Molecular and Cellular Mechanisms of Axonal Regeneration After Spinal Cord Injury*

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Following axotomy, a complex temporal and spatial coordination of molecular events enables regeneration of the peripheral nerve. In contrast, multiple intrinsic and extrinsic factors contribute to the general failure of axonal regeneration in the central nervous system. In this review, we examine the current understanding of differences in protein expression and post-translational modifications, activation of signaling networks, and environmental cues that may underlie the divergent regenerative capacity of central and peripheral axons. We also highlight key experimental strategies to enhance axonal regeneration via modulation of intraneuronal signaling networks and the extracellular milieu. Finally, we explore potential applications of proteomics to fill gaps in the current understanding of molecular mechanisms underlying regeneration, and to provide insight into the development of more effective approaches to promote axonal regeneration following injury to the nervous system. *Molecular & Cellular Proteomics 15: 10.1074/mcp.R115.053751, 394–408, 2016.*

During development, neurons extend axons throughout the nervous system, establishing connections with postsynaptic targets that are often located quite long distances away from their origin. The ability of these young neurons to robustly extend their axons is dramatically diminished in adulthood, and this reduced intrinsic growth capacity is a key mechanism underlying the inability of adult central nervous system (CNS)[1] neurons to regenerate their axons following injury (1–4). The injured adult CNS is a nonpermissive environment for axon outgrowth because of the abundance of inhibitory proteins and glycoproteins (5–7), together with a deficiency of adequate trophic support (8–10). In addition, intrinsic neuronal mechanisms initiating a growth program are limited in injured adult CNS neurons (11). Because of the failure of CNS axons to spontaneously regenerate, sensory, motor, autonomic, or cognitive deficits resulting from CNS injury are often permanent. Hence, there remains a great unmet need for therapeutic strategies to enhance regeneration of injured CNS axons and thereby improve function. Here, we will review key protein networks identified in the injured and regenerating axon and discuss progress in experimental approaches to promote repair of the injured nervous system.

Axonal Regeneration in the Peripheral Nervous System— Following axotomy, injured neurons of the peripheral nervous system (PNS) shift to a regenerative state. Injury signals are communicated to the soma, cell survival pathways are activated, and numerous regeneration-associated genes are upregulated. This injury-induced cascade of events includes neurochemical changes, functional alterations in excitability, growth cone reconstruction, local protein synthesis, and multiple signaling pathways to activate axonal regeneration. In this section, we will review molecular mechanisms that underlie axon regeneration following peripheral nerve injury.

Axotomy: Cellular and Molecular Events after Nerve Injury— An immediate influx of calcium occurs at an injured axon tip (12) that reaches above 1 mM in concentration (13). This drastic rise in intracellular calcium is necessary for triggering axon regeneration, as neurons in a calcium-free environment fail to initiate axon outgrowth (14, 15). A first-wave retrograde injury signal transmits to the cell body through a back-propagating calcium wave, which is thought to lead to chromatin remodeling (16). This is the first communication the injured tip has with the soma. Electron-micrograph images have shown that injury-induced vesicles occlude the cut ends of the axon, forming a plasmalemmal seal (17, 18). As the cytoskeleton undergoes reconstruction, the rate of sealing is dependent on calcium-regulated proteins such as synaptotagmin, syntaxin, and synaptobrevin (19). Following this initial burst of events, the soma undergoes chromatolysis, in which chromatin within the nucleus is dissipated and spread to the cell periphery as the cell body undergoes swelling. Excitatory inputs are mostly eliminated, leaving inhibitory inputs as the main communicators to the injured soma. This "synaptic stripping" is thought to involve glial cells and may prevent glutamate excitotoxicity (20).

[1] The abbreviations used are: CNS, central nervous system; PNS, peripheral nervous system; DRG, dorsal root ganglion; STAT, signal transducer and activator of transcription; HNF, hepatocyte nuclear factor; USF, upstream stimulatory factor; SILAC, stable isotope labeling by amino acids; NIF, neuroscience information network; ECM, extracellular matrix; CSPG, chondroitin sulfate proteoglycan; NCAM, neural cell adhesion molecule; PST, polysialyltransferase.

A second wave of retrograde signaling, thought to comprise the main injury signal, occurs by 4–6 h postinjury (21). The complete scope of the temporal communication between the injured axon and soma is still not fully understood (22). Local protein synthesis is necessary for the formation of the main retrograde signal, in which importins and vimentin are locally translated in the injured axon (23, 24). Perturbation of this signal attenuates the cellular response to injury (23, 24). The motor binding protein Jun-N-terminal kinase (JNK)-interacting protein JIP links axon vesicles to the injury signal and retrogradely transport back the injury signal along microtubules (25). As the membrane resting potential is restored, the axon may form either a retraction bulb or a new growth cone. Retraction bulbs are thought to be the nongrowing counterparts of growth cones, where growth failure is attributed to microtubule destabilization (26). Ertürk and colleagues showed that destabilizing microtubules both attenuated retraction bulb formation and axonal degeneration in PNS neurons, and enhanced the growth capacity of CNS neurons cultured on myelin (26). Within the axoplasm, calpain proteins are activated to cleave membrane spectrins, leading to brief proteolysis of the cytoskeleton (27). This is thought to restructure the microtubule ends, and is necessary for the transformation into a growth cone (28, 29). As microtubules repolymerize, a brief reorientation of polarities form. Vesicular transport resumes, and selective traps for anterogradely transported vesicles are established (30). For example, Golgi-derived vesicles can only undergo anterograde transport and as microtubules reorient themselves with plus-end out orientation in the axon, these vesicles remain trapped at the growth cone, allowing for growth cone reconstruction.

The newly formed growth cone begins to recycle material thereby generating an immediate supply, but local protein synthesis is ultimately necessary for growth cone formation (31). Synthesis of proteins within the injured axon is a crucial event underlying regeneration, and improper regulation of this process can cause growth deficits (32). Translation machinery and mRNAs are transported into the injured axon from the soma (33). Schwann cells have also been shown to transfer polyribosomes to the axoplasm of desomatized axons, suggesting that Schwann cells can modify protein expression of injured axons (34, 35).

After growth cone formation, injured axons start to regenerate or sprout. Earlier studies have indicated that axonal regeneration or sprouting is accompanied by activation of genes associated with developmental axon growth (36–38). Indeed, neurite outgrowth has been correlated with the ability to express regeneration-associated genes that are normally expressed during development (39); though there are distinctive regeneration programs associated with different modes of growth. Without a conditioning lesion, the dorsal root ganglion (DRG) neuron extends highly arborized neurites for a discrete period, a process that is dependent on new transcription. Within 24 h, this growth switches to elongation growth in which fewer, longer axons extend from the soma (40). In contrast, following a conditioning lesion, DRG neurons only exhibit elongation growth, which is dependent on translation for the formation of regenerating axons (41). Together, these findings show that different modes of growth are associated with different patterns of protein expression. Key studies have revealed the importance of coordinated regulation of protein expression through the activation of specific signaling networks during axonal outgrowth and regeneration (see Table I). This emphasizes a potential for combined manipulation of multiple signaling pathways to activate robust regeneration programs. Indeed, there is a need to more fully understand the interactions between protein networks, and how these interactions impact axon growth and regeneration.

Contribution of Proteomic Studies to Axon Regeneration— Proteomics can provide a systematic approach for analysis of protein expression in a cell, tissue, or tissue systems under a given condition. As quantitative proteomics has begun taking center stage for relative and absolute quantification of protein species, it has fast tracked the discovery of biomarkers, drug target identification through protein–protein interactions, and improved diagnostics by altered protein expression. For example, individuals with a range in spinal cord injury (SCI) severity were shown to express different protein profiles within their cerebrospinal fluid (42). Twenty-four hours postinjury, a variety of unique proteins were found to be up-regulated in the lesion epicenter, including 14–3-3 zeta/delta, HSP90α, peripherin, apolipoprotein A, and transferrin (43, 44). Unique phosphorylation events were also detected at the lesion site, including the phosphorylation of neurofilament light chain and fertuin-A. Further efforts to understand the proteomic profile in experimental SCI models has indicated that proteins altered after injury can be functionally categorized into metabolic homeostasis, stress response, protein and lipid degradation, development, neural survival and regeneration, and stimulus response (45, 46).

In order to investigate changes associated with injury, transcriptional profiling was performed in the cell body of DRG neurons at various time points after peripheral nerve injury (47). Transcription factor binding site analysis revealed 26 transcription factor families associated with gene expression changes 18–28 h postinjury. These included STAT (signal transducer and activator of transcription), HNF (hepatocyte nuclear factor), USF (upstream stimulatory factor), Jun, Smad, SRF, and ERα transcription families. Combining transcriptional changes with proteomic changes by performing transcription factor enrichment and ExPlain driven network analysis, revealed several hub pathways. Pharmacological inhibition of these pathways, including Abl (Abelson protooncogene), which was identified as a major hub, reduced neurite outgrowth within 24 h, but did not show sustained inhibition over time. Inhibiting Akt showed marked reductions in axon outgrowth over 72 h, whereas p38 inhibition only had transient effects. Combinatorial inhibition of Abl and Akt failed

TABLE I
Central regulators identified in axon growth after injury

Target molecule	Effected pathway	Biological outcome	Neural system
pTEN deletion	mTOR signaling	Accelerate axon outgrowth in PNS (236)	Dorsal root ganglion
SOCS3 deletion	JAK/STAT signaling	Sustained axon regeneration in vivo (165)	Retinal ganglion
pTEN & SOCS3 co-deletion	mTOR & JAK/STAT signaling	Synergistic effects on regeneration in vivo (167)	Retinal ganglion
PCAF expression	ERK-mediated signaling	Promotes acetylation of regeneration-associated promoters (237)	Dorsal root ganglion
c-myc expression	Myc signaling	Promotes survival and axon regeneration (51)	Retinal ganglion
c-Abl tyrosine kinase inhibition	BCR-ABL signaling	Transient inhibition of axon growth in vitro (50)	Dorsal root ganglion
Akt inhibition	PI3K/Akt signaling	Inhibits axon growth in vivo (50)	Dorsal root ganglion
Atf3 expression	Atf3/CREB signaling	Increased neurite outgrowth in vitro (49)	Dorsal root ganglion
STAT3 phosphorylation	STAT3/gp130 signaling	Increased regeneration after injury (117)	Dorsal root ganglion
Set-β phosphorylation	Cytoplasmic localization of set-β	Increased neurite outgrowth (118)	Retinal ganglion & hippocampal neurons
Dock6 phosphorylation	RAC1 signaling	Decreased axon regeneration in vitro and in vivo (119)	Dorsal root ganglion
DLK phosphorylation	STAT3 & c-Jun	Increased regeneration in motor and sensory neurons (106)	Dorsal root ganglion
Rho inhibition	Rho signaling	Increased axon and neurite outgrowth in vitro (238–240)	Cortical neurons, retinal ganglion, dorsal root ganglion

to have a significant effect on neurite length, indicating these two networks may not have overlapping mechanisms.

Other signaling proteins within the soma have been associated with regeneration. Following sciatic nerve crush, coordinated changes in expression were observed beginning 6 h postinjury. Some of these proteins included Atf3, neural peptide Y, Arginase I, and ankyrin repeat domain 1 (Ankrd1) (48). Indeed, when the transcription factor Atf3 was overexpressed, neurite outgrowth was enhanced; suggesting Atf3 is a central regulatory hub for regeneration (49).

In an effort to understand the proteome profile in injured axons, Fainzilber and colleagues identified 145 proteins associated with retrograde transport and 154 proteins associated with anterograde transport after injury (50). They further identified the axon phospho-proteome profile, where 268 unique phosphorylation sites were associated with injury in the retrograde compartment of the axoplasm. These proteins could be functionally classified into signal transduction, guanosine triphosphate activity, microtubule-based transport, and metabolism (47). Most recently, Steen and colleagues used a tandem mass tag (TMT) proteomics approach to uncover c-myc as a central injury-response hub in retinal ganglion cells (51). Quantitative mass spectrometry revealed several pathways altered after retinal ganglion injury including p53, MAPK, NF-κB, and Huntington protein.

To date, there have been a small number of published reports of proteomic analyses in injured spinal cord tissue (44, 46, 52–55). In these studies, 2 dimensional SDS-PAGE based mass spec identified only 61 proteins differentially expressed in injured spinal cord tissue, a relatively low number, which is not surprising because of the limited range of detection. More sensitive LC-MS/MS techniques have now allowed for a greater detection range, although mass spectrometry is not inherently quantitative in nature because of differences in proteolytic physicochemical properties (56). Hence, quantitative mass spec best compares each individual peptide between experiments. Stable isotope labeling by amino acids (SILAC) in cell culture has overcome these limitations where every protein contains at least one amino acid isotope tag, and labeled proteins are not dependent on peptide sequence. For *in vivo* applications this technique has been attempted (57, 58). Isobaric mass tagging of peptides has overcome limitations of proteome quantification where the identity and relative abundance of peptide pairs can be investigated in up to eight different biological samples (59). This is particularly useful when investigating protein changes over time or therapeutic treatments in the same experiment, for example. Detection of phosphorylated peptide- and glycosylated peptide techniques have also been developed, which delves into the post-translational modifications associated with disease and/or treatment such as SCI. Although quantitative proteomics has advanced rapidly in the past decade, limitations still remain. For example, in detecting the enrichment of proteins, only a small percentage of the proteome is detectable if

enrichment detection programs factor in the entire genome as background, decreasing the number of identified protein species. In contrast to transcriptomic analyses that typically identify thousands of differentially expressed genes following SCI, (55) quantitative proteomics cannot amplify proteins as a means to increase dynamic range.

The development of bioinformatic tools that focus on proteomic-based discovery is key to further understand these complex biological mechanisms. First order analyses have generated many free web-based tools including David, GSEA, Kegg pathway analysis, and genemania to name a few. However, these analytic programs are gene-centric rather than protein-centric. Second and third order analysis requires hypothesis driven approaches to obtain reasonable subnetworks within a framework that would directly address the scientific question at hand. For neural systems, some of the best web-based tools for hypothesis-driven discoveries are stored at the neuroscience information framework (NIF; http://neuinfo.org/). NIF has been cataloging and surveying the tool and data landscape since 2008. It currently contains the largest inventory of searchable tools and data in neuroscience (60).

Of note, transcriptomics and proteomics approaches have been pursued largely independently of one another, with researchers operating under the general assumption that for the majority of genes, changes in levels of transcript directly correlate with expression of the protein product. However, recent studies have shown that the correlation between mRNA and protein expression may be low (61). A gene's biological function may determine the relative stability of its transcript and protein levels; for example, genes that are involved in constitutive cellular processes, such as glycolysis and translation, exhibit high stability of both transcripts and protein products (62). In contrast, genes associated with processing RNAs tend to have unstable transcripts but stable proteins, whereas genes that produce stable transcripts but unstable proteins tend to be extracellular in nature. Similar discrepancies can be expected for post-translational changes in proteins and their effect on transcriptomics. Accordingly, integration of transcriptomic and proteomic findings will need to take into account the nature of the type of cell under study, and the biochemical function that is being probed, to reconcile genetic and protein control.

Importance of Proteomics in Identifying Mechanisms Involved in Regeneration—Alterations in protein expression drive metabolic changes necessary for supported axonal growth. The cell's ability to activate these transcriptional and translational switches may account for the marked differences in regeneration observed in different classes of neurons. Following a peripheral injury to an axon in the sciatic nerve, regeneration is successful and neuromuscular junctions become reinnervated. However, injury to the central branch of a DRG axon root results in no regeneration (63). However, the central branch of a DRG axon can successfully regenerate if
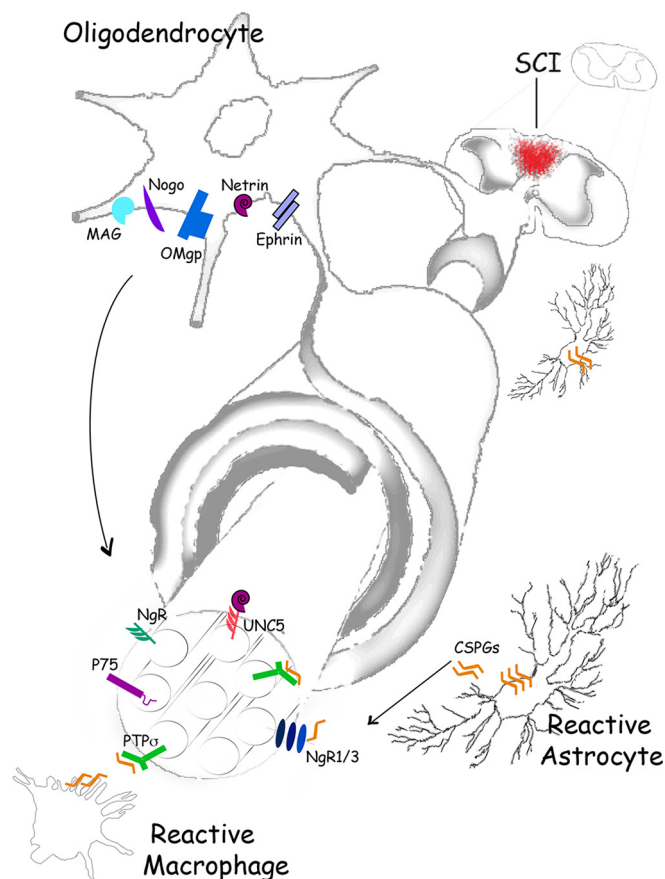

Fig. 1. **The inhibitory extracellular milieu of the central nervous system.** Following spinal cord injury (SCI), inhibitory molecules act as barriers of regeneration. These inhibitors fall into two broad classes: inhibitory molecules of the extracellular matrix such as the chondroitin sulfate proteoglycans (CSPGs); and inhibitory proteins associated with adult myelin. Myelin-associated inhibitors include Nogo, oligodendrocyte-myelin glycoprotein (OMgp), myelin-associated glycoprotein (MAG), Netrin-1, Ephrins and others. They bind to their respective receptors on the surface of injured axons, activating downstream effectors leading to inhibited axon outgrowth.

its peripheral branch was first crushed days earlier (64, 65). This is known as the conditioning effect. These observations indicate that the intrinsic state of a neuron can readily alter its regenerative ability. This difference in growth capacity has been associated with signaling pathways activated after peripheral nerve injury and relies on the coordinated expression of proteins in the axons and their somata (32, 41, 48, 66).

Successful Peripheral Regeneration and Failure of Central Regeneration—The failure of axonal regeneration in the CNS is partially attributed to the proteome profile of the CNS, especially the extracellular milieu surrounding the injured adult axons (Fig. 1). The lesion site forms cystic or trabeculated cavities to which axons are unable to attach for growth (67). These inhibitors fall into two classes: inhibitory molecules of the extracellular matrix (ECM), and inhibitory proteins associated with adult myelin. After SCI, reactive macrophages, oligodendrocyte precursor cells, and reactive astrocytes shift

the nature of the ECM at the lesion site, releasing cytokines, myelin associated inhibitors, and chondroitin sulfate proteoglycan (CSPG) molecules that potently inhibit neurite growth (68–70). These inhibitory molecules are not present within the peripheral nervous system, where axon regeneration robustly occurs after injury.

A second distinction between peripheral and central axons is microtubule stabilization. Following injury, most mammalian CNS axons retract, and only a few axons sprout for short distances. These axons exhibit dystrophic, swollen endings where axons are exposed to an inhibitory environment. They fail to initiate growth cones, leading to failure to regenerate (71). Recent studies show that retraction bulbs in the CNS have a disorganized microtubule network, whereas growth cones in the PNS contain organized bundling of microtubules (26). In addition, disruption of microtubules in growth cones using pharmacological agents transforms them into retraction bulb-like structures. Hence, microtubule stabilization prevents the formation of retraction bulbs and enhances the growth capacity of CNS neurons both *in vitro* and *in vivo* (72).

A third mechanism that may influence regeneration is a potential lack of the localization of ribosomes and mRNAs in CNS axons. Local protein synthesis and mRNA localization has been closely associated with successful peripheral nerve regeneration (73). Ribosomes actively translate proteins at the peripheral injury site that both propagate signals back to the soma and supply building blocks for new growth cone formation (31, 33, 74). Equivalent mechanisms have not been extensively reported in the CNS, and we remain unaware of their potential contribution to CNS axon regeneration (75). A mere lack of these local mechanisms in the injured axon tip could limit the cell's ability to successfully regenerate in the CNS, and the adequacy of this local response requires further study.

Lastly, the intrinsic cellular pathways that are activated after injury differ between peripheral and central neurons. This was first hypothesized when injury to the central axons of DRG neurons failed to activate regeneration programs. Injury to the peripheral axons of DRG neurons activates regeneration-associated genes and results in robust axonal outgrowth (65). Successful regeneration is coincident with prolonged expression of genes including CAP-23 and GAP-43 (76). Similarly, the up-regulation of regeneration-associated genes has been observed in corticospinal neurons following a proximal intracortical injury, but not a distal spinal axotomy (77). Overexpression of GAP-43 showed improved regeneration in the PNS, but no long-distance regeneration in the CNS (78–81). Targeting other regeneration-associated genes individually has had marginal effects on outgrowth (82, 83), illustrating that manipulating individual proteins may be insufficient to achieve robust, long-distance axonal regeneration. It is therefore likely that targeting protein regulatory networks may present a more potent strategy to activate neuronal growth programs.

Signaling Networks Associated with Regeneration—Several signaling pathways have been shown to play a role in outgrowth after injury. For example, both the PI3K and ERK pathways are essential for axon assembly (84). When inhibitors to both pathways were applied to the axon compartment, axon extension was abolished (84).

The PI3K pathway has been linked to neurotrophin-induced axonal branching where axon turning toward an NGF gradient is PI3K dependent (85, 86). PI3K-Akt regulates local protein synthesis in the axon through the mTOR pathway, where adult CNS neurons require mTOR signaling for axon regeneration (31, 87, 88). Quantitative mass spectrometry identified syntaxin13 as a protein locally synthesized by activated mTOR in the axon (89). Knockdown of syntaxin13 in culture prevented axon growth and regeneration. Interestingly, within the regenerating axon, PI3K is only activated at the distal tip, and its signaling is conveyed downstream through the inactivation of glycogen synthase kinase 3β (GSK3β) (90, 91). In adult DRG neurons, GSK3β inhibition leads to enhanced neurite outgrowth (92). In hippocampal neurons, inactivation of GSK3β is crucial for axon specification and growth through the phosphorylation of CRMP-2 (93–95). In cerebellar granule neurons, GRMP-2 is localized to dendritic branches, where its phosphorylation by GSK3β inhibits dendritic growth (96).

GSK3β is widely expressed within the adult brain and is regulated through Akt as well as ILK, and PKC (97–99). Inactivation of GSK3β stimulates axon elongation on inhibitory substrates in adult neurons, and induced corticospinal axon sprouting after injury (100). Slit2 signaling inhibits neurite outgrowth through GSK3β phosphorylation in adult DRG neurons (101). Therefore, GSK3β has been negatively linked to axon growth and sprouting. The role of GSK3β in axon growth is, however, controversial (102). Interestingly, GSK3β phosphorylation of MAP1B acts as a molecular switch to regulate microtubule dynamics in growing axons. Further, the MAPK pathway is involved in NGF mediated activation of TrkA, through phosphorylation of GSK3β during development (103). Therefore, distinct molecular switches control developmental growth, which may be perceived as inhibitory in the adult neuron.

A few MAP kinases are involved in repair mechanisms (104, 105). In dual leucine zipper kinase (DLK) knockout mice, neurons did not respond to a preconditioned lesion, suggesting this MAPKK kinase acts as a key mediator between the injured axon and somatic response to injury (106). Extracellular signal regulated kinase (ERK) phosphorylation is an essential component of the retrograde injury signal, and is thought to transmit information from the site of injury to the soma (107). ERK is thought to regulate local protein translation after injury, and may play a role in axon outgrowth (108). Growth cone collapse in response to semaphorin 3A is also regulated by ERK-mediated local protein synthesis (109). In adult DRG neurons, inhibition of RhoA stimulates neurite outgrowth, even in the presence of inhibitory substrates (110). Local

protein synthesis and ERK signaling is required for axon re-sensitization during chemotactic guidance (111), suggesting it may play a role in axon assembly (112). ERK phosphorylation is induced by neurotrophins, netrin (113), semaphorin 7A (114), and cell adhesion molecules (115). PI3 Kinase and MEK kinase is necessary for NGF-induced axon growth in sympathetic neurons, whereas MEK-signaling is required for BDNF/TrkB mediated axon elongation (84, 116). The MAP kinase pathway may therefore play a central role linking the extracellular environment to intracellular mediated responses.

STAT3 phosphorylation through gp130 signaling has also shown to increase regeneration after injury (117), whereas phosphorylation of cytoplasmic set-β abolished its inhibitory role on neurite outgrowth (118). Phosphorylation profiles in the injured cell have also been associated with inhibiting growth. The phosphorylation of Dock6, a guanine nucleotide exchange factor, inhibited its activity, which suppressed axon regeneration after injury *in vitro* and *in vivo* (119). Identifying key signaling networks associated with axon regeneration allows us to experimentally manipulate these pathways, unveiling potential therapies for regeneration.

Identification of Post-Translational Modifications in the Injured, Degenerating, and Regenerating Environment—Post-translational modifications (PTMs) of proteins are also crucial modulators of the neuron's ability to respond to its environment. During glaucomatous neurodegeneration, the 14–3-3 family of proteins have been shown to regulate apoptosis in a phosphorylation dependent manner (120). Holzbaur and colleagues showed a decrease in phospho-proteins associated with dynein retrograde signaling, including phospho-Trk receptors and phosho-ERK1/2 signaling proteins (121), suggesting that post-translational modifications play a role in ALS neurodegeneration. Intraneuronal post-translational modifications can therefore modulate the cell's homeostasis and response to extracellular cues. The activity of synaptic proteins has also been shown to be phosphorylation-dependent (122–125). Specifically, a novel motif of serine/threonine-glutamate ([S/T]-Q) containing substrates has recently been identified to localize predominantly in dendrites, synapses and the soma (126). Phosphoproteomics identified 150 proteins containing this motif, thought to bidirectionally regulate synaptic activity. Other post-translational modifications have also been shown to regulate synaptic plasticity. Sumoylation of the GluA1 AMPAR subunit is required for its surface expression during long-term potentiation (LTP) (127). Decreased sumoylation showed a marked reduction in surface expression of AMPAR, emphasizing the role of SUMO in synaptic plasticity. Sumoylation of the transcription factor MeCP2 also leads to transcriptional repression, known to play a role in synaptic development (128). Directional transport of proteins in axons has also been shown to be SUMO dependent (129); and sumoylation and phosphorylation have further shown to have synergistic effects (130).

Proteins within the environment can also modulate post-translational modifications within the injured and regenerating axon. A recent study employed isobaric tag (iTRAQ) labeling for quantitative proteomics to identify changes in the phosphoproteome of primary cerebellar granular neurons induced by culturing with inhibitory CSPGs (131). Over 100 significantly altered phosphopeptides were identified, reflecting differential phosphorylation of cytoskeletal proteins, DNA- and RNA-binding proteins, and transcription factors, among others. Post-translational modifications within the extrinsic milieu have also been shown to influence the dynamics of injured axons. Phosphorylation of the adhesion protein galectin-3 on the heparin sulfate proteoglycan (HSPG) substrate interacts with L1-NCAM on hippocampal axons, promoting axonal branching via local actin destabilization (132). Hence, phosphorylation of this extracellular protein acts as a highly functionally relevant molecular switch modulating complex axon dynamics. Recently, Ghosh and colleagues found that grafted Schwann cells expressing polysialyltransferase (PST) to modify polysialic acid residues on neural cell adhesion molecule (NCAM) displayed an enhanced ability to associate with and support very modest growth of injured corticospinal axons (133).

Post-translational modifications play a critical role in the regulation of protein activity and biological signaling activity. Quantitative proteomics have the advantage to detect phospho-peptides, sumoylated-peptides, ubiquitinated-peptides, and palmitoylated-peptides to name a few (134, 135). As more researchers employ these techniques, greater understanding of post-translational modifications related to disease will arise. At this juncture, combining high-throughput techniques and disease models will advance our understanding of spinal cord injury and help determine better therapeutic strategies (136).

Experimental Strategies to Promote Axonal Regeneration—In order to identify therapeutic targets that can promote neural repair and functional recovery following CNS injury, it is important to understand neuron-intrinsic and -extrinsic pathways underlying axonal regeneration (137).

Stimulating the Endogenous Growth State of the Neuron—Experimental approaches to enhance neuron-intrinsic growth capacity have largely been informed by the study of molecular mechanisms active in regeneration-enabled neuronal populations. Here we review several studies in which key proteins have been identified in these populations and successfully manipulated to enhance axonal regeneration in models of CNS injury.

Intrinsic Mechanisms of Axon Regeneration: The Conditioning Lesion—Enhanced spontaneous regeneration of PNS neurons is because of the activation of intrinsic signaling networks, in combination with a growth-permissive extrinsic environment, which together allow robust regeneration of injured peripheral axons (138). Though permissive extrinsic cues are important determinants contributing to successful

regeneration of the DRG axon through the peripheral nerve (139), the injured DRG neuron presents a unique model with which to investigate the cell-intrinsic growth programs activated by axotomy. In the conditioning lesion paradigm, lesion of the peripheral, but not central DRG axon induces a cascade of signaling events in the axon and neuronal soma that enhances the intrinsic growth state of the neuron (140). Notably, this conditioning lesion also enhances regeneration of the injured central axon branch if performed prior to, or shortly after the central lesion (64, 141).

Much work has been done to characterize the genetic programs recruited by conditioning lesion in the DRG neurons. Injured DRG neurons rapidly activate a transcriptional program of hundreds of genes as early as 1 day postinjury, and the majority of these exhibit sustained expression patterns by 2 weeks postinjury (141, 142) (140, 141, 143–149). Because this gene expression program is not activated upon central axotomy, many groups have endeavored to promote CNS axon regeneration via the exogenous expression of regeneration associated genes or their upstream regulators (2, 150).

Transcription Factors: Coordinators of Regeneration-Associated Gene Expression Programs—Several studies have focused on manipulating transcription factors, regulators of intraneuronal growth programs, that can coordinate the simultaneous expression of multiple regeneration associated genes.

In the spinal cord, regeneration and sprouting of dorsal column sensory axons has successfully been enhanced by overexpression or activation of transcription factors such as CREB (151), RARβ2 (152), STAT3 (153), Smad1 (154), and SnoN (155) in DRG neurons. The corticospinal tract (CST), the most important motor projection in humans, is especially refractory to efforts to promote its regeneration following injury. Partial regeneration or sprouting of the lesioned CST has recently been reported via overexpression of KLF7 (156), p53 (157), STAT3 (158), and Sox11 (159), and by conditional deletion or systemic antagonism of PTEN (88, 160) in CST motor neurons. Though fewer transcriptional regulators have been assessed in retinal ganglion cell (RGC) regeneration paradigms, it is known that KLF family members regulate regeneration in the optic nerve (161, 162), and that specific KLFs have antagonistic roles in modulating RGC growth cone dynamics (163). Additional work has shown that subcellular localization of the transcription factor Set-β plays a regulatory role in regeneration (164). Perhaps the most impressive findings to date arise from work showing that deletion of PTEN (87), SOCS3 (165, 166), or both (87, 167) substantially enhances optic nerve regeneration.

Epigenetic Mechanisms of Axon Regeneration—As epigenetic regulation of gene expression becomes more fully understood, the prospect that the epigenetic state of the neuron might significantly influence its intrinsic growth capacity becomes more compelling (168, 169). In the last few years, experimental manipulation of chromatin dynamics in injury

models has provided valuable insight into epigenetic influence on axon regeneration.

Gaub and colleagues showed that pan-inhibition of the histone deacetylases (HDACs) with the drug trichostatin A (TSA) promotes neurite outgrowth *in vitro* by enhancing activity of the histone acetyltransferases (HATs), CBP/p300 and PCAF (170). This approach increased acetylation not only of histones but also of the transcription factor p53, resulting in increased expression of p53 target genes including the classical regeneration associated genes, GAP-43 and SCG10. HDAC inhibition was also shown to enhance sensory axon regeneration in the lesioned mouse spinal cord (171). Cavalli and colleagues revealed that HDAC5 becomes exported from the nucleus following peripheral nerve injury, resulting in histone hyperacetylation and activation of a proregenerative gene expression program (16). Expression of the histone deacetylase SIRT1 was also shown to contribute to regeneration of conditioned peripheral nerve (172). Most recently, Di Giovanni's group reported that peripheral nerve injury, via ERK-mediated retrograde signaling, induces PCAF-dependent acetylation of histone H3K9 at the promoters of regeneration associated genes, triggering a transcriptional regeneration program (173). Notably, overexpression of PCAF in DRG neurons was sufficient to enhance regeneration of ascending sensory axons in the injured spinal cord, suggesting that PCAF is a central epigenetic component of the conditioning lesion effect. Together, these findings implicate a role for epigenetics in determining the intrinsic neuronal growth state.

In summary, the intrinsic mechanisms governing axon regeneration are complex and can be modulated by a host of factors acting at subcellular locations from the chromatin to the growth cone. Much of our current understanding is based on transcriptomic mechanisms associated with regeneration, where most of these studies have been done in the peripheral nervous system. A broader understanding of protein changes in response to injury and therapy is needed. Quantitative proteomics will allow us to develop a global view of the injured spinal cord's proteome. Not only will it reveal the active biological condition, but also the magnitude and complexity imparted by protein regulation and respective modifications (174). Additional perspective may be gained by characterizing the intrinsic proteomic mechanisms driving compensatory sprouting of both injured and intact CNS projections following injury (175). This includes corticospinal (176–178), rubrospinal (179), reticulospinal, and propriospinal axons (180, 181). Finally, exploring the identified signaling networks activated within injured CNS neurons that regenerate into permissive cellular grafts discussed below may yield novel insight into the molecular switches that enable growth of an axon previously incapable of regeneration.

Modifying the Injured Environment—The injured, adult CNS axon is faced with an extracellular milieu very different from that of the developing CNS and the PNS. The increased permissiveness of the peripheral nerve environment first

gained appreciation from early studies showing robust regeneration of central axons into grafted peripheral nerve "bridges" following CNS injury (182). In subsequent decades, a plethora of work has identified specific components of the CNS environment that inhibit regeneration of central axons. Here we will briefly highlight key efforts to shape the injured CNS environment in order to enhance regeneration.

Myelin-Associated Inhibitors—Myelin-associated inhibitors are a group of proteins that inhibit regeneration of injured adult axons within the CNS (Fig. 1) (5, 183). The classical myelin-associated inhibitors, Nogo (184), OMgp (185), and MAG (186), bind to transmembrane receptors on the axon (187 - 189), which modulates axon growth. We have also identified netrin-1 as a novel Myelin-associated inhibitor in the spinal cord (190). Though experimental deletions of these myelin-associated inhibitors or their receptors have thus far shown a limited effect on axonal regeneration (183), it is clear that OMgp and Nogo modulate axon sprouting following SCI (191).

Neutralizing the Inhibitory ECM—Following injury to the CNS, the lesion site becomes surrounded by reactive scar tissue, and axons interacting with this glial scar form dystrophic end bulbs and fail to regenerate; this process is reviewed in detail elsewhere (6, 192–194). Chondroitin sulfate proteoglycans (CSPGs), a class of proteins with sulfated glycosaminoglycan (CS-GAG) moieties, are deposited by macrophages, microglia, and reactive astroglia, and encompass a major component of this inhibitory environment from very early to chronic stages after injury (195). Though the inhibitory nature of these proteins has long been appreciated, the axonal receptors that bind them have only recently begun to be identified. One of these, receptor protein tyrosine phosphatase sigma (PTPσ), has been successfully targeted in experimental studies to promote axon regeneration in the optic nerve (196) and the spinal cord (197, 198). Notably, Silver and colleagues successfully promoted regeneration of serotonergic projections and enhanced functional recovery via systemic delivery of a mimetic peptide, presenting an attractive strategy for potential clinical translation. The Nogo receptors NgR1 and NgR3 were also identified as CSPG receptors by Giger's group, who showed significant regeneration of injured optic nerve axons in NgR1$^{-/-}$ and NgR3$^{-/-}$ animals (199).

Aside from inhibiting the activity of CSPG receptors, others have utilized the enzyme chondroitinase ABC (ChABC) to degrade CS-GAG moieties from CSPGs, rendering the scar environment less inhibitory to axon growth (200). These efforts have led to several reports of improved axon growth and, in some cases, functional gains in models of partial rodent spinal cord injury (195, 201–204).

Providing Permissive Substrates for CNS Axon Regeneration—Strategies to modulate the extrinsic environment to promote regeneration are not limited to the neutralization of inhibitory factors. Recent work from our group has shown the powerful potential of transplanting permissive neural stem cells (NSCs) into the lesioned spinal cord to promote host axonal regeneration (205, 206). Following neural stem cell transplantation into sites of severe SCI, we observed that injured supraspinal host axons penetrated grafts for distances up to 2 mm, and formed synaptic connections with grafted neurons. Moreover, these grafts placed in sites of SCI attenuate the reactive glial scar surrounding the lesion (205). Though the molecular mechanisms supporting CNS axon regeneration into permissive neural stem cell grafts are just beginning to be explored, it is plausible that grafted cells provide a combination of trophic support for regeneration as well as permissive extracellular matrix and/or cell adhesion molecules that attract host axonal regeneration and synapse formation with graft-derived neurons. Ongoing work in our laboratory is focused on characterizing the molecular factors in these permissive cell grafts that support regeneration of host axons. These findings have opened the door to a promising new strategy to overcome the inhibitory extrinsic environment and provide new postsynaptic targets via the reconstitution of spinal cord tissue with neural stem cells.

Stimulating Growth of the Injured Axon: Growth Factors and Diffusible Factors—The success of peripheral nerve regeneration is attributed in part to the presence of neurotrophins secreted by Schwann cells in gradients that support regeneration throughout the peripheral nerve milieu (207, 208). These proteins provide trophic support for peripheral neuron survival and axon growth; in contrast, trophic factors are not secreted in temporal and spatial gradients to enable regeneration in the adult injured CNS. We will briefly summarize studies utilizing exogenous growth factor delivery to support regeneration of injured CNS populations.

Growth Factor Effects on Spinal Cord Axon Regeneration—Spinal cord axons can be induced to regenerate by the provision of appropriate gradients of neurotrophins delivered exogenously either by genetically modified growth factor-secreting cell grafts (209). Early work from our group showed that NGF-expressing fibroblasts grafted into sites of acute and chronic SCI support extensive growth of central supraspinal and sensory axons, establishing that adult CNS axons retain the ability to regenerate if appropriate gradients of growth factors are provided (210, 211). Administration of NT-3 also promotes growth of injured sensory axons *in vivo* (212–215). Work by our group showed the ability of NT-3 gradients to enable guidance and reinnervation of appropriate brainstem targets by lesioned, ascending sensory axons (216). BDNF also promotes regeneration of multiple injured spinal cord projections, including rubrospinal (217, 218) and reticulospinal axons (219). We observed growth of ceruluspinal, serotonergic, CGRP+, and ChAT+ axons into BDNF-secreting MSC grafts, showing that BDNF exerts trophic effects on multiple axon populations (220).

Growth Factor effects on the Injured Corticospinal Projection—Efforts to elicit growth of injured corticospinal axons using neurotrophic factors have been largely unimpressive.

An early study by our group showed the failure of brain-derived neurotrophic factor (BDNF) to influence corticospinal axon growth even as BDNF supports survival of axotomized corticospinal neurons (221); however, this may be because the BDNF receptor TrkB was not trafficked from the cell body into the axon. Indeed, viral-mediated TrkB overexpression in the corticospinal soma enhanced regeneration of a modest number of corticospinal axons into BDNF-expressing grafts placed in a subcortical lesion, but not in sites of SCI (222). Neurotrophin-3 (NT-3) also enhances corticospinal sprouting (223), resulting in partial functional recovery after SCI (224). In a primate spinal cord injury model, corticospinal axons also failed to penetrate grafts of BDNF/NT-3 secreting fibroblasts, although lesioned brainstem projections did regenerate in to the grafts (225). Together, these findings show that provision of BDNF or NT-3 is not a sufficient strategy to promote regeneration of corticospinal axons; rather, it is likely that more permissive substrates, such as neural stem cell transplant, in combination with intrinsic manipulations will be needed to achieve robust regeneration of this notoriously regeneration-deficient projection.

The Role of Neurotrophins in the Injured Optic Nerve—It has been well documented that trophic factors promote the survival of retinal ganglion cells (RGCs) following optic nerve injury (226); however, the ability of growth factor treatment to support regeneration of retinal ganglion cell axons is less clear. Treatment with ciliary neurotrophic factor (CNTF) enhanced axonal regeneration after optic nerve injury into peripheral nerve grafts (227). Viral delivery of CNTF to the RGC soma was also shown to increase survival of retinal ganglion cells after optic nerve injury, and to promote regeneration of injured axons for several millimeters (228, 229).

The multitude of intrinsic and extrinsic factors influencing axon regeneration does not act in isolation. It is almost impossible to consider the effect of an intrinsic manipulation of a CNS axon without regard to how that manipulation may affect the axon's response to factors in the environment. For example, delivery of a transcription factor known to promote axon regeneration likely does not simply switch the neuron into a growth state regardless of environmental cues. Rather, such factors most likely act in synchrony, modulating the axon's cadre of growth factors, proteins, myelin- and ECM-associated molecules. It is likely that combinatorial treatments encompassing multiple intrinsic and/or extrinsic manipulations will be needed in order to promote robust axonal regeneration of injured CNS neurons. Proteomics and its post-translational modifications have the potential to unveil how proteins interact with one another in response to injury, broadening our understanding of which signaling networks are at play. It can also reveal cellular communication, which will provide insight into what determines a suitable permissive substrate for growth.

Extracellular Signals in the Environment—Extracellular matrix molecules (ECM) provide a molecular scaffold in the extracellular environment. The predominant ECM molecules present in the CNS consist of tenascin-R and hyaluronic acid (230, 231). Attached to the core protein backbones of these ECM molecules are sugar side chains that generate distinct species of glycoproteins and proteoglycans. These species in turn interact with axons, providing either permissive or repulsive binding that can alternately support axon growth and guidance.

One of the major classes of cell adhesion molecules that regulate axon growth during development and regeneration is the integrin receptor family. Integrin receptors located on the axon growth cone communicate with the ECM. Integrin mediated signals are generated through the nonreceptor tyrosine kinases, such as focal adhesion kinase (FAK) and Src. FAK activity is required for growth cone point contacts, which results in rapid neurite outgrowth (232). The Rho family GTPases Rac1 and RhoA also regulates the maturation of point contacts downstream of integrins (233). Interestingly, CNS inhibitory molecules inactivate integrins, where forced activation can abolish inhibition (234). Some of the most refractory adult CNS neurons have shown an absence of localized integrins in their axons (235). Manipulation of integrins *in vivo* for promotion of axonal regeneration could therefore be a potential therapeutic strategy.

Summary—Substantial progress has been made in understanding the basic mechanisms underlying the refractory state of the injured spinal cord to regeneration. But further progress is needed to promote understanding of basic mechanisms that can drive CNS axon regeneration. Quantitative proteomics has the potential to provide a more complete understanding of protein networks that will be necessary to enhance axon regeneration.

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