

From Animal Models to Humans: Strategies for Promoting CNS Axon Regeneration and Recovery of Limb Function after Spinal Cord Injury

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ABSTRACT

There are currently no fully restorative therapies for human spinal cord injury (SCI). Here, we briefly review the different types of human SCI pathology as well as the most commonly used rodent and nonhuman primate models of SCI that are used to simulate these pathologies and to test potential therapies. We then discuss various high profile (sometimes controversial) experimental strategies that have reported CNS axon regeneration and functional recovery of limb movement using these animal models of SCI. We particularly focus upon strategies that have been tested both in rodents and in nonhuman primates, and highlight those which are currently transitioning to clinical tests or trials in humans. Finally we discuss ways in which animal studies might be improved and what the future may hold for physical therapists involved in rehabilitation of humans with SCI.

Key Words: plasticity, SCI, regeneration, animal, human, therapies, functional recovery

INTRODUCTION

Spinal cord injury (SCI) affects hundreds of thousands of people worldwide, with massive associated health care and other socioeconomic costs.¹ Damage to the spinal cord results most notoriously in flaccid paralysis and loss of normal sensation in the limbs below the level of the lesion. Spinal cord injury may also lead to debilitating pain, spasticity, impairments in breathing and coughing, bowel or bladder problems, and reduced reproductive ability or sexual sensation.¹ People with SCI may suffer from autonomic dysreflexia and may be at increased risk for stroke, decubitus ulcers, fractures, and depression. There are no fully restorative clinical therapies for SCI.

In attempts to develop therapeutic strategies for overcoming each of these sequelae, researchers often rely upon animal models of SCI. These enhance our understanding of the cellular and molecular response of the mammalian spinal cord to injury, and they allow us to evaluate the safety and efficacy of potential therapies for improving outcome. Use of animal models has shown that dysfunction following SCI results from interruption of descending and ascending spinal axons and loss of both myelin and cells including neurons, oligodendrocytes, and astrocytes. Many axons regenerate following injury to the peripheral nervous system (PNS) but few, if any, axons regenerate long distances following injury to the central nervous system (CNS).¹ This review will discuss strategies for promoting regeneration of

CNS axon tracts and recovery of limb function in animal models of SCI.

Although beyond the scope of this review, there are other extremely important efforts aimed at restoring normal function after SCI. For example, following the initial insult to the spinal cord, further structure and function is lost through active secondary processes.² Substantial effort has been devoted to limiting this secondary damage through development of neuroprotective measures.³ Substantial effort also has been dedicated to improving upon the small degree of endogenous repair that proceeds spontaneously in the spinal cord.^{4,5} For example, strategies for improving conduction through spared axons have been tested in animals and in humans.⁶ Strategies for treating pain and sexual, bladder, or bowel dysfunction and autonomic dysreflexia are also beyond the remit of this article. Readers are directed to excellent recent reviews of these fields in this volume and elsewhere.^{1,3,6,7}

The present review will first outline different types of human SCI and will discuss how contemporary animal models of SCI attempt to simulate these pathologies. Next, we will discuss cellular and molecular strategies for improving CNS axon regeneration and recovery of limb function after SCI. Special consideration will be given to experimental strategies that have been evaluated in nonhuman primates and/or are currently transitioning to clinical studies or trials.⁷ We will highlight where strategies remain controversial, and where safety or efficacy barriers to translating experimental strategies exist. The aim is to inform readers of exciting laboratory advances, but to temper this with a realistic understanding of the challenges that remain in developing effective and safe therapies for humans.

HUMAN SCI IS PATHOLOGICALLY HETEROGENEOUS

The response of the human spinal cord to injury has been studied using imaging techniques as well as by histological staining and inspection of autopsy material.⁸ These studies reveal 4 classes of lesion. In one study of 48 specimens,⁸ in 33% of cases, contusion injuries resulted in cavity and cyst formation, typically with some sparing of white matter (or glial tissue) but with the external glial limiting membrane (the *glia limitans*) remaining intact. In 29% of cases, massive compression or maceration occurred due to vertebral displacement whereas in 21% of cases, laceration occurred due to cord penetration by foreign bodies or bony fragments. After compression, maceration or laceration,

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substantial breaks in the *glia limitans* led to the epicenter being filled predominantly with connective tissue; cysts and cavities were less prominent. In 17% of cases, solid core injuries manifested as either central cord syndrome (with loss of myelin and axons, but with preservation of gray matter), or as chronic cord compression (with loss of myelin and/or motor neurons in the epicenter, but with preservation of axonal integrity). The extent of demyelination, the invasion of host cells and the deposition of growth-inhibitory molecules (see below) at sites of human SCI also have been documented.^{9,10}

Various animal models of SCI have been developed in order to model each of these different types of human injury. The relative merits and limitations of these models will be discussed next, since these directly affect the ability of the researcher to develop safe and effective strategies for improving CNS axon regeneration and motor function after SCI.

ANIMAL MODELS OF SCI ARE DESIGNED TO SIMULATE DIFFERENT TYPES OF HUMAN SCI

The vast majority of mammalian SCI research is conducted using adult mice and rats, although some work has been done using cats and dogs, and increasing work is being done using nonhuman primates. Contusion injuries in the human are typically modeled using devices that displace^{11,12} or impact¹³ the exposed spinal cord. Compression injuries may be simulated by subdural insertion and inflation of a balloon¹⁴ or by clip compression.¹⁵ Methods for inducing contusion via vertebral displacement have also recently been developed.¹⁶ Laceration injuries are usually modeled by surgical incision, including complete transection,^{17,18} dorsal hemisection,¹⁹ or lateral hemisection.²⁰ In all cases, the aim is to produce reproducible graded (or complete) injuries.

The animal model selected by the researcher largely depends upon the hypothesis to be tested. Researchers testing neuroprotective strategies often employ contusion or compression injuries. Those testing pro-regenerative strategies typically prefer models that involve transection of all tracts (complete) or some tracts (eg, dorsal column for sensory axons ascending in the fasciculi gracilis and cuneatus) as these allow one to distinguish regenerating axons from axons that were spared or that sprouted a collateral.²¹ Cervical injuries are typically chosen to test therapies for improvement of forelimb movement while thoracic injuries are usually selected to evaluate therapies for improvement of hindlimb movement.

In the USA alone, a large number of people live with existing spinal cord injuries and a substantial number receive injuries each year (prevalence ~250,000; incidence ~11,000²²). Different temporal opportunities to intervene therapeutically therefore exist. Thus the time between the injury and the time of treatment and of evaluation in an animal model is of critical importance. 'Acute,' 'subchronic,' and 'chronic' have all been used to describe postinjury

phases, but here we prefer to specify precise numbers of hours, days, months, or years. The majority of animal studies involve interventions within minutes, hours, or days of injury. There are far fewer studies reporting improvements in CNS axon regeneration or functional outcome after intervening more than one month postinjury²³⁻²⁸ and this imbalance requires much redressing if CNS axon regeneration is to be promoted in individuals with long-standing injuries. Additionally, the majority of studies only assess outcomes over a period of weeks or months. Relatively few studies that report improvements in CNS axon regeneration and functional recovery go on to determine whether these changes remain stable beyond 2 or 3 months.

Data from animal models show that only a small percentage of CNS axons need to be spared to mediate reasonable locomotion. Partial locomotion on a treadmill is possible following lesions that spare only 5% to 10% of axons in adult mammalian thoracic spinal cord.^{29,30} For example, following spinal cord compression in the adult cat, remaining nerve fibers became myelinated by Schwann cells and were able to support spontaneous walking.³¹ These data are encouraging as they suggest that only a small percentage of axons need to regenerate and form functional connections to restore useful function.

It is important to bear in mind that rodent models only approximate normal human locomotion and dysfunction after SCI. Bipedal locomotion (humans and birds) differs radically from quadrupedal locomotion (rodents and most mammals) or partially upright locomotion (nonhuman primates) in terms of posture, kinematics, physiology, and anatomy. Further, whereas many studies show that rodent spinal cords respond to injury in ways similar to that of the human,^{32,33} it is clear that there are differences in responses to SCI between species and even between strains of a given species.³⁴ For example, whereas many human spinal cord injuries become cystic or cavitated, very little cavitation occurs following contusion injury to many strains of adult mice.³² The histopathological response of the primate spinal cord to injury has been studied only relatively little, including after injection of autologous fibroblasts into otherwise uninjured rhesus monkeys³⁵ and after contusion injury in rhesus monkeys.³⁶⁻³⁸ This situation is likely to improve since a graded model of contusion injury in marmosets has recently been developed³⁹ and a number of groups are now testing potential therapeutics in nonhuman primates (see below). Given the differences between rodent and primate nervous systems, it seems reasonable to test pro-regenerative therapies in nonhuman primates for safety and efficacy. The paucity of safety and efficacy studies using nonhuman primates makes it somewhat remarkable therefore that so many clinical studies and trials are in progress.⁷

In summary, researchers who test therapeutics designed to improve function after SCI select animal models that simulate particular aspects of the human pathology. Animal models indicate that axon regeneration by a small percentage of injured CNS neurons may be sufficient to restore par-

tial function. As will be reviewed next, factors intrinsic and extrinsic to neurons limit CNS axon regeneration.^{40,41} Modulation of these intrinsic and extrinsic factors can promote CNS axon regeneration and recovery of function in animal models of SCI. As will be described, these successes are leading to clinical tests and trials.

EXTRINSIC FACTORS

Transplants of peripheral nerve promote CNS axon regeneration and functional repair after SCI

Axon regeneration and functional recovery occurs spontaneously (if incompletely) after injury to the adult mammalian PNS. Early experiments therefore attempted to promote CNS axon regeneration by transplantation of PNS segments into the mammalian CNS. Advances in retrograde tracing techniques allowed the unambiguous demonstration that neurons in many (but not all) different regions of the CNS can regenerate axons into PNS grafts.⁴² Transplants of peripheral nerves as a strategy for promoting CNS axon regeneration and functional repair are in part attractive because autologous grafts reduce the risk of immune rejection and obviate the consequent need for immunosuppression.

There are limits, however, on the ability of PNS grafts to promote axon regeneration and functional repair after SCI. In rat models of SCI, supraspinal axons (eg, from the red nucleus and from cortex) do not extend axons into peripheral nerve grafts placed into thoracic sites of SCI.⁴³⁻⁴⁵ Further, CNS axons that do extend into peripheral nerve grafts rarely exit grafts distally to form synapses or to restore function unless routed directly to appropriate targets.⁴⁶

As a result of these limitations, combination strategies have been tested and found to enhance long distance CNS nerve regeneration and motor recovery following SCI and implantation of peripheral nerve. For example, in adult rats, following creation of a gap in the dorsal columns, transplants of peripheral nerve support regeneration of the central branch of sensory axons from lumbar dorsal root ganglia (DRG)⁴⁷ and some of these axons grow out of grafts when growth factors are infused rostrally.^{48,49}

In several studies, multiple segments of intercostal peripheral nerve have been transplanted autologously following complete thoracic transection and creation of a 5 mm gap. Nerves were routed from white matter to distal gray matter and secured using fibrin glue containing acidic fibroblast growth factor. In studies using adult rats, corticospinal (CST) axons regenerated through and beyond the nerve bridges⁵⁰ and hindlimb functional recovery was reported.⁵¹ Importantly, this work recently has been reproduced and extended independently. In one study, somatosensory and motor cortex-evoked potentials demonstrated recovery of electrophysiological connectivity from motor cortex to hindlimb muscles and from sciatic nerves to sensorimotor cortex.⁵² Extent of recovery in hindlimb function in the open field correlated positively with motor evoked potential amplitudes in hindlimbs. In some cases, weight supported stepping was reported. Transection of

grafts 6 months after implantation abolished electrophysiological and behavioral recovery, interpreted to mean that functional improvements were dependent on long tract regeneration. In another study, axons from the cortex and brainstem nuclei (including vestibular, red and raphe nuclei, and reticular formation) were also reported as regenerating beyond transplants into the distal spinal cord, accompanied by improvements in hindlimb function including instances of weight supported hindlimb stepping.⁵³

Using a similar strategy, peripheral nerves have been grafted autologously into nonhuman primates (cynomolgous monkeys) after lateral spinal hemisection²⁰ with evidence for regeneration of at least some spinal axons at 4 months postinjury. No functional differences were detected between control and experimental monkeys in this study.

This approach also has been used to treat one human case of clinically complete SCI, with a report of limited functional recovery.⁵⁴ This study did not include control subjects and therefore additional work remains to be done to determine whether therapies involving peripheral nerve bridge grafting safely and effectively improve outcome after SCI in humans.

Transplants of Schwann cells promote CNS axon regeneration and functional repair after SCI

Axon regeneration through peripheral nerves is largely due to the presence of growth-promoting Schwann cells (SCs).⁵⁵ While grafts of peripheral nerve are suited to bridging regular gaps in the injured human spinal cord, many of the cysts and cavities that often form after SCI are irregularly shaped and below the cord surface, and therefore less amenable to bridging with segments of peripheral nerve. Purified SCs have therefore attracted attention as candidates for promoting CNS axon regeneration and functional repair after SCI because they can be transplanted as suspensions. Additionally, use of mitogens allows rat or human (but, to date, not mouse) SCs from small biopsies of adult peripheral nerve to be dramatically expanded in culture⁵⁶ to produce sufficient numbers to bridge large gaps in the spinal cord.⁵⁷

Purified populations of rat SCs from peripheral nerve have been transplanted into rat models of SCI, injected as suspensions 1 week after contusion injury,⁵⁸ or implanted within channels containing extracellular matrix (Matrigel or fibrin) immediately following lateral hemisection⁵⁹ or complete transection.¹⁸ After transection and implantation of SCs, sensory and spinal axons with cell bodies near the grafts extend axons into these bridge grafts, are myelinated,¹⁸ and are electrophysiologically active.⁶⁰ After contusion and implantation of SCs, less cavitation is observed and sensory and spinal axons extend axons into grafts, and are myelinated.⁵⁸ In the transection model, regenerating axons do not leave the grafts distally to reinnervate the host: combination therapies including SCs have therefore been the subject of investigation.

Various combinations of therapies have been reported to enhance long distance CNS nerve regeneration and

recovery of hindlimb function following SCI and implantation of Schwann cells. Following transection of adult rat thoracic spinal cord, SCs have been transplanted with delivery of growth factors including brain derived neurotrophic factor (BDNF)⁶¹ or BDNF with neurotrophin-3 (NT-3).¹⁷ The SCs have also been transplanted in combination with anti-inflammatory steroids (methylprednisolone),⁶² and with other regeneration-promoting cells including olfactory ensheathing glia (OEG).⁶³ Increased regeneration of CNS axons into and beyond bridges was reported in several of these studies. Following thoracic contusion in adult rats, SCs compared to OEG were found to be less effective than SC-only transplants.⁵⁸

Purified populations of human SCs from peripheral nerve have also been transplanted into the injured rodent spinal cord (within channels containing Matrigel).⁶⁴ To avoid rejection of grafted cells, nude rats (that have attenuated immune systems and were treated with methylprednisolone) were used as hosts. Under these conditions, regeneration of brainstem-spinal axons into Schwann cell grafts was observed and propriospinal neurons regenerated up to 2.6 mm distal to grafts. Mild functional improvements were also reported, including extensive movements of ankles, knees, and hips (sweeping without weight bearing) and occasional weight bearing in stance. Weight-supported stepping was only observed in a single rat.

To date, SCs have not been transplanted into humans following SCI. A number of hurdles remain. Since transplanting SCs alone affords only a small level of recovery of function, it remains important to find the most effective and reproducible combination therapy involving SCs. It will also be important to ensure that transplantation of SCs is safe. Although mitogen stimulated human SCs do not form tumors after implantation into mouse PNS, it will be important to check this after implantation into the injured spinal cord.⁶⁵ One important step towards human clinical trials would be to test the safety and efficacy of transplanting autologous SCs into nonhuman primates following contusive SCI.³⁹

Transplants of OEG promote CNS axon regeneration and functional repair after SCI

Central nervous system axon regeneration and functional recovery also has been reported when OEG are transplanted immediately after SCI⁶⁶⁻⁶⁹ or after a delay of 4²⁶ or 8 weeks.²⁷ In one study using adult rats, following lateral hemisection of the cervical spinal cord, injection of OEG improved respiratory function and enhanced performance on a climbing task.⁶⁹ In other studies using adult rats, following thoracic transection and implantation of OEG into spinal cord stumps, regeneration of long descending tracts important for locomotion has been reported,^{26,68} even up to 2.5 cm distally, for serotonergic axons.⁷⁰ Recovery has also been reported in adult rats after transection and implantation of OEG when testing hindlimb function using an inclined plane.⁷⁰ Between 3 and 7 months post-transplantation, many transplanted rats were capable of propelling

their bodies up a wire grid inclined at 45° and onto a horizontal platform at the top.

Primate⁷² and human OEG⁷³ derived from olfactory bulb or lamina propria, are now being tested in rodent and non-human primate models of SCI and demyelination.⁷⁴ Over 500 transplants of cells from human fetal olfactory bulbs have been performed in humans in China with reports of modest rapid improvement in function.⁷⁵ Because in many cases mild recovery of function is reported to occur within 1 or 2 days, it undoubtedly does not depend upon long distance regeneration of CNS axons but perhaps on secretion of trophic factors or induction of local changes in circuitry. To date, comprehensive follow-up studies have not been performed, so it is difficult to gauge the long-term safety and efficacy of this intervention.⁷

Do transplants of immune/inflammatory cells promote or impair CNS axon regeneration and functional repair after SCI?

Spontaneous axon regeneration in the PNS also has been partly attributed to the abundance of inflammatory cells including macrophages. Macrophages in the CNS derive predominantly from resident microglia but also come from blood-borne monocytes.⁷⁶ Monocytes enter the CNS more slowly and in fewer number than in the PNS and they differ phenotypically from monocytes that have entered the PNS.^{77,78} For these reasons, it has been hypothesized that transplants of monocytes preactivated by exposure to PNS tissue may boost CNS axon regeneration and functional recovery in animal models of SCI.

In one study, homologous monocytes, activated by preincubation with PNS tissue, were implanted immediately following transection of adult rat thoracic spinal cord, resulting in improved CNS axon regeneration.⁷⁹ Partial recovery of hindlimb movements was observed during open field locomotor testing. Hindlimb muscle responses were also evoked by cortical stimulation. Finally, loss of function after re-transection was taken to indicate a dependence of recovery upon axon regeneration. In a more recent paper, monocytes were activated by preincubation with skin, and functional recovery was reported following implantation into a contusion injury model in adult rats.⁸⁰

In stark contrast to these findings, several studies indicate that inflammatory cells aggravate outcome after SCI. Subpopulations of macrophages within the injured spinal cord are found in regions that subsequently cavitate⁸¹ and intraspinal injection of a macrophage activator (zymosan) worsens cavitation.⁸² Finally, pharmacological depletion of macrophages at 1, 3, and 6 days after injury (using clodronate) actually *improves* functional recovery after contusion injury in adult rats.⁸³ These data argue that transplantation of inflammatory cells risks worsening the original injury and may increase disability after SCI.

Transplantation of inflammatory cells for treatment of SCI is therefore extremely controversial. Replication and within-experiment comparison of these rodent studies by

independent laboratories would be extremely valuable. Testing of autologous (not allografted), activated monocytes would also be informative. To date, we know of no studies investigating the safety or efficacy of transplanting immune or inflammatory cells into injured nonhuman primate spinal cord. Despite this, clinical trials involving transplantation of autologous, activated monocytes are currently in progress, supported by Proneuron Biotechnologies.⁷

Transplants of embryonic/fetal spinal cord promote CNS axon regeneration and motor recovery after SCI

Fetal spinal cord transplants have been shown to promote regeneration of CNS axons following resection lesions of adult rat spinal cord and in contused adult rat and cat spinal cord. A variety of host axons from distances up to 6 mm away grow short (<500 μ m) distances into, and innervate, the grafts including serotonergic and coeruleospinal axons.⁸⁴ Innervation of the host tissue by transplanted neurons is also observed. Despite the attenuation in scar formation induced by transplantation of immature tissue, the number of axons to cross the host/graft interfaces in either direction is usually relatively small. Modest but significant functional recovery is observed following transplantation of immature tissue in rats⁸⁴ and in cats 1 to 3 months after contusion SCI.⁸⁵ For example, although there was no improvement on inclined plane or grid walking up to 3 months postimplantation, 2 indices of gait analysis (base of support and stride length) showed statistically significant improvement.^{86,87} Improvements in locomotion were also observed following delayed transplantation of immature tissue into contusion injured adult cat spinal cord and electrophysiological evidence suggested ascending host axons made synapses with graft neurons.⁸⁸ However, recovery does not appear to depend on long distance growth into, through and beyond grafts and the authors of those studies speculated that transplants behave as relays, affording transmission of signals via the transplanted neurons which are innervated by proximal host neurons and project in turn to distal host neurons. Grafts may also provide growth factors or may improve conduction in spared axons, perhaps by remyelination or neuroprotection.⁸⁹

Transplants of embryonic tissue may also enhance function of spared circuitry caudal to the lesion thereby improving local somatic and visceral responses. One study demonstrated that serotonergic neurons from the embryonic raphe nucleus grafted caudal to a complete cord transection in the adult rat influenced penile erectile function⁹⁰ and enhanced weight support and treadmill locomotion in spinal rats.⁹¹

Since grafts of fetal/embryonic tissue promote recovery but without concomitant long distance axon regeneration beyond the graft/host interface, co-treatment strategies have been evaluated. In one study, fetal tissue transplanted 2 to 4 weeks after injury, when combined with neurotrophin delivery, promoted regrowth of axons caudal to the transection site, resulting in plantar foot placement and weight-supported stepping in adult rats.⁹²

Fetal cell grafts slightly reduce neurological dysfunction following human SCI.⁸⁹ In one study, 41 patients with clinically complete thoracic spinal cord lesions and progressive syringomyelia received multiple subpial grafts of human neocortex from 8 to 9 week old fetuses usually within 6 to 12 months of injury (cited in reference 89). A trend towards improved neurological outcome over a few dermatomal levels was observed relative to patients who had laminectomy, durotomy, and lysis of adhesions. There were no reports of morbidity, mortality, or increased pain. However, it should be noted that as yet, there is no available evidence for graft survival or CNS axon regeneration in humans. Fetal cell grafts have also been used in an attempt to prevent additional loss of function in cases of progressive syringomyelia and to date there appear to have been few negative outcomes associated with transplantation.^{93,94} Therapeutic strategies involving tissue derived from human embryos or fetuses remain ethically controversial and therefore clinical trials are additionally challenging to conduct.

Transplants of progenitor cells may improve motor recovery following SCI

Multipotent progenitor cells (cells that have the capacity to differentiate into *many* different cell types) and stem cells (cells having the capacity to self-renew indefinitely and differentiate into *any* cell type) appear to exist in adults as well as in embryos and these may have the potential to replace cells of different types that die after SCI. Since it is in practice difficult (if not impossible) to establish that a precursor cell is capable of indefinite division and of differentiation into all possible phenotypes, here we prefer to use the term progenitor. Transplants of purified progenitor cells from embryos and adult tissues are now being tested in animal models of SCI.⁹⁵ The following cell types are among those that have been transplanted in animal models of SCI: human umbilical cord progenitors,⁹⁶ bone marrow stromal cells,⁹⁷ murine cell-line cells,⁹⁸ and populations of multipotent cells derived from human fetal brain⁹⁹ and human embryonic spinal cord.¹⁰⁰

To date, however, where multipotent cells promote recovery of function in animal models of CNS injury, axons do not appear to regenerate through and beyond grafts.^{96,98} One study has shown that immortalized neural progenitors promote PNS axon growth following SCI.¹⁰¹ Alternative explanations for recovery of function include cell replacement, creation of new neuronal relays, myelination, neuroprotection, and/or plasticity.^{102,103}

Researchers also have recently begun investigating transplants of multipotent cells with restricted fates. Grafts of glial-restricted precursors promote mild ingrowth of CST axons.¹⁰⁴ Grafted neuronal-restricted precursors extend axons *de novo* after grafting into the intact spinal cord¹⁰⁵ although differentiation into mature neurons appears to be inhibited after grafting into the contusion-injured spinal cord.¹⁰⁶

Recently, neural progenitors derived from 8 week old human fetuses have been transplanted into immunosuppressed nonhuman primates 9 days following cervical contusion injury.¹⁰⁷ There was a statistically significant difference in spontaneous locomotion within the cage and in forelimb grip power between marmosets transplanted with cells relative to those transplanted with culture medium only. Eight weeks following transplantation, histopathology revealed reduced cavitation but no evidence of tumor formation. Some surviving grafted cells expressed markers characteristic of neurons or glia and there was evidence for some PNS or CNS axon ingrowth.

Autologous transplants of progenitor cells remain to be tested in rodent or primate models of SCI. Future experiments using animals may also show whether it is possible to induce regeneration and repair by stimulating the response of *endogenous* adult progenitors. Finally, several clinical studies are attempting to evaluate whether transplantation of progenitor cells improves outcome after human SCI (eg, cells from bone marrow).⁷

Delivery of growth factors promotes CNS axon regeneration and motor recovery after SCI

Growth factors potentially promote axon regeneration during nervous system development and after PNS injury. Following contusion or transection injury to the adult rat spinal cord, however, message levels for many growth factors remain almost undetectable (including neurotrophins 3 and 4), or only briefly and slightly upregulated (including nerve growth factor (NGF) and glial cell line-derived neurotrophic factor).¹⁰⁸ After SCI, message levels for neurotrophin receptors on neurons remain absent (trkA), unaltered (trkB, trkC), or only slightly upregulated (p75).¹⁰⁸ Brain derived neurotrophic factor may not be available for signaling through its high affinity receptor (trkB) since there is a large, fast, and sustained upregulation of competitor binding sites (truncated trkB receptors) that may dominate as non-functional sinks for the active neurotrophin.¹⁰⁹ In summary, after SCI, CNS neurons may lack the ability to respond to generally low levels of most growth factors.

Consequently, to promote CNS axon regeneration and functional recovery after SCI, supplementary growth factors have been delivered to neuronal cell bodies (eg, in brainstem or cortex) or SCI sites by direct injection,¹¹⁰ by osmotic minipump,¹⁷ by application within fibrin glue,¹¹¹ or by gene transfer techniques.^{61,112-114} Delivery of growth factors can enhance long distance anatomical, electrophysiological, and behavioral recovery of CNS axons *in vivo*. For example, following dorsal hemisection lesions of adult rat spinal cord and immediate implantation of NT-3-secreting fibroblasts, CST axons grew up to 8 mm distal to the injury site with reduced locomotor deficits on a gridwalk task 1 and 3 months postinjury.¹¹³ Following cervical lateral hemisection and implantation of BDNF-expressing fibroblasts (together with methylprednisolone and immunosuppression), up to 7% of rubrospinal neurons regenerated beyond

the transplant site.^{112,114} Forelimb use was also partially restored, and more so when transplants were placed early after injury rather than when delayed 6 weeks.²⁸

Although many neurons die after axotomy, severely atrophied neurons can be rescued. In one study, severe atrophy of rubrospinal neurons could be reversed by applying BDNF to the cell bodies up to one year after injury.²⁵ Further, this treatment promoted the regeneration of these rubrospinal axons into peripheral nerve transplants engrafted at the cervical SCI site.

Growth factors have also been delivered to the nonhuman primate spinal cord using autologous fibroblasts genetically modified to produce NGF.¹¹⁵ Three months after implantation into rhesus monkeys, sensory and putative coeruleospinal axons were found to regenerate into NGF-secreting grafts. Since these monkeys had not been given deliberate spinal cord injuries, further work is required to show whether delivery of growth factors promote CNS axon regeneration and functional recovery in injured nonhuman primates.

Disappointingly, clinical trials using systemic delivery of high doses of neurotrophins for various nervous system disorders have run into a number of difficulties, including induction of side effects including severe muscular pain, fever, depression, and hallucinations.¹¹⁶ Targeted delivery of physiological doses of growth factors (eg, by using viral vectors) may prove safer and more efficacious.

Scar tissue modifiers enhance CNS axon regeneration and functional recovery after SCI

Extrinsic factors that limit CNS axon regeneration also include factors associated with myelin and the injury site. Spinal cord injury sites, particularly those resulting from penetrating injury, are also typically filled with multiple layers of fibroblasts. This scar tissue represents a formidable physical barrier to CNS axon regeneration,¹¹⁷ the cells often being oriented perpendicular to the neuraxis (appearing geometrically impenetrable) and containing molecular inhibitors of growth including chondroitin sulfate glycosaminoglycans (CS GAGs).^{40,118}

In adult rats, degradation of CS GAGs by delivery of the bacterial enzyme, chondroitinase ABC, promotes regeneration of injured CNS axons¹¹⁹ and recovery of function after dorsal hemisection.¹²⁰ Following spinal cord hemisection in adult rats, delivery of chondroitinase ABC also promotes regrowth of axons from spinal cord neurons into grafts of peripheral nerve¹²¹ and regrowth of CNS axons into spinal cord beyond hemichannel bridges containing SCs and Matrigel.¹²² Following complete transection and implantation of channels containing SCs and Matrigel, delivery of chondroitinase ABC and immunoglobulin promotes regeneration of serotonergic axons beyond grafts.¹²³ Repeated intrathecal delivery of chondroitinase ABC also promotes hindlimb recovery following severe (but not moderate) thoracic contusion injury in adult rats.¹²⁴

A number of safety and delivery issues remain to be tackled. Delivery of bacterial proteins to humans carries a risk of nonspecific immune responses. Further, repeated dosing (as is typically performed in animal models of SCI using chondroitinase ABC) may not be possible if the immune system develops responses to neutralize these foreign proteins. Thus efforts are underway to develop smaller, less immunogenic yet enzymatically active variants of chondroitinase ABC. Human enzymes (eg, hyaluronidases or matrix metalloproteinases) that cleave CS GAGs might also avoid these side effects. Encouragingly, a preparation of chondroitinase ABC is currently being tested in Phase 2 clinical trials for herniated lumbar discs¹²⁵ and FDA approval for this therapy might accelerate clinical trials for SCI. In due course, chondroitinase ABC should be tested for safety and efficacy in nonhuman primate models of SCI.

Therapies that target myelin inhibitors promote CNS growth and functional recovery

Intact and injured CNS myelin contains a number of different growth inhibitory molecules.¹²⁶ These include Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp). Various therapeutics have been developed to target and overcome these inhibitors of axon growth, most notably the antibody IN-1, which binds to Nogo-A and neutralizes its inhibitory effects.¹²⁶

The CNS axon growth and recovery of limb function have been reported in various animal models of SCI following delivery of anti-Nogo therapeutics. In the original experiments, small numbers of adult rat CST axons were observed approximately 1 cm beyond sites of thoracic dorsal hemisection following implantation of hybridoma cells secreting IN-1.^{19,127} Further, between 7 and 12 weeks postlesion, low threshold stimulation of hindlimbs enhanced the incidence of paw placing responses in 8 of 10 animals treated with IN-1 antibodies but in no control animals, and this effect was abolished by bilateral sensorimotor cortex ablation.¹²⁸ Administration of IN-1 antibodies also potentiated CST axon growth after dorsal hemisection in adult rats and implantation of embryonic tissue¹²⁷ or following injection of NT-3 either immediately after SCI¹²⁹ or after a delay of 2 or 8 weeks.²⁴ IN-1 also induced CNS axons to sprout collaterals, for example, following unilateral injury of the CST at the level of the brainstem.^{130,131}

Recently, and promisingly, IN-1 has been shown to promote growth of CST axons following unilateral lesioning in 4 out of 5 marmoset monkeys tested.¹³² Recombinant, humanized fragments of IN-1 and immunoglobulin G class antibodies against Nogo-A have so far been tested in 2 rat stroke models¹³³ and in a dorsal hemisection model of SCI.¹³⁴

To date, however, no reports exist of testing anti-Nogo therapies in contusion or compression models of SCI. This is important because anti-Nogo antibodies apparently do not promote long distance CNS axon regeneration in all models tested,^{130,131,135} particularly in larger injuries such as

those involving transection.^{111,136} It will also be important to evaluate whether anti-Nogo strategies have any adverse outcomes in animal models of SCI. One study indicates no increase in tail flick (interpreted as no increase in nociception) 5 weeks following dorsal overhemisection and delivery of IN-1 in adult rats.¹³⁷ Clinical trials using antibodies against Nogo-A are currently being planned in association with Novartis.

A peptide inhibitor (NEP1-40) targeting a receptor for Nogo-A (NgR) has also been developed¹³⁸ in order to prevent neurons from responding to this inhibitor. Remarkably, *subcutaneous* treatment with NEP1-40 one week after thoracic dorsal hemisection promotes growth of CST axons and serotonergic fibers and a degree of locomotor recovery.¹³⁹ Intrathecal delivery of NEP1-40 also promotes regrowth of rubrospinal axons and a degree of functional recovery after rat SCI.¹⁴⁰ Biogen Idec Inc. holds a license for NgR-related therapeutics and clinical trials may follow.

INTRINSIC FACTORS

Cyclic adenosine monophosphate

Various strategies exist to boost CNS axon regeneration by targeting molecules intrinsic to neurons. For example, levels of cyclic adenosine monophosphate (cAMP) are higher in young, growing neurons than in older neurons. Levels of cAMP are low in neurons in a state of growth arrest, such as those cultured on growth-inhibitory MAG, and delivery of cAMP analogs relieves this growth-arrest *in vitro*.¹⁴¹

To test the hypothesis that elevation of cAMP boosts CNS axon regeneration in adult rats, cAMP analogs have been injected into adult rat DRG one week prior to thoracic dorsal column hemisection. In 2 studies, centrally projecting sensory axons regrew better within injury sites.^{142,143} Combining strategies, preinjury administration of cAMP with postinjury delivery of NT-3, promotes regeneration of sensory axons within and beyond transplants of bone marrow stromal cells.¹⁴⁴ In these studies, however, it should be noted that administration of cAMP or NT-3 alone did not promote regeneration of axons beyond grafts.

Since prophylactic treatments for SCI are inappropriate, postinjury delivery of drugs that boost cAMP levels are being tested. Postinjury delivery of a cAMP analog promotes CNS axon regeneration after SCI in fish.¹⁴⁵

A fall in the levels of endogenous cAMP after SCI in adult rats can be prevented by delivery of the phosphodiesterase inhibitor, rolipram, which prevents the hydrolysis of cAMP.¹⁴⁶ After cervical hemisection in adult rats, delivery of rolipram promotes growth of serotonergic axons into fetal tissue transplants and enhances placing of the impaired forelimb during rearing.¹⁴⁷ After spinal cord contusion, adult rats injected intraspinally with SCs and a cyclic AMP analog together with subcutaneous delivery of rolipram exhibited white matter sparing, increased numbers of spinal and supraspinal axons beyond the injury/transplant site and improved locomotion.¹⁴⁶

Prior to human clinical tests or trials, solo or combination methods for elevating cAMP levels after injury need to be evaluated for safety and efficacy, at least in rodent models of SCI and preferably also in nonhuman primates. Therapeutic windows of delivery of cAMP analogs need to be defined and doses and methods of delivery established.

Modulation of small enzymes (GTPases) boosts CNS axon regeneration and recovery of function after dorsal hemisection

Many different extrinsic factors that impact upon axon regeneration have common final pathways, many signaling intracellularly via small enzymes that interact with guanosine triphosphate (GTP; a chemical compound that is used as a source of energy during synthesis of proteins). This has been shown for CS GAGs as well as MAG, OMgp, and Nogo-A.^{148,149} These GTPases include Rho and Rac family members. Injured spinal cords of adult rats contain increased levels of message for at least 5 different small GTPases including RhoA, RhoB, and Rac1.¹⁵⁰ Rho and Rac GTPases generally act antagonistically; while Rho family members retard growth, Rac family members promote neurite growth *in vitro*.¹⁵¹ Consequently, modulators of small GTPases have been evaluated as methods for promoting CNS axon regeneration and functional recovery in animal models of SCI.

Rho GTPase protein is activated after SCI and inhibition of Rho (using C3 botulinum toxin) promotes CNS axon regeneration and a degree of functional recovery following dorsal hemisection injury in adult rats.¹⁵² Rho kinase acts as a downstream effector of Rho, and pharmacologic inhibition of Rho kinase promotes CNS axon regeneration following dorsal hemisection in adult rats.¹⁵³

Improved cell-permeable inhibitors of Rho are currently being tested. For example, BioAxone Therapeutic Inc. is developing technologies to target Rho signaling. Strategies to stimulate Rac *in vivo* remain to be developed. To date, activators of Rac and inhibitors of Rho and Rho kinase have neither been tested in rodent models of contusion injury nor in any nonhuman primate models of SCI.

Other inhibitors of CNS axon regeneration intrinsic to neurons likely exist, and with the advent of molecular knockdown methods including RNA interference, attempts to promote CNS axon regeneration through targeting of regeneration-inhibiting genes and proteins may become possible.

Overexpressing regeneration-promoting molecules in neurons promotes CNS axon regeneration

As axons elongate during development, PNS and CNS neurons express a range of genes¹⁵⁴ including growth associated protein 43 kDa (GAP-43) and cortical cytoskeleton-associated protein 23 kDa (CAP-23). On maturation, most neurons downregulate expression of these genes. After injury to the PNS, neurons that re-extend axons re-express many of these "regeneration-associated" genes.¹⁵⁵⁻¹⁵⁷ Injured CNS neurons do sprout processes locally, and this is associ-

ated with mild expression of a limited set of regeneration-associated genes,¹⁵⁸ particularly when injury occurs close to the cell body. CNS neurons that regenerate axons into transplants of peripheral nerve express various regeneration-associated genes¹⁵⁸⁻¹⁶¹

In general, however, after injury to the CNS, neurons fail to re-express the majority of these genes, and do not regenerate long distances. For example, following peripheral transection, DRG neurons express GAP-43 in their regenerating peripheral projections but not in their nonregenerating central projections following central axotomy in dorsal columns.¹⁶² It is therefore encouraging that overexpression of regeneration-associated genes causes injured CNS neurons to regenerate axons. For example, simultaneous overexpression of GAP43 and CAP23 in transgenic mice causes enhanced neurite growth from explanted DRG *in vitro* and boosts regeneration of centrally projecting axons from lumbar DRG into peripheral nerve grafts implanted in thoracic spinal cord.¹⁶³

Experiments overexpressing regeneration-associated genes in CNS neurons in nonhuman primates have not yet been performed and to our knowledge, therapies specifically designed to boost expression of pro-regenerative genes in neurons after SCI are not yet being considered for human clinical trial.

A number of hurdles remain. In the combination strategy described above,¹⁶³ relatively few neurons regenerated and there was no report of testing for recovery of function. Future experiments may identify additional, more potent regeneration-promoting genes. For example, novel regeneration-associated genes have been identified in neurons during PNS¹⁶⁴ and CNS regeneration. In addition, we have recently identified a novel set of regeneration-associated genes that are expressed by injured spinal neurons that regenerate into grafts of SCs placed in transected adult rat spinal cord.¹⁶⁵ Future experiments are underway to identify genes that potently promote neurite growth in cell culture systems. Targeted upregulation of these growth-promoting genes (eg, using viral vectors) may prove effective in promoting CNS axon regeneration and functional recovery in animal models of SCI.

Physical therapy enhances recovery of function after SCI

Although we know of no evidence yet that physical therapy or rehabilitation directly improves CNS axon regeneration after SCI, environmental enrichment, physical therapy, and motor training have been shown to improve limb function in animal models of SCI (including after transection¹⁶⁶ and contusion¹⁶⁷⁻¹⁶⁹) and in humans with SCI.¹⁷⁰⁻¹⁷³

To date, very few studies have combined rehabilitation/physical therapy with strategies for promoting CNS axon regeneration and recovery of limb function. In one recent study from our laboratory, following transection of adult rat thoracic spinal cord, OEG were transplanted with or without SC bridges.⁷¹ Motor activity was encouraged

through provision of motor enrichment housing (MEH): a large, multilevel cage filled with ramps, textured surfaces, and hanging food rewards. The return of hindlimb joint movement was assessed weekly for 22 weeks starting 1 week postinjury and was compared between animals housed in MEH and those maintained in basic housing (BH). All rats recovered a small amount of hindlimb movement, but recovery was not accelerated or enhanced by MEH. Recovered hindlimb movements were, however, sustained up to 22 weeks postinjury in most rats in MEH, whereas most rats kept in BH progressively lost recovered hindlimb movements after 9 weeks. Furthermore, MEH decreased mortality, and improved health. We conclude that motor rehabilitation can be of substantial benefit when using transplantation strategies to treat SCI.

In the future it seems very likely that rehabilitation/physical therapy will play a key role in many strategies designed to promote CNS axon regeneration and recovery of limb function. These combination strategies will likely first be tested in rodent, then primate models of SCI, and then, if safe and efficacious, in human clinical trials.

TOWARDS THE FUTURE

In cases of anatomically complete injury in animal models, the degree of recovery of hindlimb movement obtained by any single transplant therapy remains functionally slight. In general, following transection and intervention of any kind in adult rats, plantar placement and weight support on hindlimbs during stepping is not consistently observed. This includes: (1) transplantation of glia obtained from nasal mucosa or olfactory bulb;^{26,68} (2) transplantation of activated monocytes;⁷⁹ (3) implantation of peripheral nerve grafts secured across transection gaps with fibrin glue containing acidic fibroblast growth factor;⁵¹ and (4) transplantation of OEG, with or without SC bridges;⁷¹ and (5) implantation of SC bridges and OEG with delivery of chondroitinase ABC.¹²³ To our knowledge there exists only one study that reports frequent plantar placement and weight-supported stepping in adult rats with transection injuries.⁹² This study utilized delayed transplantation of fetal tissue in combination with neurotrophin delivery.

In cases of anatomically incomplete injury, the degree of recovery witnessed in the open field is typically modest and most studies remain to be reproduced independently.¹⁷⁴ Replication is extremely desirable in order to determine the general applicability of a therapy. Incomplete injuries are notoriously variable and animals should be randomized to treatment group, treated blind and evaluated blind. Where possible, baseline testing should be performed prior to intervention to ensure that groups do not differ one from another initially. Where axon regeneration is evaluated, stringent criteria should be applied to discriminate this from sprouting or sparing.²¹

Many claims have been made as to the *particular* reparative potential of transplants of a given cell type, including SCs, OEG, monocytes, genetically modified fibroblasts, and

multiple types of progenitor cells. In many cases these claims have been made relative to controls that were not transplanted with cells, but relative to injection of medium.^{63,107} Very few studies have directly compared different cell types within a single experiment,⁵⁸ so it is very difficult to know which cell type affords particular benefits over others. In the future, additional control or comparison groups should be included such as transplants of other cell types.

Worryingly, some pro-regenerative therapies have been shown to promote growth of sensory axons within the PNS or CNS. This includes progenitor cells,¹⁷⁵ neurotrophin therapies,¹⁷⁶ anti-inhibitor therapies,¹¹⁰ and regeneration-gene overexpression therapies.¹⁶³ In light of this, rigorous testing for changes in nociception should take place in animal models of SCI before moving to the human patient. This is especially important as many humans with SCI rate neurologic pain as one of the worst sequelae of SCI.¹⁷⁷ Great care should therefore be taken to ensure that any clinical therapy does not induce neurological pain as a side effect.

CODA

Increasing numbers of experimental studies using animal models of SCI are resulting in reports of improvements in CNS axon regeneration and recovery of limb function. Combinations of these strategies in animal models and in humans may lead to additive improvements in outcome after SCI. Many of these combination strategies may involve rehabilitation measures and, as clinical tests and trials proceed, it seems likely that the demand for physical therapists with a good working knowledge of pro-repair strategies for SCI will increase.

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