REGENERATION BEYOND THE GLIAL SCAR

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After injury to the adult central nervous system (CNS), injured axons cannot regenerate past the lesion. In this review, we present evidence that this is due to the formation of a glial scar. Chondroitin and keratan sulphate proteoglycans are among the main inhibitory extracellular matrix molecules that are produced by reactive astrocytes in the glial scar, and they are believed to play a crucial part in regeneration failure. We will focus on this role, as well as considering the behaviour of regenerating neurons in the environment of CNS injury.

DURA MATER
The outermost and toughest of the three layers of the meninges.

INTERMEDIATE FILAMENTS
Cytoskeletal filaments, typically 10 nm in diameter, which occur in higher eukaryotic cells and contribute to their mechanical strength.

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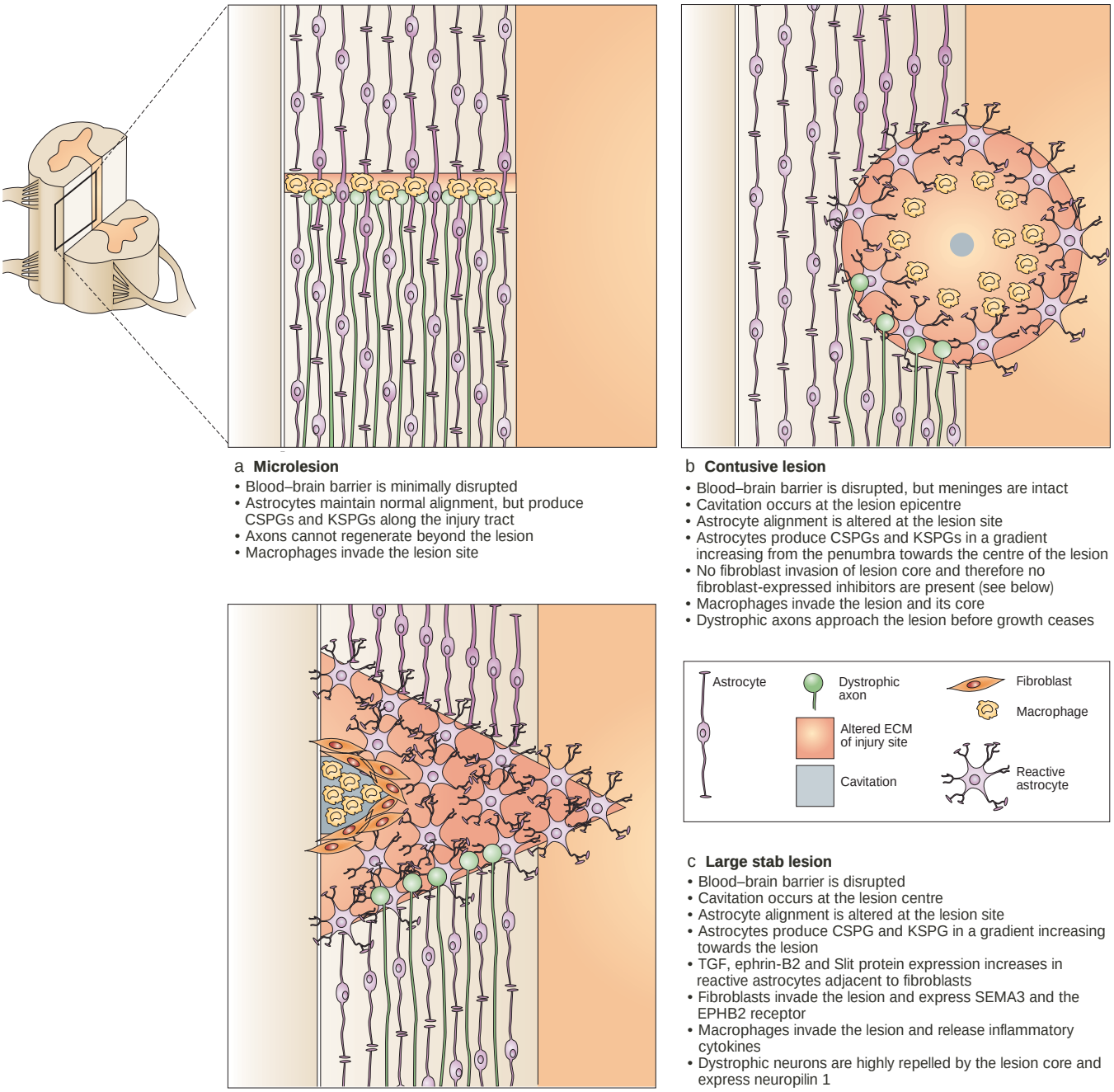
With the exception of a small pathway in the hypothalamus[1] and the olfactory sensory projections within the olfactory bulb[2,3], severed axons within long myelinated tracts of the central nervous system (CNS) are capable only of abortive sprouting that provides little functional recovery [4,5]. Injury to the CNS induces tissue damage, which creates barriers to regeneration. One of the main barriers is the glial scar, which consists predominately of reactive astrocytes and proteoglycans. Axons cannot regenerate beyond the glial scar, and they take on a dystrophic appearance of stalled growth.

Oligodendrocytes and degenerating myelin have also been identified as sources of regeneration failure in animals[6,7], and this has been the subject of many other reviews (for examples, see REFS 8–10). Here, we will briefly consider myelin's role in growth cone collapse and as an inhibitor of regeneration. However, we will focus largely on the environment of the glial scar and the cellular and molecular determinants of regeneration failure at the site of CNS injury, including a discussion of the behaviour of axons that are unable to regenerate past the lesion. We will conclude the review by considering possible strategies to enhance the regenerative capabilities of the CNS.

The glial scar and its formation

In lesions that spare the DURA MATER, the scar is composed primarily of astrocytes, but in more severe lesions that open the meninges, astroglia become mixed with invading connective tissue elements[11–15] (FIG. 1). The astrocyte response to injury is referred to as reactive gliosis (more glia) but in fact, in most types of injury, the actual amount of glial cell division is relatively small and confined to the immediate penumbra surrounding the lesion core[16]. Far more of the reactive glial response to injury is hypertrophy with increased production of INTERMEDIATE FILAMENTS[17,18]. One can identify hypertrophic reactive astrocytes by immunocytochemical methods that reveal increases in expression of glial fibrillary acidic protein (GFAP)[19–21], as well as other intermediate filament proteins, such as vimentin (reviewed by Yang[22]). Identification of enlarged and entangled reactive astrocytes surrounding dystrophic endballs at the tips of non-regenerating fibres led to the idea that the reactive glia are responsible for failed regeneration through the formation of a physical wall. Indeed, the glial scar does develop into a rubbery, tenacious, growth-blocking membrane, but this takes considerable time (FIG. 2).

In addition to preventing regeneration, recent evidence indicates that the glial scar might provide several important beneficial functions for stabilizing fragile CNS tissue after injury. The Sofroniew laboratory[16,23], used an ingenious combinatorial technique, involving avian herpes simplex viral infection of mammalian astrocytes and gancyclovir delivery, to produce targeted depletion of the subpopulation of reactive astrocytes that undergo mitosis immediately surrounding the core of the lesion. Their findings indicated that after injury, this component of the glial scar serves to repair the blood–brain barrier (BBB), prevent an overwhelming inflammatory response and limit cellular degeneration. So, it is now clear that one role of the glial scar is to



a **Microlesion**
- Blood–brain barrier is minimally disrupted
- Astrocytes maintain normal alignment, but produce CSPGs and KSPGs along the injury tract
- Axons cannot regenerate beyond the lesion
- Macrophages invade the lesion site

b **Contusive lesion**
- Blood–brain barrier is disrupted, but meninges are intact
- Cavitation occurs at the lesion epicentre
- Astrocyte alignment is altered at the lesion site
- Astrocytes produce CSPGs and KSPGs in a gradient increasing from the penumbra towards the centre of the lesion
- No fibroblast invasion of lesion core and therefore no fibroblast-expressed inhibitors are present (see below)
- Macrophages invade the lesion and its core
- Dystrophic axons approach the lesion before growth ceases

c **Large stab lesion**
- Blood–brain barrier is disrupted
- Cavitation occurs at the lesion centre
- Astrocyte alignment is altered at the lesion site
- Astrocytes produce CSPG and KSPG in a gradient increasing towards the lesion
- TGF, ephrin-B2 and Slit protein expression increases in reactive astrocytes adjacent to fibroblasts
- Fibroblasts invade the lesion and express SEMA3 and the EPHB2 receptor
- Macrophages invade the lesion and release inflammatory cytokines
- Dystrophic neurons are highly repelled by the lesion core and express neuropilin 1

Figure 1 | **Schematic representation of three stereotypical CNS lesions.** In all examples, macrophages invade the lesion, and both chondroitin sulphate proteoglycans (CSPGs) and keratan sulphate proteoglycans (KSPGs) are upregulated. **a** | Microlesion in which astrocyte alignment is not altered by the injury process, but axons are unable to regenerate past the lesion site. **b** | Contusive injury that does not disrupt the meninges, but produces cavitation and proteoglycan deposition. Again, axons are unable to regenerate beyond the lesion, but spared axons can be found distal to the injury site. **c** | Stab lesion that penetrates the meninges and allows fibroblast invasion in addition to macrophages. Axons are highly repulsed by the increasing gradient of CSPGs and KSPGs. Several other inhibitory molecules are also made in this type of injury and are especially prevalent in the core of the lesion. ECM, extracellular matrix; TGF, transforming growth factor.

seclude the injury site from healthy tissue, preventing a cascading wave of uncontrolled tissue damage[16]. Unfortunately, although the benefit of glial scarring is important to the overall survival of the animal, warm-blooded species have largely sacrificed the capacity for long-distance functional regeneration.

Formation of the glial scar occurs after the introduction of non-CNS molecules into the brain parenchyma as a result of BBB disruption[24]. The BBB remains porous to blood and serum components for up to 14 days after brain or spinal cord injury, and the areas of greatest glial scarring correlate well with areas of most extensive BBB

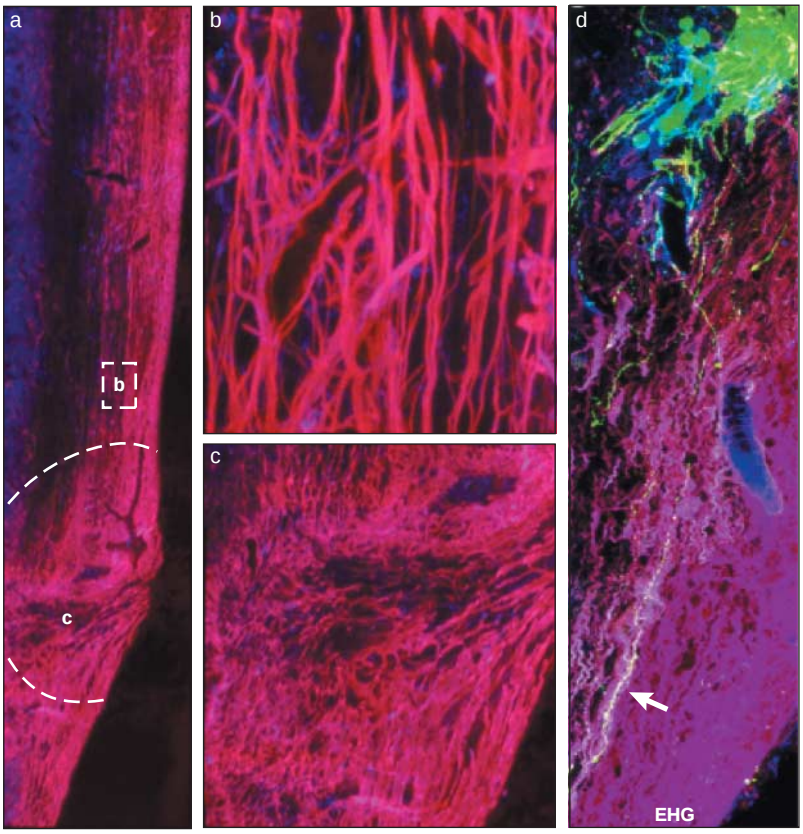


Figure 2 | **The glial scar nine months after a spinal cord stab lesion. a** | Sagittal section of the spinal cord illustrating astrocyte hypertrophy (red) and chondroitin sulphate proteoglycan (CSPG) upregulation (blue, denoted by dashed lines). Note the longitudinal, thickened bands of reactive astrocytes forming an extremely dense wall of cells. **b** | High magnification of the banded reactive astrocytes, further demonstrating the extreme hypertrophy of astroglia at this late time point after the lesion. **c** | High magnification of the injury region clearly illustrates the presence of CSPG still remaining, along with the fibrous banding of reactive astrocytes. **d** | Regenerating axons from a microtransplanted dorsal root ganglion (arrow) can grow almost everywhere within the zone of Wallerian degeneration and reactive gliosis (red) rostral to the lesion, even at nine months after injury. However, the regrowing axons are excluded from a zone of extremely dense fibrous reactive astrocytes (right of arrow). Note the lack of proteoglycans in this region of the scar. This form of axon repulsion seems to be caused by the mechanical constraints of the reactive tissue. EHG, extremely hypertrophic glia.

breakdown, as well as the largest numbers of activated MACROPHAGES. Minimally invasive microinjection techniques[25], using a non-toxic yeast wall preparation known as Zymosan to focally and aggressively stimulate macrophages, produced rapid astrocyte migration away from the inflammatory focus, and also caused CNS cavitation, glial scarring and intense proteoglycan upregulation around the cavity. Furthermore, astrocyte migration *in vitro* resulted in secondary axon stretching and axotomy in the region of inflammation, owing to aberrant neuron–glia associations. These studies indicate that blood (or a serum component), along with activated macrophages, has a crucial role in secondary axotomy, as well as the upregulation of the inhibitory extracellular matrix (ECM) components and other phenomena related to formation of the glial scar.

The search for the initial molecular inducer of inhibitory gliosis continues. One of the most interesting potential triggers is transforming growth factor β (TGFβ). In the injured brain and spinal cord, TGFβ1 expression increases immediately after injury, whereas TGFβ2 expression augments more slowly near the wound in astrocytes, endothelial cells and macrophages[26]. TGFβ2 has been shown to significantly increase the production of proteoglycans by astroglia[27]. When TGFβ1 and TGFβ2 activity is attenuated experimentally using antibodies[28], glial scarring is reduced. However, this occurs without a reduction in macrophage invasion and is insufficient to allow long-distance regeneration.

Another candidate family of scar inducers is the INTERLEUKINS. Injection of interleukin 1, a protein that is produced by mononuclear phagocytes, helps to initiate the inflammatory response in various cells, including astrocytes, which take on the reactive state[29]. So, TGFβ1 and 2 and interleukin 1 have been implicated as mediators of macrophage-induced glial scarring, but other factors might also be involved.

The interactions between two inflammatory CYTOKINES — interferon-γ (IFNγ) and basic fibroblast growth factor 2 (FGF2) — also have a role in the induction of the glial scar. In culture, preventing IFNγ activity reduces the mitogenic effects of activated T-lymphocytes, and the addition of recombinant IFNγ induces astrocyte proliferation[30]. Furthermore, administration of IFNγ to lesioned brains increases the extent of glial scarring. After injury, the levels of FGF2 increase in both the brain and spinal cord[31,32], and FGF2 has also been shown to increase astrocyte proliferation in culture[109]. It has been proposed that IFNγ and FGF2 modulate one another after injury, with IFNγ antagonizing the pro-mitogenic effects of FGF2 (REF. 33). However, it should be reiterated that in the vicinity of BBB extravasation, much of the glial scar forms without astrocyte proliferation. Instead, there is a switch to the reactive state, followed by inhibitory ECM production and hypertrophy[34]. Although we are beginning to understand the complex role of inflammation in the induction of the glial scar, there is still much to learn.

The response of axons to the scar

When regenerating axons encounter the environment of the glial scar, so-called dystrophic endbulbs form, as first described by Ramón y Cajal[5]. For many years, these unusually shaped endbulbs were considered to be sterile, and therefore incapable of extending a growth cone. Dystrophic endings can form into various bizarre shapes, ranging from small globular clusters to huge multivesicular sacs[5].

More recent research has indicated that axons with dystrophic endings do not lose their ability to regenerate, and that they can in fact return to active growth states. Chronically injured axons in the spinal cord can regenerate into implanted peripheral nerve grafts after four weeks of stagnation in the lesion environment[35]. More importantly, even in one-year-old lesions of the adult RUBROSPINAL TRACTS, cells that are treated in the vicinity of their bodies with brain-derived neurotrophic factor (BDNF) can restore the size of their soma[36] and can achieve new axon growth into peripheral nerve grafts with an accompanying upregulation of the

MACROPHAGES
Cells of the mononuclear phagocyte system that are characterized by the ability to phagocytose foreign particulate and colloidal material.

INTERLEUKINS
A generic term for cytokines originally identified as products of leukocytes.

CYTOKINES
In general terms, cytokines are proteins made by cells that affect the behaviour of other cells. They are produced mainly by the immune system.

growth-associated protein GAP43 (REF. 37). Li and Raisman[4] examined dystrophic endings in the context of a chronic lesion. They found that even 13 weeks after injury, there was a persistence of large varicosities and swollen bulbs that resembled the classic dystrophic ending. Furthermore, even at these lengthy time periods post-injury, supposedly sterile dystrophic endings were capable of sprouting, and many of the sprouts were myelinated by migrating Schwann cells that slowly invade the CNS through the damaged root entry zones.

Additional work by Houle and Yin has carefully examined the extent of axon retraction after spinal cord injury[38]. Cervical cord hemisection resulted in the formation of numerous dystrophic endbulbs. Previous studies indicated that considerable dieback occurs, but by using more modern tracing techniques, Houle and Yin showed that most of these endings actually remained close to the lesion site.

The significance of the persistence of such unusual 'growth cones' within the epicentre of the lesion implies that some type of cytoskeletal and/or membrane plasticity must be occurring to maintain axon viability and stability, even though the axons remain in one place without forming synapses. Recent work in our laboratory has begun to elucidate, both in culture and *in vivo*, the behaviour of dystrophic endings[39]. Using a specially crafted gradient of aggrecan and LAMININ as a growth substrate, classically shaped dystrophic endings can be induced in adult DORSAL ROOT GANGLION (DRG) neurons as the fibres struggle up the gradient. Time-lapse analysis revealed the surprising fact that 'dystrophic' endings are highly active structures. Although they can remain stationary for days, they constantly turn over their distal membranes, alter their cytoskeleton and change the location of their complement of integrin receptors. Why or how they do this is unclear, but it can occur as a response to certain types of proteoglycan gradients. We have evidence that these dynamic phenomena also occur *in vivo*, so it is possible that the adult sensory neuron uses this form of aberrant growth cone to maintain itself in a hostile environment.

In addition to dystrophy, growth cones can undergo a different stalled growth, which is referred to as collapse. Growth cone collapse occurs after injury when mature regenerating axons encounter mature oligodendrocytes and myelin products[40], and it can also occur normally during development when axons move from one growth-supporting substrate to another[41]. Growth cone collapse has been well characterized in cell culture[42–45] and results in stalled forward progress with a shrunken, quiescent growth cone that can restart over time, only to collapse again following renewed contact with the collapsing agent. Mature growth cones maintain their morphology and extension on their supportive substrate until membrane–membrane contact occurs with oligodendrocytes or myelin. At this point, the growth cone arrests, collapses and often retracts. Evidence of growth cone collapse has been implicated in regeneration failure, and methods that enhance regeneration by blocking the effects of myelin will be discussed later. Whether growth cone collapse occurs during the earliest phases in the progression of events that lead to growth cone dystrophy is unknown, but it is clear that the two phenomena are remarkably different. One important characteristic of dystrophic axons is that they can be stimulated to regenerate, as discussed earlier in the text.

Inhibition of the glial scar: proteoglycans

In addition to growth-promoting molecules[46,47], astrocytes produce a class of molecules known as proteoglycans[48,49]. These ECM molecules consist of a protein core linked by four sugar moieties to a sulphated GLYCOSAMINOGLYCAN (GAG) chain that contains repeating disaccharide units. Astrocytes produce four classes of proteoglycan; heparan sulphate proteoglycan (HSPG), dermatan sulphate proteoglycan (DSPG), keratan sulphate proteoglycan (KSPG) and chondroitin sulphate proteoglycan (CSPG)[50]. The CSPGs form a relatively large family, which includes aggrecan, brevican, neurocan, NG2, phosphacan (sometimes classed as a KSPG) and versican, all of which have chondroitin sulphate side chains. They differ in the protein core, as well as the number, length and pattern of sulphation of the side chains[51–53]. Expression of these CSPGs increases in the glial scar in the brain and spinal cord of mature animals[54–56].

Proteoglycans have been implicated as barriers to CNS axon extension in the developing roof plate of the spinal cord[57,58], in the midline of the rhombencephalon and mesencephalon[59,60], at the dorsal root entry zone (DREZ)[61], in retinal pattern development[62,63], and at the optic chiasm and distal optic tract[64,65]. Extensive work has demonstrated that CSPGs are extremely inhibitory to axon outgrowth in culture. Neurites growing on alternating stripes of laminin and laminin/aggrecan had robust outgrowth on laminin, but at the sharp interface between the two surfaces, growth cones rapidly turned away (unlike their stalled behaviour in a gradient, see above). The inhibitory nature of the proteoglycan-containing lanes can repel embryonic as well as adult axons, and the effect can last for more than a week *in vitro*. The turning behaviour is not usually mediated by collapse of the entire growth cone, but rather by selective retraction of FILOPODIA in contact with CSPG and enhanced motility of those on laminin[66,67]. CSPGs are potent inhibitors of a wide variety of other growth-promoting molecules, including fibronectin and L1 (REFS 68,69).

In the early 1990s, the first evidence emerged that CSPGs might have a role in the failure of regeneration in the CNS after injury. In mature mammals, CSPGs are secreted rapidly (within 24 hours) after injury and can persist for many months[54–56]. It was shown that CSPGs are produced in excess by astrocytes when they are induced to become reactive *in vivo* after small lesions of the DREZ[61]. The upregulated CSPGs were present at the right time and place to inhibit sensory axons from regenerating in the dorsal columns or through the DREZ. With the exception of those few places in the hypothalamus where regeneration can occur among TANYCYTES[70], CSPGs are now known to be upregulated and excreted extracellularly after a wide

RUBROSPINAL TRACTS
In the lateral quadrant of the spinal cord, the rubrospinal tracts are the main descending pathways from the brain stem.

LAMININ
A glycoprotein that is the main constituent of basement membranes. It mediates the attachment, migration and organization of cells into tissues during development.

DORSAL ROOT GANGLION
The cell bodies of sensory neurons are collected together in paired ganglia that lie alongside the spinal cord. These cell bodies are surrounded by satellite glial cells, which share much in common with the Schwann cells that ensheath peripheral axons. Few synapses have been observed in these ganglia.

GLYCOSAMINOGLYCAN
Any polysaccharide that contains a high proportion of amino-monosaccharide residues; that is, monosaccharides in which an alcoholic hydroxyl group is replaced by an amino group.

FILOPODIA
Long, thin protrusions that are present at the periphery of migrating cells and growth cones. They are composed largely of F-actin bundles.

TANYCYTES
A type of ependymal cell found principally in the walls of the third ventricle of the brain. The tanycytes might have branched or unbranched processes, some of which end on capillaries or neurons.

Box 1 | Immature reactive astrocytes promote regeneration

Embryonic axon pathways in warm-blooded species resemble, in certain respects, the axon highways of amphibians, in that they too allow for regeneration[128,129]. Furthermore, when embryonic tissue is implanted into the spinal cord of adult rats, far better integration and regeneration of tissues is achieved[130].

Differences in the ability of axons to regenerate in very young and old mammals seems to be due partly to changes in the astroglial reaction to injury. When nitrocellulose is impaled into the brain of both young and old animals, it is possible to remove the tightly adherent reactive astrocytes, along with other cells that respond to and become embedded in the implant. The wound tissues that have reacted to the same insult, albeit at different ages, can then be harvested and used to examine the outgrowth of identified populations of neurons *in vitro*[131]. Hippocampal neurons cultured on 'scar-in-a-dish' explants from neonatal animals could extend neurites with much greater vigour than those cultured on explants from mature animals. The results indicated that scar tissues from younger animals were growth supportive, whereas scar tissue from older animals was inhibitory, at least *in vitro*.

Similar results were obtained using astrocytes collected from the lesioned optic nerve. Retinal ganglion cells cultured on newborn reactive optic nerve astrocytes extended processes over greater distances and with greater velocity than retinal ganglion cells cultured on adult reactive astrocytes. In fact, adult reactive astrocytes completely inhibited growth of both embryonic and adult neurons *in vitro*, whereas young astrocytes promoted outgrowth from both embryonic and adult neurons[132]. Interestingly, adult reactive astrocytes[133], as well as extracted adult scar tissues[73,131], allow growth of dendrites but not axons *in vitro*. The reason for this differential effect is unknown, but it might reflect differences in susceptibility to scar-associated inhibitory molecules.

In vivo studies further illustrate the differences between immature and mature astrocytes in the context of injury. Untreated Millipore implants placed in the brains of adult acallosal mice normally produce glial scarring and tissue degeneration[134]. When the implant was placed into young acallosal mice, highly reactive astrocytes integrated into the implant. Nevertheless, even in the reactive state, immature glia supported regeneration of callosal axons across the implant, at least until the critical period. Furthermore, immature, but not mature, reactive astrocytes transplanted into adult brains on nitrocellulose[135], or directly as cell suspensions[136], suppressed glial scar formation and could also modify the glial response of the adult.

range of CNS injuries[21,71,72]. Importantly, pre-critical period embryonic reactive astroglia do not upregulate CSPGs after injury[73,74] (BOX 1), and there is minimal upregulation of CSPGs on reactive glia in cold-blooded species, with the exception of special regions where regeneration does not occur[64] (BOX 2).

Several *in vitro* assays, in which mature astrocytes were induced to be highly reactive, have confirmed that upregulated proteoglycans within the complex ECM that is made by reactive astroglia inhibit axonal outgrowth[68,73,75,76]. To further demonstrate that the GAG portion of CSPGs is inhibitory to neurite outgrowth, chondroitinase — an enzyme extracted from the bacterium *Proteus vulgaris* that selectively removes a large portion of the CSPG GAG side chain and renders CSPGs less inhibitory (see later in text) — was applied to mature glial 'scar-in-a-dish' explants (see BOX 1). Retinal ganglion cells cultured on the scar explants could only extend long neurites after the enzyme treatment, indicating that CSPGs were present in the glial scar and could potentially serve as potent inhibitors of neurite outgrowth *in vivo*[77]. Furthermore, when laminin (which is also produced by astroglia in these scar explants) was blocked with antibodies, the growth-enhancing effects of chondroitinase treatment was reduced. These findings indicate that neurite extension might depend on a balance of growth-promoting and growth-inhibiting molecules at the site of injury, or that CSPGs might be inhibiting outgrowth indirectly by interfering with the growth-promoting effects of laminin or its receptors.

Differential responses to CSPGs. Different populations of neurons respond to CSPGs with varying degrees of growth retardation *in vitro*[78]. Embryonic DRG neurons, retinal ganglion neurons or forebrain neurons were cultured on a gradient of laminin and CSPG, in which the concentration of CSPG increased gradually in a stepwise fashion while the concentration of laminin remained unchanged. Neurons were initially cultured on laminin alone, and the ability of each neuronal population to extend neurites up the step gradient was analysed. Interestingly, all neurons could extend neurites some distance up the gradient. Of the three neuronal subtypes, retinal ganglion cells could navigate the furthest, although they slowed their rate of outgrowth considerably as they negotiated each step. Not only does this work show that different populations of neurons respond to CSPGs differently, but it also shows that neurons are capable of outgrowth on CSPGs (in part, through their ability to gradually upregulate integrin receptors) until a critical threshold level is reached. Furthermore, time-lapse microscopy of adult DRG growth cones on a more smoothly constructed spot gradient of CSPG indicates that growth cones can extend along a gradient of proteoglycan until a highly inhibitory region is reached, which results in complete cessation of forward extension. Adult neurons, unlike their embryonic counterparts, cease growing and form an unusual type of dystrophic ending[39].

In the spinal cord, different neuronal populations also have differential abilities to regenerate into a proteoglycan-enriched lesion[79]. After injury, regenerating motor axons were unable to enter the lesion directly, but could sprout in the region adjacent to the lesion. Serotonin-expressing neurons were also only capable of regenerating to the edge of the lesion, whereas sensory axons were capable of deeper penetration, albeit not all the way through the lesion. These findings show that some neurons have a higher intrinsic threshold for dealing with a terrain that is laden with proteoglycans, especially when they are presented in a gradient. At some point, however, usually near the lesion epicentre, all regenerating fibres become dystrophic and finally succumb to their increasingly inhibitory environment.

At the other extreme, there are instances in which neurite extension occurs in the presence of proteoglycans. Developing thalamocortical axons traverse areas of CSPG deposition in the subplate on the way to their target, and these axons maintain a trajectory that is limited to CSPG-rich regions over much of their journey[80]. Furthermore, oversulphated DSPGs have been shown to promote, albeit minimally, neurite outgrowth from embryonic hippocampal neurons[81].

Box 2 | Cold-blooded species can regenerate their axons

Not all vertebrates suffer the same fate of failed regeneration with formation of an inhibitory glial scar. Evidence that the glial scar is differentially repulsive depending on the species was demonstrated most convincingly by Reier, who showed that regeneration can occur directly through a glial 'scar' in amphibians[137,138]. In these studies, the optic nerve of *Xenopus* was repeatedly crushed and the dense reactive glial tissues that were induced were harvested and transplanted near the freshly severed optic stump of a recipient. Regenerating retinal ganglion cell axons were capable of extending through these densely formed glial tissues. Indeed, amphibian reactive glia (as well as those of fish[139,140]) can undergo plastic changes after injury, which includes the formation of new axon growth-promoting membrane and a channel-like arrangement for guiding the new fibres[141,142].

WALLERIAN DEGENERATION
Degenerative changes in the distal segment of an axon and its myelin sheath that occur secondarily to proximal injury of the axon or cell body.

These studies indicate that the role of CSPGs as growth inhibitors might not be a simple 'all or none' phenomenon as was originally discussed in Snow *et al*[58]. However, one question remained: do axons grow differently in regions containing proteoglycans than in regions where proteoglycans are lacking? Using relatively low concentrations of CSPGs that allow for intermittent neurite outgrowth on laminin, Snow and colleagues[82] have begun to identify the growth patterns of neurons on permissive levels of normally inhibitory CSPGs. Interestingly, axons growing on CSPG fasciculated tightly (as they do in the subplate), but returned to a defasciculated state when on laminin. These studies highlight the importance of proteoglycan concentrations and their balance with other molecules during their interactions with growth cones, and also indicate that particular mixtures can help regulate the overall patterning of axonal bundling or branching characteristics.

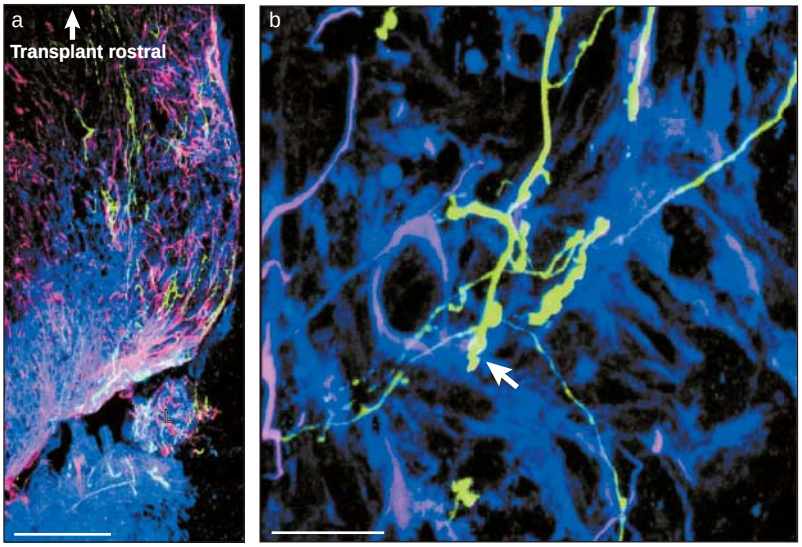


Figure 3 | **Failure of regeneration of microtransplanted dorsal root ganglion axons on reaching a proteoglycan gradient. a** | Double scanning confocal photomicrograph of transplanted adult dorsal root ganglion (DRG) neurons (green) extending axons into the periphery, but not the centre, of a spinal cord lesion (L). Axons are present in areas of chondroitin sulphate proteoglycan (CSPG) deposition (blue), but are unable to traverse regions of increasing CSPG deposition. **b** | High magnification of dystrophic axons extending from transplanted adult DRG neurons. Note the characteristic endballs[5] that form in the presence of high levels of CSPG (arrow). Scale bars: **a**, 250 μm; **b**, 25 μm. Figure reprinted from REF. 84 © Society for Neuroscience.

Excess proteoglycans prevent regeneration in vivo. The crucial role of inhibitory scar ECM in causing regeneration failure was revealed by microtransplantation experiments. Adult sensory neurons that were placed in the adult corpus callosum or lesioned spinal cord could regenerate their axons in intact or degenerating white matter, provided that minimal damage was created on placing the cells far rostral to a lesion. However, regeneration ceased as the growing axons approached the vicinity of the lesion. Here, proteoglycan upregulation occurs in a decreasing gradient, being highest in the centre of the lesion and diminishing gradually into the penumbra (FIG. 3). As regenerating fibres pass through the lesion penumbra, they alter their morphology and are strongly inhibited from moving further as they form ever increasing numbers of dystrophic endings near the core of the developing scar[83,84] (FIG. 3). Rather unexpectedly, transplanted adult sensory neurons were capable of extending axons rapidly and robustly (at the rate of 1 mm per day) among oligodendrocytes, degenerating myelin and astrocytes that are intensely reactive owing to the accompanying WALLERIAN DEGENERATION. However, it is important to reiterate that the site of transplantation must be far enough from the lesion to be beyond the zone of serum leakage through the ruptured BBB. Unknown factors that extravasate into the CNS parenchyma seem to trigger the intense, rapid upregulation of proteoglycans that occurs after injury (see earlier in text).

So, our experiments have revealed that in addition to immature astrocytes, reactive astroglia that are distal to a lesion are surprisingly growth supportive, even within purportedly inhibitory white matter. Although the molecular mechanisms through which reactive astrocytes support axon growth *in vivo* are unknown, recent evidence from our laboratory indicates that extracellular fibronectin and the longitudinal geometric alignment of the intrafascicular astroglia might be two of several important factors[85]. The remarkable capacity for regeneration distal to the lesion can last for many months, until the mechanically obstructive properties of glial hypertrophy come into play and block regeneration through more physical means (FIG. 2).

Additional inhibitory molecules in the glial scar

In addition to the inhibitory effects of CSPGs, several other molecules are now known to be upregulated in the core of the lesion and to contribute to the growth-retarding effects of the glial scar. One such molecule is the secreted protein semaphorin 3 (SEMA3), which acts as a chemorepellent through its high-affinity receptor neuropilin 1. After lesions of the lateral olfactory tract[86], cortex[86] or spinal cord[86–88], SEMA3 expression increases in fibroblasts that penetrate the lesion deeply, and neuropilin 1 expression increases in neurons that project to the site of injury. Regenerating axons were excluded from areas of damage containing SEMA3, essentially creating an exclusion zone at the heart of the lesion. These correlative results indicate that semaphorins help to prevent the penetration of regenerating neurites past the centre of fibroblast-containing CNS lesions.

Box 3 | Chondroitin sulphate proteoglycans (CSPGs) and synaptic plasticity

Within the grey matter parenchyma of the brain and spinal cord, various proteoglycans related to the aggrecan family might form a kind of 'caulk' that encapsulates synapses[143]. Removal of this aspect of proteoglycan inhibitory function might be essential for the stimulation of neuritic sprouting and synaptic plasticity. In adult cortical and subcortical regions, neurons and synapses are surrounded by a lattice-like accumulation of extracellular matrix (ECM) that is thought to stabilize synapses, control ion homeostasis and provide neuroprotection (reviewed by Steindler[44] and Celio *et al.*[145]). This ECM, which is known as the perineuronal net, consists mostly of CSPGs[146], which are produced as the animal passes through critical periods of central nervous system maturation[147,148]. Interestingly, dark-rearing prolongs the duration of the critical period and, in turn, development of the perineuronal net, implicating sensory experience in maturation of the net[148]. During juvenile periods of visual cortex development, there is great plasticity in neuronal connections. As the animal matures and the perineuronal net increases, the potential for plasticity decreases and the synaptic connections made during development become 'locked in'. When this occurs, new experiences do not alter established neuronal connectivity, which is thought to result, in part, from CSPG-mediated sprouting inhibition. This is beneficial, because it confers the adult with protection against disturbances in functional connectivity that are brought about by aberrant experience. However, in other instances, and especially after injury, it might be beneficial to restore plasticity. Pizzorusso and colleagues[149] attempted just that by treating the adult visual cortex with chondroitinase. This treatment did indeed restore plasticity to the adult visual cortex, creating the same state of plasticity that is found in juvenile animals, and implicated CSPGs in limiting sprouting in an important sensory system. The ultimate goal will be to restore ocular dominance plasticity and possibly functional vision in adult STRABISMIC, otherwise monocularly deprived animals that have been made cortically blind in one eye from birth, but are allowed to mature for long periods before enzyme treatment.

Recent work by Bundeson and colleagues[89] has also implicated ephrin-B2 and its receptor EPHB2 in the inhibition of regeneration after spinal cord transection. During normal development, this ligand-receptor pairing has diverse roles in cell migration, axon guidance and tissue patterning. The interaction of these partners becomes important again after injury, when ephrin-B2 expression increases in astrocytes and EPHB2 expression increases in fibroblasts. At first, astrocytes and fibroblasts co-mingle, but then as ephrin-B2 and EPHB2 expression increase, they signal cell-type segregation, creating bands of fibroblasts and astrocytes, and more importantly, the cellular structure of the so-called glial/mesenchymal scar.

Slit proteins, which are important regulators of axon guidance and cell migration (reviewed by Brose and Tessier-Lavigne[90]), are also increased, along with their glypican 1 receptors[91], in reactive astrocytes after cortical injury[92]. These observations have caused the Slit proteins to be implicated in regeneration failure. Together, SEMA3, ephrin-B2 and Slit proteins add additional levels of complexity to the source of regeneration failure in the adult CNS, especially after open injuries that allow mesenchymal cell infiltration.

Overcoming inhibition

Modification of sulphated proteoglycans. One strategy to overcome the inhibitory effects of CSPGs, and to further demonstrate that CSPGs inhibit regeneration, is to enzymatically digest them *in vivo* after injury to axonal pathways. The chondroitinase enzyme removes much, but not all, of the sugar chain from CSPGs, leaving the protein core and stub carbohydrate behind, and chondroitinase is effective at removing the inhibitory properties of CSPGs. In fact, a single injection of chondroitinase into the brain results in decreased levels of intact CSPG that persist for up to four weeks[93].

Treatment with chondroitinase after nigrostriatal tract lesioning enhanced the regeneration of dopaminergic neurons back to their desired targets[94]. Intrathecal application of chondroitinase to animals with bilateral dorsal column lesions resulted in degradation of CSPG at the lesion site, and allowed both ascending sensory and descending motor axon regeneration through, and perhaps even slightly past, the lesion[95]. Furthermore, this treatment resulted in recovery of certain locomotor and proprioceptive functions. Additional preliminary studies have shown that CSPG digestion can improve regeneration after spinal cord hemisection in the cat[96], and also after a compressive injury to the spinal cord of rats[97], which is a more relevant model for the typical human spinal cord injury. In another strategy, treatment with chondroitinase enhances the ability of regenerating axons to enter, as well as exit, peripheral nerve grafts transplanted into CNS lesions to provide a Schwann cell-laden highway and possible bypass of the injury site[98–100]. Last, preliminary results in our laboratory indicate that preventing the synthesis of CSPGs after injury by inhibiting the synthetic enzymes for GAG chain assembly also enhances regeneration[101]. So, work from many laboratories has shown that removal of CSPGs reduces the inhibitory environment of the glial scar *in vivo* and fosters some measure of functional regeneration. Furthermore, chondroitinase treatment has helped us to elucidate the role of CSPGs in normal inhibition of synaptic sprouting in the cortex (BOX 3).

It is important to note that enhancement of regeneration after chondroitinase treatment is not without limitations. Recent work involving the implantation of a basement membrane matrix into the spinal cord illustrates this point[102]. When the matrix alone is implanted, robust sensory axon regeneration into this substrate occurs, but the addition of aggrecan prevents regeneration into the matrix. Interestingly, when aggrecan is predigested with chondroitinase and the purified core is

STRABISMIC
A term that describes animals with a condition in which the eyes are not straight or properly aligned. The misalignment reflects the failure of the eye muscles to work together. One eye might turn in (crossed eyes), turn out (wall eyes), turn up or turn down. Although some cats are congenitally strabismic, strabismus can also be achieved by cutting the tendon of one of the eye muscles.

added to the matrix, regeneration is still inhibited, indicating that regions of the digested CSPG that are left behind after digestion still remain somewhat inhibitory to neurite outgrowth. *In vitro* work in our laboratory has also demonstrated that the protein and sugar stub remaining after chondroitinase treatment loses its inhibitory potency, but is still somewhat inhibitory to adult DRG process outgrowth[39]. Multiple regions of proteoglycans can inhibit neurite outgrowth[103], so chondroitinase digestion might not remove all aspects of CSPGs that prevent regeneration, and other strategies that more thoroughly eliminate the GAG chains are desirable[104].

Blocking the effects of myelin. In addition to enhancing regeneration by removing the inhibitory effects of CSPGs, extensive work has shown that blocking Nogo, a myelin-associated inhibitor of regeneration, improves regeneration[105]. Antibodies directed against the Nogo receptor administered into spinal cord lesion sites[106] or even systemically[107] seem to enhance regeneration, although recent work[108] has disputed whether this is truly enhanced regeneration or merely local sprouting. Indeed, it is now being suggested that most of the functional recovery that is seen when inhibitors of myelin are used occurs as a result of remodelling of local circuits, such that functional recovery is mediated along uninjured long axons[108]. This proposal, in conjunction with work from our laboratory demonstrating rapid axon regrowth from adult neurons in the presence of degenerating white matter[83,84], as well as the differences between growth cone collapse and dystrophy, indicates that myelin might not be acting fundamentally to inhibit long-distance regeneration. In fact, it has even been suggested that myelin might facilitate axon growth under certain conditions[109].

Enhancing the intrinsic growth machinery. Removal of extrinsic inhibitory cues from the glial scar with treatments such as chondroitinase might aid regeneration, but this might not be sufficient for long-range regrowth. Neurotrophin 3 (NT3) or nerve growth factor (NGF), when delivered directly to transected neurons in the dorsal columns of animals treated with peripheral nerve graft transplants, enhances growth into the graft, out the opposite end and beyond the glial scar into host tissue[110,111]. Exogenous NGF administration also induces sprouting into the lesion of crushed dorsal columns[112]. Intrathecal or adenoviral application of NT3 or NGF to the injured DREZ induces DRG neurons to cross the peripheral nervous system/CNS barrier and penetrate some distance into the spinal cord[113–117], where the regenerating fibres restore nocioceptive function. So, evidence from the injured spinal cord and DREZ indicates that regenerating axons can overcome proteoglycan barriers after neurotrophin stimulation, perhaps through induction of growth enhancing genes, offering an additional therapeutic strategy.

The intrinsic growth properties of mature neurons can be altered in other ways to enhance regeneration. Unlike mature neurons, embryonic neurons have a high capacity for regeneration and can rapidly upregulate integrin receptors for growth-promoting molecules when mixed with inhibitory aggrecan[118]. Overexpression of integrins by viral transduction endows adult neurons with enhanced regenerative capability, on a par with that of young neurons. Peripheral conditioning lesions also enhance the ability of CNS neurons to regenerate into a lesion site, in part owing to increases in cyclic AMP (cAMP) levels in the neuron cell body[119]. Furthermore, this effect can be replicated without peripheral lesion by simply injecting the cell bodies of sensory neurons with cAMP[120], which acts through protein kinase A-mediated pathways to affect cellular sprouting and outgrowth. These findings indicate that protein kinase A-mediated pathways could be exploited to enhance the ability of a neuron to overcome scar inhibitors as well as myelin inhibitors following injury.

CSPGs in the glial scar impair neuronal outgrowth by signalling through the Rho/ROCK pathway, and specific inhibition of Rho GTPase enhances process outgrowth[121–123] on proteoglycan-containing substrates, as well as on myelin-containing substrates[122]. Pharmacologically blocking the downstream signalling from CSPGs might also enhance CNS regeneration.

Last, certain reparative states of the inflammatory cascade, which must be different from those that influence glial scar formation, might also have pro-regenerative effects on neurons. Activation of macrophages (outside the CNS compartment) using Zymosan enhances retinal ganglion cell regeneration for short distances past the glial scar after optic nerve injury[124] and enhances process outgrowth of DRG neurons in a culture model of proteoglycan contained in the glial scar[125]. Interestingly, a simple sugar, mannose, seems to be one mediator that stimulates neurons to increase their ability to regenerate[126].

Combinatorial strategies. Combinatorial strategies that take advantage of the pro-regenerative effects of chondroitinase, along with strategies that foster stimulation of the intrinsic growth potential of adult neurons (for example, exogenous neurotrophins), can also be undertaken to induce regeneration and sprouting.

Lesions of the adult retina that denervate the superior colliculus produce some limited sprouting of uninjured fibres. However, this sprouting is often so minimal that it is not sufficient to restore function. Tropea and colleagues[127] attempted to increase this sprouting response by treating the superior colliculus with chondroitinase combined with retinal ganglion cell body stimulation through application of BDNF. Chondroitinase and BDNF acted synergistically to enhance sprouting to a greater extent than either therapy alone. Furthermore, markers of synaptogenesis were also identified in the newly sprouting fibres, demonstrating the potential for functional recovery. This important work further demonstrates the potential therapeutic roles of chondroitinase in enhancing functional regeneration through a sprouting response, and hints at the potential of combinatorial therapies. It will be fascinating to learn whether the function that is restored through ectopic sprouting in the colliculus is beneficial or detrimental to the animal's visually-guided behaviours.

Conclusions

Our discussion of the glial scar has led us to conclude that to overcome the inhibitory environment of the glial scar, treatments should ideally provide a growth-supportive highway across the lesion cavity, intrinsically enhance the ability of neurons to elongate and manipulate the extrinsic inhibitors that block growth in the immediate environment of the glial scar. With this combinatorial strategy, it might be possible to induce long distance and functional regeneration after CNS injury.

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Competing interests statement
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