University of Alberta

Molecular and Cellular Mechanisms Regulating Neuronal and Schwann Cell Gene Expression during Peripheral Nerve Regeneration

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Edmonton, Alberta

Fall 1996

ISBN 0-612-18135-9

University of Alberta

Library Release Form

Name of Author: Wendong Wu

Title of Thesis: Molecular and Cellular Mechanisms Regulating Neuronal and Schwann Cell Gene Expression during Peripheral Nerve Regeneration

Degree: Doctor of Philosophy

Year this Degree Granted: 1996

7580 SW 82 ST., APT.# F-114

Miami, Florida 33143-7330

USA

October 1, 1996

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled MOLECULAR AND CELLULAR MECHANISMS REGULATING NEURONAL AND SCHWANN CELL GENE EXPRESSION DURING PERIPHERAL NERVE REGENERATION submitted by WENDONG WU in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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Date August 30, 1996

Acknowledgment

To Dr. Freda Miller, my supervisor, whose high professional standards, complete understandings, wise guidance, invaluable advice, great encouragement, and constant support, have made possible the successful completion of my Ph.D. program, I owe an innumerable debt of gratitude.

To the members of my supervisory committee, Dr. Robert Campenot, for his continued encouragement and support, Dr. Richard Smith, for his invaluable advice and hard work of collaboration, Dr. Larry Guilbert and Dr. Richard Murphy, for their helpful comments and great suggestions, I express my deep appreciation.

To the students, faculty and staff, at the Department of Anatomy, especially my colleagues, Ms. Honey Chan, and everyone at Dr. Freda Miller's laboratory (now at Center for Neuronal Survival, Montreal Neurological Institute), for their cooperation and support, I offer my sincere thanks.

To my family, my wife, Fang Guo, my mother, Cuilan Wu, and my sister, Lynn Vair, for their continuing love, enduring patience, and strong support through all my endeavors, I express my heartfelt thanks.

This work was supported by a studentship from the Rick Hansen Man in Motion Foundation.

Abstract

Following peripheral nerve injury, there are series of changes in gene expression in neurons and Schwann cells. There are generally two theories for inducing these changes: macrophage invasion and inflammatory response; or loss of homeostatic interaction among neurons, Schwann cells, and target. We hypothesize that the alterations in neuronal and Schwann cell gene expression observed following peripheral nerve injury are at least partially due to the disruption of ongoing homeostatic signals. Our strategy is to attempt to interrupt normal homeostatic signals by disrupting axonal function without inducing an inflammatory injury response, and to determine whether such disruption leads to alterations in Schwann cell or neuronal gene expression that are similar to those observed following peripheral nerve injury. Cold block on intact peripheral nerve blocks the fast axonal transport and distal electrical activity without inducing macrophage invasion or Wallerian degeneration. I have been able to demonstrate that induction of at least some changes during peripheral nerve injury are independent of macrophage invasion or inflammatory response. The results are described in more detail next.

Following nerve injury, Schwann cells distal to the site of injury downregulate genes associated with myelination. We hypothesized that at least some of these alterations were due to the loss of ongoing axon:Schwann cell homeostatic signals, as opposed to loss of physical contact and/or inflammatory responses. To directly test this hypothesis, we perturbed axonal physiology by selectively blocking fast axonal transport and electrical activity via locally-cooling the sciatic nerve to 5-8° C (a cold block). We demonstrated that the nerve distal to the cold block showed no signs of Wallerian degeneration, with maintenance of normal axon and myelin profiles, and

the absence of invading macrophages. Levels of p75 neurotrophin receptor (NTR) mRNA were unaffected by the cold block, while p75 NTR protein levels were increased in the region of the nerve immediately adjacent to the cold block, presumably reflecting protein accumulation as a consequence of the block to fast axonal transport. In contrast, levels of Po mRNA were decreased in the distal nerve in a fashion that indicated modulation of Schwann cell phenotype as a function of local axonal microenvironment. Thus, Po and p75 NTR are regulated as a function of two different aspects of Schwann cell:axon communication. Furthermore, these data demonstrate that the presence of axon:Schwann cell contact alone is insufficient to maintain Po gene expression, and indicate that at least some myelin-specific Schwann cell responses are dependent upon ongoing biochemical signals generated by the axon and maintained by fast axonal transport and/or electrical activity (Wu et al., 1994a).

Axotomy of motoneurons leads to upregulation of Tα1 α-tubulin and p75 NTR mRNAs (Miller et al., 1989; Ernfors et al., 1989; Wood et al., 1990a; Koliatsos et al., 1991; Saika et al., 1991; Armstrong et al., 1991). To distinguish whether these increases are due to interruption of ongoing homeostatic signals or to positive factors derived from nonneuronal cells of the injured nerve, we use the same paradigm as in the sciatic nerve model mentioned above, to selectively block axonal transport and electrical activity. We demonstrated that a cold block induced Tα1 α-tubulin and p75 NTR mRNAs to the same level as did a corresponding nerve transection. In contrast, T26 α-tubulin mRNA, which does not increase following axotomy, was not affected by the cold block treatment. These data suggest that neurons sense their status by a constant flow of information carried, circulated, or maintained by fast axonal transport and that the axotomy-induced increases in Tα1 and p75 NTR mRNAs in

motor neurons are, to a great extent, due to loss of such homeostatic signals (Wu et al., 1993).

Another approach we used to study signaling mechanisms in the neuronal injury response was to study Tα1 α-tubulin gene expression at the transcriptional level in a Tα1:nlacZ transgenic mouse to define the molecular mechanisms underlying this pattern of gene expression. It has been previously demonstrated that expression of Tα1 α-tubulin mRNA is increased following axonal injury (Miller et al., 1989, 1990; Tetzlaff et al., 1991), and that the extent of the increase is a function of the amount of axon that is lost (Mathew & Miller, 1993). Following axotomy of facial motoneurons, the Tα1:nlacZ transgene was rapidly upregulated, transcription was maximal for 1-7 days, and, if neurons regenerated and reinnervated their target musculature, transcription returned to control levels. Moreover, if regeneration was inhibited, transgene expression remained elevated, suggesting that target contact represses expression of the Tα1 gene, and that loss of this transcriptional repression largely accounts for the pattern of Tα1 mRNA expression in regenerating motoneurons. In contrast, in sympathetic neurons of the superior cervical ganglion, differences were observed between the Tα1:nlacZ transgene, and the endogenous mRNA. While Tα1 mRNA only increased when neurons were axotomized close to, but not distal from their cell bodies (Mathew & Miller, 1993), transgene expression was increased regardless of the injury site. It is likely that this "distance effect" is due to posttranscriptional mechanisms that couple tubulin mRNA levels to cytoskeletal status; when cultured sympathetic neurons were treated with colchicine, which depolymerizes microtubules, steady-state Tα1 mRNA levels were greatly decreased. Thus, a combination of transcriptional and posttranscriptional mechanisms allow

mature neurons to closely modulate synthesis of this essential cytoskeletal protein as a function of both target contact and axonal status (Wu et al., 1996).

In summary, there are intrinsic and extrinsic mechanisms mediating neuron, Schwann cell and target interactions. Our data suggest that at least some alterations are independent of macrophage invasion or local inflammatory response. This is an important contribution to understanding the mechanisms of peripheral injury response, and suggest that peripheral nerve injury perturbs a few or many aspects of the intrinsic homeostatic interactions, thereby inducing a series of phenotypic changes that are manifested in changes in gene expression. However, the nature of homeostatic signals remains to be elucidated. In addition, our data support the hypothesis that the target influence neuronal expression of growth-associated genes at the transcriptional level, and that neurons possess the intrinsic ability to monitor the status of the axonal cytoskeleton in a dynamic fashion following axonal injury.

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CHAPTER ONE GENERAL INTRODUCTION

I Overview of peripheral nerve injury responses

Following peripheral nerve injury, there are a series of well-characterized morphological responses. These include axon and myelin breakdown, changes in the permeability of the blood vessels, proliferation of Schwann cells, invasion of macrophages, and phagocytosis of myelin fragments by Schwann cells and macrophages. The distal segment of the injured peripheral nerve thereby provides a supportive environment for the regeneration of the nerve fibers(for review, see Cajal 1991; Beuche et al. 1984; Fawcett & Keynes, 1990; Clarke & Richardson, 1994).

1. Following axotomy of the peripheral nerve, the distal nerve segment undergoes Wallerian degeneration, and the damaged neurons respond to injury as shown by the process of chromatolysis.

Wallerian degeneration

Transection of a vertebrate peripheral nerve triggers Wallerian degeneration (for reviews, see Hallpike, 1976; Allt, 1976; Sunderland, 1978) in the distal stump. Axons and myelin degenerate and disappear but connective tissue and dedifferentiated Schwann cells persist and provide a framework for subsequent axonal regeneration, myelination, and reinnervation of the peripheral target. Disruption of axonal integrity during Wallerian degeneration is associated with profound changes in myelinated fibers. Changes in the distal axon segment become apparent about 1 week after the distal stump begins to degenerate, and continue over the next 1-2 months, or until the entire distal segment is destroyed. Internodes break up into smaller myelin ovoids enclosing fragments of axonal debris (Williams & Hall, 1971). Schwann cells become hypertrophied in the paranuclear region and undergo mitosis. The rapid anterograde spread of premitotic activity in Schwann cells and fibroblasts can be detected as early as 3-4 days following injury of sciatic nerve (Oaklander et al., 1987). Schwann cell proliferation appears to be a critical event in Wallerian degeneration, since pharmacological inhibition of cell division in the distal stumps retards myelin disintegration and subsequent axon regeneration (Hall & Gregson, 1974; Pellegrino et al., 1986).

Chromatolysis

Shortly after axotomy, the cell body and its nucleus swell, the nucleus moves from its typical position in the center of the cell soma to an eccentric location, and the ordered arrays of endoplasmic reticulum, called Nissl substance, break apart and disperse to the periphery of the swollen cell body. This phenomenon is called chromatolysis, and is a reflection of altered patterns of protein synthesis (for reviews, see Cragg, 1970; Lieberman, 1971; Waston, 1974). Within a few hours, new axonal sprouts emerge from near the tip of the proximal stump and regeneration begins. If the neuron successfully reestablishes synaptic contact with a target, the cell body regains its original appearance. Failure to contact new target cells, however, leads to a variety of changes in adult peripheral neurons. Axotomized sensory and motoneurons survive but atrophy and lose some of their differentiated properties. In autonomic ganglia, axotomized ganglion cells become less sensitive to acetylcholine, shrink, and may eventually die.

2. Neurons in the peripheral nervous system can regenerate their axons.

Morphological responses of the axon

Damage to the peripheral nervous system is frequently reversible. Neurons are able to regenerate and eventually restore functional connections with their target organs. Axons begin to regenerate within a few hours of axotomy. The first sprouts in myelinated axons are generally seen coming from the terminal nodes of Ranvier, through the gap left by partial retraction of Schwann cells (Friede & Bischhausen, 1980; Meller, 1987; McQuarrie 1985). Unmyelinated axons sprout equally rapidly (Bray et al., 1972). While these sprouts are forming, the cut tip of the axon swells, inflated with smooth endoplasmic reticulum, mitochondria, and eventually microtubules. The regenerating sprouts, of which there may be several from each axon, grow down the endoneural tubes; the growth cones are usually in contact with Schwann cell basal lamina on one side, and with the Schwann cell membrane on the other (Haftek & Thomas, 1968; Scherer & Easter, 1984). At a forward rate of 1-2mm/day, the axons continue to grow in this fashion back toward their targets.

Interactions between regenerating axons and their environment

The environment through which axons regenerate in the PNS consists of Schwann cells and their basal lamina, fibroblasts, and collagen, and, earlier in regeneration, axonal debris, degenerating myelin, and phagocytic cells. Of these, the Schwann cell is the critical factor promoting axon regeneration. If the endoneural tubes and Schwann cell basal lamina are left intact, as is often the case with crush injuries (Haftek & Thomas, 1968), Schwann cells and fibroblasts may provide guidance cues (Wigston & Donohue, 1988) and the regenerating axons usually remain in their parent tubes which guide them directly back to their target. Whenever live Schwann cells are absent from the terrain confronting regenerating axons, the axons fail to grow, or their growth is much reduced(Ide et al., 1983; Hall, 1986).

In this chapter, I will review the specific changes in neuronal and Schwann cell associated molecules, with complete focus on neuronal growth associated tubulin gene and myelin specific Po gene during regeneration and the molecular and cellular mechanisms that potentially induce peripheral nerve injury responses. Understanding the mechanisms of peripheral nerve regeneration will not only benefit regeneration in the peripheral, but also in the central nervous system, where lack of molecules to facilitate growth and persistence of inhibitory substances become the major hurdles (Schwab & Caroni, 1988, 1993; Schnell & Schwab, 1990).

II. Changes in tubulin gene expression in neurons and Po myelin gene expression in Schwann cells following peripheral nerve injury

1. Tubulin gene expression in neurons of the peripheral nervous system

The onset of neuronal regeneration is associated with expression of new genes and proteins. In general, the proteins produced during regeneration are the same as those associated with axonal growth in embryos. Substances that are abundant in developing axons, such as growth associated proteins (GAPs, e.g., GAP-43, for review, see Skene, 1989; Bennowitz & Ruttenberg, 1987), tubulin (see below), and actin (for review, see Bentley & O'Conor, 1994; Heidemann & Buxbaum, 1991), have their synthesis enhanced, whereas neurofilament protein (for review, see Steinert & Roop, 1988), which mainly appears in development when axons have connected with their targets and are expanding radially (thickened), is decreased. In the following section, I will discuss one representative of regeneration associated gene, tubulin, with an emphasis at the molecular and cellular level.

Neuron specific isotypes of tubulin

Microtubules are fibrillar structures common to most eukaryotic cells. They are polymers consisting of one major protein called α and β tubulin and a variety of minor components known as microtubule-associated proteins (MAPs). Microtubules are the major component of the neuronal cytoskeleton. They establish and maintain polarized cell shape in neurons, and provide spatial and dynamic organization in the cytoplasm, through their interaction with a variety of different cellular organelles (for review, see Mandelkow and Mandelkow, 1990). Microtubules contribute to intracellular transport and secretory movements (Wallin, 1988), and provide the cellular machinery for axonal transport (Hirokawa, 1993 a&b). In mammals both α and β tubulin isotypes are encoded by large multigene families (Cleveland and Sullivan, 1985; Sullivan, 1988). At least six α-tubulin genes (Villasante et al, 1986) and five β-tubulin genes (Wang et al., 1986) are expressed in mammals. Of these, 2 distinct α-tubulin mRNAs (Lemischka et al., 1981; Ginzberg et al., 1986) and 4 β-

tubulin mRNAs (Bond et al., 1984; Joshi & Cleveland, 1989) are expressed in rat nervous system. A number of different approaches have recently revealed that βII and III-tubulin isotypes play functionally divergent roles in the nervous system (Joshi & Cleveland, 1989; Lewis et al., 1985; Sullivan & Cleveland, 1986). The two α-tubulins mRNAs, Tα1 (Lemischka et al., 1981) and T26 (Ginzberg et al., 1986) are the only members known to be expressed in the embryonic rat nervous system. The proteins encoded by Tα1 α-tubulin mRNA differs by only a single amino acid from the protein encoded by T26 mRNA (Cowan et al., 1983), with a glycine and serine at residue 232, respectively.

Tubulin expression during neuronal development and regeneration

A significant number of studies indicate that tubulin genes are differentially regulated during development of the nervous system. One each of the α- and β-tubulin genes are apparently turned on as soon as neurons commit to their fate. This has been demonstrated using an antibody, TuJ1, that is specific to class III β-tubulin (Frankfurter et al., 1986; Sullivan & Cleveland, 1986) and a transgenic mouse in which a marker gene is driven from the Tα1 α-tubulin promoter (Gloster et al., 1994a). Specifically, in the central nervous system and peripheral trigerminal system of chick embryos, the first appearance of TuJ1 immunoreactivity coincide with the terminal mitosis of neuroblasts (Moody et al., 1989; Lee et al., 1990). More recently, Kameda et al. (1994) took advantage of this early expression to demonstrate the directional movements and migration of the precursors of parasympathetic neurons from the distal vagal ganglion to the carotid body region in chick embryos. In mouse brain, the first TuJ1 immunoreactive cells appeared at E8.5, prior to neural tube closure, in the neural plate immediately caudal to the optic vesicle (Easter et al., 1994), while the first axons in the dorsal mesencephalon appeared at E9.0. This coincidence suggests that close relationship of the terminal mitosis of neuronal precursor cells and induction of neuron specific β-tubulin expression even before neurons start to extend axons. With regard to the Tα1 α-tubulin gene, transgenic mice carrying a fusion gene comprised of 1.1 kb nucleotides of the upstream Tα1 promoter region linked to a nuclear β-galactosidase reporter gene were generated (Gloster et al., 1994a). Developmentally, expression of the transgene appeared early during embryogenesis, coincident with neurogenesis (Gloster et al., 1994b). Moreover, in the

adult olfactory epithelium, a region of ongoing neurogenesis, transgene expression remained elevated.

These two genes, class II, III β-tubulin, and Tα1 α-tubulin genes are all expressed at high levels in developing neurons and subsequently downregulated in the adult. In contrast, other isotypes of β-tubulin, such as class I and IV(Hoffman & Cleveland, 1988), and other isotypes of α-tubulin, such as T26 (Miller et al., 1987 a&b) show little change during nervous system development. This downregulation of tubulin isotype expression following neuronal maturation can be quite dramatic. For example, Tα1 α-tubulin mRNA accounts for more than 95% of total α-tubulin mRNAs at embryonic day 16, whereas, upon maturation, it drops to 5-10% of the total α-tubulin mRNA (Miller et al., 1987 a&b). Interestingly, all three of these genes, Tα1, class II and III β-tubulin, are again increased in their expression following axonal injury (Miller et al., 1989; Tetzlaff et al., 1991; Hoffmann & Cleveland, 1988; Oblinger, 1989 a&b; Wong & Oblinger, 1990; Moskowitz et al., 1993). In particular, Tα1 α-tubulin mRNA increases following axotomy of motor (Miller et al., 1989), sympathetic(Mathew & Miller, 1990), and central rubrospinal neurons (Tetzlaff et al., 1991). Following crush of facial motoneurons, Tα1 α-tubulin mRNA rapidly increases within the first 12 hr and continues to increase to reach peak levels at approximately 3 to 7 days. This increase persists if axonal regeneration does not occur. However, if regeneration occurs and target contact is reestablished, Tα1 α-tubulin mRNA decreases to reach control levels sometime between 21 and 49 days postaxotomy(Miller et al., 1989). This pattern of expression is highly reminiscent of that seen during development (Miller et al., 1987).

One intriguing observation is that the induction of Tα1 α-tubulin mRNA during regeneration is a function of the amount of axon lost (Mathew & Miller, 1993; Tsui et al, 1991; Tetzlaff et al., 1994, 1996). When axons are cut or crushed close to their cell body, neurons respond with a more vigorous upregulation of Tα1 mRNA . This was shown in sympathetic neurons of the SCG, where a close axotomy evoked a much more vigorous upregulation of Tα1 α-tubulin mRNA (Mathew & Miller, 1993). When only a small amount of axon was lost, the increase in Tα1 mRNA was much lower, even though contact with target tissue was completely disrupted. The induction of Tα1 mRNA also showed a pronounced effect of distance, in both axotomized spinal motoneurons and rubrospinal neurons(Tsui et al, 1991; Tetzlaff et

al., 1994, 1996). Thus it appears that there is a consensus that the length of axon remaining after injury determines the level of Tα1 α-tubulin upregulation.

Other stimuli that lead to increased growth of mature neurons also increase expression of Tα1 α-tubulin mRNA. Selective increased expression of Tα1 α-tubulin mRNA was associated with the sprouting of intact sympathetic neurons as induced by several different environmental cues (Mathew & Miller 1990) . Expression of this growth-associated mRNA in intact neurons was increased by systemic NGF, and by changes in connectivity during collateral sprouting, presumably in response to increased target-derived NGF, since exogenous NGF can increase Tα1 α-tubulin mRNA levels in these neurons both in vivo (Mathew & Miller, 1990; Miller et al., 1991, 1994) and in vitro (Ma et al., 1992). Furthermore, intermittent stimulation of the angular bundle led to reproducible and selective induction of Tα1 α-tubulin mRNAs in specific populations of neurons in adult hippocampus (Causing et al., 1996), reflecting to some degree, sprouting or remodeling of neurons in response to high levels of excitatory input (Represa et al., 1989).

Molecular mechanisms regulating tubulin expression in neurons

Recent evidence presented in this thesis indicates that both transcriptional and posttranscriptional mechanisms are important in regulating Tα1 α-tubulin gene expression (also see chapter 3 and 4). At the transcriptional level, the Tα1 α-tubulin promoter has been demonstrated to specify gene expression as a function of neuronal growth and regeneration in transgenic mice (Gloster et al., 1994). When 1.1kb of the 5' flanking region of Tα1 gene was fused to a nuclear lacZ reporter gene and introduced into transgenic mice, marker gene expression was specific to the nervous system, and correlated temporally with neuronal commitment. Transgene expression was high during development, downregulated with neuronal maturation, and reinduced following axotomy of motoneurons and sympathetic neurons (Gloster et al., 1994; Wu et al., 1994b; Wu et al., 1996), thus providing the first direct evidence for transcriptional regulation of α-tubulin gene in neurons.

Expression of α and β tubulin can also be regulated at posttranscriptional level. Initial evidence for autoregulatory control of tubulin synthesis came from experiments using drugs that promote polymerization or depolarization of cellular

tubulin (Ben-Ze'ev et al., 1979; Caron et al., 1985; Cleveland & Havecroft, 1983; Cleveland et al., 1981; Pittenger & Cleveland, 1985). Cell cultures treated with colchicine or nocodazole showed a two fold increase in the intracellular concentration of tubulin heterodimers and a concurrent five- to tenfold specific repression of new tubulin synthesis. Similarly, treatment of cells with vinblastine or taxol, which reduce the intracellular concentration of tubulin heterodimers, resulted in a three- to fourfold increase in new tubulin synthesis (Ben-Ze'ev et al., 1979; Pachter et al., 1987). These changes in the rate of tubulin synthesis can be accounted for by corresponding changes in tubulin mRNA levels (Caron et al., 1985; Cleveland & Havecroft, 1983; Cleveland et al., 1981; Pachter et al., 1987). Transcriptional run-off experiments and experiments with enucleated cell suggest that autoregulation of tubulin mRNA abundance results from modulation of tubulin mRNA stability, rather than from changes in tubulin gene transcription (Caron et al., 1985; Cleveland & Havecroft, 1983; Pittenger & Cleveland, 1985). Cleveland and co-workers subsequently demonstrated that the element responsible for autoregulated destabilization is contained in the first 13 transcribed nucleotides of β-tubulin. Specifically, the encoded tetrapeptide sequence, Met-Arg-Glu-Ile (MREI), is the important recognition signal, rather than the nucleic acid sequence, and translation must proceed through at least 41 codons for the MREI to confer proper regulation (Yen et al., 1988 a & b; Bachurski et al., 1994). Microinjection of a monoclonal antibody that binds to β-tubulin nascent peptide selectively disrupts the regulation of β-tubulin, but not α-tubulin, synthesis (Theodorakis & Cleveland, 1992). Furthermore, mutation of the sequences encoding the tetrapeptide MREI at the amino-terminal disrupt the autoregulatory mechanisms of β but not α tubulin mRNA, suggesting that, despite similarities, α and β tubulin mRNA destabilization pathways utilize divergent determinants to link RNA instability to tubulin subunit concentrations (Bachurski et al., 1994). Despite these differences, the neuron has the ability to monitor the monomer:polymer ratio of both α- and β-tubulins and to "fine tune" their mRNA levels posttranscriptionally. The tight control of these important cytoskeletal elements at the posttranscriptional level acts in coordination with other mechanisms such as transcriptional regulation of tubulin gene expression (see chapter 3; Wu et al., 1993) and likely plays an important role during neuronal growth and regeneration.

Increased axonal transport and assembly of tubulin following injury

In neurons, tubulin is synthesized only in the cell body or dendrites, yet the growing axon requires a steady supply of this protein at the growth cone. Hence, some mechanism must exist to move tubulin from the cell body to the growth cone. Slow axonal transport moves the cytoskeletal proteins that are synthesized in the cell body of the neuron down into the axon. The slow component (SC) is represented by two rate classes, SCb and SCa, that move at approximately 1-4 and 0.2-1 mm/day respectively (Hofffman & Lasek, 1975). Whether this can account for tubulin transport during neurite extension is still controversial.

One theory proposes that the bulk of tubulin is translocated in a polymeric form, possibly by sliding of the microtubules over one another (Cleveland and Hoffman, 1991). This was examined by using flurescently labeled tyrosinated α-tubulin, which is a marker for a faster rate of turnover of microtubule subunits (Ahmad et al.,1993; Baas & Ahmad, 1993; Baas & Black, 1990). It was found that relatively short preassembled microtubules were transported from the cell body into the axon and that the microtubules elongated as they progressed down the axon, as a result of the expansion of a labile domain containing tyrosinated α-tubulin at the plus end of each tubule. The labile domain is particularly prominent in microtubules nearest the advancing growth cone, where only little of the dynamic polymer is ever converted into stable polymer. Only one study found that the bleached polymer translocated (Keith, 1987). Using a caged fluorescent coupled to tubulin, a recently developed technique, it was shown that tubulin is translocated down the axons as intact polymers in Xenopus laevis (Reinsch et al., 1991). Another group of researchers suggest that tubulin is not transported as polymers, but rather possibly through slowly growing axons by diffusion (Sabry et al., 1995). This was examined by incorporating fluorescein-labeled tubulin into exiting microtubules, and by analyzing photobleached polymers as the axon elongated (Lim et al., 1989, 1990; Okabe & Hirokawa, 1990). They suggest that tubulin movement in neurons probably occurs by movement of monomeric tubulin, either by transport or diffusion. Interestingly, it has been recently shown that in compartment cultures of sympathetic neurons, the newly synthesized tubulin from the cell bodies appeared in the distal compartment as early as 6 hours following in vitro axotomy (Campenot & Senger, 1995), suggesting a much faster rate of tubulin transport than slow axonal flow.

In spite of the controversy regulating the mechanism whereby tubulin is assembled in growing neurites, it is clear that slow axonal transport plays a critical role in axon growth. Previous studies of peripheral mammalian axons showed that axotomy resulted in decreases in the amount of labeled tubulin protein transported in slow component a (SCa) in motor and sensory axons (Hoffman & Lasek, 1980; Oblinger & Lasek, 1988). In contrast, increases in the amount and rate of labeled tubulin transported in the faster of the two slow transport components, SCb, have been documented in axotomized motor neurons (Hoffman & Lasek, 1980). In regenerating axons, the rate of outgrowth is proportional to the velocity of SCb. Significant alterations of slow transport occur during axonal regeneration. The first demonstration of this was in goldfish optic nerve (Grafstein & Murray, 1969), where the velocity of SCb and the amount of tubulin it conveys are increased dramatically following axonal injury (Grafstein, 1991). In mammalian motor axons, a similar, although smaller, change occurs, with the increase in the amount of tubulin transported in SCb. Recent studies of tubulin transport in rat motor neurons using isotype-specific antibodies have shown that transport of β_{II} and β_{III}-tubulin are increased in both amount and rate during regeneration (Hoffman et al., 1992). The leading front of the SCb wave, which is magnified in regenerating neurons, was shown to be enriched in βIII-tubulin (Hoffman et al., 1992). In regenerating sensory neurons, β_{III} tubulin expression was induced and the SCb vector of slow transport conveyed a modified cytoskeleton, enriched in βIII-tubulin, into the axons of regenerating DRG neurons (Moskowitz & Oblinger, 1995). The increased axonal transport of tubulins following nerve injury provides a molecular mechanism for supplying newly synthesized tubulins into the regenerating axons and growth cones, without excessive accumulation of tubulin monomers in the cell bodies. Whether the differences in tubulin composition also play a role in aiding regeneration is still a matter of speculation.

2. Changes in Po myelin gene expression in Schwann cells following peripheral nerve injury

Following peripheral nerve injury, Schwann cells and fibroblasts distal to the injury site undergo a complex set of changes: axonal degeneration, macrophage invasion, myelin breakdown and Schwann cell proliferation and dedifferentiation. Re-establishment of axonal contact during regeneration, results in a Schwann cell

proliferative response followed by redifferentiation. These changes are accompanied by dramatic alterations in the expression of genes associated with (1) myelination and (2) trophic responses (for review, see Lemke, 1988; Bunge, 1994). Here, I will only talk about Po myelin gene expression.

Po myelin gene expression in the peripheral nervous system

Myelin is a unique cellular organelle formed by Schwann cells, the specialized glial cells of peripheral nerve. Myelination includes the process of neuron-glia recognition, molecular assembly of myelin components, and compaction of membranes to form lamellar structures. The insulating layer formed by the myelin sheath drastically reduces the effective capacitance of the axon membrane and prevents current leakage across it. Between each segment of sheath lies the nodes of Ranvier, where the axon is not ensheathed. All of the Na^+ channels of the axon are concentrated at these nodes. This enables the action potential to conduct in a saltatory fashion, resulting in faster conduction and better conservation of metabolic energy (for review, see Bray et al., 1981; Richie, 1984). In addition to insulating axolemmal membranes of neurons, myelin has been suggested to be an important determinant in inhibition of axonal regeneration (Schwab et al., 1993).

The elaboration of myelin depends on the regulated expression of a set of genes unique to myelinating glia(Lemke, 1988). The most intensively studied of these genes have been those encoding the peripheral myelin structural proteins Po protein(Lemke & Axel, 1985), myelin basic protein (MBP)(Roach et al, 1983), 2', 3'-cyclic nucleotide 3'-phosphohydrolase (CNP)(Lewis et al, 1984), myelin-associated glycoprotein (MAG)(Lai et al, 1987), and PMP-22, a newly discovered major PNS component (Snipes et al, 1992). The most abundant protein of peripheral nerve myelin is a glycoprotein termed Po. Po is a 28 kDa protein, which comprises 40 to 50% of total protein content in peripheral myelin (for review, see Lemke, 1993a). Po is believed to be involved in the compaction of the myelin sheath and is postulated to be the closest relative to the ancestral gene for the immunoglobulin superfamily. Po consists of an extracellularly oriented immunoglobulin-like domain, a single transmembrane domain and an intracellular cytoplasmic domain (Lemke & Axel, 1985; Lai et al., 1987). The immunoglobulin (Ig)-like domain contains an asparagine-linked glycosylation site which, like N-CAM, MAG and PMP22, carries the

L2/HNK-1 carbohydrate epitope (Bollensen & Schachner, 1987), suggesting that Po may interact homotypically during myelin formation. The cytoplasmic portion of the Po molecule is highly charged and is important for stabilization of the interaction between the phospholipid head groups of the myelin membrane at the major dense line (Lemke, 1988; Ding & Brunden, 1994; Wong & Fibin, 1994).

The Po gene has been cloned and is comprised of six coding exons that appear to correspond to functional domains of the Po protein (Lemke, 1988). When Po was deleted in transgenic mice through targeted gene disruption, it led to hypomyelination, abnormal expression of recognition molecules, and degeneration of myelin and axons(Giese et al, 1992). These mice are deficient in normal motor coordination and exhibit tremors and occasional convulsions. Axons in the peripheral nerves are severely hypomyelinated and a subset of myelin-like figures and axons degenerate. The mutation also led to an abnormal regulation of some, but not all, molecules involved in myelination. Another approach to the analysis of Po gene function is analysis of the Po promoter. The 1.1 kb 5' flanking region of the Po gene has been shown to direct Schwann cell- specific expression both in vitro (Lemke & Chao, 1988) and in transgenic mice (Messing et al., 1992). When the Po promoter was used to drive expression of the genes encoding bacterial diphtheria toxin A chain (DT-A) in myelinating Schwann cells(Messing et al, 1992), one line of P0-DT-A mice developed a generalized hypomyelinating peripheral neuropathy, with Schwann cell deficiency apparent in newborn animals.

Axonal regulation of Po myelin gene expression in Schwann cells during development and following regeneration

Schwann cell expression of myelin genes shows a remarkable plasticity that is almost entirely controlled by neurons (for review, see Lemke & Chao, 1988, 1993a &b). This phenomenon has been most extensively studied for Po. The initial induction of this gene during development appears to be dependent on axonal contact. Should this contact be interrupted anytime from days to weeks following initial induction, steady-state levels of Po myelin gene products fall dramatically (Politis et al., 1982). In vivo, this effect is seen when developing peripheral nerves are transected and myelin gene expression in Schwann cells distal to the transection site (where axons degenerate) is measured. If such a transection is performed in a rat in

which the peak rate of myelination has already passed, one observes roughly a 40-fold decrease in steady-state Po mRNA levels in Schwann cells distal to the cut (Gupta et al., 1988; Trapp et al., 1988). Most of this decrease occurs within 5 days of transection, although the first decreases immediately distal to the cut site can be observed within hours of axonal injury (Toma et al., 1992). Myelin protein synthesis resumes in crush-lesioned peripheral nerves with a time course comparable to the remyelination of regenerating axons (Trapp et al., 1988; LeBlanc & Poduslo, 1990; Mitchell et al., 1990). Thus, myelin protein expression shows a similar pattern of regulation during both development and during nerve regeneration. A similarly dramatic decrease is seen in vitro when Schwann cells are dissociated from the peripheral nerves of neonates, purified by immunoselection, and placed into culture in the absence of neurons (Lemke & Chao, 1988). Coculture with purified neurons (Wood et al., 1990b), induces these Schwann cells to elaborate myelin. Whether the axonal dependence of Schwann cell gene expression is mainly transcriptional or posttranscriptional remains uncertain. However, studies of the Po promoter suggest that this regulation occurs at the transcriptional level (Messing et al., 1992).

III. Can inflammatory response and noval factors produced in peripheral nerve generate neuronal and Schwann cell injury responses?

It is possible that as a consequence of injury, novel factors or signals are produced that induce a regenerative response for both neurons and Schwann cells. For example, the blood nerve permeability barrier breaks down and axons are exposed to signaling molecules or inflammatory cytokines produced by invading macrophages. Moreover, the injured peripheral nerve produces a large number of growth factors, as discussed below. Some of these potential signals will be discussed in more detail here.

1. Extrinsic cues for neurons

a. Macrophage invasion

Macrophage invasion in the peripheral nerve and/or around neuronal cell bodies

A small population of resident macrophages is irregularly scattered within the uninjured peripheral nerve (Perry et al, 1990). These resident macrophages are like those of other tissues, expressing a range of macrophage antigens including MHC class II antigens. Following nerve injury, myelomonocytic cells (monocytes) are recruited from the blood into the degenerating part of the nerve and become activated macrophages. It is believed that a chemotactic signal from the disintegrating axons induces this recruitment (Perry et al, 1990). The monocytes differentiate into macrophages that exert important functions not only in nerve degeneration, but also potentially in regeneration.

Concomitant with the macrophage invasion in the nerve, there is an increase in the number of macrophages around the injured neuronal cell bodies. This increase is best characterized in the DRG. (Gehrmann, 1991; Lu & Richardson, 1993; Smith & Adrian, 1972). After sciatic nerve transection in rats, the number of macrophages in the DRG increase for four days to one month, and then return to normal numbers by two months (Lu & Richardson, 1993). This increased number probably represents

recruitment of macrophages (Smith & Adrian, 1972), but this has not been confirmed. Many of these reactive macrophages are closely apposed to sensory neuronal cell bodies where they may play a role in neuronal survival and regeneration. In fact, the presence of macrophages in DRGs as induced by the injection of Corynebacterium parvum enhanced regeneration of crushed axons in the corresponding dorsal spinal root towards the spinal cord (Lu & Richardson, 1993). Conversely, however, inhibition of macrophage proliferation in the DRG did not hinder the regeneration of sensory axons (Lu & Richardson, 1993, 1995). Macrophages are also unlikely to evoke early responses since they do not increase in number until 4 days postaxotomy.

A similar cell body reaction occurs for motor neurons whose cell bodies are located in the spinal cord and brainstem. Following transection of the hypoglossal or facial nerve, there is proliferation of resident microglial cells in the appropriate brainstem nucleus (For review, see Streit et al., 1988; Perry & Gordon, 1988). Although it might seem that this would benefit regeneration in some way, recent evidence suggests that preventing microglial proliferation around regenerating motoneurons does not interfere with regeneration of motor fibers, and the microglial cell reaction is not necessary for peripheral nerves to regenerate and restore target contact at a normal rate and to a normal extent (Svensson & Aldkogius, 1992, 1993 a,b,c).

Is macrophage invasion, either in the peripheral nerve and/or around neuronal cell bodies, responsible for inducing regenerative responses in injured neurons?

Although it remained questionable whether or not they play a role in initiating a regenerative response, our data strongly exclude the potential roles of macrophages in inducing at least some aspects of peripheral nerve injury response (Wu et al., 1993, 1994). However, it is clear that macrophages are essential for providing an appropriate nerve environment for axonal regeneration. These issues will be discussed here.

In mammals, intact nerves do not appear to provide a favorable environment for axon growth (Brown et al, 1991 a&b). Following injury, the distal stump of a transected nerve is invaded by macrophages which phagocytose myelin and axonal debris, and then move to a subperineural location where they are eventually

reabsorbed into the vascular system. During their invasion and residence in the endoneurium, macrophages proliferate and modify their pattern of gene expression. For example, class II major histocompatibility antigens are expressed on macrophages only during the first week of Wallerian degeneration (Perry et al., 1987; Stoll et al., 1989). The removal of old axons and discarded myelin is probably a major first step in making a distal nerve stump a suitable place for axon growth, and this step is only possible with the arrival of recruited macrophages, which not only phagocytose the debris, but also secret proteases and reactive oxygen metabolites important for degradation of dying cells (Nathan, 1987). In addition to removing the products of degeneration, macrophages release molecules that stimulate the proliferation of Schwann cells (Baichwal et al., 1988; Beuche & Friede, 1984).

Storage of lipid degradation products by macrophages also provides one of the main sources of lipids needed as building blocks to reconstruct the membranes of regrowing axons. Lipid recycling is mediated by apolipoproteins, an important class of lipid-associated proteins that are involved in lipid metabolism (Mahley et al., 1983). At the molecular level, one of the most prominent changes after transection of peripheral nerves is the enhanced synthesis and secretion of apolipoprotein E (apo E) (Mueller et al., 1985 a&b), a lipid-binding glycoprotein involved in the metabolism and transport of cholesterol and phospholipids. In the distal segment of crushed rat sciatic nerve, infiltrating macrophages were identified as the putative cellular source of apo E production during Wallerian degeneration. The accumulation of apo E in peripheral nerves is associated with regeneration of axons after injury. Recent experiments with nerve cell cultures suggest that apo E is involved in the formation of endoneural lipoprotein complexes and in mediating the uptake of this lipoprotein into the neuronal growth cones (Ignatius et al., 1987) and Schwann cells (Mueller & Rothe, 1988) for membrane biosynthesis during nerve repair.

Although these two roles, phagocytosis of myelin debris and lipid recycling, are likely necessary to produce a nerve environment conductive to axonal regrowth, they likely have no impact on the induction of neuronal regeneration. However, invading cells of the immune system produce a variety of growth factors and cytokines that are characteristic of inflammatory responses, and that may, directly or indirectly, induce a neuronal response. These factors include interleukins (IL-1, IL-6 etc.), transforming growth factor β (TGFβ), basic fibroblast growth factor (bFGF),

and tumor necrosis factor α (TNF-α). These factors can affect cell migration, expression of cell adhesion molecules by nerve resident cells and other phenomenon necessary for tissue remodeling and axonal growth (for review, see Merrill, 1992; Hopkins & Rothwell, 1995; Rothwell & Hopkins, 1995).

These cytokines that are associated with inflammation can also induce synthesis and release of a variety of other growth factors that are capable of promoting the survival and repair of damaged neurons. For example, both IL-1 and TNF induce production of NGF in the injured nerve (Lindholm et al., 1987; Hattori et al., 1993), as well as platelet-derived growth factor (PDGF). TGFβ reduces NGF production, while it promotes Schwann cell growth and activity (Ridley et al., 1989). Although the physiological function of cytokines might be to preserve or restore homeostasis, sustained or excessive production of cytokines, such as TNFα, can result in demyelination and axonal degeneration directly (Said et al., 1992). Therefore, by affecting the Schwann cells and nonneuronal cells, cytokines could promote or inhibit neuronal regeneration (also see III2a).

For some neurons, such as sympathetic neurons, cytokines such as IL-1 influence neuronal phenotype (for review, see Jonakait, 1993). In vitro, IL-1 upregulates the synthesis of leukemia inhibitory factor (LIF) in nonneuronal cells of the sympathetic ganglia (Shadiack et al., 1993). In vivo, it has been shown that the absence of LIF in the LIF nullizygous mice abolishes changes in neuropeptide synthesis in axotomized sympathetic neurons (Rao et al., 1993). Thus IL-1, either present in the ganglia or derived from macrophages, acts on IL-1 receptors present on nonneuronal cells-either Schwann cells or fibroblasts. This activation of IL-1 receptors leads to increased synthesis of LIF which, in turn, causes alterations in expression of substance P in sympathetic neurons.

Any discussion of potential role of macrophages in nerve regeneration would not be complete without a consideration of Ola mice. These mice, which are a substrain of the mouse inbred strain C57BL, demonstrate a marked delay of Wallerian degeneration: axons and myelin in the distal stump degenerate very slowly, and macrophage infiltration of the endoneurium is impaired (Glass et al., 1993; Perry et al., 1990). Less NGF is available in the regenerating nerves of Ola mice than in control strains, and regeneration is delayed (Brown et al, 1991a&b). The defect in

axonal regeneration in C57BL/6J mice appears to be a delay rather than permanent impairment and appears to involve sensory more than motor axons (Lu & Richardson, 1993). These observations support the idea that macrophages have an important function in peripheral nerve regeneration. These conclusions have, however, recently been questioned, since peripheral nerve grafting experiments between C57BL/6J and C57BL/Ola mice indicate that the primary deficit in Ola mice resides in neurons rather than in Schwann cells or macrophages (Glass et al., 1993).

b. Growth factors produced in Schwann cells and fibroblasts in the peripheral nerve

Neurotrophic factor production

Following axonal injury, Schwann cells, fibroblasts and macrophages (Lindholm et al., 1987; Toma et al., 1992) of the nerve adjacent and/or distal to the lesion, produce a variety of factors that can promote the survival and regeneration of injured neurons. Schwann cells synthesize NGF (Rush, 1984). While the adult NGF-dependent peripheral neurons are trophically supported from their peripheral target tissue in normal uninjured condition, after nerve lesion there is a massive increase in the synthesis of NGF by the sciatic nerve non-neuronal cells (Heumann et al., 1987). In the absence of nerve fiber regeneration, NGF mRNA remained high in the distal segments but fell markedly when regeneration was allowed, to attain control levels by 2 weeks after the lesions (Heumann et al., 1987). BDNF synthesis is also increased following lesion of the peripheral nerve. While highly abundant in the brain, BDNF is expressed at very low or undetectable levels in mature Schwann cells under normal conditions. Transection of the rat sciatic nerve leads to a very marked increase in BDNF mRNA, with the final levels being approximately ten fold higher than those of NGF mRNA (Meyer et al., 1992). However, the time-course and spatial pattern of BDNF mRNA expression are distinctly different. There is a continuous slow increase in BDNF mRNA in Schwann cells and fibroblasts (Funakoshi et al., 1993; Acheson et al., 1991) starting at 3 days postlesion and reaching maximal levels 3-4 weeks later. These distinct differences in synthesis of NGF and BDNF in the peripheral nerve suggest different underlying regulatory mechanisms. With regards to the other neurotrophins, investigation of their mRNAs in the intact rat sciatic nerve revealed that the levels of mRNA for NT-3 were the highest, followed by NT-4 and BDNF

mRNA (Funakoshi et al., 1993). The low or undetectable levels of NGF and BDNF mRNAs could be due to a downregulation of NGF and BDNF mRNA in Schwann cells by axonal contact. Unlike NGF and BDNF, NT-3 mRNA decreased shortly after transection of the peripheral nerve and returned to normal levels 2 weeks later.

Do any of these growth factors have a role in inducing the neuronal axotomy response? Previous investigations have revealed that the retrograde axonal transport of proteins, in general, is increased upon injury to the peripheral nerve (Bulger & Bisby, 1978; Redshaw & Bisby, 1984). One potential signal that may induce an axotomy response in the neuronal cell body is the increased production and/or abnormal entry of substances produced at the site of injury. However, there is controversy as to whether there are increases or decreases of retrograde transport of these neurotrophins (Curtis et al., 1994; DiStefano et al., 1992, 1994; Raivich et al., 1991). Furthermore, it is still unknown what are the real nature of retrograde signals: whether it is the neurotrophin-receptor complex, or secondary messengers generated in the axons at the nerve terminals (also see IV1a). Nevertheless, neurons may possess the intrinsic capacity to increase the transport of several molecules in response to damage.

Cytokine production in the peripheral nerve

Ciliary neurotrophic factor (CNTF) was discovered and named for its ability to keep embryonic ciliary neurons alive, and its actions remain largely limited to cells of the nervous system and muscle (for review see Ip & Yancopoulos, 1992). The highest levels of CNTF mRNA in adult mammals occurs in peripheral nerves, such as the sciatic nerve (Williams et al., 1984), paralleling the differentiation of Schwann cells in the nerve (Jessen & Mirsky, 1992). CNTF mRNA and protein are both apparently localized in Schwann cells (Friedman et al., 1992). After peripheral nerve lesion, CNTF mRNA drops dramatically to less than 5% within one week postlesion (Sendtner et al., 1992). The synthesis of CNTF in injured peripheral nerves is decreased when and where the synthesis of nerve growth factor is increased (Heumann et al., 1987 a&b, 1988). After nerve crush, CNTF mRNA levels slowly recover, with the first positive cells being detectable 1 week after lesion, with a significant increase in CNTF mRNA at about 4 weeks after lesion (Sendtner et al., 1992; Friedman et al., 1992). Thus Schwann cell CNTF expression is correlated with

the more mature myelinating state, and since Schwann cell differentiation is thought to be induced by axonal contact, it may be hypothesized that neuron-Schwann cell signaling is involved in the regulation of CNTF expression. The role of CNTF during nerve injury remains unclear. CNTF lacks a signal sequence common to secreted proteins, and its mechanism of release is unknown. It has been suggested that CNTF is not constitutively released, but rather acts as a "lesion factor", becoming released after nerve injury (Lin et al., 1989; Stockli et al., 1989; Thoenen, 1991). Recent immunohistochemical evidence suggests that CNTF is shed from Schwann cells following nerve injury and becomes more available to regenerating axons (Sendtner et al., 1992).

Unlike CNTF, LIF is rapidly up-regulated in Schwann cells and fibroblasts after nerve injury (Patterson & Nawa, 1993; Curtis et al., 1994). The high level of LIF expression in injured nerve is coupled with a substantially increased retrograde transport of LIF(Curtis et al., 1993, 1994), implying that LIF may act as a neurotrophic factor for sensory and motor neurons after lesion. Interestingly, LIF is encoded by two alternatively-spliced transcripts. Both forms of LIF are secreted, but one becomes immobilized in association with the ECM (Rathjen et al., 1990). This differential compartmentation could allow LIF to act as both a localized and as a long distance signal following nerve injury. Interleukin-6, which has been shown to enhance cell survival and growth of cultured neurons (Hama et al., 1989), is produced by Schwann cells and is induced within 12 hours following sciatic nerve injury(Bolin et al, 1995).

Regeneration of mature sensory and sympathetic neuron is independent of target-derived NGF

Although neurotrophins and neurokines are suggested to be potential signals for the injury response, there is good evidence that sensory neurons of adult rats regenerate and restore sensory function to the skin independently of endogenous NGF(Diamond et al, 1992a,b). Specifically, antibodies to NGF inhibit collateral sprouting but not regeneration of nociceptive axons, indicating that these two types of neuronal growth are fundamentally different. This is also true for sympathetic neurons (Gloster & Diamond, 1992). Sympathetic neurons in adult rats regenerate normally and restore pilomotor function during an anti-NGF treatment that prevents their

collateral sprouting. It should be noted, however, that these experiments do not rule out the potential involvement of other neurotrophic factors in neuronal regeneration that are not blocked by the anti-NGF antibody.

2. Extrinsic signals that can generate a Schwann cell injury response

The potential extrinsic signals that may be responsible for inducing Schwann cell response following nerve injury include those derived as a result of macrophage invasion and/or autocrine/paracrine loops established within the injured nerve. These different potential signals will be reviewed here.

a. Macrophage invasion in the peripheral nerve

As described in section III1a, macrophages invade the injured peripheral nerve and play a number of important roles. For example, Beuche and Freide (1984) detected no Schwann cell mitosis in nerves in which macrophages were excluded, although the axons degenerated and the Schwann cells extruded their myelin. This suggested that macrophages and/or the loss of axonal contact, rather than nerve breakdown products, might cause the well known proliferation of Schwann cells that accompanies normal Wallerian degeneration. Tritiated thymidine labeling of dividing cells in the distal nerve stump coupled with antibody labeling demonstrated that the mitosis of Schwann cells begins on day 2 and peaks on day 3 after nerve injury, with a sustained lower plateau for some days thereafter (Clemence et al., 1989). This time course correlates with the time course of macrophage invasion as measured using the monoclonal antibody ED1, which recognizes macrophages and monocytes. Macrophage invasion in the mouse saphenous or sciatic nerve assessed with F4/80 antibody, however, is slightly more delayed and only rises significantly after day 2 (Brown, 1991a). Our data, obtained using ED-1 antibody, indicates that macrophages invade the distal tip of the injured rat sciatic nerve as early as 1 day, and the body of the distal stump by 3 days (Wu et al., 1994). Nevertheless, macrophages that have digested myelin membrane release a soluble Schwann cell mitogen (Baichwal et al., 1988) as well as a number of cytokines known to influence Schwann cell behavior. Specifically, macrophages synthesize TGFβ, interleukin-1, interferon-gamma, and TGF-α, reviewed here.

After nerve injury, TGFβ is secreted into the local environment by invading macrophages (Assoian et al., 1987), nonneuronal cells (Ridley et al., 1989), and even injured DRG neurons (Rogister et al, 1993) (see also III1a). The TGFβ family of cell signaling molecules has been implicated in wound repair in many organ systems and includes at least five distinct isoforms (Roberts et al., 1990). Recent studies indicate that these growth factors have multiple effects on cell proliferation, differentiation, and extracellular matrix (reviewed in Barnard et al., 1990; Massague, 1990; Sporn & Roberts, 1992). TGFβ has been reported either to enhance (Eccleston et al., 1989; Rdley et a., 1989; Rogister et al., 1993) or to have no effect (Mews & Meyer, 1993) on proliferation of cultured Schwann cells (Centralla et al., 1987; Robey et al., 1987). TGF-β also apparently blocks myelination, but not ensheathment of axons by Schwann cells in vitro(Guenard et al, 1995) and may also modulate gap junctional communication in cultured Schwann cells(Chandross et al, 1995).

Macrophage also produces both interleukin-1α and β. Although IL-1 α and β do not possess a hydrophobic signal peptide sequence for secretion, they may be "leaked" out of damaged cells (Auron et al., 1984) or secreted through unconventional ways, as in the case of IL-1β suggested by Van Damme et al. (1985). Comparison of the cDNAs and proposed amino acid sequence demonstrated that IL-1 α and β are the products of two different genes and share less than 30% homology to one another (March et al., 1985), they bind to identical IL-1 Receptor and have essentially identical biological activities (De Giovine et al., 1990). For instance, both interleukin-1α & β, upregulated NGF mRNA production in nonneuronal cells in vitro (Lindholm et al, 1987). The level of mRNA for NGF rises in Schwann cells uniformly throughout the distal stump of a cut peripheral nerve(Heumann etal, 1987, 1988), with a time course that correlates well with that of recruitment of macrophages. In the C57BL/Ola mutant mice, the lack of macrophage recruitment results in very low levels of IL-1 in the distal stump of a cut nerve in vivo and matching low levels of mRNA for NGF(Brown et al, 1991b; Lindholm et al, 1987). However, IL-1 affects NGF production only in fibroblasts, but not in Schwann cells (Matsuoka et al., 1991) while an increase in cAMP triggered the upregulation of NGF mRNA in Schwann cells.

In addition to TGF-β and IL-1, the inflammatory cytokines, interferon-gamma (IFN-gamma) and tumor necrosis factor-alpha (TNF-alpha) also affect Schwann cells, by inhibiting the neurite outgrowth-promoting properties of the Schwann cells. This effect may be mediated by a downregulation of myelin-associated glycoprotein (MAG) to approximately 60% (Schneider-Schaulies et al, 1991), since antibodies to MAG inhibited neurite outgrowth on Schwann cells to the same extent as treatment with the two cytokines. Since MAG appears to be involved in axon:Schwann cell interactions, this could provide a mechanism whereby inflammatory cytokines might regulate these interactions.

b. Autocrine and paracrine interactions within Schwann cells and fibroblasts of the injured nerve

There are two explanations, which are not mutually exclusive, for the synthesis of neurotrophins in the normal and injured peripheral nerve. One is that these growth factors provide an alternative source of trophic support for peripheral neurons that have been disconnected from their targets (see III1b). The second is that these neurotrophins act in a paracrine/autocrine fashion on the nonneuronal cells of the peripheral nerve themselves. To address this latter possibility, numerous studies have focused on expression of neurotrophin receptors in peripheral nerve.

All the neurotrophins are capable of binding to the p75 NTR (Rodriguez-Tebar et al., 1990, 1992), whereas they bind more selectively to the TrkA, TrkB, and TrkC tyrosine kinase receptors (for review, see Barbacid, 1994). It is clear that many of the biological actions of the neurotrophins can be mediated directly via the Trk receptors (for review, see Bothwell, 1995; Barbacid, 1994, 1995). The role of the p75 NTR is less clear, although recent evidence indicates an important role for this receptor both in modulating the activity of the Trk receptors and in signaling on its own. Interestingly, p75 receptor may regulate Schwann cells behavior during regeneration. Schwann cell migration is an important component of nerve regeneration following injury (Fields et al., 1989; Cajal, 1991). In a recent study, antibodies to NGF or to p75 NTR strongly inhibited migration of Schwann cells on denervated nerves, while pretreatment of denervated nerve sections with NGF enhanced the rate of Schwann cell migration (Anton et al., 1994). Since Schwann cells do not express the TrkA NGF receptor, the authors suggested that this effect was

mediated through the p75 receptor. Consistent with this suggestion, the p75 receptor, which is a member of the tumor necrosis factor receptor family (Kolesnick & Gold, 1994; Rabizdeh & Bredesen, 1994), has recently been shown to signal (for review, see Chao, 1995). Specifically, activation of the p75 receptor led to ceramide production in both T9 glioma cells (Dobrowsky et al, 1994) and in PC12 cells (Dobrowsky et al, 1995). More recently, Carter et al. (1996) have shown that NGF selectively activate NF-kappa B through P75 NTR in Schwann cells. NF-kappa B is known to be one of the downstream targets by TNF receptor (Baeuerle & Henkel et al., 1994).

Both the full-length TrkC, the truncated receptor and the kinase insert form of the receptor are expressed in Schwann cells (Offenhauser et al., 1995). The full-length TrkC is expressed during the critical period of Schwann cell proliferation and nerve development, and is downregulated in adult sciatic nerve, suggesting that this receptor and its ligand, NT-3, may play a role in Schwann cell proliferation and/or differentiation during development.

Although CNTFRα, is expressed almost exclusively in cells of the nervous system, which explains the limited sites of CNTF action (Ip et al., 1993; for review, see Ip & Yancopoulos, 1994). LIF, however, may stimulate mitosis and/or trophic factor production by Schwann cells, as LIF receptor is widely expressed in a variety of cells, including neurons and glia cells, and target disruption of LIFR gene causes various defects in different tissues (Ware et al., 1995).

Thus, following nerve injury, Schwann cells not only produce many neurokines and cytokines such as NGF, BDNF, NT-3, CNTF, LIF and IL-6, but also express p75 NTR, full-length TrkC, LIFRβ, gp130. The expression of neurokines, cytokines and/or their receptors in Schwann cells may provide the molecular substrates for as-yet-undefined as an autocrine/paracrine actions in the injured peripheral nerve.

IV. Loss of intrinsic homeostatic signals induces at least some alterations in gene expression in neurons and Schwann cells

The signaling mechanisms that are potentially responsible for induction of regeneration associated gene expression in peripheral neurons are numerous. Intrinsic mechanisms include loss of target contact and target derived neurotrophic factors, loss of Schwann cell-axon contact and trophic factors normally provided by nonneuronal cells, disruption of normal turnover of axonally transported materials and disruption of normal electrical activity between neuron and target.

1. Loss of homeostatic signals that can induce a neuronal regenerative response

a. Potential signals that depend upon fast retrograde axonal transport

Because biosynthesis is largely restricted to the cell body and dendrites, there must be a constant flow of material from these portions of the cell out into the axon; this process is called anterograde axonal transport, while the return of materials towards the cell body from axons and terminals is by retrograde transport. Axonal transport is divided into two compartments; the slow transport of cytoplasmic proteins including glycolytic enzymes and cytoskeletal structures and the fast transport of membrane-bounded organelles along linear arrays of microtubules. The polypeptide compositions of the different rate classes of axonal transport have been well characterized (for review, see Hirokawa et al, 1993 a&b).

Retrograde transport, which is responsible for movement of material along the axon in the direction from the nerve terminals toward the cell body, has a velocity close to that of anterograde transport, but usually somewhat slower (Tsukita & Ishikawa, 1980; Graftein & Forman, 1980). Much of the retrogradely moving material in normal axons arises from anterogradely transported material that turns around at the nerve terminal; some also is derived from endocytotic activity at the terminal (Hollenbeck, 1993). Most of the retrogradely transported organelles are indistinguishable from those in the anterograde stream, consisting of relatively small (up to 80 nm) vesicles, tubulovesicular organelles (Smith & Synder, 1992), larger size retrograde organelles (Forman et al., 1983; Hollenbeck & Bray, 1987), multivesicular bodies and other lysosome-related structures, and mitochondria. As in the case of

anterograde transport, a certain proportion of the retrogradely directed material is deposited in the axon in the course of its transit (Smith & Synder, 1992).

Microtubules play a central role in this rapid axonal transport, by constituting the substrate for the appropriate molecular motors (for review, see Schroer et al, 1992; Hirokawa et al, 1993; Cyr & Brady, 1992). With their plus ends towards the axon terminus and the minus ends towards the cell body, they provide a basis for directional polarity. The discovery of the microtubule-based motors, kinesin and cytoplasmic dynein, and their specificity for translocating towards the plus and minus ends of microtubules, respectively, led to the hypothesis that they are the anterograde and retrograde motors of the fast axonal transport system. Both kinesin and dynein have been shown to be present in anterograde and retrograde vesicles.

More and more evidence has emerged demonstrating that a blockade of fast axonal transport alone is sufficient to initiate many neuronal regenerative responses. Two approaches have been used to induce such a block. One involves using a local application of colchicine or vinblastine, drugs that depolymerize microtubules, which are required for fast axonal transport. For example, blocking axonal transport with colchicine, without interfering with electrical activity, induces a cell body response in parasympathetic and sympathetic neurons (Daniels, 1972; Pilar & Landmesser, 1972; Purves, 1975). Colchicine application also leads to abnormal cell body neurofilament phosphorylation in DRG sensory neurons (Gold & Austin, 1991). These drugs also induce expression of a number of genes known to change after axotomy; vinblastine application to peripheral nerve induced GAP-43 mRNA in sensory neurons and led to a decrease in expression of choline acetyltransferase in hypoglossal motoneurons (Woolf et al, 1990). Application of vinblastine or colchicine on the intact vagus nerve led to induction of the immediate early genes c-jun and jun-D in both cervical sympathetic and vagal motor neurons (Herdgen et al, 1991); a response that is also induced by axotomy in spinal cord motoneurons and DRG sensory neurons after application of colchicine or vinblastine or following sciatic nerve transection (Leah et al., 1991).

However, there is controversy about colchicine studies of neuronal responses. In contrast to the data mentioned above, Singer et al. (1982) showed that colchicine injected intraneurally in intact nerve did not induce chromatolysis and delayed onset

of metabolic and morphologic changes induced by axotomy. Furthermore, one of the major problems associated with the use of colchicine and other related drugs is their potential pharmacological effects on the neurons themselves. Therefore, a second approach, which avoids this problem, is to block axonal transport by locally cooling the nerve. I have used this approach in the experiments described here (chapter 2 & 3) and have demonstrated that this leads to an increase in both Tα1 α-tubulin and p75 neurotrophin receptor mRNAs in facial motoneurons (Wu et al., 1993, 1994a).

All of these studies suggest that the perturbation of fast axonal transport either as induced pharmacologically, by local cooling, or by axonal injury, is sufficient to induce some aspects of the neuronal injury response. A large number of potential explanations could be invoked, including loss of ongoing signals derived from the target and positive or negative signals that derive directly from the transport machinery itself. I will discuss these two possibilities in more detail here. It is also possible that the loss of Schwann cell contact is responsible for some of these changes; this will be discussed in section IV1b.

The idea that the loss of ongoing signals from the target are responsible for some of the alterations seen in axotomized neurons is an old one, for which there is considerable experimental support. In particular, the loss of target-derived NGF that occurs after axonal injury is responsible for many changes seen in sympathetic and NGF-dependent sensory neurons. While some effects of NGF are elicited directly at the nerve terminals, the longer-term responses to NGF require retrograde transport of a signaling molecule to the cell body (Thoenen & Barde, 1980; Hendry, 1992b). Internalized NGF itself at the axon ends is retrogradely transported to the cell body, but is unlikely to be the active intracellular messenger, because intracellularly injected NGF does not elicit the appropriate cellular responses. Moreover, microinjection of anti-NGF antibodies into the nucleus does not block the response to exogenous NGF (Heumann et al., 1981; Seeley et al., 1983). The p75 NTR (Johnson et al., 1987) and TrkA are retrogradely transported along axons (Ross et al., 1994; Johanson et al., 1995), while the known secondary messengers downstream of tyrosine receptor kinase such as phosphoinoside 3-kinase, ERK, MEK and MEK kinase, have also been demonstrated to be retrogradely transported (Johanson et al., 1995). Thus, not only can an NGF-TrkA complex (or NGF-TrkA-p75 complex) be a likely candidate for the retrograde signal but also the generated secondary messengers activated by tyrosine

receptor kinase may play an important role in regulating neuronal cell body responses.

It has been postulated that loss of NGF from target organ induces some axotomy-induced alterations in gene expression such as neurofilament downregulation. In support of this hypothesis, NGF can reverse the axotomy-induced downregulation of neurofilament gene expression, but only in neurons with trkA receptors (Verge et al., 1990a). It is clear, however, that such a loss cannot explain all of the observed changes in gene expression, since the axotomy-induced increases in GAP-43 and Tα1 α-tubulin mRNAs are not "antagonized" by exogenous NGF (Verge et al., 1990a; Hu-Tsai et al., 1994; Mathew & Miller, 1990; Ma et al., 1992; Mohiuddin et al., 1995). However, the loss of ongoing, target-derived trophic support may account for at least some of the changes seen in injured peripheral neurons.

A second class of signals that may derive from perturbation of axonal transport involves the transport machinery itself. Lesion of peripheral nerves (and/or disruption of microtubules) leads to a conversion from anterograde to retrograde vesicular movement; organelles can be seen to reverse direction within a few minutes (reviewed in Smith & Snyder, 1992). This reversal of transport at the site of injury, which normally occurs at the nerve terminal, may provide one signal for inducing a neuronal injury response (Bisby, 1984).

The mechanisms that regulate this vesicle turnaround are currently somewhat unclear, although it appears that the motors for axonal transport in both directions are present on many vesicles. For example, antikinesin antibodies inhibit both anterograde and retrograde transport (Brady et al., 1990). Moreover, dynein co-localizes with both anterogradely and retrogradely transported vesicles (Hirokawa et al., 1990). These data suggest that both motors are present on organelles traveling in both directions, and raises the question of how the polarity of movement of an individual organelle is determined. One possibility is that each motor protein is carried in an inactive form until activated by specific local conditions in the axon.

In this regard, it has been suggested that intracellular pH and/or calcium levels at the injury site or nerve terminal are elevated, which may in turn activate a critical protease, kinase, or phosphatase, all of which have been experimentally

implicated in the process of vesicle directionality(Sahenk et al, 1988; Rozdzial 1986). The turnaround of proteins at the tip of a severed axon can be blocked by inhibition of protease (reviewed in Smith & Snider, 1992). Protein phosphorylation is another mechanism that may be involved in the modification of organelles in preparation for turnaround (reviewed in Smith & Snyder, 1992). The quality and/or quantity of turnaround vesicles at the injury site may be quite different from that observed at the nerve terminal, and may therefore act as an "intrinsic signal" for the neuronal response to injury. However, as of yet there is no evidence to directly address this possibility, although an interesting potential mechanism has been uncovered in Aplysia neurons (Ambron et al., 1992, 1993; see next).

Within the past few years, a signal sequence that mediates the retrograde transport of proteins from the axon periphery to the cell body and then into the nucleus has been identified in Aplysia neurons (Ambron et al., 1992, 1993). The authors have speculated that the retrograde signal sequence in such proteins could be masked, to become exposed in response to a transduction event (Ambron et al., 1992, 1993). Such events include nerve injury (Cragg 1970; Walters et al., 1991), the binding of a trophic factor that influences neuronal phenotype or survival (Ambron et al., 1985; Macagno et al., 1986; Schotzinger & Landis, 1990; Oppenheim, 1991; Frenh & Kristan, 1992), or the binding of a ligand that produces long-term structural changes at the presynaptic terminal (Nguyen & Atwood, 1992) such as occurs in learning and memory (Bailey & Chen, 1983). Thus, the unmasking of a transport sequence in response to injury could lead to trafficking of a novel protein(s) into the neuronal nucleus (Schmied et al., 1993), thereby potentially regulating transcriptional events.

b. Signals that derive from axon:Schwann cell interactions

As previously discussed, axons regulate Schwann cell phenotype (section II2). A number of recent studies indicate the Schwann cells also profoundly regulate neuronal phenotype at the local axonal level, and potentially, distally at the cell body level. The first set of studies demonstrating this point extends the pioneering work of Aguayo(1977) and co-workers, who used nerve transplants between wild type transplants and myelin-deficient Trembler mice, as a useful system for studying axon-Schwann cell interactions. In a recent study employing the same approach, de Waegh

et al (1992) demonstrated that host axons that have regenerated through nerve grafts transplanted from hypomyelinating Trembler mice show reduced axon calibers, slowed axonal transport, altered neurofilament densities and neurofilament phosphorylation specifically within the graft. These findings are consistent with the morphological findings that local changes in neurofilament density and axonal caliber are found at the normally unmyelinated nodes of Ranvier. Thus, Schwann cell:axon interactions regulate axonal status at a local level. The second group of studies (Cole et al., 1994) confirmed this conclusion. Using peripheral myelin protein zero gene (Po) promoter and either the diphtheria toxin A chain gene product or the SV40 (simian virus 40) large T antigen, Cole et al. (1994) demonstrated that perturbation of myelin formation in Schwann cells affects axon diameter and neurofilament phosphorylation in transgenic mice. Thus the key biological properties of axons are modulated by signals transmitted from myelinating Schwann cells to axons of peripheral nerves.

Whether such signals also regulate neuronal phenotype distally remains to be determined. Schwann cell-derived growth factors provide one candidate group of molecules that could regulate mature neuronal/axonal phenotype on an ongoing basis. As previously stated, Schwann cell-derived CNTF is expressed at highest levels in uninjured peripheral nerve (Jessen & Mirsky, 1992; Dobrea et al., 1992). Moreover, degeneration of motoneurons is observed in the CNTF nullizygous mice (Masu et al., 1993), presumably as a consequence of the loss of CNTF in the nerve. In summary, Schwann cells affect neurons and loss of signals as a result of interruption of axon:Schwann interaction could explain some axotomy-induced changes. This idea is further supported by our studies of neuronal gene expression following disruption of axonal transport by a cold block (see chapter 3; Wu et al., 1993).

c. Loss of neuronal activity as a potential injury signal

One other thing that changes after axotomy or with local cooling of the nerve is activity. Is there any evidence that activity affects expression of genes that are induced following axotomy?

Neuronal activity regulates establishment of neurotransmitter phenotype during neurodifferentiation (Agoston et al., 1994; Rao et al., 1992; Sun et al., 1992),

as well as phenotypic plasticity in the adult nervous system (Black et al., 1988). Transection of motor, sensory and central neurons induced upregulation of neuropeptide galanin synthesis (Villar et al., 1989). This lesion effect was mimicked by tetrodoxin-induced blockade of neuronal activity in septohippocampal neurons. Thus, perturbation in neuronal activity may be a potential signal for some injury-induced some neuronal changes.

Could it be that the establishment of appropriate patterns of neuronal activity downregulate Tα1 α-tubulin and GAP-43 expression and that loss of this patterned activity leads to their upregulation?

Axonal growth during development and regeneration coincides with the expression of growth-associated proteins, including GAP-43 and Tα1 α-tubulin (see also section II). Following contact with the target region and synaptogenesis, GAP-43 and Tα1 are downregulated (Hesselman et al., 1989; Reynolds et al., 1991; Miller et al., 1987b; 1989; Tetzlaff et al., 1991). In detailed studies of neuromuscular junction formation, Caroni & Becker (1992) demonstrated that GAP-43 mRNA declined rapidly at a time corresponding to the onset of synapse elimination, and nerve terminal GAP-43 immunoreactivity became undetectable with a delay of 2-3 d, while Tα1 mRNA levels declined with a similar time course, suggesting a close association between downregulation of GAP-43 and Tα1 and synapse development and elimination with target. In regenerating systems GAP-43 and Tα1 fall significantly following target reinnervation and synaptogenesis(Skene, 1989; Miller et al. 1989; Tetzlaff et al., 1991). Furthermore, if regeneration is prevented, GAP-43 and Tα1 mRNAs remain at high levels in the injured neurons. Interestingly, blockade of neuromuscular transmission with either a pre- or a postsynaptically acting toxin prevented GAP-43 and Tα1 downregulation following synaptogenesis, indicating that target activity affects GAP-43 and Tα1 mRNA levels in motoneurons(Caroni & Becker, 1992).

2. Loss of homeostatic signals that induce the Schwann cell injury response.

The signals responsible for inducing Schwann cell responses following peripheral nerve injury remain relatively speculative, and, like the neuronal response to injury, may involve intrinsic mechanisms. The intrinsic signals include the loss of

ongoing axon:Schwann cell interactions as monitored either by physical contact and/or by ongoing biochemical signals.

a. Fast anterograde axonal transport, a mediator of signal(s) regulating Schwann cell gene expression?

In the course of fast anterograde transport, there is a constant transfer of constituents from the transport stream into a stationary phase of the axon (Smith & Snyder, 1992). This "offloading" may provide a mechanism for maintaining the biochemical status of the axonal membrane, a source of growth factors for secretion from the axon and/or as yet uncharacterized signals responsible for axon:Schwann cell interactions.

In fact, direct evidence that fast axonal transport is essential for maintenance of Schwann cell phenotype derives from a number of studies, including those included in this thesis. Two different hypotheses invoke a role for fast anterograde axonal transport (FAAT) in the peripheral nerve injury response. One is the "on-signal" hypothesis which postulates that exogenous molecules enter axons at the site of nerve injury and are then transported distally by FAAT where they affect Wallerian degeneration. The fact that blockade of axonal transport just distal to a nerve transection does not retard Schwann cell premitotic activity (Rozdzial et al, 1986) argues against this hypothesis. By contrast, the "off-signal" hypothesis assumes that absence of anterogradely transported substance(s) after axotomy directly or indirectly initiates components of Wallerian degeneration. In support of the second "off-signal" hypothesis, Oaklander et al., 1988 demonstrated that cold blockade of axonal transport activated premitotic activity of Schwann cells and Wallerian degeneration; Schwann cell proliferation in distal stumps was spatially and temporally advanced by locally cooling the nerve to 10°C and blocking fast axonal transport (Oaklander et al, 1988; Ochs et al, 1975) prior to nerve transection. It should be noted that in these experiments the electrical activity was also blocked by cooling the axon at 4-10°C (see chapter 2). These observations provide strong support for the proposal that Schwann cell proliferation in Wallerian degeneration is a consequence of the loss of an axonally transported factor required to maintain normal axon-Schwann cell relationships. This hypothesis is supported further by the observation that blockade of axonal transport by chemical agents such as colchicine, induces Wallerian

degeneration distally 2 weeks after blockade (Cancalon et al, 1985). In the present thesis, we were able to demonstrate that perturbation of axonal transport and electrical activity without loss of axonal contact or macrophage invasion induced at least some aspects of Schwann cell gene expression, including Po downregulation (see chapter 2; Wu et al., 1994a). Taken together, significant amounts of evidence support the idea that loss of axon:Schwann cell crosstalk induces dramatic changes in Schwann cell gene expression and phenotype.

b. Signals that derive from axon:Schwann cell interactions

Peripheral nerve injury disrupts intimate axon-Schwann cell contacts and lead to axonal degeneration, macrophage invasion, myelin breakdown and Schwann cell proliferation and dedifferentiation. Reestablishment of axonal contact during axonal regeneration results in a Schwann cell proliferative response followed by redifferentiation. Moreover, the loss of axon:Schwann cell contact following peripheral nerve lesion is accompanied by a dramatic decrease in myelin gene expression, followed by a reinduction of these genes when myelination again occurs following reestablishment of target contact. Clearly, a complete set of axonal interactions regulate Schwann cell phenotype throughout the course of peripheral nerve regeneration. I have hypothesized that, following peripheral nerve injury, the loss of ongoing Schwann cell:axon interactions are responsible for the dramatic decrease in myelin gene expression and, possibly, for the immediate increase in Schwann cell proliferation. Here I will review the signals that could potentially be involved.

Axonal interactions that regulate myelin gene expression

As reviewed in section II2, it is clear that axonal contact is essential for maintenance of myelin gene expression. What are the axonal signals that regulate these genes? Recent evidence, including our own, indicates that it may not be axonal contact per se, but biochemical signals derived from the axon that are important. Specifically, in our own work (Wu et al., 1994), we have demonstrated the blocking fast axonal transport and electrical activity without macrophage invasion or loss of axonal contact with Schwann cells can induce changes in Schwann cell gene

expression. These data indicate that at least some aspects of the Schwann cell gene expression require a constant flow of ongoing biochemical signals from the axon.

Such a conclusion is supported by recent work of Eyer and Peterson (1994). In this study, they created neurofilament-deficient axons by introducing a neurofilament-β-galactosidase fusion protein in transgenic mice, and demonstrated that even though axonal size decreased, the thickness of the myelin was the same. Thus, it appears that expression of myelin genes and elaboration of myelin depends upon a biochemical axonal signal.

What is this signal? More definitive support for the axon:Schwann cell contact hypothesis derives from culture studies. When Schwann cells are cultured in the absence of other cell types, they downregulate Po and upregulate p75 NGF receptor gene expression (Lemke et al., 1988; DiStefano & Johnson, 1988). Furthermore, either coculture with axons or the addition of axonal membranes to Schwann cell cultures reverses these changes (Brunden et al., 1990; DiStefano and Diane, 1990). Thus, for these two genes, the presence or absence of axon:Schwann cell contacts appears to be a key regulatory factor. Using an in vitro co-culture paradigm in which primary neurons and adult Schwann cells are separated by a microporous membrane, Bolin et al. (1993) demonstrated that neurons regulate Schwann cell genes by diffusible molecules, and that axonal contact is not an absolute requirement for neuronal regulation of Schwann cell genes. In this system conditioned medium from neurons but not other cell types, repressed the expression of the Schwann cell p75 neurotrophin receptor mRNA while inducing the expression of Po and SCIP (POU-domain transcription factor) mRNAs (Bolin et al. 1993; also see next).

The diffusible factor responsible for these alterations was not defined, but it is interesting in this regard that cAMP can similarly regulate expression of myelin genes in cultured Schwann cells (Lemke & Chao, 1988). Moreover, neurons make a number of growth factors that could be potentially secreted from their axons and play an essential role in maintaining the differentiated Schwann cell phenotype (see next).

Although the factors responsible for mediating these alterations in myelin gene expression remain largely undefined, some progress has been made on the intracellular mechanisms that might be responsible. These studies have taken the

advantage of the fact that, in cultured Schwann cells, the requirement for continuous axonal contact can be overridden in part by treatment with any agent that stimulates the synthesis or mimics the action of intracellular cAMP. Thus the major and minor myelin genes are induced by cAMP analogs, cholera toxin and the reversible adenyl cyclase activator forskolin(Lemke & Chao, 1988; Taniuchi et al, 1988), and the relative sensitivity of these genes to induction by cAMP parallels their relative level of expression in myelinating Schwann cells. The Po gene, for example, is approximately 10-fold more sensitive to forskolin induction than is the MBP gene, and Po is expressed at approximately 10-fold higher levels than is MBP in actively myelinating Schwann cells(Lemke & Chao, 1988). One interesting point here is that while the positive effect of axons on Schwann cell mRNA levels can be mimicked by elevation of intracellular cAMP, their negative effect on expression of the p75 neurotrophin receptor mRNA is not, suggesting different regulatory mechanism(s) that are independent of cAMP.

cAMP induction of the Po gene is likely to result from regulation at the level of transcription, since the cloned rat Po regulatory region functions as a transcriptional activator only when transfected into Schwann cells cultured in the presence of forskolin(Lemke & Chao, 1988). In addition to cAMP regulation, this cloned regulatory region (approximately 1.1 kb of DNA) also exhibits the cell-type specificity of the endogenous Po gene. It is only expressed on transfection into Schwann cells and is not expressed in transfected fibroblast, melanoma, or muscle cell lines(Lemke & Chao, 1988). In transgenic mice, expression is confined to myelinating nerve among all tissue examined(Messing et al, 1992). Although the induction of myelin gene expression by cAMP is robust, it is neither rapid nor transient. Instead, increases in Po and MBP mRNAs are not evident until 18-24 hours after cAMP elevation, and once induced, they remain high as long as cAMP levels remain elevated(Monuki et al, 1989). These regulatory kinetics are consistent with the hypothesis that cAMP induction of major myelin gene expression occurs indirectly by a cascade of intermediate, cAMP-modulated regulatory molecules.

Some of these effects have been hypothesized to be regulated by the Schwann cell transcriptional factor SCIP (for suppressed cAMP-induced POU; also called Tst-1 and Oct-6; He et al., 1989; Suzuki et al., 1990). SCIP is a member of the POU domain transcription factor family, and rapidly accumulates in forskolin-treated

Schwann cells. The level of SCIP mRNA peaks prior to the peak in myelin gene expression both in forskolin-treated Schwann cells and in developing peripheral nerve, which led to the initial suggestion that SCIP might promote myelin gene expression (Monuki et al., 1989). In cotransfection experiments, however, SCIP inhibited the activation of Po, MBP, and p75 NGFR promoter (Monuki et al., 1990; He et al., 1991). To reconcile these data, Monuki et al. (1990, 1993) postulated that SCIP is specifically expressed in proliferating Schwann cells prior to their differentiation into postmitotic, myelinating Schwann cells, and acts to inhibit the expression of Schwann cell-specific genes as they make the transition to the myelinating phenotype (Monuki et al., 1990).

Neuronal factors that may regulate Schwann cell phenotype

Neurons make a number of growth factors that, if trafficked into their axons and secreted, could regulate Schwann cell behavior. These include the glial growth factors (GGF) (Marchionni et al., 1993; also see next), fibroblast growth factors (FGFs) (Eckenstein et al., 1991), TGFβ (Flanders et al., 1991; Unsicker et al., 1991; Scherer et al., 1993; also see next), and NT-3 (Curtis et al., 1993, 1994; Funakoshi et al., 1993). GGF, exists as multiple isoforms (Marchionni et al., 1993), many of which are made by neurons, and is probably the most potent Schwann cell mitogen of neuronal origin (Lemke & Brockes, 1984). GGF exists as three major isoforms with molecular masses of 34, 59 and 45 kDa (Marchionni et al., 1993), which are alternatively spliced products of a single gene. When transfected into mammalian Schwann cells, these isoforms promote mitotic activity (Marchionni et al., 1993). GGF mRNAs are expressed preferentially in the anterior horn of the spinal cord and in the dorsal root ganglia, the major locations for neurons that project into the PNS(Marchionni et al 1993). Interestingly, in addition to its mitogenic activity on Schwann cells(Levi et al, 1995), GGF strongly suppresses neuronal differentiation of precursor cells derived from the neural crest, and promotes their differentiation towards the glial lineage (Shah et al 1994). Thus, GGF represents an excellent candidate for a factor involved in neuron(axon)-glia interactions. GGFs are members of neuregulin gene family (Marchionni et al., 1993). The neuregulins/GGFs include both membrane-bound and secreted forms (Holmes et al., 1992; Wen et al., 1992; Falls et al., 1993). Although many of the selected variants are mitogenic for Schwann cells, those lacking the EGF-like domain present in many members of the family

lacks mitogenic activity. Marchionni and colleagues (Marchionni et al., 1993) have suggested that these variants may be potential candidates for maintaining the mature phenotype of Schwann cells by acting as natural antagonists at the GGF receptor.

The neuregulin/GGFs receptor is thought to be the p185$^{erbB/neu}$ receptor tyrosine kinase, which is related to the EGF receptor. p185$^{erbB2/neu}$ is present on Schwann cells (Cohen et al., 1992), and is expressed at high levels in the rat sciatic nerve in the early postnatal period with substantially lower levels in adulthood. Interestingly, both the mRNA and protein increase in distal nerve stumps following sciatic nerve section (Cohen et al., 1992). Toma et al. (1992) describe a similar pattern of changes in EGF Receptor of Schwann cells after peripheral nerve injury to that of p185$^{erbB2/neu}$, with substantial elevation in distal stumps commencing at the transection site and progressing distally. The mRNA for the p185$^{erbB2/neu}$ receptor is substantially increased in cultured Schwann cells by elevating cyclic AMP, implying that axons may control synthesis of the Schwann cell receptor, as well as providing the source of its ligand (Cohen et al., 1992). Whether one of the variants of GGF is itself responsible for normally controlling p185$^{erbB2/neu}$ receptor expression is an intriguing possibility. A physical interaction between the EGF receptor (EGF-R) and p185$^{erbB2/neu}$ has been demonstrated in fibroblasts where EGF binding to EGF-R leads to phosphorylation of p185$^{erbB2/neu}$ (Wada et al., 1990). Whether these two receptors and their ligand operate together or independently requires investigation.

Although there is currently great interest in the neuregulin/GGFS as axonally-derived Schwann cell factors, a number of other neuronal growth factors may regulate Schwann cell phenotype. Specifically, acidic Fibroblast Growth Factors (aFGF) were found to be produced in a variety of neuronal cell bodies (Janet et al., 1988). Interestingly, high levels of aFGF were found in normal sciatic nerves, but were downregulated following nerve transection (Eckenstein et al., 1991). Axons as well as purified FGF have been shown to promote mitogenesis in Schwann cells in vitro (Rather et al., 1988; Davis & Strootbant, 1990). Reports also indicate that exogenous application of FGFs can accelerate axonal regeneration in lesioned sciatic nerve (Cordiero et al., 1989). However, like CNTF (see section III), aFGF lacks the N-terminal signal sequence, thought to be necessary for secretion (Stockli et al., 1991; Vlodavsky et al., 1991). Thus, it was suggested that aFGF may be released in a quick

and transient burst of activity from the injured nerve, resulting possibly both in activating Schwann cells and in promoting neuronal regeneration.

Isoforms of TGFβ have also been detected in both the developing and adult mammalian CNS and PNS (Flanders et al., 1991; Unsicker et al., 1991; Scherer et al., 1993); more specifically, TGFβ has been localized in certain neurons in the CNS, dorsal root ganglion neurons, astrocytes, and Schwann cells themselves, suggesting a possible role for TGFβ in the regulation of glial cell function. Insights on the possible role of TGFβ in Schwann cell development come from in vitro studies showing that TGFβ is a mitogen for Schwann cells (Eccleston et al., 1989; Ridley et al., 1989; Schubert, et al., 1992). Interestingly, TGFβ also inhibits cAMP-induced Po mRNA expression, and downregulates p75 NTR mRNA expression in Schwann cells (Mews & Meyer, 1993). These effects were demonstrated in cultures lacking neurons. More recent data (Guenard et al., 1995) demonstrated that in cocultures of DRG neurons and Schwann cells, TGFβ blocked the formation of myelin sheaths by Schwann cells but not ensheathment of axons by Schwann cells. In addition, the expression of myelin-related molecules was blocked. Finally, TGF-β also modulates gap junctional communication in cultured Schwann cells (Chandross et al, 1995). These effects are not consistent with a role for TGFβ in regulating mature Schwann cell phenotype.

While NT-3 was shown to be expressed in a variety of neurons during development (Schecterson & Bothwell, 1992), the discovery of full-length trkC in Schwann cells (Offenhauser et al., 1995) may provide another potential mechanisms for neuron:Schwann cell signaling or crosstalk.

Cell adhesion molecules regulate Schwann cell phenotype

Although the work by Bolin et al. (1993) suggests that at least one axonal signal for Schwann cells involves a diffusible factor, other studies indicate that direct contact with axonal membranes regulates Schwann cell behavior. In particular, in co-cultures of neurons and Schwann cells, Schwann cells proliferate upon contact with axonal processes. This rapid mitogenic action of neuronal membranes is dependent upon cell-cell contact. Two alternative explanations have been invoked to explain this contact-dependent mitogenesis. One is that ligands bound to the axolemma are involved (Davis & Goodearl, 1991). Alternatively, cell adhesion molecules may

signal directly to regulate Schwann cell phenotype in a cell contact-dependent fashion. In this regard, cell adhesion molecules that are members of the immunoglobulin-gene superfamily have been implicated in axon:Schwann cell interactions. These include neural cell adhesion molecule (N-CAM), neuron-glia cell adhesion molecule (Ng-CAM)/L1, myelin-associated glycoprotein (MAG) (Owens et al, 1989)and Po(reviewed in Martini, 1994).

Schwann cells recapitulate expression of the neural cell adhesion molecules L1 and N-CAM after transection of the adult mouse sciatic nerve in a temporal sequence similar to the one during development (Nieke & Schachner, 1985; Martini & Schachner, 1986). Three CAMs, namely N-CAM, N-cadherin and the L1 glycoprotein (Doherty & Walsh, 1992; Bixby & Harris, 1991) have been suggested to be important for contact dependent axonal growth. When functioning to promote axonal growth, CAMs have been suggested to interact with the FGF receptor kinase and to influence calcium influx into neurons (For reviews, see Doherty & Walsh, 1994). These CAMs operate by a homophilic binding mechanism. Recent studies have shown that two of these CAMS, N-cadherin and L1, can also act heterophilically as neuronal receptors for other ligands (Redies & Takeichi, 1993; Kuhn et al., 1991).

MAG is also thought be functionally involved in the initial stages of myelination both because of its periaxonal localization and because of its receptor-like structure. However, the function of MAG appears to be dispensable (or redundant) for the development of the PNS, since mice lacking MAG develop near normal peripheral nerves(Li et al 1994). However, Schwann cells infected with a recombinant retrovirus expressing myelin-associated glycoprotein antisense RNA do not form myelin(Owens & Bunge et al, 1991), strongly suggesting that MAG is the critical Schwann cell component induced by neuronal interaction that initiates peripheral myelination. Interestingly, MAG has recently been implicated in a signal-transduction cascade involving direct interaction with, and activation of , the Fyn tyrosine kinase, p59fyn(Umemori et al 1994). Interestingly, Fyn-deficient mice produce decreased amounts of CNS myelin compared with wild-type animals. It will be interesting to see whether Fyn or additional kinases, or both, play a similar role in transducing an axonal signal for myelin formation in the PNS.

V. Thesis Introduction

Following peripheral nerve injury, there are series of changes in gene expression in neurons and Schwann cells. There are generally two causes that are hypothesized to induce peripheral nerve injury responses: macrophage invasion and inflammatory response; or loss of homeostatic interaction among neurons, Schwann cells, and target. We hypothesize that the alterations in neuronal and Schwann cell gene expression observed following peripheral nerve injury are independent of macrophage invasion and are at least partially due to the disruption of ongoing homeostatic signals. In the following three papers (chapters), I have studied the molecular and cellular mechanisms underlying the peripheral nerve injury response in both neurons and Schwann cells. More specifically, I have asked the following two questions: 1. Are any of the alterations in neuronal or Schwann cell gene expression that occur following peripheral injury due to the loss of ongoing homeostatic signals? 2. What are the molecular mechanisms responsible for regulation of Tα1 α-tubulin mRNA during neuronal regeneration?

I have used two different technical approaches to test our hypothesis and address these questions: local cooling of the peripheral nerve to block fast axonal transport in our attempts to examine signaling mechanisms that may play a role in the peripheral nerve injury response, and Tα1:nlacZ transgenic mice to dissect transcriptional versus posttranscriptional mechanisms that regulate Tα1 α-tubulin gene expression during nerve regeneration. I will discuss the two approaches and our data from them as follows.

The first strategy is to attempt to interrupt normal homeostatic signals by disrupting axonal function without inducing an inflammatory injury response, and to determine whether such disruption leads to alterations in Schwann cell or neuronal gene expression that are similar to those observed following peripheral nerve injury. Cold block on intact peripheral nerve selectively blocks the fast axonal transport and distal electrical activity without inducing macrophage invasion or Wallerian degeneration. I have been able to demonstrate that induction of at least some changes during peripheral nerve injury are independent of macrophage invasion or inflammatory response. The results are described in the two papers (Wu et al., 1993, 1994). In the first paper (Wu et al., 1994), I have relied upon local cooling of the

intact peripheral nerve (a cold block) to block fast axonal transport and electrical activity without inducing macrophage invasion or Wallerian degeneration. Using this experimental approach, for the intact sciatic nerve, I have demonstrated that Schwann cell gene expression distal to the cold block was selectively altered; expression of the mRNA encoding Po was downregulated, as it is following peripheral nerve injury, whereas expression of the p75 neurotrophin mRNA was unaltered. These results indicate that Po and p75 NTR are regulated as a function of two different aspects of Schwann cell:axon communication. Moreover, these experiments demonstrate that at least some myelin-specific Schwann cell responses are dependent upon ongoing axonal signals that are maintained by fast axonal transport and/or electrical activity. In the second paper (Wu et al., 1993), I have used the same approach to determine whether the loss of ongoing signals that are dependent on fast axonal transport or electrical activity can explain alterations in motor neuron gene expression observed after axonal damage. These studies demonstrated that the expression of two regeneration associated genes, Tα1 and p75 neurotrophin receptor mRNAs, were strongly induced following a local cold block to the intact facial nerve. These data suggest that neurons sense their status by a constant flow of information carried, circulated, or maintained by fast axonal transport and/or electrical activity and the axotomy-induced increases in Tα1 and p75 neurotrophin receptor mRNAs in motor neurons are, to a great extent, due to loss of such homeostatic signals.

In the second approach, we are able to further characterize the induction of Tα1 α-tubulin gene expression, the neuronal growth associated gene, at the transcriptional level during neuronal regeneration (Wu et al., 1996). Following axotomy of facial motoneurons, the Tα1:nlacZ transgene was rapidly upregulated, and, if neurons regenerated and reinnervated their target musculature, transcription returned to control levels. Moreover, if regeneration was inhibited, transgene expression remained elevated, suggesting that target contact represses expression of the Tα1 gene, and that loss of this transcriptional repression largely accounts for the pattern of Tα1 mRNA expression in regenerating motoneurons. These data are in agreement with my second paper (Wu et al., 1993; see above), which indicates that loss of homeostatic signals derived from the target results in rapid induction of this mRNA. In contrast, in sympathetic neurons of the superior cervical ganglion, differences were observed between the Tα1:nlacZ transgene, and the endogenous

Tα1 mRNA. While Tα1 mRNA only increased when neurons were axotomized close to, but not distal from their cell bodies (Mathew & Miller, 1993), transgene expression was increased regardless of the injury site. It is likely that this "distance effect" is due to posttranscriptional mechanisms that couple tubulin mRNA levels to cytoskeletal status; when cultured sympathetic neurons were treated with colchicine, which depolymerizes microtubules, steady-state Tα1 mRNA levels were greatly decreased. Thus, I propose a model where a combination of transcriptional and posttranscriptional mechanisms allow mature neurons to closely modulate synthesis of this essential cytoskeletal protein as a function of both target contact and axonal status.

GENERAL ACKNOWLEDGEMENTS

In the following three chapters (papers), as the first author, I was the principle researcher who carried out all the experiments, under the excellent co-supervision of Drs. Freda Miller and Bob Campenot. Throughout the progress of my thesis projects, I have received invaluable scientific advice and technical assistance from the co-authors on these papers. I should make particularly acknowledgment and gratitude to Dr. Dick Smith and Ms. Honey Chan for their significant contribution to the electron microscopy studies in chapter 2 (Wu et al., 1994).

CHAPTER TWO: DISRUPTION OF FAST AXONAL TRANSPORT IN VIVO LEADS TO ALTERATIONS IN SCHWANN CELL GENE EXPRESSION

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ACKNOWLEDGEMENTS:

We wish to thank Bob Campenot, Larry Guilbert, and the other members of the Miller laboratory for frequent discussions, scientific advice, and constant support. This work was supported for grants from the Canadian M.R.C., and the N.C.E. for Neural Regeneration and Recovery to F.M. and R.S. R.S. is an MRC Scientist, and W.W. is supported by a studentship from the Rick Hansen Foundation for Spinal Cord Research.

This manuscript was published in Developmental Biology (1994) 163: 423-439.

INTRODUCTION

In the mature peripheral nerve, Schwann cells are closely associated with axons, and either myelinate a single axon, or closely ensheath a number of different axons without forming myelin. The nature of the association that develops between any given Schwann cell and it's corresponding axon(s) is believed to be determined by the type of neuron and caliber of the axon(reviewed in Bray et al., 1981). For example, axonal caliber is correlated with the extent of myelination (Duncan, 1934a; 1934b; Friede, 1973a; 1973b), and the presence or absence of axons regulates gene expression in Schwann cells (Brunden et al., 1990; DiStefano et al., 1990). This communication is not one way: myelinating Schwann cells can locally regulate axonal caliber, neurofilament distribution and neurofilament phosphorylation (Waegh et al., 1992). Thus, there exist mutual interactions between axons and Schwann cells that are likely determined by the local cellular microenvironment, including potential paracrine interactions, cell:cell adhesion, and/or simple physical contact.

Much of the evidence for an axonal influence on Schwann cell gene expression is derived indirectly from studies examining the peripheral nerve injury response. Interruption of normal axon:Schwann cell interactions by nerve injury leads to a number of alterations in Schwann cell gene expression, including down-regulation of myelin genes (Trapp et al., 1988; Gupta et al., 1988; LeBlanc and Poduslo, 1990) such as that encoding the major myelin protein, Po, and upregulation of a number of trophic factors and their receptors, including NGF (Heumann et al., 1987), BDNF (Meyer et al., 1992; Acheson et al., 1991), p75 NGF receptor (Taniuchi et al., 1986; 1988), and EGF receptor (Toma et al., 1992). These changes are believed to play a key role in providing a permissive substrate for peripheral nerve regeneration. Reversal of Schwann cells to a mature phenotype is subsequently correlated with reestablishment of axon:Schwann cell contact following axonal regeneration.

Although these findings suggest that at least a subset of injury-induced alterations in Schwann cell gene expression are a function of the loss of axon:Schwann cell contact, this interpretation is confounded by a number of variables. Following nerve injury, macrophages invade the nerve at the injury site,

and the distal segment undergoes a process known as Wallerian degeneration, which involves axonal fragmentation, debris removal, and proliferation of Schwann cells and fibroblasts (reviewed in Allt, 1976; Perry and Brown, 1992). At least one axotomy-induced alteration in gene expression, the upregulation of NGF mRNA, has been attributed to the release of interleukin-1 by invading macrophages (Heumann et al., 1987; Brown et al., 1991). Thus, although the nerve injury studies indirectly support the hypothesis that Schwann cell gene expression is regulated by axonal contact, any of a number of alternative explanations could be similarly invoked.

More definitive support for the axon:Schwann cell contact hypothesis derives from culture studies. When Schwann cells are cultured in the absence of other cell types, they downregulate Po and upregulate p75 NGF receptor gene expression (Lemke et al., 1988; DiStefano et al., 1988). Furthermore, either coculture with axons or the addition of axonal membranes to Schwann cell cultures reverses these changes (Brunden et al., 1990; DiStefano and Diane, 1990). Thus, for these two genes, the presence or absence of axon:Schwann cell contacts appears to be a key regulatory factor. The cellular mechanisms whereby this occurs remain ill-defined, but elevation of intracellular cAMP levels in expanded Schwann cell cultures mimicked the axon-induced increase in Po gene expression (Lemke and Chao, 1988; LeBlanc et al., 1992).

We have previously documented spatial/temporal gradients in Schwann cell expression of Po and p75 NGF receptor mRNAs within the injured nerve. These gradients occurred as a function of distance from the lesion site, and, in light of evidence gathered from cultured Schwann cells, we hypothesized that they were due to the sequential loss of ongoing axon:Schwann cell homeostatic signals. In order to directly test this hypothesis in vivo, without all of the confounding variables associated with peripheral nerve injury, we locally-cooled the sciatic nerve, which, by blocking fast axonal transport, selectively perturbed axonal physiology without disrupting axon:Schwann contacts or inducing an inflammatory response. Results obtained using this experimental paradigm indicate that, in vivo, the Po and p75 NGF receptor genes are regulated by two different aspects of axon:Schwann cell communication. Furthermore, these data suggest that the expression of myelin-specific genes requires not only physical contact between axons and Schwann cells,

but also axonally-derived biochemical signals that are maintained by fast axonal transport.

MATERIALS AND METHODS

Animals and surgical procedures. Female Sprague-Dawley rats (2-3 months old, 250-300 grams) were anaesthetized with urethane (1.25 g/kg) subcutaneously, and the sciatic nerve on one side was carefully dissected out at midthigh level. A 10 mm segment of the nerve was then locally-cooled to 5 - 8^0 C by placing a cold cuff around the nerve sheath, taking care not to injure or stretch the nerve. Cold antifreeze was continuously run through the nerve cuff, and the temperature of the nerve itself was monitored at all times (see Wu et al., 1993). The skin was then sutured over the cuff and temperature probe, and the cold block was maintained in place for 72 hours, during which time the animal remained anaesthetized. The contralateral sciatic nerve of these experimental animals was sham-operated and, in 3 cases, a similar cuff of body temperature was implanted. Urethane was administered as required to maintain anesthesia, and animals were supplemented with lactated Ringers solution throughout the course of the experiment. A second group of positive control animals were similarly anaesthetized, and the sciatic nerve dissected and axotomized at approximately the same location. These animals were allowed to recover from the anesthesia. In total, 16 animals in each group, cold block and axotomy, were analyzed.

Following 72 hours of cold block or axotomy, animals were anaesthetized with sodium pentobarbital (35 mg/kg), and transcardially perfused with 4% paraformaldehyde. The intact, cold-blocked, and transected nerves were removed, fixed overnight in 4% paraformaldehyde in phosphate buffer at pH 7.4, and cryoprotected in graded sucrose solutions. Longitudinal cryostat sections of nerve (10 µm) were mounted on chrom-alum subbed slides, and analyzed by immunocytochemistry and <u>in situ</u> hybridization. For morphological studies, after the sciatic nerve had been cold-blocked or axotomized for three days, anaesthetized animals were vascularly perfused with 250 ml of 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) at room temperature.

To test the effectiveness of the cold block, female Sprague-Dawley rats were anaesthetized with urethane subcutaneously, and the sciatic nerve locally-cooled, as described above. Two hours after initiation of the cold block, 20 µl of a 3% solution

of Fluorogold was injected into the lower hindleg on the ipsilateral, cold-blocked side, and on the contralateral, sham-operated side. 72 hours after initiation of the cold block, the animals were perfused with 4% paraformaldehyde, and the nerves were processed as described above. 3 animals were utilized in these experiments.

Nerve morphology. A 40 to 50 mm length of the nerve with the cold block or axotomy site at its center was removed from the animal, desheathed and stored in the fixative overnight at 10^0 C. Nerves were postfixed in osmium tetroxide, dehydrated in a series of alcohols and infiltrated with Epon. Specimens for electron microscopy were embedded in a known orientation and thin-sectioned. Sections were stained with uranyl acetate and lead citrate and were viewed with a Philips 410 electron microscope. For light microscopy, small groups of myelinated axons were teased out while the nerve was in uncured Epon; these were mounted on microscope slides, cover-slipped, and viewed with bright field optics.

Hybridization probes. Antisense RNA probes specific to the p75 NGF receptor were generated from a subclone containing nucleotides 400-710 of the rat cDNA (Radeke et al., 1987), as previously described (Miller et al., 1991; Toma et al., 1992). Antisense RNA probes for Po mRNA (Lemke and Axel, 1985) were generated as previously described (Toma et al., 1992).

Antibodies. 192 IgG is a mouse monoclonal antibody first described by Chandler et al., (1984). 192 IgG-producing hybridomas were used to produce ascites fluid in mice, and IgG was isolated using the Pierce Immunopure method according to the manufacturer's instructions. This antibody, which was the kind gift of Dr. R. Murphy, (Montreal Neurological Institute) was used for immunostaining at a final concentration of 8 µg/ml. The monoclonal anti-ED1 antibody was obtained commercially from Serotec and recognizes a cytoplasmic antigen specific to mononuclear phagocytes including macrophages and monocytes (Dijkstra et al., 1985; Beelen et al., 1987). This antibody was used for immunostaining at a final concentration of 5 µg/ml.

Immunocytochemistry. Slide-mounted nerve sections were fixed by a brief 5 min exposure to 4% paraformaldehyde in phosphate buffer, pH 7.4. The sections were permeabilized with HEPES-buffered saline (HBS; 10 mM HEPES, pH 7.4, 150 mM

NaCl) containing 0.1% Triton X-100 for 5 min. The Triton was removed with two HBS washes. Nonspecific binding was blocked by a 30 min incubation in HBS containing 4% goat serum. Sections were incubated with appropriate concentrations of the primary antibody diluted in HBS plus 4% goat serum overnight at 4^0 C. Following two washes with HBS, sections were incubated with a rhodamine-conjugated affinity-purified goat anti-mouse IgG secondary antibody (Jackson Immuno Research Laboratories, Inc.) diluted to a final concentration of 2 µg/ml. Sections were washed 3 times in HBS, and mounted in Mowiol.

Controls and immunostaining. As a control for immunostaining, we used IgG from a nonspecific mineral oil plasmacytoma (MOPC21, obtained from Organon Teknika) at 8 µg/ml. Additional controls, including elimination of primary or secondary antibody, yielded little detectable staining. Occasionally, however, a small amount of non-specific immunostaining was associated with the collagenous portion of the nerve sheath located outside of the perineurium.

In situ hybridization. In situ hybridization was performed on sections of nerve as previously described (Miller et al., 1989). After hybridization, slides were air dried and apposed to Kodak XRP film for 1-5 days to obtain x-ray images. The slides were subsequently dipped in Kodak NTB-2 emulsion and exposed for 2-7 days prior to development. Hybridization with sense probes was performed to ensure specificity of hybridization.

Analysis. To minimize variability, we mounted and processed control and lesioned nerves, or control and cold-blocked nerves from the same animal on the same slides (Toma et al., 1992). Furthermore, adjacent sections of the same nerves were immunostained with ED1 and 192 IgG, and analyzed for expression of Po and p75 NGF receptor mRNAs to obtain a full profile on each individual cold-blocked animal. Comparisons were only made between sections processed at the same time, and all photographs utilized for comparisons were developed and printed using identical exposure times.

RESULTS

To test the hypothesis that maintenance of adult Schwann cell phenotype is dependent upon axon:Schwann cell signals that require normal axonal homeostasis, we developed an in vivo experimental paradigm that allowed us to selectively perturb axonal physiology without causing an inflammatory response or disrupting physical contact between Schwann cells and axons. More specifically, we locally-cooled the sciatic nerve to 5 - 8^0 C, a procedure which selectively interrupts fast axonal transport(Ochs and Smith, 1975), and determined what effect this manipulation had on the phenotype of Schwann cells associated with these axons.

The temperature profile of the cold-blocked nerve is shown schematically in Figure 1. A 10 mm segment of sciatic nerve at midthigh level was locally-cooled to 5 - 8^0 C for 72 hours by means of a temperature cuff, during which time the temperature was continuously monitored. Normal temperature (30^0 C, as measured with the probe) was reached within 5 mm of the cooling cuff on either side. Thus, for the duration of the treatment, temperature was perturbed within the cold block region itself, as well as within the adjacent 5 mm regions where a temperature gradient of 5 to 30^0 C was localized.

We have previously demonstrated that a similar cold-block effectively blocked the retrograde transport of Fluorogold from the terminals of facial motor neurons back to their cell bodies (Wu et al., 1993). To ensure the effectiveness of the block to fast axonal transport within the sciatic nerve, the nerve was cooled unilaterally, and Fluorogold was injected into the lower hindlimbs bilaterally 2-3 hours after initiation of the cold block. Seventy hours later, during which time the cold block was maintained, the nerve was examined for the distribution of Fluorogold (Fig. 2). For the control nerve, a low level of Fluorogold was detected throughout the length of the nerve segment (Fig. 2a). For the cold-blocked nerve, Fluorogold was absent from the cold block region itself (data not shown) and from the region of the nerve proximal to the cold block (Fig. 2b). In contrast, Fluorogold was concentrated in the nerve segment immediately distal to the cold block (Fig. 2c), decreasing to control levels approximately 15 mm from the cold block region (data not shown).

Thus, the cold-block treatment effectively perturbed fast axonal transport, at least as monitored by the retrograde transport of Fluorogold.

COLD BLOCK OF THE SCIATIC NERVE DOES NOT LEAD TO AN INFLAMMATORY RESPONSE

A number of injury-induced alterations in Schwann cell phenotype have been attributed to invading macrophages, and the subsequent release of inflammatory cytokines. To ensure that the cold block did not lead to such an inflammatory response, we performed immunocytochemistry using the ED1 monoclonal antibody, which recognizes cells of the mononuclear phagocyte family, including macrophages and monocytes (Fig. 3). For comparison, we immunostained sections of control sciatic nerve, and of sciatic nerve that had been transected for 72 hours. In the control nerve, staining was limited to the occasional positive cells (Fig. 3a), presumably resident macrophages (Perry et al., 1987). In the 72 hours transected nerve, large numbers of ED1-positive cells were detected at both the proximal and distal tips of the nerve (Fig. 3b), and many of these were large and vacuolated, characteristics diagnostic of macrophages. The distribution of ED1-positive cells was not uniform throughout the distal segment of the transected nerve: more ED1-positive cells were detected at the transection site than were seen 10 and 25 mm distal (Fig. 3b-d), although, relative to the control nerve, increased numbers of positive cells were detected throughout the entirety of the distal segment.

In contrast, the cold block procedure did not affect the number of ED1 positive cells within the sciatic nerve. Immunostaining demonstrated that only the occasional ED1-positive cell was detected throughout the length of the cold-blocked nerve, ranging from 30 mm proximal to, and extending 40 mm distal to the cold-block region (Fig. 3e-h). This pattern was indistinguishable from that observed in the control nerve (Fig. 3a). Immunostaining with the ED1 antibody was performed on all nerves used in this study; invasion of ED1-positive cells was never noted when the cold block was applied at midthigh level. We did, however, observe such an invasion in a number of experiments (which were not included in our analysis) where we attempted to place the cold block close to the spinal column, presumably as a consequence of stretching of the nerve and/or the more invasive nature of the surgery (data not shown).

THE EFFECT OF A COLD BLOCK ON NERVE STRUCTURE

By examining teased nerve fibers as whole mounts and transmission electron micrographs of cold-blocked and axotomized nerve, we obtained answers to two questions. First, we asked whether the cold block treatment led to invasion of macrophages and loss of the physical relationship between Schwann cells and axons. Second, we asked whether the cold block led to morphological changes that were indicative of blocked anterograde and retrograde fast axonal transport and whether changes distal to the block could be correlated with the distribution of the Fluorogold tracer described above.

Teased myelinated fibers proximal to either a 72 hours cold block (Fig. 4a) or to an axotomy lesion (Fig. 4b) had a similar fiber and nodal morphology; this morphology was indistinguishable from that of fibers in control preparations (not shown). In the distal segment of the transected nerve, the fibers had all reached an advanced stage of Wallerian degeneration (Fig. 4c). In contrast, distal to a cold block at 72 hours, teased fibers had close to normal morphology (Fig. 4d). While about 75% of the fibers distal to a cold block showed slightly crenulated margins (Fig. 4d), the ultrastructure of these fibers was normal (Fig. 5c). Thus, teased fiber morphology indicated that the cold block did not produce Wallerian degeneration over 72 hours.

Ultrastructural features of cold blocked axons were normal proximal to the cold block (Fig. 5a) and largely normal at all locations distal to the cold block (Fig. 5c) with less than 5% of distal axons showing any degenerative changes. Except for the small percentage of teased fibers showing early degenerative changes distal to the cold block, myelin profiles and axon: Schwann cell physical relationships were similar to controls at all regions examined proximal and distal to the cold block and within the cold block. Macrophages were rare or absent at all locations in the cold blocked nerve, confirming the immunostaining results obtained with the ED1 antibody. Thus, cold blocked nerve showed no evidence of widespread Wallerian degeneration.

In comparison, electron microscopy of transected nerve showed, 10 mm distal to the site of section at 72 hours, the typical characteristics of widespread Wallerian degeneration (Fig. 5f). All myelinated and non-myelinated axons were at an advanced

stage of degeneration, and non-neuronal cells, probably macrophages, containing myelin debris were common. The nerve at 10 mm proximal to the axotomy site had an ultrastructure indistinguishable from that of control nerve.

Within the cold block region, myelin profiles and axon: Schwann cell physical relationships were normal, but both myelinated and non-myelinated axons often contained increased numbers of vesicles (Fig. 5b). Very high numbers of membranous organelles were present in axons up to 10 mm proximal and distal to the cold block. Proximal to the cold block (Fig. 5d) these organelles were predominantly small vesicles with a mean ± SEM diameter of 82 ± 4 nm (n=61) and short tubules of a similar diameter. Distal to the block (Fig. 5e) the organelles were a mix of small vesicles (diameter 104 ± 5 nm, n = 48) and larger organelles (diameter 224 ± 9 nm, n=49) which had structural characteristics of mitochondria, lysosomes and prelysosomes. The organelles adjacent to the cold block resembled those known to undergo anterograde and retrograde fast axonal transport in vertebrate axons (Smith, 1980; Tsukita and Ishikawa, 1980) hence the organelle accumulations constitute evidence of blocked fast axonal transport.

p75 NGF RECEPTOR EXPRESSION WITHIN THE COLD-BLOCKED NERVE

To determine whether the cold block led to alterations in expression of p75 NGF receptor mRNA, we performed in situ hybridization on longitudinal sections that included 30 mm of nerve proximal to and 40 mm distal to the cold block. For comparison, we examined similar sections of the sciatic nerve 72 hours post-transection. As previously reported (Taniuchi et al., 1986; 1988; Toma et al., 1992), p75 NGF receptor mRNA was expressed at low levels in Schwann cells of the control nerve and, 72 hours postaxotomy, was greatly increased in the distal segment of the injured nerve (Fig. 6f,g; Fig. 7a,b). In contrast, in the cold-blocked nerve, p75 NGF receptor mRNA levels were similar to controls in both the proximal and distal segments (Figs. 6h,i; 7c-f), with no alterations being detected as far distal as 40 mm. Within the cold block region itself, levels of p75 NGF receptor mRNA were actually reduced relative to the rest of the nerve (Fig. 7d), and to control nerves (data not shown), presumably as a consequence of lowered Schwann cell metabolism.

To confirm that the cold block did not alter Schwann cell expression of p75 NGF receptor, we stained adjacent longitudinal nerve sections with the monoclonal antibody 192 IgG. In the 72-hour axotomized nerve, p75 NGF receptor was uniformly increased throughout the distal nerve segment (Fig. 8b), relative to the control nerve (fig. 8a), and was increased at the distal tip of the proximal segment (data not shown), as previously reported (Toma et al., 1992). In contrast, the distribution of p75 NGF receptor in the cold-blocked nerve differed significantly. Within the cold-block region itself, levels of IgG-192 immunoreactivity were low, and were similar to the control nerve (Fig. 8a) . Immunostaining was then greatly increased in the region extending approximately 10 - 15 mm immediately proximal (Fig. 8d) and distal (Fig. 8f) to the cold block region. By 15 mm proximal (Fig. 8c) and distal (Fig. 8g) immunostaining was again similar to that detected in the control nerve, and remained so throughout the remainder of the nerve. Thus, p75 NGF receptor protein levels were increased within the nerve immediately adjacent to the cold block, but were similar to controls in the remainder of the proximal and distal segments. This pattern of increase in p75 NGF receptor protein in the cold-blocked nerve was similar to that observed for retrogradely-transported Fluorogold in the distal nerve segment (Fig. 2). Since p75 NGF receptor is retrogradely-transported (Taniuchi and Johnson, 1985), and is presumably transported anterogradely by fast axonal transport, like other membrane-bound proteins (Hirokawa et al., 1990; Sheetz and Martenson, 1991), we interpret the increased local IgG-192 immunostaining in the absence of increased gene expression as accumulation of transported p75 NGF receptor.

Po mRNA EXPRESSION IS ALTERED DISTAL TO A COLD BLOCK

To determine whether expression of the major myelin protein, Po, was affected by the cold-block treatment, we performed <u>in situ</u> hybridization on longitudinal nerve sections adjacent to those examined for p75 NGF receptor expression. In control animals, Po mRNA was expressed at approximately the same level throughout the length of the sciatic nerve (Figs. 6a; 9a), and, 72 hours following transection, its levels were uniformly decreased throughout the distal nerve segment (Figs. 6b; 9b), as previously reported (Trapp et al., 1988; Toma et al., 1992; Gupta et al., 1988). The cold-block treatment also altered levels of Po mRNA, but the alterations were not uniform throughout the nerve. In the cold block region, Po

mRNA levels were downregulated (Fig. 6d,e; 9d), similar to what was observed for p75 NGF receptor mRNA. In the nerve proximal to the cold block, Po mRNA levels were similar to those in the control nerve (Fig. 6c; 9c). However, in contrast to p75 NGF receptor mRNA, levels of Po mRNA were dramatically altered in the segment of the nerve distal to the cold-block (Fig. 6d,e; 8e-g). In the region adjacent to the cold block, where axonal traffic had accumulated, Po mRNA levels were similar to controls and to the proximal nerve segment (Fig. 9e). However, further distal (greater than 15 mm), throughout the remainder of the distal nerve segment, Po mRNA levels were decreased to levels similar to those observed following axotomy (Figs. 6d,e; 9f,g). Electron microscopy revealed that axon:Schwann cell contacts and myelin profiles in the region of decreased Po mRNA expression were indistinguishable from regions of the same nerve where Po gene expression remained high (Fig. 5c,e). Thus, Po mRNA levels differed significantly along the length of the distal cold-blocked nerve segment, presumably reflecting local regulation of Schwann cell phenotype as a function of local variations in axonal physiology.

DISCUSSION

In the experiments reported here, we tested the hypothesis that Schwann cell phenotype is regulated as a function of the local axonal microenvironment. To pursue these studies we utilized an experimental paradigm that allowed us to perturb axonal physiology in the mature peripheral nerve without causing an inflammatory response, Wallerian degeneration, or loss of axon:Schwann cell contact. Results of these experiments support three major conclusions. Firstly, these data indicate that the genes encoding the major myelin protein, Po, and the p75 NGF receptor, both of which are influenced by the presence of axons (Brunden et al., 1990; DiStefano and Diane, 1990; Taniuchi et al., 1986; 1988; LeBlanc and Poduslo, 1990; Trapp et al., 1988; Gupta et al., 1988), are regulated as a function of two different aspects of Schwann cell:axon communication. Secondly, we provide direct in vivo evidence that at least some of the axotomy-induced alterations in the peripheral nerve are not due to macrophage infiltration and/or Wallerian degeneration. Finally, and most importantly, we demonstrate that Schwann cell phenotype, as monitored by expression of the Po gene, is regulated by the local axonal microenvironment independently of physical axon:Schwann cell contact, and that this regulation is a function of ongoing biochemical signals generated by the axon and maintained by fast axonal transport.

These conclusions rely upon the validity of the cold block paradigm as a mechanism for perturbing fast axonal transport and thereby altering the local axonal microenvironment. Three lines of evidence were presented in this paper that demonstrated the efficacy of this technique: a) the cold block effectively prevented the retrograde transport of Fluorogold (data presented here; Wu et al., 1993), thereby leading to its accumulation for 15 mm distal to the cold block region, b) the cold block led to a localized accumulation of p75 NGF receptor protein 15 mm proximal and distal to the cold block region, and c) the cold block led to accumulation of large numbers of vesicles and organelles in axons immediately adjacent to the cold block region. Thus, the cold block effectively blocked both anterograde and retrograde fast axonal transport. The functional consequence of this block was to create at least two domains within the axons distal to the cold block region. The first domain consisted of the 10-15 mm segment of nerve immediately adjacent to the cold block region,

where there was accumulation of vesicles, organelles, p75 NGF receptor and, presumably, a large number of other proteins that are trafficked from nerve terminals via retrograde transport (reviewed in Sheetz et al., 1991). The further distal segment of the nerve (ie. greater than 15 mm from the cold block) comprised a second domain where, by definition, intracellular traffic was depleted, since the cellular supply of new proteins and organelles was severed by the cold block. The situation within the proximal nerve segment is somewhat less clearcut; a similar accumulation occurred within the region 15 mm proximal to the cold block, but the remainder of the proximal segment was still presumably receiving vesicular traffic from the cell body.

Our conclusions are dependent upon the ability to eliminate a number of confounding variables that complicate the interpretation of peripheral nerve axotomy experiments. One such variable is the inflammatory response that follows nerve injury (reviewed in Allt, 1976; Perry and Brown, 1992). Data presented in this paper indicate that such a response did not occur following a cold block, as monitored by a) the lack of invasion of ED1 positive cells, and b) the lack of macrophages as detected by a second method, electron microscopy. A second confounding variable in axotomy experiments is the loss of direct physical contact between axons and Schwann cells. The morphological studies presented here demonstrated that myelin profiles and axon:Schwann cell contacts were morphologically normal throughout the nerve segment distal to a cold block. Furthermore, with the exception of local organelle accumulation, the vast majority (greater than 95%) of axons in the distal nerve segment were similar to controls. A subset of axons (less than 5%) did, however, show some degenerative changes, but these axons were observed throughout the distal segment, in regions of both high and low Po mRNA expression, and thus could not explain the global downregulation of Po mRNA in the far distal nerve segment.

Finally, potential local effects of the decreased temperature must be considered in any analysis of this data. Changes in nerve temperature were localized to the 10 mm segment of the cold block itself and to the regions 5 mm proximal and distal, where a temperature gradient existed. It is clear that the lowered temperature affected the behavior of Schwann cells, since we documented a generalized decrease in gene expression in the cold block region, as well as some local alterations in morphology (Wu et al., 1993). As a consequence, we restricted our analysis to the

distal nerve segment more than 5 mm from the cold block region, where morphology was comparable to controls, and where there was no possible misinterpretation due to local temperature effects. In this regard, it should be noted that vesicles, organelles, and proteins accumulated in a region extending approximately 10 - 15 mm distal to the cold block region, and the transition from high to low Po mRNA expression also occurred at 15 mm.

Thus, at least as measured by the parameters described here, the only factor that varied within the distal nerve segment as a function of the cold block was the local axonal microenvironment. The pattern of Po mRNA expression was therefore somewhat surprising: in the 15 mm segment of nerve immediately distal to the cold block, all positively-hybridizing cells expressed Po mRNA at levels similar to controls, followed by an abrupt transition into the remainder of the distal nerve segment, where Po mRNA was expressed at levels similar to those observed following axotomy. Thus, decreased Schwann cell expression of Po mRNA was spatially correlated with axons that were depleted of intracellular traffic.

These results are particularly striking in light of the fact that, in the same Schwann cells, p75 NGF receptor expression was unaltered; in the distal nerve segment greater than 15 mm from the cold block, both p75 NGF receptor mRNA and protein levels were similar to controls, but Po mRNA levels were greatly decreased. In contrast to these results, expression of these two genes is usually coordinately regulated. For example, in cultured Schwann cells (Lemke and Chao, 1988), and in the peripheral nerve early in development (Lemke and Axel, 1985; Heumann et al., 1987), Po mRNA levels are low, and p75 NGF receptor levels high. Maturation of the peripheral nerve is coincident with increased Po expression in myelinating Schwann cells, and a decrease in p75 NGF receptor in the same cells (Lemke and Axel, 1985; Heumann et al., 1987). Finally, following nerve axotomy, p75 NGF receptor levels increase and Po levels decrease in Schwann cells throughout the distal nerve segment (Taniuchi et al., 1986; 1988; Trapp et al., 1988; Gupta et al., 1988; LeBlanc and Poduslo, 1990). Thus, both of these mRNAs are apparently regulated in vivo as a function of axon:Schwann cell contact. Such coordinate, axon-dependent regulation is not limited to Po and p75 NGF receptor, but includes a number of other myelin genes such as that encoding myelin basic protein (Roach et al., 1983; Gupta et al., 1988). This coincident regulation of a repertoire of Schwann cell genes has led to

the concept of an axon:Schwann cell signal that shifts a Schwann cell from an immature, nonmyelinating to a mature, myelinating phenotype (Monuki et al., 1990; Lemke, 1988; Yan & Johnson, 1988; Jessen et al., 1990; Brown & Asbury, 1991). Experiments presented here demonstrate that multiple axon:Schwann cell signals are involved in these transitions and that only the axon:Schwann cell signal responsible for regulating Po gene expression was disrupted by the cold block.

Any discussion of the cellular signals responsible for regulating Schwann cell Po gene expression as a function of axonal microenvironment is, at this point, speculative. However, a number of relatively simplistic explanations can be ruled out on the basis of our data. If the axonal signal responsible for maintaining Schwann cell Po expression was an anterograde neuronal "messenger", then the cold block would deplete all of the distal axons of this factor, and all distal Schwann cells would downregulate Po mRNA. Conversely, if the signal was a retrograde factor, then the cold block would deplete proximal axons of this factor, leading to downregulation of Po mRNA in the proximal nerve segment. Neither of these outcomes was observed. However, the spatial correlation between control levels of Schwann cell Po expression, and axonal Fluorogold and p75 NGF receptor accumulation in the distal cold-blocked nerve suggests an interrelationship between vesicle/protein trafficking, and the axon:Schwann cell signal for Po. One potential explanation for such a relationship invokes constitutive axonal secretion of soluble molecules, such as growth factors, that locally regulate Schwann cell biology. Since growth factors are presumably trafficked via fast axonal transport, then secretion would be disrupted in axonal domains that were depleted of vesicular traffic, while it could conceivably be maintained in regions where vesicles were concentrated. Such a mechanism could explain the observations reported that Schwann cell Po mRNA levels can be regulated, in culture, by neuronally produced diffusible molecules (Bolin & Shooter, 1993). Moreover, neurons synthesize a number of growth factors that are known to influence Schwann cell biology, including members of glial growth factor (Marchionni et al., 1993) and fibroblast growth factor (Eckenstein et al., 1991) families. Alternatively, trafficking along the length of the axon may be monitored by some as-yet-undefined mechanism, and disruption of this trafficking may alter the axonal surface, thereby modulating axon:Schwann cell adhesion and ultimately, may result in changes in Schwann cell gene expression.

Regardless of the underlying molecular signal, these results indicate that axon:Schwann cell physical contact and/or axonal caliber are not, of themselves, sufficient to specify Schwann cell expression of Po or, by extrapolation, myelination. These results instead infer that Schwann cells are dependent upon a chemical axonal signal for maintenance of a normal, myelinating Schwann cell phenotype, and, potentially, during development, for the selection of axons for myelination.

FIGURES AND FIGURE LEGENDS

Figure 1. Temperature profile of the sciatic nerve during a cold block experiment. The local cooling cuff was placed on the sciatic nerve at midthigh level. The temperature in the region of the cuff was measured throughout the course of the experiment by a temperature probe. At the end of the experiment, temperature variations along the length of the nerve were carefully measured using the same probe. The 10 mm region where a temperature of 5-8^0 C was maintained is defined as the cold block region. In the nerve segments immediately proximal and distal to the cold block region, there was a temperature gradient ranging from 5-8^0 C (adjacent to the cold block) to 30^0 C (5 mm away from the cold block).

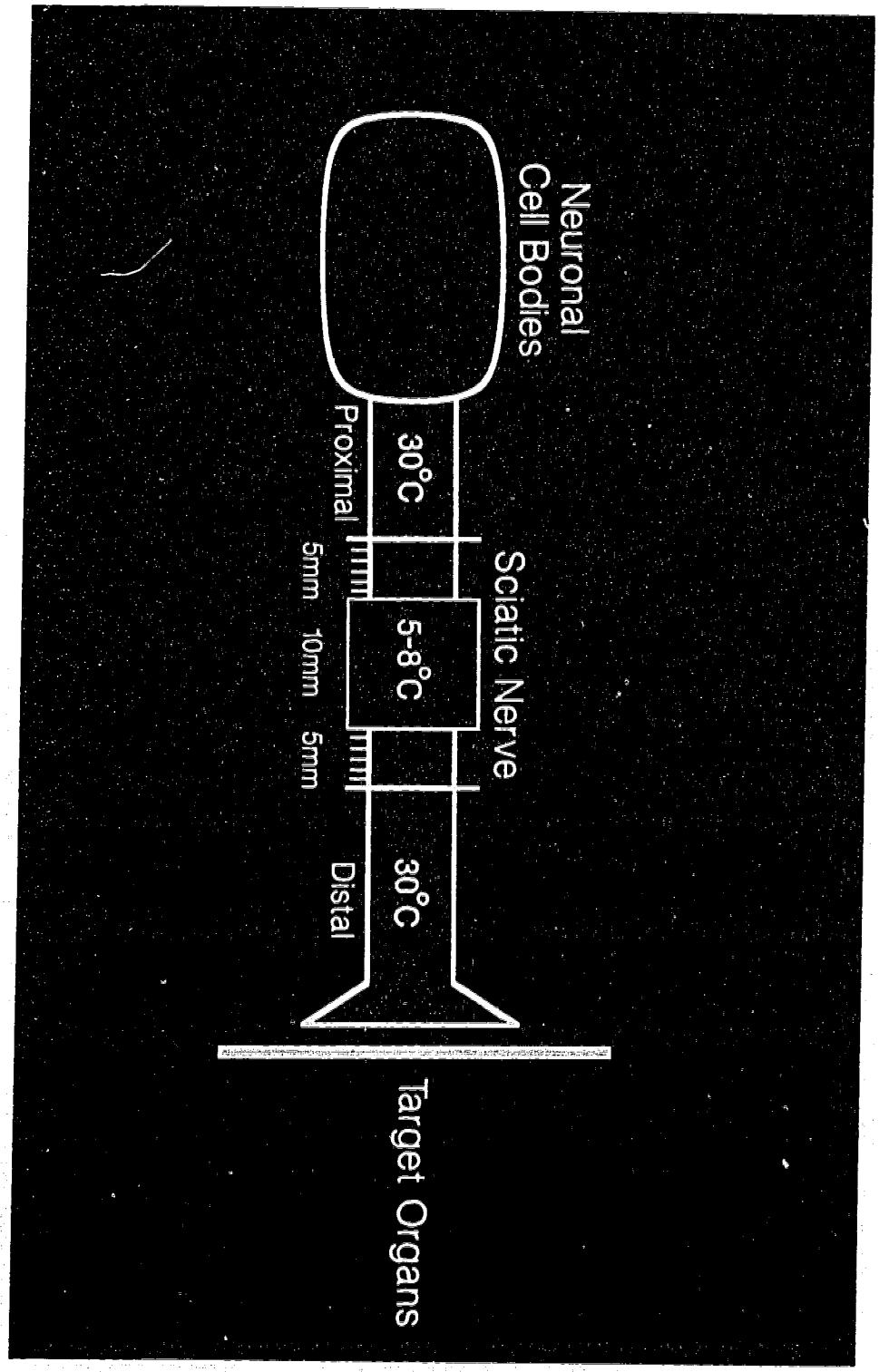
Neuronal
Cell Bodies
30°C
Proximal
5mm
5-8°C
10mm
5mm
30°C
Distal
Sciatic Nerve
Target Organs

Figure 2. Fluorogold accumulation in the cold-blocked nerve. A cold block was established unilaterally for 2 hours and Fluorogold was then injected bilaterally into the lower hindlimbs. The cold block was subsequently maintained for 70 more hours (for a total of 72 hours), and longitudinal sections of the control nerve (a), and of the sciatic nerve proximal (b) and distal (c) to the cold block were analyzed using fluorescence microscopy. a) A low level of Fluorogold was detected throughout the length of the control sciatic nerve. b) No Fluorogold was detected 12 mm proximal to the cold block region. c) Fluorogold was accumulated 12 mm distal to the cold block region, indicating the efficacy of the block to fast axonal transport. Scale bar = 100 μm.

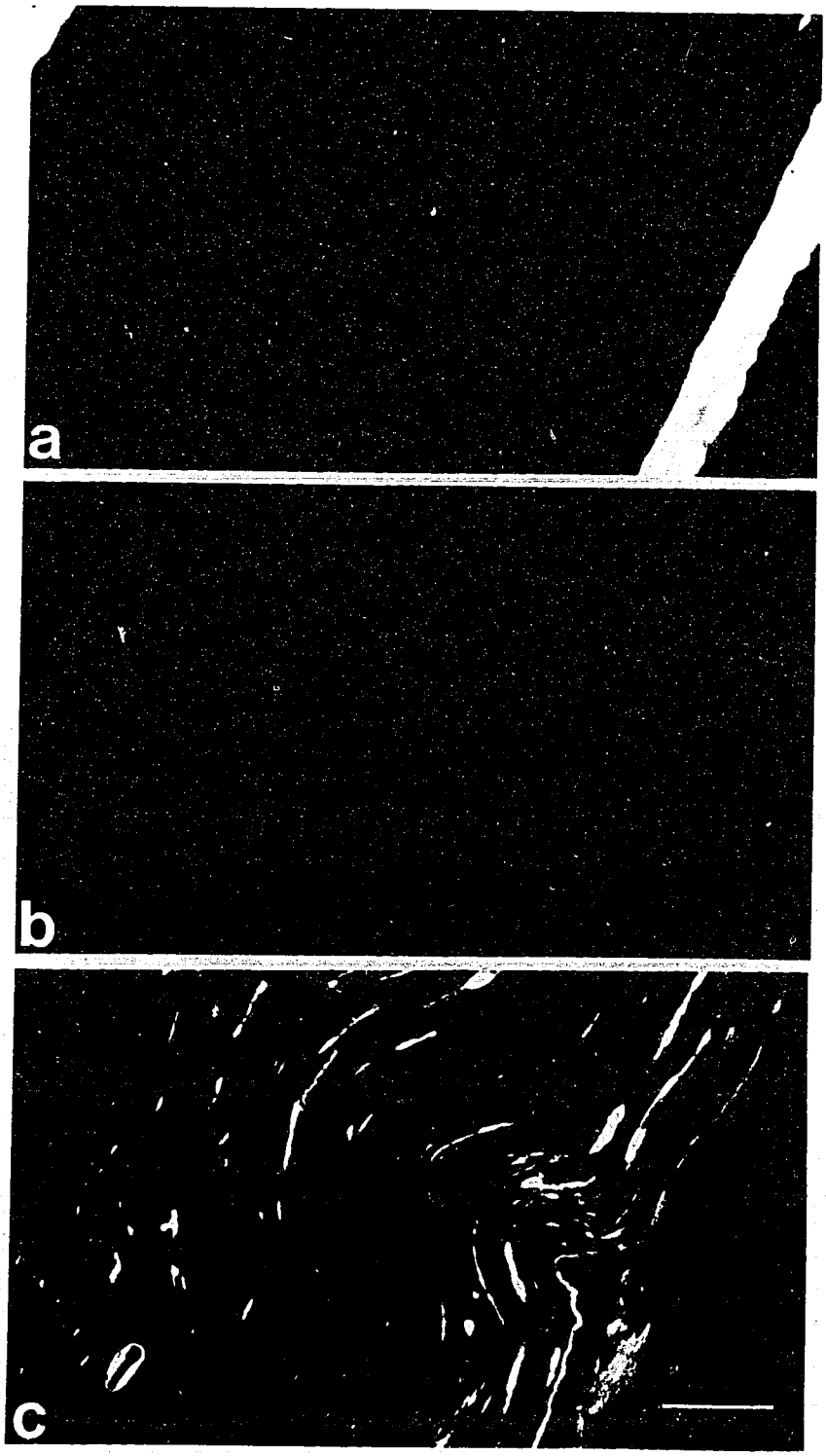
a
b
c

Figure 3. Immunostaining of macrophages in the control (a), transected (b-d), and cold-blocked (e-h) sciatic nerve, as detected using the mononuclear phagocyte-specific antibody ED1. Stained, longitudinal nerve sections were examined and photographed using fluorescence microscopy. a) A few ED1-positive cells were detected in the control sciatic nerve. Three days following nerve transection, a dramatic increase in the number of ED1-positive cells was detected throughout the distal nerve segment, with the numbers of positive cells being greater at the transection site (b) than at 10 mm (c) or 25 mm (d) distal. In contrast, the number of ED1-positive cells in the cold-blocked nerve at 10 mm proximal (e), 10 mm distal (f), or 25 mm distal (g) to the cold block region was indistinguishable from the control nerve (a). Scale bar = 50 μm.

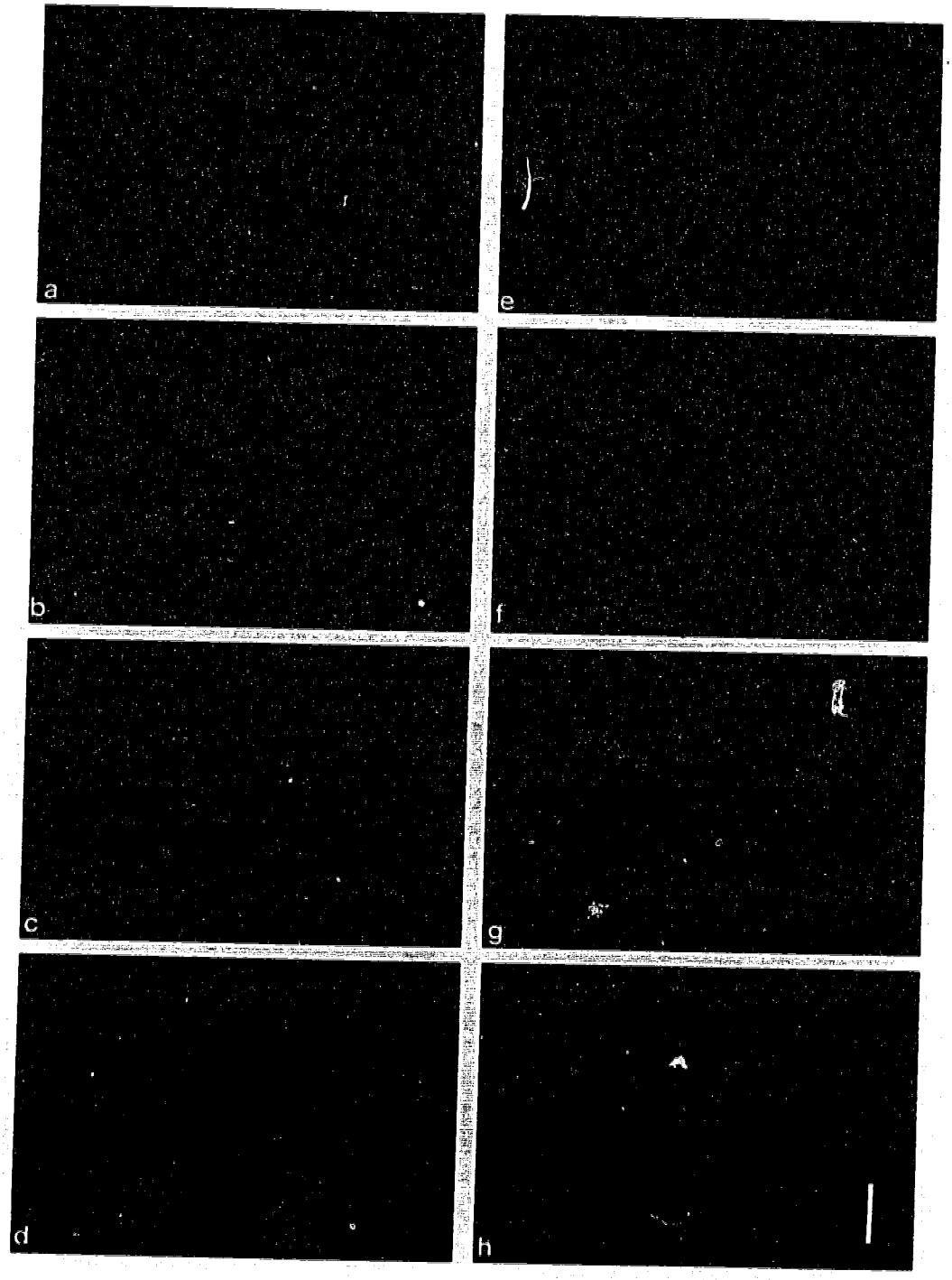
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Figure 4. Whole mounts of teased myelinated axons from 72 hours cold blocked and 72 hours axotomized sciatic nerve. a) Cold blocked nerve 10 mm proximal to the proximal edge of the cold block. Myelinated regions of axons and nodes of Ranvier (n) appear normal (indistinguishable from contralateral control fibers, not shown). b) Axotomized nerve 10 mm proximal to the axotomy site. Myelinated axons and nodes of Ranvier (n) appear normal. c) Axotomized axons 10 mm distal to the axotomy site. The myelin of all axons has broken into ovoids typical of Wallerian degeneration. d) Cold blocked nerve 10 mm distal to the distal edge of the cold block. Some fibers show a slightly crenulated myelin edge (arrows). Nodes of Ranvier appear normal (n). Scale bar = 50 μm.

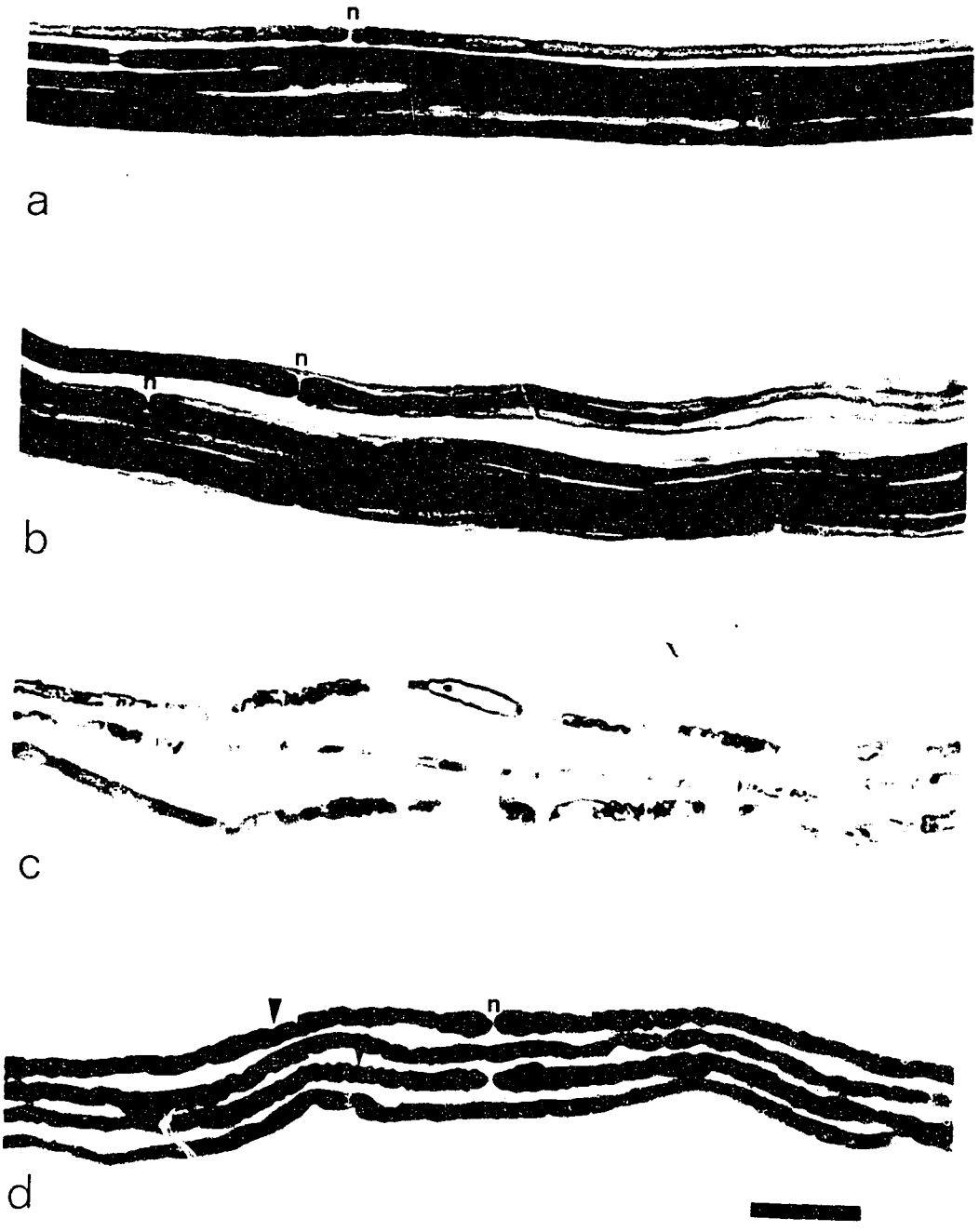
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Figure 5. Electron micrographs of cross sections of 72 hours cold blocked (a-e) and axotomized (f) sciatic nerve. a) Cold blocked nerve 15 mm proximal to the cold block. Myelinated axons and non-myelinated axons are present showing normal physical relationships to Schwann cells. b) Within the cold block the physical relationships of myelin and Schwann cells to myelinated axons is normal. The myelinated axon and the surrounding non-myelinated axons contain an elevated number of vesicles. c) 25 mm distal to the cold block myelin profiles, axon-Schwann cell physical relationships, and axoplasmic structure are normal. d) 5 mm proximal to the cold block a myelinated axon shows clusters of small vesicles (v), mitochondria (m) are also present in the axoplasm. e) 2.5 mm distal to the cold block a myelinated axon shows a very high content of membranous organelles, many of these are larger and morphologically distinct from those appearing proximal to the cold block. f) Axotomized nerve 10 mm distal to the site of section. Degenerating myelinated axons (dm) are present, the axon at the lower left appears swollen and shows a peripheral rim of axoplasmic debris. Two large non-neuronal cells (nn) are present, the lower one contains many dense bodies. At the right is a clump of cellular debris. Scale bars: c) 1 μm, applies also to a) and b); e) 1 μm, applies also to d); f), 0.5 μm

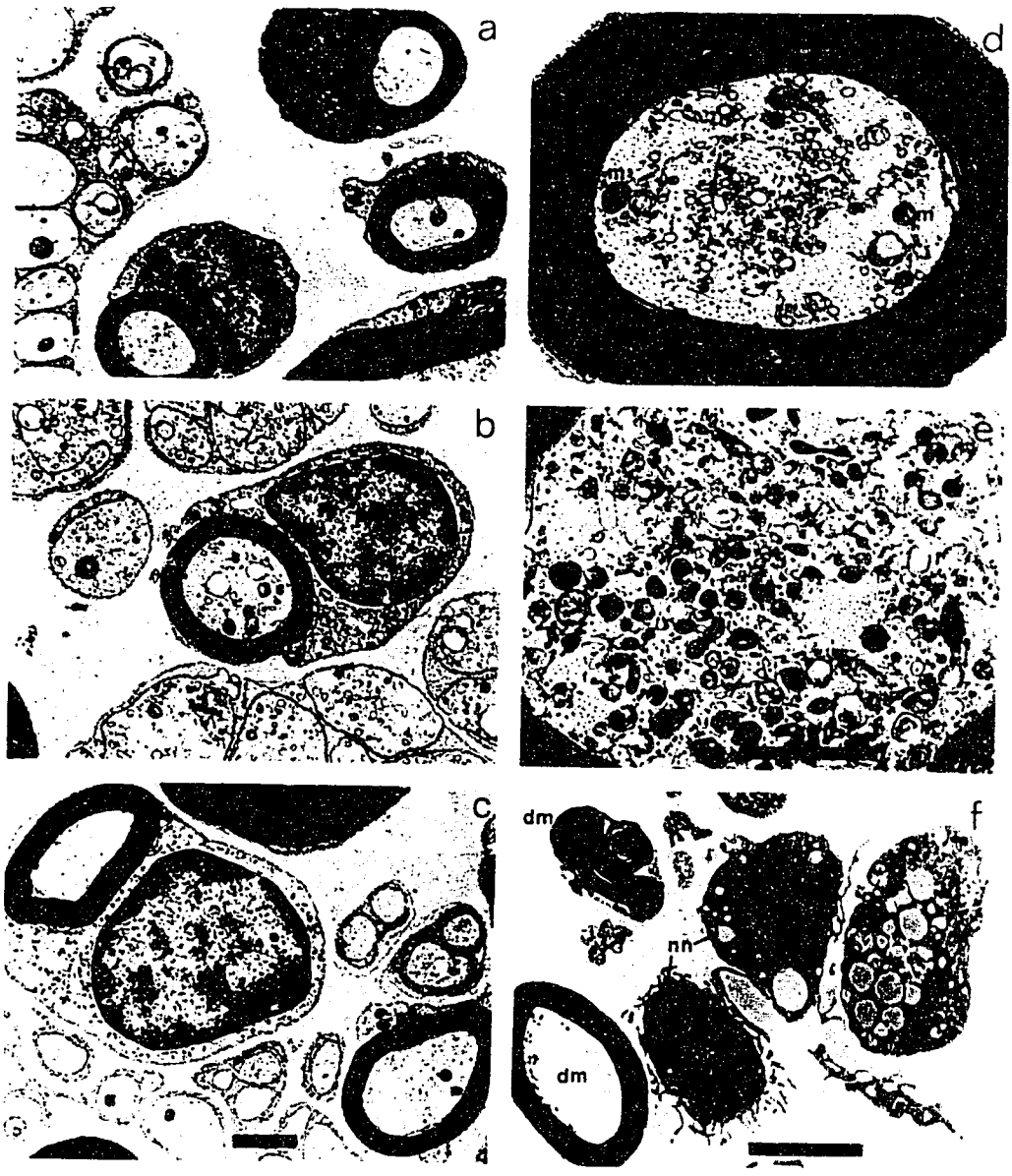
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Figure 6. Expression of Po (a-e) and p75 NGF receptor (f-i) mRNAs in the control (a,f), transected (b,g), and cold-blocked (c-e, h, i) sciatic nerve, as detected by in situ hybridization. Longitudinal sections of the sciatic nerve from animals that were cold-blocked for 3 days, or that had been transected 3 days earlier, were hybridized to probes specific for Po or p75 NGF receptor mRNAs, and apposed to X-ray film. In all cases, the portion of the nerve most proximal to the cell bodies is oriented to the top of the panel. (a-e) X-ray film pictures of nerve segments hybridized to a probe specific for Po mRNA. a) Control sciatic nerve. b) A 3 day transected nerve from the same animal and from the same slide as (a). Note that Po mRNA levels were dramatically decreased by axotomy. c) Proximal segment of a cold-blocked nerve, including a portion of the cold block region. Levels of Po mRNA were similar to those detected in the control nerve of the same animal (data not shown). Note that the nerve was cut in the middle of the cold block region to facilitate analysis, and the left-hand nerve section in this panel contains part of the cold block region (bottom of the section) which shows significantly lower hybridization. d) Distal segment of the same cold-blocked nerve as shown in (c), from the same in situ hybridization run. Note the differential distribution of Po mRNA throughout this distal segment. In the cold block region, at the top of the sections, Po mRNA levels were relatively low, and were comparable to those seen in the cold block region in (c). In the region immediately distal to the cold block region, Po mRNA levels were similar to those detected in the proximal segment of the same nerve in (c). However, further distal, the levels of Po mRNA decreased dramatically, with the magnitude of the decrease being similar to that observed in the axotomized nerve. (e) Distal segment of a second cold-blocked nerve, which also includes a portion of the cold block region at the top of the section. Note that the pattern of Po mRNA expression is indistinguishable from that seen in the nerve shown in (d). (f-i) X-ray film pictures of nerve sections hybridized to probes specific for p75 NGF receptor mRNA. All nerve sections were derived from the same in situ hybridization run, and were photographed under identical conditions. (f) Control sciatic nerve. (g) The distal segment of a transected nerve from the same animal as shown in (f). Note the large increase in p75 NGF receptor mRNA following axotomy. (h) The proximal segment of a cold-blocked nerve. Levels in the proximal segment were similar to those in controls (f). (i) Distal segment of the same cold-blocked nerve as in (h). Levels of p75 NGF receptor mRNA in the nerve

segment distal to the cold block were indistinguishable from those in the proximal segment (h) or in control nerve (f). Scale bar = 5 mm.

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Figure 7. Expression of p75 NGF receptor mRNA in the control (a), axotomized (b), and cold-blocked (c-f) sciatic nerve as detected by in situ hybridization and emulsion autoradiography. Longitudinal sections of the sciatic nerve either 3 days posttransection, or following 3 days of cold block treatment, were hybridized to probes specific for p75 NGF receptor mRNA, processed for emulsion autoradiography, and visualized using darkfield illumination. All panels illustrate nerve sections from the same in situ hybridization run that were developed and photographed under identical conditions. a) Control sciatic nerve. b) The distal segment of the axotomized nerve, 25 mm distal to the transection site. Note that p75 NGF receptor mRNA levels increased dramatically following axotomy, relative to the control nerve (a). In contrast, no alteration in p75 NGF receptor mRNA levels was detected anywhere in the cold-blocked nerve, as illustrated here, (c) 10 mm proximal to the cold block region, (e) 10 mm distal to the cold block, and (f) 25 mm distal to the cold block. p75 NGF receptor mRNA levels were, however, somewhat lower in the cold-block region itself (d). Scale bar = 100 μm.

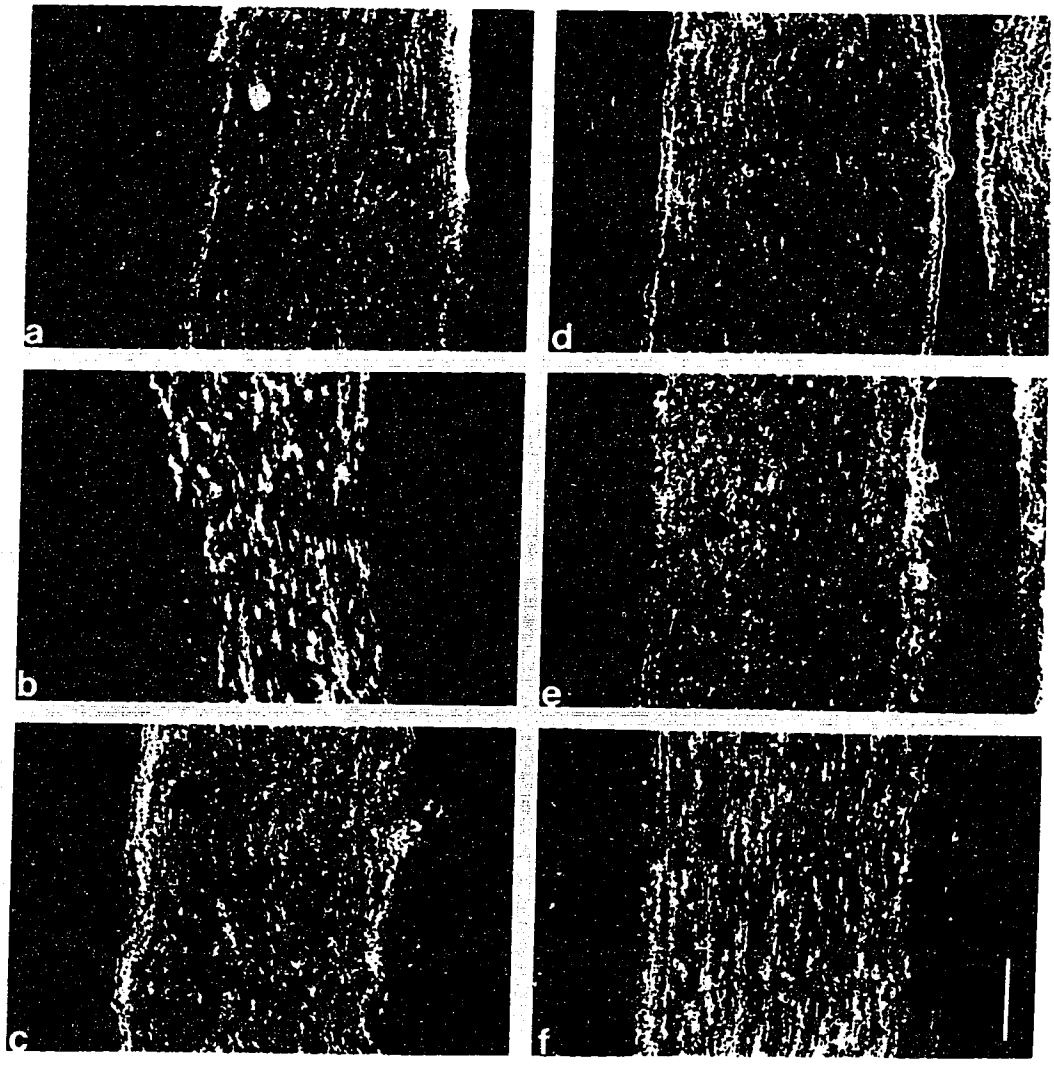
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Figure 8. Immunocytochemical detection of p75 NGF receptor in the control (a), axotomized (b), and cold-blocked (c-g) sciatic nerve. Longitudinal sections of nerve from control and cold-blocked nerves of the same animal were stained with the monoclonal antibody IgG 192, which is specific for p75 NGF receptor. Nerves from transected animals were used as positive controls. All panels are derived from nerve sections on which immunocytochemistry was performed at the same time, and which were photographed under identical conditions. Levels of p75 NGF receptor were low, but detectable in the control nerve (a), and were dramatically increased 25 mm distal to a transection site 3 days postaxotomy (b). The levels of p75 NGF receptor-like immunoreactivity within the cold-blocked nerve varied along the length of the nerve. 25 mm proximal (c) or 25 mm distal (g) to the cold block region, p75 NGF receptor levels were similar to control nerve (a). In contrast, 10 mm proximal (d) and 10 mm distal (f) to the cold block region, p75 NGF receptor levels were greatly increased. Within the cold block region (e), receptor levels we [illegible] to controls. Scale bar = 50 μm.

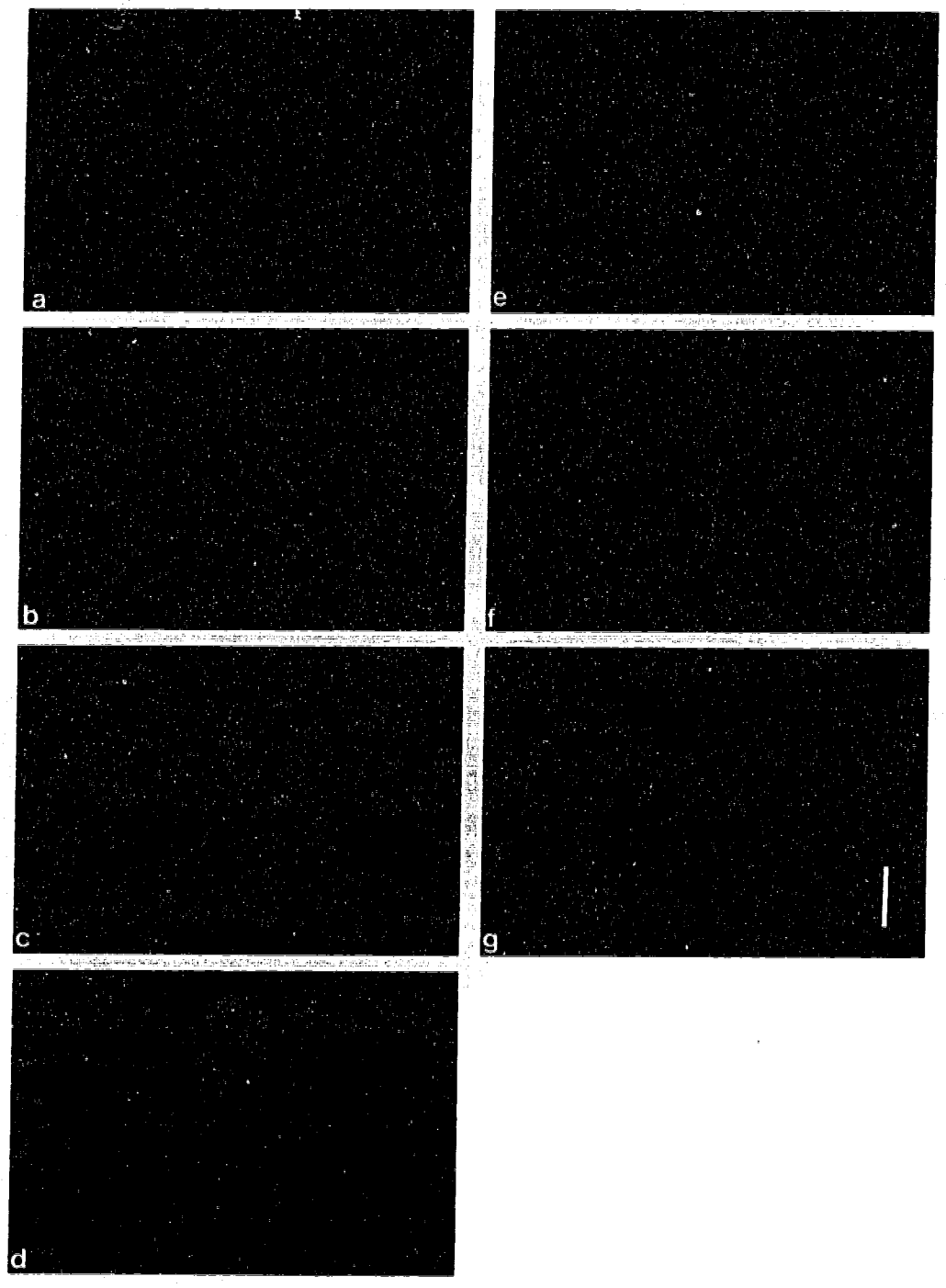
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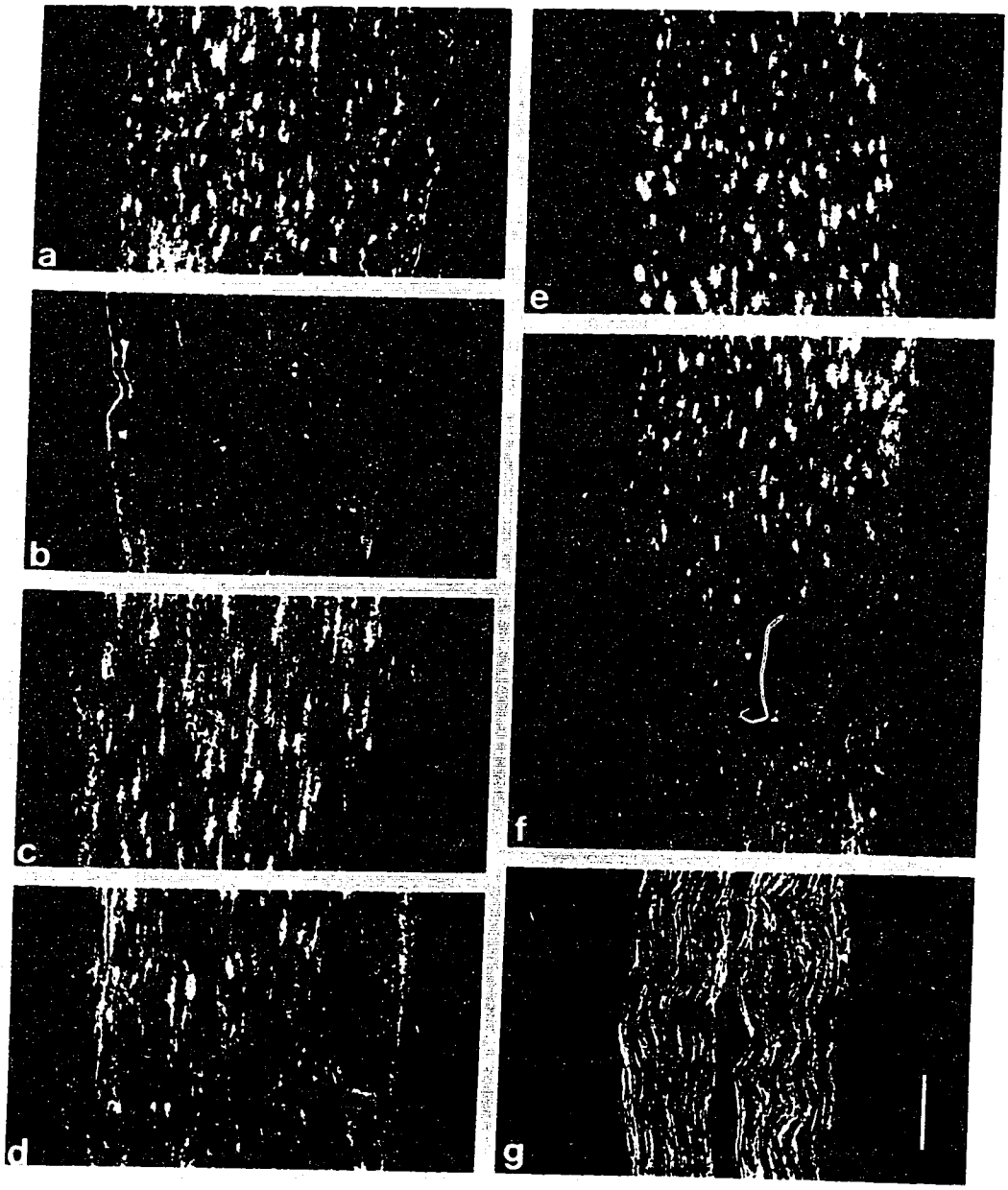
Figure 9. Expression of Po mRNA in the control (a), axotomized (b), and cold-blocked (c-g) sciatic nerve as detected by in situ hybridization and emulsion autoradiography. Longitudinal sections of the sciatic nerve adjacent to those used for analysis of p75 NGF receptor were hybridized to probes specific for Po mRNA, processed for emulsion autoradiography, and visualized using darkfield microscopy. All panels illustrate nerve sections from the same in situ hybridization run that were developed and photographed under identical conditions. a) Control sciatic nerve. b) The distal segment of the axotomized nerve, 25 mm distal to the transection site. Note that Po mRNA levels decreased dramatically following axotomy, relative to the control nerve (a). In contrast, the levels of Po mRNA varied along the length of the cold-blocked nerve. 10 mm proximal to the cold block (c), Po mRNA levels were similar to controls (a). Within the cold block region itself, Po mRNA levels were decreased, as illustrated in (d), which shows the transition from the proximal region (top of the panel) to the cold block region (bottom). 10 mm distal to the cold block region, Po mRNA levels were again similar to controls (e). However, at 15 mm distal to the cold block region (f), there was an abrupt transition from Schwann cells expressing control levels of Po mRNA (top of panel) to those expressing greatly decreased levels of this mRNA (bottom of panel). These low Po mRNA levels were observed throughout the remainder of the distal segment, as illustrated here 25 mm distal to the cold block region (g). Scale bar = 100 μm.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CHAPTER THREE: EDIVENCE THAT LOSS OF HOMEOSTATIC SIGNALS INDUCES REGENERATION-ASSOCIATED ALTERATIONS IN NEURONAL GENE EXPRESSION

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ACKNOWLEDGEMENT

We thank Ms. Audrey Speelman for excellent technical assistance, and Drs. Bob Campenot, Warren Gallin, Ann Acheson, and Richard Murphy for frequent discussions and advice. We particularly thank Dr. Dick Smith, for all of his help with image analysis and retrograde transport, Ms. Honey Chan for helping us with the morphological studies, and Dr. Bill Colmers for his help with the stimulation studies. This work was supported by grants from the Canadian M.R.C., and the N.C.E. for Neural Regeneration to F.M. F.M. is an A.H.F.M.R. Scholar, W.W. is supported by the Rick Hansen Foundation for Spinal Cord Research, and T.M. was supported by the Alberta Association for Paraplegic Research during the course of this work.

Note: This manuscript was published in Developmental Biology (1993) 158: 456-466.

INTRODUCTION

Most vertebrate peripheral neurons respond to axonal injury by undergoing a series of well-defined morphological changes. The neuronal soma progresses through a complicated series of alterations, including retraction of afferent synapses, establishment of new glial cell contacts, and reestablishment of appropriate afferent inputs (reviewed in Fawcett & Keyenes, 1990; Schwartz, 1987). In addition, axons regenerate and form functional synapses with their target organs. Microtubules, which are assembled from α- and β-tubulins, are integral components of growing neurons, both during development and during regeneration (Daniels, 1972). Of six different α-tubulin genes known to be expressed in mammals (Villasante et al., 1986), one termed Tα1 in rats, is specialized for high levels of expression during the growth of both developing (Miller et al., 1987a, 1991a) and mature (Miller et al., 1989; Mathew & Miller, 1990) neurons. In contrast, another rat α-tubulin mRNA that encodes a virtually identical protein, termed T26, is expressed constitutively in neurons, remaining unchanged throughout development and during regeneration (Miller et al., 1987a, 1989).

Tα1 α-tubulin mRNA increases following axotomy of motor (Miller et al., 1989), sympathetic (Mathew & Miller, 1990), and central rubrospinal neurons (Tetzlaff et al., 1991). Following crush axotomy of facial motor neurons, Tα1 α-tubulin mRNA rapidly increases within the first 12 hr and continue to increase to reach peak levels at approximately 3 to 7 days postlesion. At 10 to 20 days, the growing axons start to reinnervate the target musculature, and Tα1 α-tubulin mRNA starts to decrease to reach control levels sometimes between 21 and 49 days postaxotomy (Miller et al., 1989). This pattern of expression is highly reminiscent of that seen during development: Tα1 α-tubulin mRNA is high in neurons during the period of morphological development and subsequently decreases following target contact (Miller et al., 1987a; Mathew & Miller, 1990). In rubrospinal neurons, which do not successfully regenerate, Tα1 mRNA again increases following axotomy, but remains elevated as long as 7 weeks postaxotomy (Tetzlaff et al., 1991). A similar prolonged upregulation of Tα1 mRNA is seen following ligation of the sciatic nerve,

which inhibits appropriate regeneration (Miller et al., 1989). Thus, elevation of Tα1 α-tubulin mRNA appears to be a general characteristic of the cell body response to axonal injury, whereas down regulation of this mRNA, either during development or following injury, apparently requires appropriate axonal outgrowth and establishment of connections.

The p75 NGF receptor is another molecule that is expressed at elevated levels during the development of motoneurons (Erfors et al., 1989) and is subsequently reinduced following axonal injury (Ernfors et al., 1989; Wood et al., 1990; Koliatsos et al., 1991; Saika et al., 1991; Armstrong et al., 1991). The precise role of this receptor during the growth of motoneuron survival (Oppenheim et al., 1982; Yan et al., 1988). The p75 NGF receptor may, however, play a role in mediating motoneuron responses to other members of the neurotrophin family, to which it can also bind (Rodriguez-Tebar et al., 1990).

The cellular signals responsible for inducing these two mRNAs following axonal injury are unknown. A number of different signals have been hypothesized to account for the cell body response to axonal damage. These can be classified into "positive" signals, arising from the peripheral nerve injury response, and "negative" signals, derived from the loss of normal connections and interruption of homeostatic mechanisms. Candidates for positive signals include (a) novel factors produced at the site of nerve injury that may be triggered by the migration and activation of macrophages, and (b) systemic factors produced as a consequence of nerve injury. Candidates for negative signals include (a) the loss of neuron:target organ contact, as monitored by either target-derived growth factors or electrical activity, (b) the disruption of axon:Schwann cell contacts, and (c) abnormal reversal of vesicular traffic (Smith, 1980).

Evidence indicates that at least one negative signal, the loss of target-derived trophic factors, leads to a subset of axotomy-induced alterations in neuronal phenotype. Axotomy of sensory neurons leads to decreased high-affinity NGF receptors and neurofilament mRNA levels, and both of these decreases can be reversed by exogenous NGF in the NGF-responsive neuronal population (Verge et al., 1989, 1990). Furthermore, exogenous NGF can reverse some (Nja & Purve, 1978),

but not all (Hall & Wilson, 1982), of the effects of axotomy on sympathetic neurons of the superior cervical ganglia.

Exposure of the injured neuron to growth factors is one frequently considered positive axotomy signal. Non-neuronal cells of the nerve distal to an injury synthesize increased levels of NGF (Heumann et al., 1987), and injured peripheral axons may locally release FGF (Eckenstein et al., 1991), and injured Schwann cells ciliary neurotrophic factor (CNTF) (Manthrope et al., 1986; Stockli et al., 1989; Sendtner et al., 1990). Furthermore, injured axons endocytose large amounts of tissue debris at the site of a lesion (Kristensson & Olsson, 1976), providing a mechanism for transport of exogenous molecules.

In this paper, we attempted to distinguish whether the induction of Tα1 α-tubulin and p75 NGF receptor mRNAs in axotomized motor neurons is due to positive signals produced as a consequence of factors derived from nonneuronal cells following nerve injury or to negative signals arising from the interruption of normal homeostatic mechanisms. Our data support the latter of several facets of normal axonal signaling was sufficient to induce both Tα1 α-tubulin and p75 NGF receptor mRNAs in intact facial motoneurons. Thus, some axotomy-induced increases in neuronal gene expression are likely due to the loss of ongoing homeostatic signals as monitored by fast axonal transport.

MATERIALS AND METHODS

Animals and surgical procedures:

(a) Regeneration studies. Female Sprague-Dawley rats (200-300 g) were anesthetized with sodium pentobarbital (35 mg/kg), and 20 µl of a 4% solution of fluorogold (Fluorochrome Inc., Englewood, CO) was injected into the parotid region (see Fig. 1). Four to 5 days later, animals were anesthetized with urethane (120-250 mg/kg) subcutaneously or sodium pentobarbital (35 mg/kg) intraperitoneally, followed by one of the following surgical procedures: (i) the buccal and marginal mandibular branches of the facial nerve were dissected and unilaterally transected 0.5-0.7 cm from the stylomastoid foramen for 36 hr (three animals) or for 60 hr (two animals), or (ii) the main branch of the facial nerve was dissected and unilaterally transected as it exited the stylomastoid foramen for 36 hr (three animals). In all animals, the contralateral, control nerve was sham-operated at the same time. Thirty-six or 60 hr following transection, rats were anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde. The brainstems were processed for in situ hybridization.

(b) Cold block studies. (see Fig 1. for a schematic representation of this procedure.) Female Sprague-Dawley rats (200-300 g) whose facial motor neurons were retrogradely labeled with fluorogold, as detailed above, were used for all experiments, except where specified. Animals were anesthetized with urethane (120-250 mg/kg) subcutaneously, followed by one of the following surgical procedures: (i) the buccal and marginal mandibular branches of the facial nerve were dissected and unilaterally locally cooled to 4-8 °C 0.5-0.7 cm from the stylomastoid foramen for 36 hr (two animals) or for 60 hr (two animals), or (ii) the main branch of the facial nerve was dissected and unilaterally locally cooled to 4-8 °C as it exited the stylomastoid foramen for 36 hr (two animals) or 60 hr (one animal (see Fig.1) Within 1-3 mm of the cuff, the temperature was 37 °C. In all cases, the control nerve was similarly dissected, but not cooled. As an additional control, in tow animals, the cold block apparatus was put into place on the nerve, but coolant was not run through. To maintain anesthesia during the course of the experiment, 0.1-0.2 ml of urethane (1.25 g urethane in 5 ml of 0.9% saline) was injected every 9-12 hr. One to 3 ml of lactated

Ringers solution was also injected intraperitoneally every 4-6 hr. Following the cold block, rats were anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde. The brain stems were processed for in situ hybridization.

To test the effectiveness of the cold block, female Sprague-Dawley rats that had not previously been labeled with fluorogold were anesthetized with urethane (120-250 mg/kg) subcutaneously. The main branch of the facial nerve was unilaterally dissected and locally cooled. Two hours after initiation of the cold block, 20 µl of a 4% solution of fluorogold was injected subcutaneously in the vibrissal region. Thirty-six hours after initiation of the cold block, animals were transcardially perfused with 4% paraformaldehyde, and the brainstems processed and sectioned to check for retrograde labeling of facial motor neurons. To test the reversibility of the cold block, either the main branch of the facial nerve (one animal) or the marginal-mandibular and buccal branches of the nerve (two animals) were unilaterally dissected and locally cooled for 36 hr in unlabeled female Sprague-Dawley rats. Within 60 min of removal of the cold block, 20 µl of a 4% solution of fluorogold was injected subcutaneously into the appropriate vibrissal regions both ipsilateral and contralateral to the cold block. Animals were sacrificed 48 hr (two animals with a cold block of the marginal-mandibular and buccal branches) or 5 days (one animal with a cold block of the main branch) following injection of the fluorogold.

Simulation experiments. To determine whether the cold block effectively inhibited transmission of action potentials to the target muscle, the facial nerve was stimulated at 25 Hz, 1msec, 100 µA with a bipolar silver wire electrode. The nerve was stimulated (a) prior to establishment of the cold block, (b) proximal and distal to the cold block 10 min after its establishment, (c) proximal and distal to the treatment site 10 min following release of the cold block, and (d) proximal and distal to the treatment site 48 hr following release of the cold block. In all cases, contraction of the facial musculature was used as a functional assay.

Morphological analysis. The buccal and marginal-mandibular branches of the facial nerve were locally cooled for 36 hr, as described, and animals were transcardially perfused with either 4% paraformaldehyde or 4% glutaraldehyde. The appropriate branches of the nerve were then postfixed in 1% sodium tetroxide in PBS, dehydrated in graded concentrations of ethanol, and embedded in TAAB 812 resin. One-

micrometer epoxy sections were subsequently stained with a mixture of toluidine blue and azur II and examined via light microscopy. Two experimental animals were analyzed morphologically.

In situ hybridization. Brainstems from retrogradely labeled, perfused animals were cryoprotected by immersion in graded sucrose solutions and sectioned onto chrom-alum subbed slides. Antisense Tα1 α-tubulin (Miller et al., 1987a) and p75 NGF receptor (Radeke et al., 1987) probes were prepared as previously described (Miller et al., 1991 a) with [^{35}S]CTP (New England Nuclear, Boston, MA; 800 Ci/mmole) rather than [^{32}P]CTP. A rat T26 α-tubulin 35S riboprobe was synthesized from a pGEM4Z (Promega Biotec) subclone containing a 3' untranslated region insert that was PCRed out of PC12 cell. (Ma et al., 1992). In situ hybridization was performed with all of these clones as previously described (Miller et al., 1989). After hybridization, slides were airdried and apposed to Kodak XRP film for 12-24 hr to obtain X-ray images. The slides were subsequently dipped in Kodak NTB-2 emulsion and exposed for 1-3 days prior to development. Slides were mounted in glycerol and examined with a Zeiss fluorescence microscope using wide band ultraviolet excitation (emission maximum: 408 nm, excitation maximum: 323 nm) and/or dark-field illumination. Controls were done to ensure specificity of hybridization, including hybridization with sense probes.

Analysis and quantification. The quantification of in situ hybridization analysis was carried out using a computer-based image analysis system (LECO 2001, Leco Instruments, Ltd., Quebec, Canada) that quantitated the number of silver grains overlying retrogradely labeled neurons. Only those slides optimally exposed (10-100 grains/cell) were chosen for analysis, and only those neurons sectioned through the nucleus were chosen for grain counting. The periphery and area of each individual cell were defined using fluorescence illumination of the retrograde label, and the numbers of grains overlying that cell were counted using dark-field or combined dark-field/fluorescence illumination. The background level of grains was minimal and was not subtracted from the grain counts. The area of the retrogradely labeled facial motor neurons was variable, so the results are expressed in terms of grain density.

For the cold block experiments, data from different in situ hybridization runs were not pooled to obtain mean grain densities. Instead, results obtained from each run were

pooled and Student's t test was used to compare control and experimental neurons within a given in situ hybridization experiment. To compare results obtained from the same animal during different in situ hybridization runs, and to compare results obtained from different animals, we expressed the data in terms of relative increases. Relative increase was defined as [grain density (experimental)-grain density (control)]/grain density (control). For each treatment, we subsequently pooled the data by using the relative increase to define a mean and standard deviation. Student's t test was then used to compare the results.

RESULTS

To determine whether the induction of Tα1 α-tubulin and p75 NGF receptor mRNAs in axotomized motoneurons was due to a negative signal arising from the interruption of normal homeostatic mechanisms, we developed an experimental paradigm that allowed us to block some facets of normal axonal signaling without activating a peripheral nerve injury response (see Fig. 1). The paradigm is based upon the observation that locally cooling the nerve to 5-8 °C will specifically and reversibly block fast axonal transport, as well as transmission of electrical activity distal to the cold block (Ochs & Smith, 1975; Oaklander & Spencer, 1988).

To perform these experiments, we utilized the facial motor nerve, which is easily accessible. The experimental motoneurons were retrogradely labeled by injecting fluorogold bilaterally into the vibrissal region, which is innervated by the buccal and marginal mandibular branches of the facial nerve (Fig. 1). Three to 5 days later, these branches of the nerve were locally cooled (a cold block) or transected unilaterally. In all experiments, the contralateral, control nerve was sham-operated and, in some animals, a cuff was also placed on the contralateral nerve. The level of expression of Tα1 α-tubulin and p75 NGF receptor mRNAs in the labeled motoneurons was subsequently determined by in situ hybridization.

To ensure the effectiveness of the block to fast axonal transport, the nerve was cooled unilaterally, and fluorogold was injected into the musculature bilaterally 2-3 hr after initiation of the cold block. Thirty-three hours later, during which time the cold block was maintained, facial motor neurons on the control side were well-labeled, while those on the cold block were not (Figs 2C and 2D). To insure the reversibility of the cold block, the facial nerve of unlabeled rats was unilaterally cooled for 36 hr, the cold block was removed, and fluorogold was injected into the musculature. Two days following injection, both control and experimental neurons were labeled (Figs. 2A and 2B), with no obvious decrease in number of labeled cells on the cold-blocked side, thereby demonstrating the physical continuity of the majority of the axons following a cold block. Furthermore, vibrissal movement returned on the cold-blocked side.

To ensure the effectiveness of the block to distal transmission of electrical activity, the nerve was cooled unilaterally and then stimulated at 25 Hz at 1-msec intervals with a bipolar silver wire electrode. Stimulation of the nerve downstream of the cold block resulted in unilateral contraction of the facial musculature. Stimulation of the nerve upstream of the cold block had no effect. Similar results were observed throughout the duration of the cold block experiments. The block to distal transmission was reversed following removal of the cold block; stimulation of the nerve proximal to the cold block site led to contraction of the facial musculature, thereby indicating that the axons remained functionally continuous.

One of the "triggers" for the peripheral nerve injury response is macrophage infiltration (Perry et al., 1987; Xin et al., 1990; Brown et al., 1991). To ensure that the cold block protocol did not lead to macrophage infiltration, semithin epoxy sections of two facial nerves that were cold-blocked for 36 hr were prepared and stained with toluidine blue and azur II (Fig.3). No evidence either of damage to the nerve sheath or of macrophage infiltration was observed in any portion of the cold-blocked nerves. Moreover, in similar experiments with the sciatic nerves, macrophages had not infiltrated following a 72-hr cold block, as indicated by immunostaining with anti-ED1, a marker for mononuclear phagocytes (Wu et al., in press).

The morphological analysis also demonstrated that, proximal and distal to the cold block, axon and myelin profiles were histologically similar to controls (Fig.3), although there was some increase in organelle number and axoplasmic density in axons of the region immediately adjacent to the cooled region, presumably due to inhibition of fast axonal transport. Within the cold block region itself, although many profiles were normal, abnormal profiles were also observed, including some with thinning and disordering of the myelin sheath, as previously reported (Oaklander & Spencer, 1988). However, in related studies, we have observed a generalized decrease in gene expression in the cold-blocked region, making it unlikely that any positive injury factors are produced by the subset of nonneuronal cells in this region (Wu et al., 1994). Thus, the morphological data show no indication of nerve injury, or macrophage infiltration, although some axon:Schwann cell interactions were altered within the cold block region itself.

To determine whether a cold block alone was sufficient to induce Tα1 α-tubulin mRNA, we performed in situ hybridization and image analysis on retrogradely labeled facial motoneurons (Figs. 4, 5). Thirty-six hours following a unilateral cold block of the buccal and marginal mandibular branches of the facial nerve, the grain density for Tα1 α-tubulin mRNA was increased 36±5% relative to contralateral, sham-operated control neurons of the same section (Fig. 5A). Unilateral transection of these two branches of the facial nerve produced a statistically similar increase of 38±4% (Fig. 5B). Sixty hours following either a cold block or transection, the relative increases were significantly higher, being 54±4% and 58±7%, respectively (Figs. 5C and 5D). In a number of animals, we transected or cold-blocked the main branch of the facial nerve rather than the buccal and marginal-mandibular branches. Sixty hours following either transection or a cold block (Fig. 4 and 5E) of the main branch, grain densities for Tα1 α-tubulin mRNA were increased relative to that for sham-operated control neurons.

To determine whether the increase in Tα1 α-tubulin mRNA following a cold block reflected a more widespread cell response, we analyzed the expression of p75 NGF receptor mRNA. In situ hybridization indicated that, in control facial motoneurons, p75 NGF receptor mRNA was expressed at levels that were not significantly higher than background. Sixty hours following a unilateral transection of the marginal-mandibular and buccal branches of the facial nerve, the grain density for p75 NGF receptor mRNA was increased 69±15% in the axotomized versus control facial neurons (Fig. 6B). Sixty hours following a cold block of these two facial nerve branches, the grain density for p75 NGF receptor mRNA was increased 53±5%, a result that was statistically similar to that obtained following axotomy (Fig. 6A). Thus, Tα1 α-tubulin and p75 NGF receptor mRNAs were induced following either a cold block or axotomy.

To demonstrate the specificity of the response, adjacent sections from the same animals were hybridized to a probe specific for T26 α-tubulin mRNA, which does not increase during regeneration of motor neurons (Milleret al., 1989). Grain counting demonstrated that T26 mRNA was not increased following either a transection or a cold block (Figs. 4, 6C).

DISCUSSION

The cell body axotomy response has been hypothesized to result from "positive" nonneuronal cell-derived factors produced as part of the peripheral nerve injury response and/or from "negative" signals derived from the loss of normal connections and interruption of homeostatic mechanisms. To clarify the role of these two categories of signals, we utilized a cold block to ask whether interruption of the fast axonal transport and/or transmission of electrical activity to the target was sufficient to induce Tα1 α-tubulin and p75 NGF receptor mRNAs in facial motor neurons. Our studies indicate that a cold block increased expression of these two mRNAs to the same level as a transection. These results therefore indicate that a cold block increased expression of these two mRNAs to the same level as a transection. These results therefore rule out signaling models that depend directly upon activation of a peripheral nerve injury response and suggest that the neuronal axotomy response, as monitored by Tα1 and p75 NGF receptor mRNAs, is due to the loss of normal cell:cell contacts and/or ongoing homeostatic signals.

Previous studies using colchicine, which depolymerizes the labile population of axonal microtubules, to block retrograde transport have produced contradictory results (reviewed in Bisby, 1984). Axonal colchicine application alone caused a cell body reaction in parasympathetic and sympathetic neurons (Pilar & Landmesser, 1972; Purves, 1975), in agreement with the observations made in this study. However, the onset of the cell body reaction to transection in hypoglossal motoneurons was delayed if colchicine was applied proximal to the injury site (Singer et al., 1982).

Local cooling of the axon, which blocks fast axonal transport via a number of different cellular mechanisms, including a metabolic block to the microtubule motors (Ochs & Smith, 1975), circumvents the problems associated with use of a toxic compound like colchicine. Although we cannot formally rule out the possibility that a local cold block induces some facets of the peripheral nerve injury response, our reversibility data indicate that the majority of axons are not themselves physically or functionally interrupted, and our morphological data show no indication of nerve injury or macrophage infiltration. In similar studies using the antibody ED1 as a

marker for mononuclear phagocytes (Dijkstra et al., 1985), macrophages do not invade the sciatic nerve at the site of a cold block as they do at the site of a transection (Wu et al., 1994). Furthermore, in the same study, p75 NGF receptor and Po mRNAs, two markers of the Schwann cell response to nerve injury (Heumann et al., 1987; Taniuchi et al., 1986; Gupta et al., 1988; Trapp et al., 1988; Toma et al., 1992), were not altered immediately proximal or distal to a cold block, although expression of both mRNAs was downregulated within the cold block region itself, presumably as a consequence of a local temperature-induced slowdown in Schwann cell metabolism (Wu et al., 1994). Thus the most likely interpretation of the cold block data is that loss of communication with either the target muscle or the Schwann cells of the nerve was responsible for the observed upregulation of Tα1 α-tubulin and p75 NGF receptor mRNAs. However, it should be noted that our data do not completely rule out the possibility that minor disruption of the axonal or Schwann cell membranes could lead to leakage of acidic FGF (Eckenstein et al., 1991) or CNTF (Manthorpe et al., 1986; Stockli et al., 1989; Sendtner et al., 1990), respectively, and that these factors could contribute to the observed responses.

The cold block imply that expression of the Tα1 α-tubulin and p75 NGF receptor genes is normally repressed in mature motoneurons by ongoing homeostatic signals. Since expression of both of these mRNAs is high in developing motoneurons until the time of target contact (Miller et al., 1987a; Ernfors et al., 1989), it is likely that either target-derived factors or axon:target muscle contact is responsible for this repression developmentally. In the mature nervous system, there is probably an additional level of redundancy, with some contribution from axon:Schwann cell contacts.

The results obtained here suggest a number of potentially redundant explanations for the observed increase in Tα1 α-tubulin mRNA following axotomy. First, Tα1 mRNA might be increasing due to the loss of repressive homeostatic signals that arise from the target organ alone was insufficient to induce Tα1 mRNA in mature neurons (Mathew & Miller, 1993). Second, Tα1 mRNA might be increasing due to loss of homeostatic signals that arise as a function of normal axon:Schwann cell communication, mediated either via cell:cell contact or via trophic factors. For example, Schwann cells of the intact nerve synthesize CNTF (Stockli et al., 1989; Sendtner et al., 1990), which supports the survival of motor neurons (Sendtner et al.,

1990; Arakaw et al., 1990). Following axotomy, CNTF decreases in Schwann cells of the distal nerve segment, consistent with a potential role as a homeostatic factor (Friedman et al., 1992). Finally, the neuron itself may be intrinsically capable of monitoring the status of its axon. For example, anterogradely traveling vesicles will turn around at the site of a lesion (Smith, 1980) or a cold block (Tsukita & Ishikawa, 1980) and will, presumably, travel back to the cell body. Such abnormal vesicular traffic could well provide the cell body with the information necessary to induce a regenerative response.

The results presented here also support the hypothesis that neuronal regeneration and neuronal sprouting, both of which involve upregulation of Tα1 α-tubulin mRNA, are fundamentally different phenomena. We have previously hypothesized that the induction of Tα1 mRNA during the collateral sprouting of sympathetic neurons is due to increased available target-derived NGF (Mathew & Miller, 1990) and have obtained evidence supporting this hypothesis both in culture (Ma et al., 1992) and in vivo (Miller et al., 1991b). In contrast, our recent studies (data presented here; Mathew & Miller, 1993) suggest that the induction of Tα1 mRNA following axonal injury is due to the loss of ongoing homeostatic signals for these two types of neuronal growth is strongly supported by work from Diamond and colleagues, demonstrating that sprouting, but not regeneration, of sensory and sympathetic neurons is dependent upon NGF (Diamond et al., 1987, 1992a,b; Gloster & Diamond, 1992).

Although the results presented in this paper suggest that the initiation of a regenerative response is due to the loss of normal cell:cell contacts and/or disruption of ongoing homeostatic mechanisms, they do not negate the importance of changes that occur in the nerve itself following axotomy. Instead, induction and maintenance may be two different phases of a regenerative response. Thus, our data indicate that a negative signal triggers an initial response in axotomized neurons, but subsequent maintenance of this response and successful regeneration likely require establishment of appropriate trophic support and a permissive substrate by the environment surrounding the regenerating axon.

FIGURES AND FIGURE LEGENDS

Figure 1. Schematic representations of the experiments performed on the facial nerve. The facial nerve exits the cranium via the stylomastoid foramen and bifurcates into a number of smaller branches. Two of these branches, the buccal and marginal mandibular, innervate the vibrissal ipsilaterally. Facial motor neurons were retrogradely labeled by injecting fluorogold subcutaneously near the terminals of these two branches (the bottom two in the figure). The facial nerve was locally "cold blocked" either at the main branch, as shown, or at the point where the buccal and marginal-mandibular branches separate. Cold antifreeze was continuously run through the nerve cuff, and the temperature of the nerve itself was monitored at all times.

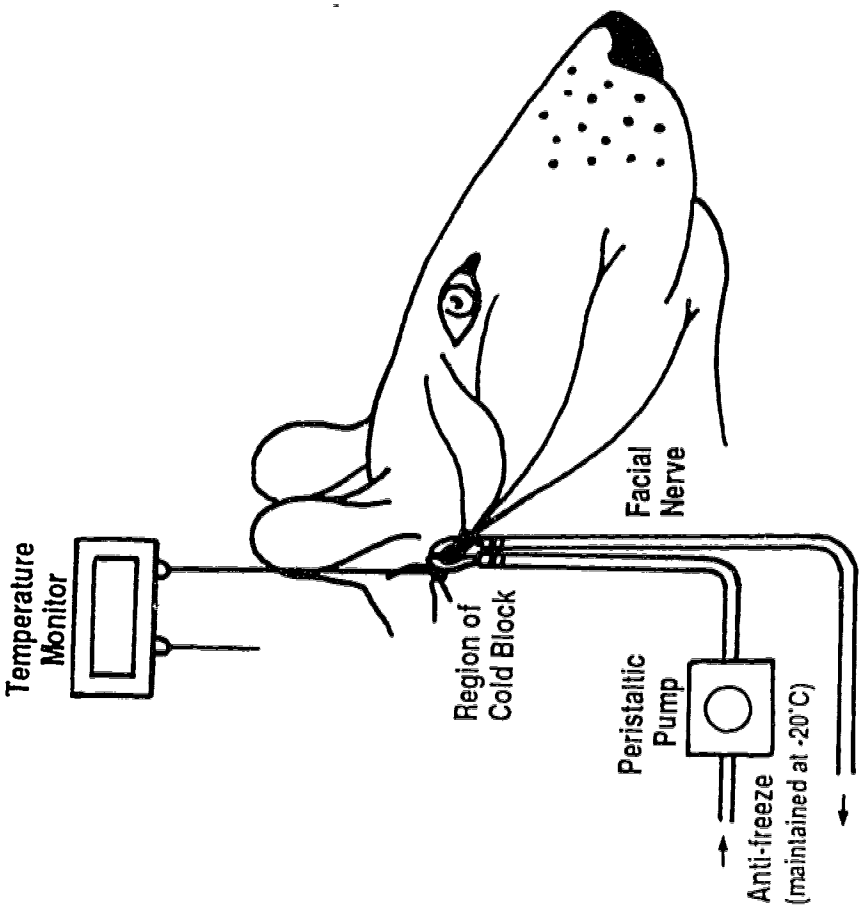

Temperature Monitor
Region of Cold Block
Facial Nerve
Peristaltic Pump
Anti-freeze
(maintained at -20°C)

Figure 2. Retrograde labeling of facial motor neurons with fluorogold to demonstrate the reversibility and efficacy of the cold block procedure. (A,B) Fluorogold was injected into the ipsilateral (B) and contralateral (A) vibrissal regions following cessation of a unilateral, 36-hr cold block to the marginal-mandibular and buccal branches of the facial nerve. Two days later the experimental side (B) was well-labeled, demonstrating the reversibility of the block to axonal transport. (C,D) Fluorogold was injected into the ipsilateral, experimental (D) and contralateral, control (C) vibrissal regions 3 hr after the onset of a unilateral cold block to the main branch of the facial nerve. Thirty-three hours later, during which time the unilateral cold block remained in place, neurons on the experimental side (D) were not labeled with fluorogold, while neurons on the control side were (C), demonstrating the efficacy of the cold block. Bar, 100 μm.

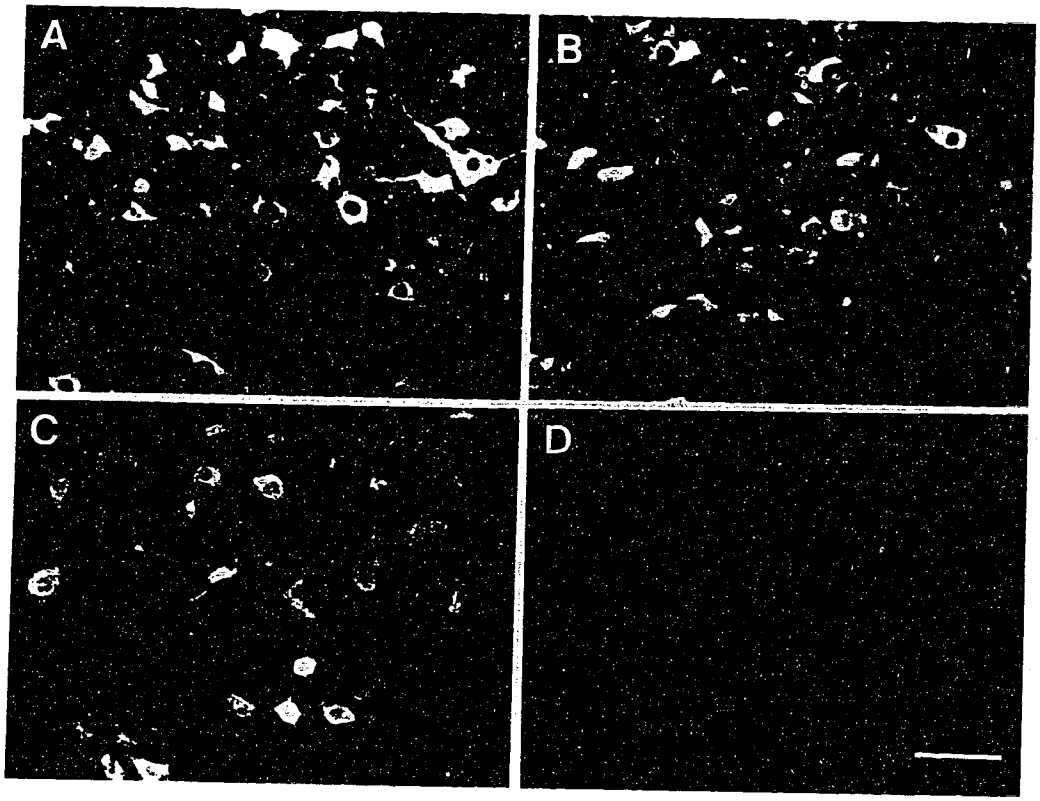

A
B
C
D

Figure 3. Morphological analysis of control (A) and cold-blocked (B,C) facial nerve. Semithin epoxy sections of a facial nerve were stained with toluidine blue and azur II and analyzed by bright-field microscopy. Axonal profiles are similar to the control (A) in segments of the nerve both proximal (B) and distal (C) to the site of a 36-hr cold block. Bar, 10μm.

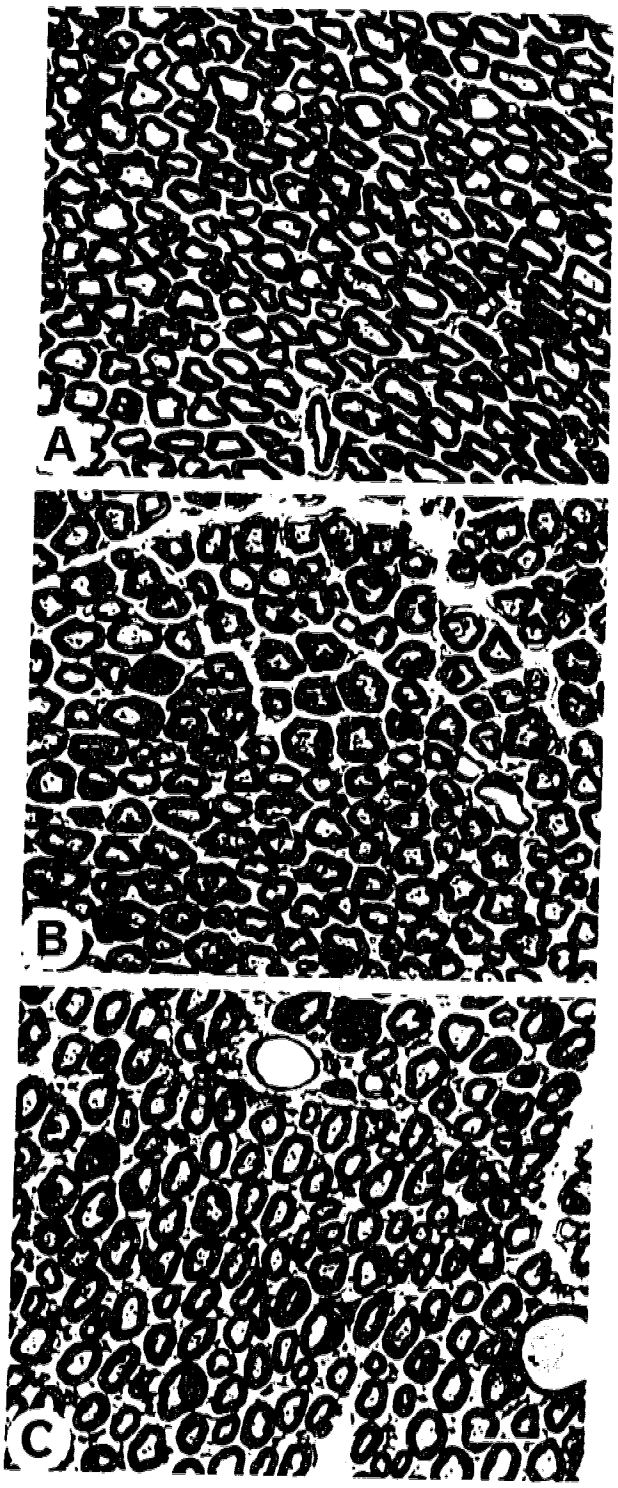

A
B
C

Figure 4. Expression of Tα1 and T26 α-tubulin mRNAs in facial motor neurons following a unilateral transection (AXO) or cold block (CB) of the buccal and marginal-mandibular branches of the facial nerve. Representative autoradiographs are shown of coronal sections through the facial nuclei of operated rats hybridized to ^{35}S-labeled probes specific to the 3'-untranslated region of Tα1 or T26 α-tubulin mRNAs 60 hr following transection or cold block. In all photographed sections, the cell bodies of neurons on the operated side are situated to the right of the midline.

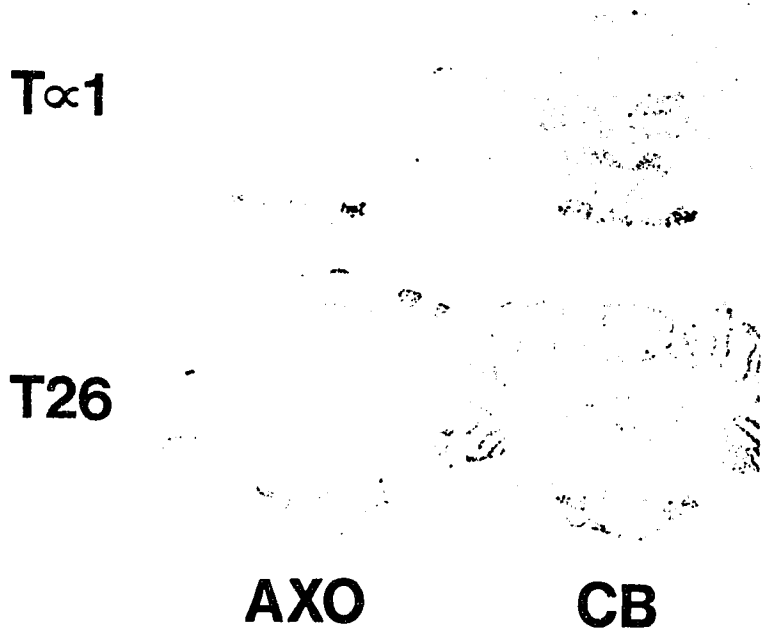

Tα1
T26
AXO
CB

Figure 5. Expression of Tα1 α-tubulin mRNA in cold-blocked or transected facial motor neurons 36 and 60 hr postsurgery, as detected by in situ hybridization. (A) Mean grain densities for Tα1 α-tubulin mRNA in experimental versus sham-operated neurons of two different animals 36 hr following a unilateral cold block of the marginal mandibular and buccal branches of the facial nerve. Neurons that project to the vibrissal region were labeled with fluorogold bilaterally, and the facial nerve was unilaterally cold-blocked for 36 hr. Grain counts of fluorogold-labeled, cold-blocked neurons were compared to those of control, fluorogold-labeled neurons from the contralateral facial nucleus. Each pair of bars represents results obtained from all of the labeled neurons from one in situ hybridization run. Each group of paired bars represents results obtained for the same animal. The error bars represent the standard error of the mean. (B) Axotomized versus sham-operated facial motor neurons 36 hr following unilateral transection of the marginal mandibular and buccal branches of the facial nerve. (C) Experimental versus sham-operated facial motor neurons 60 hr following a unilateral cold block of the marginal-mandibular and buccal branches of the facial nerve. (D) Axotomized versus sham-operated motor neurons 60 hr following a unilateral transection of the marginal-mandibular and buccal branches of the facial nerve. (E) Experimental versus sham-operated facial motor neurons 60 hr following a unilateral cold block of the main branch of the facial nerve.

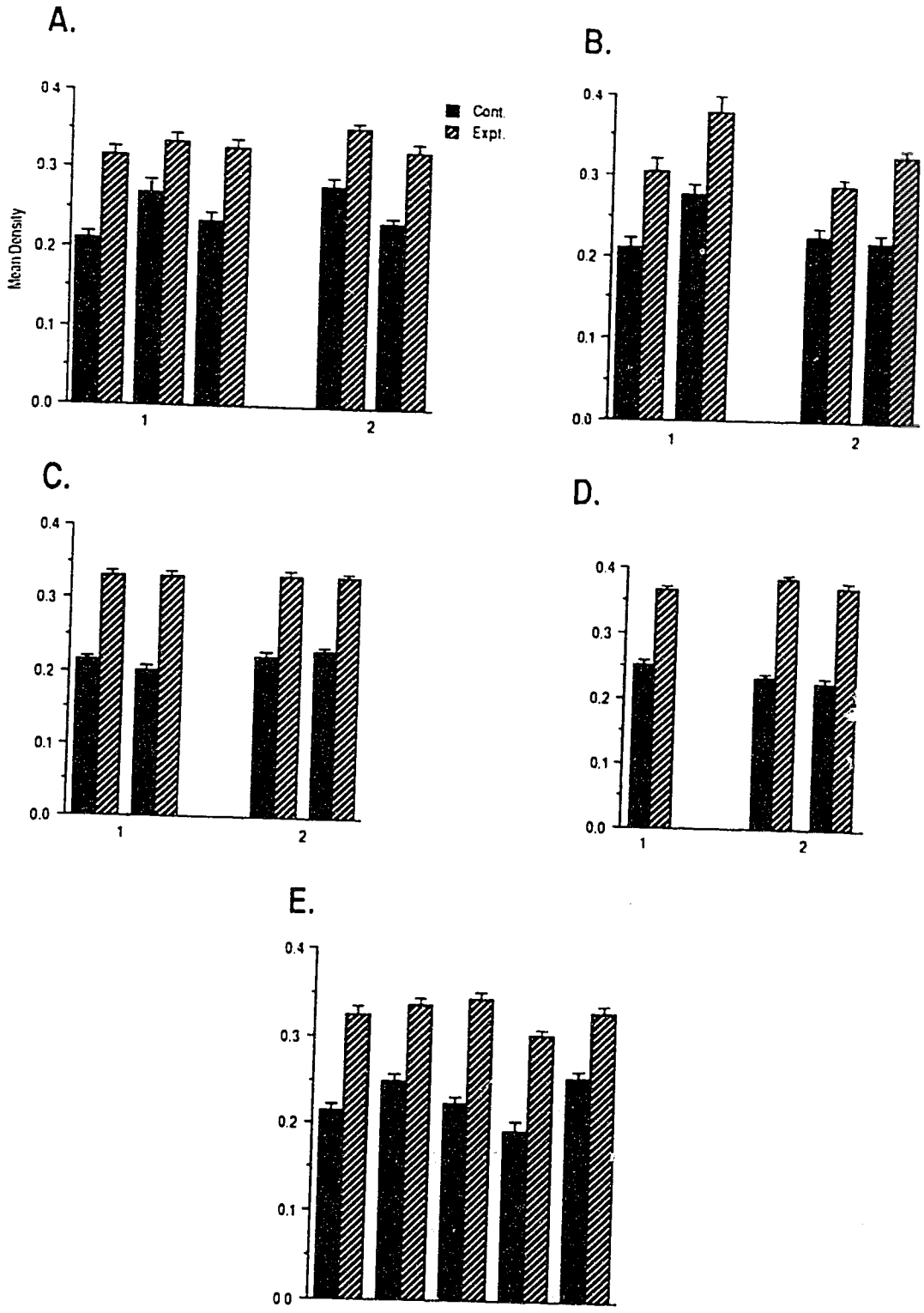
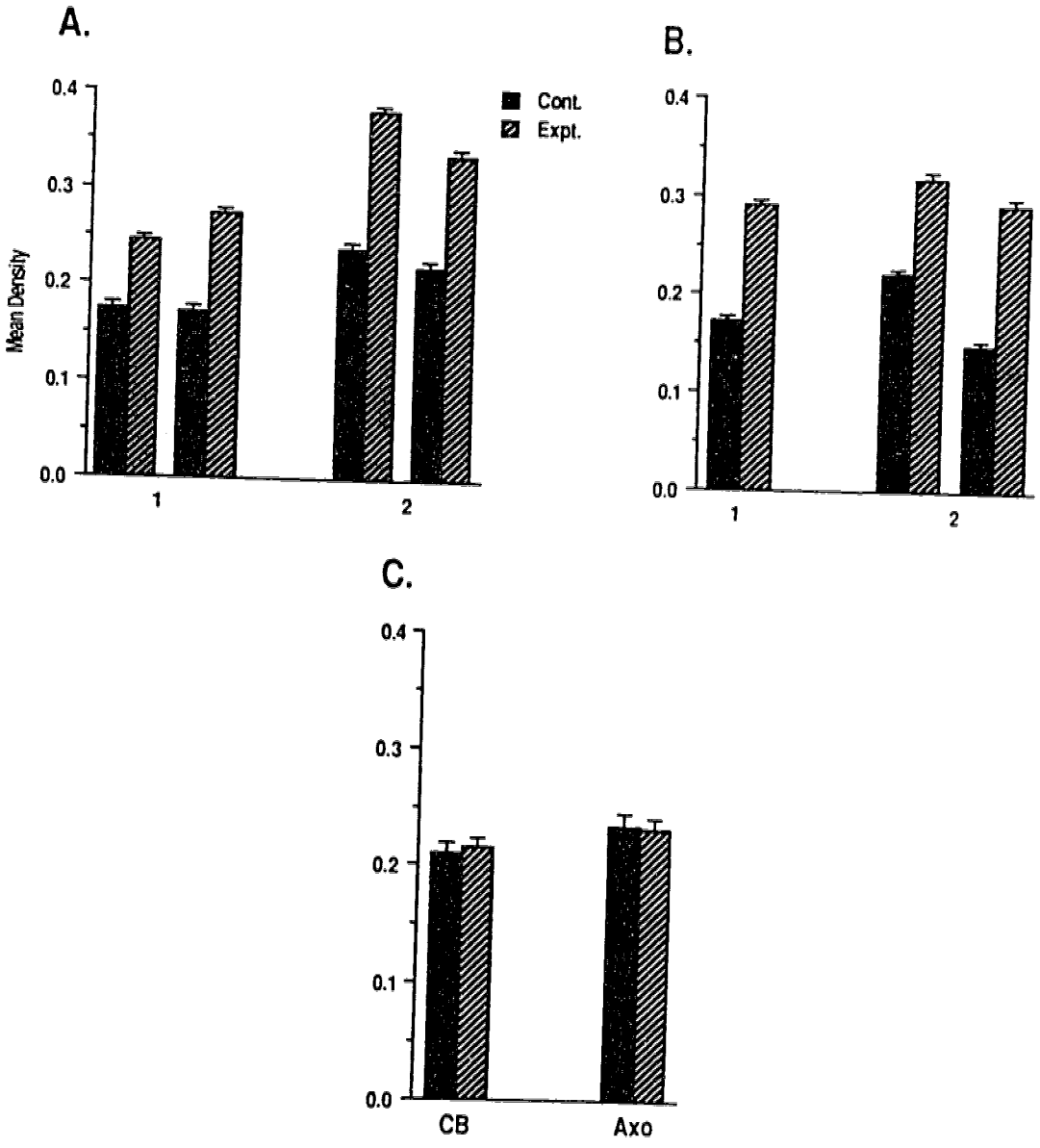

A.
B.
C.
D.
E.
Mean Density
Cont.
Expt.
0.4
0.3
0.2
0.1
0.0
1
2

Figure 6. Expression of p75 NGF receptor mRNA (A,B) and T26 α-tubulin mRNA in cold-blocked and transected facial motor neurons 36 and 60 hr postsurgery. (A) Mean grain densities for p75 NGF receptor mRNA in experimental versus sham-operated facial motor neurons of two different animals 60 hr following a unilateral cold block of the marginal-mandibular and buccal branches of the facial nerve. Neurons that project to the vibrissal regions were labeled with fluorogold bilaterally, and the facial nerve was unilaterally cold-blocked for 60 hr. Grain counts of fluorogold-labeled, cold blocked neurons were compared to those of fluorogold-labeled, sham-operated neurons from the contralateral facial nucleus. Each pair of bars represents representative results obtained from all of the labeled neurons on one slide. Each group of paired bars represents results obtained for the same animal. The error bars represent the standard error of the mean. (B) Mean grain densities for p75 NGF receptor mRNA in axotomized versus sham-operated facial motor neurons 60 hr following unilateral transection of the marginal-mandibular and buccal branches of the facial nerve. (C) Mean grain densities for T26 α-tubulin mRNA in experimental versus sham-operated facial motor neurons 36 hr following a unilateral cold block (CB) or a unilateral transection (AXO) of the main branch of the facial nerve.


A.
B.
C.
Mean Density
0.4
0.3
0.2
0.1
0.0
Cont.
Expt.
1
2
CB
Axo

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CHAPTER FOUR: MULTIPLE MOLECULAR MECHANISMS REGULATING Tα1 α-TUBULIN GENE EXPRESSION IN REGENERATING MAMMALIAN NEURONS

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Acknowledgments: We thank Dr. Bob Campenot for his guidance and advice throughout the course of this work, and Audrey Speelman and Rahul Varma for excellent technical assistance. This work was funded by grants from the Medical Research Council of Canada, the Canadian Neurosciences Network, and the MacArthur Foundation Research Network for Development and Psychopathology to FDM. FDM is a Killam Scholar, WW is supported by a studentship from the Rick Hansen Foundation for Spinal Cord Research, and AG by a Canadian Neurosciences Network fellowship.

This manuscript has been submitted.

INTRODUCTION

Microtubules, which are assembled from α and ß tubulin, comprise the major cytoskeletal component of growing neurites (Daniels, 1972). Of six different α-tubulin genes known to be expressed in mammals (Villasante et al., 1986), one, termed Tα1 in rats, is specialized for high levels of expression during neuronal growth (Miller et al., 1987; Miller et al., 1989; Mathew and Miller, 1990). Tα1 α-tubulin mRNA is highly abundant in developing neurons, with levels decreasing at least twenty-fold around the time of target contact (Miller et al., 1987). Following axotomy of motor (Miller et al., 1989), and sympathetic (Mathew and Miller, 1990) neurons, Tα1 mRNA increases rapidly, and then decreases to control levels following target reinnervation. If regeneration is unsuccessful (Miller et al., 1989), as with CNS neurons (Tetzlaff et al., 1991), Tα1 mRNA levels remain elevated. These increases appear to be due, to a great extent, to loss of repressive homeostatic signals (Mathew and Miller, 1993; Wu et al., 1993). Moreover, the increase in Tα1 mRNA levels is a function of the injury site, with maximal increases when neurons are injured close to their cell bodies, but little or no increase when neurons are injured close to their terminals (Mathew and Miller, 1993; Tetzlaff et al., 1994).

Expression of Tα1 α-tubulin mRNA is also increased during collateral sprouting of adult sympathetic neurons (Mathew and Miller, 1990), and in dentate granule cells of the hippocampus in response to elevated excitatory input (Causing et al., in press). These increases are likely due to increased available neurotrophins, since exogenous neurotrophins can increase Tα1 α-tubulin mRNA both in vivo (Mathew and Miller, 1990; Miller et al., 1994), and in culture (Ma et al., 1992; Krivko and Miller, 1994; Mohiuddin et al., 1995). Thus, expression of Tα1 mRNA is high during developmental growth, is downregulated as a function of neuronal maturation, and is then increased in response to axonal injury and to extrinsic cues like growth factors that regulate the growth of mature neurons.

It is our ultimate objective to define the mechanisms that allow a neuron to couple expression of a functionally-important gene to morphological growth. To this end, we have isolated the Tα1 α-tubulin gene, and have demonstrated that 1.1 kb of 5' upstream region from this gene conferred an appropriate pattern of marker gene

expression in transgenic mice (Gloster et al., 1994a; Bamji and Miller, 1995). In this paper, we have extended these studies, and have focused on the molecular mechanisms that contribute to regulation of Tα1 α-tubulin gene expression in regenerating neurons. Studies reported here indicate that target contact represses Tα1 α-tubulin transcription, and that its loss following axonal injury derepresses expression. However, the preferential upregulation of Tα1 mRNA following injury close to, versus far from, neuronal cell bodies appears to be due to posttranscriptional mechanisms that allow the neuron to modulate synthesis of this structural protein as a function of the cytoskeletal status of the neuron.

MATERIALS AND METHODS

Animals and surgical procedures. For the studies on regenerating facial motoneurons, adult Tα1:nlacZ transgenic mice from line K6 (described in Gloster et al., 1994a; Bamji and Miller, 1995) were anaesthetized with sodium pentobarbital (35 mg/kg), and the main branch of the facial nerve was transected or crushed as it exited the stylomastoid foramen, taking care not to injure the adjacent blood vessels. At timepoints 1 day, 3 days, and 49 days following axotomy, animals were transcardially perfused with 4% paraformaldehyde in phosphate buffer. Following dissection, mouse brains were sectioned into 1-2 mm thick sections on the mouse brain slicer (Activational Systems Inc.), and were stained for ß-galactosidase activity with the ligand X-gal. In total, 7 animals were analyzed 1 day postaxotomy, 7 at 3 days, and 10 at 49 days postaxotomy. For the studies on regenerating sympathetic neurons, neurons were labeled with a retrograde tracer prior to axotomy. Specifically, adult Tα1:nlacZ transgenic mice from line K6 were anaesthetized with sodium pentobarbital (35 mg/kg), and 5 µl of a 3% solution of fast blue (Sigma Chemical Co., St. Louis, MO) or fluorogold (Fluorochrome Inc., Englewood, CO) was injected bilaterally into the orbit of the eye to identify neurons that project to the eye via the internal carotid nerve. In some animals, 5-10 µl of a 3% solution of fluorogold was injected into the pinna of the ear to label neurons that innervate the pinna via the external carotid nerve. One week later, these transgenic mice were reanaesthetized with sodium pentobarbital, and the internal or external carotid nerve was unilaterally crushed or transected 2-3 mm from the superior cervical ganglia (ICN or ECN short cut). Alternatively, unilateral enucleation of the eye was performed to transect sympathetic axons that project to the eye near their termination (long cut) (for a schematic diagram of the anatomical organization of the system, see Fig. 5 or, for a more detailed description, Mathew and Miller, 1993). Five days following axotomy, animals were sacrificed under deep anesthesia, and the SCG were removed, along with part of the preganglionic cervical sympathetic trunk to ensure appropriate orientation. The SCG were subsequently fixed in 4% paraformaldehyde in phosphate buffer for 30 minutes prior to X-gal staining of the entire ganglia. Alternatively, ganglia were fixed in paraformaldehyde for 1 hour, cryoprotected in graded sucrose solutions, as we have previously described (Wu et al., 1993; 1994), and sectioned on the cryostat prior to X-gal staining.

LacZ staining. Dissected ganglia or brain slices were rinsed three times, for 30 minutes each, with a wash containing 0.1M NaH_2PO_3 (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% NP-40 as previously described (Gloster et al., 1994a). The staining reaction was performed by incubating the tissue at 37 ^{0}C in a reaction mix containing all the components of the rinse buffer with the addition of 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, and 5mM $K_4Fe(CN)_6$ (at pH 7.3-7.6). For the Q54 line, staining was done for 12-24 hours, and for the K6 line for 30-90 minutes. Brain slices to be used for sectioning were cryoprotected in graded sucrose solutions (12%, 16%, 18%) as previously described (Miller et al., 1987), and sectioned on the cryostat. Cryostat sections on slides were stained using similar procedures.

Neuronal cultures and RNA isolation and analysis. Primary cultures of neonatal sympathetic neurons were performed as we have described previously (Ma et al., 1992). Briefly, the SCG were removed from postnatal day 1 (P1) Sprague-Dawley rats. Ganglia were enzymatically dissociated in 0.1% trypsin (Calbiochem-Behring Corp., La Jolla, CA) for 17 min at 37 ^{0}C, then treated with 0.01 mg/ml DNAse I (Sigma Chemical Co., St. Louis, MO) for 3 min at room temperature, and finally mechanically dissociated (Campenot, 1982). Dissociated cells were plated at a density of one ganglion per a well of a 24-well collagen-coated tissue culture plate, using L15-CO_2 medium (Gibco Laboratories, Grand Island, NY) as described by Hawrot and Patterson (1979). Medium was supplemented with methylcellulose (0.6%), rat serum (2.5%), ascorbic acid (1 mg/ml), and the mitotic inhibitor cytosine arabinoside (10 μM). This method produces neuronal cultures that are free of nonneuronal cells. 2.5S NGF was added to neuronal cultures at a concentration of 200 ng/ml. Cultures were maintained in a 5% CO_2 atmosphere at 37^{0}C. The experiment was performed after 2 weeks in culture. The cultures were divided into two sets of treatments, one set served as control, and the second one was treated with colchicine (Sigma Chemical Co., St. Louis, MO) at a final concentration of 10 μM for 6 hours.

Total RNA was prepared from these cultures as previously described, and Northern blot analysis for Tα1 versus T26 α-tubulin mRNAs was performed using 32P-labeled RNA probes, again, as previously described (Ma et al., 1992).

RESULTS

Expression of a Tα1:nlacZ Transgene During Regeneration of Facial Motoneurons

We have previously shown (Miller et al., 1989) that the endogenous Tα1 α-tubulin mRNA rapidly increases in facial motoneurons following crush injury, is maintained at high levels during the period of axonal regeneration, and subsequently returns to control levels following functional reinnervation of the target musculature. To determine whether this pattern of expression could be attributed to transcriptional regulation of the Tα1 gene, we performed the same experiment in Tα1:nlacZ transgenic mice of line K6. In this particular line of transgenic mice, transgene expression is very high during development, and is downregulated following neuronal maturation (Gloster et al., 1994a, b), although it is still easily detectable in mature neurons (Bamji and Miller, 1995).

To perform this experiment, the main branch of the facial nerve was unilaterally crushed as it exited the cranium at the stylomastoid foramen. Animals were sacrificed at timepoints ranging from 1 to 49 days following this injury, and coronal sections through the brainstem at the level of the facial nucleus were stained with X-gal for 1-2 hours. Comparisons were subsequently made between injured, regenerating motoneurons on one side of the section versus control motoneurons on the other side of the same section (Fig. 1). Transgene expression was already increased 1 day following axonal injury (Fig. 1a,b), with similar increased levels observed 3 days following injury (Fig. 1c,d), as previously observed for the endogenous mRNA. By 49 days postaxotomy, following functional reinnervation of target musculature, transgene expression was decreased, and was similar in the injured versus control neurons (Fig. 1g,h). In contrast, when regeneration and reestablishment of target contact were inhibited by resection of the facial nerve, transgene expression was maintained at high levels in the injured motoneurons at 49 days postinjury (Fig. 1e,f). These observations are very similar to those previously reported for the endogenous Tα1 α-tubulin mRNA (Miller et al., 1989).

Expression of a Tα1:nlacZ Transgene in Regenerating Sympathetic Neurons of the Superior Cervical Ganglion

Tα1 α-tubulin mRNA was also upregulated during the regeneration of sympathetic neurons of the superior cervical ganglion (SCG) and, in this system, the axotomy-induced increase in Tα1 mRNA was a function of the amount of axon lost: when sympathetic neurons were injured close to their cell bodies, Tα1 mRNA levels were robustly increased, whereas when they were injured by removal of only the terminal axon, no detectable increase in Tα1 mRNA was noted (Mathew and Miller, 1993). To determine whether this "distance effect" was transcriptionally-mediated, we performed a similar experiment in line K6 Tα1:nlacZ transgenic mice.

Sympathetic neurons of the SCG that project to the eye via the internal carotid nerve (ICN; eye neurons) were bilaterally labeled by injection of the retrograde tracers fluorogold or fast blue into the posterior chamber of the eye. This protocol led to a highly-localized distribution of labeled eye neurons in the region of the SCG adjacent to the ICN (Fig. 2A), as we have previously described. One week following retrograde-labeling, eye neurons were axotomized either by crushing or transecting the ICN close to the SCG (a short cut), or by removing the terminal axon by enucleation of the eye (a long cut). Five days following axotomy, a timepoint when expression of the endogenous Tα1 α-tubulin mRNA is maximal (Mathew and Miller, 1993), sympathetic neurons of the SCG were analyzed for ß-galactosidase expression by staining with X-gal. In all cases, the relative level of staining was compared both to other populations of neurons within the same ganglion, and to neurons of control ganglia that had not been injured, but that were treated identically, and analyzed at the same time.

Using this experimental approach, when control, retrogradely-labeled SCG were stained with X-gal, a random, relatively uniform pattern of transgene-positive nuclei was observed throughout the ganglion (Fig. 2A,B; 3A). To determine whether transgene expression was increased when sympathetic neurons were injured close to their cell bodies, SCG were examined 5 days following a short cut. When the ICN was crushed or transected, increased transgene expression was observed in cells throughout the ganglion, with the highest density of positive nuclei localized to the region adjacent to the ICN (Figs. 2G,H; 3C; 5), consistent with the localization of sympathetic neurons that project their axons via this nerve. When the ECN was short cut, transgene-positive neurons were again localized throughout the ganglion, with

some increased density of positive nuclei adjacent to the cervical sympathetic trunk (CST) (Figs. 2K,L; 3D; 5), consistent with the location of neurons that project their axons via the ECN (Bowers & Zigmond 1979). Thus, as previously observed for the endogenous Tα1 mRNA, increased transgene expression were observed in appropriately located populations of sympathetic neurons 5 days following a short cut to either the ICN or ECN.

To ensure that retrograde-labeling of eye neurons did not influence the results of these experiments, neurons that project via the ECN to the pinna of the ear were retrogradely-labeled with fluorogold, and the ICN was short cut. As observed when eye neurons were retrogradely-labeled, the highest density of transgene-positive nuclei were localized in the region of the ganglion adjacent to the ICN (Fig. 2I,J). In contrast, retrogradely-labeled neurons were preferentially localized adjacent to the CST (Fig. 2I). Thus, the retrograde label had no effect on Tα1:nlacZ transgene expression in sympathetic neurons.

To determine whether the transcription of the Tα1:nlacZ transgene was similarly increased when neurons were injured far from their cell bodies, eye neurons were retrogradely-labeled, and subsequently long cut. Five days postinjury, X-gal staining revealed a large increase in transgene expression that was highly localized to the region of the SCG immediately adjacent to the ICN (Figs. 2C-F; 3B). This cluster of transgene-positive nuclei was localized in the same region as the retrogradely-labeled eye neurons (Figs. 2C-F; 3E-H; 5) and, in many cases, the retrograde label and increased transgene expression were colocalized to the same cells (see, for example, Fig. 2E). In all cases, the area containing increased transgene staining was much more limited than that observed following a short cut of the ICN. Thus, in contrast to the endogenous Tα1 α-tubulin mRNA, expression of the Tα1:nlacZ transgene was robustly increased in eye neurons five days following removal of the terminal axon.

Posttranscriptional Degradation of Tα1 α-Tubulin mRNA in Sympathetic Neurons

The discrepancy between transgene versus endogenous mRNA expression following a long cut could be due to posttranscriptional regulation of neuronal α-

tubulin gene expression. Synthesis of the two major microtubule subunits, α- and ß-tubulin, is regulated posttranscriptionally in nonneuronal cells and, at least for ß-tubulin mRNAs, this regulation occurs at the translational level (reviewed in Cleveland, 1987; 1989). More specifically, a rise in the cellular ratio of monomeric to polymeric tubulin leads to posttranscriptional degradation of tubulin mRNAs.

To determine whether steady-state neuronal levels of Tα1 α-tubulin mRNA were subject to similar posttranscriptional regulation, two week old primary cultures of neonatal sympathetic neurons were exposed to 10 μM colchicine for 6 hours, leading to microtubule depolymerization and an increase in the tubulin monomer/polymer tubulin ratio. Northern blot analysis of equal amounts of total RNA from treated versus untreated cultures revealed that colchicine treatment led to a dramatic decrease in the steady-state levels of Tα1 α-tubulin mRNA in these neurons (Fig. 4). A similar decrease in T26 α-tubulin mRNA was observed, indicating that, as described in nonneuronal cells, this posttranscriptional regulation likely generalizes to all tubulin mRNAs. Thus, neuronal steady-state levels of Tα1 mRNA are determined both by transcriptional regulation of the gene, and by posttranscriptional regulation of mRNA levels as a function of neuronal cytoskeletal status.

DISCUSSION

Although the cell biology of nerve regeneration has been intensively studied (for reviews, see Fawcett and Keynes, 1990; Aguayo et al., 1991; Carbonetto, 1991; Clarke and Richardson, 1994), little is known regarding the molecular mechanisms that allow a neuron to respond to axonal injury with an appropriate regenerative response. In studies described here, we address this issue by focusing on the Tα1 α-tubulin gene, taking advantage of a Tα1:nlacZ transgene we have previously described (Gloster et al., 1994a) to dissociate transcriptional and posttranscriptional mechanisms. These studies, together with our previous work, support a number of conclusions. First, they indicate that target contact represses transcription of the Tα1 α-tubulin gene and that the loss of target contact that occurs after axonal injury is largely responsible for increased transcription during neuronal regeneration. Second, our data suggest that the pattern of expression of Tα1 α-tubulin mRNA observed after axonal injury close to the neuronal cell body can be accounted for by transcriptional regulation. Third, experiments described here demonstrate that the modulation of Tα1 α-tubulin mRNA levels as a function of the amount of axon lost is posttranscriptionally mediated, and is likely a direct result of alterations in the cellular tubulin monomer/polymer ratio. Such a posttranscriptional feedback loop would allow a neuron to maintain high levels of Tα1 mRNA when axonal growth was robust, and downregulate it when growth was limited, as following a far distal axotomy.

A number of lines of evidence support the first conclusion that the increase in Tα1 α-tubulin gene expression during neuronal regeneration is largely a result of transcriptional derepression due to the loss of target-derived signals. During development, both the Tα1:nlacZ transgene and Tα1 α-tubulin mRNA are high in neurons, and decrease significantly around the time of target contact. Both are increased following the loss of target contact subsequent to axonal injury, remain high if target contact is inhibited, and decrease if target contact is reestablished. Although the alterations observed following nerve injury could be due to transcriptional modulation resulting from extrinsic, injury-derived cues, as opposed to derepression due to the loss of target contact, we have previously performed studies indicating that this is unlikely. When fast axonal transport and transmission of nerve impulses were

blocked by locally cooling the axons of facial motor neurons, a manipulation that did not damage the nerve or lead to a peripheral nerve injury response (Wu et al., 1994), Tα1 α-tubulin mRNA increased as it did following transection of the facial nerve (Wu et al., 1993).

Similar observations have been made for other genes whose expression is increased following axonal injury. For example, GAP-43 mRNA, which is increased in most neurons following axonal injury , is downregulated developmentally at the time of target contact, is increased following axonal injury, and remains high if target reinnervation is prevented (Benowitz & Routtenberg, 1987; Nerve et al., 1987; Hoffman et al., 1989). Vasoactive intestinal peptide (VIP) is increased dramatically in sympathetic neurons following axotomy, is downregulated following reinnervation, and is increased following a colchicine block to fast axonal transport (Knyihar-Csillik et al., 1991; Kashiba et al., 1992; Hyatt-Sachs et al., 1993; Mohney et al., 1994). Thus, there appears to be a general target-derived signal(s) that inhibits cell body growth responses and that, at least in the case of the Tα1 α-tubulin gene, results in transcriptional repression.

The nature of this repressive signal remains largely speculative, although our previous studies indicate that it involves ongoing neuronal activity and/or signals transmitted as a function of fast axonal transport (Wu et al., 1993). Such signaling could, for example, involve soluble target-derived factors or cell:cell interactions mediated by adhesion molecules and/or extracellular matrix-bound molecules that both locally inhibit growth, and retrogradely signal to inhibit cell body growth responses. Alternatively, the "stop" signal could involve ongoing patterns of neuronal activity that directly or indirectly inhibit growth responses. One group of target-derived factors that are, however, unlikely to mediate such signaling are the neurotrophins; application of neurotrophins to axotomized neurons does not reverse the axotomy-induced increase in GAP-43 (Verge et al., 1990; Hu-Tsai et al., 1994), or Tα1 α-tubulin mRNAs. In fact, Tα1 α-tubulin mRNA is increased by neurotrophins (Mathew and Miller, 1990; Ma et al., 1992; Mohiuddin et al., 1995) coincident with increased neuronal sprouting (Miller et al, 1994).

The molecular mechanisms that mediate transcriptional repression of the Tα1 gene in response to target contact are also currently unknown. In this regard, it is

interesting, however, that genomic sequences derived from the GAP-43 (Vanselow et al., 1994) and peripherin (Belecky-Adams et al., 1993; Foley et al., 1994) genes are capable of conferring patterns of neuronal gene expression similar to that observed for Tα1 promoter; expression is high during development, downregulated following target contact, and increased following axonal injury. Moreover, the Tα1 promoter fragment used in the Tα1:nlacZ transgene (Gloster et al., 1994a) contains sequences homologous to those found in the peripherin gene (Foley et al., 1994). We are currently testing the potential importance of these elements in a transgenic mouse context.

Although transcriptional regulation explains the pattern of Tα1 α-tubulin mRNA expression observed following axotomy close to the neuronal cell body, it cannot explain the "distance effect" that has been documented for this mRNA. More specifically, Tα1 α-tubulin mRNA levels are not substantially increased in either sympathetic (Mathew & Miller, 1993) or motor (Tetzlaff et al., 1994) neurons following axotomy distal to the neuronal cell bodies, whereas transgene expression is increased irrespective of the injury site (for a summary of these data, see Figure 5). Although it is possible to explain these results by invoking promoter elements that allow a neuron to monitor axonal status, and that are missing from the 1.1 kb fragment used to construct the transgene, the more likely explanation involves posttranscriptional regulation of the endogenous Tα1 mRNA. It is well-established that tubulin mRNAs are subject to an autoregulatory posttranscriptional feedback loop that modulates mRNA levels as a function of the tubulin monomer/polymer ratio; when this ratio increases, tubulin mRNAs are degraded(Cleveland et al., 1983). For ß-tubulin mRNAs, this degradation occurs during translation, whereas, for α-tubulin mRNAs, the precise mechanism is less clear, but is definitely posttranscriptional (Cleveland et al., 1983; Caron et al, 1985; Bachurski et al., 1994). Data presented here demonstrate the existence of similar posttranscriptional mechanisms in neurons.

Together, these mechanisms can explain the pattern of Tα1 α-tubulin mRNA expression observed in regenerating peripheral neurons. In the mature, uninjured nervous system, transcription of the Tα1 α-tubulin gene is repressed as a result of ongoing, target-derived signals that are dependent upon fast axonal transport and/or

ongoing electrical activity. Following axonal injury, this repressive target-derived signal(s) is lost, and transcription of the Tα1 gene is increased. When the injury is close to the neuronal cell body, the regenerative response is fast and robust, the rate of tubulin transport increases, and tubulin is rapidly recruited into the regenerating axon during the period of regrowth (Moskowitz and Oblinger, 1995). The net result is that, even though the synthesis of tubulin monomers is greatly increased, the monomer/polymer ratio in the cell body is not increased, and there is little, if any negative feedback at the posttranscriptional level. Thus, the increased Tα1 α-tubulin mRNA levels are a direct reflection of the increased transcriptional rate. In contrast, when the axotomy is distal to the cell bodies, the amount of actual regrowth is limited, and can largely be accommodated by existing transport of tubulin within the axon. In this case, synthesis of tubulin monomers within the cell body is increased with no concurrent increase in transport of tubulin into the axon, and the monomer/polymer tubulin ratio in the cell body increases, leading to degradation of Tα1 mRNA. Thus, the increased transcription is compensated by an equally large increase in Tα1 mRNA degradation rate so that there is little, if any, net increase in Tα1 mRNA levels. In this way, the cell is able to maintain the tubulin synthetic capacity required for axonal regeneration, but at the same time to ensure that the cytoskeletal status of the neuron is not disrupted. Such transcriptional/posttranscriptional fine-tuning of Tα1 α-tubulin synthesis may well not be specific to injured, regenerating neurons, but may provide a sensitive mechanism for regulating cytoskeletal synthesis as a function of the growth requirements of uninjured neurons both during development and in the mature nervous system.

FIGURES AND FIGURE LEGENDS

Figure 1. Timecourse of expression of the Tα1:nlacZ transgene in injured facial motor neurons following unilateral injury to the main branch of the facial nerve. Expression of ß-galactosidase, as detected by X-gal staining, in control (a,c,e,g) and injured (b,d,f,h) facial motoneurons 1 day following nerve transection (a,b), 3 days following nerve transection (c,d), 49 days following nerve resection, to prevent regeneration (e,f), and 49 days following nerve crush, where regeneration occurred (g,h). Note that transgene expression was robustly increased by 1 day postaxotomy, and that if regeneration was prevented, it remained high for up to 49 days, whereas if regeneration occurred, transgene expression decreased to control levels. Scale bar = 50 µm.

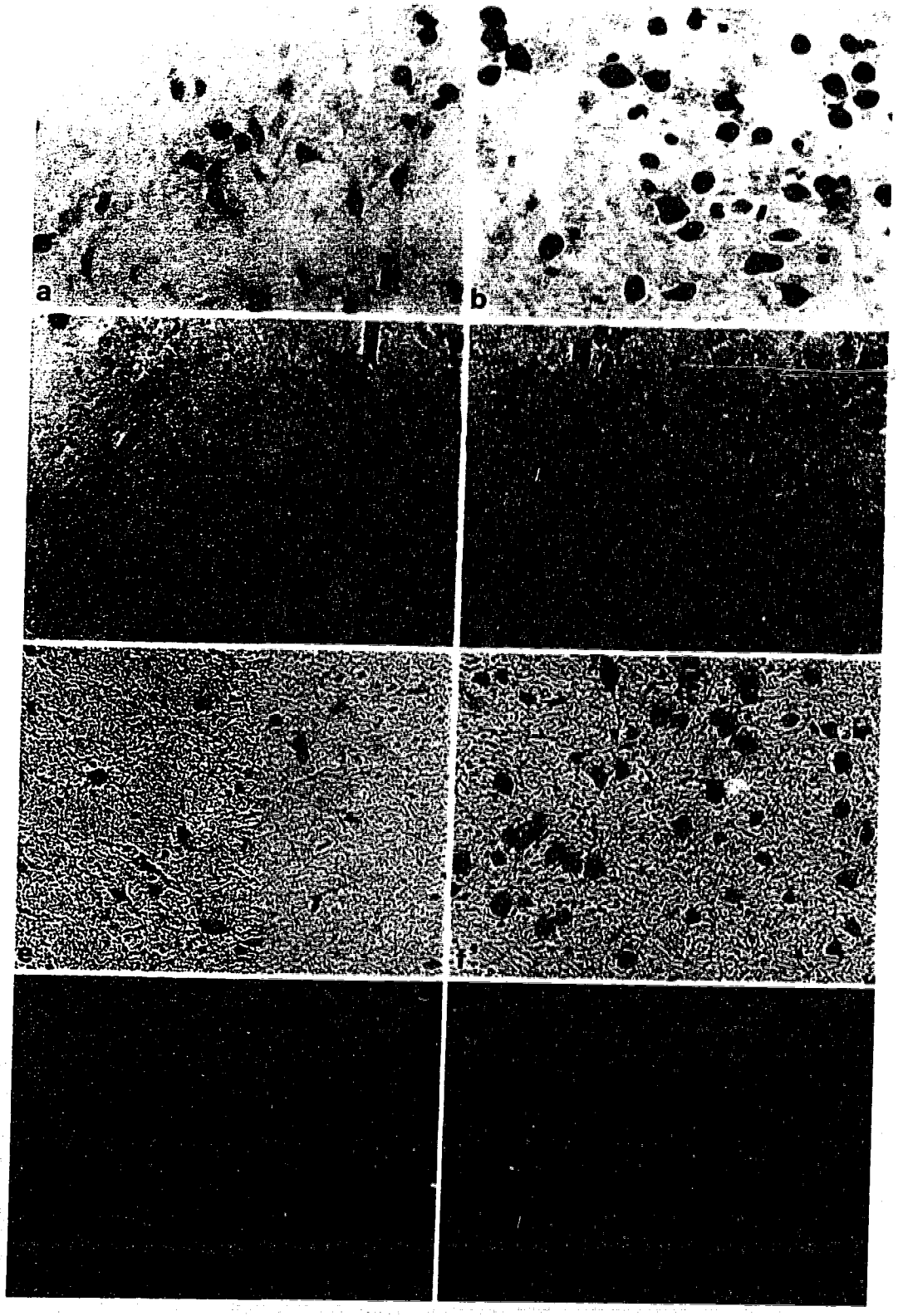
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Figure 2. Increased expression of a Tα1:nlacZ transgene 5 days following either a long cut or a short cut to sympathetic neurons of the SCG. Ganglia were sectioned, stained with X-gal, and photographed. Panels on the left are photographed under fluorescence illumination to visualize the retrogradely-labeled fast blue or fluorogold containing neurons. Panels on the right are the same field of the vision to those shown on the left, as photographed under combined brightfield/fluorescence illumination. In the panels on the right, the outline of the ganglia, as visualized under fluorescence illumination, is interposed on the picture for purposes of orientation. In all cases, the ganglia are oriented so that the entrance of the CST into the ganglion is to the right of the panel. (A,B) A control SCG that was retrogradely-labeled from the eye. Note that the retrogradely-labeled neurons are localized to the left of the ganglion, at the point of exit of the ICN, but that scattered X-gal positive nuclei are randomly localized. (C,D) An SCG that was retrogradely-labeled from the eye, and long cut 5 days previously. Note again the localization of the retrogradely-labeled neurons at the exit of the ICN, and an increased density of transgene-positive neurons in the same location. (E,F) A second SCG that was retrogradely-labeled from the eye, and long cut 5 days previously. Localization is as described for panels C and D. (G,H) An SCG that was retrogradely-labeled from the eye, followed by a short cut of the ICN 5 days previously. In this case, the density of the transgene-positive neurons is still highest at the pole where the ICN exits, but there are more positive cells localized throughout the remainder of the ganglion, consistent with previous reports of the localization of sympathetic neurons whose axons project via the ICN (Bowers and Zigmond, 1979). (I,J) An SCG that was retrogradely-labeled from the pinna of the ear, followed by a short cut of the ICN 5 days previously. In this case, the retrogradely-labeled neurons are localized closer to the CST than to the ICN, as predicted for neurons that project via the ECN to the ear. In contrast, however, the transgene-positive neurons are clustered closer to the ICN, as observed in panel (H), demonstrating that the retrograde label itself had no influence on the pattern of transgene expression. (K,L) An SCG that was retrogradely-labeled from the eye, followed by a short cut of the ECN 5 days previously. Note that, although the pattern of retrograde-labeling was similar to that observed in A,C,E, and G, the localization of transgene-positive cells differed. In this case, the transgene positive cells were localized throughout the ganglia, with a notably lower density in the region close to the ICN where the eye neurons were clustered. Scale bar = 1 mm.

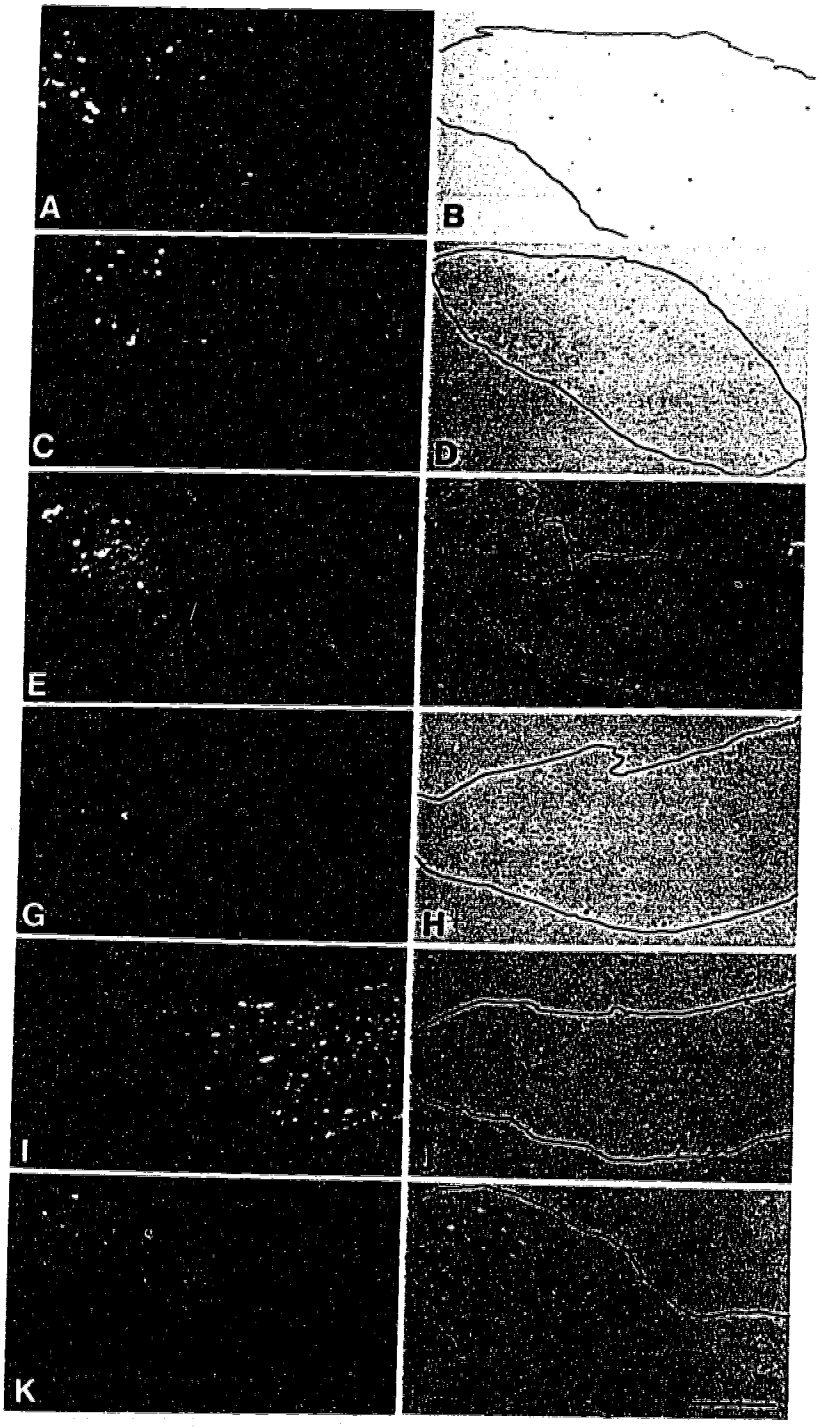
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Figure 3. Expression of the Tα1:nlacZ transgene in injured sympathetic neurons of the superior cervical ganglion (SCG). In panels A-D, whole ganglia were stained with X-gal, and photographed so that, in all cases, the arrows indicate the location of the preganglionic cervical sympathetic trunk (CST). (A) Control SCG. (B) An SCG 5 days following a long cut, stained under identical conditions to the control ganglion in (A), showing intense X-gal staining at the pole of the SCG opposite to the CST, where the majority of the eye-projecting neurons are located. (C) An SCG 5 days following a short cut of the ICN. Note that, although X-gal positive cells are localized throughout the ganglia, their density is increased at the pole of the ganglion opposite the CST, where the ICN exits. (D) An SCG 5 days following a short cut of the ECN. In contrast to the ganglion shown in (C), an increased density of X-gal positive cells are localized in the region adjacent to the CST. Panels E-H are high-magnification photographs of sectioned SCG that were long cut 5 days previously, photographed under combined fluorescence/brightfield illumination, demonstrating colocalization of increased X-gal staining with the retrograde tracer fluorogold, which was injected into the eye one week earlier. (E) A photograph derived from the region of the SCG opposite the CST, showing many intensely X-gal positive nuclei, which are, in some cases, colocalized with fluorogold (arrows). (F) A photograph of the central region of the same ganglion. Note the absence of retrograde tracer, and the relatively low intensity of X-gal staining, although the occasional more intense cell was noted (arrow). (G) A photograph derived from the region of the SCG opposite the CST from a second long-cut ganglion, again showing the similar localization of retrogradely-labeled neurons that project to the posterior chamber of the eye, and a high density of X-gal positive nuclei. (H) A photograph of the central region of the same ganglion as shown in (G). Scale bar for panels A-D = 1 mm. Scale bar for panels E-H = 50 μM.

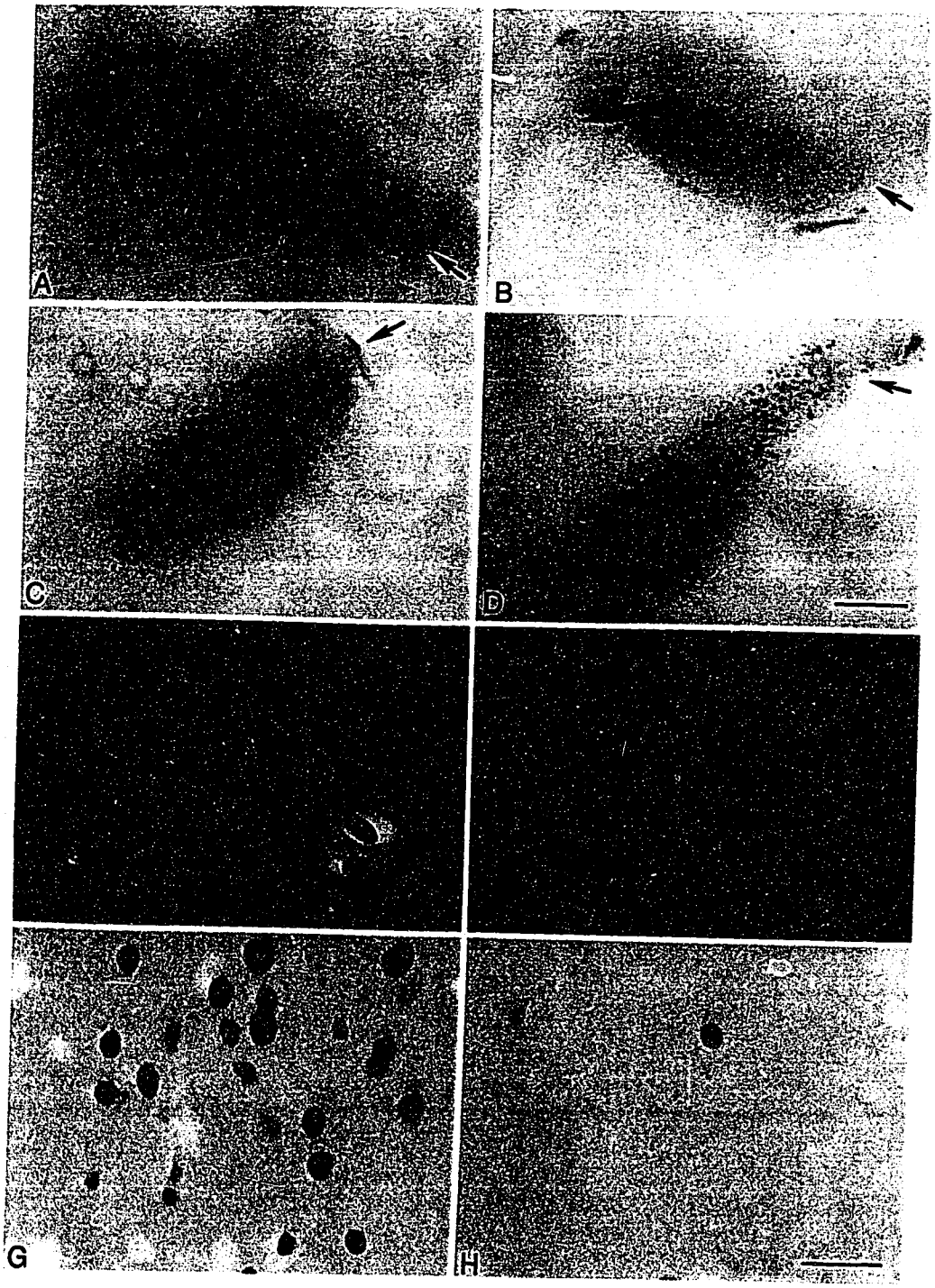

A
B
C
D
G
H

Figure 4. Northern blot analysis of Tα1 and T26 α-tubulin mRNAs in primary cultures of rat sympathetic neurons that were maintained in 200 ng/ml NGF for two weeks followed by treatment with colchicine (Col.) at a final concentration of 10 uM for 6 hours. Sister cultures that were maintained in 200 ng/ml NGF served as controls (Cont.). The upper panels are photographs of autoradiographs produced by hybridization with radiolabeled probes specific for the 3' untranslated region of each of the two mRNAs. The lower panels are photographs of the original agarose gels with the samples electrophoresed in the presence of ethidium bromide to demonstrate the amounts of total RNA loaded in each lane. Two different aliquots of total RNA from colchicine treated cultures were used in the blot that was hybridized with the probe specific for T26 α-tubulin mRNA. Note that both Tα1 and T26 mRNA levels were dramatically reduced in the colchicine-treated sympathetic neurons, relative to the control cultures.

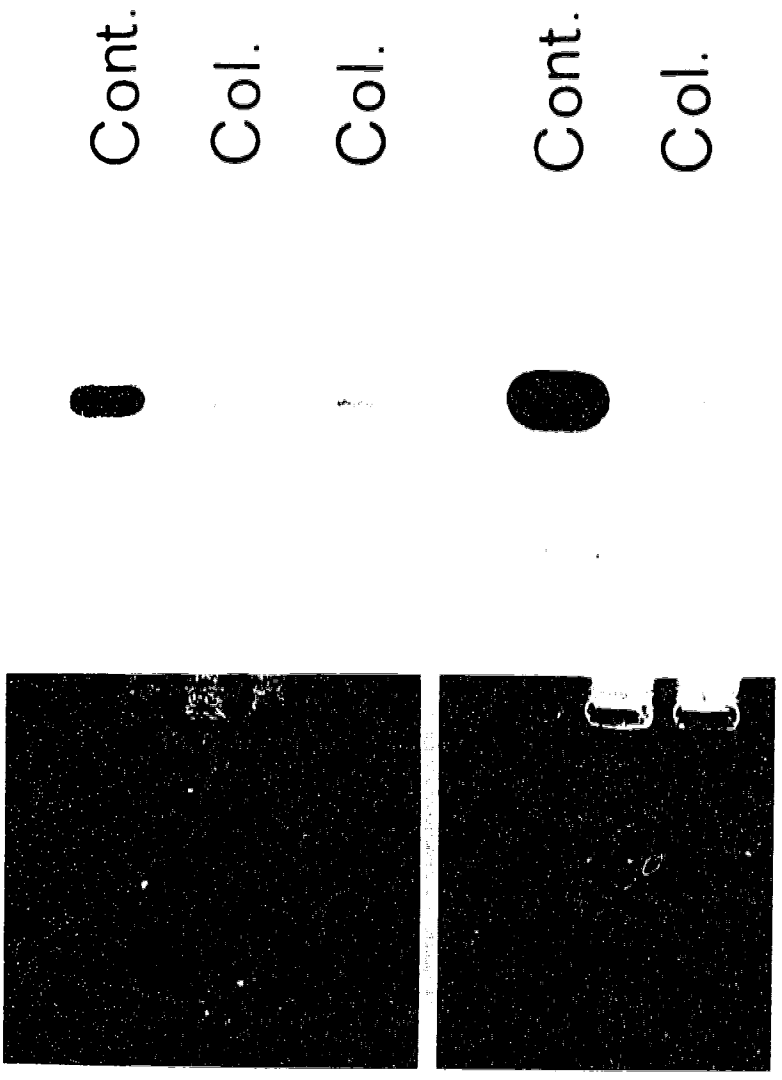
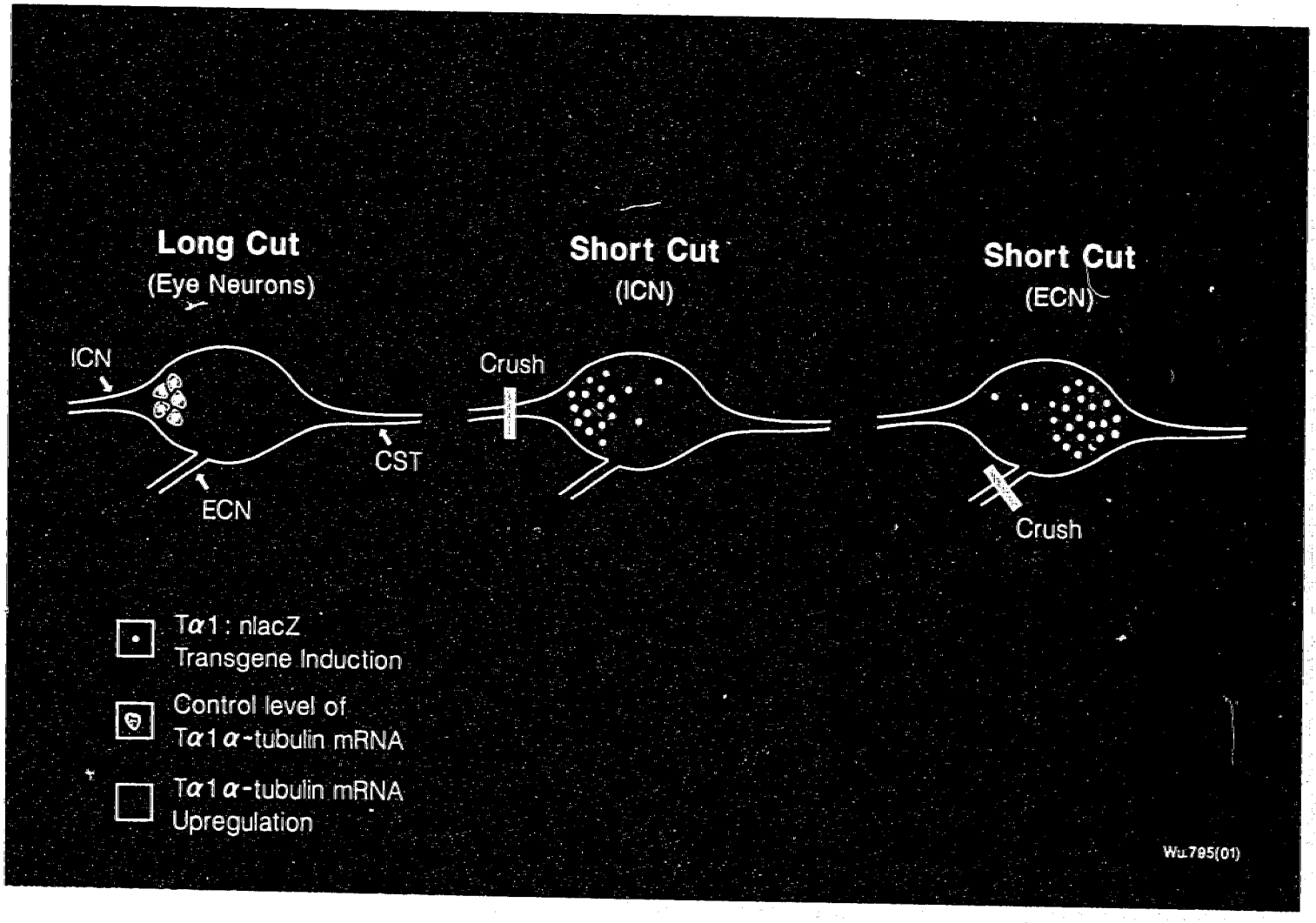

Cont.
Col.
Col.
Cont.
Col.
T26
Ta1

Figure 5. A summary of Tα1:nlacZ transgene expression versus Tα1 α-tubulin mRNA expression following a long cut or short cut to sympathetic neurons of the SCG. When sympathetic neurons are crushed or transected close to their cell bodies (short cuts of the ICN or the ECN), transcription of the Tα1:nlacZ transgene is increased, as are steady-state levels of Tα1 α-tubulin mRNA. In contrast, when sympathetic neurons are transected far from their cell bodies, and close to their terminals (a long cut to eye neurons), transcription of the transgene is increased, but steady-state levels of Tα1 mRNA are not detectably altered.


Long Cut
(Eye Neurons)
ICN
CST
ECN
Short Cut
(ICN)
Crush
Short Cut
(ECN)
Crush
Tα1 : nlacZ
Transgene Induction
Control level of
Tα1 α-tubulin mRNA
Tα1 α-tubulin mRNA
Upregulation
Wu.795(01)

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CHAPTER FIVE: CONCLUSION AND CLOSING REMARKS

I. Overviews

Following peripheral nerve injury, there are series of changes in gene expression in neurons and Schwann cells. The cellular and molecular mechanisms of induction of these genes have been extensively studied. There are generally two causes: macrophage invasion and inflammatory response or loss of homeostatic interaction among neurons, Schwann cells, and target. We hypothesize that the alterations in neuronal and Schwann cell gene expression observed following peripheral nerve injury are at least partially due to the disruption of ongoing homeostatic signals.

We used two approaches to study these mechanisms. The first approach is the cold block experiments, to study whether in the absence of macrophage invasion, the alterations in neuronal and Schwann cell gene expression observed following peripheral nerve injury are at least partially due to the disruption of ongoing homeostatic signals. Using first strategy we attempted to interrupt normal homeostatic signals by disrupting axonal function without inducing an inflammatory injury response, and to determine whether such disruption leads to alterations in Schwann cell or neuronal gene expression that are similar to those observed following peripheral nerve injury. Cold block on intact peripheral nerve blocks fast axonal transport and distal electrical activity without inducing macrophage invasion or Wallerian degeneration. With the first approach, I have been able to demonstrate that induction of at least some changes during peripheral nerve injury are independent of macrophage invasion or inflammatory response. Instead, it is the loss of as-yet-undefined ongoing biochemical and/or electrical signals from axon:target interactions that is responsible for induction of some aspects of peripheral nerve injury response.

With regards to slow axonal transport, microtubule and ATP-dependent motors have been suggested to play a role (for review, see Vallee & Bloom, 1991), especially for tubulin transport. More recently, cytoplasmic dynein is suggested to generate the movement of microtubules in slow axonal transport (Dillman et al., 1996). Furthermore, colchicine application has been shown to induce changes in slow

axonal transport, including tubulin transport in vagus nerve (Griffiths & McLean, 1980; Archer et al., 1994). Low temperature depolarizes microtubule into tubulin monomers, which may in turn perturb some qualitative and quantitative aspects of slow axonal transport, although to a less extent than fast axonal transport (for review, see Cancalon, 1985). Thus, we have to be cautious in interpreting our cold block results.

The second approach was to use a transgenic model to study the molecular mechanisms regulating the neuronal regeneration associated gene, Tα1 α-tubulin promoter, at transcriptional level. Using this strategy, we have demonstrated that Tα1 α-tubulin is induced following nerve injury at transcriptional level, and its induction is caused by target deprivation, regardless of the amount of axon loss. Furthermore, we have shown that posttranscriptional autoregulatory mechanism coexists with transcriptional regulation for Tα1 α-tubulin, which allow mature neurons to closely modulate synthesis of this essential cytoskeletal protein as a function of both target contact and axonal status. The extrinsic suppression of transcription derives from target, while neurons, like other cells, possess the posttranscriptional machinery to tightly control Tα1 α-tubulin mRNA levels as a function of the tubulin monomer:polymer ratio in the cell body.

II. Loss of intrinsic homeostatic interactions induce at least some of the axotomy-induced alterations in Schwann cell gene expression.

Neurons affect Schwann cells in different ways. For instance, depending on its mature or injured status, neurons can either maintain the myelinating phenotype of Schwann cells in the mature nerve, or promote Schwann cell proliferation during nerve regeneration. In the present study (see chapter 2), using local cooling of the peripheral nerve to perturb axonal function without macrophage invasion or Wallerian degeneration, we have demonstrated the existence of two mechanisms whereby axons affect Schwann cell gene expression. First, we have demonstrated that blockage of both electrical activity and fast axonal transport in axons of the intact sciatic nerve led to a dramatic downregulation of Po gene expression in Schwann cells distal to the blockage. Thus maintenance of Po gene expression in mature Schwann cells is dependent on biochemical signals that are dependent on constant axonal flow and/or on electrical activity derived from the neuronal cell body.

The nature of the axonal signal for Po gene expression and Schwann cell myelination remains speculative. However, the spatial correlation between control levels of Schwann cell Po expression, and axonal Fluorogold and p75 NTR accumulation in the distal cold-blocked nerve suggests an interrelationship between vesicle/protein trafficking, and the axon:Schwann cell signal for Po. This indicate that electrical activity by itself can not be responsible for maintaining Po expression in Schwann cells. One potential explanation for such a relationship invokes constitutive axonal secretion of soluble molecules, such as growth factors, that locally regulate Schwann cell biology. Since growth factors are presumably trafficked via fast axonal transport, then secretion would be disrupted in axonal domains that were depleted of vesicular traffic, while it could conceivably be maintained in regions where vesicles were concentrated. Such a mechanism could explain the observations reported that Schwann cell Po mRNA levels can be regulated, in culture, by neuronally produced diffusible molecules (Bolin & Shooter, 1993). Moreover, neurons synthesize a number of growth factors that are known to influence Schwann cell biology, including members of glial growth factor (Marchionni et al., 1993) and fibroblast growth factor (Eckenstein et al., 1991) families. For instance, there are multiple forms of GGFs, some of them may function to stimulate Schwann cell proliferation, others may be responsible for Po myelin expression (see Chapter 1 Introduction II2). Alternatively, trafficking along the length of the axon may be monitored by some as-yet-undefined mechanism, and disruption of this trafficking may alter the axonal surface, thereby modulating axon:Schwann cell adhesion and, ultimately, may result in changes in Schwann cell gene expression. It is also important to know that slow axonal transport is affected by cooling temperature (for review, see Cancalon, 1985). This may well affect the local accumulation region of cold block and complicate the interpretation of our results.

Although there are parallel correlation of Po protein and its mRNA level (Lemke & Axel, 1985), suggesting that protein expression is largely determined by the steady state mRNA level, the Po protein half-life and degradation have not yet been studied. While Po mRNA is rapidly downregulated following injury, the downregulation of Po protein level may be slightly delayed by two to three days (Gupta et al., 1988). Thus for our cold block experiments, the delays in decrease of Po and potentially other myelin proteins, together with the absence of macrophage

invasion or inflammatory response, may allow myelin to be maintained relatively intact, and Schwann cells to remain in very close relationships with axons, at least during the time course of our cold block experiments.

In contrast to Po, our data indicate that Schwann cell expression of p75 NTR is controlled by intact local axon:myelin profile and/or by environmental cues that are independent of electrical activity or axonal transport. Alternatively, p75 NTR might well be associated with mitotic activity in Schwann cells and its proliferative state, or potentially induced by macrophage invasion or cytokine production.

III. Induction of Tα1 α-tubulin gene expression following peripheral nerve injury : target derepression versus mRNA autoregulatory mechanism.

1. Loss of homeostatic signals induces regeneration-associated alterations in neuronal gene expression.

Axotomy of motoneurons leads to upregulation of Tα1 α-tubulin and p75 NGFR mRNAs. In our cold block model, we have demonstrated the induction of these genes can be mimicked by blocking axonal transport and electric activity without inflammatory responses (see chapter 3; Wu et al., 1993). Therefore, ongoing homeostatic signals derived from target cells and/or Schwann cell contact appears to play a critical role in repressing growth-associated gene expression in motor neurons. We have hypothesized that this repression is transcriptional in nature, and is a function of target contact. Support for this hypothesis derives from our studies in Tα1:nlacZ transgenic mice (see chapter 4).

2. Loss of target contact induces transcription of the Tα1 α-tubulin promoter.

We have previously demonstrated that expression of Tα1 α-tubulin mRNA is increased following axonal injury, and that the extent of the increase is a function of the amount of axon that is lost (see Chapter 1 Introduction III α-tubulin). Using a Tα1:nlacZ transgenic mouse to define the molecular mechanisms underlying this pattern of gene expression, we have demonstrated that target contact represses expression of the Tα1 gene expression at the transcriptional level: following axotomy of facial motoneurons, the Tα1:nlacZ transgene is rapidly upregulated, transcription

is maximal for 1-7 days, and, if neurons regenerate and reinnervate their target musculature, transcription returns to control levels. However, if regeneration is inhibited, transgene expression remains elevated, suggesting that target contact represses expression of the Tα1 gene. Interestingly, in sympathetic neurons of the superior cervical ganglion, unlike the endogenous mRNA (Tα1 mRNA only increased when neurons were axotomized close to, but not distal from their cell bodies), transgene expression was increased regardless of the injury site. This further supports the hypothesis that Tα1 expression at the transcriptional level is dependent on repression only from target contact and not from Schwann cell interactions.

It should be noted there are limitations of data from transgenic mice. Firstly, the 1.1 kb promoter may not contain the complete regulatory sequences or may be missing a repressor element. Secondly, due to the qualitative nature of X-gal staining, the transgene induction may be "exaggerating" the endogenous Tα1 gene expression in a long cut experiment. Direct evidence of transcriptional activation of endogenous Tα1 expression can be demonstrated by nuclear run-off experiments, although there are doubts if this is feasible. It would also be interesting to set a cold block closer to or far away from the neuronal cell bodies, although techniquely it is extremely difficult because of the smallness and extensive branching of motor nerve fibers towards its target, and it is virtually impossible to apply cold block in the sympathetic nervous system.

What is the nature of the repressive target-derived signal? Our cold block experiments block not only fast axonal transport, but also action potential propagation (Wu et al., 1993, 1994). On one hand, application of colchicine/vinblastine on intact peripheral nerve to block axonal transport without interfering the electrical activity have been shown to induce some neuronal gene expression such as c-jun, June D and GAP-43 etc. (Gold & Austin, 1991; Woolf et al., 1990; Herdegen et al., 1991; Leah et al., 1991; also see Chapter One IV1a). Thus the repressive signal is suggested to be dependent of retrograde axonal transport. For Tα1 α-tubulin, we do not believe that the signal is target-derived neurotrophins. In fact, Tα1 α-tubulin mRNA expression is increased by a number of different growth factors, including the neurotrophins. The neurotrophin-dependent sprouting process induces Tα1 α-tubulin gene expression through fundamentally different mechanism from that of regeneration process. On the

other hand, studies by Caroni et al. (1992) indicate that expression of Tα1 α-tubulin gene may well involve patterned electrical activity that occurs after synaptogenesis (see chapter One IV1c). Our data are consistent with this hypothesis; local cooling of the nerve blocks electrical contact between the neuronal cell body and the target, and it induces Tα1 α-tubulin mRNA. However, both of these two hypothesis remains to be tested and substantiated, and they may well coexist as "suppressive signals" for Tα1 α-tubulin gene expression.

3. Intrinsic autoregulatory mechanisms of Tα1 mRNA

It is well known that the synthesis of both α- and β-tubulin mRNAs is under tight control by their own protein product, with the tubulin monomer:polymer ratio being a key determinant in the stability of tubulin mRNAs (see II tubulins). In sympathetic neurons of the superior cervical ganglion, differences were observed between the Tα1:nlacZ transgene, and the endogenous mRNA (see chapter 4). While transgene expression was increased regardless of the injury site, Tα1 mRNA only increased when neurons were axotomized close to, but not distal from their cell bodies. We have hypothesized that this "distance effect" is due to posttranscriptional mechanisms that couple tubulin mRNA levels to cytoskeletal status; when cultured sympathetic neurons were treated with colchicine, which depolymerizes microtubules, steady-state Tα1 mRNA levels were greatly decreased. This additional intrinsic mechanism may allow tight control of tubulin synthesis throughout neuronal growth, remodeling and regeneration.

However, we do not know how important a role this posttranscriptional autoregulatory mechanism plays in in vivo situations such as following nerve injury. In rat brain, the half-life of total tubulins (preassembled and assembled) has been shown to be relatively stable, varying from 4 to 9 days (Forgue & Dahl, 1978; Hemminki, 1973). It is likely that the estimated half-lives determined for tubulin were influenced by the variations in the extent to which tubulin was assembled into microtubules, and their association with microtubule-associated proteins. We do not have much information on how fast tubulin monomers are degraded in injured versus normal neurons in vivo. Is there a transient accumulation of tubulin monomers following a long cut? How rapidly does it downregulate the increased synthesized mRNA? All these questions remain to be clarified in the coming future.

How do we interpret the results from our cold block experiments on Tα1 α-tubulin mRNA expression, based on our latest studies of transgenic model versus Tα1 α-tubulin mRNA autoregulation? While Tα1 α-tubulin mRNA expression is induced by target derepression of unknown inhibitory signal following a cold block, increased amounts of tubulin have to be transported from the cell body down into the axon, to avoid tubulin mRNA autodownregulation by tubulin accumulation inside the cell body. Although we have not studied the cold block effect on slow axonal transport of tubulins, there may be a local accumulation of tubulins around the cold block region which may in turn perturbs slow axonal transport. This may "entrap" the induced tubulin and "falsely" signal the neurons to transport induced tubulin down into the axons.

IV Conclusions

There exit multiple mechanisms mediating neuron, Schwann cell and target interactions. The present thesis work has made significant contributions of understanding the molecular and cellular mechanisms underlying peripheral nerve injury responses. We demonstrate that at least some alterations are independent of macrophage invasion or local inflammatory response. Furthermore, our data support the idea that peripheral nerve injury perturbs a few or many aspects of the intrinsic homeostatic interactions, thereby inducing a series of phenotypic changes that are manifested in changes in gene expression. While the nature of homeostatic signals remains unclear, our data suggest that the target influence neuronal expression of growth-associated genes at the transcriptional level, and that neurons are capable of intrinsicly monitor the status of the axonal cytoskeleton in a dynamic fashion following axonal injury.

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ABBREVIATIONS FOR INTRODUCTION AND DISCUSSION

Apo E: Apolipoprotein E

a, bFGF: acidic, basic Fibroblast Growth Factor

BDNF: Brain Derived Neurotrophic Factor

CAM: Cell Adhesion Molecule

CNP: 2', 3'-Cyclic Nucleotide 3'-Phosphohydrolase

CNS: Central Nervous System

CNTF: Ciliary Neurotrophic Factor

CNTFR: Ciliary Neurotrophic Factor Receptor

DRG: Dorsal Root Ganglion

DT-A: Diphtheria Toxin A

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

FAAT: Fast Anterograde Axonal Transport

GAPs: Growth Associated Proteins

GAP-43: Growth Associated Protein-43

GGF: Glial Growth Factor

gp130: Glycoprotein 130

IL-1, 6: Interleukin-1, 6

IFN-gamma: Interferon-gamma

LIF: Leukemia Inhibitory Factor

LIFR: Leukemia Inhibitory Factor Receptor

MAG: Myelin Associated Glycoprotein

MAPs: Microtubule-Associated Proteins

MBP: Myelin Basic Protein

MHC: Major Histocompatibility Antigens

N-CAM: Neuronal Cell Adhesion Molecule

Ng-CAM: Neuro-glia Cell Adhesion Molecule

NGF: Nerve Growth Factor

NT-3, 4: Neurotrophin-3, 4

p75 NTR: p75 Neurotrophic Receptor

p75 NGFR: p75 Nerve Growth Factor Receptor; equivalent to p75 NTR

PMP-22: Peripheral Myelin Protein-22

PNS: Peripheral Nervous System

SCa, b: Slow Component a, b

SCG: Superior Cervical Ganglion

SCIP: Suppressed cAMP-induced POU

TGFβ: Transforming Growth Factor β

Trk A, B, C: Tyrosine Kinase Receptor A, B, C

TNF-α: Tumor Necrosis Factor α