured from adult rats did not retain the ability to myelinate embryonic DRG neurons; instead, myelin in DRG cultures was formed by Schwann cells that persisted in culture despite antimitotic treatment with fluorodeoxyuridine (FUDR).[307] These data, demonstrating the propensity of SCs to myelinate axons even in the presence of OECs, have potentially important implications for interpreting the results of OEC transplantation experiments.

Focal areas of demyelination in the adult spinal cord can be created by injections of ethidium bromide or lysolecithin, both of which are toxic to oligodendrocytes. Typically, the demyelinating lesion is created in tissue that has been X-irradiated several days previously, which prevents host oligodendrocytes from repopulating the lesion site.[308] Using this model, peripheral myelin has been observed in the demyelinated dorsal columns of the adult rat subsequent to transplantation of rat, human, and canine OECs[309–314] and recently, in the dorsal columns of the monkey following porcine OEC transplantation.[315] In these experiments, peripheral myelin was morphologically and/or antigenically indistinguishable from that formed by SCs myelinating previously demyelinated CNS axons.[316,317]

When OECs and SCs were grafted at the same type of contusion injury, more myelinated axons were found within SC-containing grafts than within OEC-only grafts.[243] Importantly, peripherally myelinated axons were also found in nontransplanted lesions, demonstrating that host SCs could access the untreated contusion site, in limited numbers, and produce myelin.[243] Remyelination has also been achieved by stimulating Schwann cell immigration via cellular delivery of GDNF at the site of thoracic transection.[318] Recently, Boyd *et al*[261] used virally labeled OECs to demonstrate that peripheral myelin that formed within the OEC-transplanted site of SCI was derived entirely from invading host SCs and not from grafted OECs. Taken together, these provocative data highlight the role of endogenous SCs in accomplishing remyelination of spinal axons. While it has been reported that SC remyelination of spinal axons is a transient phenomenon, with SC myelin progressively replaced by oligodendrocyte myelin during recovery,[319] the most recent data indicates that SC myelin is stable and persists in the spinal cord.

Stage right: progenitor cells

In response to demyelination of spinal axons, a resident population of endogenous oligodendrocyte precursors proliferate and differentiate into mature

oligodendrocytes to initiate remyelination.[320–322] Progenitor cells have been grafted at the site of SCI to enhance this repair process. While grafts of SCs or OECs trigger formation of peripheral myelin, progenitor cell transplantation aims to reconstitute central myelin at the site of SCI. Neural progenitors from both mouse embryonic multipotent cells and embryonic rat spinal cord have been injected at the site of contusion injury in rat, where both types of grafted cells differentiated into oligodendrocytes (as well as astrocytes and neurons) and enhanced functional recovery,[323,282] although remyelination was not examined in either study. Stem-like cells, derived from bone marrow, have been reported to myelinate spinal axons when introduced at the site of focal demyelination.[324,325] Intriguingly, these cells also stimulated remyelination – and themselves formed myelin – when injected into the bloodstream.[325,326] If such cells can reliably overcome the blood–brain barrier, migrate to demyelinated CNS regions and differentiate appropriately, with their proliferation being controlled, then an intravenous delivery route presents an attractive alternative to direct parenchymal injection into the damaged CNS.

Now showing: treatment with 4-aminopyridine

While cellular transplantation might rebuild stable myelin to permanently restore conductivity, transplantation procedures are invasive and immunologically problematic. Pharmacological treatment, although it must be chronically sustained, may represent a more imminent solution to the problem of conduction block. Fampridine, or 4-aminopyridine (4-AP), is a potassium channel blocker that restores conduction in de- or dysmyelinated axons.[327,328] When 4-AP is added to an *in vitro* preparation of injured spinal cord, conduction across the site of SCI is improved.[329–331] In people with incomplete SCI a sustained-release (oral) formula of 4-AP (Fampridine) was reported to improve sensory and motor function and reduce spasticity during treatment.[332] As a result of these encouraging data, Acorda Therapeutics (New York, USA) initiated two large, multicenter clinical trials testing 4-AP in chronic SCI (8) and published results of these trials may soon become available.[204] Animal experimentation with 4-AP is ongoing, in an effort to clarify the optimal parameters of 4-AP treatment. Although pharmacological intervention is logistically simpler than cell transplantation, issues of effective dose and delivery may be complex; for example, recent data revealed that 4-AP more potently overcomes conduction deficit induced by stretching forces than conduction deficit induced by compression.[333] These experiments underscore the importance of animal models in optimizing treatments for SCI, even and especially after clinical trials are underway.

Rehearsing roles: rehabilitation and CNS plasticity

Even though there is little evidence for spontaneous *de novo* regeneration of damaged CNS axons over substantial distances, there is overwhelming evidence for short- and long-term changes within existing circuits.[334,335] The extent to which such plasticity is accomplished as a result of axonal sprouting or short-distance regeneration *versus* compensatory changes within existing circuits remains to be determined.[190,336] Nevertheless, the available studies point to an innate and remarkable ability for the damaged adult CNS to undergo spontaneous or activity-dependent plastic changes. A choreographed recovery after SCI is dependent on a diverse number of players, contributing their parts in many subtle, but important, ways. Recent research has been able to identify a diverse number of poorly understood mechanisms that can contribute to a remodeling of the CNS after injury. The challenge for rehabilitation after SCI is to ensure that these alterations are beneficial rather than detrimental and maximized to their fullest extent.

For example, damage to the periphery (eg, by digit amputation, retinal lesions) results in latent responses being recorded almost immediately (within minutes) from cortical regions that had previously received input from the damaged regions.[335] In other words, loss of input from the periphery can result in the rapid appearance of expanded receptive fields of the remaining cortical neurons. This phenomenon has been referred to as synapse 'unmasking', a process proposed by Basbaum and Wall to underlie receptive field expansion following partial spinal deafferentation.[337] Later, an anatomical correlate of synapse unmasking was described by Goshgarian *et al*, which occurred within 4 h of SCI, and involved astrocytic process retraction and a subsequent increase in the area of synaptic apposition. The rapidity of the changed response patterns revealed a previously under appreciated feature of CNS organization, namely that each region receives input from a much wider peripheral area than can normally be detected electrophysiologically.[334,335] Furthermore, the data suggest that much of the input to the receptive field must normally be subthreshold for detection or actively suppressed (inhibited) and that damage brings about an almost immediate expansion of the receptive field.

In addition to short-term plastic activity changes, long-term changes also occur and appear to be underpinned by anatomical sprouting or rewiring of synaptic connections (see above[338]) Small binocular retinal lesions initially silence the corresponding portion of the visual cortex. However, within weeks to months, the cortex becomes responsive to inputs from adjacent portions of the uninjured retina. Anatomical tracing studies revealed extensive axonal sprouting from the adjacent cortex, which was interpreted as arising from intrinsic neurons that had formed new stable connections within the denervated region. Likewise, recent reports using trans-synaptic tracing techniques, electrophysiology and behavioural assessments have demonstrated a significant degree of plasticity involving compensatory sprouting from corticospinal projections with concomitant functional rewiring after a dorsal hemisection injury of the adult rat thoracic cord.[339] It is important to recognize that even if regeneration of

injured axons is successful, or if synaptic spaces can become newly occupied by intact axons, the target in both cases is profoundly altered by injury. As a consequence, functional reovery will inevitably require some form of retraining.

Practice makes perfect

Given the innate capacity for the damaged nervous system to undergo plastic changes, the question arises as to whether plasticity within undamaged CNS circuits or regenerating pathways can be harnessed to improve functional outcome. Reports point to the importance of appropriate training to enhance recovery after damage. After sciatic nerve crush in rats, both sensory and motor recovery is improved by placing water bottles at heights such that animals have to extend both hind paws maximally to drink; animals with water bottles on the floor do not show improved recovery.[340] There are, however, offsetting data in the same report suggesting that overactivity or stressful regimes such as forced running or swimming can impede rather than enhance functional recovery.

Generation of movement requires the coordination of a vast network of neuronal subsets, resulting in the coordinated activation of the limbs. There is ample evidence that the intrinsic (or isolated) spinal cord circuitry has the capacity to directly generate many of the complex aspects of locomotion. Complex motor patterns are not just programmed by supraspinal systems such as the cortex, basal ganglia, and cerebellum, with the spinal cord contributing only passively or reflexively by relaying sensory and motor information to the brain or muscles.[341]

Locomotion, whether walking, running, flying, or swimming, is ultimately produced by spinal neurons that are collectively termed *central pattern generators* (CPGs). Normally, CPGs are activated by supraspinal control from the brainstem and thalamus and are also continuously modified by peripheral cutaneous and proprioceptive input. However, it has long been known that the spinal cord can act independently to produce locomotor movements. Indeed, CPGs appear to be a broad vertebrate characteristic because removal of supraspinal input in lampreys, birds, nonprimates, and primates (including humans) by complete or nearly complete spinal cord transection results in the retention of locomotor movements.[342–344] In these instances, locomotor movements can be initiated by a variety of stimuli such as certain postures, electric shocks, or exercise. Given that the spinal cord has the capacity to encode and execute both stepping movements and higher-order functions associated with locomotion, the evidence is now accumulating that these abilities can be harnessed to generate beneficial function after spinal injury.

Training and neural activity to harness spinal cord plasticity

The cat has proved to be an extremely valuable model for assessing the effect of training on the ability of the isolated spinal cord to generate stepping movements after complete spinal cord transections, which were undertaken at low levels, namely T12–13. It has now been firmly established that, with interactive training, adult spinal transected cats can rapidly develop locomotion. Initial studies showed that animals developed stepping patterns on a treadmill if their weight was partially supported and their balance stabilized.[345] Strong pinching of the perianal and abdominal regions was only necessary in the first few days of step training and could be reduced to very light stimulation over the following 3 weeks. All aspects of locomotor behavior improved over the subsequent months, and animals were able to support their body weight, often stepping on the treadmill for up to 10 min before losing their balance. However, without daily step training, performance regressed with animals dragging their hindlimbs, although locomotor performance could be re-established within a few days by step training.

The improved performance compared to spontaneous recovery was attributed to neurons being activated in a more appropriate fashion by training. Recent data obtained using a rodent exercise device indicated that a daily exercise program of moving paralyzed hindlimbs through the motions of walking prevented atrophy of spinal motor neurons.[346] Furthermore, it was suggested that neural activity facilitated the return of function in existing sensorimotor pathways rather than the generation of new pathways. Although some spontaneous recovery was seen when animals were not step trained but were tested briefly on the treadmill, these animals showed only short bouts of weight-bearing stepping and stumbled frequently.[347]

If the isolated spinal cord can learn specific tasks, how long can it remember them? Cats that underwent treadmill step training for 12 weeks still performed maximally on all criteria when tested on the treadmill for up to 6 weeks after cessation of training.[348] However, performance declined considerably if training was withheld for 12 weeks. Nevertheless, retraining after 12 weeks of withdrawal resulted in a more rapid improvement in stepping than that seen during the initial training period. The results suggest that the isolated spinal cord can learn, and it will forget a task without maintained use or practice; however, the isolated spinal cord retains trace memories that can be rapidly recovered upon renewed training.

For the majority of individuals with functionally complete injuries, there is a small band of spared tissue that bridges the gap. Thus, clinically (functionally) complete lesions are not necessarily ones where anatomically the spinal cord has been completely severed. Studies have shown that some locomotor recovery can occur in animals where only a small subset of axons, spanning a lacerating lesion, is spared.[349,350] In addition, after an initial contusion injury that spares some axons around a central cavity and that mimics the type of closed SCI most commonly seen in humans, rats recover some locomotion.[351] The surprise was that if the spinal cord was then completely cut, rats were still able to

maintain some locomotor function, something that rarely happened after spinal cord transection alone. Furthermore, animals recovered a greater range of motor movements and did so more rapidly than after transection alone. The interpretation was that even small percentages of spared axons are capable of 'instructing' the spinal cord below the lesion and that the instructions are retained.

Treadmill training has been used with considerable success in people with SCI, classified as functionally incomplete,[352] that is, with retention of some sensory or motor function below the level of injury (ASIA scale B–D). Persons were selected if they had some voluntary activity in leg muscles, had mobile joints, had no spasticity and if they lacked complications such as ulceration or infection. Treadmill training involved sessions of 30–60 min, 5 days a week for 3 weeks to 5 months, starting with low treadmill speeds. The aim was to encourage movements that mimic natural walking as much as possible and involved providing maximal sensory feedback from the muscles, joints, and skin.

Initially, partial body-weight support was provided by a harness, and the legs were moved and the feet were placed on the treadmill by physiotherapists. Persons were encouraged to put their full body weight onto the extended (say, right) leg during the stand phase and then shift their body weight onto the left leg just before swinging the right leg forward. As performance improved, persons were encouraged to swing their arms in the way they would if walking naturally. Persons were also encouraged to attempt overground walking as soon as possible. The study included comparison with persons who had previously received conventional physiotherapy only. Treadmill training, rather than conventional physiotherapy, resulted in remarkable improvements. Of 44 patients that were wheelchair-bound, over two-thirds learned to walk independently, at least for short distances, with only one person not showing any improvement; about half of these patients were capable of walking up stairs. Seven paraplegic persons classified as complete, that is, no function below the level of injury, were also included in the study and were selected because some aspects of stepping could be evoked by placing them on the treadmill. However, despite daily training, no improvements were seen, and they were not able to generate full stepping cycles. Follow-up studies in persons with incomplete injuries were encouraging and showed that improvements made during a few weeks to months of treadmill training were maintained for 6 months to 6 1/2 years after training ceased.[352,353]

The long-term effects of treadmill training have recently been compared for individuals that are functionally incomplete and complete.[354] After training (15 min daily, 5 days per week for several months), persons with incomplete injuries showed significant improvements and could use their new locomotor capabilities for overground walking for at least short distances. The benefits that had been acquired during training could thus be maintained by independent movement, although there did not appear to be any further improvements. However, all individuals with complete injuries were unable to maintain stepping movements after training stopped; thus, the stepping that had been learned during training could not be translated into overground walking and furthermore appeared to be lost in the absence of continued practice ('use it or lose it').

Rehabilitation rigor

Rehabilitation research often lacks a foundation of experimentally derived data. There have been few multicenter trials including a statistically significant number of participants with double-blind assessments and randomized controls. Rehabilitation has been susceptible to the practice of procedures developed on the basis of personal experiences of the therapist, in part because there are few catastrophic consequences if a therapeutic strategy fails. All too often, it has also been difficult to objectively measure a beneficial outcome or standardize an effective treatment strategy across multiple rehabilitation centers that is independent of the important, but confounding trial variable, 'chemistry' that intrinsically develops within a therapist–client relationship.

However, this is changing rapidly. For example, there is currently a multicenter NIH-sponsored trial, using blinded assessments of people with incomplete SCIs, investigating the benefits of weight-supported treadmill training *versus* more conventional forms of active physical therapy. Other active forms of rehabilitation such as constraint-use therapy and functional electrical stimulation are also undergoing more rigorous evaluations.

In summary, neural activity shapes the developing and adult CNS. If people with SCI can regain some function through the myriad plasticity changes in whatever small number of undamaged pathways persist after injury, then the possibility of combining active rehabilitation therapy with surgical and therapeutic interventions promises realistic opportunities for improved functional recovery after SCI in the near future.

Casting the combination

Several recent studies highlight both the potential success of combination therapy, and the complexity inherent in its development. These studies combine growth-promoting and bridging strategies in three different models of SCI in the adult rat. They are notable not only because the results are encouraging, but because they represent collaborative efforts between experts in different fields, the type of cooperation required to develop a successful combination therapy for SCI.

In one study, sensory neurons were primed for growth in some animals by injecting dibutyryl-cAMP (db-cAMP) into the lumbar DRGs 5 days prior to a cervical lesion of the dorsal columns.[156] Immediately following the injury, all animals received implants of autologous

Table 1 Combination therapies applied to SCI in adults

	Methyl-prednisolone	*Other neuro-protective**	*Myelin antago-nism*	*Demyeli-nation/ myelin clearance*[a]	*Anti-scarfor-mation/ deposition*	*Conditio-ning lesion*	*Neuro-trophic factors*[b]	*cAMP elevation*	*Nerve graft*	*Schwann cells*	*OECs*	*Synthetic bridges/ scaffolds*	*Prenatal tissue*	*Multi-potent cells*	*K^+ channel antago-nism*	*Rehabili-tation*[c]
Methyl-prednisolone		355–359,361–364		365		355	360,366,367			212,218,260	236,260	212,218			368	
Other neuroprotective*	355–359,361–364	369–372			373	355	369		374							
Myelin antagonism							211,375		180	211						
Demyelination/myelin clearance[a]	365									376,377						
Anti-scar formation/ deposition		373							378	93		379				
Conditioning lesion	355	355					380,381		136,380–383							
Neurotrophic factors[b]	360,366,367	369	211,375			380,381		156	23,27,153, 200,201,203, 380,381, 384–391	154,211, 254,392–397	245,255	151,292, 398–403	151,155, 276,400, 404,405	156,280, 406		
cAMP elevation							156		407	150			149	156		
Nerve graft		374	180		378	136,380–383	23,27, 153,200, 201,203, 380,381, 384–391	407								
Schwann cells	212,218,260		211	376,377	93		154,211,254, 392–397	150			243,260	212,214–217, 219,408–414				
OECs	236,260						245,255			243,260						
Synthetic bridges/ scaffolds	212,218				379		151,292, 398–403			212,214–217, 219, 408–414			151,392, 400,415	285		
Prenatal tissue							151,155, 276,400, 404,405	149				151,392, 400,415				416,417
Multipotent cells							156,280,406	156				285				
K^+ channel antagonism	368															
Rehabilitation[c]													416,417			

*Other neuroprotective treatments include therapies aimed at reducing tissue damage or loss related to inflammation and/or excitotoxicity; hypothermia is considered a neuroprotective treatment
[a]Demyelination includes T-cell-based vaccination, but not X-irradiation or ethidium bromide-induced lesions
[b]Neurotrophic factors include all treatments thought to exert or stimulate trophic effects on spinal and/or supraspinal neurons
[c]Rehabilitation does not include studies using treadmill training solely as a means of assessing locomotor recovery

bone marrow stromal cells (BMSCs), and some animals received injections of NT-3 at the lesion site. At 1 week following injury, some animals received injections of NT-3 rostral to the lesion. In order to dissect the effects of each treatment, this study included five experimental groups. While sensory axon growth into the BMSC graft was improved by any combination of cAMP and/or NT-3 injections, significant growth beyond the graft was only observed in animals that received both cAMP injections prior to surgery and NT-3 injections at and beyond the lesion following injury. No significant recovery of function was observed in any of the animals, likely because regenerating sensory axons did not reach their targets in the nucleus gracilus. Although stimulating regeneration through preconditioning is not clinically applicable, this study illustrates that growth-promoting interventions aimed at different parts of the neuron – the axon and soma – can have additive effects.

In another study exploiting the growth-promoting effects of cAMP, a cervical hemisection was treated with embryonic spinal tissue, and (in experimental animals) rolipram.[149] The graft was implanted immediately following injury, and rolipram was administered 2 weeks later, for a duration of 10 days. Rolipram-treated animals exhibited both increased axonal growth into the embryonic transplant and evidence of improved forepaw placement compared to vehicle-treated controls. While this experiment tested the efficacy of different concentrations of rolipram, it did not examine the effects of rolipram treatment alone, without concomitant grafting, or of administering rolipram immediately following SCI.

The effectiveness of acute rolipram was examined in a third study, in which a moderate thoracic contusion injury was treated with various combinations of a SC graft, a single injection of db-cAMP near the graft, and systemic administration of rolipram over 2 weeks.[150] The timing of intervention is of interest in this experiment, as rolipram treatment was initiated either at the time of injury or 1 week following injury, while SC grafts and db-cAMP injection were administered only 1 week following injury. Even without testing all combinations of acute *versus* delayed interventions, this study involved seven different experimental groups. Rats that received rolipram immediately following injury plus SC grafts and db-cAMP exhibited increased sparing and growth of supraspinal axons, as well as improved locomotor recovery, compared to rats that received only SC transplants; delaying rolipram administration generally reduced treatment efficacy. By most outcome measures reported, the combination of SC graft plus acute rolipram also enhanced axonal sparing/growth and functional recovery. Interestingly, acute rolipram alone was sufficient to augment both sparing/growth and functional recovery relative to untreated controls.

While combination therapy has achieved star status in recent years, attempts to synergistically combine treatments aimed at different components of SCI have been ongoing for more than a decade. Table 1 summarizes many (but by no means all) of the attempts at combination therapy applied in adult spinal cord. In addition to the sheer number of studies combining different treatments, the distribution of combinations are of interest. For example, cell transplants and neurotrophic factors have been combined many times, while combinations testing rehabilitative treatments are comparatively scant. This is unexpected, given that most SCI patients have access to some form of rehabilitation and that any treatment that moves to clinical trial is likely to be tested on a background of some form of rehabiliation. A review of combination therapies also serves as a warning that unforseen interactions between treatments may preclude the application of some in combination. For example, a recent study revealed that acute delivery of methylprednisolone and interleukin-10 worsened the behavioral outcome of SCI in animals that received SC transplants.[260]

The complexity of combination therapy will only escalate in prospective clinical trials where randomized control groups and blind assessment will be required to test safety and efficacy of candidate therapies. Some components of SCI therapy may entail less risk, and thus move to clinical trial sooner, than others. For example, pharmacological treatments may entail less risk than surgical interventions, although both may be required to achieve recovery after SCI. Such risk–benefit considerations will vary between patients according to the type, level, and extent of injury to further complicate approval for and analysis of clinical trials. These and other issues surrounding clinical trial design, including an urgent need for communication between regulatory agencies and the SCI research community, were raised at the International Clinical Trials Workshop on SCI held in Vancouver in 2004.[204]

Despite these intrinsic difficulties, the show must go on, and clinical trials must be guided by animal experiments that rigorously test neuroprotective, growth-promoting, bridging, remyelinating and rehabilitative therapies for SCI in isolation and in combination. The successful combination therapy will consider the interactions between players to ensure that they take the stage at the correct place and time to maximize efficacy and minimize risk, and will reveal that there are, truly, no small parts.

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