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Changes in Cytoskeletal Protein Synthesis Following Axon Injury and During Axon Regeneration

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Contents

Abstract

Injury to the axons of facial motoneurons stimulates increases in the synthesis of actin, tubulins, and GAP-43, and decreases in the synthesis of neurofilament proteins: mRNA levels change correspondingly. In contrast to this robust response of peripheral neurons to axotomy, injured central nervous system neurons show either an attenuated response that is subsequently aborted (rubrospinal neurons) or overall decreases in cytoskeletal protein mRNA expression (corticospinal and retinal ganglion neurons).

There is evidence that these changes in synthesis are regulated by a variety of factors, including loss of endoneurially or target-derived trophic factors, positive signals arising from the site of injury, changes in the intraaxonal turnover of proteins, and substitution of target-derived trophic support by factors produced by glial cells. It is concluded that there is, as yet, no coherent explanation for the upregulation or downregulation of any of the cytoskeletal proteins following axotomy or during regeneration.

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In considering the relevance of these changes in cytoskeletal protein synthesis to regeneration, it is emphasized that they are unlikely to be involved in the initial outgrowth of the injured axons, both because transit times between cell body and injury site are too long, and because sprouting can occur in isolated axons. Injury-induced acceleration of the axonal transport of tubulin and actin in the proximal axon is likely to be more important in providing the cytoskeletal protein required for initial axonal outgrowth. Subsequently, the increased synthesis and transport velocity for actin and tubulin increase the delivery of these proteins to support the increased volume of the maturing regenerating axons. Reduction in neurofilament synthesis and changes in neurofilament phosphorylation may permit the increased transport velocity of the other cytoskeletal proteins.

There is little direct evidence that alterations in cytoskeletal protein synthesis are necessary for successful regeneration, nor are they sufficient in the absence of a supportive environment. Nevertheless, the correlation that exists between a robust cell body response and successful regeneration suggests that an understanding of the regulation of cytoskeletal protein synthesis following axon injury must be a part of any successful strategy to improve the regenerative capacity of the central nervous system.

Index Entries: Regeneration; cell body reaction; axonal transport; conditioning lesion effect; actin; tubulin; neurofilament; GAP-43; nerve injury.

Introduction

A complete understanding of the role of cytoskeletal proteins in axon regeneration requires that we know what happens to:

1. The synthesis and posttranslational modification of these proteins in the cell body following injury and during regeneration, and how these changes are regulated;
2. The axonal transport of the proteins from cell body to the growth cone, including information about velocities and transported state of the proteins (as polymers or soluble molecules); and
3. The dynamics of the cytoskeletal proteins within the growth cone itself, including the role of these proteins in the extension of the axon shaft, the forward movement of the growth cone, and their involvement in the steering of the growth cone in response to environmental cues.

We will focus on the first of these issues. It is worth stating at the outset that the relevance of cell body changes in cytoskeletal protein synthesis to axon outgrowth is surprisingly uncertain, nor must we regard the axon as a passive conduit taking what the cell body gives it and feeding it to the growing distal end of the axon. Recent work shows that axonal injury provokes changes in the transport of cytoskeletal proteins already in transit within the axon (Tashiro and Komiya, 1991; Bisby and Reynolds, 1991). Furthermore, interactions between Schwann cell and axon produce profound local changes in the structure of the cytoskeleton (Reles and Friede, 1991), and in the phosphorylation state and axonal transport of neurofilament protein and tubulin (deWaegh and Brady, 1991), which may influence regeneration (deWaegh and Brady, 1990).

Changes in Cytoskeletal Protein Synthesis Induced by Axon Injury in Peripheral Neurons

Following a lesion made on the facial nerve at its exit from the stylomastoid foramen, the facial nucleus responds robustly with an increase in tubulin and actin synthesis, and a decrease in neurofilament synthesis (Tetzlaff et al., 1988). *In situ* hybridization with labeled cDNA probes permitted us to distinguish between glial and neuronal contributions to the overall response of the facial nucleus, and there was good agreement between the changes in synthesis of these proteins and changes in the expression of their mRNAs (Tetzlaff et al., 1991). Furthermore, use of more specific probes showed that for α-tubulin, the increased synthesis was partly owing to reexpression of a developmentally regulated gene (Tα1) normally turned down in the adult (Miller et al.,

1989). Similarly, there is increased expression of a developmentally regulated type II β-tubulin gene following axon injury (Hoffman and Clevelend, 1988).

These basic findings—increased tubulin and actin synthesis and/or mRNA levels, and decreased neurofilament levels—are quite consistent across neurons with peripheral axons (Hall et al., 1978; Hall, 1982; Quesada et al., 1986; Hoffman et al., 1987; Wong and Oblinger, 1987; Goldstein et al., 1988; Hoffman and Cleveland, 1988; Oblinger and Lasek, 1988; Hoffman, 1989; Oblinger et al., 1989a,b; Muma et al., 1990), as is the increased expression of GAP-43, which although not a cytoskeletal protein (being membrane-associated and rapidly transported [Skene, 1989]), has been included in many studies as a specific marker for the cell body reaction to injury, since its increase following injury (10–50-fold for mRNA levels) is considerably greater than for any of the cytoskeletal proteins.

Actin, tubulin, and neurofilament proteins do not exhaust the list of cytoskeletal proteins that may be involved in axon regeneration. Studies on the type III intermediate filament protein peripherin show that, unlike neurofilament, synthesis and axonal transport increase following axon injury (Oblinger et al., 1989b; Troy et al., 1990), a finding that remains to be placed in a functional context. Alterations in the synthesis of microtubule-associated and microfilament-associated proteins, with their potential for altering the polymerization/depolymerization dynamics of the axonal cytoskeleton, may be significant, and this is an area where much work remains to be done. A study on tau expression showed that although tau immunoreactivity and mRNA levels fell after axotomy, there was no shift to the low-mol-wt tau isotype characteristic of immature, growing neurons (Oblinger et al., 1991). This result is important, because it shows that the cell body reaction to injury is a distinct state, not merely a return to an embryonic state.

Cytoskeletal dynamics will also be affected by posttranslational modification of cytoskeletal proteins. Axotomy induces changes in the phosphorylation of neurofilament proteins, tubulin, and microtubule-associated proteins in cell body and axon of both peripheral and central neurons (Bignami and Gambetti, 1986; Goldstein et al., 1987; Rosenfeld et al., 1987; Larrivee and Graftstein, 1987a,b; Watterson et al., 1989; Mansour et al., 1989; Pestronk et al., 1990; Larrivee, 1990), and more study of the effects of axotomy on the responsible kinases is required.

Changes in the synthesis of cytoskeletal proteins induced by axotomy usually persist if no regeneration of the axons occurs, but gradually return to normal if regeneration and target contact are permitted (Hoffman and Cleveland, 1988; Goldstein et al., 1988; Tetzlaff et al., 1988; Miller et al., 1989). This shows that most of these cell body changes are not governed by an intrinsic cell body mechanism, but are regulated by the events that occur subsequent to injury. It should be noted, however, that the return to normal synthesis patterns does not occur simultaneously for all cytoskeletal proteins, suggesting that each is differentially regulated. We found that recovery of neurofilament synthesis began prior to the regrowth of axons to their targets, whereas the decrease in tubulin synthesis to normal levels occurred over a longer time-course, as did the downregulation of GAP-43 mRNA levels. Actin behaved differently: Its synthesis decreased slowly to normal levels, irrespective of regeneration (Tetzlaff et al., 1988,1991). Factors responsible for these changes in synthesis will be discussed in more detail later.

Central Neurons

The reaction of rubrospinal neurons to axotomy produced by spinal cord lesions at C3 was studied (Tetzlaff et al., 1991). The initial responses were qualitatively similar to those of the facial motoneurons: a decrease in mRNA for neurofilaments, and an increase in those for tubulin (including Tα1), actin, and GAP-43. However, after 8–14 d, a downregulation occurred in the tubulin and actin mRNA levels, so that they fell to subnormal values. This phenomenon was

never seen in the case of peripheral nerves, and parallels the appearance of atrophy in the rubrospinal neurons (Barron, 1983). However, during the time that the precipitous drop occurred in actin and tubulin mRNA levels, the expression of GAP-43 and Tα1 mRNAs remained elevated. We concluded that the rubrospinal neurons received the "axotomy signal" and responded to it initially by mobilizing a program of gene expression appropriate for axon regeneration. Indeed, there is evidence of sprouting by the lesioned rubrospinal axons during this period (Barron et al., 1989). After about a week, a secondary process intervened, causing a general down-regulation of cytoskeletal gene expression, but not suppressing specific features (Tα1, GAP-43) of the cell body reaction to axotomy. We do not know what is responsible for the later downregulation, but it seems likely that factors operating at the site of the lesion are ultimately responsible, because the cell bodies of both rubrospinal and facial neurons (which never showed the downregulation) are located within the same CNS environment, at least at the time the lesion is made, although subsequently differences in glial reaction may produce very different environments (Barron et al., 1990; Tetzlaff and Harrington, 1991).

Our finding of increased GAP-43 mRNA expression in injured CNS neurons seemingly contradicted earlier reports (Skene and Willard, 1981; Kalil and Skene, 1986; Reh et al., 1987), where no such increase was observed. This discrepancy may be the result of the distance between the lesion site and the cell body. Distal rubrospinal lesions failed to induce increased GAP-43 mRNA levels (Tetzlaff et al., 1990), and earlier work also showed that the cell body response of rubrospinal neurons depended on distance to the lesion (Egan et al., 1977). Corticospinal neurons only responded to axotomy by increasing their levels of GAP-43 mRNA if the axotomy was made very close to the cell bodies, by undercutting the cortex. Lesions within the spinal cord or at the medullary pyramids did not increase GAP-43 mRNA in corticospinal neurons (Tetzlaff and Giehl, 1991). Similarly, in retinal ganglion cells, GAP-43 immunoreactivity was only increased after close lesions (Doster et al., 1991). Rather puzzling, however, is the more recent report that GAP-43 mRNA levels in retinal ganglion cells were increased equally by close or distant lesions (Jones and Aguayo, 1991) (*see* GAP-43).

Corticospinal neurons are reluctant to upregulate not only GAP-43 synthesis, but also tubulin mRNA after axotomy. Lesions at the medullary pyramid, although they induce downregulation of neurofilament mRNAs, do not induce upregulation of α-tubulin mRNAs (Bisby et al., 1990; Mikuki and Oblinger, 1991). Again, very close lesions are necessary before an increase in tubulin mRNAs can be detected (Tetzlaff W., unpublished observations).

Increases in actin and tubulin synthesis (Heacock and Agranoff, 1976; Giulian et al., 1980; Tesser et al., 1986; Burrell et al., 1979) and mRNA levels (Neumann et al., 1983; Mizobuchi et al., 1990) occur in retinas of species whose optic nerve axons regenerate after injury, and in goldfish optic nerve, there is also increased transport of neurofilament and other intermediate filament proteins. In comparison, axotomy of rat optic nerve produces delayed decreases in mRNA levels for both neurofilament and tubulin (McKerracher et al., 1991), and instead of the increase in velocity and amount of cytoskeletal protein transport, which occurs in regenerating retinal ganglion cell axons (Grafstein and Murray, 1969; McQuarrie and Grafstein, 1982), the quantity and transport velocity of both tubulin and neurofilament proteins are reduced (McKerracher et al., 1990b). When rat retinal ganglion cell axons are persuaded to regenerate into peripheral nerve grafts, there is an increase in the transport velocity for tubulin and neurofilament, but a decrease for actin, so that all three major cytoskeletal proteins are traveling at approximately the same velocity (McKerracher et al., 1990a). Taken together, these observations on retinal ganglion cells and corticospinal neurons strongly suggest that changes in both synthesis and axonal transport of cytoskeletal proteins are prerequisites for successful regeneration of these axons.

Regulation of the Changes in Synthesis Following Axotomy

Perhaps the only real advance that has been made in our understanding of the signals regulating the cell body reaction to injury since Cragg's seminal review (Cragg, 1970) is the certain knowledge that some features of the cell body reaction, in NGF-sensitive cells, can be reversed by application of NGF (Fitzgerald et al., 1985; Rich et al., 1987; Verge et al., 1989). There are, as well, hints that other trophic factors may act in other cell types to rescue injured neurons from the effects of axotomy (Sendtner et al., 1990). Furthermore, factors responsible for the regulation of synthesis of any protein during the regeneration phase may not be simply the reverse of those factors responsible for the initial reaction to axon injury.

The observation that in PNS neurons many of the features of the cell body reaction return to normal if regeneration occurs, but not if it is prevented, restricts somewhat the range of signaling mechanisms that might be involved. There remain at least the following possibilities:

1. Restoration of target contact and recovery of normal supplies of target-derived retrograde trophic factor;
2. Elongation of axons through endoneurial cells restoring trophic factors normally provided by the nerve;
3. Substitution of normal target-derived retrograde trophic factor with trophic factors either from cells of the endoneurium encountered by regenerating axons or from glial cells surrounding the cell bodies of the injured neurons;
4. Recovery of the normal turnover of axonally transported materials consequent on axon elongation and conversion of growth cones into sensory or motor endings, subsequent to receipt of the "physiological stop signal" (Liuzzi and Lasek, 1987); and
5. Elimination, through reconstruction of the normal structure and permeability barriers, of signaling molecules produced outside the axon as a consequence of injury, that is, removal of a positive signal for axotomy, to be contrasted with 1, 2, and 3, which require restoration of a negative signal that suppresses the response to axotomy.

There are few hard data available to distinguish between these possibilities for the various cytoskeletal proteins, and for the other growth-associated proteins, perhaps because our experimental approaches are rather limited. These include: application of trophic factors to see if these can reverse or prevent axotomy-induced changes; application of colchicine or vinblastine blocks to nerve, hopefully to inhibit retrograde axonal transport selectively without injuring the nerve or affecting impulse transmission; application of cold blocks similarly to block axonal transport; and use of the "near/far" paradigm. This is used to differentiate between an event stimulated by loss of target contact (1) (which should not be affected by whether the lesion is made near to or far from the cell body) and an event stimulated by loss of normal axoplasmic turnover (4), or by loss of trophic factors derived from the endoneurial cells through which the axon passes (2) (which will be more severely affected by a lesion close to the cell body than by a more distal lesion, where the normal axoplasm and contact with the endoneurium are preserved to a greater extent).

Neurofilament Proteins

The fact that neurofilament synthesis begins to recover *before* axons reach their targets (Tetzlaff et al., 1988) suggests that endoneurial factors or intraaxonal feedback play an important role. However, results from PC12 cells implicate NGF as a putative regulator of neurofilament synthesis (Lee et al., 1982; Lindenbaum et al., 1987), and studies on the postnatal development of the axotomy-induced downregulation of neurofilament synthesis point to nerve- or target-derived trophic factors as being responsible for maintaining the normal high level of neurofilament synthesis in DRG neurons (Schwartz et al., 1990). The simple hypothesis that the initial downregulation of synthesis following axotomy is due to loss of target-derived or endoneurially derived trophic factors (e.g., NGF) and that recovery during regeneration is due to restoration of trophic factors

provided by endoneurial cells is not as yet definitively supported by the experimental evidence. In support, NGF treatment of transected sciatic nerve stumps increased the neurofilament content of dorsal root axons, but was without effect on ventral root axons, and treatment of normal adult rats with antiserum to NGF produced a reduction in sensory axon caliber and neurofilament content (Gold et al., 1991a). These results strongly suggest that NGF plays a role in the normal regulation of neurofilament synthesis, as well as in the axotomy-induced downregulation. *Au contraire*, a second study in which NGF was applied to axotomized nerve stumps failed to find any effect of NGF on the downregulation of neurofilament synthesis or on mRNA levels after axotomy (Wong and Oblinger, 1991). A third study did reveal an effect of NGF in stimulating recovery of neurofilament mRNA levels following axotomy, but only on a small subpopulation of DRG cells possessing high-affinity NGF receptors (Verge et al., 1990b). This might explain why no effect of NGF was detected in the study of Wong and Oblinger (1991), since in that study, only small numbers of cells were analyzed. Since there was no correlation between the possession of high-affinity NGF receptors and expression of high levels of NF mRNA in normal cells, it was concluded by Verge et al. (1990b) that NGF (and perhaps other retrograde trophic factors for the nonNGF responsive cells) was not directly responsible for regulating the synthesis of neurofilament protein, but served to maintain the neurons in a facilitatory state where other, unknown factors were responsible for expression of a high- or low-neurofilament-synthesizing phenotype.

If NGF is involved, even indirectly, in the response of neurofilament synthesis to axotomy, then the amount of NGF delivered to the cell body following axotomy and during subsequent regeneration should parallel the changes in neurofilament synthesis or mRNA levels. Raivich et al. (1991) showed that there was a gradual recovery of normal retrograde transport of NGF in sciatic nerve over the first 4 wk following crush injury. This occurred in spite of the increased synthesis of NGF by the distal nerve stump (Heumann et al., 1987) and was a result of profound axotomy-induced downregulation of axonal NGF receptors in dorsal root ganglion axons. If nerve regeneration was impeded by a resection lesion, the recovery of NGF transport did not occur. The time-course and magnitude of these changes in delivery of NGF to the dorsal root ganglion neurons closely parallel changes in their neurofilament mRNA expression.

Near/far experiments in sciatic nerve showed that distance is without effect in determining postlesion NFM mRNA levels in spinal motoneurons (Tsui et al., 1991) implicating a target-derived factor in the regulation of NFM synthesis rather than one produced by the endoneurium of normal nerve. Conversely, in retinal ganglion cells (McKerracher et al., 1991) and in rubrospinal neurons (Tetzlaff W., unpublished observations), neurofilament levels were far more reduced by near lesions. The recent demonstration of neurofilament accumulations at proximal ends of distal nerve stumps suggests that neurofilaments are bidirectionally transported in axons (Glass and Griffin, 1991), allowing for the possibility that neurofilament synthesis may be regulated by feedback of retrograde transported neurofilament protein or breakdown products at the cell body (Schlaepfer et al., 1984).

Regulation of the increased cell body neurofilament phosphorylation, which follows axotomy, has recently been studied (Gold et al., 1991b,c). Colchicine applied to sciatic nerve induced abnormal cell body neurofilament phosphorylation in dorsal root ganglion neurons, suggesting that increased phosphorylation is induced by loss of a retrograde trophic factor (Gold et al., 1991b). In a novel approach, acrylamide was used to inhibit regeneration of axotomized dorsal root ganglion neurons following crush injury of the sciatic nerve: This treatment resulted in a reduction in the number of neurons exhibiting abnormal neurofilament phosphorylation. It was concluded that two separate trophic signals were involved, one that was responsible for initiating the abnormal neurofilament phosphorylation

following axotomy and a separate signal, dependent on axonal elongation, that maintains it during the regeneration phase (Gold et al., 1991c).

Tubulin

Although in studies on retinal ganglion cells (McKerracher et al., 1991) and corticospinal neurons (Mikucki and Oblinger, 1991; Tetzlaff, W., unpublished observation), axotomy has provoked a reduction in tubulin mRNA levels, studies on other neuronal types have shown that there is an initial increase. NGF administration has been reported not to reduce this upregulation (Wong and Oblinger, 1991), whereas near/far experiments show a pronounced effect of distance, both in spinal motoneurons (Tsui et al., 1991) and in corticospinal neurons (Bisby et al., 1990; Tetzlaff W., unpublished observations). Regulation of the specific Tα1 tubulin isotype seems under control by retrograde factors, since it can be induced in adult facial motoneurons not only by axotomy, but also by blockade of axonal transport with cold block (Miller F. D., personal communication). Near/far experiments show that in superior cervical ganglion, a close axotomy evokes a much more vigorous upregulation (Mathew and Miller, 1991).

Thus, it appears that there is a consensus that the length of axon remaining after injury determines the level of tubulin upregulation. There are several possible explanations for length dependency. "Sustaining" collaterals, which are lost with a proximal, but not with a distal axotomy and which are sources of retrograde-transported trophic factor, are possible explanations for corticospinal neurons, but are unlikely to be important in sympathetic postganglionic or spinal motor neurons. More likely is the loss of retrograde trophic factor synthesized by the endoneurial cells and taken up by the axon along its length, but candidate factors are unknown. Although peripheral nerve synthesizes ciliary neurotrophic factor (CNTF) (Stockli et al., 1989), it is not retrograde transported (Smet et al., 1991), and its production by Schwann cells is decreased in the degenerating distal nerve (Friedman et al., 1991; Seniuk et al., 1991; Tetzlaff and Harrington, 1991), but increased in the vicinity of the axotomized neuron cell bodies (Tetzlaff and Harrington, 1991). The function of CNTF in peripheral nerve is unknown: released by tissue damage, it may play a role in the initial stages of regeneration at the site of injury (Smet et al., 1991). It prevents the death of injured neonatal facial motoneurons (Sendtner et al., 1990), and the greater survival of these neurons in the adult may be a consequence of the perineuronal postaxotomy increase in synthesis (Tetzlaff and Harrington, 1991). It seems that CNTF does not function as an informative molecule providing the cell body with information about the axon terminals and does not conform to the stereotype established by NGF.

GAP-43

Upregulation of GAP-43 mRNA expression is independent of lesion distance in spinal motoneurons (Tsui et al., 1991), but highly distance-dependent in corticospinal and rubrospinal neurons (Bisby et al., 1990; Tetzlaff et al., 1990; Tetzlaff and Giehl, 1991). Surprisingly, although expression of GAP-43 mRNA in retina following optic nerve injury is not length-dependent (Jones and Aguayo, 1991), expression of GAP-43 immunoreactivity is (Doster et al., 1991), implying perhaps some translational modification of GAP-43 synthesis. Blockade of axonal transport by vinblastine application to sciatic nerve induced GAP-43 mRNA in dorsal root ganglion (Benowitz et al., 1990), but if the stimulatory effect of vinblastine is owing to the blockade of a retrograde-transported trophic factor, it is unlikely to be NGF. NGF application to axotomized DRG did not diminish the usual increase in GAP-43 mRNA expression. Moreover, in normal DRG cells, there is a good correlation between neurons expressing high-affinity NGF receptors and their levels of GAP-43 mRNA expression, suggesting that in their normal, uninjured state, NGF serves to upregulate GAP-43 mRNA in these cells (Verge et al., 1990a).

Multiple Signals

After wading through this catalog, perhaps all that can reasonably be concluded about the postaxotomy changes in cytoskeletal protein synthesis is that the different proteins appear to be differentially regulated by a variety of factors. Three examples suffice to emphasize this point. First, *the same gene product is differentially regulated in different cells.* After facial nerve transection, there is a fivefold increase in CGRP and in CGRP mRNA in the facial nucleus. In contrast, section of the sciatic nerve induces a reduction, to about one-half, of the CGRP content and mRNA levels in the L5 dorsal root ganglion (Dumoulin et al., 1991). Reduction in NGF transport may be the regulatory factor in the dorsal root ganglion (Lindsay and Harmar, 1989), but the factor responsible for the increase in motoneurons is unknown, although this response can also be elicited by colchicine application (Rethelyi et al., 1991).

Second, *axotomy of different processes of the same neuron evokes different cell body responses.* Sciatic nerve section results in increased GAP-43, and decreased neurofilament protein and mRNA levels in the dorsal root ganglion. Dorsal root section does not affect GAP-43 levels (Woolf et al., 1990), but does downregulate neurofilament mRNA levels (Wong and Oblinger, 1990). The stimulus for neurofilament mRNA downregulation is evoked by injury to either dorsal root or peripheral nerve, whereas only peripheral nerve injury generates the signal necessary for upregulation of GAP-43.

Third, *different manipulations of the same axons can generate different aspects of the cell body reaction.* Neurotrophin receptors (NTR—also known as low-affinity nerve growth factor receptors) are expressed on motoneurons following axotomy (Ernfors et al., 1989; Wood et al., 1990; Koliatsos et al., 1991; Armstrong et al., 1991). Unlike almost every other component of the cell body reaction, a nerve crush (which allows rapid regeneration) evokes a more pronounced increase in NTRs than nerve section (Wood et al., 1990; Saika et al., 1991). Vincristine block of axonal transport induced loss of choline acetyltransferase in hypoglossal motoneurons without inducing NTRs, whereas mild trauma to the nerve induced NTRs with little effect on choline acetyltransferase. Axonal damage itself may be responsible for upregulating NTR expression, whereas loss of retrograde-transported trophic factor may signal choline acetyltransferase downregulation (Hayes et al., 1991). It should be noted, however, that axonal cold block induces NTR expression in facial motoneurons (Miller F. D., personal communication).

Twenty-one years postCragg, we are still mired in the phenomenonological phase of examining the cell body response to axotomy. A change of strategy would seem in order. We may discover more by working backwards from regulatory sequences in the genes for the cytoskeletal proteins, and transgenic animals bearing regulatory sequences attached to reporter genes provide one rational new approach to this difficult old problem.

Contributions of Changes in Cell Body Synthesis of Cytoskeletal Proteins to Axon Regeneration

Direct evidence that these changes in cell body synthesis are necessary for axonal regeneration is indeed scarce. It is clear that the initiation of axonal sprouting following injury does not require new protein synthesis, since even axons isolated from the cell body will sprout (Rotschenker, 1981) and formation of growth cones does not require connection with the cell body (Shaw and Bray, 1977). Consideration of the average velocity of axonal transport of cytoskeletal proteins and the similar velocity of outgrowth of regenerating axons lead to the conclusion that the cytoskeletal proteins entering regenerating axons were derived from the parent axons and not synthesized subsequent to the axotomy (McQuarrie and Lasek:, 1989). We might well ask whether the cell body synthesis changes are even relevant to axon regeneration, since they are not required for sprouting or for the outgrowth of the new axon.

In defense of the hypothesis is one direct item and other circumstantial evidence. Protein synthesis inhibition in the regenerating goldfish retinal ganglion system leads to the cessation of axon outgrowth (McQuarrie and Grafstein, 1983). In frogs cooled to 15°C, there was no detectable cell body response to axotomy, and although axon outgrowth began, it was not sustained (Carlsen et al., 1982). In our studies on rubrospinal and corticospinal neurons, we found a correlation between the initial expression of the cell body response (neurofilament decrease, tubulin and actin increase) and the previously reported ability of the injured axons to regenerate into a peripheral nerve implant (Richardson et al., 1984). It seems that the potential for axons to regenerate is correlated with the characteristic sequence of cell body responses, and although this is necessary for regeneration, it is not sufficient in the absence of a supportive environment. This point is well demonstrated in facial motoneurons of the C57BL/01a mouse, where peripheral axon regeneration is severely impaired because of failure of the normal process of Wallerian degeneration (Lunn et al., 1989). The initial cell body response to injury, in terms of decreased neurofilament and increased GAP-43 mRNA expression, is similar to that of normal mice (Bisby and Quarrington, 1992).

The most substantial body of evidence in support of the concept that changes in cell body synthesis influence axon regeneration comes from work on the conditioning lesion phenomenon. The increased regeneration rate of axons growing from a test lesion, subsequent to a previous conditioning lesion, was ascribed to the stimulation of the cell body response produced by the conditioning lesion, specifically its effects on cytoskeletal protein synthesis (McQuarrie, 1984). When the test lesion was made close to the cell body so that proteins synthesized following the conditioning axotomy would reach the site of the test lesion within the conditioning-test interval, the ratio of neurofilament/tubulin in the conditioned sprouts was decreased, reflecting the cell body synthesis changes induced by the conditioning lesion (Oblinger et al., 1989a). The test lesion itself does not provoke a further increase in tubulin, actin, or GAP-43 synthesis, but does produce a further decrease in neurofilament synthesis (Tetzlaff et al., 1987). Unfortunately, a conditioning effect can be demonstrated with conditioning-test intervals of insufficient duration to allow the postconditioning lesion alterations in cell body cytoskeletal protein synthesis to reach the site of the test lesion (Jacob and McQuarrie, 1991), so the accelerated outgrowth of conditioned axons cannot be the result only of changes in the *synthesis* of cytoskeletal proteins.

Jacob and McQuarrie (1991) suggest an alternative explanation. They observed that conditioning accelerated the velocity of slow component b (SCb) axonal transport, consistent with earlier observations that regeneration rate was correlated with SCb velocity under a variety of circumstances (Wujek and Lasek, 1983; Cancalon, 1983; McQuarrie et al., 1989). They inferred that SCb proteins already in transit at the time of the conditioning lesion must have been accelerated: Indeed, such a direct effect of axotomy on SCb tubulin and actin has been reported (Tashiro and Komiya, 1991; Bisby and Reynolds, 1991). Jacob and McQuarrie (1991) suggest that the changes in cell body cytoskeletal protein synthesis influence the rate of SCb: The increases in actin and tubulin synthesis that follow axotomy might somehow increase the rate of their transport (even of already synthesized actin and tubulin) all along the axon and account for the higher regeneration rate in conditioned axons. A problem with this intriguing idea is that after a single lesion, the increase in tubulin synthesis does not reach a maximum until 7 d after injury (Tetzlaff et al., 1988), yet the regeneration rate remains constant after the first 3 d (Sjoberg and Kanje, 1990).

There is thus little direct evidence that changes in cytoskeletal protein synthesis following axotomy directly influence the process of axon outgrowth. Even the conditioning lesion phenomenon is more satisfactorily explained on the basis of an injury-induced acceleration of axonal transport SCb, containing actin and tubulin synthesized prior to axotomy. Indeed, this axonal

response to injury might be vital in providing adequate amounts of preexisting tubulin and actin to the regenerating sprouts after axon injury. Tashiro and Komiya (1991) showed that there is both an increase in the amount of soluble tubulin and actin in SCb, and an acceleration of the leading edge of the SCb wave following axonal injury. Bisby and Reynolds (1991) similarly showed that injury caused a shift of tubulin, already undergoing transport in the more slowly moving slow component a (SCa) into SCb. There is a lively controversy about the manner in which microtubules extend into the newly found axon, closely related to the equally vigorous debate about the physical form of the tubulin molecules transported along the axon (Hollenbeck, 1989; Okabe and Hirokawa, 1990; Lim et al., 1990; Cleveland and Hoffman, 1991; Tanaka and Kirschner, 1991; Reinsch et al., 1991). If SCb tubulin is present in a soluble, unpolymerized form and SCa tubulin is polymerized tubulin, then the shift from SCa to SCb represents mobilization of polymerized tubulin for rapid movement to the growth cone and its reassembly there onto the plus end of microtubules. Alternatively, SCb tubulin might still be primarily polymerized, but somehow freed from impeding neurofilament interactions (Cleveland and Hoffman, 1991) *(see below)* and able to move rapidly to the growth cone in support of its forward advance. Similar arguments can be made for actin, which is present in unpolymerized forms in the axon to a greater extent than tubulin (Tashiro and Komiya, 1989), and which assembles into microfilaments in the growth cone (Okabe and Hirokawa, 1991).

The increased transport of actin and tubulin in SCb applies also to these proteins synthesized after axotomy (Hoffman and Lasek, 1980; Oblinger and Lasek, 1988; Tashiro and Komiya, 1991). Thus, owing both to the increased average velocity of transport and increased synthesis, the delivery of tubulin and actin to the axon is greatly increased. It seems reasonable to suppose that this increased delivery is necessary to support the massive increase in axonal volume, which occurs as the regenerated axon sprouts mature. Whether the specific α and β tubulin isotypes that are synthesized by injured neurons (Miller et al., 1989; Hoffman and Cleveland, 1988) are specialized so that they are more likely to be associated with SCb than with SCa is not known. Specific isotypes are preferentially associated with SCb (Denoulet et al., 1989). Other suggestions for the increased proportion of newly synthesized tubulin in SCb include the intriguing idea that local "disruption of the axonal cytoskeleton might produce a self-propagating phase transition which could reach the cell body cytoskeleton without the mediation of retrograde axonal transport" (Grafstein, 1991). Obviously, such a mechanism could also be a candidate for the effector of the direct injury-induced acceleration of tubulin transport, as well as the postaxotomy increase in tubulin synthesis. Testing this hypothesis will be an interesting experimental challenge. Alternatively, as discussed in the following, the downregulation of neurofilament synthesis may play a role.

The downregulation of neurofilament synthesis, a universal response to axon injury, is not yet understood in terms of its importance to subsequent axon regeneration. One working hypothesis for why it is desirable to downregulate NF synthesis after axotomy is that interactions between neurofilament and microtubules retard the movement of tubulin into the regenerating axon (Tetzlaff et al., 1988; Oblinger et al., 1989a; Cancalon, 1990). Labeled tubulin is conveyed down the axon both in SCa (<1.5 mm/d) at the same velocity as neurofilaments (Black and Lasek, 1980; Tashiro et al., 1984), and in SCb (~4 mm/d) where, as mentioned previously, a much higher fraction is soluble. Tubulin may be trapped in SCa, either by crossbridge formation between microtubules and neurofilaments (Hirokawa et al., 1988), or simply as a result of steric hindrance between microtubules and neurofilaments (Oblinger et al., 1989). A reduction in neurofilament synthesis following axotomy may allow a greater proportion of newly synthesized tubulin to enter SCb, accounting for the observed shift of tubulin into SCb that occurs after axotomy. In facial motoneurons, a second axon injury, which caused a condi-

tioning lesion effect, produced a further down-regulation in neurofilament synthesis and mRNA levels (Tetzlaff et al., 1987). Furthermore, the conditioning paradigm also produced a further shift of transported actin and tubulin into SCb, and faster velocities (Bisby and Reynolds, 1991).

The phosphorylation state of neurofilament protein may be key to interactions between microtubules and neurofilaments (Hirokawa et al., 1988). Dephosphorylation of the 200-kDa neurofilament protein (NFH) results in increased interactions between neurofilaments and microtubules (Hisanaga and Hirokawa, 1990; Hisanaga et al., 1991). Thus, after axotomy, when cell body neurofilament phosphorylation is increased, interactions with microtubules may be reduced, both allowing more tubulin to be transported into the axon in SCb and causing an accumulation of neurofilaments within the cell body, in spite of the decrease in neurofilament protein synthesis (Sinicropi and McIlwain, 1983; Moss and Lewkowicz, 1983; Goldstein et al., 1987). Following initial phosphorylation in the proximal axon, NFH phosphorylation decreases along the length of the axon, and there is a correlation among the tendency of axons to sprout after injury, the length of the axon, and the degree of NFH phosphorylation in those axons. Shorter axons sprouted to a greater extent, had a higher degree of NFH phosphorylation (Pestronk et al., 1990), and presumably reduced interactions between neurofilaments and microtubules. In corticospinal axons, where there is no regeneration after injury, there are low levels of NFH phosphorylation, and most tubulin is in SCa (Oblinger, 1988). Novel tests of the hypothesis that neurofilaments constitute an impediment to axonal regeneration may be provided by transgenic animals, where neurofilaments are overexpressed (Monteiro et al., 1990; Vidal-Sanz et al., 1991).

Concluding Remarks

Injury to the axon provokes a multiplicity of metabolic changes in the neuron soma, including changes in synthesis of the major cytoskeletal proteins. In successfully regenerating neurons, these generally include increases in actin and tubulin synthesis, and decreases in neurofilament synthesis. These changes in cell body synthesis, coupled with changes in the velocity of actin and tubulin synthesis into the injured axon, are likely to increase the supply of actin and tubulin to the regenerating axon. The transport changes may be consequent on changes in the synthesis of these proteins, but there is recent evidence that intraaxonal changes are also involved, acting on actin and tubulin already synthesized and in transit. Since the transport state and polymerization of cytoskeletal proteins are highly dependent on posttranslational modifications and the binding of associated proteins, an understanding of these phenomena requires a more thorough study of the impact of axonal injury on synthesis of these associated proteins and the posttranslational enzyme systems.

The ultimate rationale for undertaking research into the effects of injury on neuronal metabolism is to devise strategies to foster and direct axon regeneration in the adult human central nervous system (knowledge for its own sake is apparently no longer a politically acceptable goal). For this reason, improved understanding of the mechanisms regulating the changes in cell body metabolism seen after injury is a priority. Even when lesioned central nervous system axons are provided with an appropriate environment, we cannot help but be impressed by the small number of axons capable of regrowth (Vidal-Sanz et al., 1987). Treatments that enhance the cytoskeletal components of the cell body reaction to injury seem to be a necessary component of any attempt to improve the regenerative capacity of injured central nervous system axons.

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