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Department of Anatomy and Cell Biology

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ABSTRACT

Title of Dissertation: Alterations in the Local Axonal Environment Influence Target Reinnervation and Neuronal Survival after Postnatal Axotomy

Hugh M. Dainer, Doctor of Philosophy, 2000

Dissertation directed by: Rosemary C. Borke, Ph.D.
Professor
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Following peripheral nerve injury in adult animals, Schwann cells (SC) proliferate and provide guidance in the local axonal environment by generating the infrastructure along which regenerating nerves grow. A portion of the SC in the peripheral nerves of postnatal rats undergo apoptosis during normal development, and this apoptosis is augmented at the injury site and the neuromuscular junction (NMJ) following sciatic nerve axotomy. The current work determined that SC apoptosis occurs in the distal but not the proximal nerve segment after postnatal transection of the hypoglossal nerve, suggesting that SC apoptosis is a general, age-related and location-specific response to peripheral nerve injury. Apoptotic SC were found in two strategic locations for guiding axonal outgrowth during peripheral nerve regeneration: a) capping the transected end adjacent to the injury site and b) just proximal to the bifurcation of the nerve into medial and lateral branches. Electron microscopic (EM) analysis identified apoptotic and mitotic SC in close proximity within the distal nerve segment, supporting apoptosis and mitosis as closely related phenomena.

Poor rates of neuronal survival and tongue musculature reinnervation following

hypoglossal nerve transection in the postnatal rat may be related to the increased apoptosis of SC in the distal nerve segment after injury. Insulin-like growth factor I (IGF-I) and basic fibroblast growth factor (bFGF) are neurotrophic factors which influence SC apoptosis *in vitro*. In an attempt to reduce SC apoptosis after postnatal axotomy, the effects of *in vivo* IGF-I, bFGF and vehicle treatments were examined in the distal nerve segment after postnatal hypoglossal nerve transection. All three treatments rescued SC, with the inclusion of bovine serum albumin (BSA) in the vehicle solution accounting for some of the decrease in SC apoptosis. While SC apoptosis was reduced with growth factor application, IGF-I treatment was deleterious to tongue musculature reinnervation and neuronal survival compared with bFGF and placebo treatments. Long-term bFGF treatment reduced neuronal survival compared with placebo treatment. These results imply that while SC in developing animals can be rescued by growth factor treatment, SC and motoneurons have not yet established the proper interactive relationship to translate this rescue into improved target musculature reinnervation or long-term motoneuron survival.

Alterations in the Local Axonal Environment Influence Target Reinnervation and Neuronal Survival after Postnatal Axotomy

by

Hugh M. Dainer
ENS, MC, USNR

Dissertation submitted to the faculty of the Department of Anatomy and Cell Biology graduate program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2000

To Rupa,

my wife,

my companion

and my best friend

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ABBREVIATIONS

ANOVA	Analysis of Variance
BDNF	Brain-derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CN	Cranial Nerve
CNS	Central Nervous System
CNTF	Cytokine Ciliary Neurotrophic factor
DAB	3,3'-diaminobenzidine
DAPI	4,6-diamino-2-phenylidole
DPN	Days Postnatal
DPO	Days Post-Operation
DRG	Dorsal Root Ganglion
E(number)	Embryological day (number)
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EM	Electron Microscopy
FGF	Fibroblast Growth Factors
bFGF	Basic Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
GAL-C	Galactocerebroside
GGF	Glial Growth Factors
HRP	Horseradish Peroxidase
Ig	Immunoglobulin-like
IGF-I	Insulin-like Growth Factor I
IGF-II	Insulin-like Growth Factor II
IGFBP-1	IGF-binding Protein 1
LARB	Laboratory Animal Review Board
LM	Light Microscopy
MBP	Myelin Basic Protein
NGF	Nerve Growth Factor
p75NGFR	low affinity NGF receptor
NGS	Normal Goat Serum
NMJ	Neuromuscular Junction
NRG	Neuregulin
NT-3	Neurotrophin-3
P(number)	Postnatal day (number)
PBS	Phosphate-buffered Saline
PCD	Programmed Cell Death
PI3K	Phosphatidylinositol 3-kinase
PMR	Physical Medicine and Rehabilitation
PNS	Peripheral Nervous System
P^o	Myelin zero protein
RER	Rough Endoplasmic Reticulum

ABBREVIATIONS (CONTINUED)

RITC	Rhodamine Isothiocyanate
SC	Schwann cell
SEM	Standard Error of the Mean
SNK	Student-Newman-Keuls
SSC	Sodium Citrate-Sodium Chloride
TGF-beta	Transforming Growth Factor-beta
TMB	Tetramethyl Benzidine
TR	Transection
TUNEL	Terminal dUTP Nick-end Labeling

INTRODUCTION

Peripheral Nerve Injury

Necessity of Further Research

A telling statement of the necessity for further research in peripheral nerve regeneration is contained in Chapter 2 of the 2nd Edition of the comprehensive Management of Peripheral Nerve Problems. The author discusses briefly the improvements of the last 40 years in microsurgical repair techniques and electrical analysis of nerve impulses. He notes that intraoperative microscopes and surgical ingenuity have had the greatest impact on successful peripheral nerve treatment during this period (Spinner, 1998). Although he is a physician heavily invested in healing nerve injuries by surgical intervention, Dr. Spinner recognizes the limitations of surgical repair.

> *One of my fervent wishes for the future is the discovery of a neurotrophic substance that would encourage axon regeneration, especially in a proximal nerve lesion. One Nobel prize has already been awarded for a nerve growth factor, it is hoped that there is another on the way for solving this problem* (Spinner, 1998).

Traumatic Peripheral Nerve Injury and Military Medicine

The treatment of peripheral nerve injuries is an important part of military medicine. In World War I, the French surgeon Tinel noted the surprise discovery of military physicians that 1 of every 5 limb wounds resulted in a lesion to at least one of the

nerve trunks (Tinel, 1917). During World War II, the high numbers of peripheral nerve injuries led more than a hundred of the neurosurgeons serving in the US Army Medical Corps to collect and organize over 3,500 peripheral nerve cases (Woodhall and Beebe, 1956). During the Persian Gulf War, 44% of casualties referred to Physical Medicine and Rehabilitation (PMR) services suffered from nerve injuries (Dillingham et al., 1993). Most recently, physicians in the former Yugoslavia reported that the use of cluster bombs and dumdum bullets had led to 20% of the injuries treated at the University Hospital Laboratory of Neurophysiology located in Split, Croatia (Vrebalov-Cindro et al., 1999). While the civilian practitioner might see one or two major peripheral nerve injuries in a clinical lifetime, military physicians can accumulate lifetimes of experience in a few years (Krekorian, 1971).

Other Causes of Peripheral Nerve Injury

While trauma leads to sensory and motor deficits in an otherwise healthy patient population, the majority of peripheral neuropathies result from medical illnesses (Greenberg, 1994). Non-traumatic causes of peripheral nerve injuries include diabetes, alcohol abuse, Guillain-Barré syndrome, nutritional deficiencies, genetic diseases, renal insufficiencies, radiation exposure, tumors, amyloid deposition, entrapment syndromes, infections, post-infection syndromes, surgical complications and toxic exposures. A retrospective study of carotid endarterectomy patients at Duke in the 1970's demonstrated that approximately 15% of patients suffered from cranial nerve damage (Massey et al., 1984). One-third of the patients suffering from iatrogenic hypoglossal nerve injuries and

two-fifths of the patients suffering from iatrogenic cervical branch of the facial nerve injuries demonstrated deficits a full year after carotid artery surgery. Though peripheral nerve trauma has historically been less prevalent in civilian than military populations (though this may be changing), patients suffering from all types of peripheral neuropathies will benefit from techniques that improve nerve regeneration.

Current Treatment Strategies

Peripheral neuropathies caused by medical illnesses have been targeted by preventive practices. Effective prevention includes reducing repetitive activities that compress nerves, avoidance of exposure to neurotoxic agents and encouragement for physicians to pre-operatively review the nervous innervation of surgical areas. For traumatic peripheral nerve injuries, primary nerve suture remains the standard of care, especially for proper recovery from motor nerve injuries. However, a number of factors, such as the delay between injury and surgical repair and the age of the patient, limit the effectiveness of primary nerve suture. Other more life-threatening trauma, such as head injuries, may obscure peripheral nerve injuries (Noble et al., 1998; Rakolta and Omer, 1969). Intense combat environments may also prevent immediate surgical intervention (Cramer, 1972), as necessitated by proper triage of injured soldiers. If repair is delayed following injury, peripheral nerves display reduced ability to reinnervate, most likely due to reduced effectiveness of SC in the distal stump and atrophy of end-organs (Bradley et al., 1998; Young, 1993). Unfortunately, nerve suture tends to be most successful in facilitating nerve regeneration in patients less than 20 years old (Omer, 1974).

Rates of Peripheral Nerve Regeneration

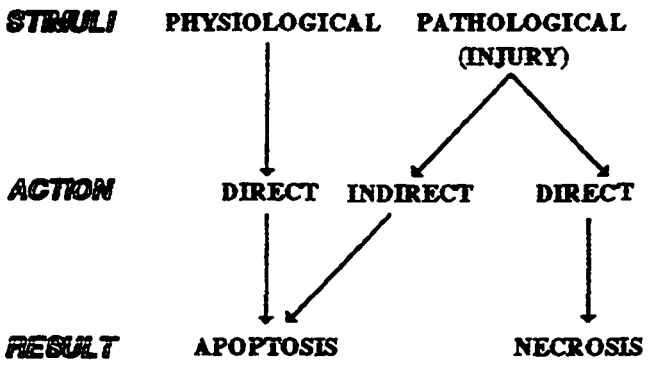
The Handbook of Neurosurgery reports that human peripheral nerves regenerate at a rate of approximately 1 mm per day (Greenberg, 1994). However, actual rates of regeneration are more difficult to predict, as functional recovery demands that nerve fibers reach their destination, mature, increase in diameter and myelinate, and each stage has independent rates of progress (Young, 1993). Although overly simplistic, the textbook figure gives us an appreciation for the slow recovery inherent in peripheral nerve injury. When proximal lesions occur, slowly growing axons may fail to reinnervate before distal targets have atrophied.

Neuronal Survival

Apoptosis and Programmed Cell Death

Experiments conducted in the 1940's and 1950's by Hamburger and Levi-Montalcini first demonstrated conclusively that massive cell death is a normal developmental event (Oppenheim, 1991). Over time, the recognition of programmed cell death (PCD) as a necessary process has shifted the study of PCD from pathologists to the general scientific community (Gerschenson and Rotello, 1992). Although used synonymously by most researchers, PCD refers to naturally-occurring developmental cell death, while apoptosis refers more generally to the cell death mechanism seen not only in

developmental cell death, but also in disease and injury (Narayanan, 1999). Although apoptosis can be induced by a variety of toxic stimuli, developmental PCD appears to have 4 main functions: 1) elimination of neurons without functional synaptic contact to targets, 2) elimination of embryonic layers during sheet fusion and shape changing, 3) elimination of transient neural populations and 4) elimination of lineage-derived cells (Narayanan, 1999). Exposure of developing cell populations primed for PCD to abnormal conditions may confuse cell signaling and cause inappropriate cells to enter the apoptosis pathway. Apoptosis, including the apoptotic subset PCD, is distinct from necrosis, which is always due to pathological insult. Apoptosis, which occurs following withdrawal from the cell cycle, constitutes a lack of inflammation and a restriction of lysosomal enzyme release. Nuclear breakdown is an early change during apoptosis. Nuclear fragmentation (karyorrhexis) is controlled and isolated, affected cells can be identified (Gerschenson and Rotello, 1992). Following nuclear breakdown, apoptotic cells fragment into smaller, membrane-bound portions termed apoptotic bodies. Necrosis involves groups of cells in which inflammation, release of lysosomal enzymes, and nuclear disappearance (karyolysis) occur.



Pathways to Apoptotic and Necrotic Cell Death
(Gerschenson and Rotello, 1992)

Factors Influencing Motoneuron Survival during Development

Research into the survival of motoneurons in embryonic and postnatal animals has led to the hypothesis that neurons of developing animals compete for limited quantities of target-derived (skeletal muscle-derived) neurotrophic factors (Oppenheim et al., 1982; Raff et al., 1993). Other studies have demonstrated that although motoneurons innervate multiple motor end plates at birth, the ratio of motoneurons to motor end plates declines to 1:1 by the end of the third week (Brown, M. C. et al., 1976). Together, these studies support the role of PCD in the normal development of peripheral motoneurons.

Neuronal Survival Following Axotomy

The credibility of the target-derived neurotrophin hypothesis has been supported by the observation that axotomy, which disrupts the retrograde transport system of neurons, produces different rates of motoneuron death in neonatal, juvenile and adult animals (Lowrie and Vrbova, 1992). Cranial nerve transection in rats one week old produced the death of 60% of hypoglossal motoneurons, whereas transection in animals 3 weeks old produced the death of 30% of hypoglossal motoneurons (Snider and Thanedar, 1989). The 3-week-old animal response was equivalent to the adult animal (Borke, 1983). The differences in neuronal survival between the 1-week-old and 3-week-old animals may be due to a feedback loop operating developmentally to prevent neurons from innervating a muscle that has already been properly innervated. Innervation of the target may induce a transition from the immature or growing neuron to a mature or

transmitting neuron (Greensmith and Vrbova, 1996).

Trophic Factor Influence on Neuronal Survival

The theory that axotomy causes motoneuron death through neurotrophin interruption is historically based on the following observations: 1) severely injured neurons will survive when cultured in medium including appropriate factors, 2) axotomy effects can be mimicked by the interruption of axonal transport, 3) manipulations, such as deafferentation, during development which do not injure neuronal axons also lead to neuronal cell death and 4) replacement of target-derived growth factors can rescue neurons that would ordinarily die after axotomy (Snider et al., 1992). With this hypothesis, the most direct solution for a potential deficiency in neurotrophic factors would clearly be exogenous application. Unfortunately, experiments employing this technique in combination with axotomy have had limited success. Although brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), cytokine ciliary neurotrophic factor (CNTF) and a variety of combinations are temporarily effective (Oppenheim, 1991), they fail to achieve the goal of long-term neuronal survival (Eriksson et al., 1994; Vejsada et al., 1995). In addition, experiments using "knockout" animals lacking the gene for NGF or other neurotrophins have shown substantial sensory-related neuron deficits but very limited motoneuron deficits (Snider, 1994). This result demonstrates that the target-derived neurotrophin hypothesis serves as only a partial explanation (Friedman et al., 1995).

Glial Cell Influence on Neuronal Survival

In addition to connections with targets and afferent inputs, neurons also communicate with glial cells (Lieberman, 1971). The glial cells of the peripheral nervous system (PNS) are Schwann cells. SC provide infrastructure to assist neurons as they grow to innervate distal structures during development and provide trophic support before neurons reach distal targets (Davies, 1998). In addition, SC possess survival factors, which aid in the *in vitro* culture of embryonic neurons (Bunge, 1993; Bunge, 1994; Raabe et al., 1996). Following injury, SC provide basal lamina, neurotrophic factors, extracellular matrix (ECM), cell adhesion molecules and receptors that enhance communication between SC and neurons (Fawcett and Keynes, 1990). In essence, the injury site becomes a "substitute target" following axotomy and proliferating SC form columns or bands of Büngner which are crucial for successful peripheral nerve regeneration (Ide, 1996). Further experiments have shown that basal lamina or grafts provide limited assistance in regeneration without viable SC present (Gulati, 1988). Another interesting finding is that both the proximal and distal nerve stumps must be living, as freeze thawing of the either nerve stump (which kills all resident SC) prevents infiltrating SC from completely bridging the gap (Madison and Archibald, 1994). Conversely, transplants of adult rat SC into other adult rats have been shown to improve regeneration of the sciatic nerve across a normally prohibitive distance of 18 mm (Ansselin et al., 1997). As current transplantation protocols often employ Matrigel (synthetic basement membrane) as a matrix for SC, it is possible that the acellular transplants have allowed SC to partially migrate into the graft (Osawa et al., 1990).

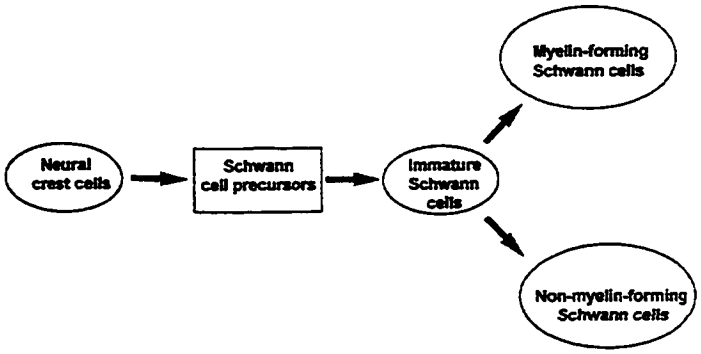
Interestingly, Matrigel also has been shown to promote SC differentiation and activate expression of myelin zero protein (P^o) and myelin basic protein (MBP), both of which are characteristic of myelinating SC (Cheng, H. L. and Feldman, 1997).

Schwann Cells

Schwann Cell Derivation

On embryological day 14 or 15 (E14-15) in developing rats, multipotential neural crest cells give rise to intermediate cells called SC precursors (Mirsky and Jessen, 1996; Mirsky et al., 1996). SC precursors divide actively, maintain a flattened appearance and express low affinity nerve growth factor (NGF) receptor (p75NGFR), N-CAM and GAP-43 markers. They fail, however, to express the distinct SC S100 protein (Mirsky et al., 1996). Experiments conducted *in vitro* have shown that SC precursors will only produce SC if cultured in the presence of media conditioned by co-cultured neurons or enriched with neu-differentiation factors or neuregulins, which are expressed in high levels by developing neurons (Jessen et al., 1994). *In vivo*, SC precursors rapidly become immature SC. By E17, these SC exhibit a bipolar morphology and express S100 protein, while maintaining p75NGFR, N-CAM and GAP-43 markers. This transformation into immature SC is apparently irreversible (Mirsky and Jessen, 1996). Newly committed immature SC proliferative rapidly during the late embryogenesis, migrate to axons and extend processes which segregate axons into bundles (Jessen and Mirsky, 1992). Between postnatal day 2 and 4 (P2-4), immature SC stop proliferating.

The cessation of proliferation appears to trigger differentiation of immature SC into myelinating and non-myelinating forms (Brown, M. J. and Asbury, 1981) under the influence of axon-associated signals (Jessen et al., 1987). Differentiation at this stage appears to be reversible (note that the cited figure improperly contains only one-way arrows),

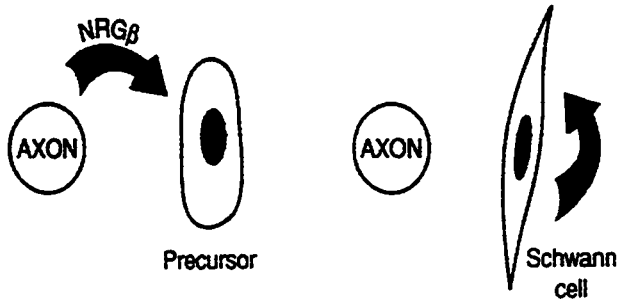


Schwann cell lineage schematic (Jessen and Mirsky, 1998)

with both myelinating and non-myelinating SC retaining the ability to dedifferentiate into the immature form both *in vivo* and *in vitro* when deprived of axonal contact (Mirsky and Jessen, 1996; Mirsky and Jessen, 1999). Non-myelinating SC retain phenotypic markers of immature SC (GAP-43, p75NGFR and S-100) and gain the expression of galactocerebroside (GAL-C) in the third postnatal week (Zorick et al., 1996). Myelinating SC express GAL-C during the late embryonic-early postnatal period, but do not express GAP-43 or p75NGFR once fully differentiated (Zorick et al., 1996). Myelinating SC can be identified by markers of myelin-associated protein, PMP-22, P^o, and MAG (Zorick et al., 1996). Transforming growth factor ß (TGFß) appears to inhibit myelination by SC both *in vivo* and *in vitro*, while IGF-I promotes myelin formation (Mirsky et al., 1996).

Postnatal Schwann Cell Apoptosis

SC adjacent to neuromuscular junctions (NMJ) in the rat undergo PCD during the first two postnatal weeks (Trachtenberg and Thompson, 1996). The current thought is that PCD is due to competition among SC for a limited amount of axonally derived trophins. SC during this postnatal period when apoptosis is prevalent apparently do not express myelin-related proteins but do express p75NGFR and erbB2, two receptors which are not expressed by myelinating SC (Cohen et al., 1992). Postnatal SC apoptosis coincides with axonal remodeling and synapse elimination of immature neurons (Matteoli et al., 1990). Axotomy at postnatal day 5 results in a 10-fold elevation in SC apoptosis in the distal nerve segment by 1 day after sciatic nerve injury (Grinspan et al., 1996). If the injury is delayed until postnatal day 20, there is little or no apoptotic SC death (Grinspan et al., 1996). Axotomy at postnatal day 14 results in the loss of 85% of SC at the NMJ via apoptosis by 3 days postoperative (DPO) (Trachtenberg and Thompson, 1996). If the injury is delayed until postnatal day 25, only 5% of SC are lost at the NMJ (Trachtenberg and Thompson, 1996).



Axonal to autocrine survival (Jessen and Mirsky, 1998)

The SC populations present in the postnatal day 0 and 5 animals possess neuregulin (NRG) receptors, erbB2 and erbB3, which are absent in the adult animals. These receptors are phosphorylated *in vivo* in response to endogenous NRG (Grinspan et al., 1996). Exogenous NRG abolishes the increase in apoptotic SC induced by sciatic

nerve axotomy in the postnatal rat (Grinspan et al., 1996; Trachtenberg and Thompson, 1996).

More recently, researchers have associated glial survival in the dorsal root ganglia (DRG) with neuronal survival by demonstrating a lag between the apoptosis of neurons and the apoptosis of glial cells following sciatic nerve axotomy of neonatal animals (Whiteside et al., 1998). In adult cats, transected distal ends of axotomized nerves were prevented from connecting with proximal segments for extended periods (80 weeks). Following eventual reattachment to the proximal segment of a newly transected neighboring nerve, they showed an absence of myelin (Pellegrino and Spencer, 1985). This result was attributed to the slow, progressive atrophy of SC in the distal stump of injured adult animals. Glial cell death in adult animals following prolonged lack of contact with axons shows that the switching of SC dependence from axon-derived factors to SC autocrine factors remains only a partial conversion. Limited independence from axon-derived survival factors is also supported by another series of experiments using adult cats in which cold blockade of anterograde axonal transport activated SC premitotic activity (Oaklander and Spencer, 1988). SC begin to switch their survival dependency around the time of birth from axonal signals such as NRG to autocrine signals (Cheng, L. et al., 1998; Jessen and Mirsky, 1997).

Schwann Cell Role following Adult Axotomy

SC proliferation and interaction with regenerating axons are critical for neuronal survival and proper target reinnervation following nerve injury in adult rats. There are

two waves of SC proliferation in the damaged nerve. The first is the proliferation of SC in the distal segment of the transected nerve during the degenerative phase. There is a 40-fold increase in SC by 4 days post-axotomy that declines to a 3-fold increase by 5 days post-axotomy (Liu et al., 1995; Pellegrino et al., 1986). Blocking of fast anterograde transport demonstrates that SC proliferation may be directly or indirectly associated with an unidentified anterograde signal transported by the neurons (Oaklander and Spencer, 1988; Wu et al., 1994). In addition to stimulating mitosis, injured axons may signal SC to resume the developmental task of phagocytosis (Liu et al., 1995). SC assist in axon and myelin debris removal and remain within the basal lamina that supported the axons before injury. The processing of myelin debris by SC may induce and attract resident macrophages, which in turn secrete cytokines that stimulate SC proliferation (Liu et al., 1995). SC displaying the non-myelinating antigens p75NGFR and GAP-43 form the bands of Büngner, which allow regenerating axons to reinnervate the distal nerve segment. Entry of the regenerating axons into the distal nerve segment appears to stimulate the second proliferation of SC and eventual myelination of the nerve (Pellegrino and Spencer, 1985).

Schwann Cell Role following Postnatal Axotomy

Whether or not SC actually form the pathways along which developing axons initially course to innervate target muscles has been a controversial issue (Davies, 1998; Son et al., 1996). However, neonatal nerve damage leads to fewer functional neuromuscular junctions than after a comparable nerve injury in adults, and recent studies

suggest that the increased apoptosis of SC near the NMJ or in the distal nerve segment following postnatal axotomy of the sciatic nerve may be partially responsible (Grinspan et al., 1996; Trachtenberg and Thompson, 1996). The large increase in neuronal death (Snider and Thanedar, 1989) and reduction in tongue musculature reinnervation (Blake-Bruzzini et al., 1997) following postnatal hypoglossal nerve transection could also be related to increased SC apoptosis in the postnatal animal. An important first step in exploring this research is determining whether the apoptosis of SC increases following postnatal hypoglossal nerve transection. Hypothesis I of the current project concerned this topic: *Schwann cell apoptosis is an age-related response to postnatal transection of the hypoglossal nerve.* Specific Aim I addressed this hypothesis: *To determine if Schwann cell apoptosis is an age-related response to postnatal transection of the hypoglossal nerve.*

Growth Factors

Neuregulin

The glial growth factors (GGF) or neuregulins compose a family of signaling proteins with at least 12 members showing structural similarities to epidermal growth factor (EGF) (Lemke, 1996; Marchionni et al., 1993). Alternate splicing of 13 or more exons of a single gene located on the short arm of human chromosome 8 produces all of the NRG isoforms involved with neuron-target interactions, SC-differentiating peripheral nerve development and neuromuscular synaptogenesis (Chen et al., 1994; Lemke, 1996;

Tabernero et al., 1998). These GGF induce tyrosine phosphorylation of the *neu* proto-oncogene, hence the collective name neuregulins. Structurally, the neuregulins possess a single immunoglobulin-like (Ig) fold and a single epidermal growth factor-like domain. Alternate splicing of the epidermal growth factor-like domain creates the alpha- and beta-isoforms, which differ in their potency as mitogens and survival factors for SC lineage (Grinspan et al., 1996). NRG effects are routed through a subfamily of tyrosine kinase receptors including erbB2, erbB3 and erbB4, which are related to the epidermal growth factor (EGF) receptor family (Carraway and Burden, 1995). The creation of a functional NRG receptor involves the heterodimerization of the signal transducer erbB2 and either of the other 2 NRG binding components, erbB3 or erbB4 (Syroid, Daniel E. et al., 1996). NRG act in the following SC processes: 1) signals for differentiation of neural crest cells into neuronal or glial cells (Shah et al., 1994; Topilko et al., 1996), 2) survival factors for SC precursors that promote their transformation to immature, committed SC (Dong et al., 1995) and 3) signals to allow axon-SC association during the differentiation of SC to non-myelinating and myelinating forms (Mirsky et al., 1996).

IGF-I as Schwann Cell Survival Factor

Insulin-like growth factor I is a polypeptide with approximately 50% structural homology with insulin (Lewis et al., 1993a). IGF-I and the closely related insulin-like growth factor II (IGF-II) act on cells by binding to the high-affinity IGF-I receptor, causing the autophosphorylation of an intercellular tyrosine kinase domain (Lewis et al., 1993a). Attempts to link axonal sprouting and neuronal survival to axonal uptake and

retrograde transport relying on indirect evidence are less than convincing (Kanje et al., 1991). Radioactive IGF-I is not transported retrogradely from axons (Caroni and Grandes, 1990; Ferguson et al., 1991). Uptake of IGF-I by motorneuron cell bodies does not appear to be the mechanism of *in vivo* IGF-I action either. Although facial nerve axotomy causes a strong increase in IGF-I immunoreactivity of hypertrophic astrocytes near injured motor neurons, no IGF-I receptor immunoreactivity occurs in the neurons of the facial nucleus following injury (Gehrmann et al., 1994). While IGF-I does not appear to function by direct action on neurons *in vivo*, the possibility remains that IGF-I contributes indirectly by enabling the effective response of glial cells to motor neuron injury. If IGF-I affects neurons through glial intermediaries, *in vivo* administration of IGF-I may improve neuronal survival and axonal regeneration by increasing SC survival following axotomy.

When serum-free *in vitro* SC cultures plated from 3 days postnatal (DPN) rats are provided with IGF-I, 80% of SC survive in a response equivalent to NRG supplementation (Syroid et al., 1996; Syroid et al., 1999). As 15-25% of plated SC die on the first day, 75-85% survival appears to be the maximal value obtained *in vitro* (Cheng, L. et al., 1998). IGF-I *in vitro* rescue of SC has been duplicated with the application of a caspase-3 inhibitor and prevented with the application of a phosphatidylinositol 3-kinase (PI3K) inhibitor, demonstrating that IGF-I prevents caspase-mediated SC apoptosis through interaction with the PI 3-K signaling (Delaney et al., 1999). SC express IGF-I receptor *in vitro* (Schumacher et al., 1993; Stewart et al., 1996), but IGF-I production in early postnatal SC appears to be deficient (Syroid, D. E. et al., 1999). In adult animals, sciatic nerve crush injury led to an intense increase in IGF-I

gene expression (between 4 and 6 days post injury) at the site of injury (Pu et al., 1995). Distal to the injury site, IGF-I gene expression reached a maximum at 10 days following the crush injury and returned to the baseline level 20 days after the injury, following axon regeneration (Pu et al., 1995). In adult rats, SC become intensely IGF-I immunoreactive following sciatic nerve transection, with a maximal level reached in 2 weeks (Hansson et al., 1986). SC appear to be the source of this increased IGF-I present both in local SC and extracellularly following nerve injury (Hansson et al., 1986; Pu et al., 1995). More recently, IGF-I activity in the distal segment following nerve transection was shown to be produced mainly by SC for the first 7 days post-injury with invading macrophages producing high levels of IGF-I after 7 days (Cheng, H. L. et al., 1996). Collectively, *in vitro* and *in vivo* studies support IGF-I as an autocrine survival factor in mature SC, replacing axonally-derived NRG support of immature SC and SC precursors (Jessen and Mirsky, 1998). Hypothesis II of the current work concerned the potential benefits of IGF-I and other growth factors: *The application of growth factor treatments will reduce Schwann cell apoptosis after postnatal transection of the hypoglossal nerve.* Specific Aim II addressed this hypothesis: *To determine if the application of growth factor treatments reduces Schwann cell apoptosis after postnatal transection of the hypoglossal nerve.*

IGF-I in Neuronal Survival and Target Reinnervation

Work examining the effects of IGF-I on motoneuron survival *in vitro* used cells from embryonic animals, with results varying from 15 to 50% neuronal survival in the

presence of exogenous IGF-I (Ang et al., 1992; Arakawa et al., 1990; Hughes et al., 1993). Lower survival rates occur when the cultured motoneurons are grown without supporting glial cells, whereas higher survival rates are found when cultured motor neurons are plated with supporting glia. Although one study with high survival rates claimed to be using pure motor neuron cultures, they attempted to purify via the low-affinity NGF receptor, which has also been identified on glial cells (Yokoyama et al., 1993). Therefore, this method of neuronal isolation could not have prevented the inclusion of glial cells. When Gelfoam soaked in IGF-I diluted in phosphate-buffered saline (PBS) was transplanted at the site of the lesion into axotomized newborn rat pups, 29% of the motoneurons in the facial nucleus were rescued up to 7 DPO by the treatment compared with 19% in vehicle animals (Hughes et al., 1993). In addition to improving neuronal survival, IGF-I appears to affect axonal regeneration. Subcutaneous injection of IGF-I or IGF-II into the adult rat or mouse gluteus muscle elevates intramuscular nerve sprouting 10-fold over unexposed and control animals (Caroni and Grandes, 1990). Once again, this effect could not be well separated from SC involvement, as attempts to confirm the IGF-I receptor on motoneurons *in vitro* with ^{125}IGF-I also demonstrated extensive non-neuronal binding (Caroni and Grandes, 1990). In addition to sprouting effects, IGF-I applied systemically or to the site of injury via osmotic pumps has been shown to enhance the regeneration of peripheral nerves following crush injury (Contreras et al., 1993; Ekstrom et al., 1989; Kanje et al., 1989; Lewis et al., 1993a; Sjoberg and Kanje, 1989). Hypothesis III of the current project concerned the potential long-term regenerative benefits of IGF-I and other growth factors: *The alteration of the local axonal environment will influence target reinnervation and neuronal survival after postnatal*

transection of the hypoglossal nerve. Specific Aim III addressed this hypothesis: *To determine if alteration of the local axonal environment influences target reinnervation and neuronal survival after postnatal transection of the hypoglossal nerve.*

Basic FGF in SC Survival, Neuronal Survival and Target Regeneration

Increased SC numbers following bFGF application appear to be induced mainly by SC proliferation, as opposed to direct SC survival effects (Davis and Stroobant, 1990; Jessen and Mirsky, 1992; Mirsky and Jessen, 1996; Mirsky et al., 1996). However, adult rat SC express bFGF within 2-3 days after sciatic nerve injury (Liu et al., 1995), and studies on central and peripheral neuronal populations have demonstrated that bFGF most likely functions through autocrine or paracrine pathways (Weise et al., 1993). Basic fibroblast growth factor is a member of the large (15 or more member) fibroblast growth factor (FGF) family, characterized by selective binding to sulfated glycosaminoglycan heparin and mitogenic or neurotrophic effects (Eckenstein, 1994; Kuzis et al., 1999). The ability of bFGF to bind proteoglycan allows SC to condense bFGF in the basal lamina, where it will contact both SC and regenerating axons (Ide, 1996). bFGF then acts through FGF receptors, by stimulating an intracellular tyrosine kinase domain (Wanaka et al., 1991).

Application of bFGF to the proximal end of lesioned sciatic nerve in adult rats prevents neuronal death (Otto et al., 1987). In addition, transgenic mice created to overexpress bFGF showed a statistically significant increase in motoneuron survival following postnatal nerve crush injury to the facial nerve (Kuzis et al., 1999). Basic FGF

treatment following spinal cord injury in rats doubles the survival rate of ventral horn neurons adjacent to the injury site (Teng et al., 1999). When bFGF has been applied distal to crush lesions of rat sciatic nerves, nerve regeneration is improved (Vergara et al., 1993). In dogs, bFGF has been shown to improve nerve regeneration in the presence of acellular SC basal laminae grafts (Ide et al., 1998). Early experiments demonstrating the ineffectiveness of bFGF to enhance neuronal survival *in vivo* may have been handicapped from an inadequate method of bFGF delivery (Li et al., 1994). Gelatin hydrogels that have been created to slowly release bFGF *in vivo* were effective in enhancing bone regeneration in rabbits, by allowing bFGF to function at the site of injury for an extended period of time (Yamada et al., 1997). Inconsistencies in bFGF improvements to motoneurons have led to delay in human trials of bFGF for treatment of neurodegenerative disorders (Lefaucheur and Sebille, 1997). However, recent successful *in vivo* experiments demonstrate the possibility that glial intermediaries may be important for modulating the effects of bFGF on motoneurons (Ide et al., 1998; Kuzis et al., 1999; Teng et al., 1999; Yamada et al., 1997). The potential benefits of bFGF treatment to the local axonal environment after postnatal transection of the hypoglossal nerve led to the inclusion of bFGF in Specific Aims II and III of the current work.

MATERIALS AND METHODS

Animal Population

Pilot Studies

Two pilot studies were initially performed for this investigation: 1) a study to determine the postnatal age and survival time for apoptosis of SC and 2) a study to establish a timetable of reinnervation of the tongue musculature after hypoglossal nerve transection. The short-term apoptosis study consisted of 27 rats of three postnatal ages: 10, 21 and 30 DPN. Three survival periods were used: 1, 2, and 3 days DPO following hypoglossal nerve transection. The long-term pilot study included ten 10 DPN rats at five survival times after nerve transection: 14, 17, 20, 30 and 40 DPO. Collectively, the pilot studies required 37 animals.

Major Studies

The results of the two pilot studies defined the ages and survival times of rats used for the specific aims of the study. A three-part investigation was carried out involving 10 DPN rat pups: 1) short-term fluorescent light microscopic terminal dUTP nick-end labeling (TUNEL) assays for SC apoptosis, 2) short-term EM assays for SC apoptosis and 3) long-term reinnervation and neuronal survival assays. A total of 41 rat pups was used for this three-part investigation.

TUNEL Assay for Apoptosis

Short-term experiments (10 DPN + 2 DPO) were performed to estimate apoptosis of SC in the distal segment of the hypoglossal nerve following nerve transection. A total of 20 rats was used for this portion of the investigation. The following five groups each required 4 animals: 1) 12 DPN uninjured control rats, 2) Cranial nerve (CN) XII transection (TR) only rats, 3) CN XII TR and Gelfoam soaked with vehicle solution rats, 4) CN XII TR and Gelfoam soaked with IGF-I solution rats and 5) CN XII TR and Gelfoam soaked with bFGF solution rats. The distal nerve segments from 2 animals (one TR-only and one vehicle) were not evaluated due to poor tissue penetration of labeling solutions during the TUNEL assay.

Qualitative EM for Apoptosis

Qualitative EM was carried out to substantiate SC apoptosis in the hypoglossal nerve 2 days after nerve transection in 8 rats. The contralateral hypoglossal nerves from injured and both nerves from an additional uninjured animal served as controls. Nine animals were used for the entire EM portion of the study.

Reinnervation and Neuronal Survival Assays

Long-term experiments were carried out in 12 rat pups to determine the degree of axonal reinnervation of the tongue musculature and hypoglossal nucleus neuron survival at 17 DPO. These assays were performed in 4 animals for each of the following three groups: 1) CN XII TR and placebo pellet pups, 2) CN XII TR and IGF-I pellet pups and 3) CN XII TR and bFGF pellet pups. One animal (placebo) was removed from the study due to abnormal weight gain after hypoglossal nerve transection.

Surgical Procedures

Surgical Anesthesia

While hypothermia serves as an effective method for anesthesia in neonatal rats, animals older than 4 DPN respond more effectively to ether. Due to Laboratory Animal Review Board (LARB) concerns about the reactivity of ether, the alternate anesthetics isoflurane and methoxyflurane were attempted via the "nose cone" method. Inconsistency in both depth of anesthesia and surgical survival led to further research and the important concepts of oxygen support, anesthetic vaporization and an open (non-rebreathing) inhalation device system. The Spartan system manufactured by Matrx Corporation vaporizes isoflurane at a steady rate with oxygen support in an open system. With the purchase and use of the anesthesia machine, surgical deaths of rat pups were virtually eliminated.

Before anesthetizing animals for surgery, each litter of pups was separated from the rat dam. Rechargeable chemical heating pads and heat lamps kept the pups warm. Pups were removed from the group one at a time and placed on an electric heating pad to maintain body temperature during the surgery. At the start of each case, oxygen flow and isoflurane vaporization began at 400 cc/min and a setting of 3.5 respectively. Oxygen and isoflurane adjustments were made throughout each surgical case to ensure that pups remained deeply anesthetized while maintaining regular breathing. During the final stages of surgery, isoflurane dosage was slowly decreased so that pups awoke and responded to stimuli within minutes following completion.

Nerve Transection

Once a pup was deeply anesthetized, an incision on the anterior of the neck was made with a sterile number 11 scalpel blade. The incision was begun just caudal to the interramal vibrissae and extended no more than 1 cm caudally. Following the initial incision, the surgery was performed using a stereomicroscope. Fascia and salivary glands were bluntly dissected and reflected from the field. The right hypoglossal nerve was identified beneath the posterior belly of the digastric muscle, separated from the surrounding tissue with a nerve hook, and transected with microscissors at a location 2 mm proximal to the bifurcation of the nerve into its medial and lateral branches. The cut ends were placed in apposition. However, no attempt was made to secure the cut ends of the nerve with sutures. Short-term or long-term treatments were applied to the transected nerve before the reflected salivary glands were replaced and the midline incision was

closed with 6 to 8 discontinuous 6.0 silk sutures. A bead sterilizer was used to maintain the sterility of surgical instruments between animals.

Animal Treatments

Short-term Treatments

For short-term treatments, a 2-3 mm^3 piece of Gelfoam soaked with 10 µl of vehicle, IGF-I or bFGF solution was placed just proximal to the transected end of the distal segment of the hypoglossal nerve. The vehicle solution consisted of 0.1% w/v BSA (Boehringer Mannheim) and 0.1% v/v gentamicin (Quality Biological Inc.) in PBS (pH 7.4). BSA served as a carrier protein and gentamicin served to prevent infection (Dr. Martin Oudega demonstrated gentamicin use as a standard laboratory procedure for non-sterile rat implants at the Miami Project for the Cure for Paralysis). IGF-I (Boehringer Mannheim) was used at a concentration of 50 ng/ml in the vehicle solution. bFGF (Boehringer Mannheim) was used at a concentration of 10 ng/ml in the vehicle solution. Gelfoam blocks were prepared and soaked with solution just before implantation into the rat pups.

Long-term Treatments

Gelfoam delivery was effective for short-term treatments, but long-term delivery of substances required a more consistent and sustained delivery system. In preliminary experiments, model #1007D osmotic pumps produced by ALZA were used to deliver total volumes of 100 µl of each solution at the steady flow rate of 0.5 µl/hour. Osmotic pumps were filled with vehicle, IGF-I or bFGF solution of the same concentration as that used for the Gelfoam implants. Osmotic pumps containing the appropriate solutions were soaked in Ringer's solution overnight at 37°C. Following nerve transection, a length of sterile tubing (filled with the same solution as the osmotic pump) was extended from a subcutaneously implanted osmotic pump to the tendon of the digastric muscle. The tubing was cut at an angle to prevent torque on the digastric muscle and two discontinuous 10.0 sutures secured the tubing in place. The pump discharged its solution at a constant rate over the course of 7 days, after which it was exchanged for a fresh pump without changing the tubing. While the pumps were effective in discharging solutions at a constant rate, their size and weight proved to be a hardship for the 10 DPN pups. In addition, the pumps and tubing caused inflammation in many of the rat pups.

The osmotic pumps were replaced with Matrix-Driven Delivery pellets manufactured by Innovative Research of America. These pellets can be manufactured to release a variety of compounds at a constant rate over a 21, 60 or 90-day period. Although the matrix composition of the pellets is proprietary, little inflammation occurred at the site of implantation. The IGF-I pellets were specially ordered to release 12.6 ng over 21 days at a constant rate of 0.6 ng/day. The bFGF pellets were specially

ordered to release 2.52 ng over 21 days at a constant rate of 0.12 ng/day. Both pellet types were designed to release an amount of growth factor equivalent to the amount released by osmotic pumps during preliminary experiments. The bFGF pellets also included 0.1% heparan sulfate to prevent rapid breakdown of bFGF *in vivo* (Fujimoto et al., 1997; Saksela et al., 1988). Placebo pellets were also manufactured and used for long-term control animals. Following nerve transection, a pellet was placed just proximal to the transected end of the distal segment of the hypoglossal nerve and deep to the posterior belly of the digastric muscle. Each pellet measured 3 mm in diameter and 1 mm thick, weighed 15 mg and fit easily within the injury site.

Animal Care

Once surgeries had been completed and all pups had recovered from the anesthesia, the rat dam was returned to her pups. To help prevent litter rejection, Vick's Vapor Rub was applied to the nose of the rat dam. In addition, experimental animals comprised no more than 50% of each litter. Transection of the right hypoglossal nerve prevented injured rat pups from nursing as effectively as uninjured pups. Each injured pup was weighed daily to monitor weight loss. Pups found to be losing weight were fed a mixture of oatmeal, whole milk and sugar by hand with a bulb syringe up to 4 times daily. Animals for the long-term experiments were fed by hand until weaning at 21 DPN. At this age, a standard diet of animal pellet *ad libitum* was supplemented with small amounts of oatmeal.

Tissue Collection

Short-term Tissue Collection

The distal segment of the hypoglossal nerve, from the site of the nerve transection to just distal to its bifurcation into medial and lateral branches, was used for short-term apoptosis assays at the light (LM) and electron microscopic levels. Pups were overdosed with isoflurane at 2 DPO. Rats used for LM apoptosis assays were then transcardially perfused with a fixative solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Both right (experimental) and left (control) hypoglossal nerves were collected and post-fixed for 2 hours in the same fixative. Animals used for EM apoptosis assays were perfused with a fixative solution of 4% paraformaldehyde and either 0.2% or 2% glutaraldehyde in 0.1 M cacodylate buffer. Both right (experimental) and left (control) hypoglossal nerves were collected and post-fixed for 1 hour in the same fixative solution.

After post-fixation, nerves collected for LM apoptosis assays were transferred to 20% sucrose in 0.1 M phosphate buffer and left overnight at 4°C. The following day, nerves were removed from the sucrose solution and placed into a flat-bottomed rubber EM embedding mold filled with Tissue-Tek® OCT Compound. Nerves were oriented in a uniform direction and the embedding mold was slowly lowered into liquid R134a (Freon substitute). The OCT froze within a minute, changing from transparent to opaque white. When bubbling of the R134a stopped, frozen OCT blocks were removed from the embedding mold and placed into marked plastic vials pre-chilled with dry ice. The plastic vials were then stored at –80°C until cryostat sectioning.

Long-term Tissue Collection

Long-term tissue collections were performed at 17 DPO for reinnervation and neuronal survival assays. Preliminary studies had determined that 17 DPO was the shortest time period to detect differences in reinnervation based on treatments. At 36 and 24 hours before euthanasia, rats were anesthetized and a 10% solution of horseradish peroxidase (HRP; Boehringer Mannheim) in 0.1 M Tris buffer (pH 7.6) was injected slowly via a Hamilton syringe into the tongue musculature. Each rat received 10 µl of HRP solution at 3 locations on each side of the tongue for a total of 60 µl per injection time. With two injection times, each rat had received a total of 120 µl of HRP solution equally distributed to the left and right sides of the tongue. Young rats were deeply anesthetized with 7% chloral hydrate (5 ml/kg). A pneumothorax was produced and 1000 units of heparin were injected into the left ventricle to eliminate endogenous peroxidase activity. Following this procedure, the right atrium was cut and a cannula inserted into the left ventricle. A prewash solution of 50 ml Lactated Ringers was delivered initially via the cannula into the left ventricle, followed by a fixative solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brain was carefully removed from the cranial cavity and the medulla was isolated, notched on the left side of the anterior median sulcus (to mark the uninjured side) and postfixed for 2 hours in the same fixative. The tissue was cryoprotected overnight at 4°C in 0.1 M phosphate buffer with 20% sucrose before cutting on the freezing microtome the following day.

Tissue Sectioning and Reactions

Short-term Tissue Sectioning and Reactions

Distal nerve segments stored at –80°C for LM apoptosis assays were transferred to a cryostat chamber and allowed to equilibrate to –20°C. Longitudinal sections were cut at 15 µm and collected onto 1% gelatin-chrome alum slides. Statically charged slides, silanized slides and 0.5% gelatin-chrome alum slides were also tested. However, 1% gelatin-chrome alum slides resulted in the smallest amount of tissue loss during subsequent reactions. To randomly select the first section to be included in the series, a coworker, with no knowledge of the tissue source, was asked to pick a number between 1 and 3. This number of 15 µm longitudinal sections was cut and discarded. Serial longitudinal sections through the entire thickness of the nerve were then collected alternately onto 4 slides. Each slide contained a series of longitudinal sections at 60 µm intervals through the entire nerve segment. Slides with serial sections were stored at -20°C until TUNEL triple-label reactions were performed.

The TUNEL triple-label fluorescent-stained protocol stained apoptotic nuclei green (fluorescein = FITC), SC cytoplasm red (rhodamine = RITC) and all nuclei blue (4,6-diamino-2-phenylidole = DAPI). To begin the reactions, slides of longitudinal nerve sections were removed from storage at -20°C and allowed to equilibrate to room temperature before each section was circled with a black HistoPrep® ether pen. Sections were immersed into coplin jars filled with 4% paraformaldehyde (pH 7.4) for 15 min to improve tissue adherence to the chrome-alum slides. Following permeabilization with 20

μg/ml Proteinase K from Promega, sections were again immersed in 4% paraformaldehyde for 5 min. PBS solution (pH 7.4) was used between steps for immersion rinses. TUNEL solutions from Boehringer Mannheim were used in a modification of the TUNEL assay (Gavrieli et al., 1992) to identify apoptotic nuclei. During the TUNEL reaction, TdT enzyme attaches a modified nucleotide to the blunt, double-ended DNA strand breaks caused by the orderly enzymatic destruction of DNA strands during apoptosis (Trauth and Keesey,). No endogenous peroxidase-blocking step was performed as the nucleotide supplied by Boehringer Mannheim was directly conjugated with a fluorescein molecule. Plastic cover slips were used to spread the TUNEL solutions evenly and prevent evaporation during incubation at 37°C for 60 min. The TUNEL reaction was stopped by immersion into 2x sodium citrate-sodium chloride (SSC) solution at room temperature.

Following the TUNEL reaction, sections were incubated for 1 hour at room temperature in rabbit anti-cow S100 antibody (1:100; Dako) diluted with PBS containing 10% normal goat serum (NGS; Cappel) and 1% BSA (Boehringer Mannheim). S100 antibody binding identifies SC in the peripheral nervous system (Brockes et al., 1979; Grinspan et al., 1996; Salonen et al., 1988; Trachtenberg and Thompson, 1996). Sections were rinsed by immersion in PBS and incubated for 1 hour at room temperature in the secondary antibody, rhodamine (RITC)-conjugated goat anti-rabbit $F(ab')^2$ antibody fragment (1:400; ICN) diluted with PBS. Sections were coverslipped using VectaShield™ with 4,6-diamino-2-phenylidole (DAPI; Vector) to counterstain nuclei and stored flat at 4°C. For long-term storage, coverslips were secured with clear nail polish and slides were boxed and stored at –20°C.

Long-term Tissue Sectioning and Reactions

Brainstems were removed from 20% sucrose solution, cut using a freezing microtome into 30 μm transverse sections and collected in 0.1M phosphate buffer (pH 7.4). Every third free-floating section through the length of the hypoglossal nuclei was processed by the tetramethyl benzidine (TMB) method (Mesulam, 1978; Mesulam et al., 1980), mounted in serial order on 0.5% gelatin-chrome alum slides and allowed to dry overnight at room temperature. The sections were counterstained with 1% neutral red and mounted in Permount® (Fisher Scientific Company). Preliminary experiments also tested processing with the 3,3'-diaminobenzidine (DAB)-glucose oxidase method (Itoh et al., 1979). However, the TMB method was found to be more sensitive by allowing for lightly labeled motoneurons to be easily identified during LM analysis. The remaining unreacted free-floating sections were transferred into an ethylene glycol-glycerin cryoprotectant solution and stored at –20°C.

Electron Microscopy Processing

Nerves collected for qualitative EM analysis were removed from post-fixative solution after 1 hour and washed overnight at 4 °C in 0.1 M cacodylate buffer with 20% sucrose. The distal nerve segment was then embedded in 10% gelatin. Longitudinal sections through the entire distal nerve segment were cut at 75 μm on a vibratome and collected in 0.1 M cacodylate buffer. These sections were post-fixed in 2% osmium

tetroxide, stained *en bloc* with uranyl acetate and processed in a routine manner for Araldite epoxy resin embedding. Plastic sections were cut at 1 μm and stained with Toluidine Blue-Pyronin Y for LM identification of apoptotic cells. Ultrathin sections of silver interference were cut and collected on Formvar-coated grids for qualitative EM analysis.

Quantitative Analysis

Area Fraction Measurements

Sections reacted with the TUNEL triple-label protocol were illuminated and viewed with an epifluorescent Nikon Labophot microscope. The TUNEL triple-label protocol stained apoptotic nuclei green (FITC), SC cytoplasm red (RITC) and all nuclei blue (DAPI). A 4-way slider facilitated switching between single-band and multiple-band filters. FITC and triple-band filter images were captured at 10x magnification with a Sony DKC-5000 "CatsEye" camera and imported with a Twain 32 Photoshop plug-in via a SCSI card onto a Windows '98 PC. Captured images were then transferred by Zip disk to a Power Macintosh and locked to prevent changes during evaluation in Photoshop.

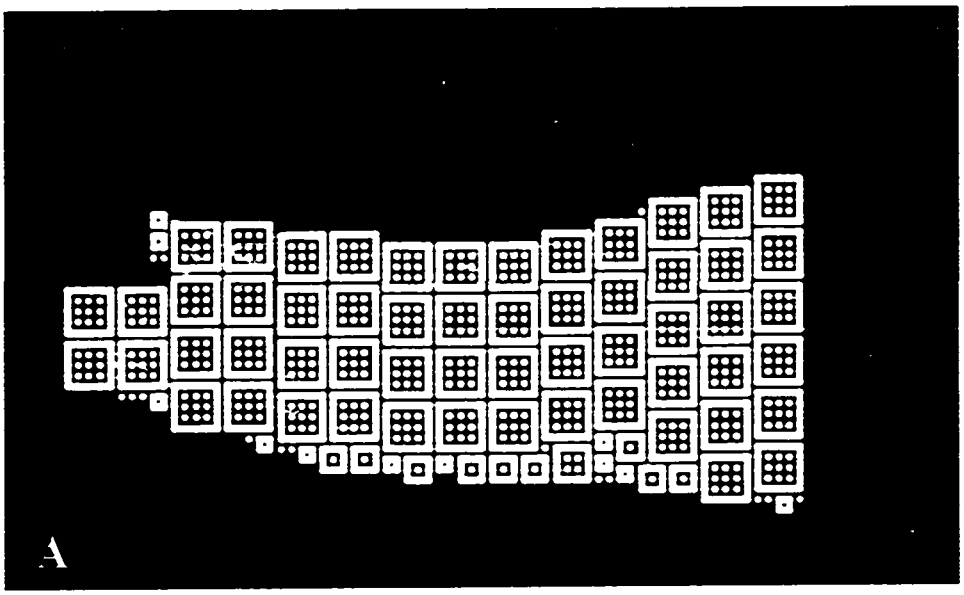
For preliminary quantitative studies, a transparency grid was generated with evenly spaced '+' marks derived from Microsoft Word. This grid was taped to the monitor overlaying the image. However, when the image was enlarged via the Photoshop zoom feature, the appropriate spacing ratio of the grid was altered. In

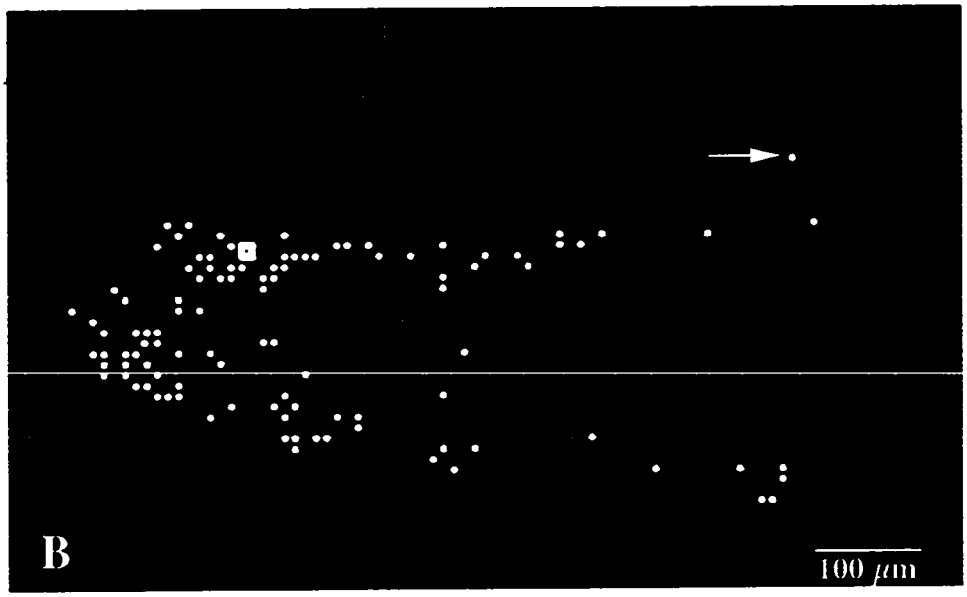
addition, counts conducted on overlain grids could not be saved for later review. To overcome these shortcomings, a modification of the taped-grid method was created. A locked grid of one-pica intervals was superimposed on the image using the Photoshop preferences menu (superimposed grids are not printed in images). This grid maintained the appropriate spacing ratio when the zoom feature was used. Images altered for quantitative analysis as well as the original locked screen captures were saved.

Images captured using the triple-band filter were analyzed initially to determine the total reference area. Grid points falling inside the region of the nerve from the site of transection to the bifurcation were marked with the Photoshop eraser tool on each section and counted as nerve area grid points (Fig. 1A). The total number of nerve area grid points for each nerve section in the series was tabulated to obtain the total reference area. The number of apoptotic grid points was determined from FITC images. The triple-band images used to determine the total reference area were also used to ascertain if FITC labeling represented apoptotic nuclei within the reference area. Grid points contacting FITC labeling within the reference area were marked with the Photoshop eraser tool and counted as apoptotic grid points (Fig. 1B). The total number of apoptotic grid points for each nerve section series was used as the total apoptotic area. The area fraction for each nerve section series was computed by the formula:

Area Fraction = Total Apoptotic Area/Total Nerve Reference Area

Figure 1. Area fraction counting method displayed using a TR-only 10 DPN + 2 DPO animal. Figures were modified in Adobe Photoshop™ by marking areas falling on grid points. (A) total nerve area; (B) apoptotic area; (arrow) apoptotic figure falling on a grid, but uncounted as it lies outside of nerve reference area.


A


B
100 μm

Stereology

Hypoglossal neurons have large somata ranging from 15 to 60 μm and distinctive dendritic arborization. TMB reaction product was readily identified in the somata and processes of neurons in the hypoglossal nuclei whose axons innervated the tongue musculature. Unlabeled neurons in the hypoglossal nuclei could be easily identified by neutral red counterstaining. In recent years, classical methods of counting cells have been criticized and counting methods collectively called unbiased stereology have been strongly recommended (Coggeshall, 1992; Coggeshall and Lekan, 1996; Saper, 1996; Sterio, 1984; West et al., 1991).

To overcome the potential bias of classical counting methods, an unbiased stereology software program was purchased and used to estimate neuronal populations within the hypoglossal nuclei. With the Stereologer software (Systems Planning and Analysis, Inc.) a Power Macintosh computer was programmed to systematically move between optical disectors in a raster pattern with an MC-XYZ stage controller (Applied Scientific Instrumentation, Inc.). Optical disectors allow for a relatively thick section of tissue to serve as both the counting and reference (exclusion) planes by focusing up and down through the section (Gundersen, 1986; Gundersen et al., 1988). Objects can be counted on-screen with real-time images imported from a Sony DXC-151A digital camera attached to a Nikon Biophot microscope onto the Power Macintosh via a Targa 2000 PCI-slot video card (Truevision, Inc.). Images were viewed on a 17-inch AppleVision monitor during counting.

The appropriate parameters for counting labeled and total neurons within the

hypoglossal nuclei were determined by preliminary studies. A number between 1 and 3, generated randomly by the Stereologer program selected the first section. A systematic sample of stained sections through the hypoglossal nuclei was used for the analysis. For each optical disector selected by the Stereologer program via a raster pattern, the user identified the neurons in focus and inside the inclusion boundaries for HRP-TMB labeled neurons (Fig. 2) or total neurons including unlabeled neurons visible via Neutral Red counterstaining (Fig. 3). Estimations of the neuronal populations were computed from the neurons tabulated in each hypoglossal nucleus. Four separate runs were performed for each animal to estimate the number of labeled and total neurons for both the injured and uninjured hypoglossal nuclei.

For the determination of tongue musculature reinnervation, the total neuronal population of the contralateral (uninjured) hypoglossal nucleus served as the reference to prevent overestimation due to neuronal cell death. Tongue musculature reinnervation was estimated by the formula:

% Tongue Musculature Reinnervation = (Total labeled neurons in the injured hypoglossal nuclei / Total neurons in the intact hypoglossal nuclei) x 100

Neuronal survival was estimated by the formula:

% Neuronal Survival = (Total neurons in the injured hypoglossal nuclei / Total neurons in the intact hypoglossal nuclei) x 100

Statistical Analysis

The mean and standard error of the mean (SEM) values of all animal groups were

Figure 2. One optical disector in the Stereologer program showing HRP-reaction product-labeled neurons. Red lines are exclusions and green line is inclusion. Green X's indicate neuron cell bodies touching the inclusion line.

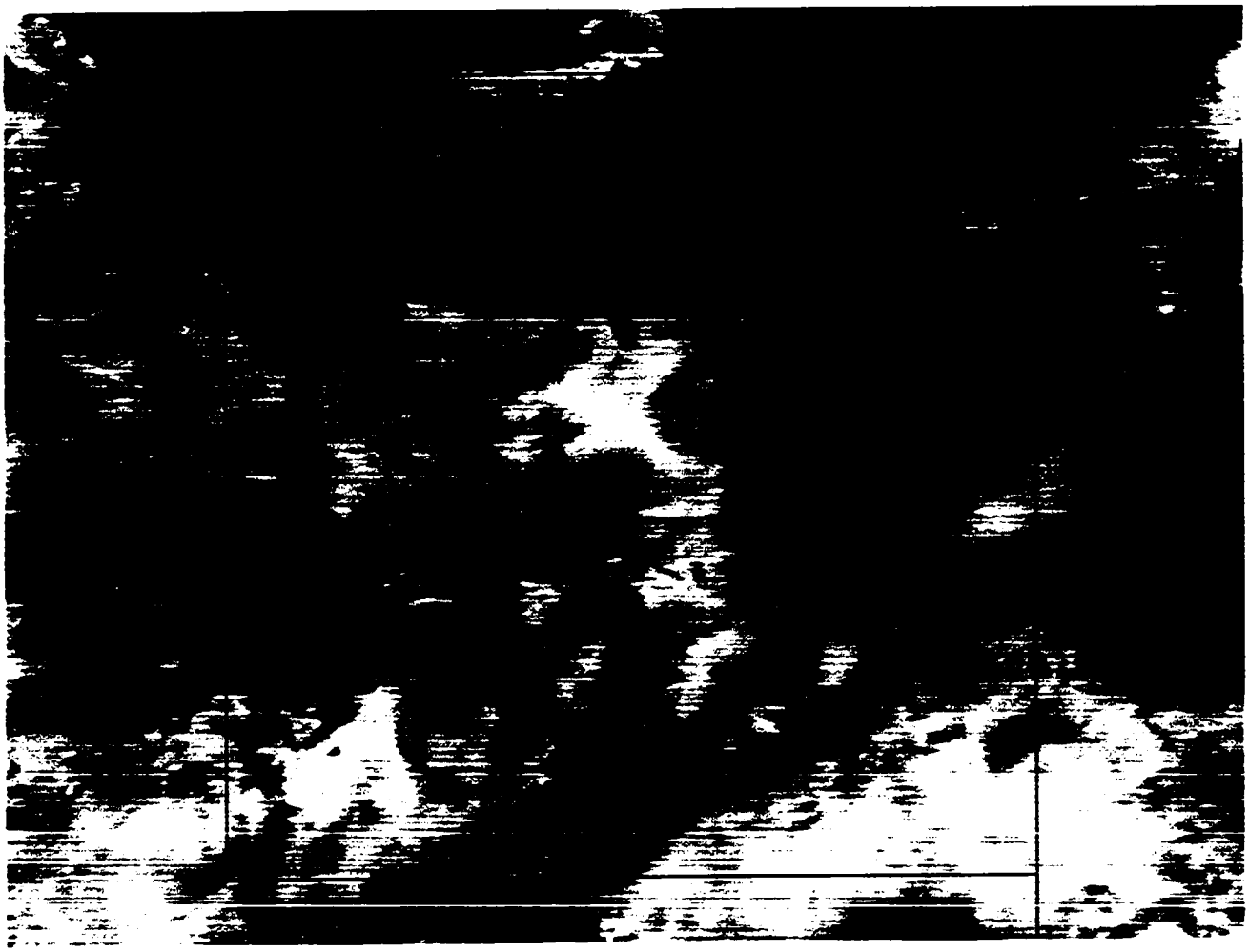
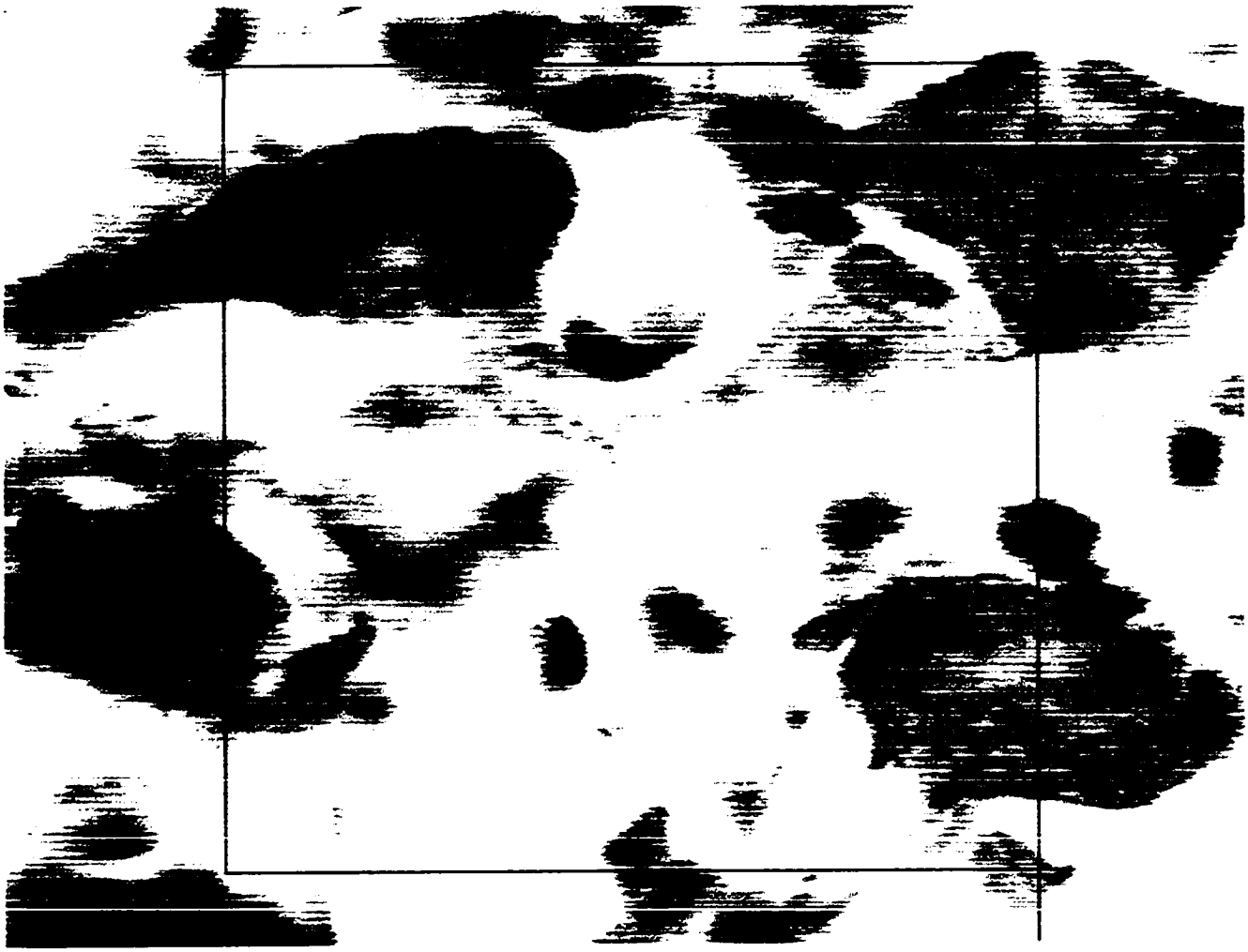


Figure 3. One optical disector in the Stereologer program showing unlabeled neurons. Red lines are exclusions and green line is inclusion. Green X's indicate neuron cell bodies touching the inclusion line.



calculated. Control and treatment groups were compared using one-way analysis of variance (ANOVA). When ANOVA demonstrated significant effects ($p < 0.05$) among groups, the Student-Newman-Keuls (SNK) test was applied *post hoc* to inspect differences between mean pairs (Snedecor and Cochran, 1980). Additionally, two-way ANOVA with repeated measures was used to assess potential differences in the percent of innervation as a function of growth factor treatments. When significant interaction effect (growth factor treatment x side) indicated there was significant difference ($p < 0.05$) in the percentage of innervation between the injured and uninjured sides of the brainstem as a result of growth factor treatments, the SNK test was applied *post hoc* to assess changes in the percentage of innervation. All computations were performed with the StatView® (SAS Institute Inc.) statistical software package.

RESULTS

Pilot Studies

Short-term Apoptosis as Determined by TUNEL Assays

The hypoglossal nerve segment from the site of transection to the attachment of the nerve to the ventral medulla was designated the proximal nerve segment (Fig. 4). The segment of the nerve from the site of transection to the bifurcation of the nerve into medial and lateral branches was referred to as the distal nerve segment (Fig. 4). TUNEL-positive nuclei labeled with FITC were observed in the following two locations along the length of the distal nerve segment: 1) at the transected end adjacent to the injury site (Fig. 5) and 2) just proximal to the bifurcation of the nerve (Fig. 6). The majority of these nuclei appeared to be within SC cytoplasm labeled with RITC (Fig. 7).

In contrast, apoptotic nuclei were scarce in the proximal nerve segment of the transected nerve. Apoptotic nuclei were also infrequent in the contralateral distal nerve segment of axotomized animals (Fig. 8).

Apoptotic nuclei were identified by TUNEL assay in the 10 and 21 DPN rats at 1 to 3 DPO, but were rarely detected in 30 DPN rats at any survival time. The incidence of apoptosis appeared to be greatest in the 10 DPN rats at 2 DPO after nerve transection.

Short-term pilot studies concerned Specific Aim I: *To determine if Schwann cell apoptosis is an age-related response to postnatal transection of the hypoglossal nerve.* These studies also provided a framework for the evaluation of the results of Specific Aim

Figure 4. Schematic of the proximal and distal nerve segments as defined by the study. Proximal segment extends from the medulla to the site of injury. Distal segment extends from the site of injury to the bifurcation of the hypoglossal (CN XII) nerve into the medial and lateral branches.

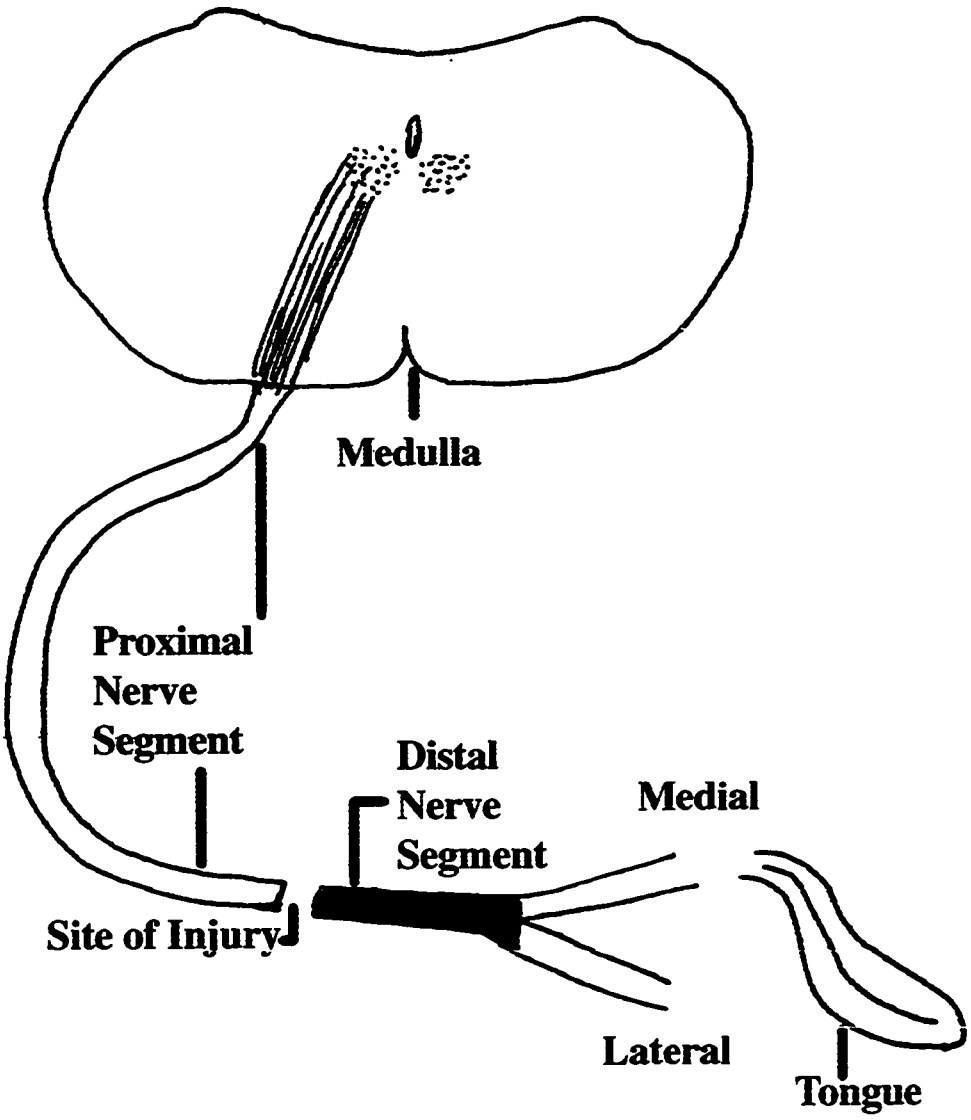

Medulla
Proximal
Nerve
Segment
Distal
Nerve
Segment
Medial
Site of Injury
Lateral
Tongue

Figure 5. TUNEL-positive apoptotic nuclei in the distal nerve segment of a 10 DPN + 2 DPO pilot study animal. Halo surrounding nerve segment (arrowhead) is an artifact from the use of a PAP pen. (A) Fluorescent double-labeling in the distal nerve segment with TUNEL-positive nuclei demonstrating fluorescein green (FITC) and SC cytoplasm showing rhodamine red (RITC). (B) The same distal nerve segment viewed through a single filter (FITC). Red blood cells (arrows) seem to be labeled but are actually autofluorescent. Bar represents 50 microns.


A
50 μm

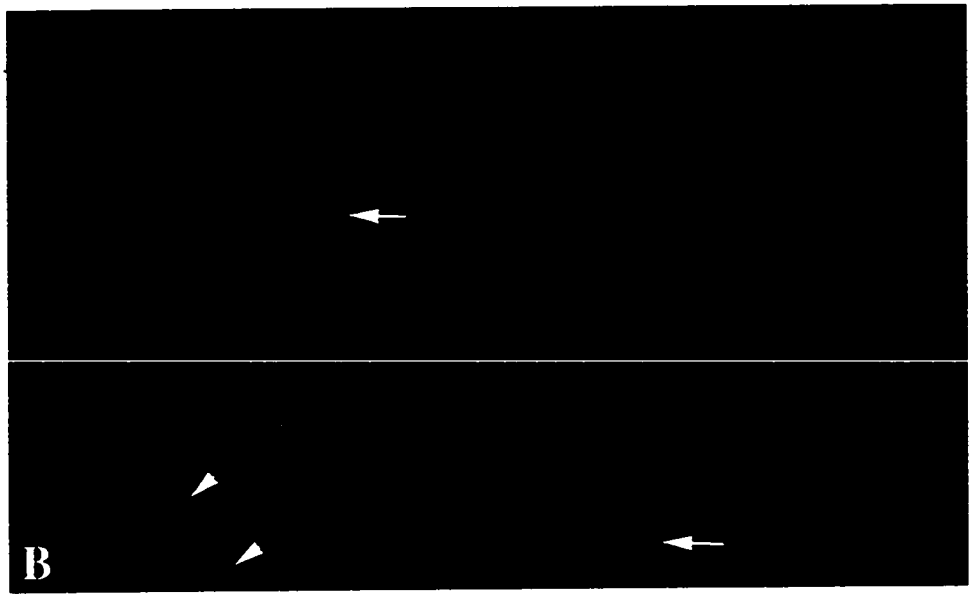
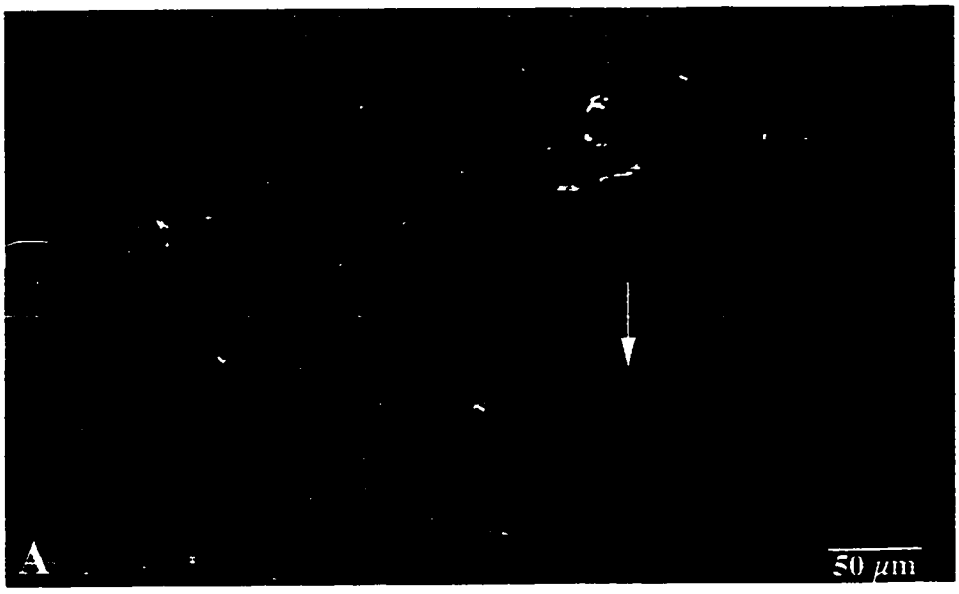

B

Figure 6. Apoptotic nuclei near the site of bifurcation (arrow) of hypoglossal nerve in a 10 DPN + 2 DPO animal. (A) Triple-labeled TUNEL-positive apoptotic nuclei are green (FITC), SC cytoplasm is red (RITC), and all nuclei stain blue (DAPI). (B) Same section shown through a single filter (FITC). Bar represents 50 microns.


A
50 μm

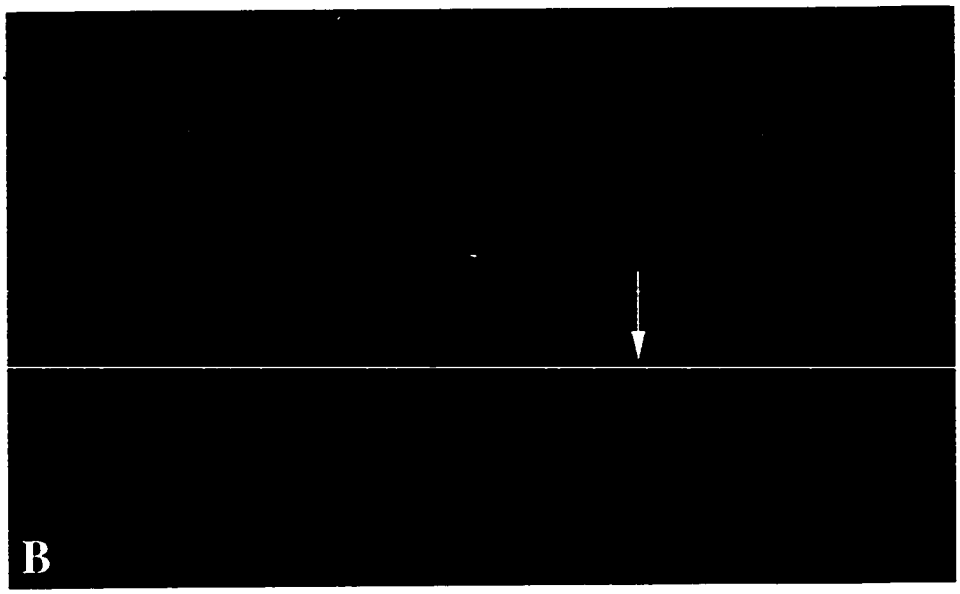
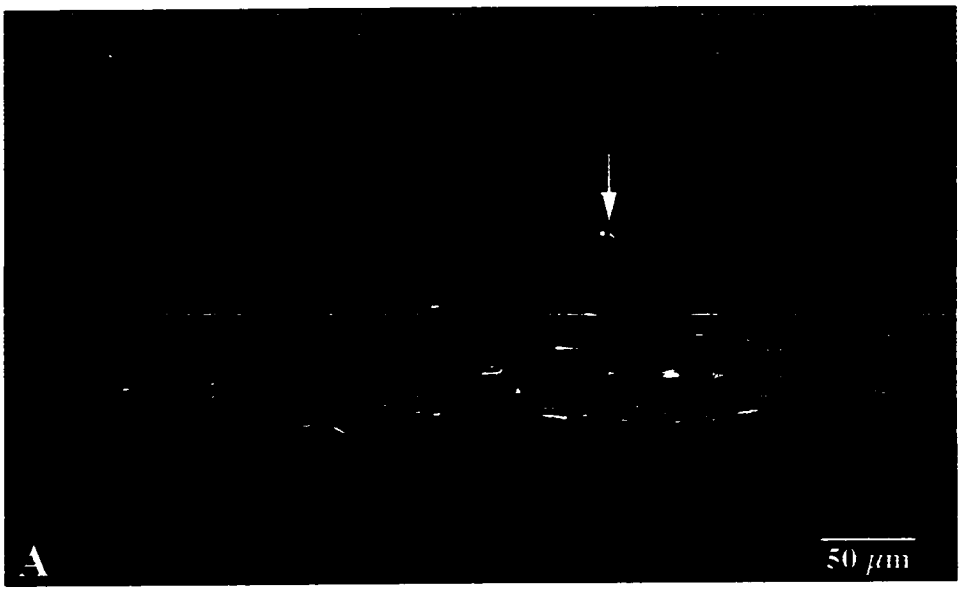
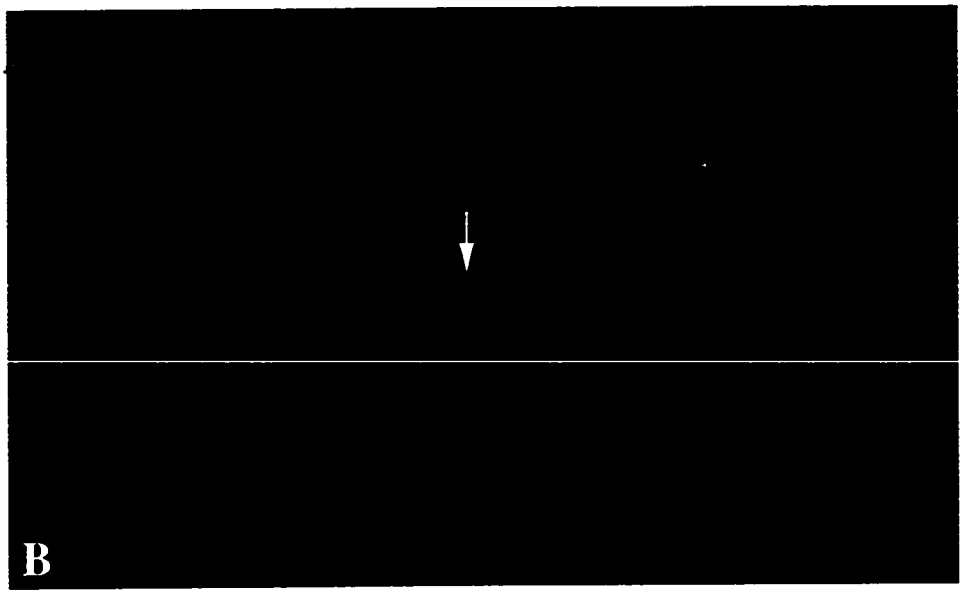

B

Figure 7. Distal nerve segment adjacent to the site of injury in a 10 DPN + 2 DPO pilot study animal demonstrates that the TUNEL-positive apoptotic nuclei (FITC) are located within SC cytoplasm (RITC). Autofluorescent red blood cell (arrow) and PAP pen artifact (arrowheads) are also seen. Bar represents 10 microns.


10 μm

Figure 8. Contralateral (uninjured) distal nerve segment of a 10 DPN + 2 DPO pilot study animal showing isolated apoptotic nuclei (arrow). (A) Distal nerve segment viewed through triple-filter. (B) The same distal nerve segment viewed through single filter (FITC). Bar represents 50 microns.


A
50 μm


B

II: *To determine if the application of growth factor treatments reduces Schwann cell apoptosis after postnatal transection of the hypoglossal nerve.* Animal ages for short-term major studies were chosen based on pilot study results, which demonstrated the age group and survival time in which SC apoptosis was greatest. Qualitative EM studies were also planned to substantiate the identity of apoptotic cells by ultrastructural analysis in animals of the same age and survival time. The observation of apoptotic nuclei in two distinct areas, separated by a portion of the nerve not involved in apoptosis, necessitated the development of a modified method of area fraction analysis to estimate apoptosis in the distal nerve segments (Dr. Peter Mouton, Department of Pathology at Johns Hopkins University, provided consultation on quantitative methods).

Long-term Regeneration as Determined by HRP Assays

Control Observations

Neurons in the injured and uninjured hypoglossal nuclei of animals with axons transected at 10 DPN contained HRP reaction product. However, no series of injections into the tongue musculature on the uninjured side resulted in HRP labeling of all neurons. This finding is in agreement with previous studies of HRP labeling in hypoglossal nuclei (Blake-Bruzzini et al., 1997; Borke et al., 1993; Hall and Borke, 1988). Unlabeled neurons in the nuclei of uninjured hypoglossal nerves following HRP injections represent a distinct interneuron population that does not project to the tongue musculature (Boone and Aldes, 1984).

Nerve Transection Observations

HRP injections into the tongue musculature on the side of the nerve transection in rats injured at 10 DPN produced some retrograde labeling of hypoglossal motoneurons at all survival times of the pilot study. Few neurons were labeled with the TMB reaction product for HRP at 14 DPO, a moderate number of neurons were labeled at 17 DPO and the majority of motoneurons were labeled by 40 DPO.

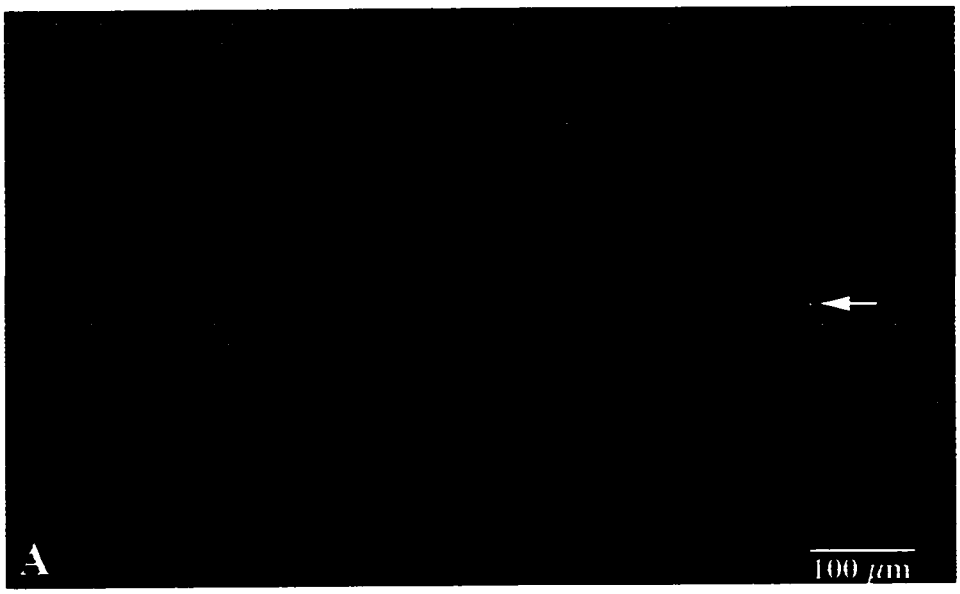
Long-term pilot studies provided a framework for the evaluation of the results of Specific Aim III: *To determine if alteration of the local axonal environment influences target reinnervation and neuronal survival after postnatal transection of the hypoglossal nerve*. For long-term major studies, 17 DPO was selected as the survival time when differences between the various treatment groups would be most apparent when evaluated by unbiased stereology.

Evaluation of SC Apoptosis after Nerve Transection in 10 DPN Rats

Quantitative TUNEL Assay for Apoptosis

Distal hypoglossal nerve segments from 12 DPN uninjured rat pups showed few TUNEL-positive apoptotic nuclei (Fig. 9). The mean area fraction of apoptotic nuclei area to nerve area in 12 DPN animals was 0.002 (± 0.001; Fig. 10). The area fraction for these uninjured animals was considered to be an estimation of naturally occurring

Figure 9. Apoptotic TUNEL-positive nuclei (arrow) in a 12 DPN uninjured animal. (A) Triple-labeled section. (B) Same section through single filter (FITC). Bar represents 100 microns.


A
100 μm

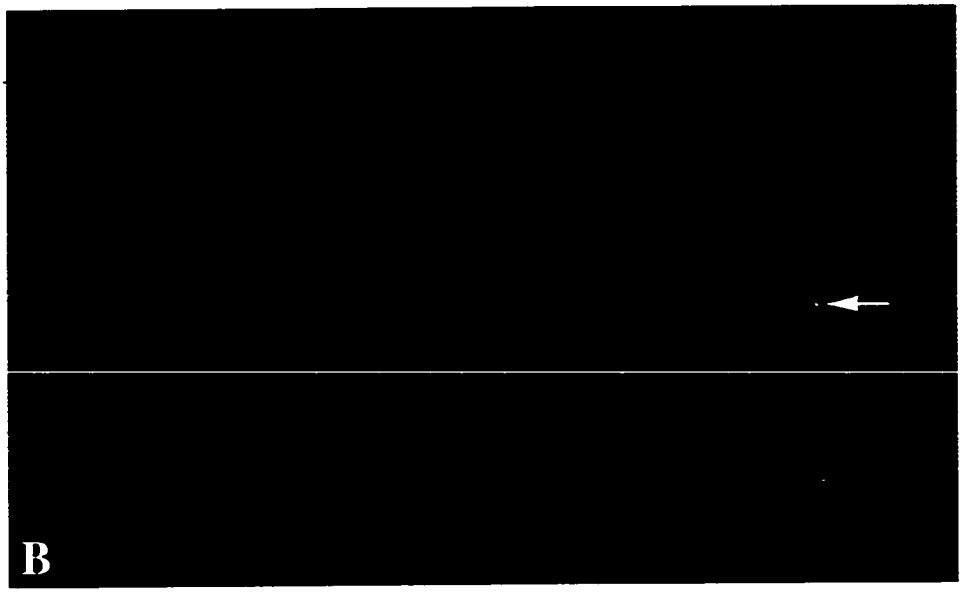
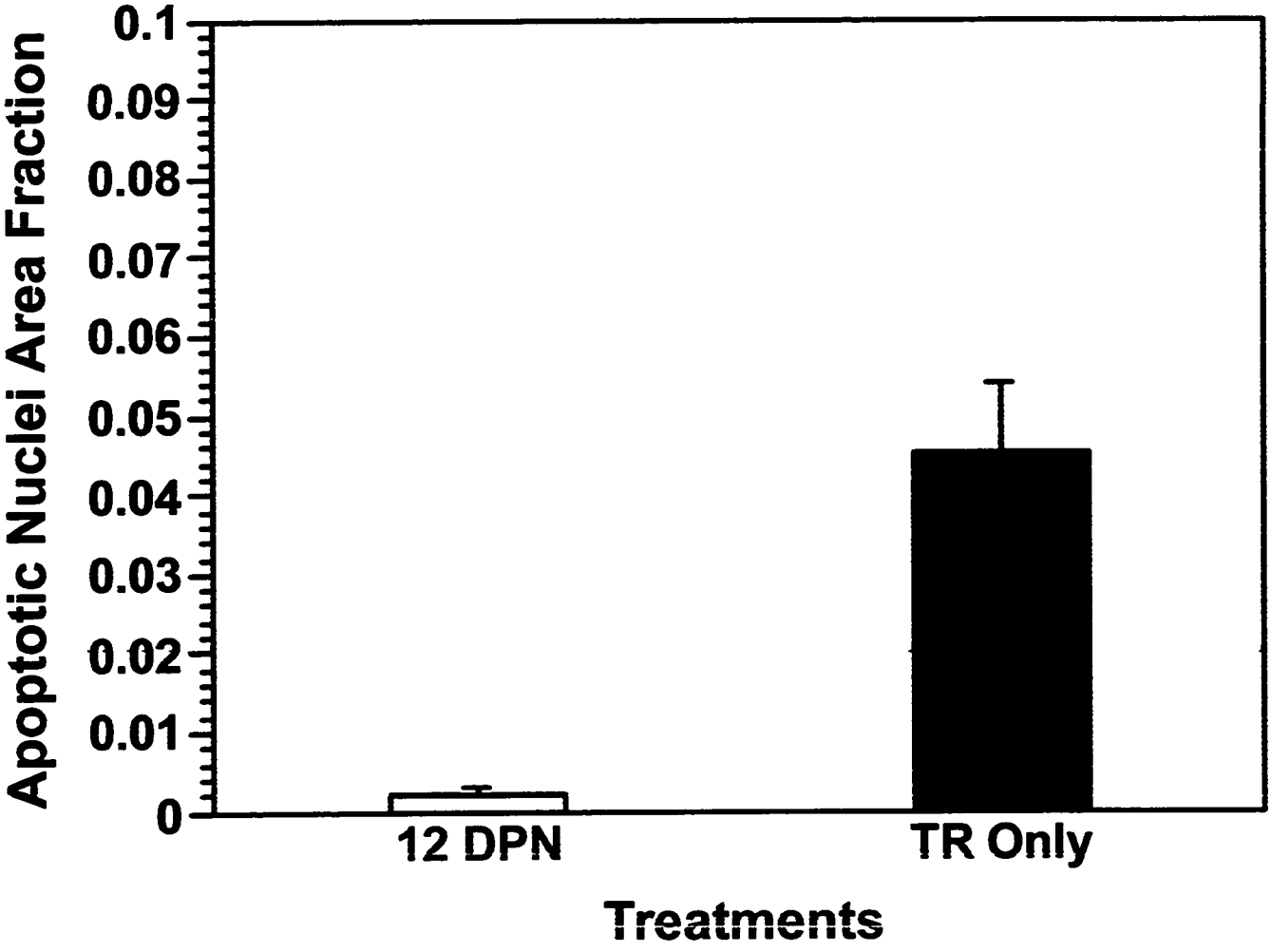

B

Figure 10. Area fraction of TUNEL-positive apoptotic nuclei in 12 DPN uninjured and 10 DPN + 2 DPO TR-only animals. Mean ± SEM are reported.


Apoptotic Nuclei Area Fraction
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
12 DPN
TR Only
Treatments

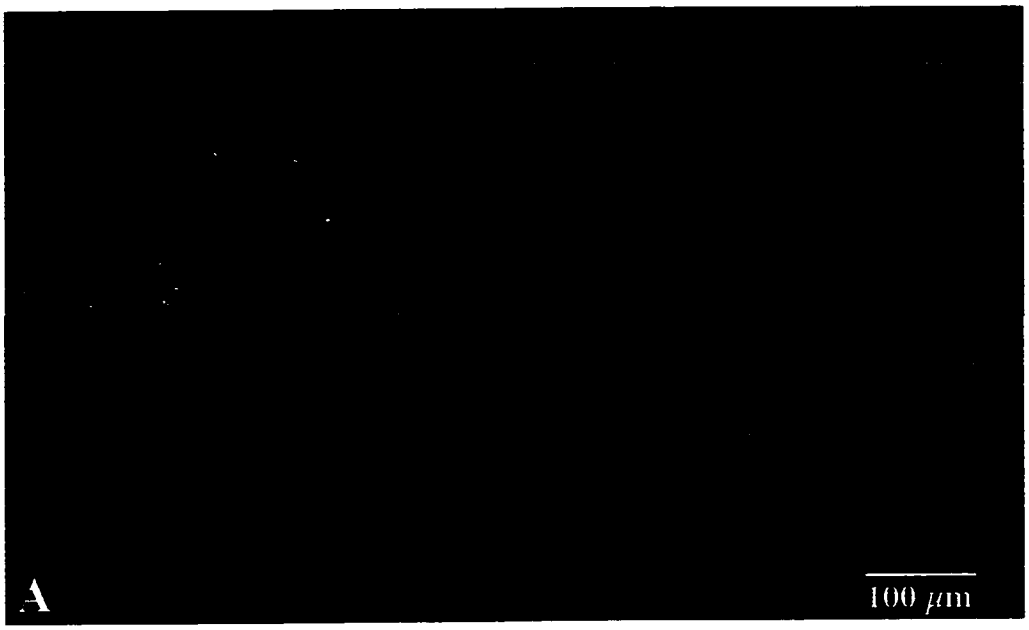
apoptosis. TUNEL-positive apoptotic nuclei (Fig. 11) were increased 20-fold in 10 DPN rat pups at 2 DPO after nerve transection (Fig. 10). The mean area fraction of apoptotic nuclei to nerve area was 0.045 (± 0.009; Fig. 10). This area fraction of apoptotic nuclei was increased significantly ($p < 0.01$) from that of the 12 DPN uninjured value.

Qualitative Electron Microscopic Evaluation of the Distal Hypoglossal Nerve

Uninjured Hypoglossal Nerve

EM examination of uninjured hypoglossal nerves demonstrated axons in longitudinal and oblique planes that were invested in myelin sheaths of varying thickness (Fig. 12). SC nuclei and varying amounts of their cytoplasm could be identified as they aligned in a segmental manner along the length of the myelinated axons. The nucleus of the SC was elongated and contained one or more nucleoli (Fig. 13). Chromatin had a patchy distribution within the nuclei. SC cytoplasm contained Golgi complexes, rough endoplasmic reticulum (RER), mitochondria and filaments. The plasma membrane of the SC was invested with a basal lamina. Interspersed between the myelinated axons were constituents of the endoneurium, including collagen fibrils, fibroblasts, mast cells and an occasional macrophage containing lysosomes. In contrast to SC, all of these endoneurial cells lacked a basement membrane.

Figure 11. Section adjacent to site of injury in distal nerve segment of a 10 DPN + 2 DPO TR-only animal. (A) Distal nerve segment viewed through triple-filter. (B) The same distal nerve segment viewed through single filter (FITC). Bar represents 100 microns.


A
100 μm


B

Figure 12. The uninjured hypoglossal nerve consists of axons (A) in longitudinal and oblique planes wrapped with myelin sheaths. Schwann cells (SC) forming the myelin sheaths are aligned along the myelinated axons. Bar represents 1 micron.


A
SC
A
1 µm

Figure 13. Nuclei (N) of Schwann cells in the uninjured hypoglossal nerve are elongated, have one or more nucleoli (arrowhead) and patches of chromatin. The cytoplasm contains mitochondria, Golgi complexes and RER. A basal lamina (arrows) invests the plasma membrane of the Schwann cell. Collagen fibrils (C) are oriented longitudinally in the endoneurium. Bar represents 1 micron.

C
N
1 µm

Injured Hypoglossal Nerve

At 2 days after 10 DPN nerve transection, axonal degeneration and myelin breakdown were evident in the distal segment of the hypoglossal nerve. Debris from degenerating axons, myelin figures (laminated myelin bodies) and lipid ovoids were seen as inclusions within the cytoplasm of SC (Fig. 14) and macrophages. Phagocytic macrophages (Fig. 15) could be distinguished from Schwann cells by the presence of numerous finger-like processes at the cell surface and the lack of a basal lamina.

In agreement with the TUNEL findings, EM confirmed the presence of apoptotic cells in the distal nerve segment. Apoptotic cells were observed in the distal nerve segment at the following sites: 1) at the proximal tip adjacent to the site of nerve transection (Fig. 16) and 2) just proximal to the bifurcation of the nerve into medial and lateral branches.

Various stages of apoptosis were recognized in cells at both locations in the distal nerve segment. In what appeared to be an earlier stage of apoptosis, most of the chromatin was aggregated in compact masses abutting portions of the nuclear membrane. (Fig. 17). Indentations of the nuclear membrane were often detected and nuclear pore regions were prominent (Fig. 17). Few morphologic changes were found in the cytoplasm of these early-stage, apoptotic cells. Mitochondria and cisterns of RER were intact (Fig. 17). Cells in early-stage apoptosis sometimes contained myelin figures, axonal debris or lipid droplets.

The majority of the apoptotic cells displayed characteristic features of late-stage apoptosis (Wyllie et al., 1980). The nucleus was broken up into a number of discrete

Figure 14. Debris from degenerating axons (asterisk), myelin figures (M) and lipid bodies (L) are inclusions in Schwann cells situated in the distal segment of the hypoglossal nerve two days after nerve transection in 10 DPN rats. Bar represents 5 microns.


L
M
M
L
5 µm

Figure 15. A macrophage in the endoneurium of the hypoglossal nerve at 2 days after axotomy in a 10 DPN rat has a kidney-shaped nucleus that is more electron dense and has coarser clumps of chromatin than a Schwann cell nucleus. Laminated myelin and lipid body inclusions indicate phagocytosis of the degenerating nerve sheaths. Macrophages have no basal lamina, but numerous invaginations of the plasma membrane typically form finger-like processes (arrows) along its surface. Bar represents 1 micron.

1 µm

Figure 16. The proximal tip of the distal nerve segment is shown in the longitudinal plane in this Toluidine Blue-Pyronin Y-stained thick section for electron microscopy at 2 days after hypoglossal nerve transection. The clear area at the far left of the micrograph denotes the site of the transection. Notice numerous apoptotic cells (arrows) within the nerve stroma close to the injury site. Bar represents 50 microns.

Figure 17. Two Schwann cells in the distal segment of the hypoglossal nerve at 2 days after axotomy in a 10 DPN rat. The lower Schwann cell demonstrates characteristics of a cell in an early stage of apoptosis. Most of the chromatin is aggregated against the nuclear membrane, the nuclear pore regions are prominent but morphologic changes do not involve cytoplasmic organelles such as RER and mitochondria. The presence of a basal lamina (arrows) indicates that the upper cell is also a Schwann cell. This cell shows features similar to those of early prophase mitosis typified by condensing areas of heterochromatin within a nuclear membrane that is intact. Bar represents 1 micron.


1 µm

fragments. Nuclear fragments often displayed condensed chromatin arranged in crescent-like caps (Fig. 18). The nuclear membrane enclosing these nuclear fragments was considerably dilated (Fig. 19). The cytoplasm of cells in late-stage apoptosis appeared more condensed or compact with increased electron density (Fig. 19). Cisterns of RER were sparse and the cytoplasm was replete with clusters of single ribosomes and polyribosomes (Fig. 19). In some cells, the electron dense cytoplasm contained translucent vacuoles. The presence of a basal lamina on these cells confirmed their identity as SC (Fig. 19). Occasionally, a cell in late-stage apoptosis was engulfed in the cytoplasm of another cell containing lipid bodies.

An interesting finding was the close proximity of apoptotic SC to mitotic figures (Fig. 20). Various stages of mitosis could be identified in cells close to the transection site and just proximal to the bifurcation of the distal nerve segment. These cells displayed the following SC characteristics: 1) myelin debris and lipid bodies in the cytoplasm (Fig. 21) and 2) basal lamina (Fig. 22). Late-stage mitosis was often apparent in Toluidine Blue sections, as demonstrated by the formation of daughter cells (Fig. 23). Occasionally, cells in late-stage apoptosis appeared to have stopped mitotic division during a stage in which karyokinesis but not cytokinesis had occurred (Fig. 24). In these instances, both of the forming daughter cells were apoptotic.

Figure 18. In the middle of the field, a Schwann cell displays features of late apoptosis: fragmented nucleus, dilatation of the nuclear membrane and increased electron density of the cytoplasm. Contrast the nuclear and cytoplasmic features of this apoptotic Schwann cell with the other normal-appearing Schwann cells in the micrograph. Bar represents 5 microns.

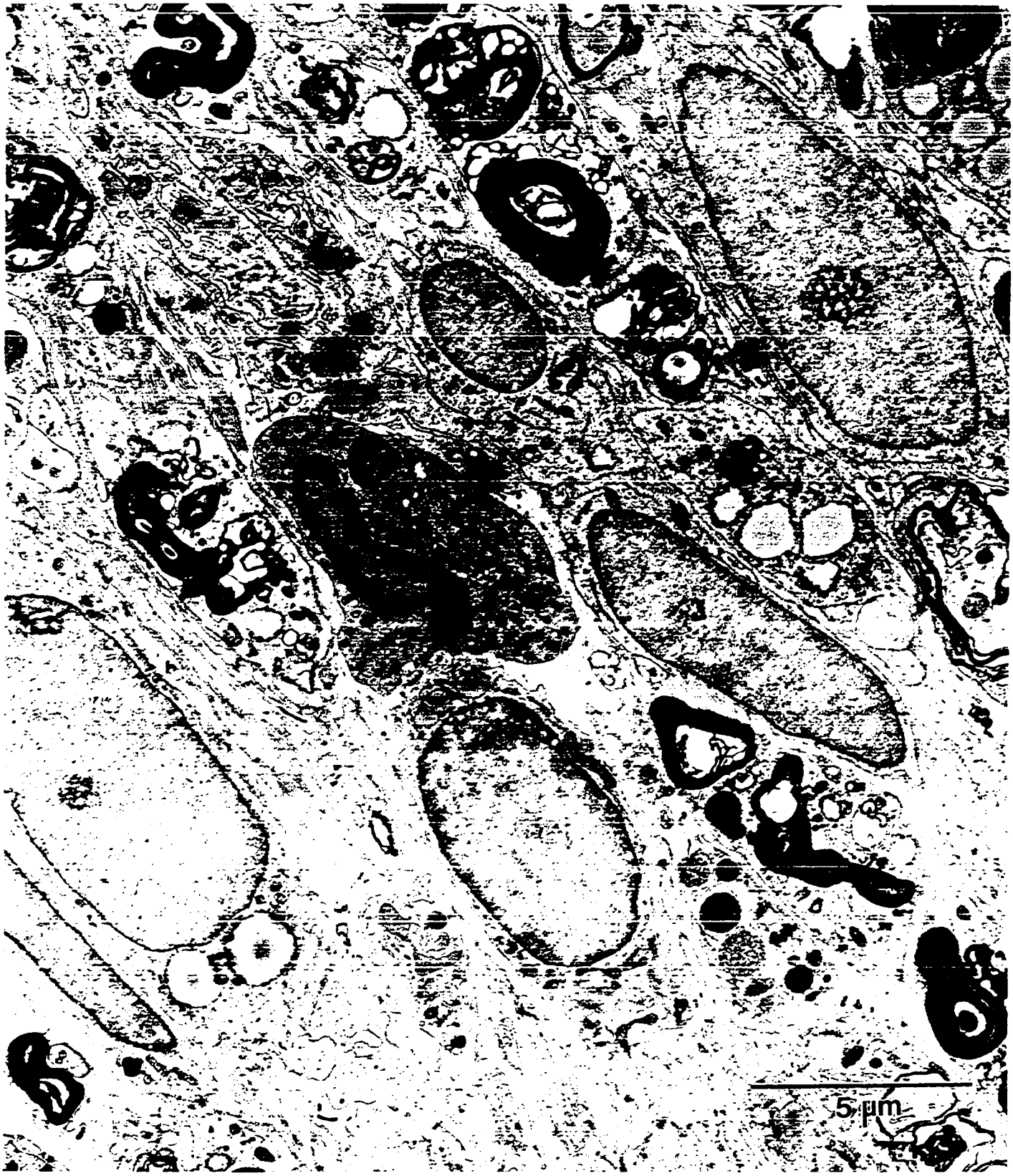

5 µm

Figure 19. A higher magnification of the apoptotic Schwann cell in Fig. 18 demonstrates crescentic caps of condensed chromatin surrounded by a dilated nuclear membrane. Notice the cisterns of RER have been largely replaced by clusters of single ribosomes and polyribosomes. Autophagic and translucent vacuoles can be distinguished in the cytoplasm. The basal lamina (arrows) identifies this cell as a Schwann cell. Bar represents 1 micron.

1μm

Figure 20. Many Schwann cells are aligned along the longitudinal axis of the distal nerve. Most of these cells had elongated nuclei and contained lipid bodies (clear vacuoles) and dark-purple myelin degeneration products in their cytoplasm. Two Schwann cells demonstrate stages of mitosis (large arrows). Another nearby Schwann cell (small arrow) is apoptotic with a deep-stained cytoplasm surrounding nuclear fragments. A Toluidine Blue-Pyronin Y-stained distal nerve at 2 days after hypoglossal nerve transection. Bar represents 10 microns.

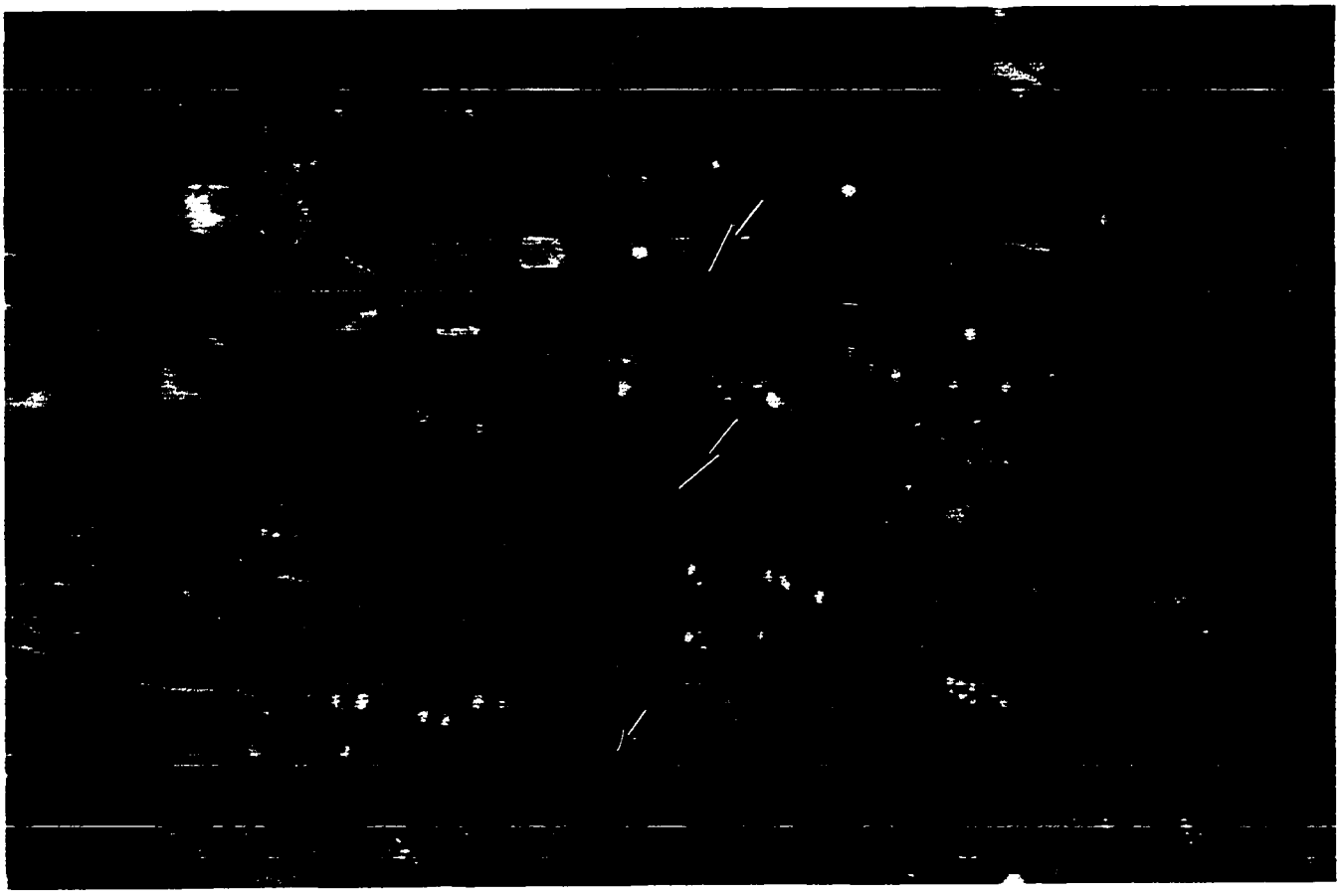


Figure 21. A Schwann cell identified by its basal lamina (arrows) and containing lipid (L) and myelin bodies (M) demonstrates features of one of the phases of mitosis at 2 days after nerve transection in a 10 day old rat. A portion of the mitotic spindle consisting of microtubules (arrowheads) directs the movements of the chromosomes in the middle of the cell and suggests that the Schwann cell is in metaphase. Bar represents 1 micron.

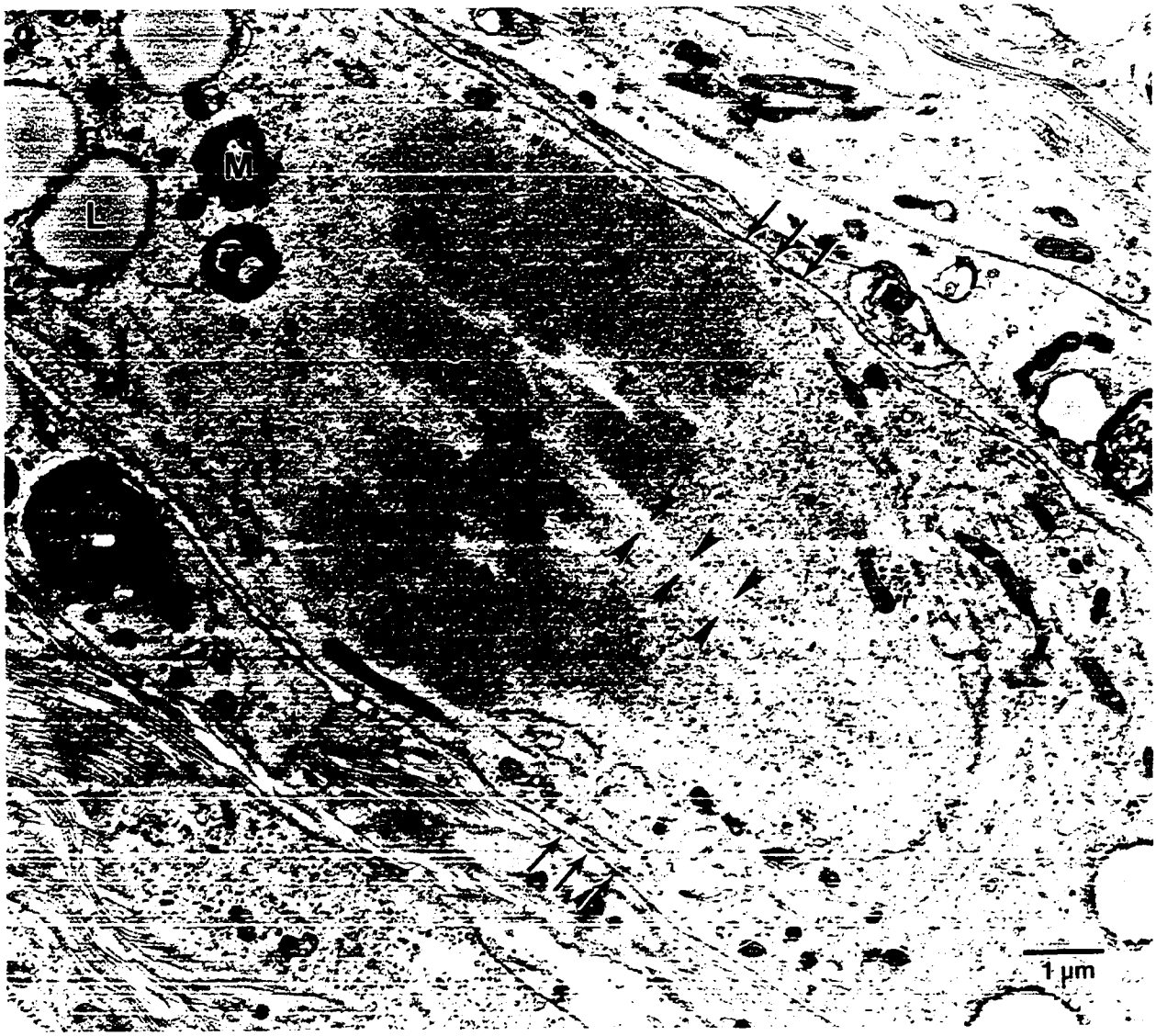

M
L
1 µm

Figure 22. Another mitotic figure in a hypoglossal nerve at 2 days after nerve transection in a 10 day old rat. The prominent basal lamina (arrows) provides confirmation of its Schwann cell origin. Bar represents 1 micron.

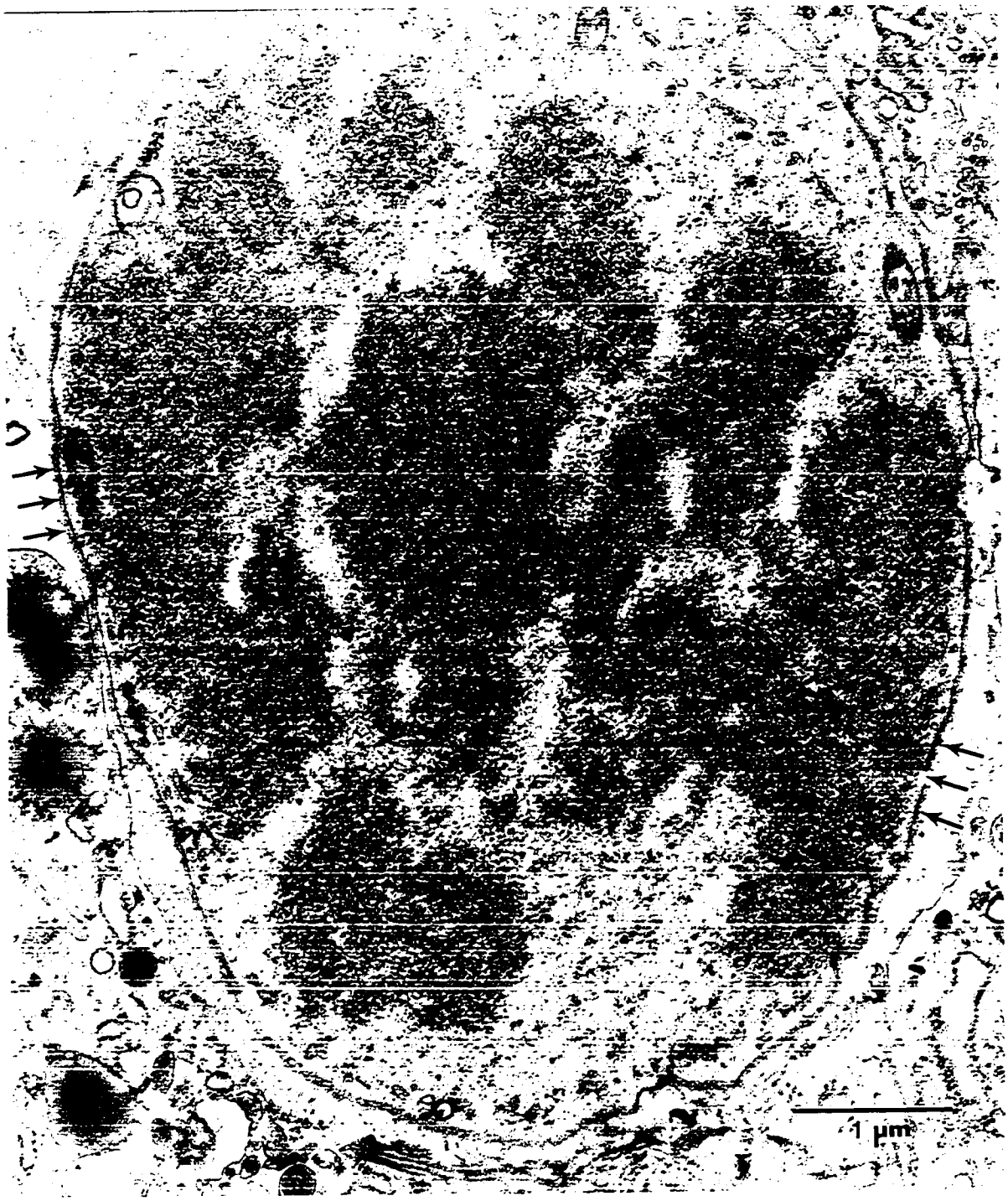

1 µm

Figure 23. The distal portion of the hypoglossal nerve at 2 days after axotomy shows Schwann cells containing lipid and myelin debris. One Schwann cell appears to be forming two daughter cells (small arrows). Another nearby Schwann cell (large arrow) demonstrates features of early prophase. A Toluidine Blue-Pyronin Y-stained distal nerve at 2 days after hypoglossal nerve transection. Bar represents 10 microns.

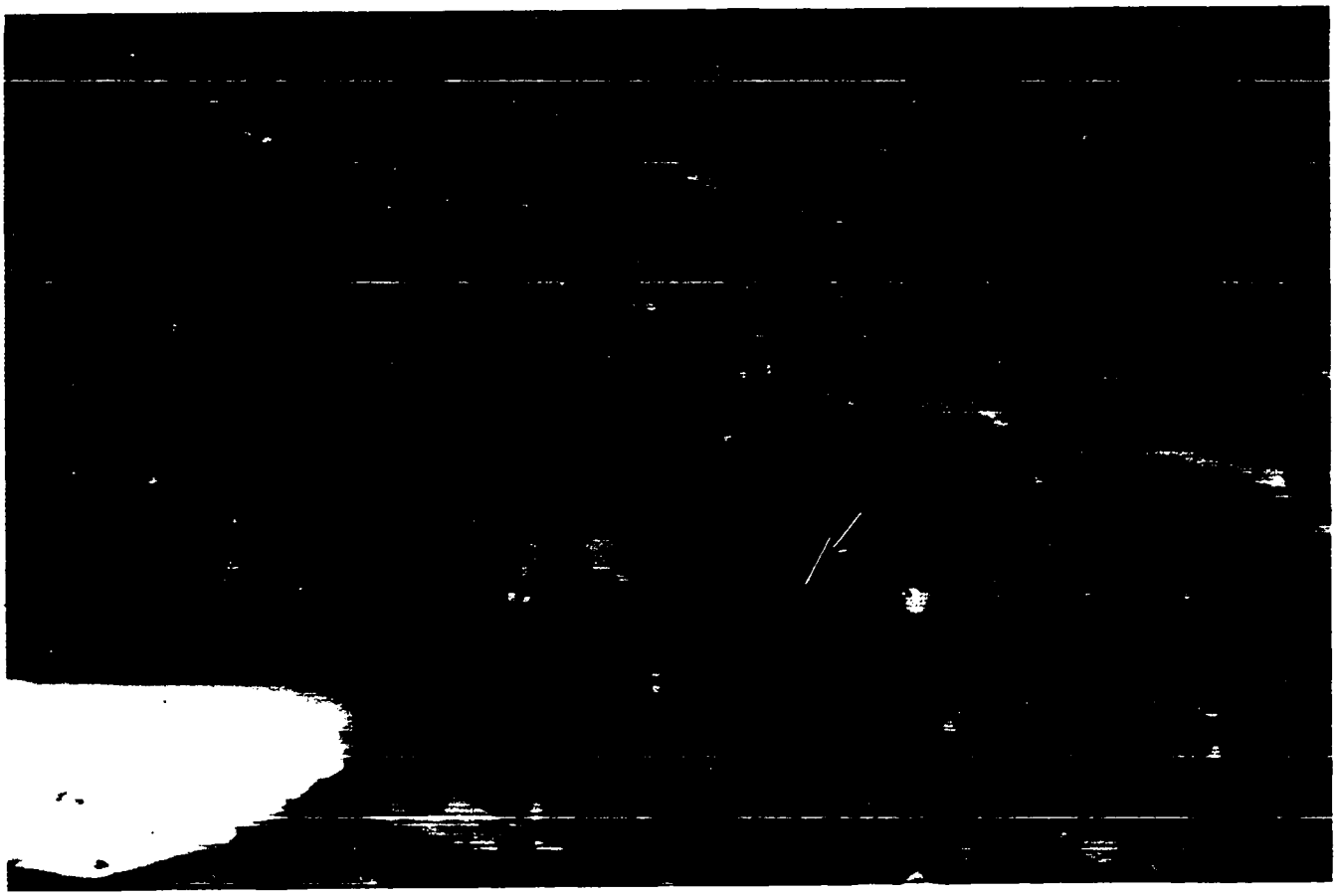


Figure 24. Two apoptotic nuclei (N) are seen in a profile that appears to be in a phase of mitosis in which karyokinesis has taken place but cytokinesis has not occurred. This profile was seen 2 days after nerve transection in a 10 day old rat. Bar represents 1 micron.

N
N
1 µm

Short-term Evaluation of SC Apoptosis after Treatment with Growth Factors

The hypoglossal nerve of 10 DPN rats was transected and either IGF-I or bFGF was applied in a vehicle solution. Apoptosis in the distal nerve segments of these rats was compared to that obtained after nerve transection and treatment with vehicle solution alone or without any treatment (TR-only). Apoptosis of SC appeared to be considerably less after all treatment groups. Apoptotic nuclei were apparent in the distal nerve segment of vehicle and bFGF animals at the site of injury (Fig. 25), more distally along the nerve (Fig. 26) and near the bifurcation (Fig. 27). In contrast, IGF-I rescued more SC all along the distal nerve segment (Fig. 28). Quantitative analysis with ANOVA substantiated that the apoptotic area fraction of all three treatment groups was significantly reduced ($p < 0.01$) from that of the TR-only group. IGF-I treatment reduced the area fraction of apoptotic nuclei 75% to a value of 0.012 (± 0.003; Fig. 29). Treatment with bFGF or vehicle only reduced the area fraction of apoptotic nuclei 50% to a value of 0.021 (± 0.003 and ± 0.005 respectively; Fig. 29). However, the trend of increased reduction of apoptosis with IGF-I compared with the other treatment groups was not statistically significant.

Figure 25. Numerous apoptotic nuclei in the distal nerve segment adjacent to the site of injury in a 10 DPN + 2 DPO bFGF treatment animal. (A) Distal nerve segment viewed through triple-filter. (B) The same distal nerve segment viewed through single filter (FITC). Bar represents 100 microns.


A
100 μm

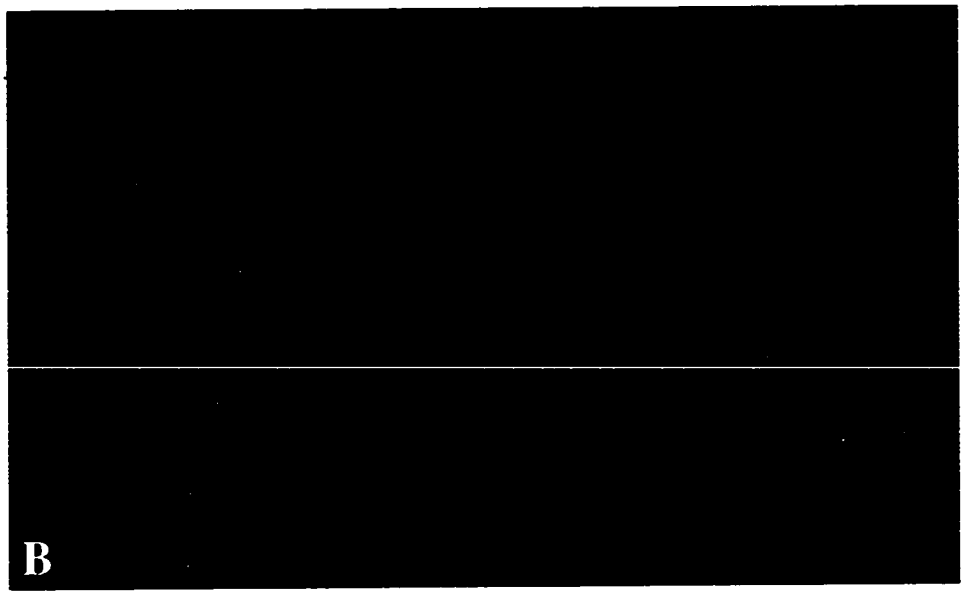

B

Figure 26. Apoptotic nuclei in the distal nerve segment a short distance from the site of injury in a 10 DPN + 2 DPO vehicle treatment animal. (A) Distal nerve segment viewed through triple-filter. (B) The same distal nerve segment viewed through single filter (FITC). Bar represents 100 microns.


A
100 μm

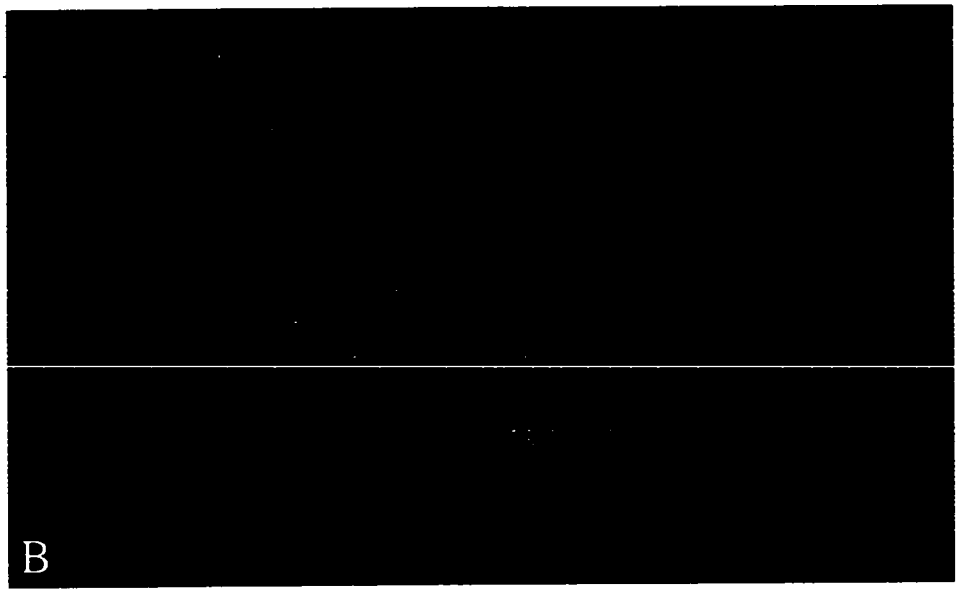

B

Figure 27. Abundant apoptotic nuclei in the distal nerve segment near the bifurcation (arrow) in a 10 DPN + 2 DPO vehicle treatment animal. (A) Distal nerve segment viewed through triple-filter. (B) The same distal nerve segment viewed through single filter (FITC). Bar represents 100 microns.


A
100 μm

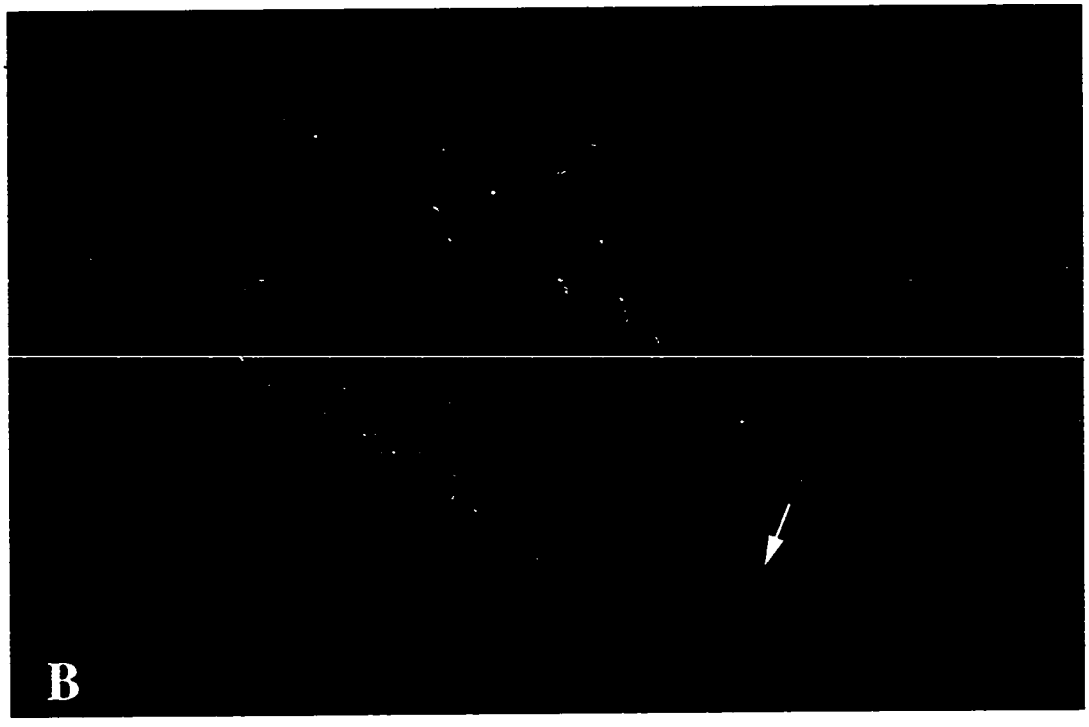

B

Figure 28. Reduced apoptotic nuclei all along the distal nerve segment of a 10 DPN + 2 DPO IGF-I treatment animal from the site of injury to the bifurcation (arrow). (A) Distal nerve segment viewed through triple-filter. (B) The same distal nerve segment viewed through single filter (FITC). Bar represents 100 microns.


A
100 μm

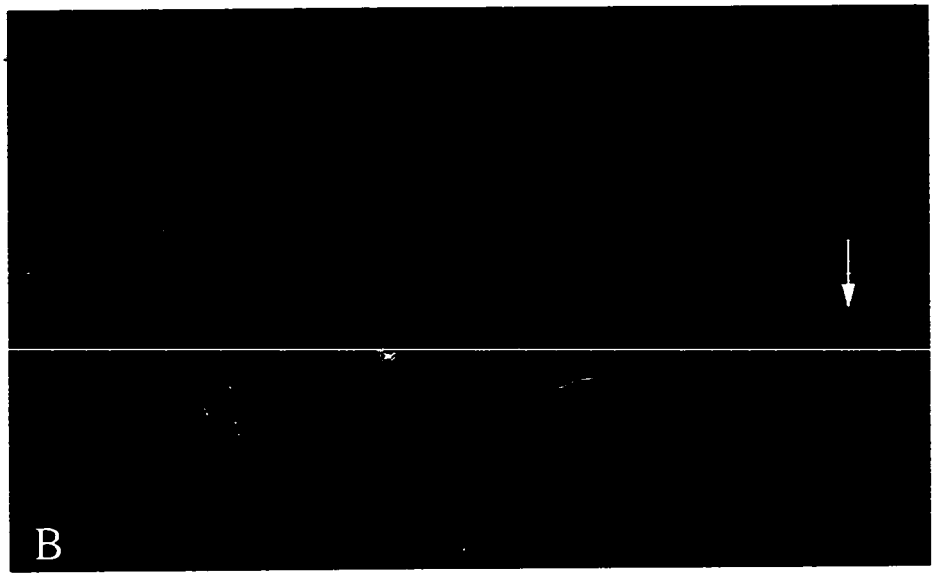
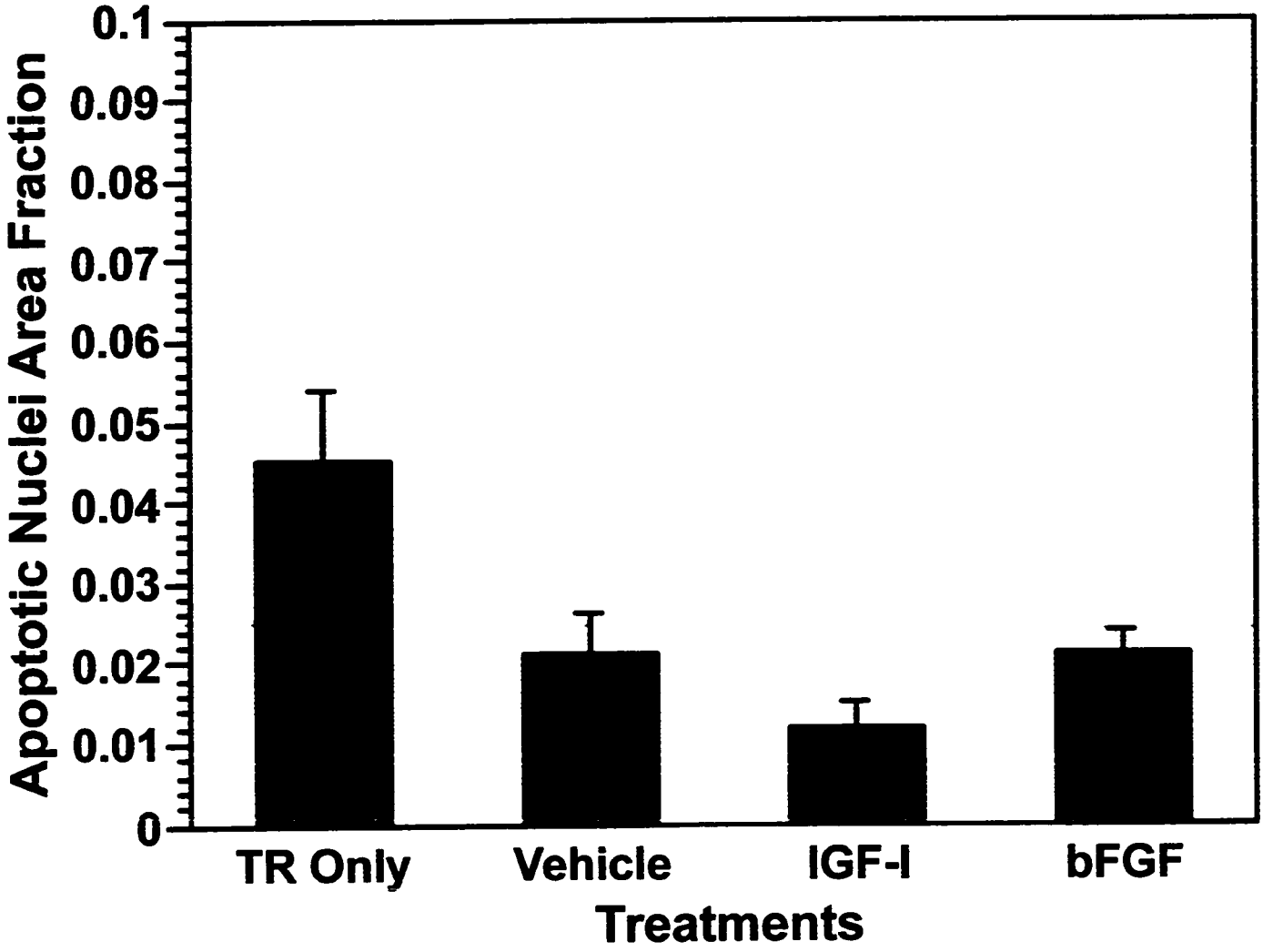

B

Figure 29. Area fraction of apoptotic nuclei in TR-only, vehicle, IGF-I, and bFGF treatment animals. Mean ± SEM are reported.

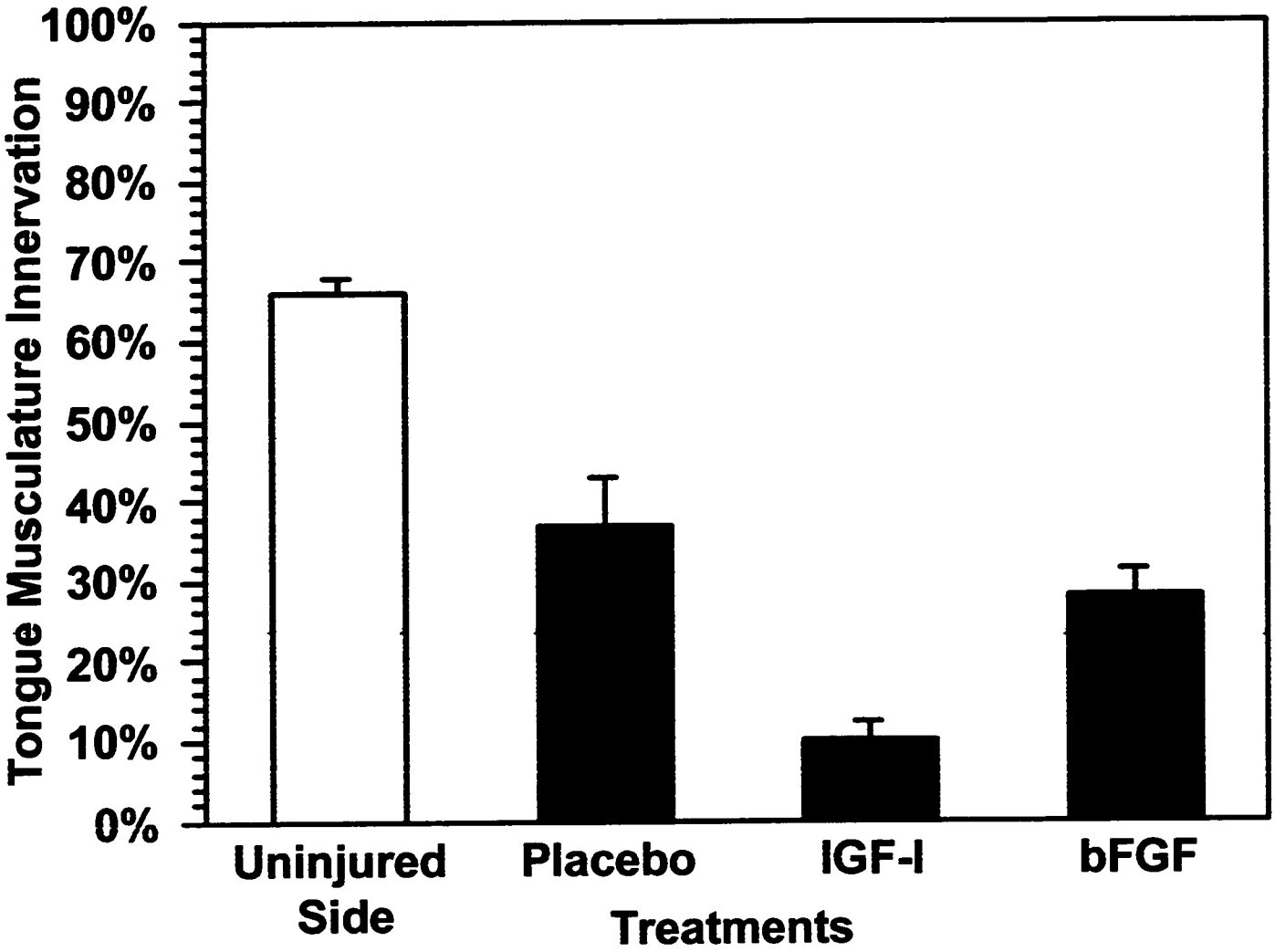

Apoptotic Nuclei Area Fraction
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
TR Only
Vehicle
IGF-I
bFGF
Treatments

Long-term Evaluation of Regeneration after Treatment with Growth Factors

Tongue Musculature Reinnervation

The incidence of HRP-labeled neurons in the injured and uninjured hypoglossal nuclei of rats was evaluated at 17 DPO after IGF-I, bFGF or placebo pellets were implanted at the site of nerve transection in 10 DPN rats. To clarify the combined effects of treatment groups and injury on tongue musculature reinnervation, a two-way ANOVA with repeated measures was performed on tongue innervation data and an interaction line chart was generated (Fig. 30). A significant growth factor x side interaction effect ($F = 25.36$, $p < 0.001$) indicated that growth factor treatments had a differential effect upon the innervation pattern of the hypoglossal nucleus on the injured and uninjured sides. Paired comparisons indicated that the innervation patterns of the hypoglossal nucleus on the uninjured side did not differ as a function of growth factor treatments ($p > 0.05$; Fig. 30). However, treatment with IGF-I resulted in a significant reduction of the degree of reinnervation in the hypoglossal nucleus on the injured side compared to bFGF or placebo treatment ($p < 0.01$; Fig. 30). IGF-I treated animals had an average reinnervation of the tongue musculature of 9.8% (± 2.3; Fig. 30). This amount was significantly reduced ($p < 0.01$) from the reinnervation of placebo animals (36.7% ± 5.9; Fig. 30) and the reinnervation of bFGF animals (27.5% ± 3.4%; Fig. 30). Although the animals receiving bFGF treatment had less reinnervation than placebo animals, this difference was not statistically significant.

Figure 30. Frequency of tongue musculature innervation in 10 DPN + 17 DPO placebo, IGF-I and bFGF treatment animals. Comparisons showed no significant difference between the percent innervation on the uninjured side in the placebo, IGF-I and bFGF treatment animals. Treatment with IGF-I, however, significantly reduced the degree of reinnervation after transection as compared to treatments with bFGF and placebo. The reinnervation pattern of the bFGF and placebo groups was significantly different. Mean ± SEM are reported.


100%
90%
80%
70%
60%
50%
40%
30%
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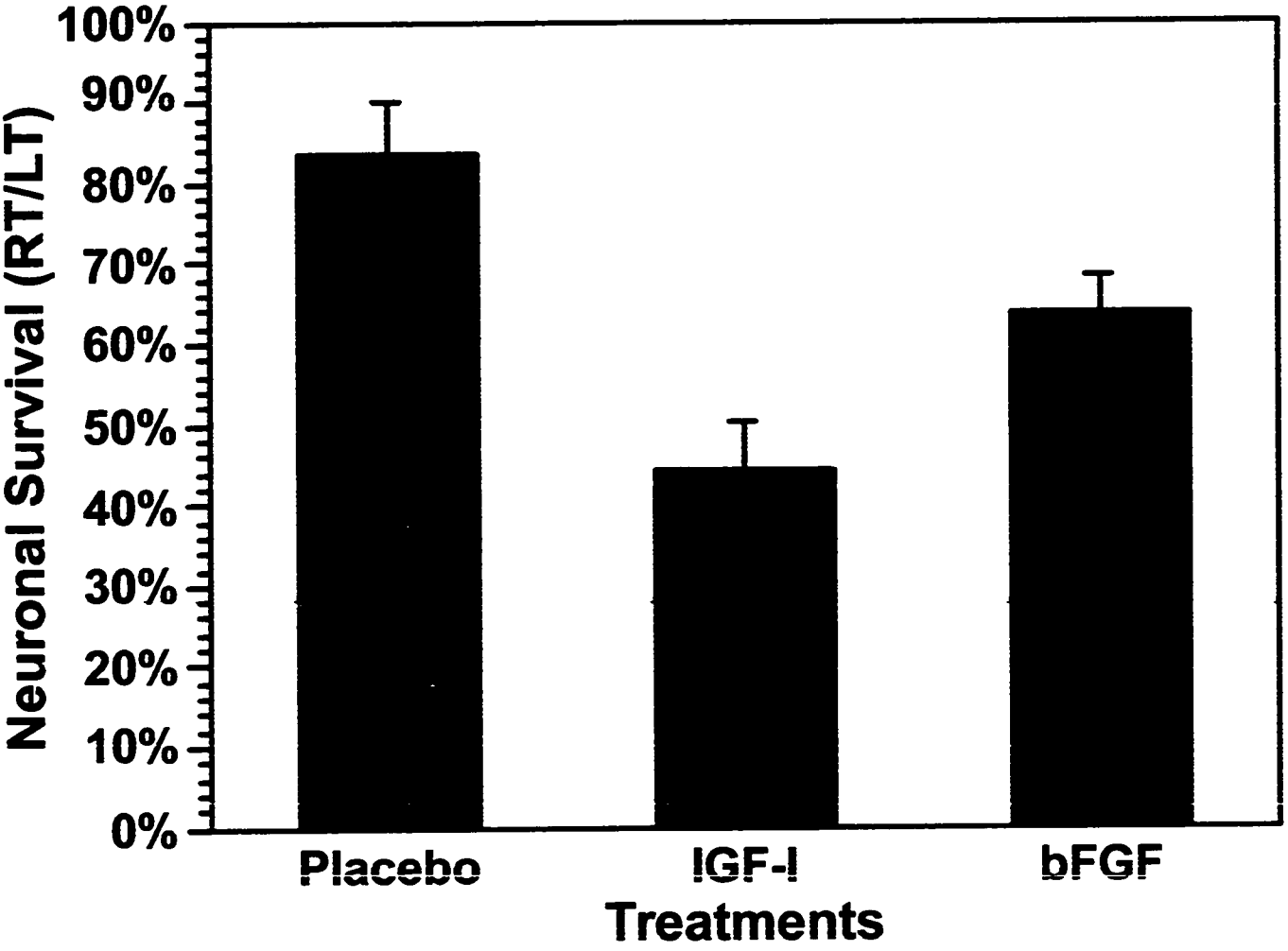
10%
0%
Tongue Musculature Innervation
Uninjured Side
Placebo
IGF-I
bFGF
Treatments

Neuronal Survival

The average number of neurons in the injured and uninjured hypoglossal nuclei of rats was evaluated at 17 DPO after IGF-I, bFGF or placebo pellets were implanted at the site of nerve transection in 10 DPN rats. An average of 4979 (± 173) neurons made up the neuronal population of uninjured hypoglossal nuclei. Neuronal cell loss occurred in the hypoglossal nucleus on the injured side after each treatment. The average neuronal survival was greatest in the placebo animals where 83.7% (± 6.6; Fig. 31) of the nucleus remained (4154 ±356). The greatest reduction in the total number of neurons on the injured side occurred in the IGF-I treated animals, where 44.4% (±5.8; Fig. 31) of the nucleus remained (2003 ± 213). In bFGF treated animals, more than 3/5 (63.9% ± 4.6; Fig. 31) of the neurons in the nucleus remained (3423 ± 162). Neuronal survival following IGF-I was significantly ($p < 0.01$ and $p < 0.05$ respectively) less than neuronal survival following placebo or bFGF treatment. In addition, bFGF pellet animals had significantly ($p < 0.05$) less neuronal survival than placebo pellet animals.

Figure 31. Frequency of neuronal survival in 10 DPN + 17 DPO animals. The percentage of neuronal survival was greatest after placebo treatment compared to treatment with either IGF-I or bFGF. In addition, treatment with IGF-I leads to an even greater loss of hypoglossal neurons since the percentage of neuronal survival after IGF-I treatment was significantly less than with bFGF treatment. Mean ± SEM are reported.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Neuronal Survival (RT/LT)
Placebo
IGF-I
bFGF
Treatments

DISCUSSION

Major Findings

The current investigation demonstrated these major findings, which are reviewed in detail in the sections that follow:

1) Apoptosis is an age-related response of Schwann cells to postnatal transection of the hypoglossal nerve. This reaction decreases as postnatal development proceeds and is discontinued in animals axotomized at one month of age.

2) The apoptosis is not only age-related, but also locality-specific. The apoptosis occurs only in that portion of the hypoglossal nerve distal to the site of the nerve transection. Within the distal nerve segment, the apoptotic Schwann cells occur in two strategic locations that are pivotal for guiding axonal outgrowth: a) capping the transected end adjacent to the injury site and b) just proximal to the bifurcation of the nerve into medial and lateral branches.

3) The application of IGF-I and bFGF at the transection site produced a reduction in apoptosis of Schwann cells in the distal nerve segment following injury. However, treatment with the vehicle containing bovine serum albumin also resulted in less apoptosis of Schwann cells.

4) The application of IGF-I had a deleterious long-term effect on the outcome of nerve transection in 10 DPN rats. Few axons reinnervated the tongue musculature and less neurons survived after IGF-I treatment as compared with bFGF or placebo treatment.

Schwann Cell Apoptosis as an Age-Related Response to Injury

The current work agrees with other reports that a low level of baseline SC apoptosis occurs postnatally in peripheral nerves (Grinspan et al., 1996; Syroid, Daniel E. et al., 1996; Trachtenberg and Thompson, 1996). SC apoptosis increased above this baseline following peripheral nerve injury as in previous reports (Grinspan et al., 1996; Trachtenberg and Thompson, 1996). The dramatic increase of SC apoptosis following hypoglossal nerve transection in 10 DPN animals but not in 21 or 30 DPN animals also confirms that SC apoptosis following injury is an age-related response in rats (Grinspan et al., 1996; Trachtenberg and Thompson, 1996). In the current work, markedly fewer SC were apoptotic following injury at 21 DPN versus 10 DPN, but SC did not become resistant to apoptosis until 30 DPN. SC at the NMJ are largely resistant to apoptosis after sciatic nerve transection by 25 DPN (Trachtenberg and Thompson, 1996). Other authors have reported that the age-related susceptibility of SC to apoptosis in the distal nerve segment disappeared by 20 DPN (Grinspan et al., 1996). However, the current work examined a larger portion of the distal nerve segment than previous studies. SC apoptosis in adult mice following peripheral nerve crush has been described in the literature, but the authors acknowledged that oxidative stress (ischemia of local tissues) during the experiment may have been a factor in this response (Ekström, 1995).

Cultures from developing rats have demonstrated that SC are a heterogeneous population of terminally differentiated, myelin-forming cells and other less differentiated nonmyelinating cells (Dashiell et al., 2000). Immature glia are more sensitive to

apoptosis than mature glia (Barres et al., 1992). Thus, differences in SC survival following injury at different postnatal ages or in different peripheral nerves may be due to differences in the population of SC present. This conclusion was supported in the current work by EM analysis of 12 DPN uninjured nerves. While most SC in adult hypoglossal nerves are myelinated, the SC of 12 DPN rats were seen to be in various stages of development with none, few and many thicknesses of myelin surrounding axons.

Previous reports of SC apoptosis following injury in postnatal animals were limited to mixed spinal nerves containing motor and sensory fibers (Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996). The current investigation is the first to show this response in a cranial motor nerve and suggests that SC apoptosis following peripheral nerve injury is a general, age-related response to nerve injury that occurs in all nerves whose neuronal cell bodies reside centrally or peripherally.

Schwann Cell Apoptosis as a Locality-Specific Response to Injury

The importance of SC proliferation and guidance at strategic points during peripheral nerve regeneration has been well-documented. The majority of the research involving SC apoptosis following postnatal injury has focused on the NMJ (Trachtenberg 1998; Trachtenberg and Thompson, 1996; Trachtenberg and Thompson, 1997). Other experiments have examined SC apoptosis in the distal nerve segment adjacent to the injury site (Grinspan et al., 1996; Syroid et al., 1996). Both of these locations are important areas for SC guidance of regenerating axons. However, the current work is the first to show that apoptosis occurs simultaneously at the strategic points that are crucial

for SC proliferation and guidance of regenerating axons down the length of the nerve to distal targets.

Within the distal nerve segment, apoptotic SC were found in two strategic locations that are pivotal for guiding axonal outgrowth: a) capping the transected end adjacent to the injury site and b) just proximal to the bifurcation of the nerve into medial and lateral branches. The large number of apoptotic SC capping the nerve just distal to the site of transection are within the region of SC proliferation critical for directing axonal sprouts that reconnect the proximal and distal nerve segments across the injury site. The second grouping of apoptotic SC occurred in the region pivotal for proper axonal routing just proximal to a branching point along the hypoglossal nerve. With transection injury, all axons are severed. Regenerating axons innervating the hyoglossus and styloglossus muscles must be directed into the lateral branch of the hypoglossal nerve while those innervating the genioglossus and geniohyoid muscles must be directed into the medial branch.

Apoptotic and mitotic SC were found in close proximity to each other within distal nerve segments following hypoglossal nerve transection. Mitotic cells were seen close to the injury site and just proximal to the bifurcation of the distal nerve segment into medial and lateral branches. In adult rats, there are two waves of SC proliferation following peripheral nerve injury. The first wave occurs within days following the loss of axonal contact (Liu et al., 1995; Pellegrino et al., 1986). Mitosis increases 7-fold over control nerves by 3 DPO, peaks to a 43-fold increase by 4 DPO and declines to a 3-fold increase by 5 DPO (Pellegrino et al., 1986). This first wave of proliferating SC are non-myelin-forming SC (Clemence et al., 1989). The second wave of proliferation occurs

when regenerating axons reenter the distal nerve segment (Pellegrino and Spencer, 1985). Reestablishment of axonal contact is critical for the initial events of myelination (Maurel and Salzer, 2000) and the second wave of axonally stimulated SC proliferation is a prerequisite for myelination during nerve regeneration (Pellegrino and Spencer, 1985). Total cell populations in the injured peripheral nerves of adult rats support this proliferation research. It is of interest that a second wave of axonally stimulated SC proliferation did not occur in nerves in which regeneration was prevented (Salonen et al., 1988).

The importance of the coincidence of apoptosis and mitosis was first described in cancer research. While the rate of cancer cell mitosis has been linked to the growth of tumors, basal cell carcinoma has a conspicuously slow rate of growth in the presence of a high rate of mitosis within lesions (Willis, 1948). This anomaly has been linked to the continuous death of cancerous cells via apoptosis, often in close proximity to mitotic cells (Kerr and Searle, 1972). Mitosis and apoptosis are now thought to be closely associated phenomena (Philpott et al., 1996). In fact, while the application of bFGF *in vitro* to oligodendrocyte progenitors stimulates rapid proliferation, the application of bFGF to mature oligodendrocytes initiates apoptosis (Muir and Compston, 1996). The apoptotic response to proliferative signaling may be due to the fact that SC have not yet reached the level of differentiation that they can adequately support themselves with growth factors in the absence of axonal contact (Reid et al., 1993). The SC in the distal nerve segment of postnatal rats may receive the same initial proliferative signal as the SC in the distal nerve segment of adult animals following peripheral nerve transection. The lack of appropriate supportive growth factors may then cause many stimulated SC to undergo apoptosis

instead of completing the cell cycle. The current work supports this concept, as EM analysis demonstrated pairs of daughter cells entering apoptosis during late-stage mitosis.

Reduction of Schwann Cell Apoptosis Following Treatment with Growth Factors

Treatment at the site of injury with IGF-I in the current study reduced SC apoptosis by 75% as compared with TR-only following hypoglossal nerve transection in 10 DPN rats. Similarly, IGF-I has also been shown to rescue cultured postnatal rat SC from apoptosis following serum withdrawal (Syroid et al., 1996; Syroid et al., 1999). This rescue of cultured SC has been duplicated with the application of a caspase-3 inhibitor and prevented with the application of a PI3K inhibitor, demonstrating that IGF-I prevents caspase-mediated SC apoptosis through interaction with the PI3K signaling pathway (Delaney et al., 1999). The reduction of SC apoptosis *in vivo* following IGF-I treatment in the current study supports the *in vitro* prediction that IGF-I inhibits apoptosis. However, while IGF-I treatment significantly reduced SC apoptosis following injury, so did bFGF and vehicle (PBS with BSA and gentamicin) treatments (Fig. 29). The lack of statistical significance between the three treatment groups implies that an additional component of the treatment system actively reduced SC apoptosis.

Albumin is a large plasma protein often used as a carrier protein in cell culture and antibody experiments. However, albumin has recently been shown to enhance the survival of mouse renal tubular cells and macrophages in cell culture through the inhibition of apoptosis (Iglesias et al., 1999). This protection occurs through at least two mechanisms: 1) binding of bioactive phospholipids such as lysophosphatidic acid which

themselves prevent apoptosis and 2) scavenging of reactive oxidant species via the oxidation of multiple free hydroxyl groups and a single free sulfhydryl group (Iglesias et al., 1999). The intrinsic cell survival mechanism of albumin apparently does not operate through activation of PI3K to prevent caspase-mediated apoptosis (Iglesias et al., 1999). This implies that the survival effects of albumin may be additive with those of cytokines and growth factors which operate through PI3K activation. In the current study, treatment with a vehicle solution (PBS with BSA and gentamicin) reduced SC apoptosis 50% as compared with TR-only animals following hypoglossal nerve transection in 10 DPN rats. There was no qualitative difference in SC apoptosis between the bFGF and vehicle treatment groups. One possible interpretation is that BSA is rescuing SC, but that bFGF has no effect on apoptosis.

Deleterious Long-Term Effects of Growth Factor Treatment Following Injury

Long-Term Effects of Growth Factor Treatment on Reinnervation

Treatment with IGF-I following hypoglossal nerve transection in 10 DPN rats led to substantial decreases in target reinnervation by 17 DPO as compared with placebo or bFGF treatment. This result contrasts with multiple studies that have demonstrated improvements in peripheral nerve regeneration with IGF treatment following crush or freezing nerve injuries (Contreras et al., 1993; Glazner et al., 1993; Kanje et al., 1989; Lewis et al., 1993b; Near et al., 1992; Sjoberg and Kanje, 1989; Vergara et al., 1993). Some of these studies delivered IGF systemically (Contreras et al., 1993; Lewis et al.,

1993b), others delivered IGF locally via osmotic pumps (Glazner et al., 1993; Kanje et al., 1989; Near et al., 1992) or via soaked nitrocellulose paper (Vergara et al., 1993). In contrast to these studies, IGF-I has recently been shown to be ineffective in improving reinnervation or functional recovery following peripheral nerve transection (Lutz et al., 1999). Transection injuries are inherently more complex than crush injuries, and often result in poor recovery due to improper reinnervation of target musculature (Lutz et al., 1999).

IGF-I promotes the formation of myelin in the nervous system. Transgenic mice overexpressing IGF-I have a significant increase in central nervous system (CNS) myelination, due to an increase in the amount of myelin produced per oligodendrocyte (Carson et al., 1993; Ye et al., 1995). Conversely, transgenic mice overexpressing IGF-binding protein-1 (IGFBP-1), a potent inhibitor of IGF-I function at high concentrations, show a significant decrease in brain myelination (Ye et al., 1995). Exogenous IGF-I application into the lateral ventricle of rats doubled the number of myelinating oligodendrocytes in the anterior medullary vellum (AVM) without depleting source promyelinating oligodendrocytes, which may also help to explain the increased myelination of transgenic mice overexpressing IGF-I (Goddard et al., 1999). Though less *in vivo* work has been done in PNS models, IGF-I also increases myelin production peripherally, apparently by the direct action of IGF-I on SC (Oudega et al., 1997). IGF-I selectively enhances the proliferation of SC surrounding myelinated nerve fibers, again showing a direct correlation between the CNS and PNS effects of IGF-I on glia (Sondell et al., 1997). The myelinating effects of IGF-I may be associated with the reported differences in peripheral nerve regeneration following crush and transection injuries in

the PNS with IGF-I treatment (Lutz et al., 1999). The deleterious effect in postnatal rats may be a more severe response related to the finding of no improvement in adult animals. In the current study, IGF-I treatment immediately following postnatal transection of the hypoglossal nerve may have "put the cart before the horse" by encouraging myelin-forming SC to begin the myelination process before proliferating non-myelin-forming SC could properly guide regenerating axons into the distal nerve stump.

A major effect of bFGF in the PNS is glial proliferation. In the CNS, bFGF application doubles the number of promyelinating oligodendrocytes in the AVM via either increased survival or proliferation (Goddard et al., 1999). In the PNS, bFGF induces the proliferation of SC *in vitro* (Davis and Stroobant, 1990; Jessen and Mirsky, 1992; Mirsky and Jessen, 1996; Mirsky et al., 1996). While bFGF is a known SC mitogen, forskolin or other compounds are necessary to activate SC adenylate cyclase and maximize proliferation (Rosenbaum et al., 1997). In addition to forskolin, IGF-I may actually be required for bFGF to induce SC proliferation (Schumacher et al., 1993; Stewart et al., 1996). In the current study, there was no statistical difference in tongue musculature reinnervation between the bFGF and vehicle treatment groups. The long-term effects of combined bFGF and IGF-I treatment following the postnatal transection of the hypoglossal nerve would make an excellent series of follow-up experiments to the current work. The synergistic effects of bFGF and IGF-I may increase the number of glial cells available at the site of injury and promote differentiation to the mature, myelinating form (Goddard et al., 1999), avoiding the deleterious effects of IGF-I treatment alone.

Long-Term Effects of Growth Factor Treatment on Motoneuron Survival

Motoneuron survival in the hypoglossal nucleus of placebo animals in the current study was comparable to that in previous reports. When 10 DPN animals were transected and treated with placebo pellets until 17 DPO in the current work, 84% of motoneurons survived. In a previous study, only 60% of motoneurons in the hypoglossal nucleus of 10 DPN animals survived until 20 DPO (Blake-Bruzzini et al., 1997). Another study in which 10 DPN animals were injured and allowed to survive until 13 DPO resulted in 80% survival (Borke, 1983). The slight improvement in neuronal survival in the current study may be explained in several ways. First, all of the pellets used for long-term delivery in the current study were composed of a proprietary matrix by Innovative Research of America. The matrix of these pellets could have an effect on the local axonal environment that translated into improved neuronal survival. Second, all of the surgeries in the current experiment were performed under isoflurane anesthesia provided with continuous oxygen support by an anesthesia machine. The previous study used ether to anesthetize animals during surgery without oxygen support. Recent evidence suggests that oxidative stress induces apoptosis in neurons and may contribute to neuronal losses in diabetes, Alzheimer's Disease and peripheral axotomy (Al-Abdulla and Martin, 1998; Behl et al., 1994; Rosenfeld et al., 1997; Russell et al., 1999; Tan et al., 1998). Oxidative stress during surgical procedures (Ekström, 1995) may have placed more stress on hypoglossal motoneurons than in the previous study. Greater motoneuron survival in the current study may be related to the use of supportive oxygen during surgical procedures. The third and most likely possibility is that counting with unbiased

stereology provided a more accurate estimation of the total neuron population than previously used assumption-based methods. In the current work, optical disectors allowed for a relatively thick section of tissue to serve as both the counting and reference (exclusion) planes by focusing up and down through the section (Gundersen, 1986; Gundersen et al., 1988). Specialized computer hardware and software permitted optical disector selection to progress systematically through a raster pattern, removing potential user error. Unbiased stereology is now the expected method of cell counting for publication unless explicit justification is made for the use of assumption-based correction methods (Saper, 1996).

Motoneuron survival in the present study was closely related to target musculature reinnervation. Treatment with IGF-I following hypoglossal nerve transection in 10 DPN rats led to substantial decreases in neuronal survival (44%) by 17 DPO compared with placebo or bFGF treatment. This result mirrored the low rate of target musculature reinnervation in IGF-I treatment animals. Differing rates of motoneuron survival have been associated with the age of the animal at the time of injury, the method of nerve injury and the distance of the site of injury from the neuron cell body (Lowrie and Vrbova, 1992). Postnatal animals appear to be especially vulnerable to injury, with 7 DPN rats losing twice the number of hypoglossal neurons following cranial nerve transection as 21 DPN rats (Borke, 1983; Snider and Thanedar, 1989). Failure to reinnervate the tongue musculature may be the cause of a large portion of postnatal motoneuron death in the hypoglossal nucleus after axotomy.

Innervation of the target musculature by developing motoneurons appears to be necessary to initiate the change from an immature growing neuron into a mature

transmitting neuron (Greensmith and Vrbova, 1996). Neurons which fail to properly establish a connection with target musculature may be considered redundant (Greensmith and Vrbova, 1996), marking them for elimination along with axons that polyinnervate motor end-plates (Brown, M. C. et al., 1976). Innervation of the target musculature may also allow neurons access to target-derived neurotrophic support (Raff et al., 1993). However, while the exogenous delivery of combinations of neurotrophic agents are temporarily effective (Oppenheim, 1991), they fail to achieve long-term neuronal survival (Eriksson et al., 1994; Vejsada et al., 1995). Proper neurotrophic support for motoneurons following injury may depend on the type of nerve injury involved (Near et al., 1992).

IGF-I treatment has been previously shown to improve motoneuron survival at 7 DPO after facial nerve transection in newborn rat pups (Hughes et al., 1993). Treatment with IGF-I after sciatic nerve transection in 5 DPN mice eliminated spinal motoneuron death at 7 DPO (Li et al., 1994). However, neither of these studies examined motoneuron death at later survival times that corresponded with the timing of target reinnervation. While neurotrophic factors applied to the injury site after sciatic nerve transection have improved motoneuron survival at the 7 DPO survival time in previous experiments, this benefit deteriorated rapidly with high levels of motoneuron death during the second week after injury (Vejsada et al., 1995). The transient improvement in motoneuron survival previously reported with IGF-I treatment in postnatal animals therefore cannot be extrapolated (Hughes et al., 1993; Li et al., 1994) to long-term survival or improvement in peripheral nerve regeneration after axotomy. The deleterious long-term effects of IGF-I treatment in the current study support this conclusion.

Treatment with bFGF also had a deleterious effect in the current study on long-term motoneuron survival following postnatal transection of the hypoglossal nerve. While less pronounced than the effect of IGF-I treatment, reduction in motoneuron survival with bFGF treatment (64%) was significantly reduced from placebo animals. Previous studies have shown that bFGF treatment at the site injury after hypoglossal nerve transection of 7 DPN rats can partially rescue motoneurons at 7 DPO (Grothe and Unsicker, 1992). SC begin to produce bFGF within 2 days of sciatic nerve transection in adult animals (Liu et al., 1995). Retrograde transport of bFGF has been demonstrated by radioactive bFGF studies and bFGF immunoreactivity is apparent in the hypoglossal nucleus of adult animals after injury (Grothe and Unsicker, 1992). However, the rapid movement of bFGF *in vivo* away from areas of application may limit its effectiveness as a therapeutic agent without co-administration of a binding glycosaminoglycan (Yamada et al., 1997). In the current study, bFGF was administered with heparan sulfate. This compound is closely related to the anticoagulant heparin sulfate, and may actually be required for activation of the FGF receptor complex (McKeehan et al., 1999). However, as with IGF-I treatment, bFGF may be ineffective in promoting long-term motoneuron survival in the absence of proper target musculature reinnervation.

SUMMARY AND CONCLUSIONS

The current study determined that apoptosis is an age-related response of Schwann cells to postnatal transection of the hypoglossal nerve. This suggests that Schwann cell apoptosis following peripheral nerve injury is a general, age-related response to peripheral nerve injury. In addition, Schwann cell apoptosis was locality-specific. The apoptosis occurred only in the distal nerve segment and in two strategic locations for guiding axonal outgrowth: 1) capping the transected end adjacent to the injury site and 2) just proximal to the bifurcation of the nerve into medial and lateral branches. Electron microscopic analysis demonstrated that apoptosis and mitosis of Schwann cells was coincidental within the distal nerve segment, supporting the theory that the two processes are closely related phenomena. Postnatal Schwann cells may receive the same proliferative signals following injury as adult animals, but some mitoses may falter or break down in the absence of proper neurotrophic support and result in apoptosis.

Treatment with IGF-I reduced Schwann cell apoptosis in the distal nerve segment after transection of the hypoglossal nerve in 10 DPN rats compared with injured, untreated animals. However, bFGF and vehicle treatment also reduced apoptosis compared with injured, untreated animals. While IGF-I produced the greatest reduction of apoptosis both qualitatively and quantitatively, the difference from the other treatment groups was not statistically significant. The absence of statistical significance between the three treatment groups may be related to the inclusion of bovine serum albumin in the

vehicle solution. The results of the current study support the role of bovine serum albumin as an *in vivo* cell survival factor.

Long-term treatment with IGF-I had a deleterious effect on the outcome of nerve transection in 10 DPN rats. Few axons reinnervated the tongue musculature and less neurons survived 17 DPO after IGF-I treatment as compared with bFGF or placebo treatment. Myelination-stimulating effects of IGF-I on Schwann cells may have altered the local axonal environment during peripheral nerve regeneration by interfering with proliferation of non-myelinating Schwann cells necessary for the proper guidance of regenerating axons. High rates of neuronal death may have followed as a consequence of reduced reinnervation of the tongue musculature. Long-term treatment with bFGF also reduced neuronal survival as compared with placebo treatment.

In conclusion: Although SC in developing animals can be rescued by growth factor treatment, SC and motoneurons have not yet established the proper interactive relationship to translate this rescue into improved target musculature reinnervation or long-term motoneuron survival.

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