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Transport Characteristics of Cytoskeletal Proteins During Regeneration of Rat Sciatic Sensory Axons

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, **"Transport Characteristics of Cytoskeletal Proteins During Regeneration of Rat Sciatic Sensory Axons"** submitted by **Brent A. Reynolds** in partial fulfillment of the requirements for the degree of **Master of Science.**

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ABSTRACT

Injury to mammalian nerve cell axons typically results in the formation of axonal sprouts and elongation of the nerve fibre till it reaches an appropriate target. Movement of the cytoskeleton, from its site of production in the cell body, through the axon is an important event during regeneration. The aim of this study was to understand better the change in the transport of two major cytoskeletal proteins, tubulin and actin, during regeneration of the peripheral nervous system (PNS).

Specifically, the following two hypotheses were tested:

i. the increased rate of regeneration seen in conditioned lesioned (CL) nerves is due to the accelerated axonal transport of tubulin and actin and

ii. newly regenerating fibres receive all their actin and tubulin from those present in the axon prior to the injury.

A significant change was found in the transport profile for tubulins and actin following a distal (55-60 mm) crush injury. Following a single lesion (SL) the proportion of tubulin and actin being transported in the slow component a (SCa) phase is decreased, while the proportion of actin in the slow component b (SCb) phase is increased. A CL, given 7 days prior to the test lesion (TL), enhances this effect such that there is a further decrease in the proportion of tubulins and actin in the SCa phase and an increase in the proportion in or ahead of the SCb phase. This effect was seen for tubulins and actin produced after the TL and for tubulins which were in transit prior to the TL. These results demonstrate that an increase in the rate of regeneration, for CL nerves, is related to a greater proportion of tubulins and actin travelling at a faster velocity, supporting the first hypothesis.

Tubulin, actin and calmodulin, synthesized 3 days following a distal crush lesion, were rapidly transported (approximately 34 mm/day) past the crush site and into the regenerating fibers. This demonstrates that cytoskeletal proteins, which are produced in response to injury, are transported to, and probably used by, regenerating fibers. Hence, regenerating axons do not derive all their structural proteins from those present in the axon prior to the injury, thus refuting the second hypothesis.

The transport of actin and tubulins to regenerating axons reveals that cytoskeletal proteins are transported at velocities exceeding those recognized for SCb. Tubulin, actin and calmodulin, in unlesioned nerves, was found to be transported at a velocity of at least 240 mm/day. In regenerating nerves the upper limit could not be calculated, however, a velocity of at least 180 mm/day was determined. This demonstrates that actin, tubulin and calmodulin can be transported at a velocity in the range of fast axonal transport.

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TO KAREN AND MERCEDES

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GENERAL INTRODUCTION

In the mammalian peripheral nervous system (PNS) injury to axons is usually followed by the restoration of nerve fiber structure and function? In contrast, in the adult mammalian central nervous system (CNS), similar injuries, although followed by production of some axonal branches from proximal segments of the severed axons, and collateral sprouting from adjacent uninjured neurons, does not result in significant axonal elongation and regeneration is aborted.

One approach in trying to understand why the CNS will not regenerate is to study a system that does regenerate and compare this to a non-regenerating system. This thesis is concerned with the role played by the transport of the cytoskeleton in relation to regeneration of the PNS. Such a study will hopefully contribute to the overall understanding of the necessary events which occur in a regenerating system. Such information may bring us further to understanding why the CNS does not regenerate and suggest possible ways in which it can be enticed to do so.

INTRODUCTION

The basic functional unit of the nervous system is the neuron. Neurons vary considerably in size, shape and have an unique morphology in relation to other cells in the body, and are generally composed of 3 principal components. The cell body is the enlarged portion of the neuron which contains the nucleus

and the majority of the cell's synthetic machinery (Golgi apparatus, endoplasmic reticulum, rough endoplasmic reticulum). It is here where protein synthesis occurs. Each neuron bears two types of processes - usually several dendrites and a single axon. Both function to transmit electrochemical signals: in general, dendrites passively carry information towards the cell body while axons carry it away in the form of nerve impulses. Axons typically contain greater than 90% of the neuronal cytoplasmic volume.

Neuronal proteins are produced exclusively in the soma and dendrites. Hence, axons are dependent upon the cell body for essential components, and on the axonal transport system which moves these elements from the cell body to the most distal portions of the axon. The role which the cell body plays in maintaining the axon, in both the normal and injured condition, and especially the way in which it transports the needed elements, has been extensively studied and comes under the general heading of axonal or axoplasmic transport.

AXOPLASMIC TRANSPORT

The idea of components being transported from the soma through the axon towards its terminals is an old one. Santiago Ramón y Cajal (1928), in explaining the dependence of an axon on its cell body, suggested that the neuronal soma produces trophic substance which are involved in the maintenance of axon and the end organ which the axon innervates. Paul Weiss (Weiss, 1943) demonstrated that axoplasm is continuously being generated in

the soma and is subsequently transported down the axon. This was based on his observation that following local constrictions nerve fibers exhibit a characteristic conformational asymmetry with a proximal dilation and distal shrinkage. Weiss suggested that the axon acts as a feeder column carrying structures from their sites of production in the cell body, to distant peripheral sites where they serve to replace catabolic losses from the axon and from terminal discharge of transmitters, neurohumors, and trophic agents. The widely-cited Weiss and Hiscoe paper of 1948 concluded that the rate of transport was approximately one millimeter per day. This paper is considered to be the starting point for the modern study of axonal transport.

Axonal transport is conveniently divided into three components according to the velocity and direction of the transported materials. There are two anterograde components, slow and fast, and one well documented fast retrograde component. In addition there have been several reports of a slow retrograde component (Gainer and Fink, 1980; Mata et. al., 1985).

FAST AXONAL TRANSPORT (FAT)

Fast anterograde transport (FAT) was first reported by Miani (1963) who used radioactive phosphate and amino acids to label phospholipids and proteins. His study indicated a rate of transport greater than 70 mm/day. In the mid and late sixties investigators classified varying velocities of FAT (120 - 500 mm/day) in different systems (Dahlström, 1967; Lasek, 1968a,b). FAT carries membranous axonal components including a wide variety of proteins (Barker et.

al., 1976; Bisby, 1977), amino acids (Sturman, 1979), calcium (Neale and Barker, 1977), gangliosides (Forman and Ledeen, 1972), nucleic acids (Por et. al., 1978), neurotransmitters and their enzymes (Laduron, 1984). In mammalian nerves, FAT advances at a velocity of 200 - 400 mm/day. For fish and amphibia the rate is considerably slower because of the lower body temperature (McEwen and Grafstein, 1968).

The mobile force for FAT is provided by a cytoplasmic protein, kinesin, which can hydrolyse adenosine triphosphate (ATP) and is simultaneously attached to transported organelles and microtubules along which they move (Weiss et. al., 1986). Electron microscopy of squid axoplasm has revealed the kinesin protein projecting from vesicles like side arms (Langford et. al., 1986). It is thought that the vesicles are propelled down the microtubule by alternating binding, pivoting and detaching of the kinesin side arms from the microtubule (Langford et. al., 86).

Retrograde axonal transport is responsible for the movement of axonal constituents towards the cell body at a velocity of approximately 200 mm/day (Graftstein and Forman, 1980). Retrograde transport is thought to act as an informational transport system carrying information about the status of the axon terminals to the cell body (Vallee et. al, 1989) in addition to recycling anterogade materials which have reached the terminal (Bisby, 1987). A separate ATPase, dynein, is responsible for fast transport in the retrograde direction (Vallee et. al., 1989; Schnapp and Resse, 1989).

SLOW AXONAL TRANSPORT (SAT)

The third type of transport is referred to as slow axonal transport (SAT) and represents movement of the cytoskeleton and cytoplasmic matrix at a velocity of between one and four mm/day. SAT was probably first observed by van Leeuwenkoek in 1717 when he reported the out flow of a pearly fluid from the ends of freshly cut optic nerve (Ochs, 1987). Based on his observations of oozing axoplasm from the cut sections of giant nerve fibers Young (1936,1944) suggested that the continuous production of axoplasm in the cell body creates a positive pressure which propels axoplasm down the nerve fibers. Young's observation and interpretation forms the basis of the popular "pushing" hypothesis of axonal growth (Bray, 1987).

The findings of Weiss and colleagues (Weiss and Hiscoe, 1948; Young, 1936,1944) and the formulation of the "axonal flow" theory by Weiss encouraged other investigators to look for direct evidence by using radioactive substances which could be taken up by the nerve cell body and transported down the axon (Samuels et. al., 1951; Ochs and Burger, 1958). With the appearance of tritiated amino acids in the early 1960's, Droz and LeBlond (1963) provided strong evidence for the hypothesis that neuronal proteins were produced in the soma and transported into the axon.

With positive evidence that slow transport did exist investigators began studying the dynamics and intricacies of axonal transport. Lasek (1968a,b) demonstrated that when the slow component of axonal transport is labelled with

[H3] leucine a distinct peak of labelled proteins migrates down the axon without diminishing or flattening even after periods as long as 60-90 days. This stability of the slow component proteins led some investigators to hypothesis that this transport component is composed of a relatively stationary portion of the axoplasm, such as the neurofilaments and microtubules (McEwen and Grafstein, 1968). This hypothesis was supported by electron microscopic radioautographic studies which indicated that the slowly migrating proteins were associated with neurotubules and neurofilaments (Droz, 1969). Karlsson and Sjöstrand (1971) were the first to report the existence of multiple rates of transport. With the use of gel electrophoresis Willard et. al. (1974) and Hoffman and Lasek (1975) were able to demonstrate the complexity of the different phases of axonal transport. Electrophoretic separation of labelled transported proteins has shown that there are at least five rate components or classes of axonal transport (Lasek and Brady, 82) [see table 1]. Group 4 and 5 are the slow transported proteins which have been termed slow component b (SCb) and slow component a (SCa), respectively.

CLASSES OF TRANSPORT••

Transport Group	Type of Structure	Rate	Direction	Protein Composition
Membranous Elements				
FAT (group 1)	Vesicles, granules	400mm/d	Orthograde	Gylcoproteins, gylcolipids peptides, catecholamines neurotransmitters, membrane associated vesicles
Fast retrograde (group 1)	Multivesicular bodies	200mm/d	Retrograde	Endocytosed and recycled elements
Group 2	Mitochondria	40mm/d	Orthograde	Mitochondria proteins, fodrin
Cytoplasmic elements				
Group 3	Myosin containing complex	15mm/d	Orthograde	Myosin-like protein
SCb (Group 4)	Microtrabecular Matrix (MTL)	2-5mm/d	Orthograde	Actin, tubulin, enzymes, calmodulin, clathrin, fodrin
SCa (Group 5)	Microtubule-neuro-filament network	0.2-1mm/d	Orthograde	Tubulin, neurofilament triplet, tau proteins fodrin

•• Adapted from Lasek and Brady, 1982

Table 1. Classes of axonal transport.

Slow Component a (SCa)

Approximately 75% of the label transported in SCa resides in five polypeptides (Hoffman and Lasek, 1975). Two of these proteins are alpha and beta tubulin while the other three are the neurofilament (NF) triplet proteins. Microtubules are composed of alpha and beta tubulin but also include a number of other proteins termed microtubule-associated-proteins (MAP's), which affect the properties of microtubules. Interestingly, not all the MAP's found in whole brain microtubules are transported with SCa (Tytell et. al., 1981). Only a subset of the tau class of MAP's (58,000 - 70,000 daltons) move with the microtubules in the axon (Matus et. al., 1981). Remaining MAP's are found in the soma and dendrites. A fourth type of protein transported in this group is a doublet of molecular weight 240,000 and 250,000 daltons which is called fodrin (Lasek et. al., 1984; McQuarrie et. al., 1986). It is believed to be the neuronal form of spectrin and is the most likely candidate for the structural protein that links the axonal cytoskeleton to the axolemma (Levine and Willard, 1981; Hirokawa et. al., 1984). This is supported by studies which demonstrate that axonal fodrin interacts with actin microfilaments and that erythrocytic spectrin attaches actin microfilaments to the cytoplasmic face of the plasma membrane (Levine and Willard, 1981). Actin, the other major cytoskeletal protein, was initially thought not to be transported with SCa (Black and Lasek, 1980; Willard et. al., 1979), however, recently it has been shown to be transported with both SCb and SCa (McQuarrie et. al., 1986; Oblinger et. al., 1987). Furthermore, two other proteins, clathrin (a protein which interacts with membranous structures

and has a well documented role in endocytosis [Goldstein et. al., 1979]) and calmodulin (a calcium binding regulatory protein which interacts with a wide variety of proteins and cellular structures [Cheung, 1980]), both previously thought to be transported exclusively in SCb (Brady and Lasek, 1982), have been found moving with SCa in rat spinal axons (McQuarrie et. al., 1986).

Many of the identified proteins conveyed in SCa are structural proteins. The coherent transport of this group of proteins suggests that the cytoskeletal elements in SCa may play a key role in maintenance of the neuronal cytoacrchitecture (Lasek, 1980; Lasek, 1981).

Slow Component b (SCb)

While the polypeptide composition of SCa is relatively simple, the composition of SCb is very complex. Two dimensional gel electrophoresis and fluorography reveals the presence of hundreds of different proteins transported in this group. Of the three major cytoskeletal proteins, actin, neurofilaments, and tubulins, only the neurofilaments are not transported with SCb (McQuarrie et. al., 1986; Oblinger et. al., 1987). Fodrin is transported with this group along with clathrin and calmodulin. Other proteins which have been found to move as a part of SCb are soluble metabolic enzymes such as nerve specific enolase, pyruvate kinase and creatine phosphokinase (Brady and Lasek, 1982; Oblinger et. al., 1987).

Differences Between Proteins in SCa and SCb

The appearance of some proteins in both SCa and SCb, specifically actin and tubulins, has raised the question of whether or not these proteins are identical

and why they travel at two different velocities. With respect to actin, Oblinger et. al. (1987) found no obvious differences between the morphology of the fluorography spot of actin being transported with SCa or SCb. However, Morris and Lasek (1984) have found differences in the solubility of the two actin populations and Fath (1985, cited in Oblinger et. al, 1987) noted a difference in polymer length. These results suggest that the two populations of actin do differ and that these differences may explain why actin travels in both phases.

Tubulin is present in SCa and SCb in both rat ventral motor neurons (VMN) axons and dorsal root ganglion (DRG) axons but is not found in SCb of optic axons (McQuarrie et. al., 1986; Oblinger et. al., 1987) or axons of the corticospinal tract (Oblinger, 1984). Although Oblinger et. al. (1987) did not find any differences in electrophoretic properties of SCa and SCb tubulins when comparing profiles from 2D gels, Tashiro and colleagues found an associated protein which suggests a difference between SCa and SCb tubulins, or at least provides an explanation for why the two velocity groups exist. Analysis of SCa and SCb tubulin revealed distinct protein compositions (Tashiro et. al., 1984). A polypeptide with a pI (isoelectric point) similar to beta-tubulin and molecular weight slightly lower than alpha-tubulin is present only in the SCa fraction. This polypeptide, called NF-associated protein (NAP), is present only in the nervous system, has been shown to be different from alpha- and beta-tubulin by peptide mapping, and is tightly associated with the NF-enriched cytoskeleton (Tashiro and Komiya, 1987). Hence it is hypothesized that the association of tubulin with SCa is mediated by the NAP and its link to the slow moving neurofilaments. This

is supported by a study of the vagal nerve which contains few neurofilaments so that the interaction with microtubules is low. In this system tubulin transport occurs as a single symmetrical wave with a velocity of 3.4 mm/day, comparable to SCb (Tashiro and Komiya, 1983b).

In addition to the different subunit composition between SCa and SCb tubulins, contrasting solubilities between the two groups has also been suggested to be responsible for differing rates. Preliminary studies of Brady and Black (1986) indicate that a greater proportion of tubulin moving with SCb is soluble relative to the proportion being transported with SCa. This suggests that a greater portion of tubulin transported with SCa is assembled into microtubules, while the majority of SCb tubulin is unpolymerized. Hence, SCa tubulin may demonstrate a slower velocity relative to SCb not only because of its interaction with the neurofilaments and the presence of NAP but it may also be related to the fact that the majority of SCa tubulin is assembled into microtubules while a large proportion of SCb tubulins are unassembled, perhaps, allowing freer movement in association with the cytoplasmic matrix.

Denoulet et. al. (1989) have demonstrated, for motor axons of the rat sciatic nerve that the same isoforms of tubulin are transported in SCa and SCb. However, the distribution of transported proteins in soluble and cytoskeletal fractions of the nerve are differentially distributed. This suggests that specific isotubulins may be involved in the stability and function of the cytoskeleton.

MECHANISMS FOR SLOW TRANSPORT: STRUCTURAL VS. UNITARY HYPOTHESIS

While many theories have been put forth to explain how cytoskeletal and related elements are transported through the axon, basically two remain today. These two theories are opposed primarily in two areas:

i) the form in which materials are transported, and

ii) the motor responsible for movement of the transported material.

Structural Hypothesis

The structural hypothesis has been directly explained as follows; "If, as we propose, each group of proteins corresponds to a specific structure, then the movement of the proteins in each group will be determined by the motile properties of that structure" (Tytell et. al., 1981). Since SCa is primarily composed of neurofilaments and tubulins (Hoffman and Lasek, 1975) it has been proposed that SCa corresponds to a microtubule-neurofilament network (Black and Lasek, 1980; Tytell et. al., 1981). Ultrastructural studies demonstrate crossbridging between these structures (Hirokawa, 1982; Tsukita et. al., 1982). SCb is thought to represent movement of the microtrabecular lattice (this is a term used by Wolosewick and Porter [1979] in referring to the microfilaments, microtubules and associated proteins) (MTL) and cytoplasmic matrix (Black and Lasek, 1979, 1980; Brady and Lasek, 1982; Ellisman and Porter, 1980; Lasek et al, 1984). Biochemical and ultrastructural evidence for interactions between the MTL and axolemma support the hypothesis that this is a coherent structure

moving as a unit (Brady and Lasek, 1981; Ellisman and Porter, 1980; Lasek et. al., 1984; McQuarrie et. al., 1986). For example, fodrin which moves with SCb has a subaxolemmal location, and like its erythrocytic counterpart, spectrin, interacts with actin microfilaments and the axolemma (Hirokawa, 1982; Levine and Willard, 1981). In addition, actin microfilaments interact with microtubules (Griffith and Pollard, 1982). Therefore, it is assumed that SCb proteins interact with one another and that this is why they are transported at the same velocity (McQuarrie et. al., 1986).

According to the structural hypothesis all proteins conveyed by the SAT system are synthesized on free polysomes and assembled into their appropriate structures in the region of the cell body before being transported to the axon terminal (Hoffman and Lasek, 1975; Lasek, 1981; Lasek et. al., 1984). Upon reaching the axon terminal the structures are disassembled and degraded via cytoplasmic proteases, which are regulated by calcium (Lasek and Hoffman, 1975; Lasek, 1986).

The mechanism for the movement of these structures is not well defined and rarely is addressed. McQuarrie et. al. (1986) has suggested that a SCb motor is responsible for the movement of the microtubules and that the movement of other structures travelling with SCb is due to their interaction with the microtubules. This "motor' is indirectly responsible for the movement of SCa proteins. The rate of the movement of SCa components is related to the extent to which SCa structural proteins interact with the proteins of SCb (primarily the neurofilaments and microtubules). Fodrin has been suggested to be one of the

essential links in this process since it demonstrates peaks in both the SCa and SCb phases (McQuarrie et. al.,1986).

Lasek (1986) has proposed a polymer sliding hypothesis which is very similar to that discussed by McQuarrie et. al. (1986). In this hypothesis Lasek refers to SCa polymers and SCb polymers. Like the structural hypothesis, transported materials are synthesized into structures, or in this case polymers, near the cell body. Since, the SCb polymers move more rapidly than the SCa polymers, interactions between the two cause a transfer of force which propels the SCa polymers in the anterograde direction. In turn, this interaction also slows down the SCb polymers. "In this way interaction between the slow moving SCa neurofilaments and the SCb microtubules may produce the large population of slow moving microtubules that lags behind the SCb wave" (Lasek, 1986). Although this theory does support the large body of evidence which Lasek and colleagues have accumulated its major weakness is that no actual mechanism has been proposed for the "motor".

The structural hypothesis is not without further problems. The theory proposes that once the structures are assembled near the cell body they pass through the axon as a coherent interacting group. Although this is supported by electron micrographs which show extensive cross-bridging between neurofilaments and microtubules, it is important to remember that these are static images which do not reflect the dynamic nature of cross-bridging structures (Nixon, 1987). One of the strongest pieces of evidence for the structural hypothesis is that groups of structures have similar rates of

movement. For example, NFs and a subset of tubulins travel at the same velocity (SCa, 0.2 - 1.0 mm/day) while actin, clathrin, fodrin, calmodulin and a second subset of tubulins travel at a different velocity (SCb, 2.0 - 5.0 mm/day). This coherence between certain proteins to travel at similar velocities suggests the proteins are somehow associated. However, this could reflect a similar mode of transport for these polypeptides rather than a stable association between them.

As mentioned previously the structural hypothesis states that tubulin proteins are polymerized at the site of synthesis. The polarity of microtubules is such that their "plus" or fast growing ends are located distally from the cell body (Heidemann et. al., 1981). This would mean, according to the structural hypothesis, that tubulin proteins are added on to growing microtubules at the "minus" or slow growing end, for which there is little evidence (Nixon, 1987). Bamburg et. al. (1986) have demonstrated that the axon tip is the major site of microtubule assembly during axonal growth, a finding which does not support the structural hypothesis. Furthermore, if the neurofilaments and microtubules are transported as a cross-linked matrix this network should remain relatively uniform throughout the length of the axon. However, the number of microtubules per unit density of cross-sectional area has been shown to decrease in a proximal to distal direction (Logvinenko and Nixon, 1986), while neurofilaments show the reverse distribution (Nixon, 1987). This opposing change in density of two components does not support the structural hypothesis. In addition, the structural hypothesis asserts that the cytoskeleton is

constantly moving (Lasek, 1986), however, Nixon and Logvinenko (1986) have demonstrated that there are stationary neurofilaments and Watson et. al. (1986) have reported a pool of stationary microtubules.

In summary, the structural and sliding polymer hypothesis pictures cytoskeletal and related elements being transported as assembled structures beginning near the cell body. The interactions between different polymers (i.e. actin, fodrin, tubulin) allow them to travel as a group (i.e. SCb). The motor for the transport of the cytoskeleton is contained in SCb. The interaction of SCb structures with SCa polymers (neurofilament-microtubule network) not only drags the SCa proteins through the axon at a slower velocity (approximately 1.0 mm/day) but also limits the velocity of SCb.

Unitary Hypothesis

While the structural theory must rely on at least two different motors to explain slow and fast anterograde transport the unitary hypothesis of Sidney Ochs (or the microstream hypothesis of Weiss) relies on one. While the Ochs and Weiss theories differ in their mechanism for slow transport they complement each other in their description of the form in which proteins are transported. In contrast to the structural hypothesis, where the majority of the proteins are assembled into their respective polymers in the region of the cell body and moved as a cross-linked cytoskeletal structure, the unitary and microstream hypothesis proposes that the material is conveyed in soluble subunit form or as small fragments which can assemble into stationary structures at different points along the axon (Weiss and Gross, 1982). Individual components are hooked

onto the same transport system which is responsible for FAT and moved down the axon until they are dropped off. Slowly transported materials have a lower affinity for the transport mechanism and are associated with the mechanism for a lower proportion of the time than fast transported materials (Ochs, 1984). The leading and trailing edges of SCb peaks would support this idea: these could be accounted for by a range of affinities of the protein in question for the transport mechanism. The apparent result would be a wave-like movement of the bulk of tubulin which is leaving the cell body at any one time. Hence, the SCb wave for tubulin appears as a direct result of tubulin's affinity for the FAT carrier. SCa tubulins would have an even slower velocity because its affinity for the FAT carrier would be less than SCb tubulins'. A major advantage of this theory is that SAT is regarded as an epiphenomenon of fast transport and hence does not require an additional force generating mechanism (Weiss and Gross, 1982).

Okabe and Hirokawa (1988) have demonstrated that tubulin subunits are incorporated at the distal ends of microtubules along the axon. They did this by microinjecting biotin-labelled tubulin into the cell body of a PC12 cell and using an anti-biotin antibody to find the location of the injected tubulin. Their results revealed that injected tubulin subunits are not incorporated into microtubules near the cell body but rather throughout the entire neurite. They propose that free tubulin subunits are moved into neurites and incorporated locally into the cytoskeleton at the "plus" ends of the microtubules along the neurite. The result of their study provides strong support for the unitary hypothesis.

In summary, the unitary hypothesis suggests that cytoskeletal elements are transported through the axon as free subunits and are incorporated into the existing cytoskeleton at various positions. The generating force is the same as is responsible for the FAT system. Cytoskeletal elements move slower than fast transported elements due to their lesser affinity for the transport system. Although this theory is supported by indirect evidence (much like the structural hypothesis) it tends to provide a framework for many of the experimental findings which the structural hypothesis cannot explain, however, the unitary hypothesis has not gained popular acceptance. This is probably due to the overwhelming amount of work which has been published by Lasek and colleagues.

EFFECTS OF INJURY ON THE NERVE CELL AND AXONAL TRANSPORT

Following injury to nerve cell axons distinct changes occur both proximal and distal from the site of injury. The sequence of events which occur distal to the injury site is referred to as Wallerian degeneration and is characterized by an initial dissolution of axoplasmic components and loss of the axolemma, formation of myelin ovoids, reabsorption of degenerating myelin, mitosis of Schwann cells, and formation of Schwann cell columns (bands of Büngner) which act as guides for regenerating nerve fibers (Spencer et. al., 1981). Oaklander and Spencer (1988) have recently provided evidence which suggests that the progressive anterograde failure of FAT, distal from the injury site, is

responsible for Schwann cell proliferation. The nature of this fast transported substance which suppresses Wallerian degeneration is unknown.

Bernice Grafstein (1983) has suggested the following classification of events occur proximal to the injury site. "Disruptive" events which comprise the nonspecific consequences of injury; "regulative" events which bring about a turning on of homeostatic mechanisms;"restorative" events whose function is reconstruction of the new axon. An example of a disruptive event may be the influx of excess Ca++ (Schlaepfer and Hasler, 1979) or entry of other exogenous materials (Sparrow and Kiernan, 1979) as a direct result of the destruction of the axonal cellular cytoplasm. The influx of such materials would have a nonspecific effect on the axon and its soma in that it would disrupt a broad range of metabolic events (Grafstein, 1983). Regulative events involve more specific metabolic functions which are concerned with the restoration of intracellular homeostasis. An example of this may be the decreased production of neurotransmitter related materials which are probably not needed in the same quantities following injury as they were prior to the injury (Ross et. al., 1978). A further example of a regulative event would be the change brought about by cutting off the supply of retrograde transported trophic factors which originate in the axon's target tissue and maintain normal metabolic functions (Varon and Adler, 1980). Restorative events may include those that are geared towards axonal growth and reestablishment of synaptic function, and it is on these that I would like to concentrate.

FAT

Analysis of restorative changes has been studied extensively in the retinal goldfish optic nerve and rat sciatic nerve. Both these systems exhibit robust axonal regeneration and their cell bodies are located in defined, easily accessible locations. The rate of FAT is between 40 - 100 mm/day in normal goldfish optic nerves, but doubles between the second and tenth day following injury to the optic tract (McEwen and Grafstein, 1968; McQuarrie and Grafstein, 1982). In addition, there is also an increase, within 24 hours after axotomy in the amount of protein being transported by FAT system (Grafstein and McQuarrie, 1978; McQuarrie and Grafstein, 1982; Perry et. al., 1987). A detailed study of the optic nerve and tract following an optic nerve crush by Perry et. al. (1987) revealed that although there is an increase in the amount of labeled protein being transported by the FAT system there are no novel fast transported proteins, but rather an increase in the uptake of label by certain ones. One of these proteins is a growth associated protein with an apparent molecular weight of 43 KDa (GAP-43) (Skene and Willard, 1981a,c). The goldfish analogue of GAP-43 shows the greatest increase of label uptake for FAT protein in both the parent and daughter axons of the regenerating optic tract (Perry et. al.,1987).

In the mammal FAT is not altered in the regenerating peripheral nervous system (Griffin et. al., 1976; Bisby, 1978). Although, like the goldfish retinal ganglion cells, there are alterations in the incorporation of label into certain proteins (Bisby, 1978), especially GAP-43 (Skene and Willard, 1981c).

SAT

Non-Mammalian Nerves

In both regenerating and normal goldfish optic nerve axons SCa advances at a velocity of approximately 0.1 mm/day (McQuarrie, 1984; McQuarrie et. al., 1986). In regenerating axons, SCb increases from 0.4 mm/day to 1.0 mm/day (McQuarrie and Grafstein, 1982; Grafstein, 1986), a velocity greater than the rate of regeneration (0.2-0.4 mm/day) (McQuarrie and Grafstein, 1981). More dramatic than the change in the rate of SCb transport is the increase in the amount of label incorporated into SCb proteins. McQuarrie and Grafstein (1982) have demonstrated an increase in label of SCb proteins beginning at one day and reaching a maximum of seven times normal 15 days post-lesion. It is interesting to note that the onset of increased protein synthesis does not begin until four to five days, yet there is an increase in the amount of protein being transported out of the cell body in the SCb phase by day one. This is thought to be due to the diversion of proteins from the soma (McQuarrie, 1988b). With respect to individual protein changes neurofilaments show a decrease in labelling (McQuarrie and Lasek, 1981), while SCb proteins, specifically tubulins and actin show an increase (Giulian et. al., 1980; Heacock and Agranoff, 1982).

Mammalian Nerves

While the transport rates for SCa and SCb in mammalian nerves proximal from the injury site remains the same for both injured regenerating axons and non-injured axons, there are changes in the amount of transported protein

(Hoffman and Lasek, 1980). In regenerating motor fibers Hoffman and Lasek (1980) have shown, for SCa, a 30-40% decrease in labelling for neurofilaments along with a 20-25% reduction in tubulin labelling. In contrast they discovered, for SCb proteins, a two-fold increase in the amount of tubulin over normal while actin remained the same. This effect is probably a direct result of changes in protein synthesis which occur in the cell body following axotomy. Tetzlaff et. al. (1988) found, in the facial nucleus, an increase in the labelling of actin and tubulin, and a decreased neurofilament labelling following crush injury of the facial nerve. If the nerve was cut rather than crushed (not allowing regeneration to occur) the effect was enhanced and prolonged. A similar reduction in neurofilament production was found by Hoffman et. al. (1985).

CONDITIONING LESION EFFECT

The conditioning lesion effect is referred to as "the earlier formation and/or accelerated outgrowth of axonal sprouts in response to a second growth stimulus as compared to a single growth stimulus" (McQuarrie, 1984). This provides an opportunity to investigate regulatory mechanisms for nerve regeneration. Since the subject of the conditioning lesion has been extensively reviewed by Grafstein and McQuarrie (1978) and McQuarrie (1984), I will not concern myself with the manifestations of the conditioning lesion effect but rather with the metabolic and environmental hypotheses which attempt to explain why it occurs.

Following injury to goldfish retinal ganglion cell axons or rat sciatic nerve, fibers regenerate distally from the injury site at a velocity of .4 mm/day or 3-4 mm/day, respectively. If a second lesion is made more than two days later, the fibers regenerate faster (Forman et. al., 1980). In this case the initial lesion is referred to as the conditioning lesion (CL) while the second is called the test lesion (TL). Such treatment has been shown not only to increase the rate of regeneration but also to decrease the initial delay and the time between the TL and functional recovery (McQuarrie, 1984). In general two hypotheses have been put forward to explain the CL effect.

Environmental Hypothesis

Following a CL Wallerian degeneration of the distal axons occurs. When the test lesion is made at the same site or distal from the CL site the outgrowing fibers regenerate over a pre-degenerated nerve. This is the central event for the "environmental" hypothesis which states that the pre-degenerated segment somehow acts as a favourable environment for the growing axons (McQuarrie, 1984). The pre-degenerated segment could influence growth in the following ways:

i) a trophic substance (e.g. NGF, Schwab et. al., 1981) may be produced by the degenerated segment which is then picked up via endocytosis and transported back to the cell body through the RT system where it effects protein synthesis,

ii) a trophic substance in the degenerated segment may act locally on the growth cone stimulating a more rapid rate of elongation,

iii) the degenerated segment may act as a more favourable substrate for the growth cone (this could be accomplished by the production of neural cell surface glycoproteins which have been shown to effect the extension of neurites on axons [Chang et. al., 1987]), and

iv) degeneration products may be re-utilized by the regrowing axons (Skene and Shooter, 1983).

Although this theory sounds plausible there is recent evidence that regenerating axons do not increase their rate of elongation when they cross into a pre-degenerated region of nerve (Bisby, 1988).

Metabolic Hypothesis

While the environmental hypothesis places the onus for increased rate of regeneration on the environment which the axon must traverse, the "metabolic" hypothesis predicts that altered cell body metabolism and delivery of cytoskeletal elements is responsible for faster outgrowth. Central to this hypothesis is the idea that the supply by axonal transport, of proteins that are essential for growth cone function (actin and tubulins), are the determining step controlling the regeneration rate (McQuarrie, 1983a). Since there is a correlation between outgrowth rate and SCb velocity in different types of axons (Lasek et. al., 1981; McQuarrie and Grafstein, 1982; McQuarrie, 1983,1984; Wujek and Lasek, 1983), a good candidate for the CL effect would be increased production of actin and tubulins that are transported with SCb (McQuarrie and Lasek, 1989). One would predict that in conditioned axons the velocity of SCb or the amount of SCb tubulin or actin that it carries would increase according to the

increase in the regeneration rate (McQuarrie, 1986; McQuarrie and Lasek, 1989).

While an increase in the rate of translocation and amount of labelling for the SCb wave in conditioned nerves of the goldfish retinal ganglion cells has been found (McQuarrie et. al., 1982), a similar result has not been unequivocally demonstrated for mammalian peripheral nerves.

McQuarrie has claimed the acceleration of axonal outgrowth is associated with an increase in the supply of tubulins and actin to the regenerating axon and that this supply is the rate limiting step (McQuarrie, 1986, 1988a, 1988b, 1989), however, the statistical significance of this increase in the supply of structural proteins is questionable. With respect to an increase in velocity -- "acceleration of outgrowth appears to be a result of increases in SCb transport that are initiated by the conditioning axotomy. In part, this is a 'diagnosis of exclusion' brought about by an inability to account for the increase in outgrowth rate by any of the changes in [FAT] or SCa" (McQuarrie, 1989). Hence, the increase in the rate of regeneration seen in conditioned nerves, according to McQuarrie and colleagues, is a result of an increase in the SCb velocity which would correspond to the regeneration rate or an augmentation of the amount of SCb proteins transported to the regenerating fibers.

It has also been suggested (Tetzlaff et. al., 1988b) that the interaction between neurofilaments and tubulins (and possibly actin) is a key event for understanding why fibers regenerate faster in conditioned nerves. This is based on the finding that in conditioned axons a TL causes a further decrease in the

synthesis of neurofilaments, and that this reduction in neurofilaments will result in a decrease in the interaction between microtubules and the slow moving neurofilaments (Tetzlaff et. al., 1988b). This would permit an acceleration of microtubules and ultimately increase the rate of regeneration. While this hypothesis is conceptually reasonable it lacks direct evidence, primarily the finding that tubulin and/or actin transport velocity is increased.

Therefore, while the environmental hypothesis is supported by indirect evidence and is missing direct experimental support that axons do grow faster over predegenerated segments, the metabolic hypothesis is hampered by the lack of experimental results demonstrating an increase in the velocity of SCb proteins, relative to the rate of regeneration, in addition to questionable statistical evidence revealing that increased amounts of structural proteins do indeed enter and participate in the regenerating of new axons.

The first experiment of this thesis has been devised in an attempt to test directly the metabolic hypothesis by determining if the increase in the rate of regeneration is related to the velocity of tubulins and actin. The second experiment was conceived as a result of a finding from the first experiment and provides the basis for the formulation of a hypothesis to explain the role which the structural proteins, tubulins and actin, may play in regeneration and how this role may be related to the conditioning lesion phenomena.

CHAPTER 1. TRANSPORT PROFILES OF TUBULINS AND ACTIN IN REGENERATING RAT SCIATIC NERVE

INTRODUCTION

Following injury to nerve cell axons in the PNS the cell body undergoes a series of changes referred to as the cell body response (Lieberman, 1971). Changes in RNA metabolism alters protein synthesis (Aldskogius et. al., 1980) in a manner which is believed to be important in determining whether or not regeneration will occur. I was particularly interested in the prominent changes which occur in the synthesis of the major cytoskeletal proteins, specifically tubulins and actin. These changes, which begin at about 12 hours post-axotomy and have been reported to last for at least 21 days, are characterized by an increase in synthesis for actin and tubulins in the cell body (Hoffman et. al., 1985; Tetzlaff et. al.,1988). While the cell body is upregulating its production of tubulins and actin the injured axon is undergoing changes characterized by growth cone formation and movement distally from the injury site. The time between the lesion and the beginning of regeneration is commonly referred to as the initial delay and lasts one to three days (Black and Lasek, 1979; Forman and Berenberg, 1978). It is important to note that the determination of the initial delay is not a direct measure but rather a backward extrapolation of the outgrowth distance to zero (Grafstein and McQuarrie, 1978). While sprouting

has been reported to occur within a few hours after nerve injury it is generally accepted that definitive sprouting of the majority of axons does not occur before 24 hours (Grafstein and McQuarrie, 1978). Once the fibers do begin to regenerate they do so at a velocity of approximately 4 mm/day (for motor and sensory neurons in the rat sciatic nerve) (Black and Lasek, 1979; Forman and Berenberg, 1978).

Two major cytoskeletal proteins of the growth cone are microtubules and microfilaments (Landis, 1983), both of which are believed to be essential for its motility (Bunge, 1986). Since microfilaments and microtubules are composed of actin and tubulins, respectively, the growth cone and the trailing axon is dependent upon a supply of these two major cytoskeletal components. Chemical disruption of the supply or assembly of these proteins (e.g. taxol, cytochalasin or colchicine) results in growth cone retraction and aborted regeneration (Yamada et. al., 1971). For the mammalian PNS the velocity of SCb tubulin and actin is usually reported to be between 3-4 mm/day (Hoffman and Lasek, 1980; McQuarrie et. al., 1986), a velocity comparable to the rate of outgrowth of regenerating fibers (Black and lasek, 1979; Forman and Berenberg, 1978). This has raised the hypothesis that SCb tubulins and actin supply the regenerating fibers with needed structural proteins and that, the SCb velocity limits the rate at which growth cones can move (i.e. they can only extend as fast as structural materials can be supplied) (Lasek and Katz, 1987; McQuarrie, 1983, 1984,1986; McQuarrie and Lasek, 1989; Wujek and Lasek,

1983). It would appear then that the supply of actin and tubulins to the growth cones and trailing axon are important events.

It should be pointed out that this hypothesis is based on the correlation between the velocity of SCb tubulins and actin and the rate of regeneration of sciatic motor and sensory fibers. It is possible that the relationship between the two velocities is purely coincidental and that the SCb velocity has nothing to do with the regeneration rate. This theory can be tested by conditioning nerves and submitting them to a subsequent TL. Conditioning would increase the rate of regeneration, one could then measure the velocity of SCb tubulins and actin and determine if the SCb velocity has increased proportionally to match the rate of regeneration. Such a finding would certainly strengthen the hypothesis. Conversely, if no increase in velocity of SCb actin and tubulins in conditioned nerves occur this would show that, at least in the conditioned situation, regeneration is not rate limited by SCb velocity.

Increases in the rate of slowly transported proteins during regeneration have been reported, however only two of these studies were concerned with conditioned nerves. In 1969 Grafstein and Murray demonstrated a two to three fold increase in the rate of slow flow following a single TL in the goldfish optic nerve, while in 1982 McQuarrie and Grafstein reported an increase in the slow flow velocity of conditioned nerves following a TL using the same system. With respect to mammalian PNS fibers, Frizell and Sjöstrand (1974) reported an increase in the velocity of slowly transported proteins in the regenerating hypoglossal axons, however, they did not look at specific proteins. For the rat

sciatic nerve, McQuarrie (1981) has reported that the transport velocity of SCb actin and tubulins reflects the rate of regeneration for single lesioned nerves, while for conditioned sciatic nerve fibers, an increase in neurofilament and tubulin velocity has been reported, however, this result has yet to be published in a refereed journal. (McQuarrie, 1983b).

Hence, for the mammalian PNS, only one study has been published (an abstract) suggesting an increase in the velocity of SCb proteins and rate of regeneration in conditioned nerves. **The experiments reported here were undertaken to test the hypothesis that the faster regeneration rate seen in conditioned lesioned nerves is due to an acceleration of tubulins and actin.** Rat sciatic sensory neurons were used because the cell bodies are located in a defined easily accessible location in the dorsal root ganglion (DRG) and their axons are long enough to allow complete separation of SCa and SCb proteins. The transport profile of SCb tubulins and actin synthesized in the cell body 3, 7 and 14 days following a TL were assessed in single-lesioned and conditioned plus test-lesioned nerves. In addition, the profile of SCb tubulins and actin which were already in transport prior to the TL were studied. Because lesions were made up to 60 mm from the cell body it was relevant to ask if tubulins and actin already in the axon prior to the TL demonstrate a change in transport which may explain the increase in the rate of regeneration seen for conditioned nerves.

METHODS

1. Animals

Male Sprague-Dawley rats (250 - 350 grams) were obtained from the University of Calgary Biosciences breeding colony. All animals were weighed prior to any surgical procedure. Eight animals were randomly chosen and were weighed before being killed and weight change was used as an indication of general health following surgery

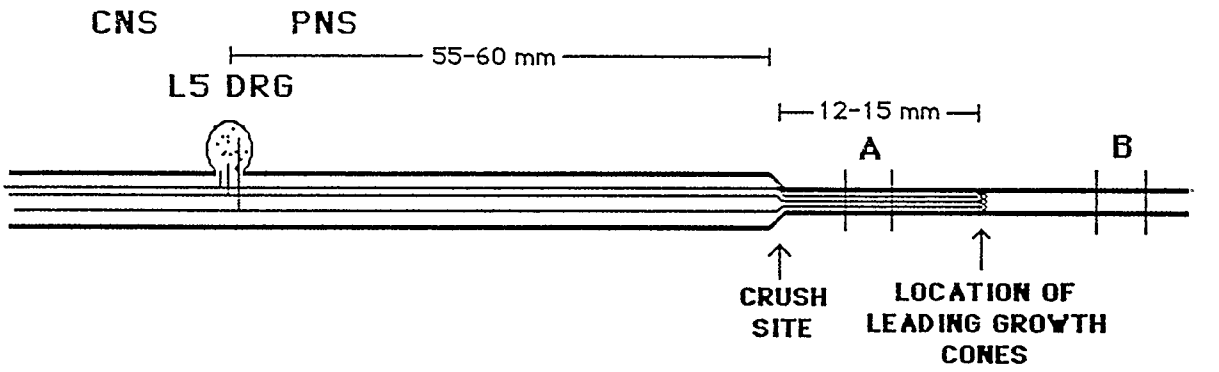
2. Anaesthesia

Prior to any surgery all animals were deeply anaesthetized with a sodium pentobarbital (25 mg/Kg) and chloral hydrate (25 mg/Kg) mixture which was injected intraperitoneally along with Atropine (10mg/Kg). Surgery was performed under aseptic conditions.

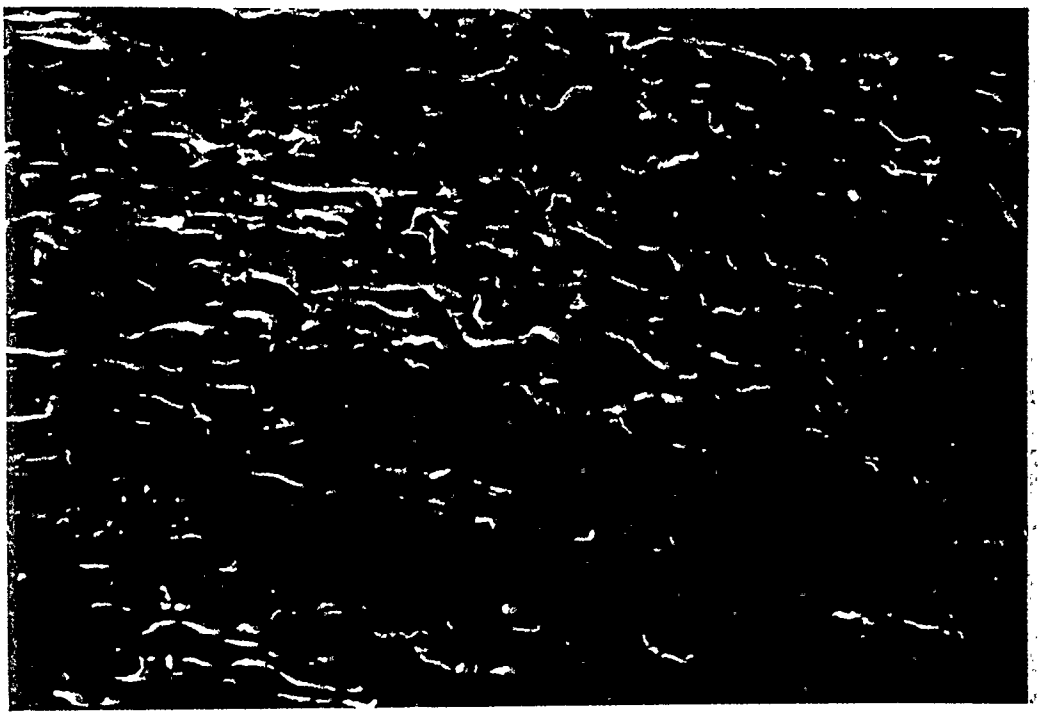
3. Lesions

i. Conditioning Lesions (CL) were performed by exposing the right sciatic nerve at the bifurcation of the common peroneal and tibial nerves (knee). Double 0 silk thread was passed under the nerve and pulled tight against a glass rod for 15 - 20 seconds severing all axons (Figure 1) The injury site was marked with a loose ligature, the wound sutured and the animal returned to its home cage.

Figure 1. Sciatic nerve 5 days following crush lesion. Figure 1A and 1B were probed for neurofilament (anti-NFM) immunoreactivity. Note the presence of NF-immunoreactivity in the segment which contains regenerating neurites and the absence of NF-immunoreactivity in the segment which contains no regenerating axons.


CNS
PNS
55-60 mm
L5 DRG
12-15 mm
A
B
CRUSH SITE
LOCATION OF LEADING GROWTH CONES

A



B

ii. Test Lesions (TL) not preceded by a conditioning lesion were performed in exactly the same manner as the CL. Those following a CL were carried out seven days post CL and in the same manner as the CL, except the injury site was 1 - 5 mm proximal (to avoid scar tissue) from the initial lesion site. The maximal rate of outgrowth has been shown to occur with a seven day condition-test delay (Bondoux-Jahan and Sebille, 1986). Sham operated controls were not used as I believe the exposure of the nerve caused an insignificant amount of stress relative to the laminectomy, which was performed on all animals.

4. Injections

A partial laminectomy was performed on the right side of the animal exposing the fourth and fifth lumbar dorsal root ganglion (DRG). One hundred μCi of L-[35S] methionine (Amersham, specific activity 1120 Ci/mmol), dissolved in 1 μl of Locke's solution, was injected through a glass micropipette into the centre of the ganglia. The injection pipette was left in place for three to five minutes to reduce leakage from the swollen ganglia. The spinal cord was covered with a piece of saline soaked Gelfoam, back muscles were sutured with 4-0 silk thread and the skin closed with wound clips. Animals recovered on a hot plate set at 37 degrees celius, after which they were returned to their home cage and given free access to food and water. For the first week following surgery the drinking water contained tetracycline, acetaminophen (0.5 mg/ml) and sugar, to increase the palatability. Although surgery was performed under aseptic conditions and antibiotics were used post-surgery some animals

demonstrated a slight infection. Severely infected animals were killed prior to completion of the experiment. Less than 10% of the animals exhibited autotomy of their foot on the lesioned side. These animals were killed immediately and not included in the results.

5. Time Intervals and Tissue Removal

Experimental animals were randomly divided into four experimental groups (Figure 2):

i. those which received L-[35S] methionine injections three, seven or 14 days following a TL and were killed five days later;

ii. those which received isotope injections three, five, seven or 14 days after a TL, which was preceded by a CL seven days earlier;

iii. animals which received isotope injections three days prior to the TL and were killed seven days after the injection and;

iv. those which received a CL, and then four days later were injected with isotope, followed by a TL three days later, and were killed four days after the TL.

Two different sets of non-lesioned controls were used, those which were injected and then killed 5 days later and those which were killed 7 days following the injection. Two different controls were used so experimental groups, which had a five or seven day injection - kill interval had a corresponding five or seven day injection - kill interval control group.

EXPERIMENTAL GROUPS

i - TEST CRUSH $\xrightarrow{3,7,14\,D}$ INJECT (L5 DRG) $\xrightarrow{5\,D}$ KILLED

ii CONDITIONING CRUSH $\xrightarrow{7\,D}$ TEST CRUSH $\xrightarrow{3,7,14\,D}$ INJECT (L5 DRG) $\xrightarrow{5\,D}$ KILLED

iii INJECT (L5 DRG) $\xrightarrow{3\,D}$ TEST CRUSH $\xrightarrow{4\,D}$ KILLED

iv CONDITIONING CRUSH $\xrightarrow{4\,D}$ INJECT (L5 DRG) $\xrightarrow{3\,D}$ TEST CRUSH $\xrightarrow{4\,D}$ KILLED

CONTROL GROUPS

INJECT (L5 DRG) $\xrightarrow{5\text{ or }7D}$ KILLED

Figure 2. Time course for lesioning and injecting the four experimental groups (i, ii, iii, iv) in addition to controls.

Following a lethal injection of chloral hydrate the thoracic cavity was opened and the right atria cut so as to drain the blood from the animal. The right sciatic nerve, from the injected DRG to the foot, was quickly removed, cleaned, straightened, placed on a card strip and immediately frozen on dry ice. Nerves were stored at -70 degrees celsius.

6. Calculation of the Rate of Regeneration

Eight days following the test lesion a subset of animals were tested to determine the location of the fastest-growing sensory axons by the pinch-reflex test (Gutmann et. al., 1942). The animals were anaesthetized and the sciatic nerve was exposed from the site of injury distal to the ankle. Using Dupont #5 forceps the sciatic nerve was pinched, beginning at the most distally exposed section of the nerve. The animal was observed for a leg movement and changes in respiration in response to pinching of the sensory axons. If a reflex was not observed the pinch site was moved 1-2 mm proximal and the procedure repeated till a pinch reflex was observed. This was taken as the location of the fastest growing axons and the distance from the original test lesion site (marked with a ligature) was recorded. The same procedure was carried out for animals with conditioned nerves. A Mann-Whitney U-test was used to determine significance.

7. Preparation of Samples for One-Dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (1-D SDS PAGE)

The frozen sciatic nerve was cut into 3mm segments. Each segment was placed in a stainless steel mortar at -70 degrees celius and pulverized. Each

individual segment was placed in a 1.5 ml Eppendorf centrifuge tube along with 60μl of a SDS-sample homogenization buffer (10% [v/v] glycerol; 5 % [v/v] 2-mercaptoethanol; 0.0625 M Tris; 3.6 % [w/v] SDS). The sample was homogenized for 30s with a machined Teflon pestle in a Eppendorf tube and centrifuged for 10 min at 10,000 RPM. A 10μl aliquot of each supernatant was taken, added to 5 ml of scintillation fluid (Scintiverse, Fisher) and counted in a scintillation counter for 5 min. From this the total activity of the entire supernatant was determined. This was done so as to determine if the injection was succesful and whether activity levels were high enough to continue with processing the tissue. A similar procedure (McQuarrie et. al., 1986) has demonstrated that greater than 95% of the radioactivity in the homogenate is in the supernatant.

8. 1D SDS PAGE and Fluorography

(Redshaw and Bisby, 1984)

Beginning with the supernatant from the ganglion and continuing with each successive 3 mm segment, 30 - 40 μl aliquots were run on 1D 5 - 15% gradient polyacrylamide gels with a 5% stacking gel. In the left hand lane of each gel 5 μl of low molecular weight protein standards in 3.6% SDS (Biorad) was run. Maximum voltage was set at 350V with a starting current of 25 mA per gel. Gels were run for approximately four hours.

Following electrophoresis, gels were stained with 0.1% w/v Coomassie Brilliant Blue R250 in 5% v/v acetic acid and 47.5% v/v ethanol. After staining overnight, gels were destained in 40% v/v methanol/5% v/v acetic acid until the

background was clear. Stained gels were observed to determine if they were correctly loaded with protein and that proper separation of proteins had occurred. Satisfactory gels were processed for fluorography according to the method of Laskey and Mills (1975) which involves impregnating the gels with a fluor (PPO) by initially removing water from the gel with dimethyl sulphoxide (DMSO), then placing it in a solution of PPO in DMSO (20% w/v) for two hours. The PPO is then precipitated with distilled water. Gels are dried onto filter paper under a vacuum, placed in contact with a sheet of Kodak XAR film and placed in the dark at -70oC. Films were developed after two to four weeks. Figure 3 is a representative 1-D gel illustrating five prominent cytoskeletal proteins in a regenerating nerve.

After development the fluorographs were used as templates to remove gel regions containing tubulins and actin. These were determined from the molecular weight of the bands and cross referencing with previously published identification studies (Lasek et. al., 1984; Oblinger et. al., 1986; McQuarrie et. al., 1986). Excised bands were rehydrated with a drop of water, solubilized in Protosol (New England Nuclear), 5 ml of scintillation fluid was added, and the samples were counted in a liquid scintillation counter.

9. Determination of Tubulin and Actin SCb Peaks and Data Comparison

For tubulins and actin the leading edge of the SCb peak was used as a reference point. The leading edge was considered to be the segment in the SCb region which demonstrated a maximal change in slope. Velocities were calculated by taking the distance between the ganglion and the segment

containing the leading edge divided by the number of days (5 or 7) since the administration of the label. Velocities for each group were averaged, standard error of the mean (S.E.M.) determined and the means compared with other groups. A Mann-Whitney U-Test was used to determine significance as data were assumed to be non-parametric due to small sample size.

10. Standardization of Data and Production of Transport Profiles

Data were standardized by converting the number of counts in each segment to a percentage of the total counts for that protein throughout the entire nerve. Tubulin and actin profiles were produced by calculating the mean (± S.E.M.) for corresponding segments of identically treated animals the results were presented in a line graph (distance from ganglion vs. % of total counts).

Data were standardized because of the large variation between animals when counts per minute (cpm) were used. While all animals were injected with the same amount of radioactivity, leakage of the label from the ganglion could not be controlled. The result was a difference between animals with respect to the amount of label available for incorporation into newly synthesized proteins and hence a significant variation. The standardization procedure changed the data so comparisons of the absolute amounts of labeled protein in individual segments could not be made. For example, I could not compare absolute amounts in two corresponding segments from different groups and conclude that one group contained a greater amount of labeled protein than the other. However, what could be compared between two groups is the difference in the transport characteristics of a specific protein and what type of distribution the

protein shows within the nerve. This can be clarified with a further example; if SCb in control nerves contains 25% of the labeled tubulin for the entire nerve and in lesioned nerves this value rises to 35%, I can conclude that there has been a shift in the distribution of transported tubulin relative to the total proportion of tubulin in the nerve such that a greater percentage of tubulin is transported in SCb for the lesioned group than the control group. I cannot conclude that there were more labeled tubulin subunits in SCb as I am comparing relative not absolute proportions. While the importance of this will become apparent in the discussion, let it suffice to say at this point that the standardization procedure eliminates the influence of changes in protein synthesis on the transport characteristics for a given protein. Hence, in interpreting the results one need not take into account what is happening in the cell body, rather changes in the profiles are a direct result of alterations in the transport of the protein through the axon.

11. Statistical Analysis of Transport Profiles

Data were analyzed by using an analysis of variance (ANOVA) (Practical Statistics [Version 2]) followed by Duncan's New Multiple Range test (same program). For all cases reported, there was a significant group by position interaction, demonstrating that the two profiles were different and that the difference was position dependent. This reflects an underlying change in the transport of the labelled cytoskeletal protein within the axon. Duncan's multiple comparison was used to locate the segments which differed significantly ($p < .05$) between the two groups.

RESULTS

1. Body Weight Loss

Mean loss of body weight for a set of animals (randomly chosen) (n=8) killed five days following isotope injection was 30 ± 11 grams ([mean ± S.E.M.] with a mean starting weight of 295 grams. Rats of this starting weight normally show less than a 5% increase in body weight over a five day period (Bisby, 1980). Therefore, experimental animals demonstrated a weight loss of 15 %, reflecting the surgical trauma which the animals underwent. General activity and grooming behaviour appeared to be normal, indicating the animals were not in continuous pain.

2. Rate of Regeneration

For nerves receiving a single lesion the mean pinch test distance (±S.E.M.) after eight days was 13.3 ± 1.4 mm (n = 8) while for conditioned nerves the average distance was 18.5 ± 1.3 mm (n = 8). This represents a 40% increase in the regeneration distance which is significant ($p < .05$). Given a 1.5 day delay, for SL nerves, (Forman et. al., 1980; Bisby, 1988) before the growth cones crossed the lesion site, the SL regeneration rate was estimated as 3.8 ± 0.4 mm/day, in good agreement with previous studies (McQuarrie et. al., 1977; Bisby, 1978; Forman et. al., 1980). For CL fibers the initial delay is shortened by approximately 40 % (Forman et. al., 1980), giving an estimated regeneration rate of 4.5 ± 0.3 mm/day.

3. Velocity of SCb Tubulins and Actin

Table 2 illustrates the mean velocities for the leading edge of SCb tubulins and actin. SCb tubulins and actin produced 3, 7 and 14 days post-axotomy (single lesioned nerves) do not demonstrate a significant change in velocity relative to the controls. In previously conditioned nerves the velocity of the leading edge of SCb tubulins and actin produced 3,7 and 14 days after the TL are not significantly different from the control nerves. For SL and CL nerves, SCb tubulins and actin already in the axon prior to the TL do not show a significant change in their velocity relative to the controls.

From these data I conclude that the velocity of the leading edge of SCb tubulins and actin do not change significantly in single and conditioned lesioned nerves. This also applies to SCb tubulins and actin which are in the axon prior to the TL.

4. Transport of Tubulins and Actin

Figure 3 illustrates the transport of proteins, produced at the time of injury, through the DRG axons. Actin, tubulins and NF's (NF1, 2, 3) can be clearly identified by molecular weight in addition to calmodulin (C) and fodrin (F). Labelled tubulin and actin can be seen extending from the cell body past the crush site. Neurofilaments are not transported as far, rather they can be seen at a maximal distance of 12 to 15 mm from the DRG.

A.

	Control (5D)	3 days	7 days	14 days
Tubulins	3.9 ± 0.5	3.6 ±0.4	3.3 ±0.5	4.0 ±0.8
Actin	3.8 ±0.5	3.7 ±0.5	3.3 ±0.5	4.0 ±0.8

B.

	Control (5D)	3 days	7 days	14 days
Tubulins	3.9 ± 0.5	3.8 ±0.7	4.0 ±0.4	3.6 ±0.3
Actin	3.8 ±0.5	3.7 ±1.0	4.1 ±0.5	4.0 ±0.6

C.

	Control (7D)	SL^+	CL^+
Tubulins	3.9 ±0.5	3.5 ±0.6	3.4 ±0.5
Actin	3.8 ±0.5	3.9 ±0.5	3.6 ±0.6

^+SL – single lesioned nerve

^+CL – conditioned lesioned nerve

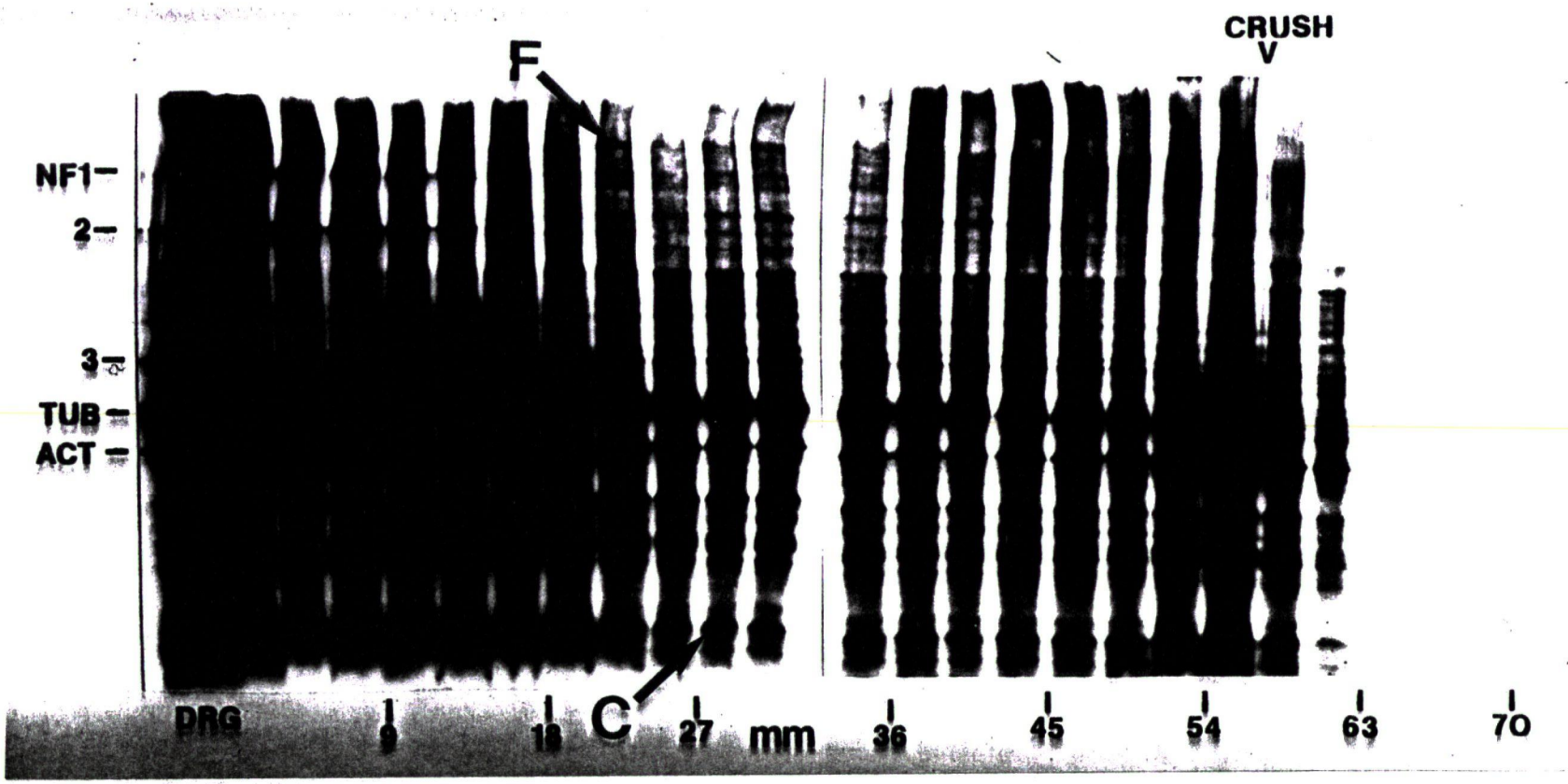
Table 2 [A,B,C]. The above three tables represent the mean velocities for the leading edge of SCb tubulins and actin (± S.E.M). None of the groups differ significantly (n=6).

Table 2A. Tubulins and actin produced 3, 7, and 14 days following a single crush injury.

Table 2B. Tubulins and actin produced 3, 7, and 14 days following a crush lesion in previously conditioned nerves

Table 2C. Tublins and actin in transit prior to the TL, for single and conditioned lesioned fibers

Figure 3. A 1-D fluorograph from a nerve which was lesioned and injected with isotope on the same day. Five days later, beginning with the injected ganglion, 70 mm of the sciatic nerve was removed, sectioned into 3 mm segments and each successive segment was run on 1-D 5 - 15% polyacrylamide gels. Gels were processed for fluorography and exposed to X-ray film for 2 - 8 weeks. Five prominent cytoskeletal proteins can be identified - three neurofilaments (NF), NF1, NF2, and NF3; tubulins (α and β-tubulin) (TUB); actin (ACT); in addition to calmodulin (C) and fodrin (F).


CRUSH
F
NF1
2
3
TUB
ACT
PRG
C
mm
9
18
27
36
45
54
63
70

i. Transport Profile for Tubulins Produced 3, 7, and 14 days Post-Axotomy

Figure 4 compares the transport profile for tubulins from control nerves and for tubulins produced three days following a single crush lesion. A significant interaction effect exists ($F = 5.941$, $p < .001$) and Duncan's multiple comparison indicates that three segments differ significantly (those located 6 mm [$p<.01$], 9 mm [$p<.05$] and 21 mm [$p<.05$] from the ganglion). Hence, for tubulins produced three days after a crush injury, there is a significant reduction in the proportion of tubulins transported with SCa and a significant increase in the proportion of tubulins transported in the region of SCb. At 7 days post-injury (figure 5) there exists an interaction effect ($F = 4.839$, $p < .001$) between the two groups with one segment differing significantly ($p < .01$). This corresponds to a decrease in the proportion of tubulins being transported in the SCa region.

The profile for tubulins produced 14 days post-axotomy (Figure 6) demonstrates an interaction effect ($F = 7.263$, $p < .001$). Duncan's multiple comparison reveals that two segments differ significantly (3 mm and 6 mm from the ganglion, both $p < .01$). This corresponds to a decrease in the proportion of tubulins being transported in the SCa region for the lesioned group relative to the control.

In conclusion, there is a decrease in the proportion of tubulins being transported in the SCa region for all three time points. For tubulins produced three days post-injury there is also an increase in the proportion being transported in the SCb region, and at other times the profiles indicate that the same effect occurs, though this is not statistically significant.

ii. Transport Profile for Tubulins Produced 3, 7, and 14 Days Post-Axotomy in Previously Conditioned Nerves

Comparison of the profiles for tubulins produced 3 days following a TL in single and conditioned nerves (figure 7) reveals an interaction effect ($F = 6.856$, $p < .001$). Duncan's multiple comparison indicates a significant increase in the proportion of SCa tubulins (6 mm from the ganglion, $p < .05$) and an increase in the proportion of tubulins transported just ahead of the SCb peak (27 mm from the ganglion, $p < .05$). The profile for tubulins produced three days post-axotomy in SL nerves from figure 7 differs from the profile in figure 4. While both profiles were produced from the same data, for figure 7 percentages were recalculated for the SL group as the first segment (3mm from the cell ganglion) for the CL group was not used. The activity in this segment for 3 of the 6 nerves was very large. Because this was not a consistent finding it is highly probable that the high activity in this segment was the result of experimental error, hence, the numbers were considered to be artifacts.

At seven days (Figure 8) there is an interaction effect ($F = 9.132$, $p < .001$) in comparing the single and conditioned lesioned groups. There is a significant reduction in the proportion of tubulins transported in and just ahead of the SCa region (3 mm and 9 mm, [both $p < .01$] and at 15 mm [$p < .05$] from the ganglion) and an increase in the proportion being transported ahead of the SCb region over a 9 mm range (24 mm-30 mm from the ganglion, all $p < .05$). Therefore, a CL 7 days prior to the TL causes a further reduction in the proportion of tubulin being transported with SCa and allows more tubulin to be

transported ahead of the SCb wave, relative to the TL alone.

By 14 days the effect seen at 7 days is reduced (figure 9), however, the two profiles do demonstrate an interaction effect ($F = 3.821$, $p < .001$). Duncan's follow-up test reveals a significant change at 9 mm ($p<.01$), 12 mm ($p<.01$) and 24 mm ($p <.05$) from the ganglion. These correspond to a decrease in the proportion of tubulin being transported just ahead of the SCa region and an increase in the proportion being transported just ahead of the SCb wave.

In summary, relative to a SL, a CL 7 days prior to a TL causes a decrease in the proportion of tubulins being transported in the SCa region, for tubulins produced 7 and 14 days post-injury and an increase in the proportion being transported ahead of the SCb peak (for all three time points). This effect seems to be maximum at 7 days following the TL.

iii. Transport Profile for Tubulins in Transit Prior to the Test Lesion

Figure 10 compares the transport profiles for fibers which contained labeled tubulin already in transit prior to the TL and controls (note: there was a 7 day delay between isotope injection and removal of the nerve, previous groups had a 5 day delay). Comparison of the two profiles reveals an interaction effect ($F = 2.639$, $p < .01$), with two segments differing significantly. These correspond to a decrease in the proportion of tubulin transported with the SCa wave (12 mm from the ganglion, $p < .05$) and an increase in the proportion being transported in the SCb region (21 mm from the ganglion; $p < .05$).

For previously conditioned fibers, relative to SL fibers (Figure 11), there is an interaction effect ($F = 4.895$, $p < .001$). The data indicate that a prior CL causes a decrease in the proportion of tubulins being transported in the SCa region (at 6 and 9 mm from the ganglion, $p < .01$ and $p < .05$, respectively) and an increase in the proportion being transported in the SCb region (27 mm from the ganglion, $p < .01$).

To summarize the above two experiments; nerve injury causes a shift in the transport profile for tubulins already synthesized and in transport prior to a single TL (decrease in SCa and an increase in SCb) and this shift is enhanced by a prior CL.

iv. Transport Profile for Actin Produced 3, 7, and 14 days Post-Axotomy

Figure 12 compares the profiles for actin produce 3 days post-lesion with the control group. A significant interaction effect ($F = 5.917$, $p < .001$) exists and a Duncan's follow-up test reveals that three segments differ significantly (those located 6 mm [$p < .01$], 9 mm [$p < .05$], and 12 mm [$p < .01$] from the cell body). Hence, the data indicates, for actin produced three days post-axotomy, a significant reduction in the proportion of actin transported with SCa over a six mm region and a significant increase in the proportion of actin being transported in one segment in the region of SCb.

At 7 days post-injury (figure 13) the two actin profiles show an interaction effect ($f = 6.265$, $p < .001$). There is a significant increase ($p < .05$) in the proportion of actin transported at the beginning of the SCa region and a significant decrease ($p < .05$) in the proportion of actin transported at the leading edge of

SCa. There was no other significant difference at any other point along the nerve.

The profile for actin produced 14 days following a single crush injury (Figure 14) reveals an interaction effect ($F = 4.232$, $p < .001$) with three segments differing significantly. At 6 mm and 9 mm ($p < .01$) from the cell body there is relatively less actin transported in the lesioned group than the control while more actin is being transported at 18 mm ($p < .05$) from the cell body. This corresponds to a decrease in SCa and an increase in SCb actin for the lesioned group relative to the control.

The consistent finding from the three above experiments is that a single crush lesion reduces the proportion of actin travelling in the SCa region. This occurred for actin produced 3, 7, and 14 days after the injury and was most pronounced at 3 days followed by 14 and then 7. In addition there was an increase in the proportion of actin travelling in the SCb region at 3 and 14 days group but not for the 7 day group.

v. Transport Profile for Actin Produced 3, 7, and 14 Days Post-Axotomy in Previously Conditioned Nerves

In comparing the profiles for actin produced 3 days following a TL in single and conditioned lesioned fibers (Figure 15) there is a significant interaction effect ($F = 5.124$, $p < .001$), Duncan's follow-up test reveals that only one segment differs significantly ($p < .01$). A greater proportion of actin is travelling in the segment 6 mm from the cell body in the CL nerves relative to the SL nerves.

For actin produced 7 days after a TL in single and conditioned lesioned fibers (Figure 16) there is no significant difference between the two groups ($F = .742$, $p = .395$) however there is a significant interaction effect ($F = 7.276$, $p < .001$). The proportion of actin 3 and 9 mm from the ganglion is reduced in addition to a significant increase 24 mm from the ganglion (all $p < .01$). This corresponds to a decrease in the proportion of actin travelling in the SCa region and an increase in the proportion travelling just ahead of the SCb peak.

For actin the most striking difference is seen at 14 days after the TL (figure 17). Analysis of variance reveals an interaction effect ($F = 7.776$, $p < .001$). There is a significant decrease in the proportion of actin being transported between the SCa and SCb waves (this occurs for a 12 mm region), an increase in the proportion of actin transported just ahead of the SCb peak (at 24 mm [$p < .05$]), and an increase in the proportion of actin being transported far distal from the SCb wave (33 - 39 mm).

In summary, a CL does not appear to affect the transport profiles for actin produced 3 days after the TL except for a large increase in the proportion of actin leaving the cell body, however, at 7 days a prior CL stimulates a decrease in the proportion of actin travelling in the SCa region and an increase in the proportion moving just ahead of the SCb peak. At 14 days after the TL the transport profile for actin from CL nerves is considerably different than in the single lesioned fibers. The most striking differences are the large reduction in the proportion of actin moving between the SCa and SCb wave and the increase in the proportion being transported well ahead of the SCb wave.

No significant change was found between groups with respect to the transport profile for actin in transit prior to the TL. Hence, the data is not shown.

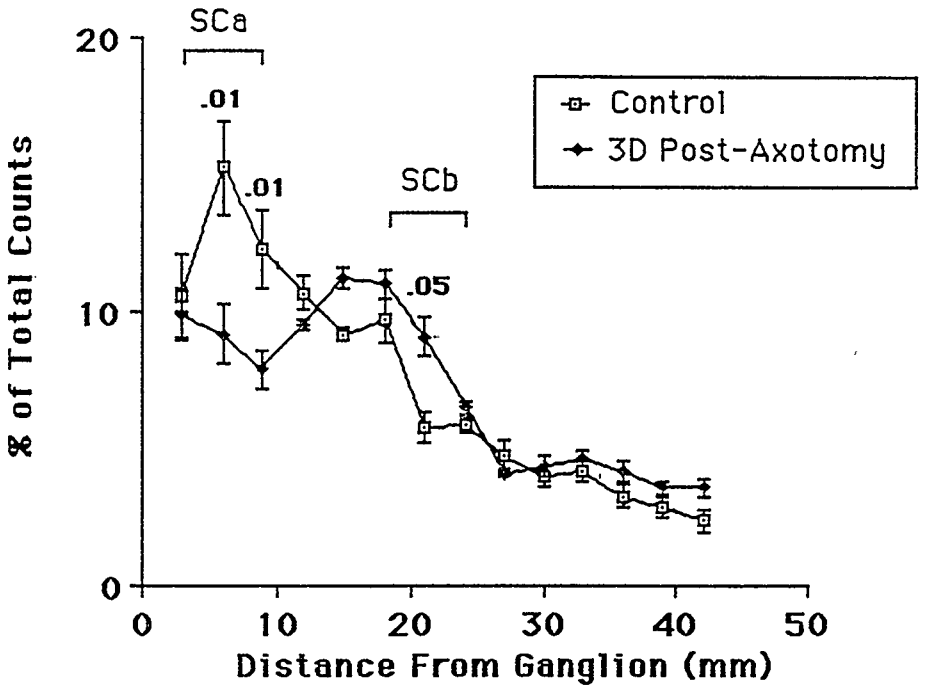


Figure 4. Comparison of the transport profiles for tubulins from control nerves (-□-) and for tubulins produced 3 days following a crush injury (-◆-) (control vs. group 'i' 5 days following precursor injection). Three segments differ significantly (p value is indicated on graph). Data for each 3 mm segment were expressed as a percentage of the total counts in the segment relative to the total activity for the protein in the nerve. "SCa" and "SCb" indicates the position of the SCa and SCb wave, determined from individual nerves.

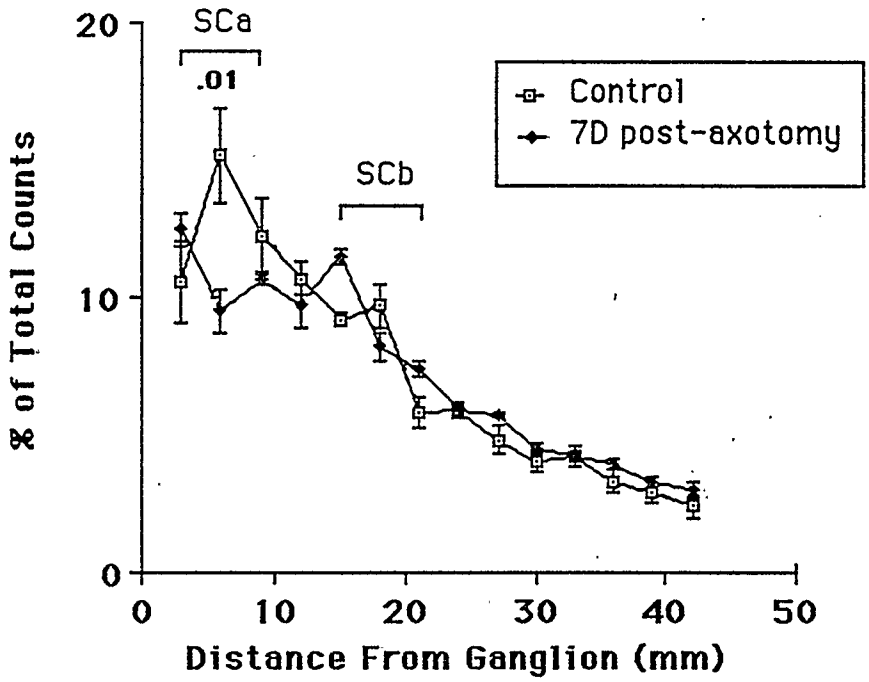


Figure 5. Comparison of the transport profiles for tubulins from control nerves (-□-) and for tubulins produced 7 days following a crush injury (-◆-) (control vs. group 'i'). One segment differs significantly.

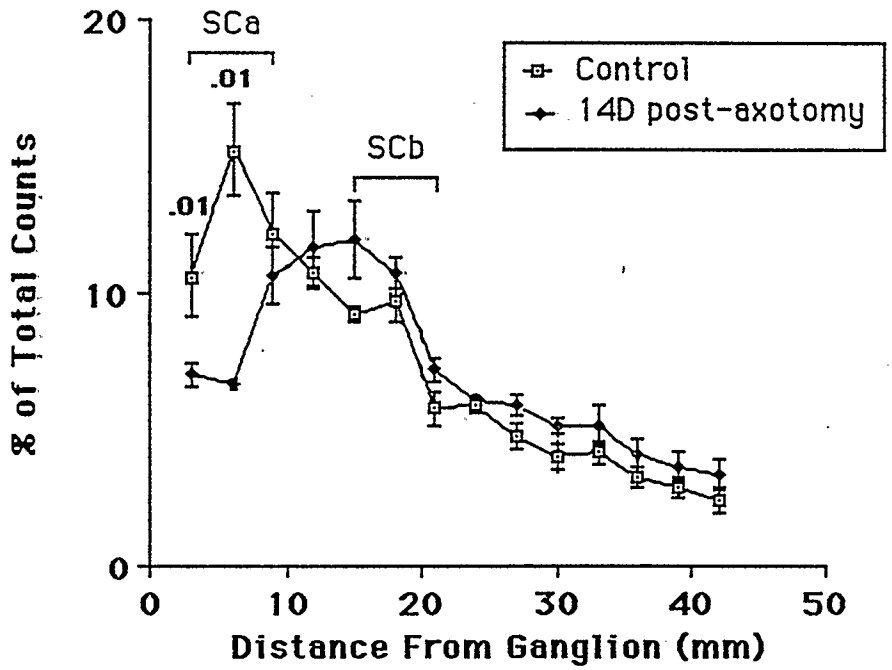


Figure 6. Comparison of transport profiles for tubulins from control nerves (⊡) and for tubulins produced 14 days following a single crush lesion (✦) (control vs. group 'i'). Two segments differ significantly.

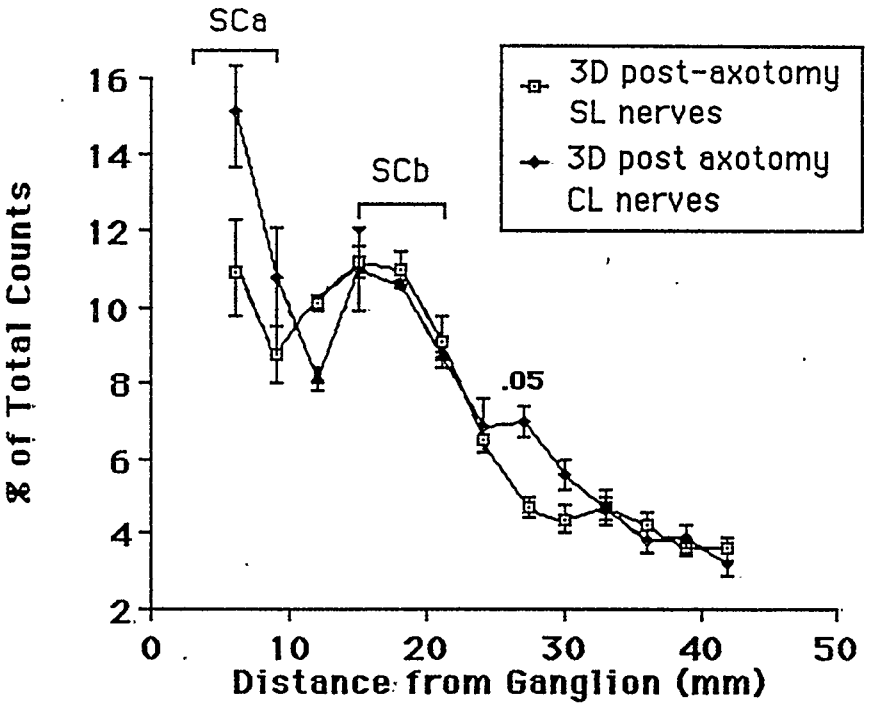


Figure 7. Transport profiles for tubulins produced 3 days post-TL for SL nerves (-□-) and CL nerves (-◆-) (group 'i' vs. group 'ii') five days following precursor injection. One segment differs significantly.

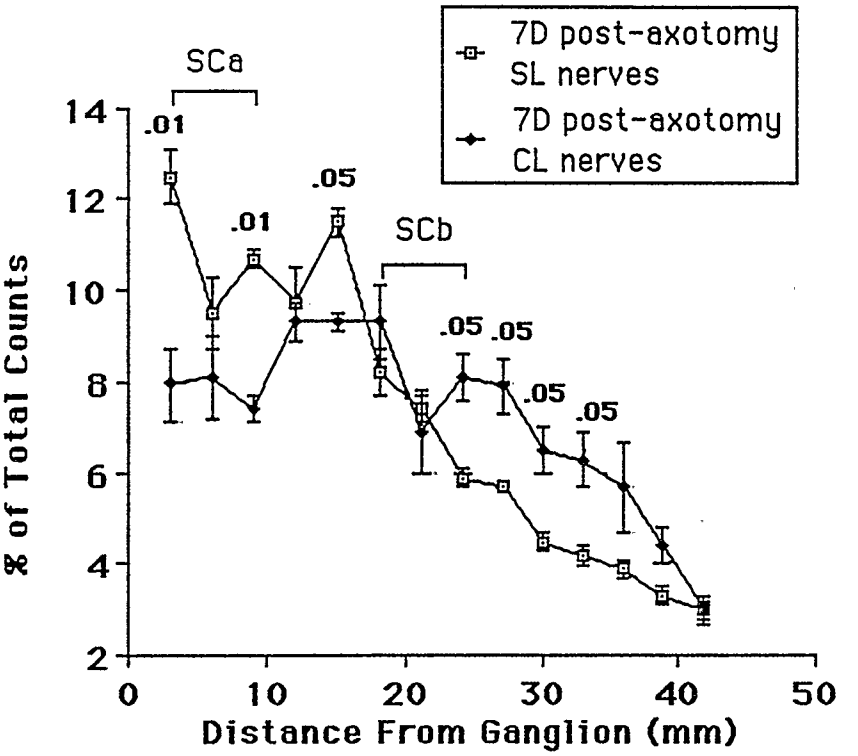


Figure 8. The transport profiles for tubulins synthesized 7 days post-TL for SL (-▫-) and CL (-✦-) nerves (group 'i' vs. group 'ii') contains numerous corresponding segments which are significantly different. The change represents a significant decrease in the proportion of tubulins transported in and ahead of the SCa region and a significant increase in the proportion of tubulins being transported ahead of the SCb wave.

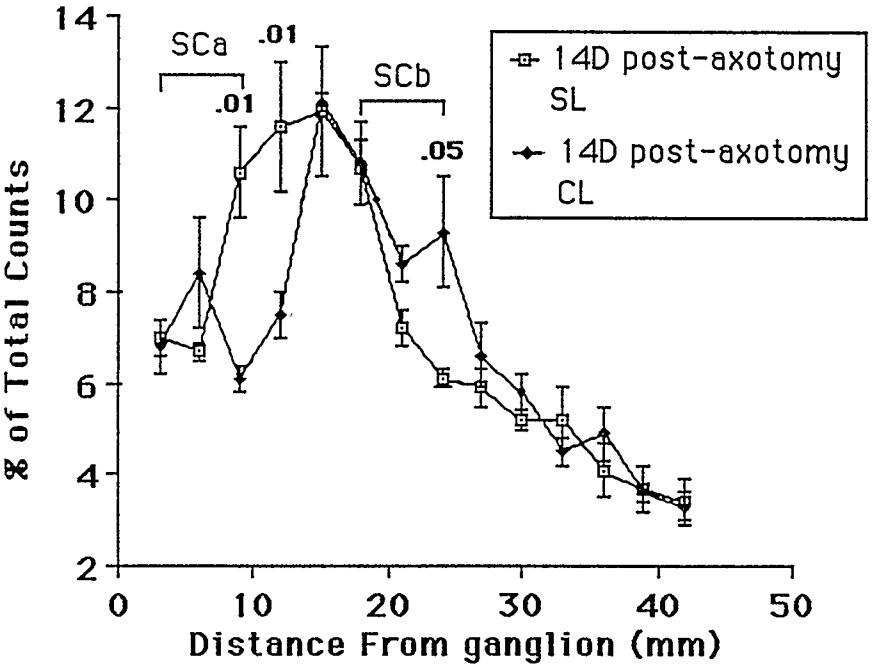


Figure 9. Comparison of the transport profiles for tubulins produced 14 days post-TL for SL (⊡) and CL (✦) nerves (group 'i' vs. 'ii') indicates that a prior CL decreases the proportion of tubulins transported at the edge of and ahead of the SCa wave in addition to increasing the proportion of tubulins being transported at the edge of the SCb wave.

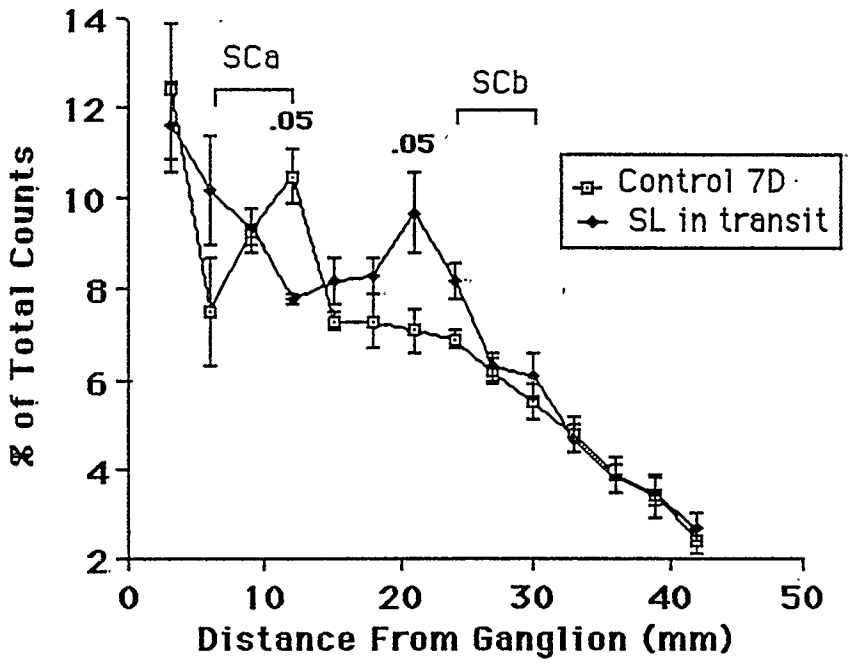


Figure 10. Transport profiles for tubulins from controls (-□-) and for tubulins in transit prior to the TL (-✦-) (7day control vs. group 'iii'). Two segments differ significantly.

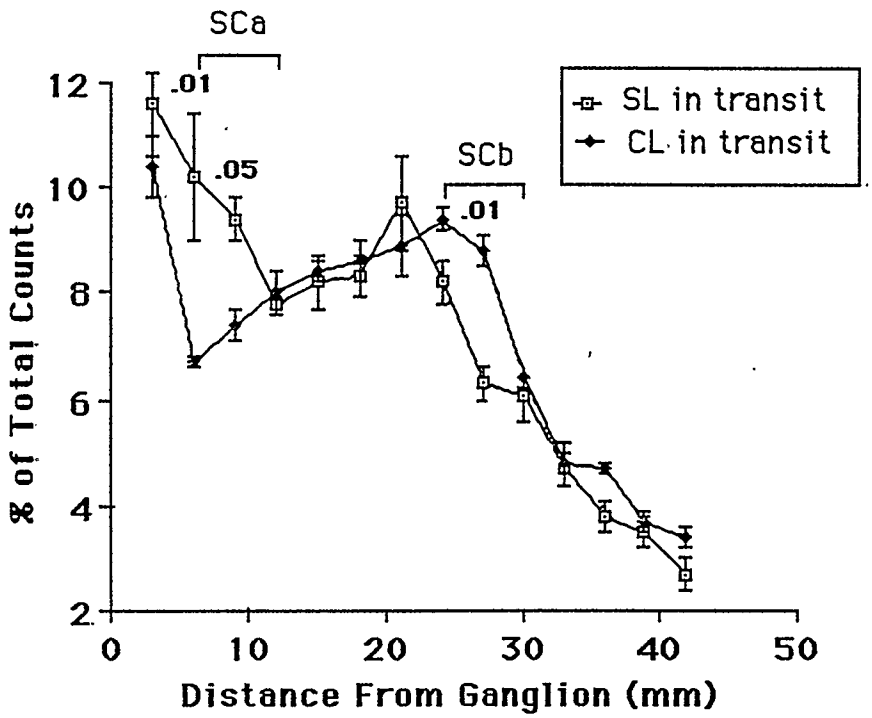


Figure 11. Comparison of transport profiles for tubulins in transit prior to the TL for SL (⊡) and CL (✦) nerves. With respect to CL fibers relative to SL nerves there is a significant decrease in the proportion of tubulins transported in SCa region and a significant increase in the proportion of tubulins transported in the SCb region.

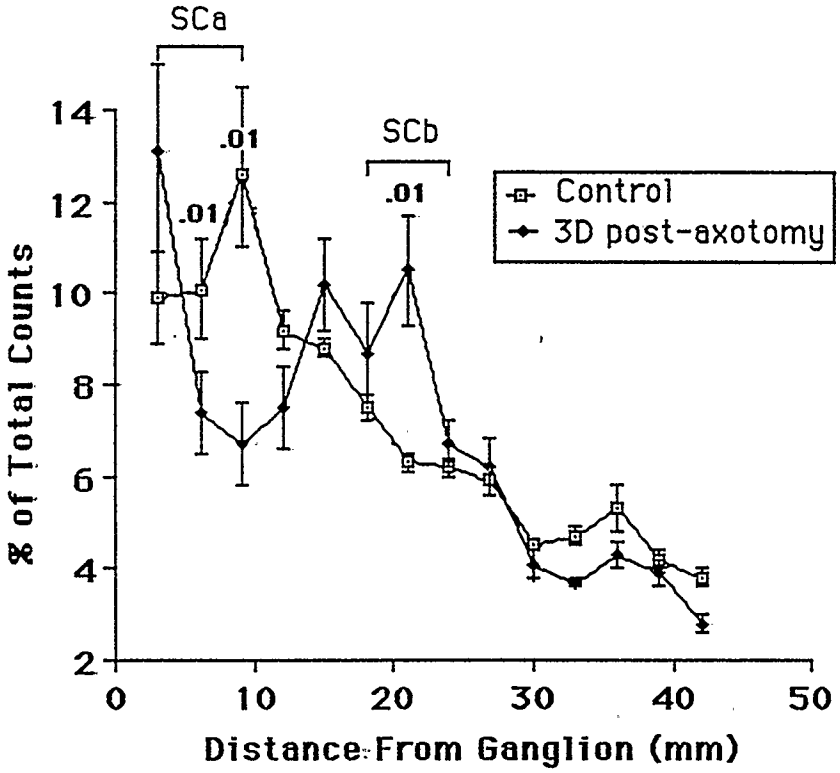


Figure 12. Comparison of the transport profiles for actin from control nerves (-□-) and for actin produced 3 days following a SL. (-•-) (control vs. group 'i'). Three segments differ significantly.

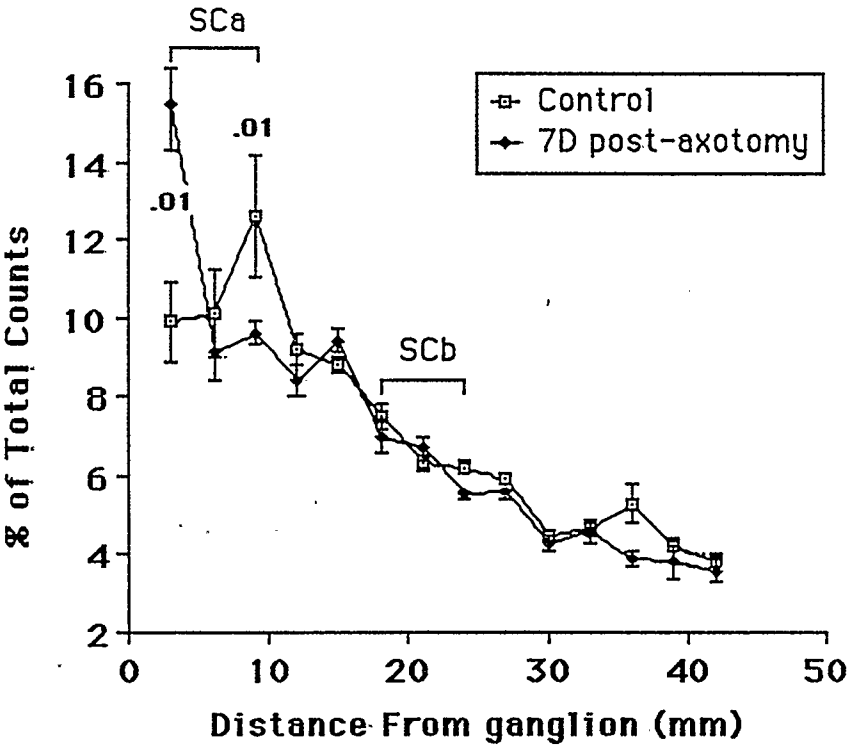


Figure 13. Comparison of transport profiles for actin from controls (-□-) and for actin produced 7 days following a single crush injury (-◆-) (control vs. group 'i'). Two segments differ significantly.

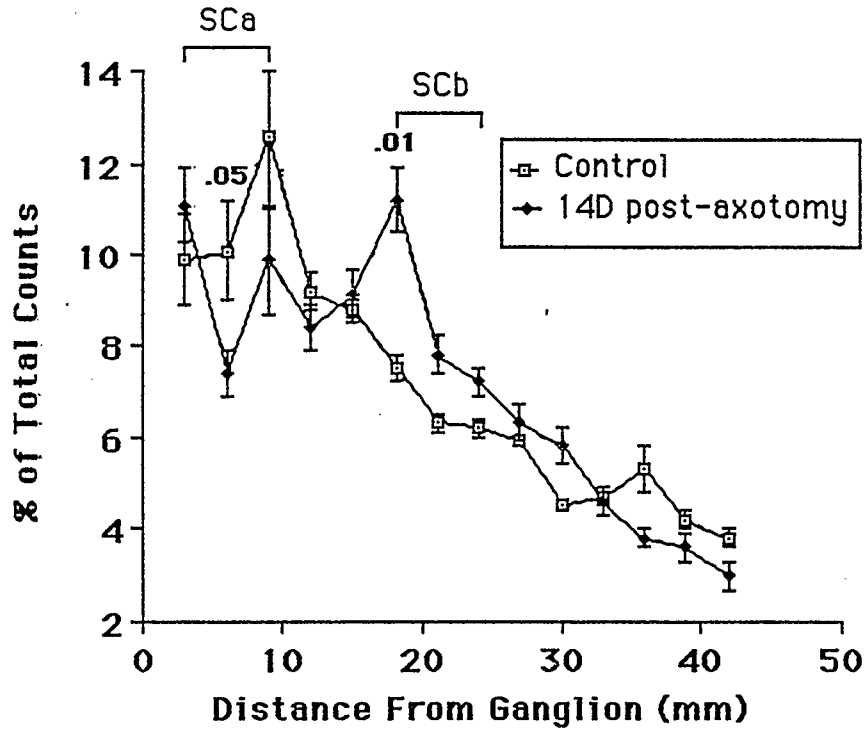


Figure 14. Comparison between the transport profiles for actin from control nerves (-□-) and actin produced 14 days following a SL (-✦-) (control vs. group'i') reveals a significant decrease in the proportion of actin being transported in SCa and a significant increase in the proportion being transported in the SCb region.

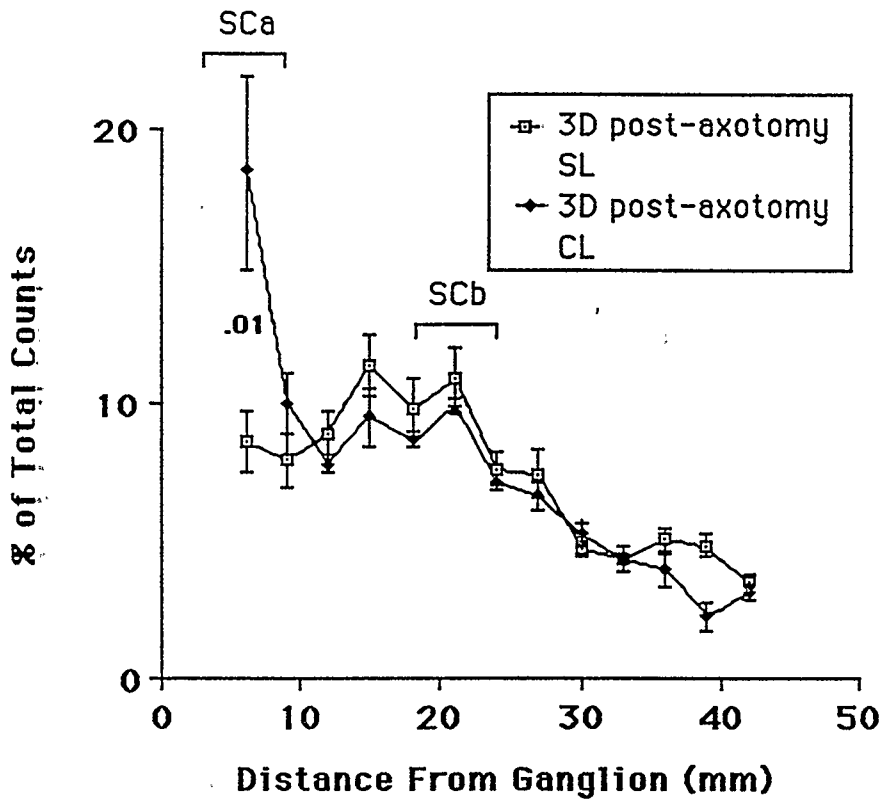


Figure 15. Comparison of the transport profiles for actin produced 3 days following a TL in SL (⊡) and CL (✦) nerves.

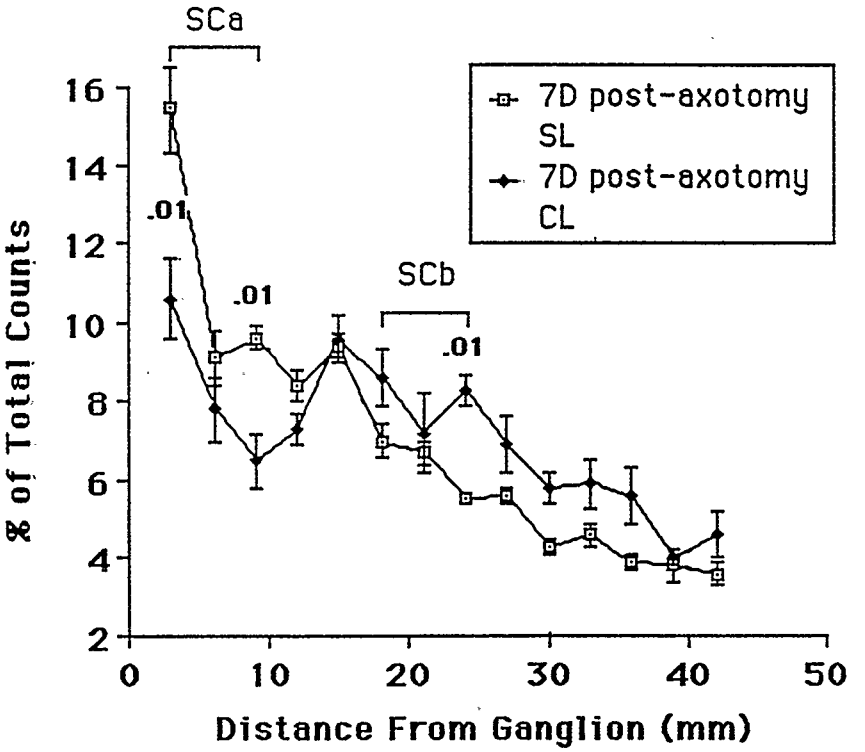


Figure 16. Comparison of transport profiles between actin produced 7 days post-TL for SL (⊡) and CL (✦) nerves reveals a significant decrease in the proportion of actin being transported in the SCa region and an increase in the proportion being transported in the SCb region for the CL nerves relative to the SL fibers.

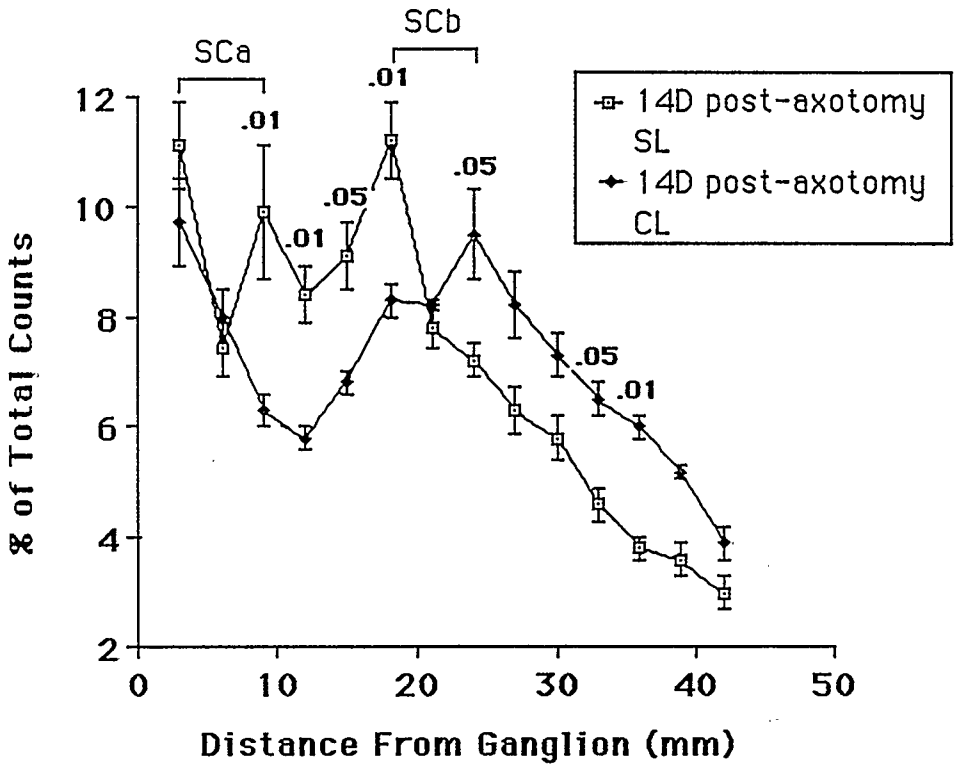


Figure 17. Relative to the transport profile for actin produced 14 days following a TL in SL nerves (-□-) the profile for CL nerves (-•-) appears quite different (group 'i' vs. group 'ii'). There is a significant decrease, in the CL group, for the proportion of actin being transported in and ahead of the SCa wave and a significant increase in the proportion of actin being transported in and ahead of the SCb wave.

DISCUSSION

1. Summary of Changes in the Transport Profiles for Tubulins and Actin Synthesized Pre/Post Axotomy

i. Changes in the Transport of Tubulins

For tubulins produced 3, 7, and 14 days post-axotomy, there is a decrease in the proportion of tubulins being transported in the SCa phase with no other consistent significant change in any other areas of the axon, however there appears to be a non-significant increase in the proportion of tubulins in the SCb region. When neurons received a CL 7 days prior to the TL and the profiles are compared to SL fibers a further decrease in the SCa component is seen (at 7 and 14 days but not 3) along with an increase in the proportion of tubulins being transported in and ahead of the SCb region. For tubulins in transit prior to any injury, the TL lesion caused a redistribution of the transported tubulins with less being transported in SCa and more in the SCb region. A prior CL enhanced this effect relative to the SL group.

ii. Changes in the Transport of Actin

For actin produced 3 days after a single crush lesion there is a decrease in the proportion of actin being transported in the SCa region and an increase in the proportion transported in the SCb phase. For actin synthesized 7 days post-axotomy the increase in the SCb phase is lost and replaced by a large

increase in the proportion of actin near the cell body. By 14 days the increase in SCb reappears.

A CL 7 days prior to the TL alters the transport profile for actin produced 7 days post-axotomy such that a decreased percentage of actin is being transported in the SCa region and an increase in the SCb region (relative to the SL group). For actin produced 14 days after the TL, the prior CL, substantially alters the transport profile by causing a decrease in the proportion of actin in and ahead of the SCa wave and an increase the proportion of actin being transported ahead of the SCb wave. For actin produced three days after the TL the two profiles are very similar except for the large proportion of actin near the cell body for the CL group. There is no effect on the transport profile for actin in transit prior to TL for both SL and CL nerves (data not shown).

The overall impression one gets from these results is that a SL decreases the proportion of tubulins and actin being transported in the SCa phase and increases the proportion of actin being transported in the SCb region. A CL enhances this effect such that there is a further decrease in the proportion tubulins and actin in the SCa phase and an increase in the proportion in the SCb phase, relative to the SL group. This holds for tubulins and actin synthesized after the TL and for tubulins in transit prior to the TL. With respect to the velocity of SCb tubulins and actin I found no change in their velocity in any of the experimental conditions.

My results indicate that a CL increases the velocity of tubulins and actin such that a greater proportion of tubulins and actin are transported

in or ahead of the SCb region. My data does not directly support the hypothesis that the "SCb velocity" limits the rate of regeneration as I found no change in the velocity of the SCb wave. This may have been due to the difficulty in determining the SCb peak for tubulins and especially actin. In some profiles the SCb peak was easily located (typical for tubulins), in others, the peak was barely noticable (typical for actin). Hence, at times it was necessary to determine the location of the peak in a subjective manner. This may have covered over a change in the velocity of the SCb peak for tubulins and actin for the CL group. However, my results imply that an increase in the rate of regeneration may be related to a greater proportion of tubulins and actin travelling at a faster velocity.

McQuarrie (1986,1988a,1988b,1989) has investigated the availability of SCb proteins for conditioned fibers distal from the injury site. McQuarrie suggests that "increased proportions of SCb tubulin and actin reached daughter axons, and that these increases are associated with a sustained acceleration of outgrowth" (McQuarrie, 1989). While his conclusions are promising, the lack of any statistical analysis by McQuarrie necessitates caution in accepting the validity of his interpretations. However, the data from my experiments would support McQuarrie's hypothesis of an increase in the availability of structural proteins being responsible for the increased distance of regeneration found in CL nerves.

An interesting finding in this series of experiments was that tubulins in transit prior to injury show a change in their transport profile (decreased

proportion in SCa region; increased proportion around the SCb region) following a single lesion, and that this effect is enhanced in fibers which have been previously conditioned. This result demonstrates that injury can alter the transport characteristics of proteins which are already in the axon and implies a degree of plasticity in the axon.

2. Possible Mechanisms for Changes in Transport Profiles

i. Tubulins

For tubulins synthesized after the injury, a decrease in the proportion of tubulins transported in the SCa region following a SL and a further decrease in the proportion of tubulins in the SCa phase along with an increase in the proportion in the SCb region for CL nerves was found. For tubulins in transit prior to the TL a SL caused a decrease in the proportion of tubulins in the SCa region and an increase in the proportion transported in the SCb region. A CL prior to the TL enhanced this effect.

Until recently the axonal cytoskeleton was believed to be assembled near the cell body and transported down the axon as stable assembled cytoskeletal polymers (Lasek, 1986). While this hypothesis is supported by a large body of indirect evidence, and more directly by studies which demonstrate that the majority of tubulins and neurofilaments enter the axons in an insoluble form (Black et. al., 1984; Morris and Lasek, 1984), it has recently been challenged.

Studies in developing neurons suggest that the assemble of MT occurs distally from the cell body (Bamburg et. al., 1986) and that tubulins in the growth cone region may have never been polymerized (Robson and Burgoyne, 1989; cited in Hollenbeck, 1989). Development of a new technique which involves microinjection of a labeled tubulin monomer has allowed investigators to observe the axonal transport of tubulins. Okabe and Hiuokawa (1988) have used this technique and concluded that tubulin is moved down the axon in nonpolymeric form and is added onto the distal ends of existing microtubules. Brady and Black (1986) have presented preliminary evidence for DRG cells indicating that a greater proportion of SCb tubulin is soluble (relative to insoluble) while for SCa tubulin the majority is insoluble. Denoulet et. al. (1989) have shown a differential distribution of α and β-tubulins isotypes in SCa and SCb suggesting that distinct subsets of axonal microtubules may have different properties with respect to stability, transport and possibly function. Therefore, the emerging hypothesis (Hollenbeck, 1989) is that tubulins travel in two different forms, a slow moving polymeric component which is more stable than the faster moving soluble, mono or oligomeric component. SCa tubulins may represent the polymerized form of tubulin which is hyperstable and slower moving, while SCb is composed of less stable microtubules and contains more mono, di, and oligomeric tubulin subunits. This increase in solubility and decrease in stability may be directly responsible for the faster velocity of SCb.

Using this new hypothesis of axonal transport three general mechanisms can be proposed to explain the changes in tubulins transport seen in my

experiments:

i. changes in synthesis of major cytoskeletal proteins,

ii. posttranslational modifications of tubulins, and

iii. alterations in other proteins which effect the polymerization of tubulins.

A. Changes in the Synthesis of Neurofilaments and Tubulins

As mentioned in the introductory chapter, following a single crush injury to facial nerve axons there is an increase in α and β-tubulins and a decrease in neurofilament synthesis (Tetzlaff et. al., 1988b). The increase in α-tubulin is believed to be solely due to the increase in an isotype coded by the Tα1 mRNA (Tetzlaff et. al., 1988a). Tα1 is an embryonic form of α-tubulin and differs from the adult form (T26) by the substitution of a serine for a glycine (Miller et. al., 1989). Tetzlaff et. al. (1988a) have proposed that the Tα1 isotype is less susceptible to interaction with the slow moving NF, hence in the developing and regenerating nervous system less α-tubulin is bound to the neurofilaments and more is able to travel at a faster velocity. Therefore, the increased expression of an embryonic α-tubulin with a missing phosphorylation site, following a single crush lesion and the possibility that it may be less susceptible to interactions with the SCa neurofilaments may partially explain why less tubulins are transported in the SCa wave following a crush injury (a similar phenomena may occur for β-tubulin). In addition, the fact that neurofilament synthesis is significantly decreased following a crush injury may also explain the decrease in

the proportion of tubulins in the SCa region (less neurofilaments to bind and slow down the tubulins).

In CL nerves a second lesion does not cause a further decrease in tubulin and actin synthesis but does induce a further decrease in neurofilament production (Tetzlaff et. al., 1988b). Therefore, the further reduction in the proportion of tubulins transported in the SCa phase, seen in CL fibers, may be due to the further decrease in the number of NF proteins in the SCa region. The increase in the proportion of tubulins in the SCb region for CL fibers may come about as a result of the decrease in NF synthesis. As less tubulins are bound up by the NF in SCa more is able to travel at a faster velocity (in the SCb phase). Therefore, the enhanced reduction in NF synthesis for CL nerves frees up more tubulin allowing it to move ahead of the SCa wave.

For tubulins in transit prior to the TL, for SL nerves, the change in the profile cannot be explained by the above hypothesis since the change must affect tubulins synthesized prior to the SL, hence, the alteration in transport must involve post-translational change or some other effect on the polymerization of tubulins already in the axon. For CL nerves, which received a TL after the isotope injection, the change in tubulin transport may be partially explained by the increase in Tα1 expression and decrease in NF synthesis. As discussed this may result in less tubulin being transported in the SCa and more in the SCb wave.

B. Posttranslational Modifications of Tubulins

Numerous subtypes of α and β tubulins occur in the brain as a result of

the expression of multiple genes (Cleveland et. al., 1985). In addition, posttranslational modifications of tubulins account for further different axonal isotypes and may explain the shift in the transport of tubulins for regenerating fibers.

It has been shown that:

i. The posttranslational phosphorylation of ß-tubulin is correlated to assembled microtubules and dephosphorylation to the disassembly of microtubules (Gard and Kirschner, 1981),

ii. acetylated α-tubulin is related to the polymeric form while deacetylation is coupled to depolymerization (Black et. al., 1989; Black and Keyser, 1987),

ii. Tyrosinated α-tubulin is associated with depolymerization or the soluble form of α-tubulin while detyrosinated α-tubulin is associated with the polymerized or stable form (Sherwin and Gull, 1989), and

iv. phosphorylation of the carboxyl-terminal tyrosine of α-tubulin prevents its polymerization (Wandosell et. al., 1987).

Hence, the polymerization state of α and ß-tubulins can be altered by at least four posttranslational mechanisms.

The general trend seen from my results on the transport of tubulins was a decrease in the proportion of tubulins being transported in the SCa region following a SL and a further decrease for CL fibers, in addition to an increase in the proportion of tubulins transported in the SCb region. A single crush lesion may cause either or any combination of - dephosphorylation of ß-tubulin, deacetylation, tyrosination of α-tubulin or phosphorylation of the

carboxyl-terminal tyrosine - all of which would destabilize the microtubules and increase the proportion of soluble tubulins. This could be brought about by altering cell body synthesis of the enzymes responsible for the posttranslational modification of tubulin. This may possibly allow more tubulins bound up in the SCa region to travel at a faster velocity, thereby, decreasing the proportion of tubulins being transported in the SCa region. For CL nerves the further decrease in the proportion of tubulins transported in the SCa region, relative to SL nerves, may be caused by the same mechanism. The second lesion could enhance any of the suggested mechanisms which were in effect following the SL and/or incorporate those not used following the SL.

Although the shift in the transport profile for tubulin in transit prior to a single crush lesion could not be explained by changes in the synthesis of NF or tubulins, the posttranslational modifications covered in this section could all play a role in causing the decrease in SCa and increase in the proportion of SCb tubulins. Enzymes which are involved in these reaction may be fast transported and able to influence the stability of the cytoskeleton within hours after axotomy. For SL axons in which labeled tubulins were in transit prior to the TL, the further decrease in SCa and increase in SCb tubulins may be due to an enhancement of the proposed posttranslational modifications.

C. Alterations in the Synthesis or State of Proteins Which Influence Microtubule Stability

In this section two different sets of proteins which affect microtubule

stability will be considered - MAP's and calmodulin.

Microtubule Associated Proteins (MAP'S)

In the adult mammalian brain the major MAP's are MAP1, MAP2, and tau. MAP1 is found in both axons and dendrites (Bloom et. al., 1984), MAP 2 is primarily located in dendrites but found to a much lesser degree in axons (Bernhard and Matus, 1984), while tau is most prominent in axons (Binder et. al., 1985). In addition to MAP1 and tau, which are of interest as they are located in mature axons, MAP3 and MAP5, both which are abundant during development, are relevant in searching for possible mechanisms.

The decrease in the proportion of tubulin transported in SCa and increase in SCb for CL fibers and the decrease in SCa tubulins for SL fibers may be regulated by MAP's in two ways:

1. The expression of juvenile MAP's (MAP3 and MAP5) may occur in regenerating adult fibers. This would alter the plasticity of the system possibly by increasing the solubility of SCa tubulins and allowing them to travel at a faster velocity (Riedener, et. al., 1986; Bruss and Matus, 1988; Tucker et. al., 1989) and

2. Phosphorylation of MAP2 and MAP1.2 are correlated to neurite outgrowth, plasticity and microtubule dissassembly. This may be another mechanism which decreases the proportion of SCa tubulin by unbinding them from the slow moving SCa phase (Marsolis and Wilson; 1979; Jameson and Caplon; 1981; Aletta et. al., 1988).

In addition, the phosphorylation of MAPs is a posttranslational event and

could be responsible for the shift in the profile seen for tubulin in transit prior to a crush lesion.

Calmodulin

Calmodulin, a calcium binding and slow transported protein interacts with a wide variety of cytoskeletal structures (Cheung, 1980). It has been reported to inhibit assembly and to promote disassembly of microtubules in a calcium dependent manner (Marcum et. al., 1978). It is believed to act by binding to tau and MAP2 (Lee and Wolf, 1981; Sobuent et. al., 1981).

Altered synthesis of calmodulin, as a result of injury, could affect microtubule stability and be partially responsible for the findings mentioned in this section. For regenerating sciatic motor neurons a crush injury results in an increase in the proportion of labeled calmodulin being transported for SL nerves (relative to controls) and CL fibers (relative to SL nerves) (McQuarrie, 1988; McQuarrie and Lasek, 1989). With respect to the change seen in tubulins in transit prior to the TL, fast transported calmodulin (see next chapter) may decrease microtubule stability allowing more tubulin to be transport with SCb.

ii. Actin

The general trend for the findings on actin transport was that a SL caused a reduction in the amount of SCa actin and an increase in the amount travelling with SCb (exception was the 7 day group). A CL given 7 days prior to the TL enhanced this effect which was maximal for actin produced 14 days post-TL. This phenomena of a decrease in SCa and an increase in SCb actin, as a result of a crush lesion may be due to:

i. altered synthesis of actin capping proteins causing dissassembly of f-actin or inhibiting the assembly of g-actin to f-actin in the SCa region, thereby, freeing more actin to travel at a faster velocity (Isenberg, 1986),

ii. the phosphorylation or dephosphorylation of actin binding proteins in the cell body or in the axon may be activated by the crush injury changing the degree of binding, this may free up actin in the SCa area, allowing a greater proportion to travel ahead of SCa (Isenberg, 1986), and

iii. altered synthesis of different MAP's along with their phosphorylation (which may decrease cross-liking of MAP's to microtubules and actin) may cause an increase in the amount of actin liberated from the slow moving phase SCa phase (Griffith and Pollard, 1978, 1982; Seldon and Pollard, 1983, 1986).

Any combination of the above mechanisms may explain the shift in actin from the SCa to the SCb phase. The effect of a CL is to enhance this phenomena and may be due to a further augmentation of these mechanisms of the incorporation of those not utilized following a SL.

3. SUMMARY AND CONCLUSIONS

This chapter demonstrates that:

i. a single crush lesion decreases the proportion of newly-synthesized tubulins and actin transported in the SCa region and increases the proportion in SCb,

ii. a CL 7 days prior to the TL causes a further decrease in the

proportion of tubulins and actin in the SCa region and increase in the proportion in of actin and tubulins ahead of the SCb region, and

iii. a crush injury causes a similar shift for tubulins already in transit, this is also enhanced by a prior conditioning lesion.

These changes may be due to the depolymerization of actin and tubulins (i.e. posttranslational modifications) or disassociation of these proteins from the more stable SCa components (i.e. NF). The result is an increase in the solubility of the SCa actin and tubulins allowing them to travel at a faster velocity. When the amount of tubulins and actin dissociated from SCa is great enough a significant increase in the amount of SCb tubulins and actin is seen.

In using the findings of this study to formulate a hypothesis for why CL fibers regenerate faster it is important to keep in mind that my results reflect the proportion of tubulins and actin in successive 3 mm segments, not absolute amounts of labeled proteins.

Following a SL, the upregulation of actin and tubulin and down regulation of NF probably causes an increase in the amount of tubulins and actin being transported in SCb (this is supported by Hoffman and Lasek (1975) who found a two fold increase in the amount of labeled tubulins in SCb following a SL). When one takes into consideration what is happening in the cell body together with the shift in transport of tubulins and actin following a SL it is reasonable to suggest (if one accepts the notion that a significant proportion of the upregulated tubulins and actin are transported out of the cell body) that a greater amount of actin and tubulins are in the SCb region. If the second (or

test) lesion is given at a specific time or a particular region of the nerve, such that the increased amount of actin and tubulins are available for incorporation into the newly regenerating fibers, then the increased availability may explain the shortened initial delay ($\approx$ 40 %) and the increased rate of regeneration.

After the second lesion tubulin and actin synthesis does not change however, there is a further shift in the transport profile causing a greater proportion of actin and tubulins to be transported ahead of the SCb region, relative to the SL nerves. This probably means a further increase in the amount of tubulins and actin travelling ahead of the SCb wave. For CL nerves if the TL is made very close to the cell body, such that the enhanced proportion of tubulins and actin are available for use by the regenerating fibers, regeneration may occur sooner and at a faster velocity. However, if the TL is made far distal from the ganglion it is unlikely that the regenerating fibers will receive an enhanced amount of tubulins and actin produced as a result of the second lesion.

Therefore, my data, in conjunction with what is known about tubulin and actin synthesis in the ganglion, suggests that the increase in the amount of tubulins and actin travelling in the SCb region following a SL may be responsible for the increased rate of regeneration for CL nerves. This applies under the conditions when the second lesion is made at a time or location on the nerve fiber such that the elevated amount of tubulins and actin are near the crush site and are available for use by the regenerating fibers. The further increase in SCb tubulins and actin following the TL (in CL fibers) would only participate in

formation of new neurites if the TL were made near the cell body. Hence, this eliminates a probable role for the elevated SCb tubulins and actin in CL fibers when the TL is made far distal from the ganglion (i.e. 60 mm) or very soon after injury.

The obvious question to ask is how can the CL effect be explained when the TL is not made at the appropriate location on the nerve or at the appropriate time following the CL. Forman et. al. (1980) have demonstrated a conditioning lesion effect with as little as a two day CL-TL interval and with the lesion site 30 mm from the ganglion. In this case the TL is made far distal from the approaching SCb wave and soon after the CL such that the SCb wave, with elevated tubulins and actin, cannot reach the crush site and therefore cannot be used by the regenerating fibers. Why then do the fibers regenerate faster? My data on the transport of tubulins in transit prior to the TL indicate that a shift in the transport profile of pre-synthesized tubulins can occur within four days of the lesion and this shift increases the proportion of tubulins travelling in the SCb wave . Hence, when the TL is given there is an elevated amount of tubulins available for use by the regenerating fibers.

Therefore, as originally suggested by McQuarrie (1986,1988a,1988b,1989), the increased supply of structural proteins may be responsible for the enhanced distance of regeneration. My experiments suggest that this hypothesis may be able to explain why CL fibers regenerate further regardless of the location and time of the CL-TL intervals.

CHAPTER 2. INTERMEDIARY AND FAST TRANSPORT OF TUBULINS AND ACTIN IN REGENERATING RAT SCIATIC NERVE

INTRODUCTION

Anterograde axonal transport has traditionally been divided into two mutually exclusive components - slow and fast. While fast anterograde transport (FAT) is usually reported to advance at a velocity of between 200 - 400 mm/day and carry membranous components, slow anterograde transport (SAT) travels at a velocity of between 1 - 5 mm/day and carries cytoskeletal structures as well as soluble enzymes (Oblinger et. al., 1987; McQuarrie et. al., 1986; Bisby, 1977).

The exclusivity of these two transport groups, with respect to velocity and material transported, is related to the form and mode in which transport is believed to occur. For the FAT system the transport of vesicles is thought to occur along microtubules via the binding and detaching of kinesin side arms which are attached to the cytoplasm of the vesicle (Langford et. al., 1986). In this manner vesicles travel from the cell body to the terminal portion of the axon along existing microtubules. SAT on the other hand, according to the popular structural and polymer sliding hypothesis, conveys assembled cytoskeletal structures and associated proteins at a much slower velocity by, an as yet, undetermined mechanism (McQuarrie et. al., 1986; Lasek, 1987).

A central difference between these two hypotheses is the form in which the cytoskeletal material is being transported. The structural hypothesis would state, for FAT the material is packaged into relatively small vesicles and transported on microtubules while SAT material is transported in relatively large assembled units. The unitary hypothesis of axonal transport bridges these two opposing views of transport by suggesting that cytoskeletal material is conveyed in a soluble subunit form composed of small fragments which are assembled into stationary structures along the nerve fiber (Weiss and Gross, 1982). Cytoskeletal materials are packaged into vesicles, placed onto the AT system and transported down the axon, getting off at an appropriate location. Hence, as its name implies, the unitary hypothesis proposes one mechanism to explain two, seemingly different, forms of transport.

The apparent slow transport wave of cytoskeletal proteins (SCa, SCb), seen in radioactive labelling experiments, does not contradict the unitary hypothesis if one accepts the idea that the wave reflects the exchange of subunits between a dynamic and stationary pool of proteins in which the soluble portion is being placed onto and removed from the FAT system. Such a suggestion is gaining support from recent studies: Okabe and Hirokawa (1988) have demonstrated that tubulin is transported in a monomeric form, while others have demonstrated that the axonal cytoskeleton may be stationary and that newly made NF and tubulins, travel down the axon in an unassembled or partially assembled form (Tashiro and Komiya, 1987; Nixon, 1987; Nixon and Logvinenko, 1986).

As mentioned in the last chapter, Hollenbeck (1989) has put forth a hypothesis suggesting that tubulins travel in two different forms, a slow, stable polymeric form and a more soluble, faster mono or oligomeric form. This and the unitary hypothesis share the belief that at least some, if not all, of the cytoskeletal proteins are transported in a soluble non-polymerized form which interacts with a more stable cytoskeleton.

If cytoskeletal proteins are transported in a soluble form and do hook onto the FAT system by being packaged into vesicles then one would expect to find cytoskeletal proteins in vesicles and to observe the fast transport of these proteins with traditional radiolabelling fast transport studies.

For regenerating goldfish optic axons Perry et. al. (1987) have reported tubulins and actin being transported at a velocity in the FAT range. Hollenbeck and Bray (1987) and Koenig et. al. (1985) have revealed, immunocytochemically, the presence of cytoskeletal proteins (tubulin, actin, fodrin, and calmodulin) in vesicles. Therefore, there is evidence that cytoskeletal proteins are fast transported and that they can be found in vesicles.

According to the structural and sliding polymer hypothesis (Lasek, 1986; Lasek and Katz, 1987) following a crush injury regenerating nerve fibers receive all their structural elements from those present in the axon prior to the injury (this is also referred to as the conservative model [McQuarrie and Lasek, 1989]). This is based on the idea that cytoskeletal material is assembled near the cell body and pushed down the axon in distinct coherent groups. It would follow then, if a nerve fiber were severed, that the steady pushing of the

cytoskeleton from the cell body would generate an anterograde force resulting in cytoskeletal material, proximal from the injury site, forming new neurites which would elongate at a rate controlled by the pushing cytoskeleton. However, a new hypothesis emerging on SAT (Hollenbeck, 1989 [see last chapter]) suggests that the cytoskeleton is not transported as an assembled coherent group. This new idea strongly contradicts the long standing structural hypothesis and stimulated me to test some of the current ideas of axonal transport.

The experiments reported in this chapter were undertaken:
i) to determine if newly regenerating fibers do receive all their structural proteins from those present in the axon prior to the injury or whether cytoskeletal proteins, synthesized post-injury, enter the newly formed axons and
ii) to determine how fast tubulins and actin are transported in sensory neurons of the rat sciatic nerve. It is obvious from figure 3 that labelled actin and tubulin are present in the nerve distal to the location of SCb. This presumably represents a faster transported component.

METHODS

1. General Procedure for Intermediary Transport Experiments

The set of experiments in this section were designed to determine if cytoskeletal proteins, produced after a TL, are transported past the crush site to the regenerating axons.

Selection of animals, TL's and injections were carried out in the same manner as described in the previous chapter, however, rather than receiving injections of 100 μCi of [S35] - methionine, animals were injected with 500 μCi of [S35] - methionine. The amount of label was changed so as to increase the sensitivity of the experiments, thereby, allowing me to investigate the movement of small amounts of labeled protein.

Animals received injections three days following the TL and were killed two days later. Nerves were removed and frozen in the same manner as described in the previous chapter.

2. Fast Transport Experiments

The fast transport experiments in this section were undertaken to calculate how fast tubulins and actin are transported. Three days following a TL animals were injected with 100 μCi [S35] - methionine into the L-5 DRG. Four hours later collection crushes were made 30 mm, 40 mm and 50 mm from the ganglion. Two hours after the collection crush the animals were killed and the nerves removed and frozen at -70 degrees celcius.

3. Processing of Tissue

i. 1-D SDS PAGE and Fluorography

Nerves were processed, run and fluorographed in the same manner as described in the preceding chapter. Bands were not excised and counted, since the objective was to obtain a qualitative assessment of the furthest distance transported.

ii. Two Dimensional SDS PAGE (2-D SDS PAGE)

(Tetzlaff et. al., 1988b)

The 2-D SDS PAGE experiments were performed to positively identify rapid and fast transported tubulin and actin.

A) Intermediary Transport Experiments

Nerves were divided into three 6 mm segments (figure 18):

a) immediately proximal to the crush site,

b) immediately distal from the crush site, containing regenerated axons and

c) far distal from the crush site, approximately 10 mm beyond the estimated position of the fastest growing axons.

B) Fast Transport Experiments

Six millimeter segments were taken proximally from each of the three collection crush sites (Figure 19).

Individual segments were pulverized in a stainless steel mortar frozen to -70 degrees celius and individually homogenized in 100μl of lysis buffer (9.5 M Urea; 5.0 % 2-mercaptoethanol; 2.0 % carrier ampholytes [0.1 ml pH 4-6, 0.1 ml pH 6-8, 0.05 ml pH 3.5-10]; 2.0 % NP-40). The sample was centrifuged for 10

minutes at 10,000 rpm and a 10 μl aliquot was taken, added to 5 ml of scintillation fluid (Scintiverse, Fisher), and counted in a liquid scintillation counter. The activity in the supernatant was counted and used to determine the amount of aliquot to load on to each gel.

Samples were separated in the first dimension on 10% urea isoelectric focusing gels. Equal volume aliquots were loaded onto 3 mm diameter tube gels and focused for 15 hours at a constant voltage of 400V, and for a further one hour at a constant voltage of 800V. The tube gels were placed on top of 5-15% polyacrylamide gradient gels with a 5% stacking gel. Gels were sealed to the top of the gradient gels with a 1% solution of agarose and 3μl of low molecular weight protein standard, in 3.6% SDS (Biorad), was applied to the corner of the gradient gel. These second dimension gels were run at an initial current of 25 mA per gel, with a maximal voltage of 350 V for approximately 4 hours. Gels were stained and fluorographed as described in the preceding chapter.

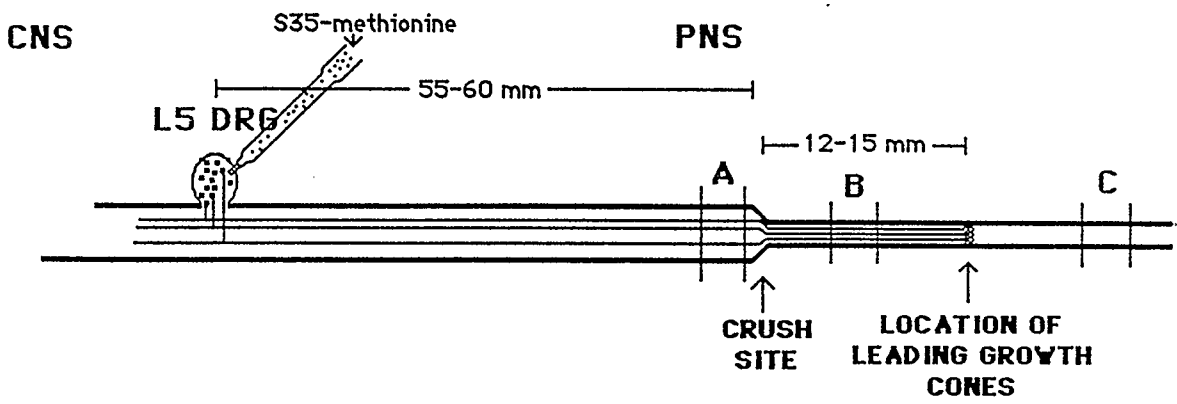


Figure 18. Three days following a crush lesion (55 - 60 mm from the ganglion) animals received an injection, into the L5 ganglion, of 500µCi of [S35]-methionine. Two days later the animal was killed, the nerve removed and three 6 mm segments were cut from the nerve and processed. "A", "B", and "C" represent the three segments with segment "A" being immediately proximal to the crush site, segment"B" immediately distal and segment "C" far distal from the crush site. Segment "C" was taken far distal so that no intact axons would be present.

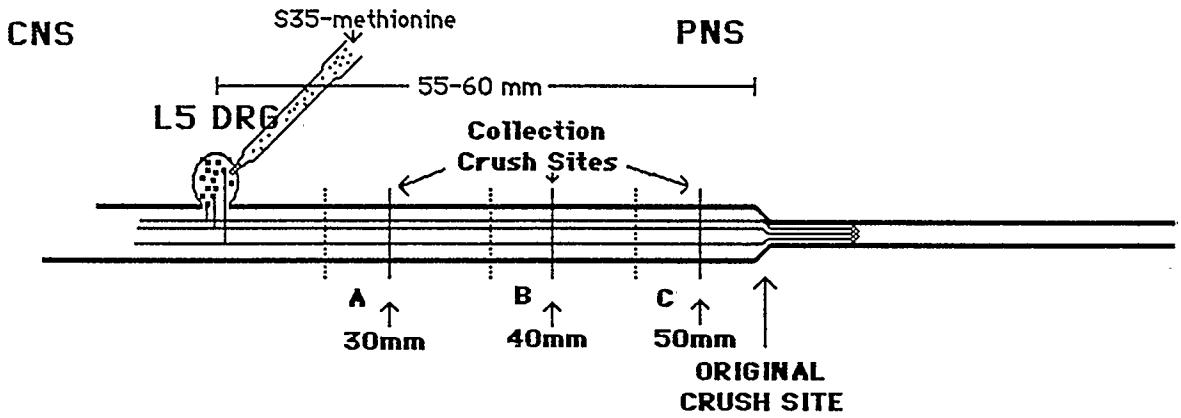


Figure 19. Experimental animals received a crush lesion 55 - 60 mm from the ganglion. Three days later animals were injected with 100 µCi [S35] - methionine. Four hours post-injection collection crushs were made approximately 30 mm, 40 mm, and 50 mm from the ganglion and the nerve was removed two hours later. Three 6 mm segments were excised from the nerve: segment "A", "B", and "C". Control animals were treated in the same manner except they did not receive a prior TL.

RESULTS

1. 1D SDS PAGE

Figure 20 shows a 1-D gel for proteins synthesized three days post-injury and after two days of axonal transport. Proteins corresponding to the molecular weight of tubulins and actin can be seen in the regenerating nerve fiber up to 69 mm from the ganglion. These proteins have travelled 69 mm in two days giving them a velocity of at least 34 mm/day.

These rats received a TL five days before they were killed, given a 1.5 day delay (Bisby, 1988), before the regenerating fibers crossed the lesion site, the fibers had 3.5 days in which to elongate distal from the crush site. Given a regeneration rate of 4.0 mm/day (Bisby, 1978; Bisby and Pollock, 1983), leading growth cones should be located 14.0 mm from the crush site. From fig. 20 one can see labeled proteins corresponding to the molecular weight of tubulins and actin up to 15 mm from the crush site. This indicates that cytoskeletal proteins synthesized 3 days post-lesion are rapidly transported to the growth cone region of regenerating fibers and that cytoskeletal proteins required for axonal regrowth are not derived exclusively from proteins present in the axon prior to the crush injury.

The most distal segment (marked by *) is located too far distal to contain any growth cones or regenerating nerve fibers. This region would be composed of Schwann cells, fibroblasts, invading macrophages and degenerating nerve fibers. This region is devoid of any labeled proteins implying that the label seen

12 mm from the crush site is not locally synthesized by non-neuronal cells from circulating label, but rather from axonally transported proteins. Figure 3 supports this result. Animals received a TL at the time of injection and as in figure 20 had five days in which to regenerate prior to being killed. Given the same delay and rate of regeneration as mentioned in figure 20, leading growth cones should be located 14 mm from the injury site. A small amount of labeled protein (corresponding to the molecular weight of actin) can be seen 12 mm from the TL site (label corresponding to the molecular weight of tubulins can be seen 3 mm proximal from this region). As in figure 20 a far distal segment containing no growth cones, has no label. Therefore, the label seen 12 mm from the lesion site is axonally transported not locally synthesized.

Hence, figure 3 and 20 demonstrate that proteins corresponding to the molecular weight of tubulins and actin, which are produced after or at the time of the TL, are transported to the regenerating fibers and to the region of the growth cones. This would correspond to a velocity of at least 34 mm/day (distance/time = 69/2 = 34 mm/day).

2. 2-D SDS-PAGE Identification of Intermediary And Fast Transported Tubulins and Actin

i. Intermediary Transported Tubulins and Actin

Figures 21 a, b, and c are Coomassie stained 2-D gels from a nerve which was injected three days after being crushed and removed two days after the injection. Comparison of the three photographs illustrates that near equal amounts of protein were loaded onto each gel. Actin along with α and β-tubulins

can be identified in all three segments. Of interest is the appearance of two new ß-tubulin isotypes in the immediately distal segment (segment "B") containing newly regenerated axons, which are not seen in the proximal or far distal segments ("A" and "C").

Figures 22, 23 and 24 are fluorographs of the same gels from figure 21. Labeled tubulins and actin had two days to be transported down the axon and can be seen in the near proximal and distal segments (figures 23 and 24), in addition to labeled calmodulin. Visual comparison of the three fluorographs reveals substantial differences between the amount of labeled tubulins and actin and the subtypes of labeled tubulins. Figure 24 represents the labeled proteins of a nerve segment distal from the leading growth cones. The appearance of labeled actin and tubulins in this region cannot be due to axonal transport as intact nerve fibers are not present (supported by the absence of GAP-43), rather the labeled proteins in this region are the result of local synthesis by Schwann cells, macrophages, and fibroblasts which have picked up blood-borne label as a result of leakage of the label from the ganglion during injection. This suggests that labeled actin and tubulins seen in figure 22 and 23 are not solely a result of transported proteins but are partially due to local synthesis. However, the amount of label in the proximal and near distal segments is much larger than the far distal one, even though near equal amounts of protein was loaded, implying that much of the label in figure 22 and 23 is from axonally transported proteins.

Figure 25 (a and b) are enlargements of figure 22 and 23, contrasting

actin and tubulins from the proximal and near distal segments (these results were consistent in three of four gels). The following comparisons can be made. Three different α-tubulin isotypes can be identified in both figures - α1, α2 and α3. While only one ß-tubulin can be seen proximal to the crush site, three can be identified distally (ß, ß1, ß2). For α-tubulins the α1 subunit is most prominent in the proximal segment. Distal from the injury site α3 is the most heavily labeled α-tubulin and the amount of α1 is substantially reduced while α2 is barely visible. For ß-tubulin only one isotype is visible proximally, however, two new isotypes can be seen distally. Therefore, while the same isotypes of α-tubulin are seen both proximal and distal from the injury site the relative amounts within the two segments differs with the most pronounced alterations being the relatively large amount of α1 proximally, the prominence of α3 and near total loss of α2 distally. With respect to ß-tubulin the most interesting difference is the appearance of two new isotypes distal from the injury site (α1 and α2) which are not present either proximally or far distally (thereby, ruling out the possibility that these two isotypes are a product of local synthesis).

In summary, actin, tubulins and calmodulin produced after the TL was made are transported to regenerating fibers located 60 - 70 mm from the cell body within 2 days. Three different α-tubulin isotypes are seen in both the proximal and near distal segments with differences existing between the relative amounts of the α-tubulin isotypes. Two new ß-tubulin isotypes appear distal from the crush site suggesting that these are post-translationally modified ß-tubulins which are specific for regenerating nerve fibers. In addition, the

appearance of a large amount of GAP-43 in the distal segment implies a routing of this protein to regenerating nerve fibers.

ii. Fast Transported Tubulin and Actin

Figure 26 (a and b) represents 2-D fluorographs from three different segments of the regenerating sciatic nerve. Labeled tubulins, actin and calmodulin are present 30 mm and 40 mm from the ganglion six hours after injection. This would convert to a velocity of at least 180 mm/day placing these proteins in the range of fast transport (since the collection crushes were made after 4 hours, proteins accumulating 40 mm from the ganglion must have been at least 30 mm from the ganglion at the time of the collection crush, therefore, velocity would be 30mm/4 hours * 24 hours = 180 mm/day). The segment located 50 mm from the cell body was not included for two reasons. First, it contains only small amount of labelled actin and tubulin and secondly, the quality was poor. Because this collection crush was made close to the crush site, labeled proteins in this segment may represent fast-transported protein, or locally-synthesized actin and tubulin made by non-neuronal cells in the nerve. In this case non-neuronal cells (macrophages and fibroblasts), had three days in which to invade the crush site and 6 hours to pick-up blood-borne label and synthesize tubulin and actin. Hence, because of the small amount of label I am not able to determine a maximal velocity for transported actin and tubulin for the regenerating nerves.

Figure 27 (b and c) shows two 2-D gels from a control animal which was injected with the label six hours prior to being killed. Labeled tubulins and actin

can be seen in nerve segments 40 and 50 mm from the cell body. This gives these proteins a velocity of at least 240 mm/day (using the same formula as above). The distal segment does not contain a large amount of labeled protein, relative to segment b, making it possible that the labeled proteins in this segment are locally synthesized. I do not believe this to be the case as any cells invading the nerve as a result of injury had only 6 hours to do so (this is compared with three days for figure 26) and had only 2 hours to pick-up blood-borne label and synthesize tubulin and actin (compared to 6 hours for figure 26). Hence, the label proteins in this segment are probably axonally transported (this is supported by the appearance of S1, a fast transported protein). This figure demonstrates that the fast transport of tubulins and actin (in addition to calmodulin) occurs in lesioned and non-lesioned nerves. Although the velocity was found to be 240 mm/day this is not in the range of the fastest component of transport (400 mm/day) which includes GAP-43 and S1.

In summary, some tubulin, actin and calmodulin are fast transported in both regenerating and non-regenerating rat sensory axons in sciatic nerve (but not in the fastest component).

Figure 20. Three days after receiving a crush lesion (57 mm from the ganglion) 100µCi [S35] - methionine was injected into L5 DRG, the nerve was removed 2 days later and processed for 1-D gel electrophoresis. Proteins corresponding to the molecular weight of actin and tubulins can be seen in the regenerating fibers. Growth cones would be located in segments which are 12 mm to 15 mm from the crush site. Label corresponding to the molecular weight of tubulins and actin can be identified 12 mm from the crush site. The * indicates the most distal segment which contains no growth cones. No label is seen in this region, suggesting the label seen in the 12 mm segment is axonally transported, not locally synthesized. (tubulin [T]; actin [A]; dorsal root ganglion [DRG]; □ – location of most distal growth cones).

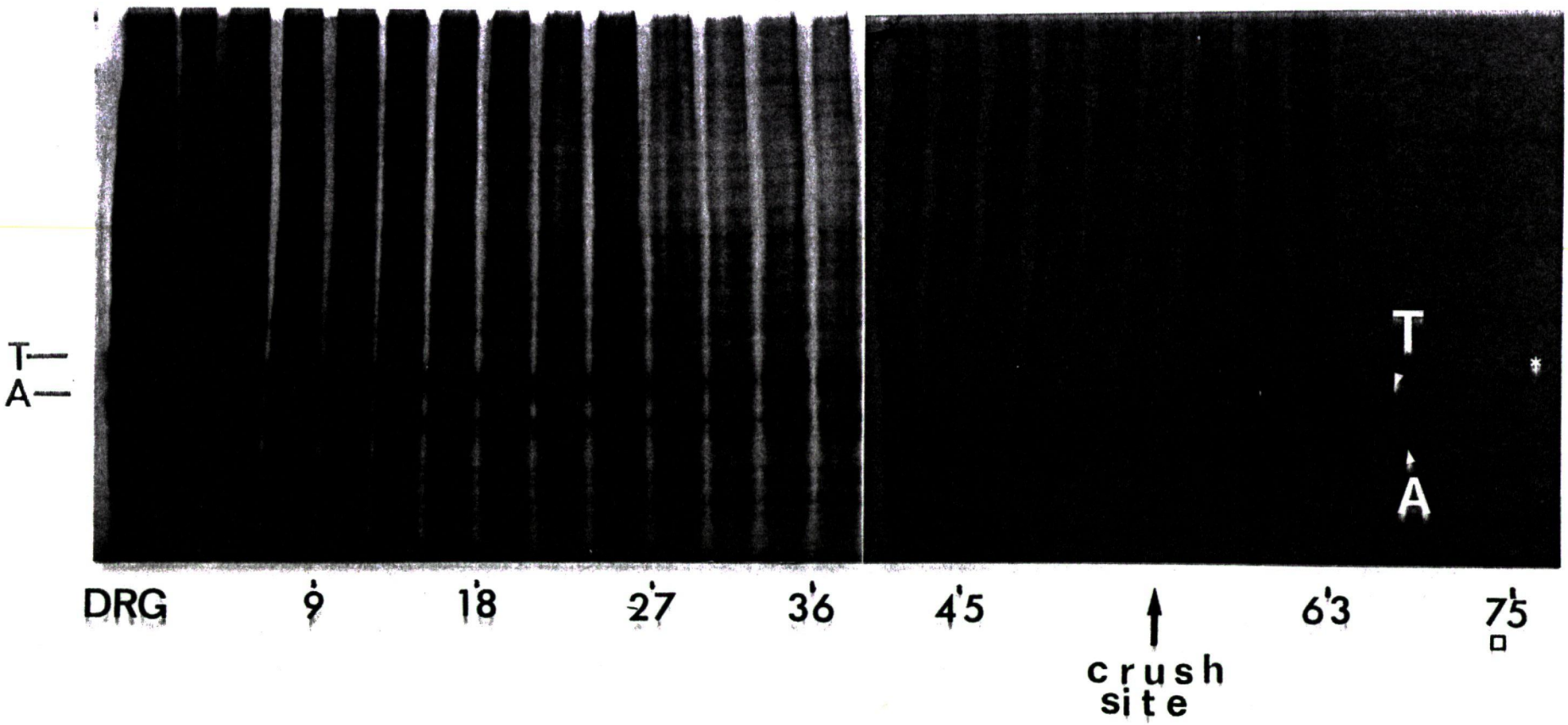
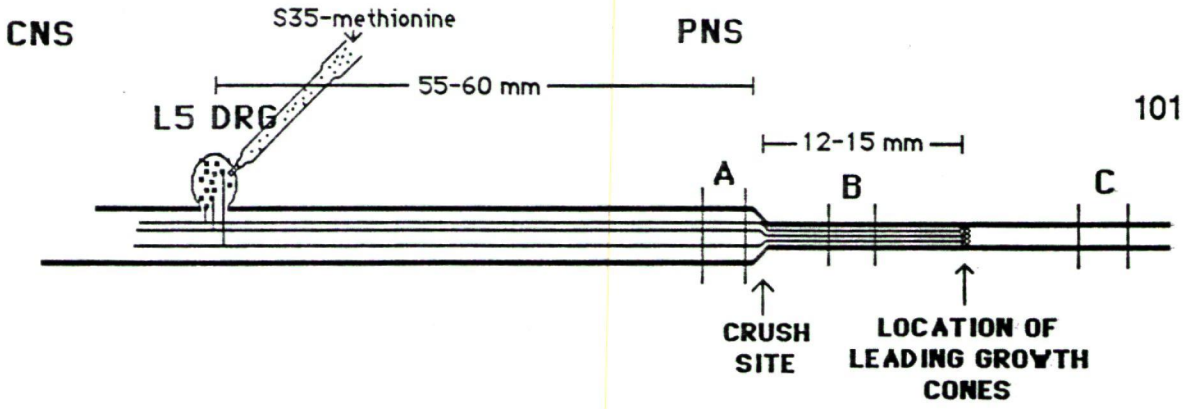
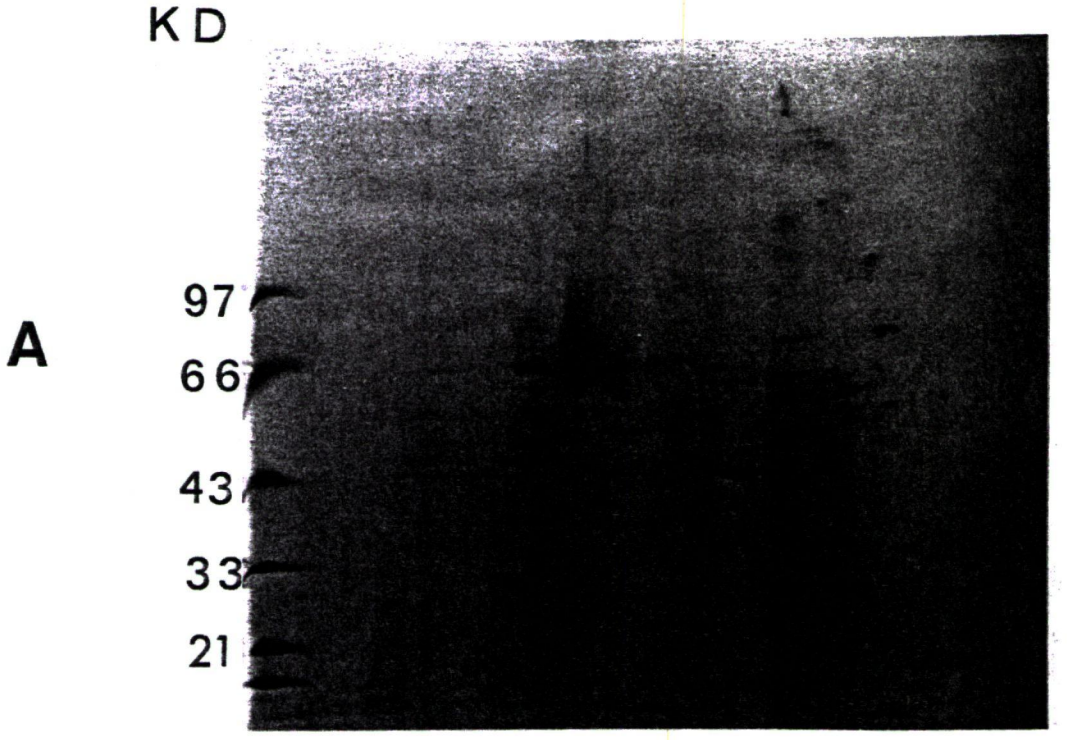
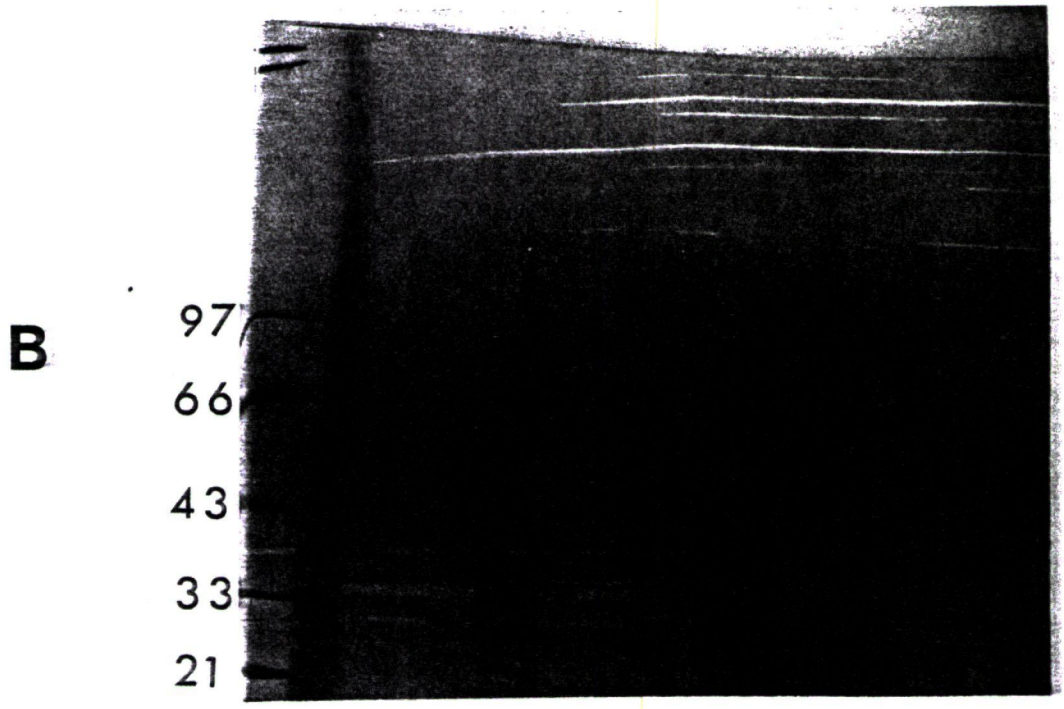

T
A
T
A
DRG
9
18
27
36
45
63
75
crush site
mm from DRG

Figure 21 Coommasie stained 2-D gels from nerves which were lesioned 3 days prior to being injected and removed two days after the injection. Three 6 mm segments were removed - an immediately proximal (segment "A"), immediately distal (segment "A"), and a far distal segment ("C"). Equal amount of aliquots were loaded onto each gel. While near equal amounts of protein were loaded from each segment, there does appear to be a small difference with segment "C" receiving more protein than segment "A" followed by segment"B". Actin (A), ∂-tubulin (∂-T) and ß-tubulin (ß-T) are identified.


CNS
S35-methionine
PNS
55-60 mm
L5 DRG
12-15 mm
A
B
C
CRUSH SITE
LOCATION OF LEADING GROWTH CONES


KD
97
66
43
33
21
A


97
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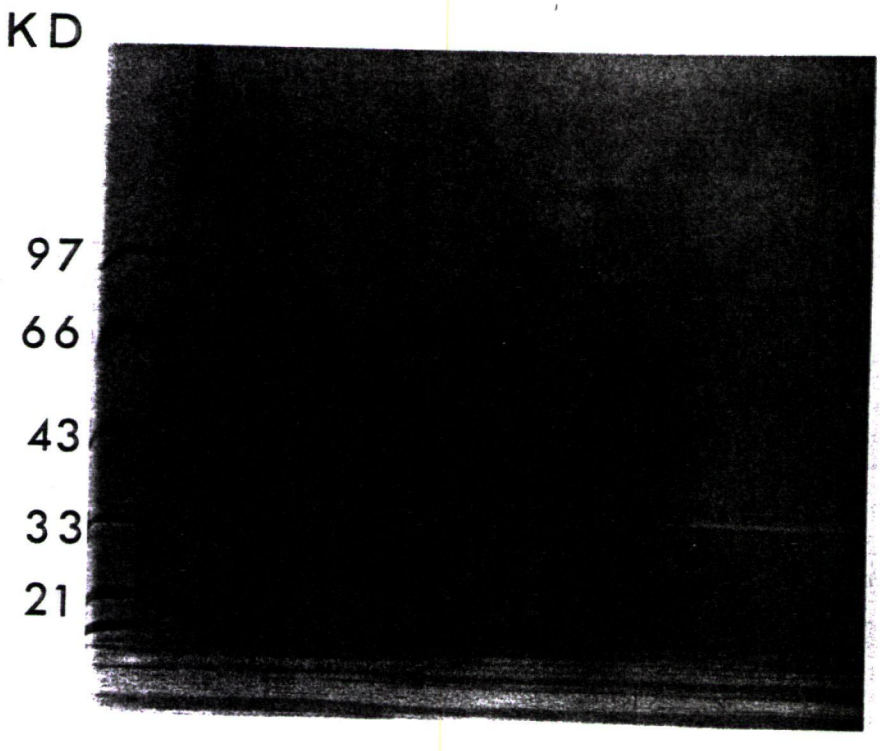
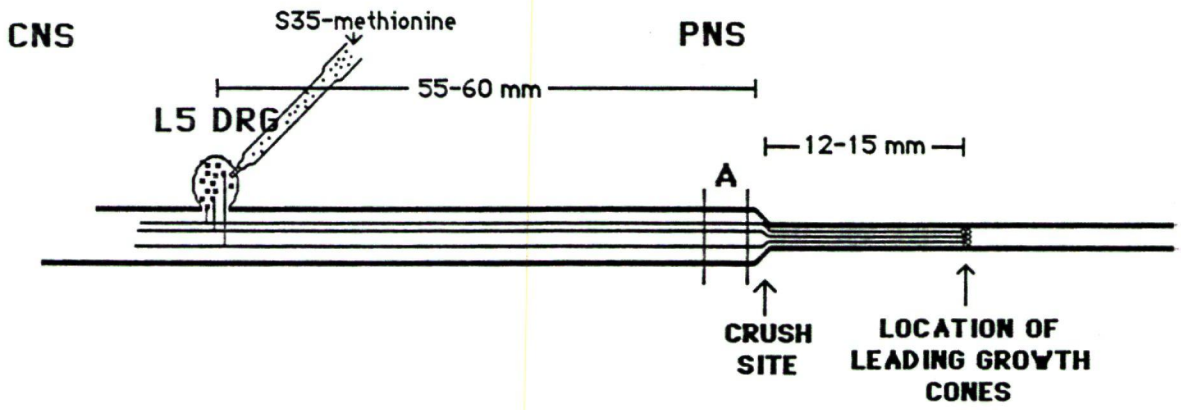


Figure 22. Fluorograph of a 2-D gel from segment "A" (produced from the commasie stained gel in 21 "A"). Labeled proteins were synthesized three days after the TL and had 2 days to be transported. Actin (A) along with ∂-tubulin (∂-T) and ß-tubulin (ß-T) can be positively identified. Calmodulin (C), another slow transported protein is prominent in addition to GAP-43 a fast transported protein.


CNS
S35-methionine
PNS
55-60 mm
L5 DRG
12-15 mm
A
CRUSH SITE
LOCATION OF LEADING GROWTH CONES

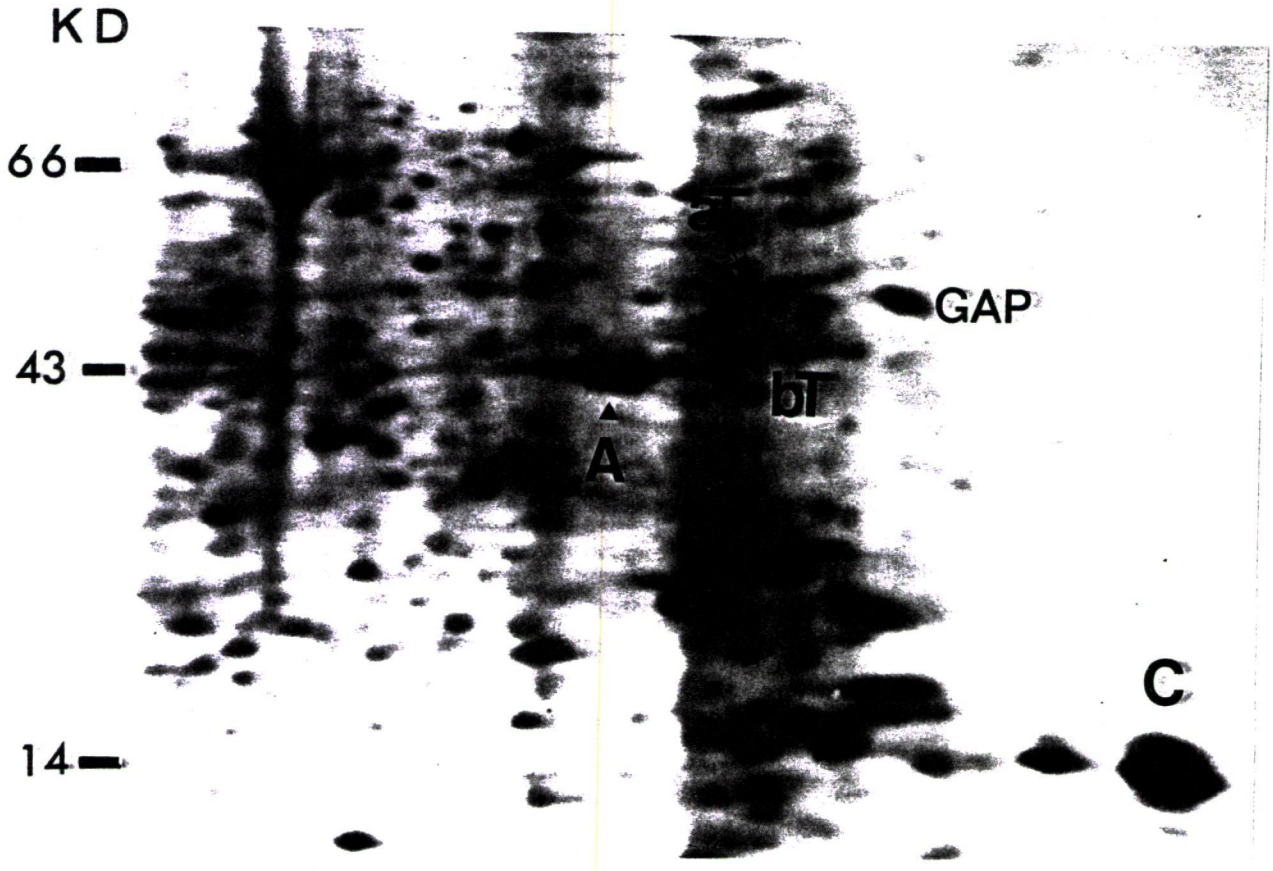
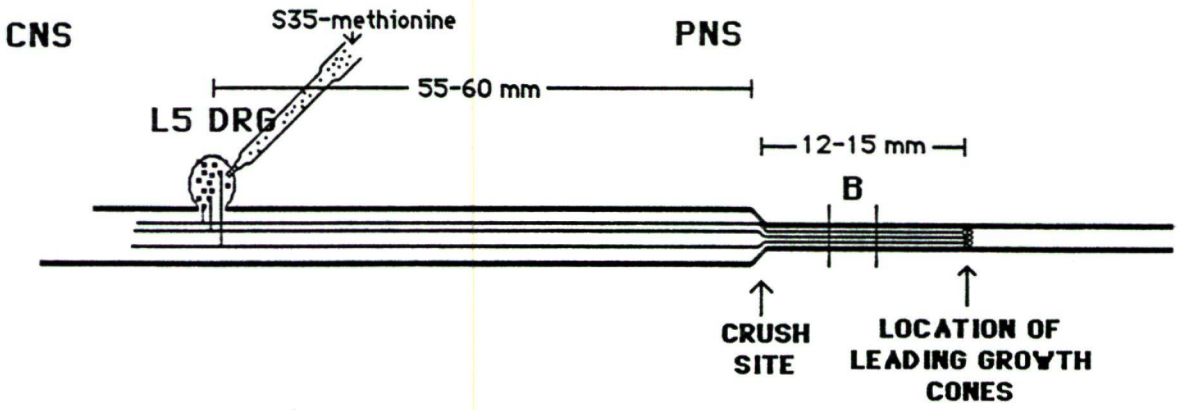

KD
66
43
14
GAP
bT
A
C

Figure 23. Fluorograph of an immediately distal segment ("B") from the same nerve a figure 22 (corresponds to the coommasie stained gel in figure 21"B"). This figure demonstrates that proteins synthesized three days following a distal crush injury are rapidly transported to the regenerating nerve fibers (distal from the crush site) in two days. Actin (A), three ∂-tubulins (ß-T) and three ß-tubulins (ß-T) can be identified. As in figure 22 calmodulin (C) and GAP-43 are present.


CNS
S35-methionine
PNS
55-60 mm
L5 DRG
12-15 mm
B
CRUSH SITE
LOCATION OF LEADING GROWTH CONES

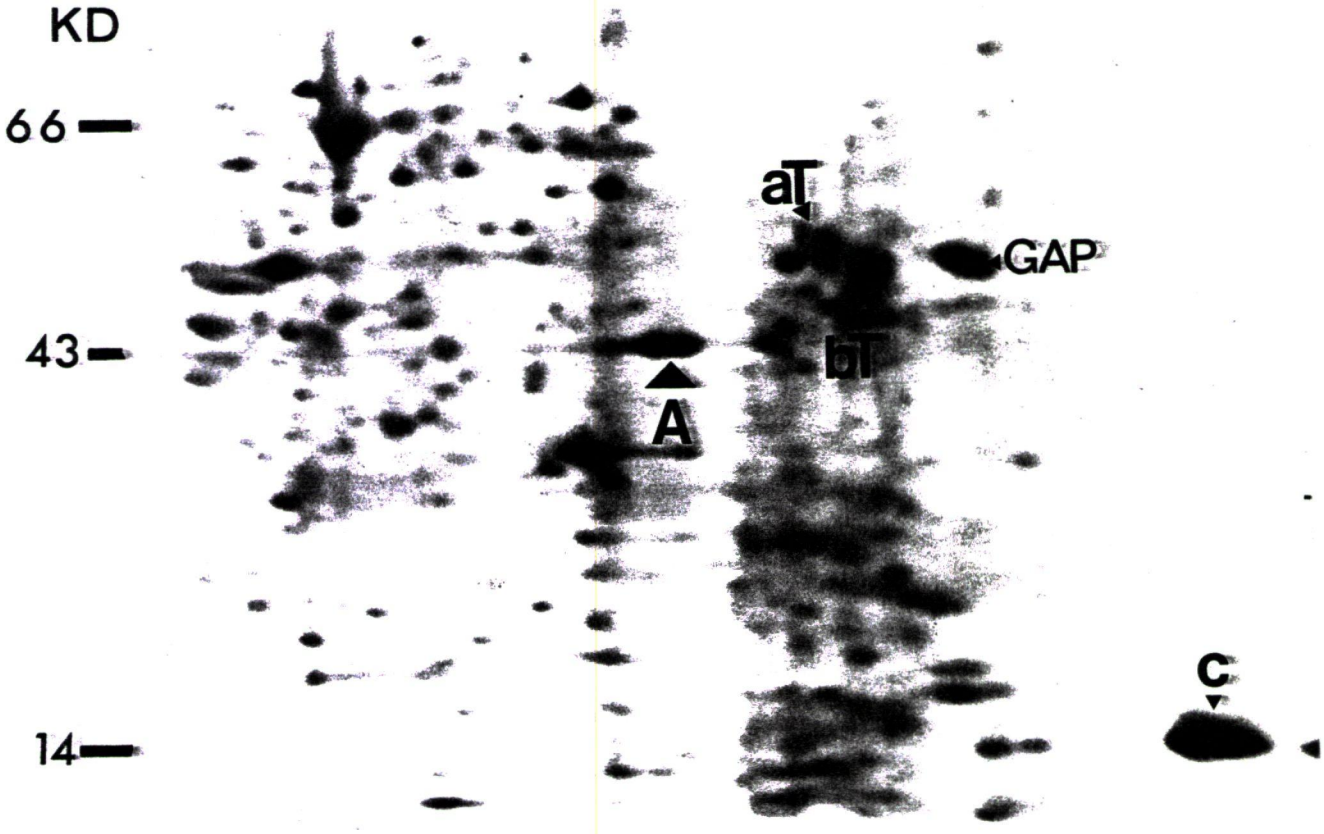
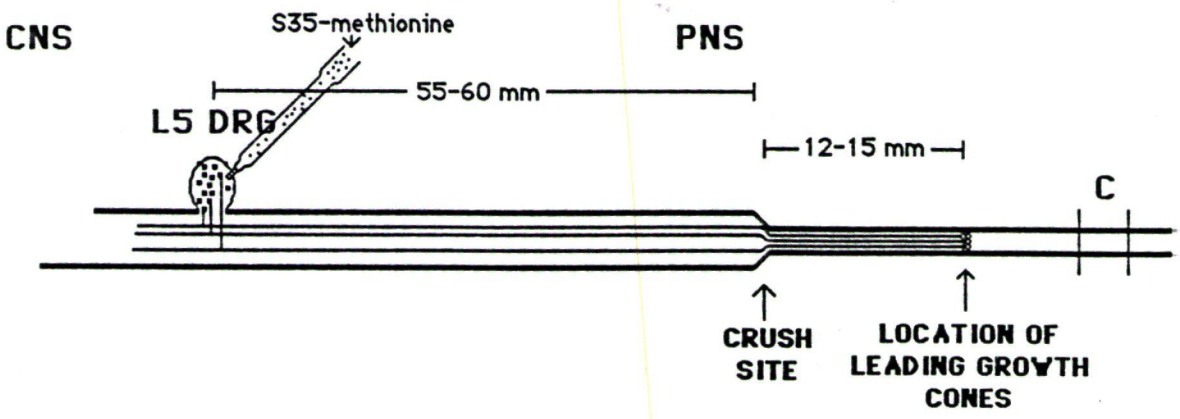

KD
66
43
14
aT
bT
GAP
A
C

Figure 24. Fluorograph of a 2-D gel of a far distal segment from the same nerve as figure 22 and 23. Because this segment contains no intact nerve fibers labeled proteins are the result of local synthesis. Actin (A), three ∂-tubulin isotypes (∂-T) and one ß-tubulin (ß-T) can be identified. Note the absence of GAP-43.


CNS
S35-methionine
PNS
55-60 mm
L5 DRG
12-15 mm
C
CRUSH SITE
LOCATION OF LEADING GROWTH CONES

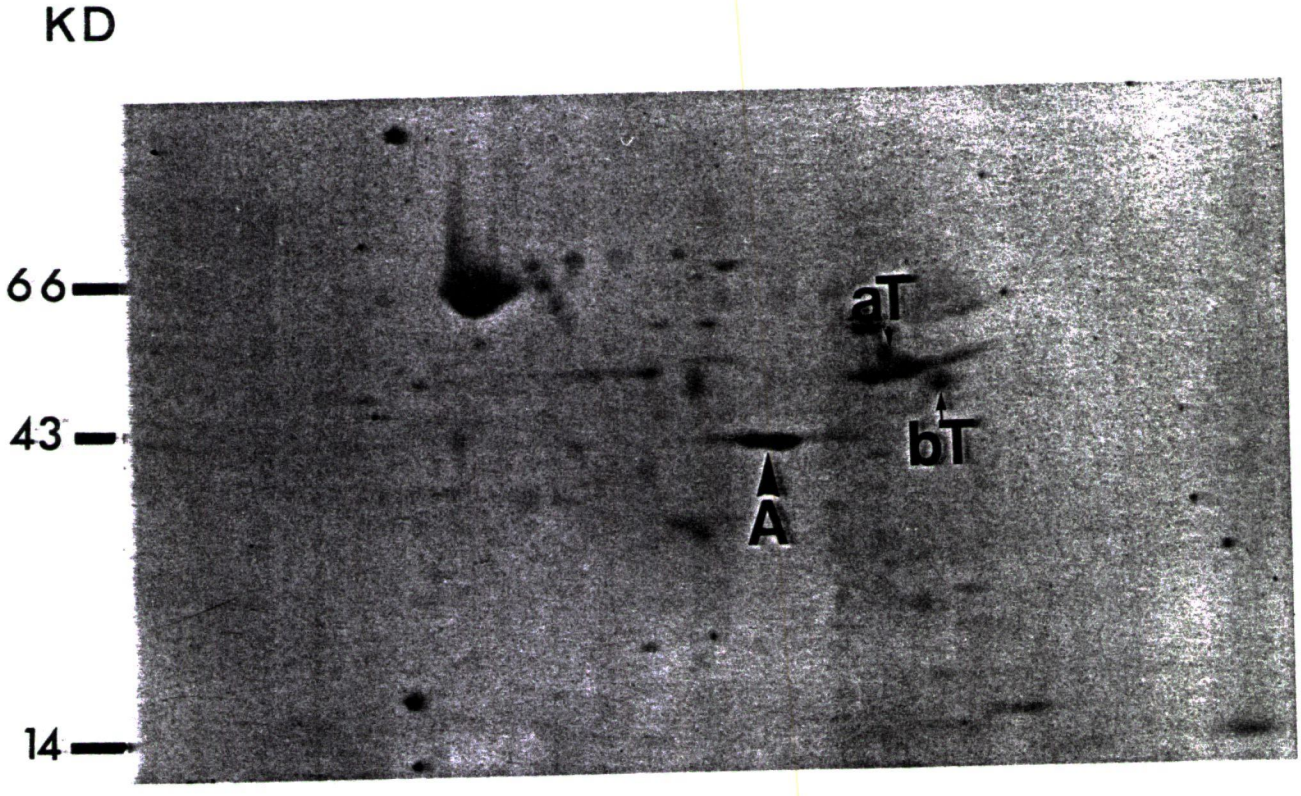
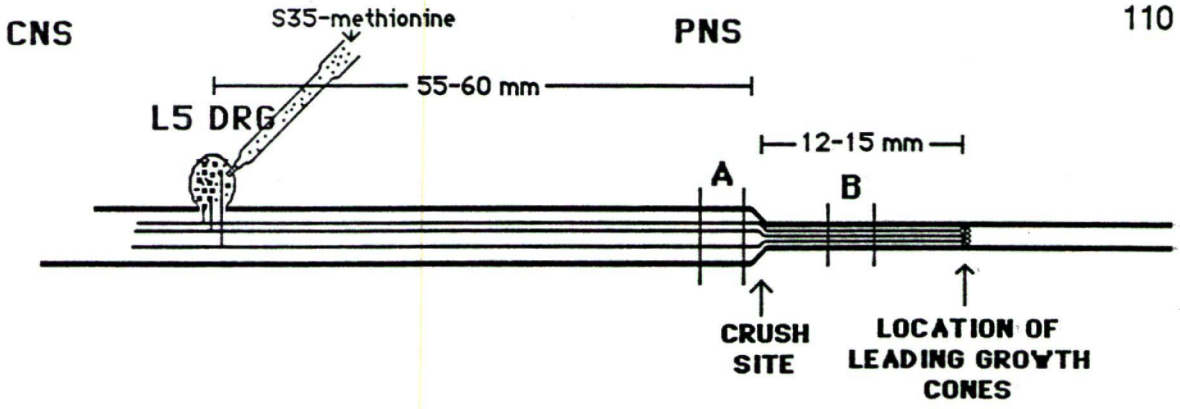

KD
66
43
14
aT
bT
A

Figure 25. Comparison of an immediately proximal (A) and immediately distal (B) segment from the same nerve ("A" and "B" are enlargements of figure 22 and 23, respectively). The consistent findings which appear in this figure, is the greater amount of labelled GAP-43 in the distal segment relative to the proximal one, the appearance of three alpha-tubulin isotypes (a1, a2, a3) in the proximal and distal segment with alpha1 being prominent in the proximal segment and alpha3 being dominant distal from the crush site, and the appearance of two beta-tubulin isotypes (b1, b2) in the distal segment which are not present in the proximal segment.


CNS
S35-methionine
PNS
55-60 mm
L5 DRG
12-15 mm
A
B
CRUSH SITE
LOCATION OF LEADING GROWTH CONES

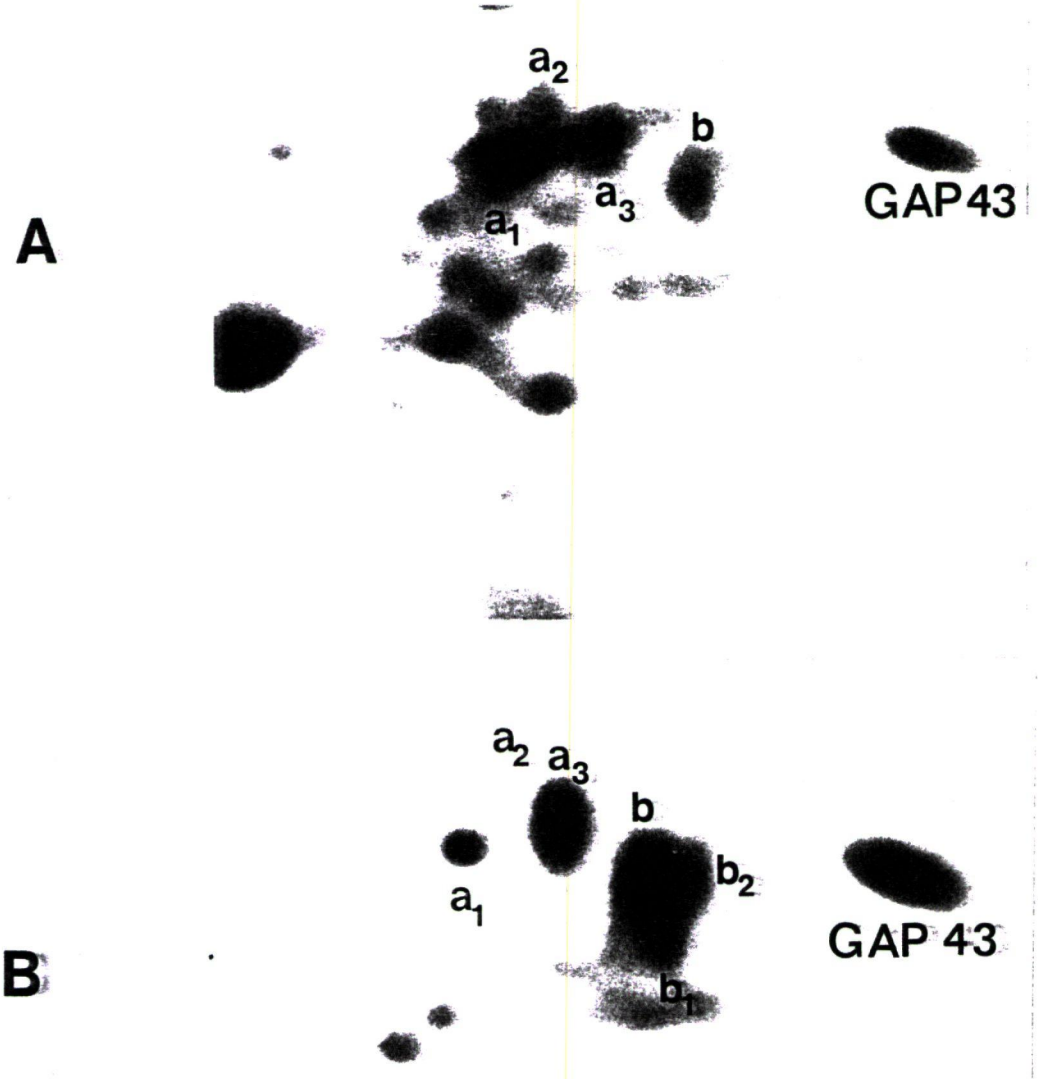
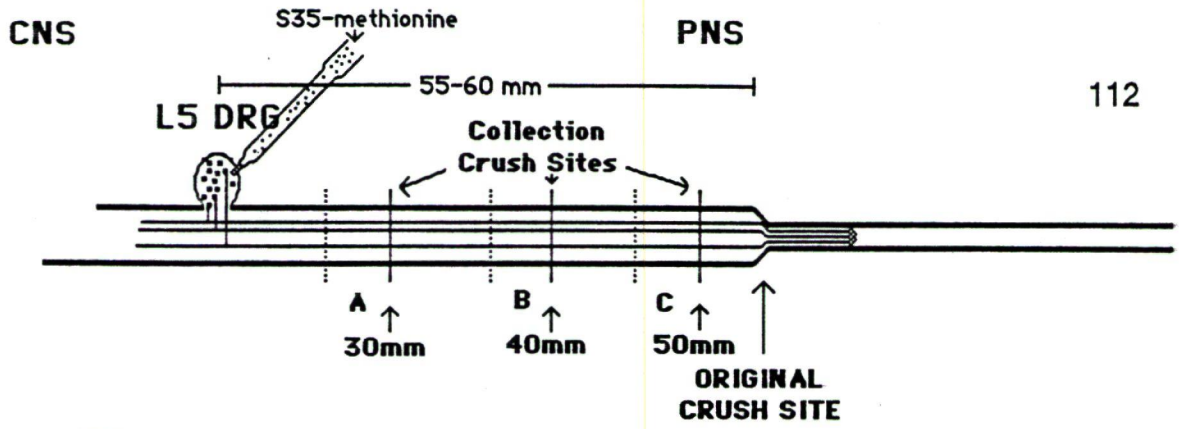
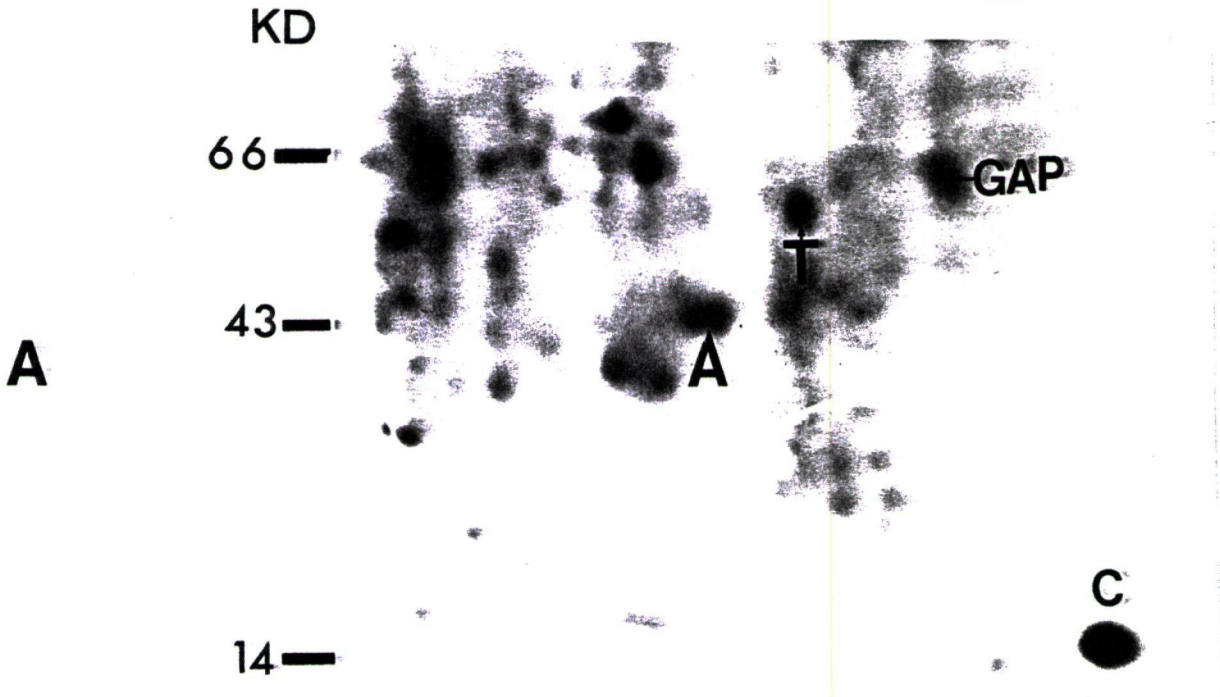

A
a2
b
a3
a1
GAP43
B
a2
a3
b
b2
a1
GAP 43
b1

Figure 26. These two fluorographs represent 2-D gels of fast transported proteins. Three days after a TL animals were injected (L5 DRG) with 100 µCi [S-35] methionine, four hours later collection crushs were made 30 mm, 40 mm, and 50 mm from the ganglion and the nerves were removed two hours following the collection crushes. Labeled actin and tubulin can be identified in both segment "A" and "B"giving them a respective velocity of at least 140 mm/day and 180 mm/day. GAP-43, a well characterized fast transported protein, is also present in both segments. Surprisingly, calmodulin (C), another protein thought to only be slowly transported, is also present in both segments. Note that segment "C" was left out. While labeled actin and tubulin were present the amount of label was much smaller than segment "B", due to its close proximity to the crush site (5 - 10 mm) it is possible that the small amount of label found is due to local synthesis and not axonal transport.


CNS
S35-methionine
PNS
55-60 mm
L5 DRG
Collection
Crush Sites
A
30mm
B
40mm
C
50mm
ORIGINAL
CRUSH SITE


KD
66
43
14
A
T
GAP
A
C

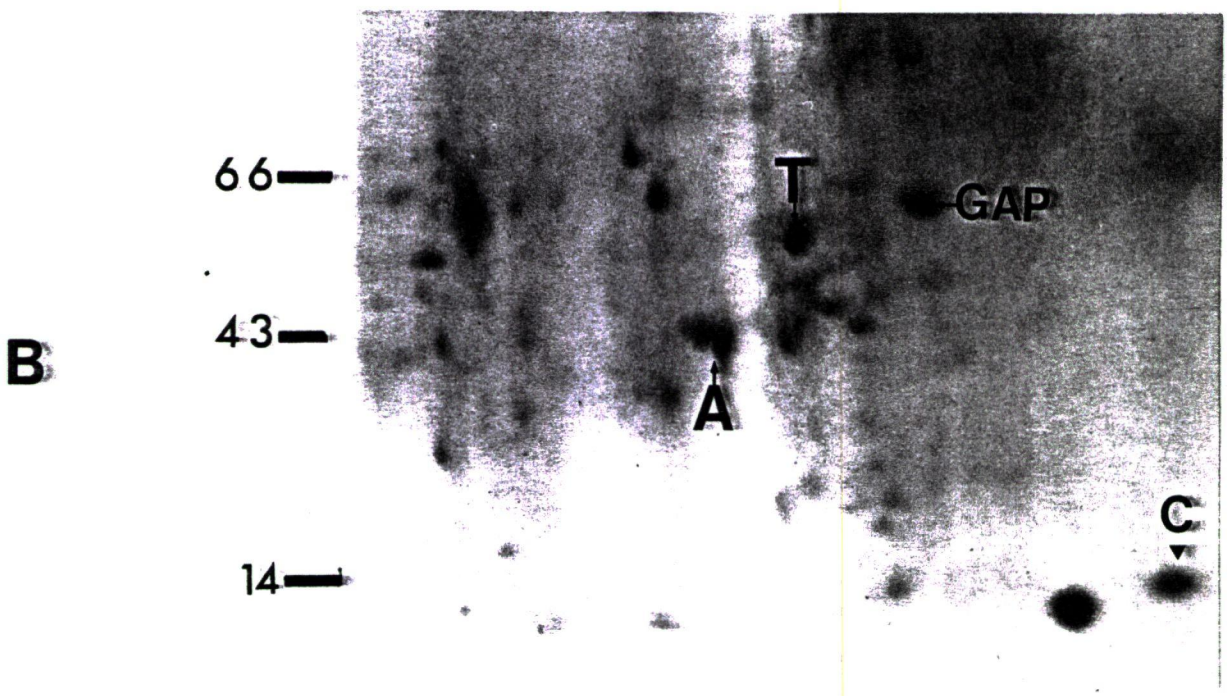
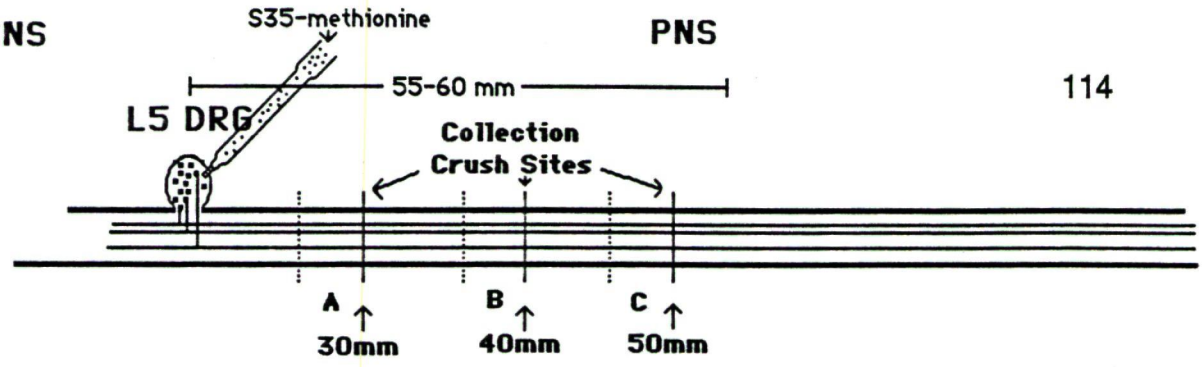
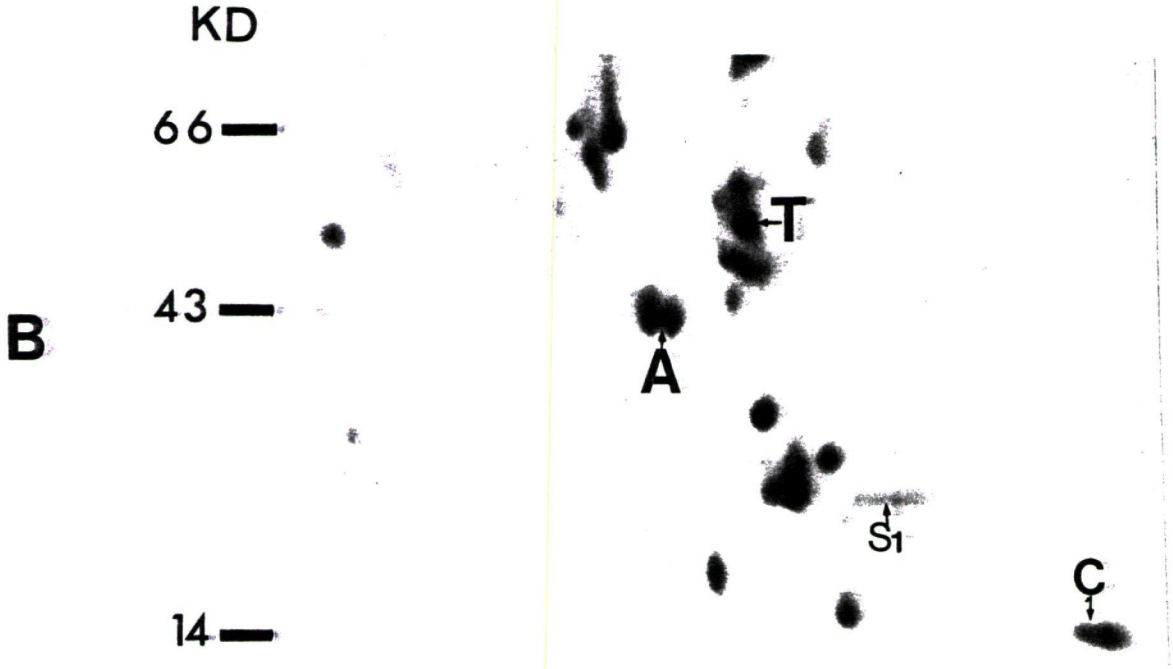
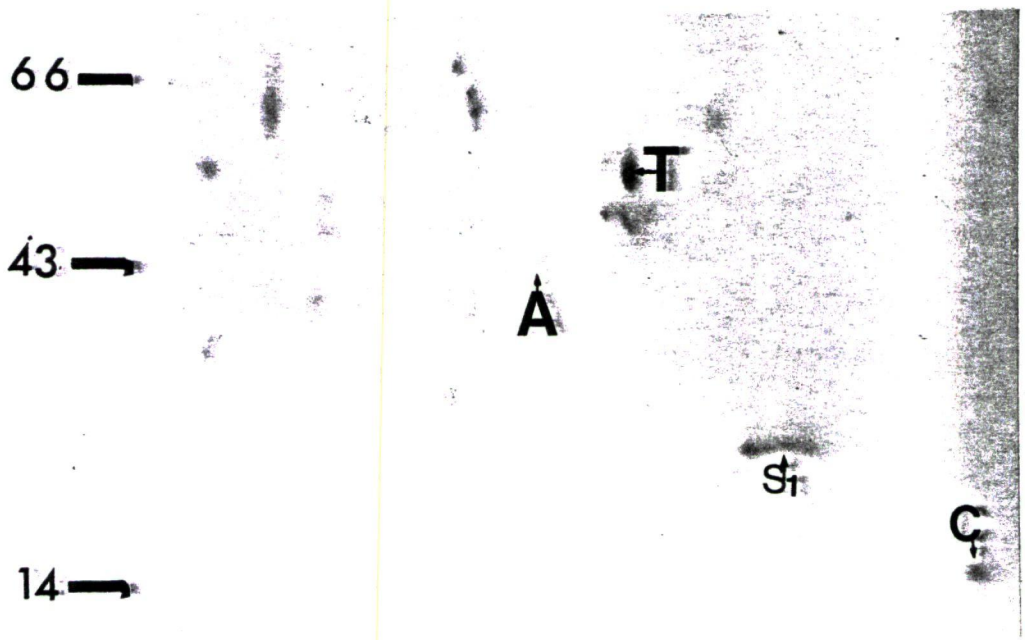

66
43
14
B
T
GAP
A
C

Figure 27. The following two fluorographs represents 2-D gels of fast transported proteins for control nerves. Animals were injected with 100 µCi [S-35] methionine, four hours later collection crushes were made 30 mm, 40 mm, 50 mm from the ganglion and two hours later the nerves were removed. Labeled actin (A), tubulin (T), and calmodulin (C) can be seen in segments 40 mm and 50 mm from the ganglion. Given that labeled proteins had six hours to be transported this corresponds to a velocity of at least 240 mm/day. Note that GAP-43 is not present, as this is a non-regenerating nerve. However, S1, a fast transported protein is present.


CNS
S35-methionine
PNS
55-60 mm
L5 DRG
Collection
Crush Sites
A
30mm
B
40mm
C
50mm


KD
66
43
14
T
A
S1
C

B


66
43
14
T
A
S1
C

C

DISCUSSION

The experiments reported in this chapter reveal that structural proteins (tubulins and actin), in addition to calmodulin, produced in the ganglion at the time of the TL or three days after the TL, are transported to the regenerating nerve fibers, hence, **newly regenerating fibers do not receive all of their structural proteins from those present in the axon prior to the injury. In addition, tubulin, actin and calmodulin are fast transported in regenerating and control sensory axons of the rat sciatic nerve.**

1. TRANSPORT OF CYTOSKELETAL PROTEINS TO REGENERATING FIBERS

Lasek and Katz (1987) have proposed the following model for cytoskeletal transport and axonal regeneration. In the nonelongating mature axon the cytoskeleton is continually advancing from the cell body to the axon tips. When cytoskeletal structures reach the axonal terminal they are degraded by calcium activated calpain, which may be controlled by the interaction of the axon terminal with its target (Lasek and Katz, 1987). Axotomy disconnects the axon from its target, deactivating the degrading mechanism, thereby, allowing growth cone formation and regeneration of new fibers. Central to this model is the idea that the continual moving of the axonal cytoskeleton in the anterograde direction is responsible for the regeneration of the axon and that the rate of regeneration is related to the amount of cytoskeletal material transported or its velocity (McQuarrie, 1988). In parallel with this hypothesis is the conservative model which was originally put forth by Weiss and Hiscoe (1946) and has

recently been used by McQuarrie and Lasek (1989) as a framework to explain their experimental results. These two models are very similar and both suggest that the cytoskeleton of the new axons is composed of cytoskeletal material which is in the axon prior to the TL.

Figures 3 and 20 do not support the idea that regenerating fibers derive all their structural material from proteins present in the axon prior to the TL. It is clear that proteins, corresponding to the molecular weight of actin and tubulins, synthesized at the time of and three days after the TL, are transported to the regenerating fibers. Closer examination, by 2-D gel electrophoresis of the regenerating region, positively identifies the labeled proteins as actin and tubulins (Figure 23). While local synthesis accounts for some of the label seen in the near distal segment it does not account for all of it (compare figure 23 and figure 24). This is supported by the lack of label in the segment just distal from where the leading growth cones are found. Therefore, according to my findings, Lasek and Katz's hypothesis of regenerating fibers receiving all their structural material from the cytoskeletal proteins present in the axon prior to the TL is not correct. While new axons do receive material which is in transit prior to the injury (what percentage is unknown) proteins synthesized in response to the injury are used by the regenerating fibers.

This result corresponds to what is happening in the cell body. As mentioned in the previous chapter, following injury to nerve cell axons the cell body responds by altering its expression of various proteins, notably, upregulating actin and tubulin and downregulating NF synthesis. Some of these

changes can be seen within hours following the injury. It would seem odd that the cell body would alter its synthesis of these major cytoskeletal proteins, soon after injury, when according to Lasek and Katz's hypothesis, these proteins will never reach the regenerating fibers, and hence play no direct role in regeneration. The results of my experiments indicate that tubulins and actin produced in response to the injury do reach the regenerating fibers suggesting that they may be important for axonal elongation.

Comparison of labeled proteins in the immediately proximal and distal segment reveals some surprising differences. With respect to the labeled α-tubulins in the proximal and distal segment, near equal amounts are present in both areas, with differences existing between isotypes. In the proximal segment α1 is dominant while in the distal segment α3 is dominant, α1 is substantially decreased and α2 is barely visible. My labelling scheme for α-tubulins corresponds to that used by Denoulet et. al. (1989). While my α1 and α3-tubulins appear very similar to Denoulet's, my α2 runs at a higher molecular weight. This may be due to the tyrosination or phosphorylation of the α2 seen in my experiments. While α1 is a primary translational product (Denoulet et. al., 1986), α2 and α3 are posttranslational modifications of α1 (Denoulet et. al., 1986; Eddé et. al., 1987). Therefore, a large proportion of the primary translational product (α1) is posttranslationally modified into α3 in the distal segment with very little being changed into α2 form.

For β-tubulin, an increased amount of label is seen in the distal segment relative to the proximal one, suggesting a greater amount of β-tubulin is

transported to the regenerating fibers. The appearance of two ß-tubulin isotypes (ß1 and ß2) in the distal segment is probably a posttranslational product of ß-tubulin (i.e. dephosphorylation). In SCa and SCb, Denoulet et. al. (1989) reported, in addition to ß-tubulin, the appearance of a ß1' and ß2' which ran at a slightly higher molecular weight than that of ß-tubulin. ß1' is a distinct primary translational product while ß2' is a posttranslational modification of ß1' (Denoulet et. al., 1986; Eddé et.al., 1987) Neither ß1' or ß2' is seen in my experiments. This suggests that not all tubulin isotypes are rapidly transported. Rather, the rapid transport of tubulin to the regenerating region is specific for the different isotypes. This implies that the transported isotypes may be important for regeneration.

The biological relevance of different isotypes being present in the proximal and near distal segments is probably related to their specific role in maintenance of the cytoskeleton in the regenerating fibers. With respect to rapidly transported α-tubulin the primary translational product, α1, is dominant in the proximal segment while the posttranslationally modified α3 is the most heavily labeled α-tubulin in the distal segment. As the axon is incapable of protein synthesis, posttranslational modification of proteins in transport provides a means of protein modification along the axon allowing local rapid modulation of the cytoskeleton. Hence, the abundance of α3 (thought to be a posttranslational acetylation [Eddé et. al., 1987; Denoulet et.al., 1989) in the regenerating fiber, is likely related to the need for this α-tubulin isotype for proper cytoskeletal arrangement and regeneration. The same can be said for the appearance of ß1

and ß2-tubulins. The precise role which these isotypes play in the formation of the cytoskeleton for regeneration fibers is not known. However, acetylation of α-tubulin is related to the stable polymeric form of tubulin while dephosphorylated ß-tubulin is associated with depolymerized microtubules (Black et. al., 1989; Black and Keyser, 1987; Gard and Kirschner, 1981). Therefore, rapidly transported α-tubulin when modified to α3 isotype may play a role in stabilizing the cytoskeleton of regenerating fibers. While rapidly transported ß-tubulin when posttranslationally modified in the regenerating region instills a degree of plasticity to the cytoskeleton by increasing the amount of soluble tubulin.

Calmodulin was also rapidly transported to the regenerating fibers. While calmodulin does not have a direct structural role, it may have an indirect effect on the cytoskeleton by its calcium binding properties. A more exciting role for calmodulin in the regenerating region is related to its ability to bind GAP-43 (Skene, 1989). While the physiological role of GAP-43 is not known its persistence in the regenerating fibers (labeled GAP-43 is seen in the regenerating region two days after injection) suggests that it may have some function in this region. Interaction with or some form of modulation over the cytoskeleton, mediated through calmodulin, is a reasonable possibility.

2. Fast Axonal Transport (FAT)

As mentioned in the introduction, two separate groups; Hollenbeck and Bray (1989) and Koenig et. al. (1985) have demonstrated, immunocytochemically, the appearance of tubulin, actin and calmodulin in

vesicles. Furthermore, Perry et. al. (1987), using traditional radiolabelling experiments to study fast transported proteins in regenerating goldfish optic axons, observed the increased labelling of α-tubulin, ß-tubulin and actin. These studies suggest that not all cytoskeletal proteins are slowly transported. My experiments confirm and extend these conclusions by demonstrating that tubulin, actin and calmodulin are fast transported in regenerating and control mammalian peripheral axons.

While these results strongly contradict the structural hypothesis, which views the cytoskeleton as being transported in assembled structures and occurring at a velocity of 1-4 mm/day, it does support the unitary hypothesis where cytoskeletal material is thought to be transported in a soluble subunit form by the FAT system.

3. SUMMARY AND CONCLUSIONS

The results in this chapter demonstrate that:

i. two major structural proteins, actin and tubulins, along with the calcium binding protein, calmodulin, produced at the time of a crush injury and three days after are rapidly transported to the regenerating fibers;

ii. immediately proximal and distal from the crush site differences exist with respect to the appearance of α and ß-tubulin isotypes - α1 is more prominent in the proximal segment while two ß-tubulin isotypes (ß1 and ß2) appear in the distal segment and

iii. actin, tubulin and calmodulin are fast transported in control and regenerating sensory sciatic nerve.

The conservative hypothesis would downplay the role of the cell body, with respect to its expression of cytoskeletal and associated proteins, during regeneration as it supports the idea that all structural proteins used by the daughter axons are derived from those present in the parent axon prior to an injury. The relatively rapid change in the expression of cytoskeletal proteins would be irrelevant as these proteins never reach the regenerating fibers. My results do not support such an idea, but rather, suggest that cytoskeletal proteins produced in response to a distal crush injury are used by the regenerating fibers and that the role of the cell body is important.

Furthermore, the appearance of different tubulin isotypes proximal and distal from the crush site suggests that rapidly transported tubulins can be post-translationally modified near the crush site. This suggests that the axon has some form of local control over the tubulin isotypes present in the regenerating fibers. Such a mechanism would probably occur so as to provide the regenerating fibers with isotypes needed for regeneration.

Hence, overall my results reveal both a local (post-translational modification at the crush site) and distal (rapid transport from the cell body) control over the supply of cytoskeletal proteins to regenerating fibers. In addition they demonstrate that actin, tubulin and calmodulin are fast transported, a finding which is consistent with the unitary hypothesis and strongly contradicts the structural and sliding polymer hypothesis of axonal transport.

CONCLUSIONS

The broad goal of this study was to investigate the role played by the transport of the cytoskeleton with respect to regeneration of the PNS. The first chapter investigated changes in the slow transport profiles of tubulins and actin under two different experimental conditions - transport in regenerating nerves following a single crush injury and transport in regenerating nerves which received a conditioning lesion prior to the test lesion. The second chapter tested the hypothesis that newly regenerating fibers receive all their actin and tubulin from those present in the axon prior to injury.

1. Changes in the Transport Profiles of Tubulins and Actin for Single and Double Lesioned Regenerating Nerves

The hypothesis which was tested in this set of experiments (chapter 1) was -- is the faster rate of regeneration, which is seen in conditioned lesioned nerves, concomitant with an acceleration in the transport of tubulins and actin? The answer was yes.

The experiments in this section demonstrated:

i. following a SL there is a decrease in the proportion of tubulins and actin transported in the SCa phase and an increase in the proportion of actin in the SCb phase,

ii. when the TL is preceded by a CL (in which case the fibers show an increased rate of regeneration) there is a further decrease in the proportion of tubulins and actin transported in the SCa phase in addition to an increase in the proportion

of actin and tubulins transported in or ahead of the SCb wave and,
iii. tubulins, but not actin, which are in transit prior to the TL demonstrate a change in their transport profile. This reorganization is similar to what is seen for tubulins and actin synthesized after the injury - a decrease in the proportion transported in the SCa phase and an increase in the proportion transported in the SCb phase. A prior CL enhances this effect.

Hence, in the conditioning lesion situation (where fibers are regenerating faster) a greater proportion of tubulins and actin are transported at faster velocity than in regenerating nerves which received a single lesion. In a recent abstract McQuarrie (1989) states that in conditioned lesioned nerves there is no change in the velocity of SCb proteins, but an increase in the amount of proteins being transported ahead of the SCb wave. This confirms my results.

An unexpected finding from this set of experiments was that tubulins in transit prior to the TL show a change in their transport profile. This demonstrates a degree of plasticity in the axon with respect to the transport of the cytoskeleton. Interestingly, this effect is enhanced in previously conditioned nerves suggesting the shift in tubulin transport may play a role in increasing the rate of regeneration for CL nerves.

The question to be asked is how this increase in the proportion of cytoskeletal proteins in and ahead of the SCb wave could be responsible for an increase in the rate of regeneration? If the increase in the proportion of tubulins and actin reflects an increase in the amount of tubulins and actin, hence, my results demonstrate an increased amount of tubulins and actin are transported

in and ahead of the SCb wave. For conditioned lesioned nerves this would mean a greater amount cytoskeletal proteins reach the crush site and are available for use by the regenerating fibers. The increased availability would then be responsible for the increase in the rate of regeneration.

2. Rapid and Fast Transport of Cytoskeletal Proteins

The hypotheses addressed in this section were:

i. regenerating fibers receive all their structural proteins from those present in the axon prior to injury and

ii. actin and tubulin are only slow transported.

The experiments in chapter 2 demonstrate that regenerating fibers do receive structural proteins which are produced in the cell body after the lesion and that tubulins and actin are transported at a velocity which is in the range of fast transport:

Labelled tubulins, actin and calmodulin, which were synthesized 3 days following a crush lesion, were transported to the regenerating region within 2 days. This result strongly contradicts Lasek and Katz's hypothesis which suggests that cytoskeletal proteins produced after injury play no direct role in regeneration. This idea downplays the role of the cell body during regeneration with respect to its regulation of cytoskeletal protein synthesis. My results challenge their hypothesis and emphasize the importance of the cell body and its production of cytoskeletal proteins in response to injury. Tetzlaff (1989) has demonstrated that the cell body alters its expression of cytoskeletal proteins soon after injury. His findings fit well with my results. Together they suggest that

rapid modulation of cytoskeletal protein synthesis, in response to injury, is a necessary event as some of these post-injury synthesized proteins are transported to regenerating fibers where they may be needed for successful regeneration.

A differential distribution of rapidly transported tubulin isotypes was found in the near proximal and near distal segment. The primary difference was the prominence of alpha1-tubulin in the proximal segment and the appearance of two novel beta-tubulin isotypes in the distal segment. This finding demonstrates posttranslational modification of tubulin at the crush site probably reflecting the need for different isotypes in the regenerating axons.

The finding of rapidly transported tubulin and actin (34 mm/day) reveals that tubulin and actin are not solely slow transported. I was able to demonstrate tubulin, actin and calmodulin transport at a velocity of at least 240 mm/day. While this is not as fast as other fast transported proteins (i.e. GAP-43) it certainly is within the range of fast transport. Such a finding lends support to the unitary hypothesis of axonal transport, arguing against the structural and sliding-polymer hypothesis of Lasek and colleagues.

In summary, this thesis has shed some light on the dynamics of cytoskeletal transport during regeneration:

i. by confirming a possible hypothesis for why conditioned fibers regenerate faster than non-conditioned nerves and

ii.by emphasizing the importance of cell body synthesis of cytoskeletal proteins in response to injury.

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