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Priming Events and Retrograde Injury Signals

A New Perspective on the Cellular and Molecular Biology of Nerve Regeneration

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Abstract

Successful axon regeneration requires that signals from the site of injury reach the nucleus to elicit changes in transcription. In spite of their obvious importance, relatively few of these signals have been identified. Recent work on regeneration in the marine mollusk *Aplysia californica* has provided several insights into the molecular events that occur in neurons after axon injury. Based on these findings, we propose a model in which axon regeneration is viewed as the culmination of a series of temporally distinct but overlapping phases. Within each phase, specific signals enter the nucleus to prime the cell for the arrival of subsequent signals. The first phase begins with the arrival of injury-induced action potentials, which act via calcium and cAMP to turn on genes used in the early stages of repair. In the next phase, MAP-kinases and other intrinsic constituents activated at the injury site are retrogradely transported through the axon to the nucleus, informing the nucleus of the severity of the axonal injury, reinforcing the earlier events, and triggering additional changes. The third phase is characterized by the arrival of signals that originate from extrinsic growth factors and cytokines released by cells at the site of injury. In the last phase, signals from target-derived growth factors arrive in the cell soma to stop growth. Because many of these events appear to be universal, this framework may be useful in studies of nerve repair in both invertebrates and vertebrates.

Index Entries: Axon injury; regeneration; excitability; signal transduction; CREB; NF-kB; *Aplysia*; MAP-kinases; cytokines; nuclear import; retrograde transport.

Introduction

The vast complexity and heterogeneity of the vertebrate nervous system imposes significant constraints on investigations of gene regulation during nerve regeneration. Thus, although there is ample evidence that nerve regeneration is associated with altered excitability (Titmus and Faber, 1990; Walters, 1994), an upregulation of the machinery for the syn-

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thesis and processing of proteins, and changes in the cytoskeleton (Bisby and Tetzlaf, 1992), relatively little is known about how the transcriptional and translational programs that underlie these changes are regulated. In particular, the signals arising from the site of injury that influence individual genes have not been identified. This knowledge will be important for formulating therapies to ameliorate the effects of nerve injury and ultimately to restore nerve function.

Recently, the marine invertebrate *Aplysia californica* has emerged as a useful model to study long-term adaptive changes after nerve injury (Walters et al., 1991; Gunstream et al., 1995; Ambron et al., 1995). *Aplysia* affords several advantages for studies of regeneration and compensatory plasticity. First, many of the neurons are identified and large enough so that studies can be carried out on single cells of known function. Second, axoplasm free of glial cell and connective tissue proteins can be readily isolated and analyzed (Schmied et al., 1993; Ambron et al., 1995); molecular signals in the axon that are activated by injury can, therefore, be studied independently of those in the neuronal cell body or in glia. Third, the neuronal circuits controlling several behaviors of the animal have been substantially characterized (Frost and Kandel, 1995; Kupfermann, 1994) so that changes in a defined behavior can be correlated with changes in gene regulation in the neurons responsible for the behavior. Especially pertinent to studies of regeneration is the recent link between injury reactions and some of the cellular mechanisms of learning and memory (Walters, 1994; Ambron et al., 1995; Walters and Ambron, 1995). These seemingly disparate phenomena share a number of features that suggest a convergence of some regulatory pathways. Consequently, the wealth of information on the molecular basis of long-term sensitization in *Aplysia* can be retrofitted into models of nerve regeneration.

In this article, we have integrated results from studies of nerve injury in *Aplysia* with new data on signal transduction mechanisms in vertebrates. What has emerged is a novel framework for future investigations into how signals generated by axon injury trigger and prime transcription-dependent responses, such as growth and long-lasting compensatory changes in excitability. References to reviews, rather than original reports, are provided for background information whenever possible.

Signals to the Nucleus After Nerve Injury: Four Phases

The repair of an injured axon is initiated, maintained, and completed in response to signals that regulate specific transcriptional programs. The signals can be ionic or macromolecular, external to the axon or intrinsic, and they may act independently to bring about a particular transcriptional event, or may act synergistically so that the concurrence of two or more signals is necessary for the event to be expressed. An important goal is to identify the signals that elicit each transcriptional event. We believe that this formidable undertaking can be facilitated by first defining the temporal phases of the neuron's response to injury; the signals involved in eliciting each phase can then be identified.

Early Signals (Seconds to Minutes After Injury)

The first signal to reach the cell body after axon injury is a high frequency burst of action potentials generated at the injury site (injury discharge). Within the soma, this discharge will have two important sequelae: First, since most, if not all neuronal somata have voltage-gated calcium channels, there is an influx of calcium that can directly activate calcium-sensitive protein kinases, such as CaM kinase II and IV, and indirectly activate others, such as protein kinase A (PKA), protein kinase C (PKC), and MAP kinase (MAPK) (Ghosh and Greenberg 1995). These kinases regulate various transcription factors *(see below)*. For the injury-induced discharge to be an informative signal of periph-

eral injury it must be distinguished from the activity generated during the cell's normal sensory or motor function, activity that regulates the turnover of ion channels, receptors, and so forth. (Morgan and Curran, 1991). In neurons that are normally silent, such as high threshold nociceptors, the injury discharge should be particularly effective in regulating the synthesis of these and other proteins. Also, neural injury sometimes leads to delayed, long-lasting spontaneous activity in sensory neurons *(see below)*.

A second effect of injury discharge in sensory or motor neurons is extrinsic neuromodulatory input. For example, intense activation of mammalian nociceptor axons causes the release of neuropeptides that directly modulate nearby nociceptor terminals in the spinal cord (Willis and Coggeshall, 1991). Local interneurons excited by the nociceptor terminals release GABA and neuropeptides that rapidly inhibit primary afferent synapses. These and other modulators (such as NO) released in the spinal cord by peripheral injury may also have slower, metabotropic effects on nociceptor terminals. An interesting possibility is that the presynaptic effects may be linked to the generation of axoplasmic signals *(see below)* that are conveyed by retrograde transport to nociceptor nuclei in dorsal root ganglia (DRG). Whereas the effects of injury discharge are considered from a neuronal perspective, axotomy alters protein synthesis in glial cells as well, and in at least one case a glial product then regulates the formation of proteins in adjacent neurons (Sun et al., 1994).

Hormones can also be neuromodulators. Nerve injury under natural (unanesthetized) conditions is highly stressful and causes the release of peptide hormones, such as adrenocorticotropic hormone (ACTH) and α melanocyte-stimulating hormone (MSH), which have access to peripheral regions of sensory and motor neurons and to sensory neuron somata in DRG. Both of these hormones stimulate the regeneration of peripheral nerves in vivo, and enhance neurite outgrowth of sensory and motor neurons in vitro (Strand et al., 1993). α MSH increases cAMP in DRG cells (Hol et al., 1994) and the subsequent activation of PKA might participate in transcriptional regulation in sensory neurons (*see* Epitope Signals).

The sequelae to rapid injury signals have been studied in considerable detail in nociceptive sensory neurons of *Aplysia*. Noxious stimulation of the body wall, or brief, intense stimulation of nerves containing nociceptor axons, causes heterosynaptic facilitation and hyperexcitability of the nociceptors (Walters et al., 1983; Walters and Byrne, 1985; Walters, 1987; Clatworthy and Walters 1994; Illich and Walters 1995). These effects involve serotonin (5HT) released onto sensory neurons since 5HT added to the bath produces identical effects (Brunelli et al., 1976; Walters et al., 1983), as does stimulation of the 5HT-containing interneurons that are activated by noxious stimulation (Mackey et al., 1989). Moreover, depletion of 5HT with the neurotoxin 5,7 DHT reduces heterosynaptic facilitation produced by noxious stimulation (Glanzman et al., 1989). Neuromodulators, such as 5HT, might also be released directly by cell rupture at a site of nerve injury. As discussed below, the neuromodulators released after injury can trigger a variety of intracellular cascades in target sensory and motor neurons.

Intermediate Signals (Hours to Days After Injury)

We propose that the events triggered by action potentials promote repair, but are not sufficient to maintain the neuron in a regenerative mode. Consequently, a second set of signals, conveyed by retrograde transport, is required. In principle, these signals can be negative or positive. Negative signals originate in target tissues and repress the transcriptional programs for regeneration (Cragg, 1970; Wu et al., 1993). Axonal injury prevents the transport of these signals to the nucleus, thereby derepressing (activating) the appropriate programs. Positive signals, on the other hand, originate at the site of injury. They include signals elicited by extrinsic factors from glial and

other supporting cells, as well as intrinsic macromolecular signals activated directly by the injury. The positive signals are then transported to the cell body. Until recently, most considerations of nerve repair focused on negative signals, largely because of difficulties in identifying positive signals. As described below, there is now considerable evidence that intrinsic positive signals participate in several key events during regeneration. The targets of these signals are transcription factors, such as c-Jun and NF-kB, that exert control over the synthesis of proteins necessary for regeneration.

Late Signals (Days to Weeks After Injury)

A damaged nerve attracts inflammatory cells to the site of injury. These cells release cytokines, growth factors, and other substances that promote the removal of the damaged tissues, facilitate growth of new cells (Bartfai and Schultzberg, 1993; Rothwell and Strijbos, 1995), and increase the sensitivity of the damaged region by producing hyperalgesia (Walters, 1994; Watkins et al., 1995). Macrophages that release interleukin-1 (IL-1) are among the cells recruited by the injured tissue. IL-1 has either beneficial or detrimental influences on regeneration, depending on the extent of injury, but the mechanisms by which these outcomes are effected are not understood (Rothwell and Strijbos, 1995). Nevertheless, the fact that cytokines and other mediators (e.g., prostaglandins) released in the inflammatory response can influence neurons means that they are yet another source of signals that, because of their late release, could prolong the regenerative response in injured neurons.

Completion Signals

Regenerative growth almost always stops after a damaged neuron has reconnected to its target. In principle then, renewed binding or internalization of target-derived trophic factors by a regenerating axon should provide efficient signals for the completion of growth.

Reactions of Aplysia Neurons to Injury

Given the premise that neurons receive injury signals in four distinct temporal phases, the question arises regarding what events are regulated by each set of signals. To answer this question, we are using the nociceptive mechansosensory neurons discussed briefly above. *Aplysia* react strongly to aversive stimuli via these neurons, whose somata lie in discrete clusters within the pleural and abdominal ganglion (Walters et al., 1983; Fig. 1A). Axons of the pleural sensory neurons course through peripheral nerves going to the body wall. Damage to these axons causes central and peripheral sprouting that eventually leads to re-established contact with targets (Dulin et al., 1995; Noel et al., 1995; Steffensen et al., 1995). Nerve injury also elicits persistent synaptic facilitation, spike broadening, and hyperexcitability of the soma that is expressed as a decrease in spike threshold, spike accommodation, and afterhyperpolization (Walters et al., 1991; Clatworthy and Walters, 1994). Similar changes occur in *Aplysia* motor neurons (Dulin and Walters, 1992, 1993). These effects can last more than a month and are restricted to the neurons that receive the injury. Long-lasting increases in soma excitability following axonal injury are common among vertebrate and invertebrate neurons (Titmus and Faber, 1990; Walters, 1994). Although the persistent hyperexcitability and regenerative growth triggered by nerve injury may represent independent responses with distinct functions (compensation for lost function and enhancement of defensive responses in the case of hyperexcitability; restoration of lost function in the case of regeneration), the possibility exists that increased electrical activity resulting from hyperexcitability might modulate regenerative processes. For example, the initial stages of neural regeneration are dependent on the levels of intracellular calcium; moderate activity might promote regeneration whereas excessive activity could increase the levels of Ca^{2+} to the point where they are inhibitory (*see* Rehder et al., 1992).

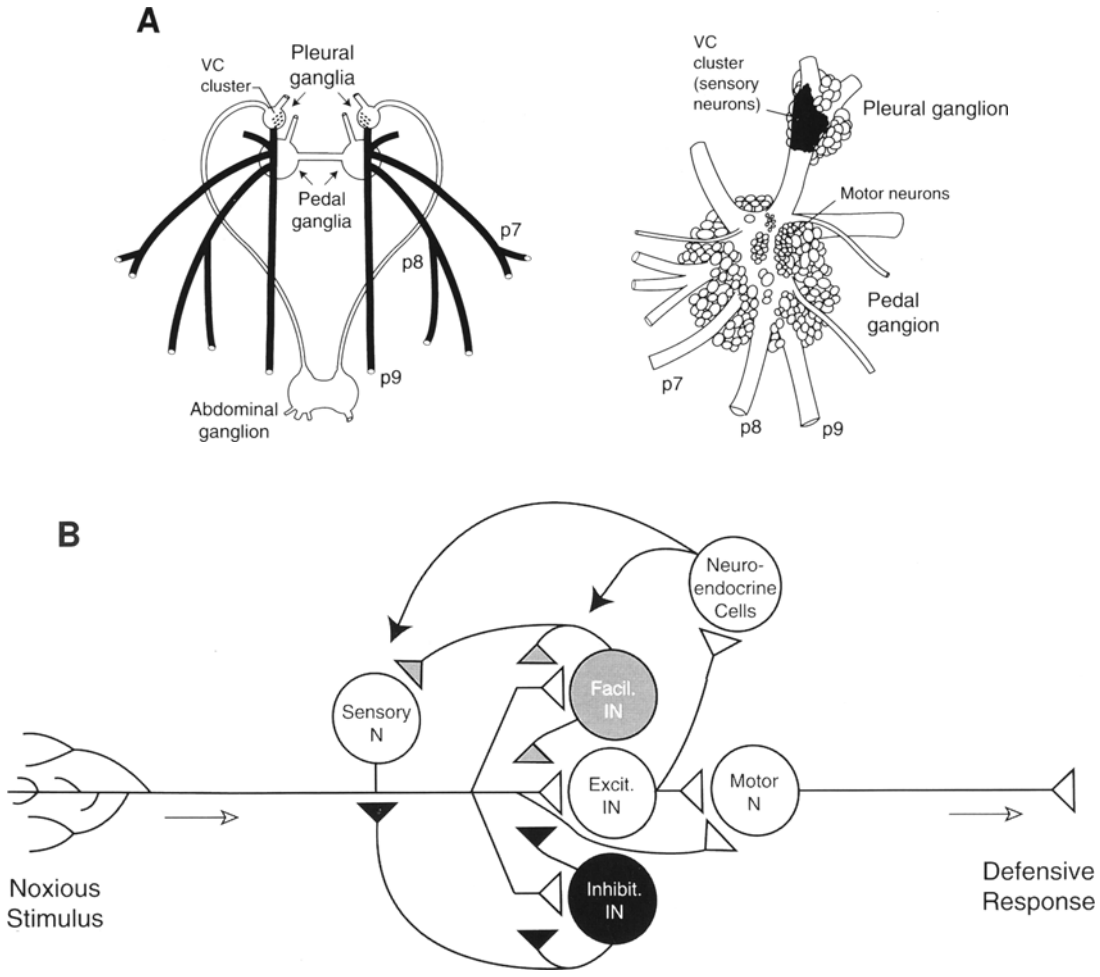

Fig. 1. Schematic diagrams of the neural components utilized in experiments on injury-induced alterations of *Aplysia* neurons. **(A)** Location of VC nociceptive sensory neurons. (Left) Sensory neuron somata (dots) lie in symmetrical clusters on the ventrocaudal surface of each pleural ganglion. Their axons (indicated by dark lines) go through the ipsilateral pedal ganglion, where they synapse with pedal motor neurons (not shown), and then exit into ipsilateral pedal nerves (only p7, p8, and p9 are indicated). No VC cell axons enter the pleural-abdominal connectives. In some experiments mixed populations of neurons were isolated from the abdominal or pedal ganglia and maintained in cell culture. (Right) Closer view of VC sensory neuron somata (~200/cluster) and pedal motor neurons. **(B)** General organization of circuits mediating defensive reflexes in *Aplysia* (*see* Walters 1994; Cleary et al., 1995; Frost and Kandel, 1995). Nociceptive sensory neurons excite three classes of interneurons: excitatory, facilitatory, and inhibitory. They also directly excite motor neurons (not shown), but most of the excitation of motor neurons comes from excitatory interneurons (Trudeau and Castellucci, 1992). The major neurotransmitter of the sensory neurons is probably glutamate (Dale and Kandel, 1993). An important neurotransmitter of the facilitatory interneurons is 5HT (Glanzman et al., 1989), which facilitates synaptic connections of the sensory neurons.

The ease with which growth and the electrophysiological changes can be monitored in the sensory neurons prompted a search for the signals that initiate these changes. The pleural mechanosensory neurons, and their homologs in other ganglia, excite and are modulated by many other neurons (Fig. 1B; Brunet et al., 1991; Trudeau and Castellucci, 1992; Walters, 1994; Cleary et al., 1995; Frost and Kandel, 1995). As is likely with mammalian primary afferents,

these interactions help regulate protein synthesis under normal conditions and are probably recruited after injury *(see above)*. Particularly important for the present discussion are the facilitatory serotonergic interneurons that synapse on the terminals (Mackey et al. 1989) and cell bodies (Zhang et al., 1991) of the sensory cells. These and other facilitatory interneurons (Hawkins and Schacher, 1989) are activated by noxious peripheral stimulation, and thus would be activated during nerve injury under normal (unanesthetized) conditions.

The Early-Phase Signals

5HT Triggers Many Events in Sensory Neurons and Primes the Cell for Later Signals

Knowledge of the defensive reflex circuits involving the sensory neurons provided a strong basis for examining the responses of these cells to 5HT at the cellular and molecular levels (Byrne et al., 1993; Walters, 1994). In brief, a short pulse of 5HT activates adenylate cyclase in the sensory neurons, leading to the formation of cAMP, which causes the dissociation of protein kinase A (PKA) into its catalytic and regulatory subunits. The catalytic subunit then phosphorylates proteins associated with potassium and calcium conductances, and with vesicle mobilization (Byrne and Kandel, 1996). These modulatory effects last for minutes to tens of minutes, and result in increased neurotransmitter release onto motor neurons and interneurons. Prolonged or repeated application of 5HT or cAMP also facilitates synaptic transmission, but this lasts for days, requires both transcription and translation (Montarolo et al., 1986), and involves growth of the presynaptic terminal (Bailey and Chen, 1988; Nazif et al., 1991) and removal of N-CAM-like cell surface glycoproteins (Bailey et al., 1992). Under these conditions, the catalytic subunit of PKA is thought to be translocated into the nucleus (Bacskai et al., 1993). How the catalytic subunit at the presynaptic terminals reaches the cell body is not known. However, there are serotonergic endings on the sensory neuron soma (Zhang et al., 1991) where 5HT can initiate the same cascade and where the catalytic subunit can diffuse the short distance to the nucleus. Once inside the nucleus, the catalytic subunit phosphorylates the transcription factor CREB (Dash et al., 1990), which then binds to the CRE enhancer sequence on the DNA, resulting in the activation of the immediate early gene for C/EBP (Alberini et al., 1994). The C/EBP is thought to regulate the synthesis of various proteins, including calmodulin, BIP, tubulin, actin, calreticulin, aldolase, and so forth (e.g., Kennedy et al., 1992; Kuhl et al., 1992; Noel et al., 1993). These proteins increase the biosynthetic capabilities of the cell and expand the cytoskeleton to accommodate new growth. In addition, synthesis of a tolloid-like protein is significantly enhanced shortly after 5HT treatment (Liu et al., 1995). This is the only protein found thus far that shows both long-term regulation by 5HT in *Aplysia* and developmental regulation in other species.

The effects of 5HT have three features that would be useful for responding to injury and to the threat of further injury. First, the convergent excitatory input onto identified serotonergic interneurons from large parts of the body surface (Mackey et al., 1989) suggests that the degree of activation of these interneurons, and consequently the amount of 5HT released onto the sensory neurons, reflects the spatial extent of peripheral injury (*see also* Walters, 1991). If so, cellular resources can be allocated for regenerative growth only to the extent required, as indicated initially by the amount of 5HT (and perhaps other modulators) released during injury. Second, the 5HT effects are amplified by paired spike activity in the sensory neuron (Ocorr et al., 1985; Abrams et al., 1991). This provides a mechanism for preferentially influencing cells whose peripheral branches are damaged, since injury will strongly activate these normally silent sensory neurons (Clatworthy and Walters, 1993). Third, 5HT causes long-lasting effects that tend to enhance subsequent injury signals should noxious stimulation occur

again. In particular, the long-lasting increase in sensory excitability caused by 5HT will enhance subsequent injury discharge, calcium influx, and calcium-dependent cAMP synthesis. Ongoing experiments (Liao et al., 1995) are investigating the possibility that prior exposure to 5HT also primes the nucleus to respond more rapidly and effectively to the arrival of axoplasmic injury signals *(see below)*.

The events just described have been examined largely in the context of learning, and are widely recognized as defining molecular mechanisms of reflex sensitization, which is an elementary form of learning (Krasne and Glanzman, 1995). Although these intracellular transduction pathways may also be affected by noninjurious external cues, the activation of the nociceptive sensory neurons and probably the release of 5HT are greatest after peripheral injury severe enough to cause nerve damage. Thus, the maximal activation of these intracellular pathways will occur during the induction of a "memory of injury." Indeed, many of the proteins that are synthesized following exposure to 5HT, or after learning-like nerve stimulation protocols, are associated with a general mobilization of the cellular machinery for growth. Not surprisingly, some of these proteins are also induced by axon injury (Noel et al., 1993, 1995).

It is tempting to speculate that in mammalian sensory neurons the cAMP synthesized in response to the injury-induced release of stress hormones, such as α MSH, leads to the early activation of CREB. This could trigger adaptive cellular reactions lasting for several days and could prime the cell to respond to later injury signals. Interestingly, experiments in mammals have shown a downregulation of CREB activity within days after nerve injury (Herdegen and Zimmermann, 1994). This time frame is about the same as the duration of the longest cAMP-induced effects in *Aplysia* (about 2 d). It suggests that CREB activation (perhaps involving synergism between injury discharge and stress hormones) is either unnecessary for, or potentially disruptive to, the effects of later signals that follow peripheral injury. Most of the studies on the axotomy-induced downregulation of CREB have utilized anti-CREB antibodies, including one that recognizes CREB that has been phosphorylated at Ser-133 (Herdegen et al., 1994). Phosphorylation at this site is thought to activate the factor.

The contribution of CREB to transcriptional regulation after injury is complicated by the recent finding of additional members of this family, including several that do not respond to PKA, and one, CREB2, that antagonizes the function of the original CREB (now called CREB1) (Yin et al., 1994). The interplay between the various forms of CREB will be a major focus of future research into the regulation of the CRE enhancer element after injury. Immunocytochemistry can prove useful for studies of CREBs in the mammalian CNS provided that the specificity of the antibodies to the various CREBs is rigorously defined. CREB2 was recently detected in the *Aplysia* pleural sensory neurons (Bartsch et al., 1995) where more molecular techniques can be easily applied.

Intermediate Phase Signals

The electrophysiological effects of 5HT in *Aplysia* appear to last for several days, whereas analogous effects of nerve injury last for weeks. Moreover, regeneration of *Aplysia* sensory neuron axons takes many weeks (Dulin et al., 1995; Noel et al., 1995; Steffensen et al., 1996). The question then arises regarding what maintains the sensory neurons in a prolonged regenerative state. Here we review evidence that prolonged reactions to injury are maintained by the transport of signal proteins from the injury site to the cell body.

Positive Injury Signals Induce Hyperexcitability and Increase Growth and Cell Survival

As discussed in Intermediate Signals, retrogradely transported injury signals fall into two categories, negative and positive. Observations consistent with a role for negative

signals include: the prevention of responses to axotomy by prior application of NGF locally to adult guinea pig sympathetic ganglia (Nja and Purves, 1978); and the induction of some of the changes seen after nerve injury by exposure of nerves to colchicine to block retrograde transport (Purves, 1976). Other experiments with colchicine have produced the opposite effect, however (Singer et al., 1982), and more recent studies indicate that the effects of colchicine are more complex than previously recognized *(see below)*. In addition, NGF added to sympathetic neurons acts as both a trophic and a tropic factor, i.e., it promotes survival and induces growth. How it represses growth in mature neurons in vivo is not explained by the proposed models. Finally, signals derived from target cells that repress growth need not be continuously present. Target-derived factors are almost certain to have a role in regeneration, but they are not the only signals that inform the cell body that its axon is injured.

Compelling evidence for positive injury signals has been recently obtained in *Aplysia*. Earlier studies established that crushing the axons of the pleural nociceptive neurons, under conditions in which fast activity-dependent signals are blocked, led to the induction of long-lasting hyperexcitability in the cell soma and facilitation of the sensory-motor synapses (Walters et al., 1991). Unlike similar effects of 5HT, which are expressed immediately, the effects of nerve crush were not expressed until 1–3 d after the injury, depending upon how far the crush site was from the sensory neuron somata. The hyperexcitability was not induced by exposure to colchicine; indeed, if the nerves were crushed and then exposed to the drug, hyperexcitability was prevented (Gunstream et al., 1995). These observations indicated that the signals responsible for inducing the hyperexcitability after injury are retrogradely transported to the cell body and will, therefore, accumulate on the distal side of a ligation placed between the crush site and the cell soma. Taking advantage of this fact, axoplasm was extruded from the nerve segment adjacent to the ligation on crushed nerves and was injected into the cell bodies of uninjured sensory neurons (Ambron et al., 1995). One day later, the cells expressed the same increase in excitability as produced by axonal injury. In contrast, axoplasm collected distal to a ligation on uncrushed nerves had no significant effect when injected into the cells.

A crush injury to *Aplysia* axons in vivo also leads to regenerative outgrowth after a delay (Dulin and Walters, 1993; Steffensen et al., 1995). This suggests that signals for growth, as well as hyperexcitability, are retrogradely transported after injury. To test this idea, we again used the crush/ligation paradigm. Axoplasm collected from injured nerves was injected into *Aplysia* neurons in vitro. Remarkably, the cells not only grew new processes, but they lived far longer than did the cells that were injected with axoplasm from ligated nerves that had not been crushed (Ambron et al., 1994; Ambron et al., submitted).

Some Positive Injury Signals Are Intrinsic to Axons

Although it is clear that (positive) injury signals in axoplasm can induce hyperexcitability and growth and prolong survival, the experiments described above did not address the source of the signals; they could be constitutively expressed in the axoplasm or they might be released from glial cells and be taken up into the axons at the crush site. Ciliary neurotrophic factor (CNTF) is thought to behave in the latter way after injury to mammalian axons (Curtis et al., 1993). To distinguish between these possibilities, *Aplysia* neurons were placed in culture in the absence of soluble growth factors or glial cells and their axons were severed 100–200 μm from their somata. Twenty-four hours later, the cells exhibited hyperexcitability (Ambron et al., submitted; Salim and Glanzman, 1995). Cells injured in vitro also regenerated their cut neurites and lived longer than uninjured cells in the same dish. Hence, glia or other cells are not required to generate retrograde signals underlying these injury-induced effects and at least some positive injury signals

must be intrinsic to the axon. These results are consistent with the idea that the growth and hyperexcitability induced by rapid, activity-dependent extrinsic injury signals (such as 5HT) are reinforced by the intrinsic injury signals that arrive later from the injury site. Normally, axonal injury occurs in or near the body wall, which can be as far as 15 cm from the somata, so that the arrival of the intrinsic signals is delayed for at least 1–3 d. An interesting possibility is that the early extrinsic signals trigger modest amounts of growth, but that a major cellular commitment to massive regenerative growth is not made until the retrogradely transported intrinsic signals arrive with information about the actual severity of the damage to the neuron's peripheral arbor. The early, modest growth phase may have a facilitating effect on subsequent growth signals, similar to the effects seen in conditioning lesion experiments in which prior injury enhances subsequent regeneration (e.g., Jacob and McQuarrie, 1993). It will be important to see if the early injury signals prime the nucleus to respond more rapidly or effectively to the later phase injury signals.

Intrinsic Injury Signals Contain Nuclear Localization Signals and Are Conveyed via the Retrograde Transport/Nuclear Import System

Synaptic terminals and axonal processes undergo modifications in response to injury and other external cues. Because some of these modifications require protein synthesis, yet the perinuclear manufacturing centers of the neuron are often far away, it was hypothesized that a signaling system communicates the needs of the periphery to the nucleus in the cell body (Ambron et al., 1992). There is now evidence that such a system exists. Some of the signals are proteins that contain a nuclear localization sequence (NLS). The NLS provides access to the retrograde transport/nuclear import pathway that conveys the proteins rapidly through the axoplasm to the cell body and then into the nucleus (Ambron et al., 1992; Schmied et al., 1993).

A search for proteins that use the pathway after nerve injury utilized an antibody to the NLS of the SV-40 large T-antigen (Ambron et al., 1992). When nerves were crushed, the antibody recognized a 97 kDa axoplasmic protein that reversed direction at the crush site. This constituent, named sp97 because of the NLS signal peptide it contains, is a prime candidate for an intermediate-phase injury signal (Ambron et al., 1995; Walters and Ambron, 1995). A model that describes how this system might work is as follows: Proteins containing an NLS are made in the cell body, enter the axon, and move by slow axonal flow toward the terminals. The NLS is hidden, either because of the way in which the protein is folded or by an association with another protein. In nonneuronal cells, proteins with one or the other of these motifs are often transcription factors or their effectors *(see below)*. On injury to the axon, the protein is modified, exposing the NLS, and the protein enters the pathway to the nucleus. As mentioned, sp97 has these characteristics and is enriched in the axoplasm that induces injury-related responses when injected into the soma of uninjured cells, but its function is not yet known.

Other Candidates for Intrinsic Intermediate Injury Signals

According to the ideas just discussed, some of the proteins activated by injury and transported to the nucleus as signals for regeneration will have an NLS. Consequently, several proteins with NLSs are being investigated, as are a number of others that enter the nucleus but in ways that are not yet understood.

MAP Kinases, JN Kinases, and the Activation of c-Jun

The p42/44 MAP kinases (i.e., ERK 1,2; MAPK 1,2) are attractive as injury signals since they participate in a cascade that is initiated by growth factors and that results in the synthesis of proteins associated with regeneration. In addition, the transcription of MAPK and its activator kinase, MAPK kinase (MEK) is upregulated after rat facial nerve transection

(Kitahara et al., 1994). The MAP kinases comprise a family of proteins that, when phosphorylated, can enter the nucleus to phosphorylate the constitutive transcription factors c-Jun and Elk. Recently a tentative link was found between MAP kinases and the synthesis of ion channels. As noted above, changes in neuronal excitability are common after axon injury. These studies utilized a transformed mouse fibroblast line that stably expresses a constitutive form of $p21^{Ras}$ (Huang and Rane, 1994). Exposing the cells to epidermal growth factor caused a sustained increase in the synthesis of a novel calcium-activated potassium channel. This indicates that the synthesis of ion channels can be regulated via cell-surface receptors linked to ras proteins. Similar events may occur in neurons since the synthesis of calcium channels was blocked in PC12 cells that expressed a dominant negative *ras* gene (Pollock et al., 1994). Although the proteins downstream from ras remain to be identified, the penultimate step is likely to be activation of a MAPK (Borasio et al., 1989).

Another, perhaps more rapid response to injury could be mediated by members of the family of kinases that are activated by cellular stress (Derijard et al., 1994; Kyriakis et al., 1994). One of these, the Jun amino-terminal kinase (JNK), phosphorylates Ser-63 and Ser-73 in the amino-terminal activation domain of the transcription factor c-Jun. Phosphorylated c-Jun forms homodimers, as well as heterodimers with c-Fos, FosB, JunB, and JunD, via a leucine zipper motif. The dimers then bind to a consensus DNA sequence known as the AP-1 site. Although the pathway responsible for the activation of JNK has not been characterized, it appears to be completely intracellular. (Derijard et al., 1994). Should it respond to injury, JNK would be a very attractive candidate for a retrogradely transported injury signal.

Consequently, we explored possible roles for MAPK and JNK in response to nerve injury in *Aplysia*. Using an antibody specific to phosphorylated MAPK, we identified a 43-kDa protein whose phosphorylation in axons increased after nerve crush (Zhang, Povelones, and Ambron, unpublished observations). We also found a MEK in axoplasm and, since ras is present as well (Swanson et al., 1986), many of the known components of the MAPK cascade are present in *Aplysia* axons. In light of our findings it is significant that Michael et al. (1995) reported finding a MAP kinase in *Aplysia* that is activated by 5HT and phosphorylates the *Aplysia* transcription factors C/EBP and CREB-2. It appears that the MAPK family will have important and perhaps overlapping roles in regulating proteins required for regeneration and learning. Using a peptide corresponding to amino acids 1–79 of c-Jun as a substrate, we also found that JNK is present and active in *Aplysia* axoplasm after nerve injury. Since both MAPK and JNK can phosphorylate c-Jun (although at different sites), we now want to know how transcription in vivo is affected by activating each kinase.

So far we have emphasized the activation of constitutive c-Jun, but many studies have shown that c-Jun is also synthesized after injury. In fact, it is the only transcriptional factor so far identified whose levels consistently increase in neurons of the PNS and CNS following nerve injury (Herdegen and Zimmerman, 1994). Befitting a regulator of axon repair, the appearance of c-Jun precedes the synthesis of proteins prominently involved in regeneration (Herdegen and Zimmermann, 1994), most notably GAP-43: The region upstream of the TATA box on the GAP-43 gene contains an AP-1 site (Weber et al., 1995). An AP-1 site is also found in the upstream region of the gene for c-Jun *(c-Jun)* so it is likely that c-Jun regulates its own synthesis. Parenthetically, c-Jun can be induced by the constitutive transcription factor CREB, but studies mentioned above have convincingly shown a decline in the levels of CREB mRNA and protein following axotomy and that the required phosphorylation of CREB at Ser-133 does not occur.

The regulation of *c-Jun* and c-Jun is often intertwined with the transcription factor c-Fos. The levels of both factors increase in response to a variety of insults to the nervous system, including ischemia and seizures (Morgan and

Curran, 1991). c-Fos induction under these conditions is driven by activation of synaptic inputs (Morgan and Curran, 1991). Interestingly, c-Fos is not induced by nerve injury (Herdegen et al., 1994), implying that the signals that activate c-Jun after injury are independent of synaptic input. The levels of c-Jun increase in DRG neurons after blockade of axonal transport with colchicine, which has been interpreted to mean that the signal is a retrograded transported target-derived growth factor. This explanation is not compelling, however. The expression of c-Jun in DRG neurons in vitro was not influenced by adding NGF or BDNF (De Felipe and Hunt, 1994), so *c-Jun* is either constitutively regulated by an unknown factor from the target or the colchicine block may itself induce c-Jun transcription, perhaps by activating intrinsic injury signals in the axon.

NF-kB

The issue of the role of colchicine raises the broader question regarding how damaging an axon initiates an injury cascade. A provocative study on HeLa cells recently showed that disrupting microtubles activates the transcription factor NF-kB (Rosette and Karin, 1995). This factor is a dimer composed of a widely conserved family of proteins that include the rel factors (Liou and Baltimore, 1993; Verma et al., 1995). The prototype NF-kB is the p50/p65 heterodimer that is found in the cytoplasm complexed with an inhibitory subunit, IkB. Colchicine, nocodazole, and similar agents lead to the phosphorylation of IkB, causing it to separate from the complex (Rosette and Karin, 1995). This exposes the NLS and NF-kB translocates into the nucleus, where it binds to kB enhancer sequences on the DNA. NF-kB causes the transcription of a wide variety of messages, including those for interleukins and cell surface adhesion proteins (Verma et al., 1995). Neurons contain NF-kB/ IkB complexes and there are indications that they are present in axons and synapses in the mammalian CNS (Kaltschmidt et al., 1993). Hence, NF-kB released from IkB by colchicine could travel along the axon to the cell body, enter the nucleus, and initiate transcription. This action would be interpreted as if the transcription was regulated by the loss of a signal from a target-derived growth factor whose transport had been interrupted by the colchicine.

This possibility prompted us to look for NF-kB in *Aplysia*. Axoplasm extruded from nerves was subjected to an electrophoretic mobility shift assay using kB DNA sequences as probes. We found a protein-DNA complex that migrated with mammalian NF-kB (Povelones et al., submitted). These results are intriguing since they provide the first unequivocal demonstration of a transcription factor within axons. Furthermore, because it can be activated by cytoskeletal disruption, at least in HeLA cells, such activation of NF-kB could play a pivotal role in axon regeneration. A complication here is that we found that NF-kB activity was lost after nerve crush, probably a result of the action of axoplasmic proteasomes (Chain et al., 1995). This suggests that inactivating mechanisms triggered by nerve injury in *Aplysia* take precedence over possible activating effects of cytoskeletal disruption in regulating NF-kB activity.

Some Intermediate-Phase Signals Are Extrinsic

Glial cells at the site of injury can also release factors that control aspects of regeneration. The best characterized is CNTF, which has generated considerable interest recently because it rescues damaged motor neurons (Sendtner et al., 1992). The protein does not have a signal sequence and is not secreted, but is thought to be released when the glial cells are damaged. Some credence to this idea comes from studies that show receptor-mediated uptake and retrograde axonal transport of CNTF in adult sensory and motor neurons after peripheral nerve lesion (Curtis et al., 1993). An involvement of CNTF in the injury response is important because it is one of several growth factor-like molecules that increase neuronal survival in vitro.

Late Phase Signals: Cytokines and Additional Glial Factors

Cytokines in the vertebrate CNS are found in microglia and some neurons (Bartfai and Schultzberg, 1993), but are also released at sites of PNS injury by macrophages and other inflammatory cells that are attracted to the damaged tissues. Although the role of cytokines in the nervous system is a relatively new area of research, there is good evidence that cytokines can have neurotrophic effects after injury (Bartfai and Schultzberg, 1993). For example, when an inflammatory reaction was provoked at rat DRG, there was a marked enhancement in the number of fibers that regenerated in response to a subsequent nerve crush (Lu and Richardson, 1991). Some of the effects are indirect, as when IL-1 induces the synthesis of nerve growth factor in astrocytes (Rothwell and Strijbos, 1995). Clearly, determining the molecular events responsible for these effects in the vertebrate nervous system is important, but will be difficult.

Invertebrates do not have a sophisticated immune system, yet they do mount effective host defense responses to microbial intruders and other foreign bodies. Inflammation near peripheral nerves in *Aplysia*, like crush injury, elicited hyperexcitability in the sensory neurons after a long delay (about 5 d), presumably related to the time required to mount the inflammatory response by macrophage-like amebocytes (Clatworthy and Walters, 1994a). Immunocytochemistry indicates that the amebocytes attracted to the site contain IL-1, and incubating the nervous system in medium containing IL-1 induces sensory neuron hyperexcitability (Clatworthy et al., 1994b). In mammalian preparations cytokines evoke their actions through membrane receptors that are linked to the NF-kB signal transduction system. The finding of NF-KB in *Aplysia* axoplasm, therefore, suggests a way in which cytokines released after axon injury can influence events in the cell body and, based on findings presented above, indicates that NF-kB has more than one role in injured axons.

Completion Signals

Regenerative growth usually ceases once the target has been contacted, which is thought to be caused by signals present in the target tissue. Some of these signals probably initially influence local events in the growth cone so that the growth ceases rapidly. Others influence gene expression in the regenerating neuron. A good example of the latter is the finding that the synthesis of GAP-43, which increases markedly after injury, stops once the target is contacted (*see* Skene, 1989 for review). Another example is c-Jun. The levels of this factor increase in goldfish retinal ganglion cells after transection of the optic nerve and begin to decline after the target is innervated (Herdegen et al., 1993). Adding a peripheral nerve graft to the proximal stump diverts the regenerating fibers, prolonging both growth and the expression of c-Jun (Herdegen and Zimmermann, 1994). These observations reaffirm that c-Jun has a role in regenerative growth and support the notion that target-derived stop signals can terminate the expression of growth-related proteins. These stop signals are viewed as repressors, although whether they need to be present constantly, or only for an initial short period, is not known.

A Model for Gene Regulation After Axon Injury

There is increasing evidence to support the idea that regenerative and compensatory responses to axonal injury result from a series of overlapping intracellular signal cascades that trigger and reinforce one another until stop signals are received. The different phases are well illustrated by the differences in the latency and duration of hyperexcitability seen in *Aplysia* sensory neurons when different sets of injury-related signals are permitted to reach the soma (Walters, 1987; Walters et al., 1991; Clatworthy et al., 1994; Dulin et al., 1995; Gunstream et al., 1995). Figure 2 presents a model that summarizes how a series of signals

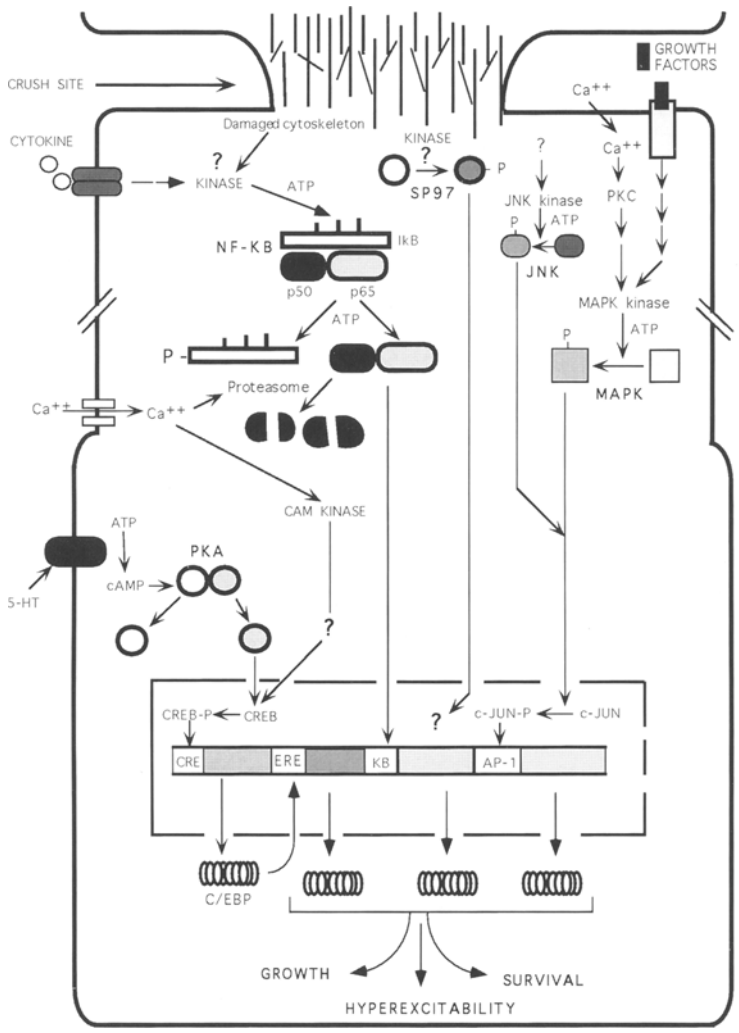

Fig. 2. Schematic depicting some of the signal transduction pathways that are proposed to participate in the temporal phases of regeneration after nerve injury in *Aplysia*. In Phase 1, action potentials propagate from the site of injury to the cell body opening calcium channels, thereby activating CAM kinase. 5HT, released from interneurons, causes an increase in cAMP, which frees the catalytic subunit of PKA to enter the nucleus and phosphorylate CREB. C/EBP is synthesized, followed by the activation of genes regulated via the ERE enhancer sequence. C/EBP may also be a substrate for a MAP kinase (not shown). Phase 2 is initiated by the activation of NF-kB at the site of injury by disruption of the cytoskeleton and by a calcium-initiated cascade that leads to the phosphorylation of a MAP kinase. Intra-axonal stress caused by the injury activates JNK. The active NF-kB and the kinases use the retrograde transport/nuclear import pathway to reach the nucleus where they regulate genes containing kB and AP-1 sites, respectively. Alternatively, elevated calcium levels activate axoplasmic proteasomes that destroy NF-kB, thereby activating the genes that are normally repressed by NF-kB. Sp-97 is also phosphorylated and transported, but its function is not known. Phase 3 begins with the release of cytokines from cells attracted to the injury site. The cytokines reinforce the NF-kB pathway. Once the target has been reinnvervated, a signal from growth factors inhibits the MAP kinase pathway (and probably others not shown), shutting off the synthesis of proteins that are activated by c-Jun and stopping growth. *See text* for discussion.

involving different regulatory pathways might initiate and maintain complex cellular responses to naturally occurring axonal injury in *Aplysia* sensory neurons.

Phase 1

Action potentials from the site of axonal injury propagate to the soma where they increase the intracellular levels of calcium, thereby activating CAM kinases. The kinases enter the nucleus, where they phosphorylate constitutive transcription factors, such as CREB. The injury-induced action potentials in damaged nociceptors excite interneurons, causing the release of neurotransmitters, such as 5HT. The 5HT binds to receptors linked to adenylate cyclase on the sensory neuron soma. The PKA cascade is initiated, resulting in both the phosphorylation of ion channels in the soma membrane and the activation CREB, which induces the synthesis of C/EBP (Alberini et al., 1994). These cascades trigger transcriptionally dependent and independent hyperexcitability that lasts 1–2 d (Dale and Kandel, 1987; Gunstream et al., 1995; *see also* Ghirardi et al., 1995). The cell is now prepared to fire vigorously to subsequent stimulation. This will again increase calcium influx, cause another round of cAMP synthesis, and initiate some growth (Bailey and Kandel, 1993). In addition, these early transcriptional events prime the nucleus to respond maximally to the slower injury signals that arrive later. This hypothesis is supported by preliminary observations that 5HT enhances long-term hyperexcitability induced by retrogradely transported axonal injury signals (Liao et al., 1995).

Phase 2

Calcium enters the axon at the injury site and initiates a cascade that leads to the activation of MEK and the phosphorylation of MAPK. Factors activated by the stress of the injury result in the activation of JNK, whereas an unknown kinase phosphorylates sp97, exposing the sp. MAPK, JNK, and the sp97, enter the retrograde transport/nuclear import pathway to the nucleus. These events result in the activation of c-Jun, elk, and other, as yet unknown, transcription factors. Activated c-Jun dimerizes, binds to the AP-1 site, and causes the synthesis of additional c-Jun and initiates the synthesis of mRNAs for the proteins needed for axonal growth. Damage to the cytoskeleton can free NF-kB for transport to the nucleus and activation of mRNA synthesis directed by kB enhancers. Opposing this is a proteasome-mediated loss of NF-kB, which would result in the transcription of genes that are normally repressed by NF-kB. The effects initiated by the events in Phase 1 are now augmented, hyperexcitability is maintained, and more extensive growth is promoted. This phase can last for many days (Gunstream et al., 1995).

Phase 3

Cytokines and growth factors are released by amebocytes attracted to the injury site and probably by glial cells along the injured nerve. Some of these factors bind to receptors on the axonal membrane that activate NF-kB, which is then transported to the nucleus. Cytokines and growth factors may be released from amebocytes for over a week if a "neuroma" forms, or if foreign bodies (e.g., bacteria) remain in the injured area. Hyperexcitability has been observed to last 2 mo after nerve transection (Dulin and Walters, unpublished observations). After amebocyte-derived injury signals end, longer-lasting production of injury signals might come from glia (positive signals) or from the absence of constitutive, target-derived factors (negative signals).

Phase 4

Once the injured axons reinnervate their targets, trophic signals from the targets resume their influence on the injured neuron, stopping the regenerative growth and hyperexcitability. This effect could be direct, by the transport of target-derived factors to the injured neuron's soma to repress transcriptional controls over the regenerative state, or indirect, by inhibiting the production of positive injury signals in the damaged region of the nerve.

Conclusions

We have presented a working model that provides a focus for future research. The model is overly simplistic because, by emphasizing the signals that are initiated at the site of injury, it ignores the many signal transduction pathways that are potentially activated in the cell body. In addition, there are several members of each kinase family that can be compartmentalized within the neuron. For example, each member of the NF-kB family can potentially be regulated uniquely so that specific responses can be elicited depending on the stimulus and the intracellular location of the factor. The fact that C/EBP, c-Jun, CREB, and so forth, can form heterodimers with members of other transcription families greatly magnifies the range of influence that these factors can have on the synthesis of proteins. Also, other kinases, phosphatases, and proteases that have not been mentioned are probably involved in the injury response. Nevertheless, a simplified initial focus on the events that originate at the site of injury should be helpful in guiding the search for signals that inform the nucleus of an axonal injury.

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