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Signaling the Pathway to Regeneration

Minireview

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Robust axon regeneration occurs after peripheral nerve injury through coordinated activation of a genetic program and local intracellular signaling cascades. Although regeneration-associated genes are being identified with increasing frequency, most aspects of regeneration-associated intracellular signaling remain poorly understood. Two independent studies now report that upregulation of cAMP is a component of the PNS regeneration program that can be exploited to enhance axon regeneration through the normally inhibitory CNS environment.

The neuronal response to peripheral nerve crush or transection (axotomy) has been intensively studied for more than a century and is certainly the best characterized example of neuronal plasticity in mature vertebrates. Axotomy switches a fully differentiated adult neuron functioning in sensory transduction, motor control, or autonomic regulation into a growth mode, where differentiated functions are shut down and an axon growth program is activated. A specific and orchestrated sequence of gene expression is induced to initiate and maintain axon growth. The response underlies the robust ability of the peripheral nervous system (PNS) to regenerate in all vertebrates.

For decades, efforts have been ongoing to elucidate the essential elements of this PNS regeneration program. Protein purification, unbiased genetic methods, and assessment of candidate genes and proteins have all been undertaken, resulting in identification of many injury-regulated genes and proteins. Neuronally expressed regeneration-associated genes span the gamut from regulatory proteins such as growth factor receptors and transcription factors through cytoskeletal building blocks of growth cones and axons, up to proteins of largely or completely unknown function.

Although the description of gene expression during regeneration is gradually growing more detailed, the current view is far from complete. It can be expected that the arsenal of genomic, gene expression profiling and proteomics methods available today will define the regeneration program, at least in broad outline, over the next few years. It is important to emphasize, though, that the functional roles even of already identified regeneration-associated genes remain largely uncharacterized. Overexpression studies have been hampered by the fact that peripheral regeneration is so robust, it is difficult to demonstrate any significant degree of enhancement in vivo. In loss-of-function experiments, mutations in regeneration-associated genes may disrupt axon growth during development. Inducible, neuron-specific disruption of these genes in mouse models will be necessary to reveal their roles in regeneration.

We may be able to gain more immediate insights by determining to what extent the PNS regenerative response is regulated by known signal transduction pathways. As underscored by two papers in the June 13 issue of *Neuron* (Qiu et al., 2002; Neumannn et al., 2002), this approach will likely yield insights into mechanisms that can be pharmacologically activated to enhance regeneration in the CNS. Indeed, these papers demonstrate that a single injection of the classical second messenger, cAMP, is sufficient to cause significant axon regeneration after a spinal cord injury.

Harnessing PNS Regeneration Signaling

The most favorable neuronal population in which to address interactions between peripheral regenerative mechanisms and CNS growth are the sensory neurons of the dorsal root ganglia (DRG; see Figure 1). Like all peripheral neurons, DRG neurons mount a robust regenerative response after nerve injury that can result in functional recovery. In contrast, lesioning the central processes of DRG neurons in the dorsal columns does not normally result in significant regrowth. However, if peripheral axotomy occurs prior to dorsal column injury (conditioning lesion), and thus the peripheral regeneration program is activated in advance, subsequently axotomized central processes of DRG neurons are capable of growing long distances inside the spinal cord (Neumann and Woolf, 1999). An important point is that this growth occurs without neutralization of inhibitory signals such as chondroitin sulfate proteoglycans (CSPGs) or Nogo-A.

A favorable property of DRG neurons for regeneration studies is that they can be readily cultured from adult animals, allowing in vitro investigation of the cell biological and signaling events associated with regenerative axon growth. Smith and Skene (1997) proved that peripheral axotomy one week prior to culture triggers a transcription-dependent program that activates an intrinsic growth capacity of adult DRG neurons. Taken together, these in vivo and in vitro results show that the axon growth program induced by a peripheral axotomy provides adult DRG neurons with both intrinsic growth capacity and the ability to overcome a hostile environment.

Signaling Developmental Axon Growth: Lessons for Regeneration?

We might expect that the explosion in knowledge about development of axon projections would contribute importantly to an understanding of regenerative axon growth. Recent studies have revealed an absolute requirement for neurotrophins for peripheral axon outgrowth during development (references in Markus et al., 2002 [this issue of *Neuron*]; see also Goldberg et al., 2002a). Furthermore, it is well known that expression of several neurotrophin, GDNF, and IGF family members is upregulated in distal peripheral nerve after injury. Therefore, effectors of Trks and other receptor tyrosine

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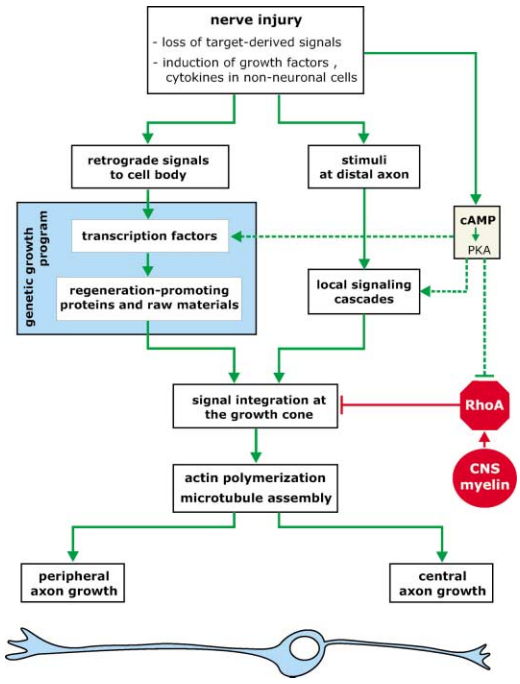

Figure 1. PNS Regeneration Signaling

Peripheral nerve injury interrupts target-derived signals and locally activates Schwann cells and macrophages to synthesize a cocktail of growth factors, cytokines, and growth-promoting surface molecules. These induce retrograde signaling and elevation of intracellular cAMP in the lesioned neurons, resulting in the transcription-dependent activation of an intrinsic growth program. The growth program includes production of growth-specific proteins such as the actin-modulating SPRR1A and Gap43. The actin- and tubulin-regulating machineries in the growth cone integrate signaling from the growth program as well as stimulatory and inhibitory signals from the local environment and translate them into axon growth. Elevated cAMP levels enable lesioned DRG central processes to overcome inhibitory CNS myelin signals, as demonstrated by Qiu et al. and Neumann et al. (2002). This finding suggests that PNS regeneration signaling can be harnessed to promote axon growth after spinal cord injuries.

Green arrows denote growth-promoting, red arrows growth-inhibitory signaling. Dotted arrows indicate possible mechanisms of cAMP action.

kinases are important to consider as mediators of regenerative axon growth.

Loss-of-function studies have recently shown requirements for several Trk effectors in mediating axon growth of developing peripheral neurons in vitro (see Markus et al., 2002 and references therein). In a gain-of-function survey, the small GTPase Ras and one of its downstream targets, the kinase Raf-1, appeared to be the most powerful mediators of axon elongation in embryonic sensory neurons (Markus et al., 2002). These findings implicate the Raf-Erk pathway as both necessary and sufficient to direct peripheral axon elongation during development.

The situation with regenerative axon growth of adult sensory neurons appears to be more complex. Application of neurotrophins to DRG neurons in vitro does not reproduce the axon growth induced by a conditioning lesion (Cafferty et al., 2001). Furthermore, a pharmacological study has demonstrated that regenerative DRG axon growth is far less dependent on Raf-Erk activation than developmental axon growth (Liu and Snider, 2001). Finally, cutaneous sensory axons have been shown to regenerate normally in vivo even when endogenous NGF is neutralized with a specific antiserum (see references in Liu and Snider, 2001). Thus, although it is tempting to speculate on the basis of developmental studies that a mechanism similar to the Raf-ERK pathway, alone or in combination with other signals, could be pivotal in regeneration, evidence at this moment is lacking.

Cytokine Signaling—Inflammatory and Regenerative?

Cytokines are classical injury-induced mediators that play multiple roles in the regulation of immune responses, hemopoiesis, and inflammation. Almost all the cytokines of the IL-6 family (except CNTF) are strongly upregulated after nerve injury, and expression levels change in non-neuronal cells distal to the lesion, as well as in glial cells and even in neurons in DRGs and motor pools. Some aspects of the axotomy response are lost in LIF null mice and the capacity for axon growth in vitro after a preconditioning lesion was significantly reduced in a subset of LIF mutant DRG neurons (Cafferty et al., 2001). Furthermore, it has been shown that sciatic regeneration is delayed in mice lacking IL-6 (Zhong et al., 1999) or LIF (Cafferty et al., 2001); However, these data are somewhat difficult to interpret because IL-6 family cytokines may also affect PNS development (see Zhong et al., 1999 and references therein). Again, inducible knockout models of either the IL-6 family cytokines or their common signaling receptor gp130 will be required to reach final conclusions.

Many cytokine signaling mediators show upregulated expression or enhanced phosphorylation after peripheral nerve injury. The transcription factor STAT3 is a major target for IL-6 and LIF signaling, and activation of STAT3 in axotomized adult DRG and motor neurons has been demonstrated (see Liu and Snider, 2001 and references therein). Schweizer et al. (2002) investigated a conditional knockout of STAT3 and found that it is required for survival of lesioned motoneurons. The effects of the STAT3 conditional inactivation on nerve regeneration have not yet been reported. In sum, although cytokine signaling is strongly implicated as an important component of peripheral nerve regeneration, its precise role in regulating axon growth remains to be defined.

Signaling from the Surface

Another essential factor for successful regeneration is expression of the proper cell adhesion molecules. In this regard, integrins are major players in the PNS because of the abundance of laminin isoforms in peripheral projection pathways. An interesting recent study has shown that when integrins are expressed in adult neurons at levels comparable to those seen in newborn neurons, the regenerative performance of their axons is likewise restored to that of newborn neurons (Condic, 2001). Furthermore, overexpression of integrin $\alpha 1$ in adult neurons is sufficient to promote axon growth on substrata containing inhibitory CSPGs and low levels of laminin. Finally, in a knockout model, loss of the integrin subtype $\alpha 7$ negatively impacts the ability of facial nerve axons to regenerate after a lesion (Werner et al., 2000).

Though little is known about the integrin signaling pathway in axon growth, studies in other systems suggest a major role for focal adhesion kinase (FAK) in the

regulation of growth factor signaling. Integrins, through FAK, can regulate the Raf-ERK signal transduction cascade. Moreover, integrins have long been known to play an important role in regulating cell motility through focal adhesion. By direct binding to paxillin, integrins serve to transduce signals from the extracellular matrix, as well as from growth factors, to the reorganization of the actin cytoskeleton.

Signaling Growth at the Growth Cone

The best-known example of a regeneration-associated protein is GAP43, an important regulator of growth cone motility during nervous system development. Recent results from Skene and colleagues (Bomze et al., 2001) have shed considerable light on the role of GAP43 and related proteins in regeneration. Coexpression of GAP43 and a functionally related protein, CAP23, which is also upregulated by injury, activated an intrinsic growth capacity of DRG neurons in vitro similar to that induced by a conditioning lesion. In vivo, transgenic overexpression of both proteins in DRG neurons enabled growth of lesioned central processes into a peripheral nerve graft.

Spatio-temporal regulation of actin dynamics in response to axon growth signals is crucial for long distance axon elongation. GAP43 and CAP23 sequester the membrane phospholipid PIP2 in discrete microdomains and thus regulate PIP2 availability. Phosphorylation of GAP43/CAP23 by PKC in response to extracellular signals abolishes its binding to PIP2, which leads to PIP2 release and subsequent actin polymerization (Laux et al., 2000 and references therein). Upregulation of both GAP43 and CAP23 may help sequester PIP2 along the axon shaft to stabilize axon cortical actin and thus inhibit axon branching, while phosphorylation of these proteins by growth signals may promote actin polymerization at the leading edge of the growth cone.

Another protein upregulated in DRG neurons after axotomy that has the ability to promote axon growth when overexpressed is small proline-rich repeat protein 1A (SPRR1A; Bonilla et al., 2002). Interestingly, SPRR1A is not expressed during development and may therefore be a truly regeneration-specific protein. SPRR1A is colocalized with specific actin structures, the lamellipodia, at the growth cone leading edge, and presumably has the ability to promote membrane ruffling through stimulating actin polymerization. Based on its proline-rich sequence, it may act in a similar manner to other known proline-rich proteins, many of which are actin binding proteins, such as Mena/VASP and WASP. These have been localized to the growth cone, and are implicated in regulating growth cone motility by controlling the activity of profilin, an actin monomer binding protein that provides G actin for actin polymerization. These results raise the possibility that overexpression of other actin binding proteins that regulate profilin activity may promote axon regeneration as well. Indeed, it is reasonable to hypothesize that multiple pathways induced by peripheral axotomy may converge on a few cytoskeletal regulatory proteins to promote regeneration.

Signaling Change: Overcoming Growth Cone Inhibitions

In addition to containing the machinery that powers axon elongation, the growth cone also receives and interprets the extracellular cues that the regenerating axon encounters. Signals that impede regeneration presumably inhibit actin assembly, resulting in growth cone collapse followed by retraction of microtubules (Zhou et al., 2002 and references therein). Thus, the growth cone integrates signaling from the intrinsic growth program and signaling transduced from environmental cues. Several growth cone collapsing molecules are present in CNS myelin, and CNS injury also induces the development of a glial scar that contains potent axon growth inhibitory molecules. Approaches aimed at neutralizing these inhibitors have achieved some degree of regeneration after CNS lesions (Bradbury et al., 2002; GrandPré et al., 2002). One appealing idea is that inhibitory components of myelin may suppress intrinsic growth programs of CNS neurons. However, a recent study has suggested that the intrinsic axon growth ability of CNS neurons is lost during the course of normal maturation (Goldberg et al., 2002b).

Of relevance to our arguments here is that activation of a regeneration program is also sufficient to allow axon regeneration inside the CNS environment. In addition to the conditioning lesion-induced regeneration discussed above, Davies et al. (1999) showed that dissociated (and therefore axotomized) adult DRG neurons readily extend axons in dorsal columns of both intact and injured adult spinal cord. Finally, application of neurotrophin and GDNF family members allows growth of DRG central processes into the dorsal columns after a dorsal root crush (Ramer et al., 2000). Taken together, these results suggest that inhibition by CNS myelin can be overcome if an intrinsic growth program is activated.

How the growth cone interprets signals from CNS inhibitors to halt axon growth remains largely unknown. Studies of axon guidance during development have identified the small G protein Rho as an important mediator that can translate a repulsive guidance signal into growth cone cytoskeletal reorganization leading to collapse or repulsive turning (Patel and Van Vactor, 2002). Because some repulsive cues present during development are also expressed after CNS injury, Rho GTPases may well be important transducers of inhibitory signals from the injured CNS environment to the growing axon, impeding regeneration. Indeed, a recent study showed that myelin-associated glycoprotein (MAG), an important component of CNS myelin, activates Rho through the neurotrophin p75 receptor (Yamashita et al., 2002). Furthermore, inhibition of RhoA has been shown to promote some axon regeneration after optic nerve crush (Lehmann et al., 1999). There is no consensus as to how Rho regulates cytoskeletal elements to stop axon growth. It may do so by directly preventing actin polymerization through LIM kinase and thus inhibiting subsequent microtubule assembly, by activating myosin II leading to axon retraction, or through as yet undefined mechanisms (Patel and Van Vactor, 2002).

Although the effect of the peripheral axotomy on Rho activation is as yet undefined, knowledge gained from neural development suggests another potential mechanism by which a conditioning lesion could overcome inhibitory signaling. Recent studies on the mechanism of axon guidance during development have revealed that cyclic nucleotides (cAMP and cGMP) can act as switches changing negative growth cone responses to positive responses and vice versa (see references in

Neumann et al., 2002). High levels of cAMP/cGMP are associated with growth cone attraction. The studies by Neumann et al. (2002) and Qiu et al. (2002) both address this function of cyclic nucleotides in central axon regeneration. Both groups show that the responses of regenerating axons to inhibitory influences can be modulated by cAMP signaling. Furthermore, they both show that increasing cAMP levels is able to mimic the effect of a conditioning lesion on growth of DRG central processes after a dorsal column lesion. Qiu et al. (2002) also demonstrate that a conditioning lesion induces an elevation of cAMP in DRG neurons and that inhibiting PKA activity can prevent conditioning lesion-induced axon growth on CNS myelin. These findings may have relevance to CNS neurons as indicated by the fact that high levels of intracellular cAMP markedly enhance the ability of retinal ganglion cells to extend axons in response to neuronal growth factors (Goldberg et al., 2002a).

Several potential mechanisms have been presented to explain the effect of cyclic nucleotides. One is that cAMP directly prevents the signal transduction from inhibitory cues to growing axons by inhibiting Rho GTPase. Another is that many environmental cues can be either positive or negative regulators of growth cone dynamics depending on the intracellular level of cAMP/cGMP. High levels of cAMP/cGMP stimulate growth cone motility, while low levels result in growth cone collapse. Indeed, MAG promotes axon growth of embryonic DRG neurons, which have higher cAMP levels than adult neurons (Qiu et al., 2002 and references therein). It is possible that cAMP may also initiate a transcription-dependent genetic program that affects axon growth in an inhibitory environment (Qiu et al., 2002).

Peripheral axotomy regulates both of the distinct components required for successful regeneration, intrinsic axon growth capacity, and positive response to the environment that growing axons encounter. The question of whether cAMP specifically regulates the response of regenerating neurons to environmental cues or whether it acts more as a general booster of growth remains open. Neumann et al. (2002) report that both dbcAMP treatment in vivo or forskolin addition in vitro impressively increase axon growth on poly-D-lysine, arguing that cAMP enhances the intrinsic growth capacity of mature DRG neurons. Qiu et al. (2002), on the other hand, find that increasing the cAMP level alone does not enhance axon growth on a CHO cell monolayer. Importantly, Qiu et al. (2002) also found that PKA inhibition does not prevent conditioning lesion-induced axon growth on a favorable substrate. Thus their work suggests that elevation of cAMP levels does not mediate the enhanced growth state activated by peripheral axotomy, but specifically regulates the response to inhibitory cues.

Conclusion

Although an impressive body of data is being assembled on changes in neuronal gene expression after PNS injuries, our current understanding of the cell biological mechanisms underlying peripheral axonal regeneration may not be sufficient to fully utilize the new information. Until recently, surprisingly little effort had been devoted to defining the intracellular pathways involved. Whether regeneration uses primarily the same signaling pathways underlying the well-described growth responses to neurotrophins and GDNF family members or whether entirely uncharacterized pathways are employed is unknown. Furthermore, our understanding of the intracellular signal transduction associated with regulation of the cytoskeletal structures that are the essential components of axon regrowth is rudimentary. Indeed, knowledge in this area is limited even for developmental paradigms of axon growth.

Defining signaling processes associated with PNS regeneration is clearly worth a major effort. Not only will this knowledge reveal mechanisms underlying a major form of neural plasticity, but it may also enable us to switch on pharmacologically an intrinsic growth state of injured CNS neurons.

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