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A role for thrombospondin in peripheral and central nervous system regeneration

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A ROLE FOR THROMBOSPONDIN IN PERIPHERAL AND CENTRAL NERVOUS SYSTEM REGENERATION

by

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To Lesley, for all her loving support and understanding

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CHAPTER I

INTRODUCTION

Regeneration of both the central and peripheral nervous systems is possible to varying degrees across animal classes. After trauma, functional regeneration in the mammalian peripheral nervous system (PNS) is often observed, but is seldom seen in the central nervous system (CNS). Lower vertebrates, such as newts and fish, are capable of undergoing central nervous system regeneration in response to injury (Windle, 1954). The loss of functional recovery of the CNS is prevalent in mammals, birds, reptiles and some adult amphibians. The goal of many investigators is to determine how and why the mammalian CNS does not regenerate while the nervous systems of other species and the PNS do.

To determine if providing a suitable environment will enhance CNS regeneration, tissue known to have regenerative capacity, such as the PNS or embryonic neurons, has been grafted into the brain or spinal cord. Early work demonstrated growth of numerous cerebral axons growing into grafts of rabbit peripheral nerve implanted into the brain (Tello, 1911, cited from Ramon y Cajal 1928). Later investigators performed comparable experiments and mis-identified fibers growing into the grafts as vascular nerves accompanying cerebral blood vessels (Le Gros Clark, 1943). The reported lack of intrinsic brain fibers entering the grafts led to the erroneous conclusion that fibers of the rabbit brain show no capacity for regeneration.

Implantation of peripheral nerve grafts into brain and spinal cord produced mixed results until recently, when it was conclusively demonstrated that CNS neurons are intrinsically able to regenerate (Clemente, 1964). When a segment of

adult rat spinal cord was removed and replaced with a small piece of sciatic nerve and the retrogradely transported marker, horseradish peroxidase (HRP) was applied, a limited number of axons projected into the graft from cell bodies in the spinal cord (Richardson *et al.*, 1980). Axons originating from neurons within the spinal cord were able to grow a distance of approximately 10 mm into the peripheral nerve (PN) graft. Axonal growth into the peripheral nerve environment demonstrated that CNS neurons are intrinsically capable of growth. Further work demonstrated that CNS neurons extended processes through the peripheral nerve grafts and into CNS tissue (David and Aguayo, 1981, 1985). This re-entry was limited to about 2 mm, was ensheathed by Schwann cells, and ended near CNS neurons as identified by electron microscopy. The limited entrance of neurons 2 mm into the CNS may not be significant, as nerves associated with glial processes could grow 1.3 mm into an acellular stainless steel tube inserted into the brain through a hole in the skull after 4 months (Azzam and Brightmon, 1985).

Further studies examined other potential permissive environments and characterized the neurites that grew through them (Oorschot and Jones, 1990). Adult rat optic nerve (Politis and Spencer, 1986), adult rat spinal cord (Fernandez *et al.*, 1984), and adult rat brain (Dum and Salame, 1986) were capable of regenerating through sciatic nerve grafts. In addition processes from adult rat dorsal root ganglia (DRG) are able to grow into embryonic but not adult spinal cord implants (Tessler *et al.*, 1988).

In order to understand the conditions that are permissive or restrictive for nerve regeneration, regenerating and non-regenerating systems must be analyzed. A variety of possible conditions could control the success or failure of nerve regeneration (for review see, Chiquet, 1989). For instance, a factor or factors present in the CNS may block neurite outgrowth. This type of inhibitory factor would normally prevent regeneration; removing or blocking its action would create a

permissive environment for regeneration (Schwab *et al.*, 1991). Alternatively regeneration-promoting factors present in the PNS may be absent in the CNS.

Benfey and Aguayo (1982) concluded that the microenvironment surrounding the graft insertion site plays a decisive role in the success or failure of regeneration. This conclusion implies that non-neuronal PNS components influence the process of regeneration and that similar components are lacking or have a diminished effect in the CNS. One aspect of the local axonal environment that may influence regeneration is the interaction of cells with the extracellular environment.

The extracellular matrix (ECM) surrounding cells is composed of a tissue-specific combination of collagens, non-collagenous glycoproteins, glycosaminoglycans, proteoglycans, ions, and water (Hay, 1981). Sequestered within the ECM are proteases, growth factors and hormones that can influence sprouting, growth and survival of cells and their processes. Collagens and related molecules provide structure, and the glycosaminoglycans and proteoglycans interact within the matrix to form a hydrated gel-like substance (Alberts *et al.*, 1989). Non-collagenous glycoproteins are mainly adhesive molecules, such as fibronectin, laminin, and thrombospondin, which are often found in the basement membrane, a compaction of extracellular matrix molecules.

The presence of a basement membrane appears to be important for establishing nerve regeneration. The adult CNS lacks a basement membrane except in the meninges and surrounding blood vessels, and appears to contain less fibronectin and laminin in comparison with the PNS (Carbonetto, 1984, Liesi *et al.*, 1984). Regenerating axons preferentially grow along the basement membrane (Ide *et al.*, 1983), and the presence of a basement membrane directs and increases the rate of neurite outgrowth (Glasby *et al.*, 1986). Interestingly, the ability to promote neurite outgrowth is not limited to only the basement membranes of the nervous system, as regenerating peripheral nerve axons grow along the basement membranes

of muscle (Keynes *et al.*, 1984), and rat or human amnionic membranes (Schwab and Thonen, 1985; Gage *et al.*, 1988; Danielson *et al.*, 1988)

The distribution of ECM molecules, including collagen, laminin, and fibronectin, within the adult nervous system after injury has been investigated extensively. In the brain and spinal cord, laminin and fibronectin immunoreactivity is normally confined to endoneurium and vessel walls. Brains lesioned with kainic acid showed a transient increase in anti-laminin but not anti-fibronectin immunoreactivity (Liesi *et al.*, 1984). Similarly anti-laminin reactivity demonstrated a distinct boundary between the PNS and CNS following DRG crush, and no regenerating axons were observed in the laminin-negative intramedullary division of the root (Bignami *et al.*, 1984). Laminin is found in the regenerating frog and goldfish optic nerves, but not in the non-regenerating rat, guinea pig, or chick optic nerves (Liesi, 1985). The data indicate a strong correlation between the presence of laminin immunoreactivity and successful regeneration. Previous accounts of transient laminin-immunoreactivity in other parts of the CNS after injury (Liesi *et al.*, 1984) may help to explain aborted regeneration, as an important substrate (laminin) is lost just as neurite outgrowth begins.

Laminin alone is unlikely to be sufficient to support nerve regeneration. While several studies that concluded that laminin was a necessary factor for regeneration (Hopkins *et al.*, 1985; Madison *et al.*, 1985; Liesi, 1985), peripheral nerve grafts which have been frozen and devitalized to kill the Schwann cells would not support regeneration in adult hamster brain or rat retinal ganglion cells, even though laminin immunoreactivity remained (Smith and Stevenson, 1988; Berry *et al.*, 1988). These studies demonstrate that a suitable substrate produced in response to injury is required for neurite outgrowth. Regenerating neurites will follow a laminin tract, but in systems where laminin is present, such as the frog and goldfish optic nerves, axons are stable. Therefore the requirement for laminin appears to be secondary, as it provides an extracellular path that growth cones follow after the

initiation of regeneration. It appears that other factors must first be present to stimulate neurons to regenerate.

The role of extracellular matrix components in supporting neurite outgrowth has also been investigated *in vitro*. Neurite outgrowth is observed on many types of ECM components including; collagen type I, II, IV, fibronectin, and laminin (Carbonetto *et al.*, 1983; Schistine and Cornbrooks, 1988). However, laminin supports neurite extension from CNS as well as PNS neurons, while fibronectin supports only PNS neurite extension (Rogers *et al.*, 1983). Recently, dissociated human fetal DRG were plated on various substrates and the results confirmed data derived from chick explants that laminin and fibronectin were permissive substrates (Yong *et al.*, 1988).

Thrombospondin is a recently identified extracellular matrix molecule that is associated with peripheral nerve development (O'Shea and Dixit, 1988). Thrombospondin was originally identified as a 420 kDa glycoprotein released by blood platelets exposed to thrombin (Baenzinger *et al.*, 1971). TSP was observed in a variety of extracelluar matrices in adult (Wight *et al.*, 1985) and embryonic tissues (O'Shea and Dixit, 1988). It is present throughout the developing CNS and PNS, but is largely restricted to the adult PNS (O'Shea and Dixit, 1988). Within the nervous system, the presence of TSP is temporally and spatially correlated with neural crest cell migration (Boyne *et al.*, 1989), cerebellar granule cell migration (O'Shea *et al.*, 1990), and peripheral nerve outgrowth (O'Shea and Dixit, 1988). In addition, TSP supports neurite outgrowth *in vitro* from DRG explants, PC12 cells, isolated sensory neurons, and chick ciliary ganglionic neurons (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991).

Thrombospondin has specific domains which may play an important role in neurite outgrowth. TSP is a trimeric, globular protein with an amino-terminal heparin binding domain seperated from the carboxy-terminal cell binding domain by a rod-like stalk, and appears as a lopsided barbell in rotary shadow electron microscopy. The stalk of TSP (figure 1) contains a procollagen-like region; three type I repeats which are homologous to region II of malaria circumsporozoite protein and human complement protein properdin (Goundis and Reid, 1988); three EGF-like repeats, often refered to as type II repeats (Haverstick *et al.*, 1984); and a highly repetitive calcium-binding region, referred to as type III repeats (Lawler and Hynes, 1986). Several regions of the molecule have been reported to facilitate cell adhesion, including the amino-terminal heparin-binding domain (Murphy-Ullrich and Mosher, 1987), the carboxy-terminal platelet binding domain (Roberts *et al.*, 1987), two VTCG sites in the type I repeats (Prater *et al.*, 1991), and the RGDA sequence within the calcium binding (type III repeats) region (Lawler *et al.*, 1988). Three cell binding regions were identified with domain specific antibodies, the fourth with RGD peptides. It is likely that specific cell types will only express receptors for specific regions of TSP, and that the conformation of the molecule may determine which regions are accessible to cell surfaces. In particular, the RGDA sequence within the type III repeats may be hidden by differing levels of calcium (Lawler *et al.*, 1988).

Thrombospondin may precisely regulate the extracellular environment available for neurite ourgrowth. In addition to serving as a potent substrate for neurite outgrowth *in vitro* (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992), TSP interacts with a wide range of other potentially-adhesive ECM molecules: type I collagen (Lahav *et al.*, 1982), type V collagen (Mumby *et al.*, 1984), laminin (Mumby *et al.*, 1984), fibronectin (Lahav *et al.*, 1982, 1984),

heparin (Lawler *et al.*, 1978), and glycosaminoglycans (Dixit *et al.*, 1984; Lawler *et al.*, 1985). TSP can also control proteolysis by binding fibrinogen, plasminogen (Silverstein *et al.*, 1984), and both tissue- and urokinase-type plasminogen activators (Silverstein *et al.*, 1986); in addition, binding by TSP increases the synthesis of plasmin by 40-fold (Silverstein and Nachman, 1987). TSP may therefore both stimulate and direct localized proteolysis, at the same time protecting proteases from matrix inhibitors. Penetration of neurites through a cell- and ECM-filled environment likely requires a rapid alternation between controlled proteolysis and attachment to an adhesive substrate (Moonen *et al.*, 1982; Pittman *et al.*, 1989; McGuire and Seeds, 1990), which may be facilitated by TSP.

Preliminary data demonstrate that TSP is a molecule with the potential to be involved in nerve regeneration in the adult. Thrombospondin is present extensively in both the developing CNS and PNS, but only in the adult PNS (Wight *et al.*, 1985; O'Shea and Dixit, 1988). This observation correlates with results of transplantion studies in which adult peripheral and embryonic central nervous tissue grafts have been shown to support axonal regeneration (Tessler *et al.*, 1988). In addition, TSP supports neurite outgrowth *in vitro* (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992) and is present at times of peripheral nerve outgrowth *in vivo* (O'Shea and Dixit, 1988). These results demonstrate that TSP is capable of supporting neurite outgrowth and that it has an expression pattern which parallels observed regeneration permissive environments.

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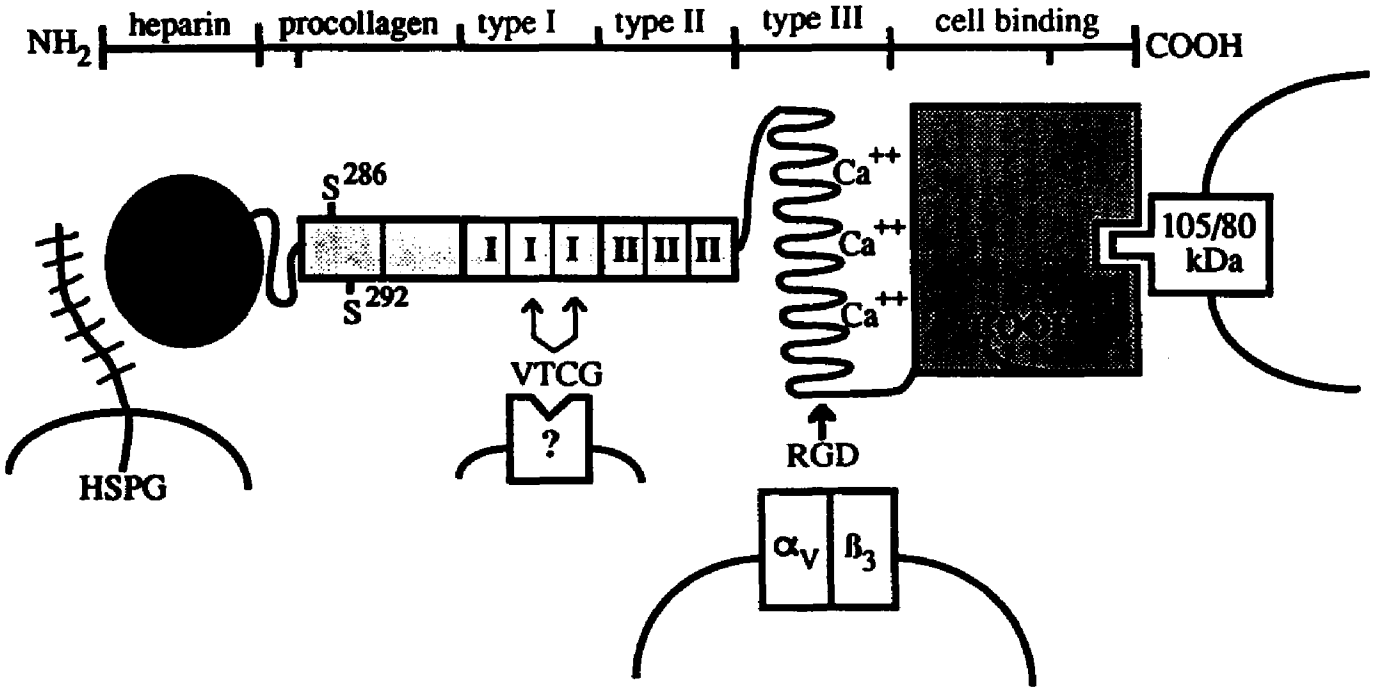

Figure 1.1. Schematic representation of a thrombospondin subunit (after Laherty, 1992).

CHAPTER II

THE EXPRESSION OF THROMBOSPONDIN IS CORRELATED WITH REGENERATIVE POTENTIAL OF NERVOUS TISSUES.

Introduction

A crucial issue in the field of neurobiology is the cellular and molecular basis of the differential regenerative potential between central and peripheral nervous systems (PNS), and across species. The mammalian peripheral nerves are capable of regenerating, but the mammalian central nervous system (CNS) does not regenerate (Ramon y Cajal, 1928, trans. R. May, 1991). Animals in so-called lower vertebrate classes, such as fish and urodele amphibia, are capable of both central and peripheral nerve regeneration (Windle,1954; Clemente, 1964). Anuran amphibia are capable of CNS and PNS regeneration as juveniles, but as adults they are only capable of optic nerve regeneration (Bohn *et al.*, 1982; Clemente, 1964); spinal cord regeneration is unsuccessful.

Theories regarding why some species are capable of CNS regeneration, while others are not, include variable glial response (Wujek and Reier, 1984; Reier and Houle, 1988), the presence of inhibitory factors (Schwab, 1991), or lack of supportive factors in the mammalian CNS (Liesi, 1985). Since each theory has supporting evidence, it is likely that an interaction of a variety of such factors may be required for optimal regeneration. Manipulation of a single factor may make it

possible to achieve neurite outgrowth, but the interaction of several variables is likely essential in promoting successful CNS regeneration *in vivo*.

Thrombospondin (TSP) is an extracellular matrix molecule with an expression pattern and physical properties which suggest that it may be involved in nerve regeneration. TSP is a 420 kDa trimeric glycoprotein, that was originally identified as being released by activated platelets (Baenziger *et al.*, 1971). TSP has since been shown to be involved in cell attachment (Varani *et al.*, 1986; Roberts *et al.*, 1987), migration (O'Shea *et al.*, 1990; Majack *et al.*, 1986), and neurite outgrowth (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992). TSP may act *in vivo* by organizing the extracellular matrix through binding of other ECM components including type I collagen (Lahav *et al.*, 1982), type V collagen (Mumby *et al.*, 1984), laminin (Mumby *et al.*, 1984), fibronectin (Lahav *et al.*, 1982, 1984), glycosaminoglycans (Dixit *et al.*, 1984; Lawler *et al.*, 1985), plasminogen (Silverstein *et al.*, 1984), and tissue- and urokinase-type plasminogen activators (Silverstein *et al.*, 1986). TSP has been sequenced in the human (Lawler and Hynes, 1986), mouse (Laherty *et al.*, 1992), chicken (Lawler *et al.*, 1991), and Xenopus (Urry *et al.*, 1991), and functional domains of TSP show a high degree of conservation of amino acid sequences across species. Although initial studies demonstrated that the distribution of TSP is highly developmentally regulated in the murine nervous system (O'Shea and Dixit, 1988), little is known about the expression pattern of TSP in adult nervous tissue. This study examines the pattern of TSP expression in the nervous systems of mice, goldfish, frogs, and newts.

Materials and Methods

Tissue: Nervous tissue was collected from a variety of animal classes. Species used in this study were adult CD-1 mice (*Mus musculus*), adult common goldfish (*Carassius auratus*), adult frogs (*Xenopus laevis*), adult newts (*Taricha torosa* and *Taricha rivularis*), as well as pre-metamorphic juvenile frog (*Xenopus laevis*) at the fore-limb bud stage. Mice were anesthesized with methoxyfluorane (Metofane, Pitman-Moore, Washington Crossing, NJ), while goldfish, frogs, and newts were anesthesized using MS-222 or submerged in ice water. Brain, spinal cord, optic nerve, and sciatic nerves then were removed and rapidly frozen in Optimal Cutting Temperature embedding compound (OCT; Miles Laboratories, Inc., Elkhart IN) in hexane cooled over an acetone-dry ice slurry. Ten micron frozen transverse and sagittal sections were collected on 0.1% poly-L-lysine coated slides, and stored at $4^{o}C$ until processing for immunocytochemistry. For the survey of brain tissue, one coronal section was collected every 200 microns; in brains cut sagittally one section was collected every 200 microns (newt, frog), 300 microns (goldfish), or 400 microns (mouse).

Antibody Specificity: The specificity of the rabbit anti-human TSP antibodies used in these experiments was tested by western blotting. The anti-TSP antibody has previously been shown to be specific for mouse TSP (O'Shea and Dixit, 1988). Brain tissue from each species was frozen in liquid nitrogen. Proteins were extracted from the tissue, run on a polyacrylamide gel, transferred to nitrocellulose, and detected using a standard enhanced chemoluminescence kit (ECL; Amersham Corp., Arlington Heights, IL). Purified platelet TSP was run as a control lane.

Immunocytochemistry: Selected sections were washed in PBS; non-specific antibody binding was blocked with normal goat serum (1:20), followed by rabbit anti-human TSP primary antibody (1:20) or normal rabbit serum. Visualization of antibody binding was conducted with either FITC conjugated goat anti-rabbit IgG secondary antibody (1:20, Sigma Chemicals, St. Louis MO) or by using an anti-rabbit StrAviGen Super Sensitive Universal Immunostaining kit (BioGenex Labs., San Ramon, CA). Slides exposed to the FITC-labeled secondary antibody were coverslipped with glycerol containing 0.1% phenylenediamine, then viewed and photographed in a Leitz Aristoplan microscope with a L-1 fluorescence cube. Slides stained with the supersensitive kit were counterstained with hematoxylin, coverslipped with Crystal/Mount (Biomeda Corp., Foster City CA),and then viewed and photographed in the Leitz Aristoplan microscope.

Results

Western Blot: The rabbit anti-human TSP antibody used in these studies reacts specifically with TSP in each of the species examined in this paper. The specificity of this antibody to mouse TSP has been shown previously (O'Shea and Dixit, 1988). The results of an immunoblot of whole brain proteins of adult goldfish, newt, and Xenopus indicate that the rabbit anti-human TSP antibodies detect a protein that comigrated with purified human platelet TSP (Figure 2.1A, 2.1B). Pre-immune serum showed no reactivity to goldfish, newt, or Xenopus proteins (Figure 2.1C).

Brain: To carry out this survey, an effort was first made to examine as many regions in each species as possible, identified from a variety of sources for mouse (Sidman *et al.*, 1971; Diefenbach *et al.*, 1991), goldfish (Peter and Gill, 1975; Easter *et al.*, 1978), frog (Kemali and Braitenberg, 1969; King and Custance, 1982;

Opdam *et al.*, 1976), and newt (Herrick, 1948; Roth, 1987). Once regions of the brain were identified completed, a comprehensive survey (Diefenbach *et al.*, 1991) was prepared and phylogenetic homoloques examined (Romer and Parsons, 1977; Sarnat and Netsky, 1981; Wake, 1979).

The results of studies of comparative brain regions are summarized in Table 2.1, abbreviations are provided in Table 2.2, and sample regions are illustrated for mouse (figure 2.2), goldfish (figure 2.3), Xenopus (figure 2.4), and newt (figure 2.5). It was surprising to note that in all species examined, TSP was often found extracellularly in association with neuronal cell bodies.

All the species examined have an olfactory bulb of varying degrees of complexity. TSP was found primarily in the white matter of the bulb, with neurons staining slightly. The neuropil of the mouse granule cell layer had less TSP than the external plexiform layer, in contrast with that of the frog, newt, and goldfish where the granule cell layer exhibited higher levels of TSP immunoreactivity than the external plexiform layer.

The cerebral hemispheres were more variable between species in overall architecture than the olfactory bulb. However, the cerebrum can be subdivided into roughly analagous structures, based upon the relationship to the ventricles. The cells in the region of the roof of the ventricle, the pallium, includes structures such as the mouse hippocampus and newt pyriform pallium. The floor of the ventricle, the subpallium, includes the septum (medial) and corpus striatum (ventral and lateral).

In the mouse hippocampus, the dentate molecular layer was negative for TSP, the hilus of dentate gyrus was slightly positive, and the granule layer was intensely positive. In the goldfish, the neuropil of the hippocampus was intensely positive, with similar regions in the frog and newt hippocampus only slightly less so. Neurons of the frog hippocampus were also TSP-positive. TSP was present in neurons but absent in the white matter of the mouse subpallium, which corresponds to the caudate putamen, while the neuropil and neurons in the goldfish, newt, and frog exhibited TSP immunoreactivity.

In the diencephalon and mesencephalon neurons were positive for TSP immunoreactivity, with less immunoreactivity in the white matter. Newt neuronal cells typically exhibited less immunoreactivity than those of other species, and both the frog and goldfish had more staining in the neuropil than the mouse.

The cerebellum exhibited a great deal of diversity both in size and complexity in the animals examined. The size of the cerebellum changed from the small structure present in the frog and newt, to the large structure present in the goldfish, where the cerebellum protrudes into the mesencephalon. Complexity ranged from a simple bilayer in the newt to the multi-layered structure of the mouse. In the goldfish, frog, and newt, TSP was present in the neuropil of both the molecular and granule layers, but only slight immunoreactivity was observed in the neurons of the cerebellum. In the mouse, TSP was present in the molecular layer, much less intense in the granule cell layer, and was completely absent in the deep white matter. The neurons of the mouse molecular layer are slightly TSP-positive, as were the cell bodies of Golgi epithelial cells.

Nerve tracts exhibited considerably greater species differences. Fiber tracts in the mouse such as the anterior commissure, corpus callosum, fornix, stria medullaris of the thalamus, and mammilothalamic tract contained little TSP immunoreactivity. Conversely, tracts in the goldfish such as the optic tract, horizontal commissure, and medial longitudinal fasiculus exhibited varying degrees of TSP immunoreactivity. We were unable to identify conclusively tracts in the frog and newt, but believe that there was very little difference between the levels of TSP immunoreactivity in neuropil and fiber tracts.

Spinal Cord: In the mouse spinal cord, TSP was present in the cell bodies of the ventral horn (motor neurons) cells and inside the axons of the motor neurons (Figure 2.6A, 2.6B), but no TSP immunoreactivity was observed in the neuropil surrounding the motor neurons or associated with the glial cells of the cord. TSP immunoreactivity was observed at high levels surrounding and within the cells of the dorsal root ganglia (DRG). There was some non-specific staining of the gray matter, when comparing the anti-TSP stained tissue with NRS stained tissue, accounting for the diffuse fluorescence in the gray matter of the cord.

In the goldfish spinal cord, TSP immunoreactivity was observed at very high levels surrounding the ependymal cells lining the central canal and in the neuropil of Rexed's lamina X (Figure 2.6C). TSP was also observed in the cell bodies of the ventral horn cells, in radial glia, and at the glia limitans (Figure 2.6D).

In the adult Xenopus spinal cord, TSP was present in the pia mater, and in peripheral nerves leaving the spinal cord, but was largely absent from the cord itself (Figure 2.6E). Upon examination at higher magnification, TSP was observed in some glial cell bodies (Figure 2.6F) and random neurons (probably motor neurons

as identified by their nuclei). The frog provides an interesting model for nerve regeneration studies as the frog is capable of spinal cord regeneration, which is lost following metamorphosis. TSP was present at high levels in the spinal cord of the frog tadpole (2.7 A,B), but was virtually absent from the spinal cord of the adult frog (2.7 C,D).

In the newt spinal cord, TSP was present in the pia mater surrounding the spinal cord and surrounding the cells of the DRG (Figure 2.6G). TSP also surrounds the cells within the gray matter, and was diffusely present throughout the white matter (Figure 2.6H).

Optic Nerve: In the mouse optic nerve, TSP immunoreactivity was virtually absent; a very low level was associated with scattered glial cell bodies. There was a limited amount of TSP in the pia-arachnoid (Figure 2.8A). In the goldfish optic nerve, TSP was present in higher levels than the mouse optic nerve. Its presence appears to be mainly within the connective tissue septae surrounding nerve fascicles as well as in the pia-arachnoid layer (Figure 2.8B). In adult Xenopus, TSP was present in glial cell bodies, diffusely throughout the nerve, and in the pia-arachnoid layer (Figure 2.8C). In the juvenile Xenopus optic nerve, TSP was present at higher levels than the adult nerve, appearing in connective tissue septae (Figure 2.8E) in a pattern similar to that observed in the goldfish. In the newt optic nerve, TSP was found in the pia-arachnoid and throughout the optic nerve (Figure 2.8D).

Sciatic Nerve: In the mouse sciatic nerve, TSP was observed within axons, in the Schwann cell endoneurium, perineurium, and epineurium (Figure 2.8F). The goldfish does not have well defined peripheral nerves that can be isolated for this type of study. There was a great deal of TSP within the adult Xenopus sciatic

nerve, in the endoneurium as well as the epineurium (Figure 2.8G). In the newt sciatic nerve, TSP was present in the epineurium and endoneurium. TSP staining was observed within the neurons, but its appearance was more coarse than in the mouse sciatic nerve (Figure 8H).

Discussion

Thrombospondin is a recently identified 140 kDa trimeric, extracellular matrix molecule that appears to be involved in a wide range of neuronal cell migrations and neurite outgrowth during development (O'Shea and Dixit, 1988; O'Shea *et al.*, 1990; Boyne *et al.*, 1989, 1990, in prep), and that supports neurite outgrowth *in vitro* (Neugenbauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992). Its appearance correlates strongly with adult nerve regeneration *in vivo* (Hoffman *et al.*, 1991, 1990, in prep.). Developmental studies showed that TSP in the central nervous system dramatically decreased at end stages of nervous system development (O'Shea and Dixit, 1988). The distribution of TSP is highly restricted in adult human tissues (Wight *et al.*, 1985), but previously little was known about the expression of TSP in the adult nervous systems. TSP immunoreactivity was present in potentially regenerative systems, such as the goldfish and newt spinal cords; goldfish, Xenopus, and newt optic nerves; and mouse, Xenopus, and newt sciatic nerves (Table 2.3). TSP immunoreactivity was absent in the neuropil of the mouse and Xenopus spinal cords, and the mouse optic nerve. Interestingly, TSP immunoreactivity was present in association with neurons of the brain in all species examined. However, TSP was absent from fiber tracts in the mouse brain, and present in nerve tracts of the goldfish brain, which are capable of regeneration. Therefore, the presence of TSP immunoreactivity in the neuropil or extracellular matrix is correlated with the regenerative potential in adult nervous systems.

The cellular microenvironment within the nervous system is of particular importance in determining the ability or inability of neurons to regenerate following trauma. Grafting of adult peripheral nerve segments into regions of the brain or spinal cord demonstrate that CNS neurons are capable of extending neurites into a permissive environment (David and Aguayo, 1981). However, grafts of adult CNS tissues, such as optic nerves (Politis and Spencer, 1986) or spinal cord (Fernandez *et al.*, 1984), are unable to support neurite growth. Furthermore, the inability of adult CNS tissue to support neurite outgrowth changes with development, as the ability of embryonic CNS tissue to support regeneration is comparable to that of the adult PNS (Tessler *et al.*, 1988). These studies lead to the conclusion that some factor present in the developing CNS, but absent in the adult CNS is required for neurite outgrowth (Liesi, 1985b) or, that some factor absent in the developing CNS is present in the adult CNS and blocks neurite outgrowth (Schwab, 1991; Schwab and Thonen, 1985); or more likely, an interaction of factors leaves the adult CNS non-permissive to neurite outgrowth and neuronal regeneration.

The presence of TSP in the neuropil of the mouse brain was somewhat unexpected, given that most regions of the brain do not support nerve regeneration; however, CNS gray matter will support limited neurite outgrowth (Savio and Schwab, 1989). In addition, CNS neurons undergo an initial sprouting in response to trauma, but this attempt at regeneration is unsucessful and shortlived (Ramon y Cajal, 1928). The absence of TSP reactivity in mouse fiber tracts, in contrast to goldfish fiber tracts, was predicted for a molecule involved in regeneration, given the much greater capacity for nerve repair of the goldfish.

There was a much greater correlation between TSP deposition and regenerative potential in spinal cords than in brains. High levels of TSP in the white matter of the spinal cords correlates with the species ability to support nerve regeneration (Windle, 1956; Clemente, 1964). In particular, the presence of TSP in the ependymal layer is interesting as massive changes in the structure of the ependymal tube proceeds and may guide regenerating neurites following spinal cord trauma in newts (Anderson *et al.*, 1983). This correlation is reinforced by the dramatic decrease in levels of TSP in the frog spinal cord following metamorphosis, but persistence of TSP in the regenerative adult optic nerve.

Like TSP, fibronectin and laminin are present in the developing nervous system. Fibronectin is associated with migrating neural crest cells (Thiery *et al.*, 1982; Le Dourain, 1984; Boucaut *et al.*, 1984) and laminin in regions of neuronal migration within the CNS (Liesi, 1985a). Other ECM molecules such as type IV collagen, glycosaminoglycans, and proteoglycans, while present in the adult CNS (Aquino et al., 1984), have a minimal or inhibitory role in neurite outgrowth (Carbonetto *et al.*, 1983; Manthorpe *et al.*, 1983; Snow *et al.*, 1990a,b). Both TSP (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992) and laminin (Rogers *et al.*, 1983) are potent substrates for CNS and PNS neurite extension *in vitro*, but fibronectin supports neurite extension only in the PNS (Rogers *et al.*, 1983). However, TSP, fibronectin, and laminin are virtually absent in the adult CNS, yet present in the adult PNS. This dichotomy suggests that factors missing from the adult CNS, may support nerve regeneration in the adult PNS.

The ability of TSP to interact with a wide variety of ECM molecules, growth factors, proteases, and protease inhibitors, suggests that TSP may play a role in precisely regulating the structure of local environments following nerve trauma to

control the nature and extent of process extension. Neurite outgrowth is dependant upon protease activity to allow passage of growth cones (McGuire and Seeds, 1990; Pittman *et al.*, 1989). Binding of or adhesion to TSP has been reported to increase the synthesis of plasmin by 40-fold (Silverstein and Nachman, 1987) and protects it from deactivation by matrix protease inhibitors.

This work demonstrates that TSP is expressed in regions capable of undergoing nervous system regeneration following trauma. Previous work has shown that TSP is present in regions of neuronal migration and neurite elongation during development (O'Shea and Dixit, 1988; O'Shea *et al.*, 1990), and will support neurite outgrowth *in vitro* (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991). While it is unlikely that TSP alone is responsible for determining the success or failure of regeneration, its localization in potentially regenerative systems and during regeneration suggest that TSP plays a crucial role in nerve regeneration.

References for Chapter II

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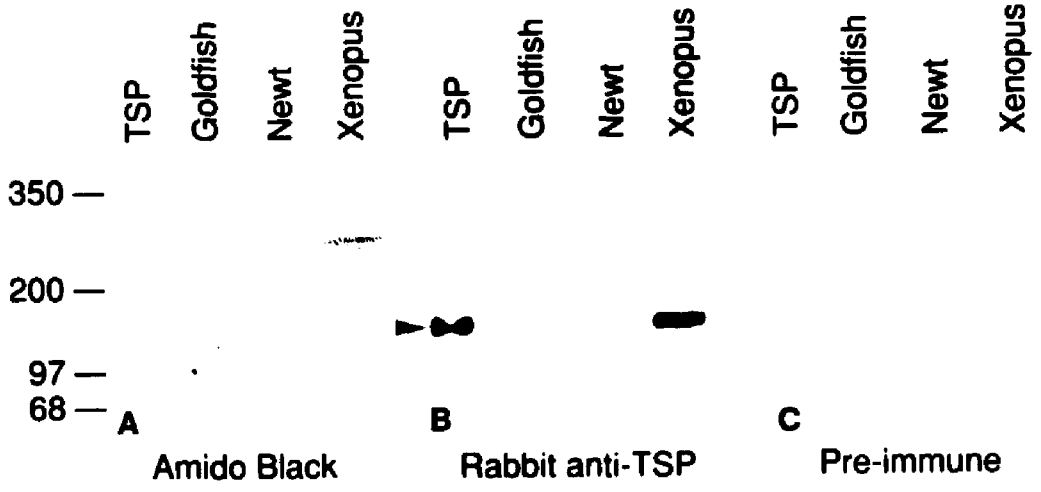

Figure 2.1. Immunoblot illustrating the specificity of the rabbit anti-human TSP antibody. Purified human platelet TSP (lane 1), and whole brain protein from goldfish (lane 2), newt (lane 3), and frog (lane 4) were stained with Amido Black (A), and indicate the specific detection of proteins co-migrating with purified human platelet TSP (arrowhead; B), and lack of immunostaining with pre-immune serum (C).

REGIONS	MOUSE		GOLDFISH		FROG		NEWT	
	neuropil	cells	neuropil	cells	neuropil	cells	neuropil	cells
external plexiform layer	++		+/-		+	+	+	
mitral cell layer	++	+/-	+	-	++	+/-	++	+/-
granule cell layer	+	+/-	++	-	++	+/-	++	+/-
medial pallium/hippocampus	*		+++	+/-	++	++	++	+/-
lateral pallium	N/A		N/A		++	++	++	+/-
subpallium/caudate putamen	-	++	++		++	+/-	++	+/-
thalamus	+	++	N/A		++	+	++	+/-
habenulae	+	++	++	+++	++	+	+	-
hypothalamus	-	++	+	++	+		++	+/-
mammillary region	+/-		++	++	N/A		N/A	
preoptic nucleus	N/A		++	++	++	++	++	+/-
optic tectum/inf. & sup. collicus	+	++	++	++	++	+	+	+/-
molecular layer of cerebellum	+	+	++		++		+	
granule layer of cerebellum	+/-		++	+/-	+++	+/-	++	+/-

Table 2.1. Survey results of levels of TSP immunoreactivity in various brain regions.

-, +/-, +, ++, +++ represent increasing levels of chromogen deposition.
N/A indicates regions not present or not identified.
* results of mouse hippocampus: dentate molecular layer, - ; hilus of dentate gyrus +; granular layer +++.

Table 2.2. Abbreviations of brain regions.

ac	anterior commissure
bs	brainstem
cb	cerebellum
cc	corpus collosum
ce	cerebral cortex
cp	caudate putamen
drg	dorsal root ganglion
e	ependymal layer
epl	external plexiform layer of olfactory bulb
f	fornix
g	glomeruli
gc	granule cell layer of cerebellum
gl	granule cell layer of olfactory bulb
h	hippocampus
ha	habenula
hy	hypothalamus
lp	lateral pallium
ml	molecular layer of cerebellum
mp	medial pallium
mr	mammillary region
nhy	neural hypophysis
ob	olfactory bulb
olt	olfactory tract
ot	optic tract
p	pons
phy	primary lobe of hypophysis
pn	peripheral nerve
po	nucleus preopticus
pvl	periventricular layer
scg	stratum centrale griseum
sm	stria medullaris
so	stratum opticum
sp	subpallium
th	thalamus
tl	torus longitudinalis
to	optic tectum
V	ventricle
wm	white matter of the cerebellum

Figure 2.2. Diagram of a sagittal section of the mouse brain (A, after Sidman et al., 1971). Dashed boxes indicate regions of the corresponding photomicrographs, arrow indicates level of the coronal section. Intense TSP deposition was present in the neuropil of the external plexiform (epl), outlining individual glomeruli (g), and in a concentric pattern within the granule cell layer (gl) of the mouse olfactory bulb (B). There was intense staining restricted to the hippocampal granular layer (arrows; C). In the cerebral cortex there was diffuse immunoreactivity in the deep cortical layers, and absence of immunoreactivity in superficial cortex (ce) and the fiber tracts of the corpus callosum (cc; D). In the mouse cerebellum (E), staining was present in the molecular layer (ml), in cell bodies of Golgi epithelial cells; there was slight staining in the granule cell layer (gl), but none in the white matter (wm). Scale bars = 100 um.

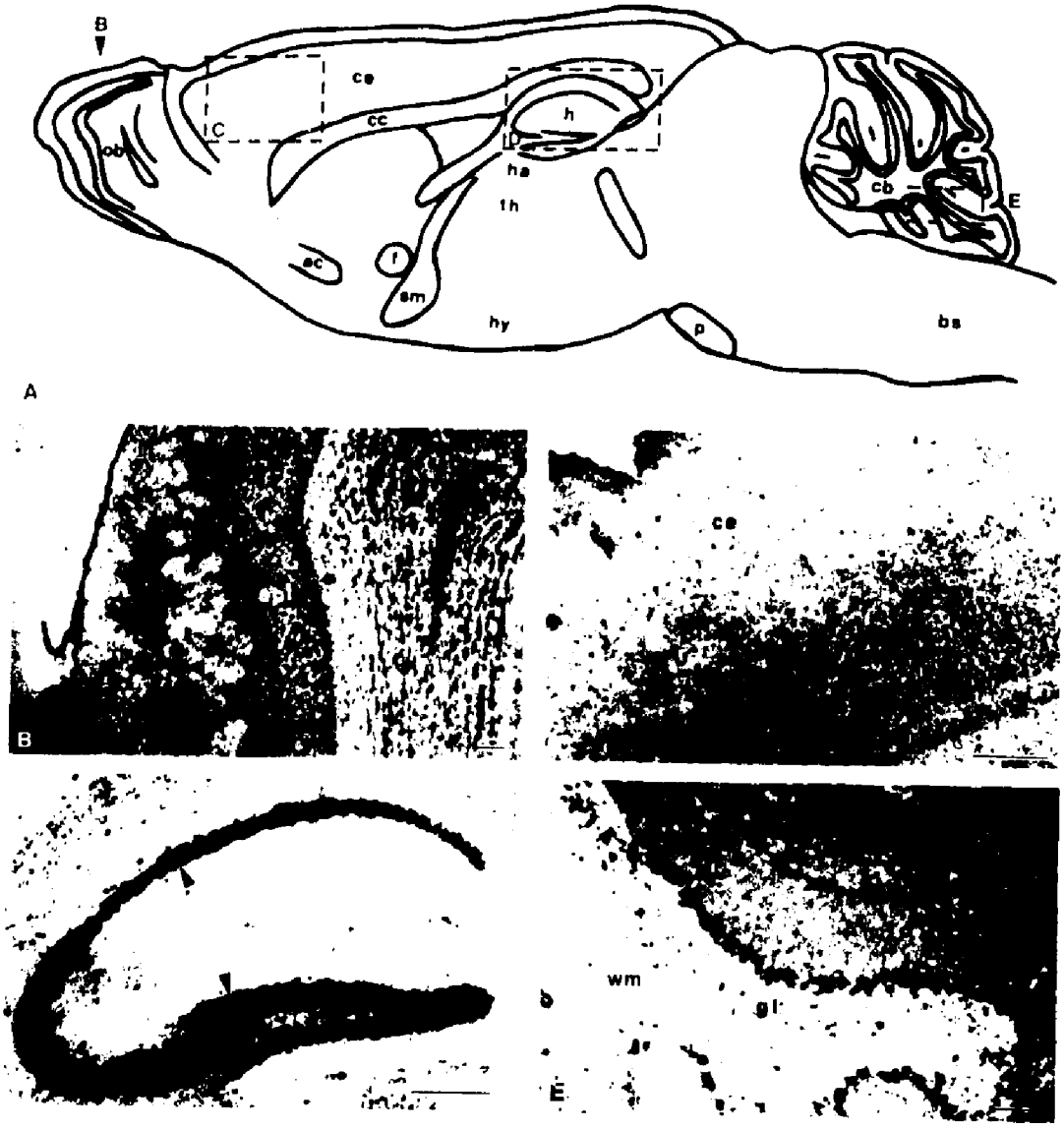

Figure 2.2.

Figure 2.3. Diagram of a sagittal section of a goldfish brain (A, after Romer and Parsons, 1977). The dashed box indicates the region corresponding to photomicrograph (E), arrows indicate the level of coronal sections. (B) Immunostaining was present in the granule cell layer (gl), but not the external plexiform layer (epl) of the goldfish olfactory bulb (B). Intense reaction product was present throughout the hippocampus (h; C). There was intense immunoreactivity in the periventricular layer (pvl) of the optic tectum. Staining was also present in the stratum centrale griseum (scg) and granule cell layer (gc) of the cerebellum (D). (E) Higher magnification view of D illustrating the lack of immunoreactivity in the stratum opticum (so), and cell associated staining in the periventricular layer (pvl). Scale bars equal = 100 um.

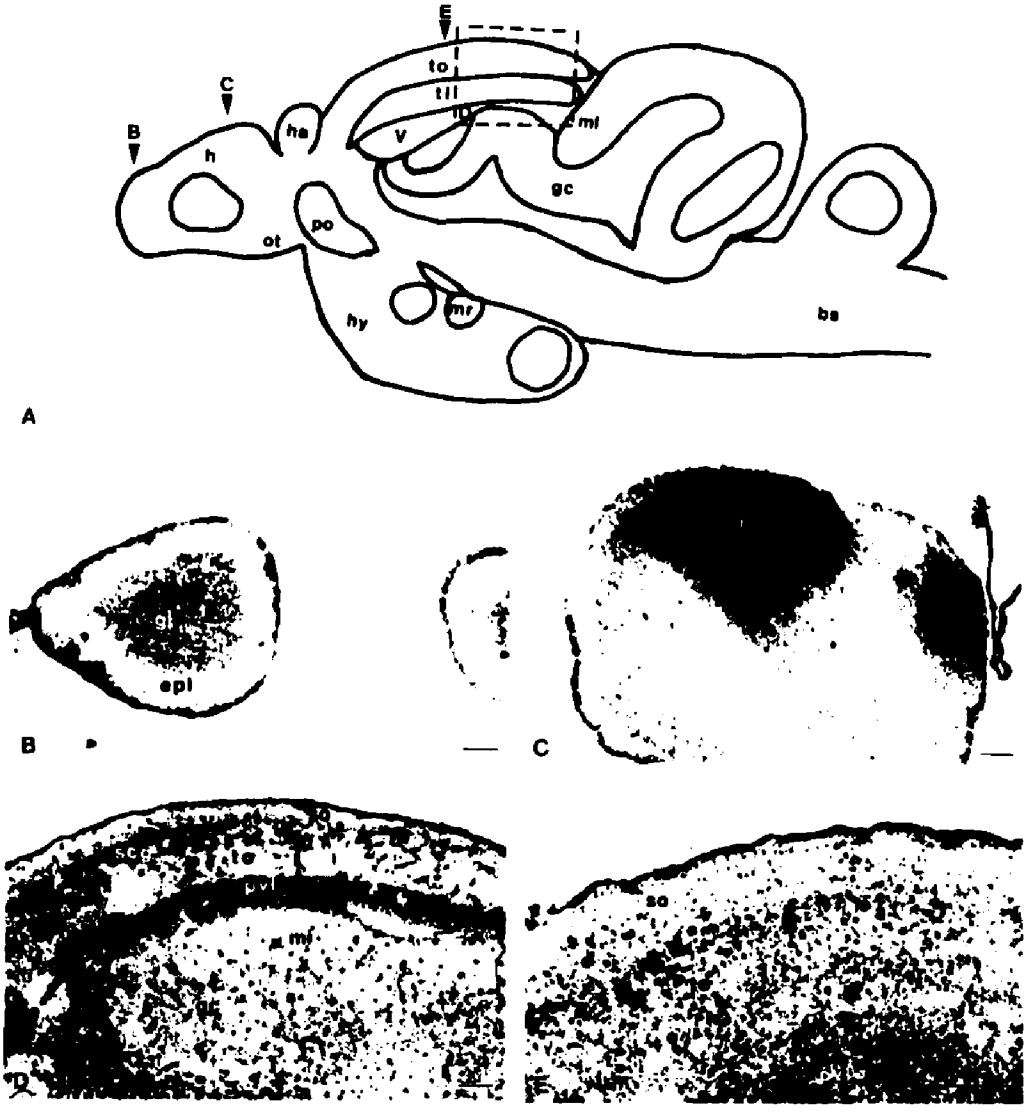

Figure 2.3.

Figure 2.4. Diagram of a sagittal section of adult Xenopus brain (A, after Kemali and Braitenberg, 1971), arrows indicate the level of coronal sections in the photomicrographs below. Immunoreaction product was found in the granule cell (gl) and external plexiform layers (epl) of the olfactory lobe (B). TSP was present in the medial pallium, and more intensely in the lateral pallium (lp; C). It was also present in the periventricular layer (pvl), but not in the stratum opticum (so) of the optic tectum (to; V=ventricle). TSP was densely deposited in the primary lobe of the hypophysis (phy), but not in the neural lobe of the hypophysis (nhy; E). Scale bars = 100 um.

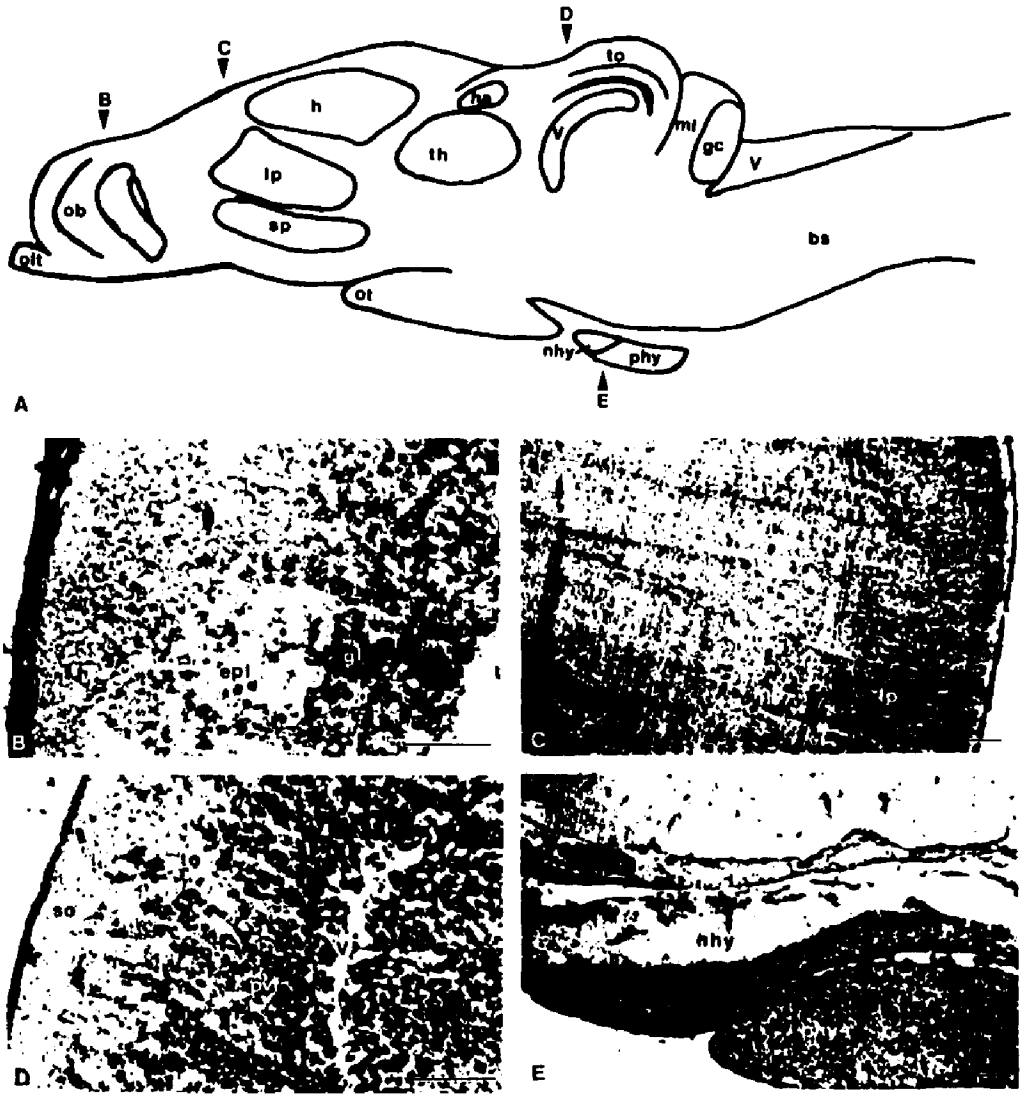

Figure 2.4.

Figure 2.5. Diagram of a sagittal section of a newt brain (A, after Herrick, 1948). Dashed boxes indicate corresponding photomicrographs below, arrows indicate levels of coronal sections. Immunoreaction product was densely deposited in the granule cell layer (gl), with diffuse staining of the external plexiform layer (epl) of the olfactory lobe (B). Anti-TSP reaction product was intense in the subpallium (sp), with less in the white matter (wm; C). TSP was present in the optic tectum (to) and granule cell layer of the cerebellum (cb) and diffusely in the white matter (wm; D). Immunoreactivity was prevalent in the periventricular layer (pvl), and was present at lower levels in the white matter (wm) of the optic tectum (E). V = Ventricle. Scale bars = 100 um.

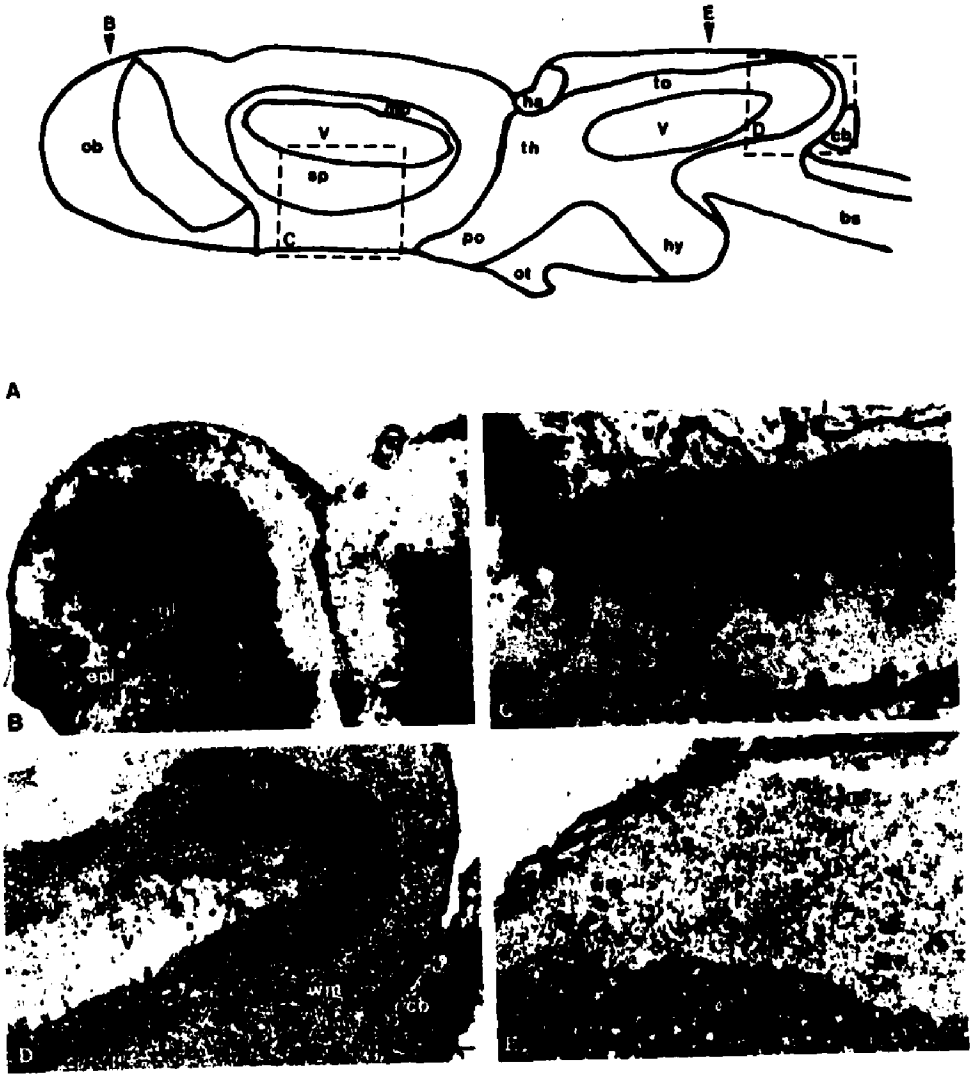

Figure 2.7.

Figure 2.6. Thrombospondin localization in spinal cords using FITC-conjugated secondary antibodies. In a transverse section of mouse spinal cord, TSP immunoreactivity was intensely present in the dorsal root ganglion (drg) and associated peripheral nerve (pn; arrow indicates dorsal; A). Higher magnification of mouse spinal cord illustrating staining in cell bodies of motor neurons (arrow; B). In goldfish spinal cord, TSP was present in the ependymal layer (e), and radial glia cells (arrow; C). Higher magnification of goldfish spinal cord, illustrating staining of motor neurons (arrow; D). Low (E) and higher magnification (F) views of the frog spinal cord, illustrating the presence of TSP in scattered neurons and glial cells. In the newt spinal cord, TSP was present in dorsal root ganglia (drg; G), surrounding cells of the grey matter, and surrounding ependymal cells (e). Higher magnification of G, illustrating the deposition of TSP on ependymal cell surfaces (H). Scale bar in A = 200 um; in B,C,F,G = 100 um; in D,H = 60 um in E = 500 um.

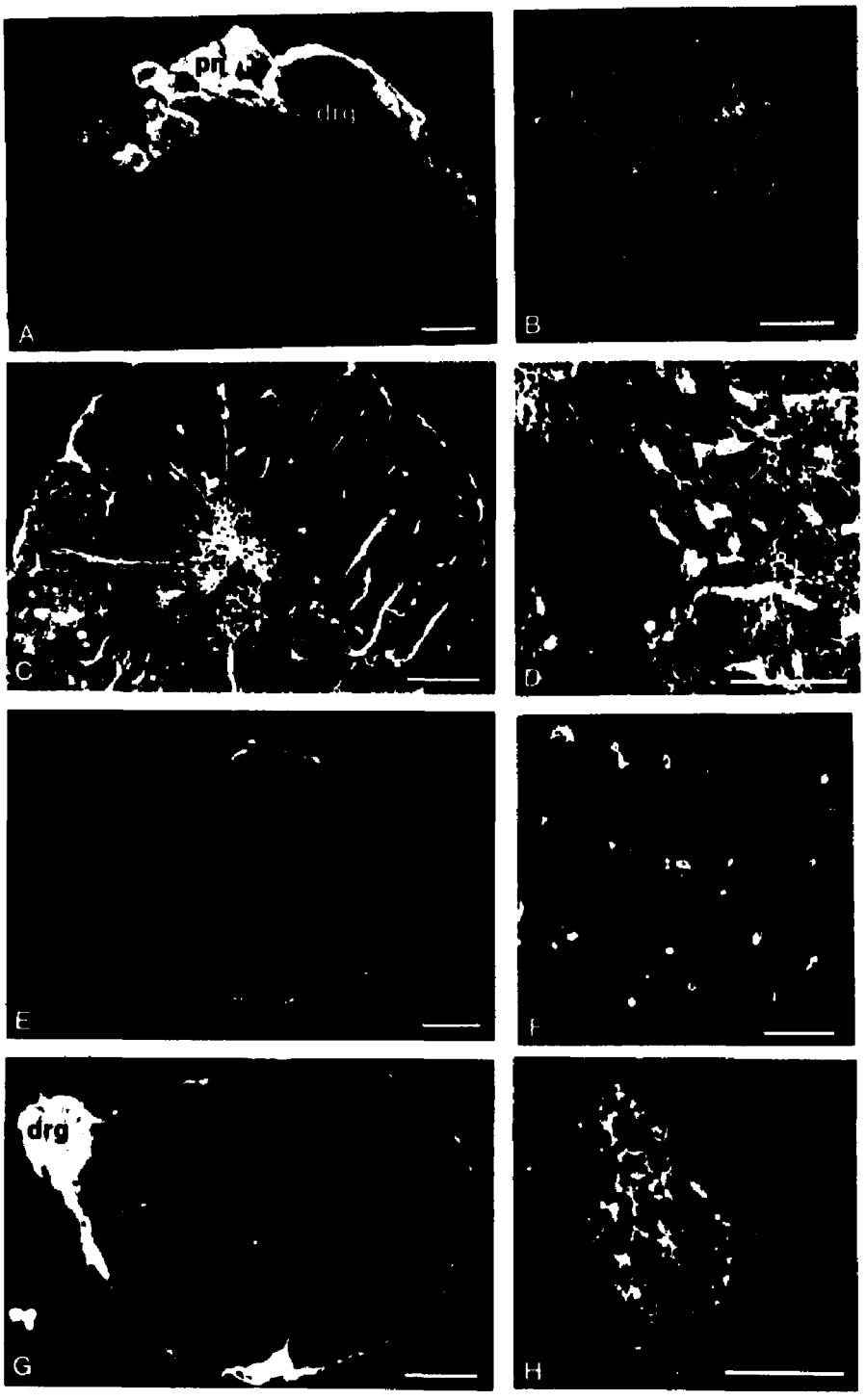

Figure 2.6.

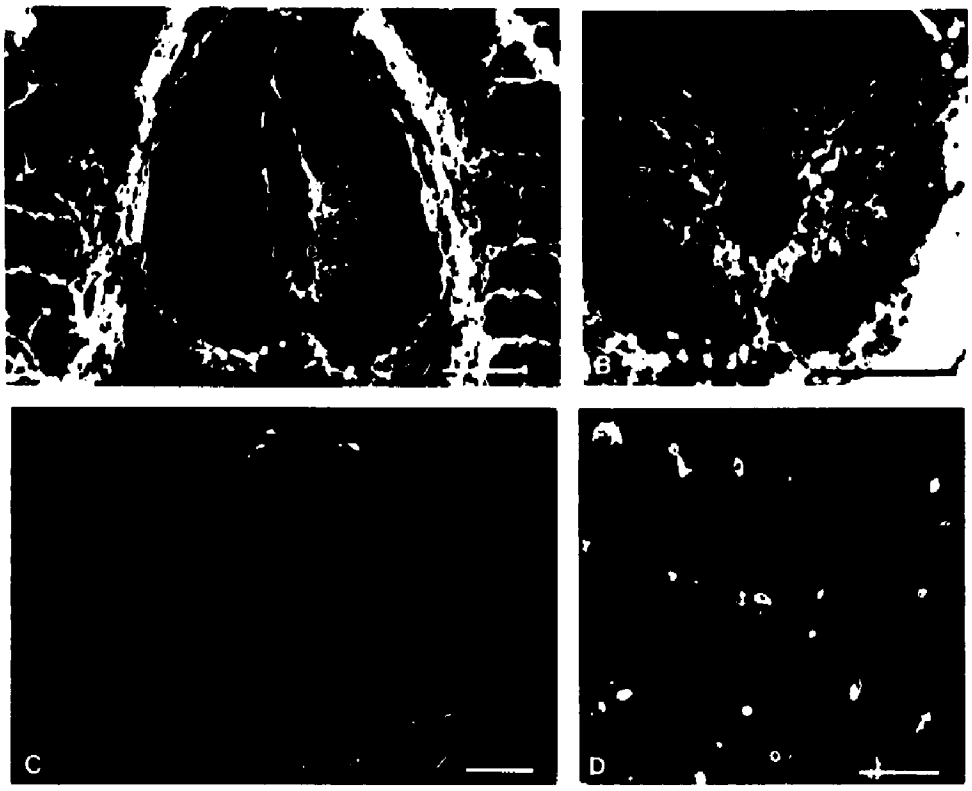

Figure 2.7. Low (A,C) and higher magnification views of the spinal cord of juvenile (A,B) and adult (C,D) Xenopus. In the juvenile spinal cord, thrombospondin immunoreactivity was present throughout both the ependymal layer (e) and in glial fibers (A,B). TSP was virtually absent from the adult spinal cord (C) and was present only in isolated glial cell bodies (D), random neurons, and pia (arrow). The secondary antibody was conjugated to FITC. Scale bars in A,D = 100 um, in B = 60 um ; C = 500 um.

Figure 2.8. Thrombospondin localization in optic and sciatic nerves. TSP was virtually absent from the mouse optic nerve, but was present in the connective tissue pia-arachnoid layer (arrow; A). It was present in glial cells, in the pia-arachnoid layer (arrows), and neuropil of the goldfish (B), adult Xenopus (C), newt (D), and juvenile Xenopus (E) optic nerves. TSP was present in the epineurium, perineurium, and axons of the mouse (F), Xenopus (G), and newt (H) sciatic nerves. Scale bar = 100 um.

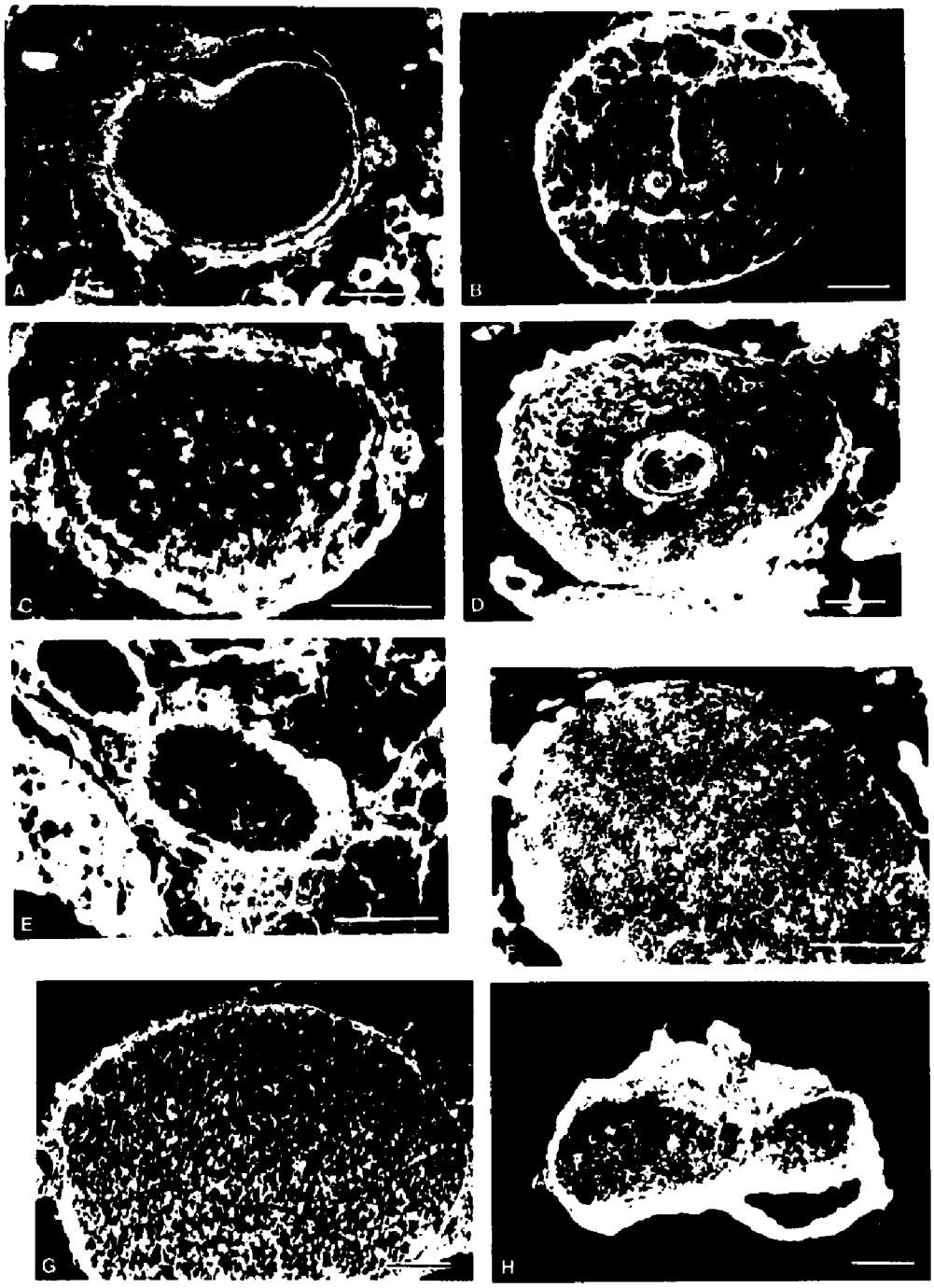

Figure 2.8.

		MOUSE		GOLDFISH		XENOPUS		NEWT	
		TSP	REG	TSP	REG	TSP	REG	TSP	REG
BRAIN	cells	YES	NO*	YES	YES*	YES	VAR**	YES	YES*
	neuropil	YES		YES		YES		YES	
	white	NO		YES		YES		YES	
SPINAL CORD	cells	YES	NO	YES	YES	YES	NO	YES	YES
	neuropil	NO		YES		NO		YES	
	white	NO		YES		NO		NO	
OPTIC NERVE		NO	NO	YES	YES	YES	YES	YES	YES
SCIATIC NERVE		YES	YES	N/A	N/A	YES	YES	YES	YES

Table 2.3 . TSP immunoreactivity in the brain, spinal cord, and optic and sciatic nerves.

N/A indicates region not present; TSP, thrombospondin; REG, regenerative potential.
* indicates regenerative potential of majority of brain regions.
**Xenopus brain regeneration is highly variable between animals (Clemente, 1968).

CHAPTER III

INCREASED THROMBOSPONDIN EXPRESSION IN REGENERATING MOUSE SCIATIC NERVE.

Introduction

Regeneration in the mammalian nervous system is highly dependent upon the environment encountered by a growing neurite. Regeneration occurs in the peripheral nervous system (PNS), but not typically in the central nervous system (CNS); however, CNS axons grow into peripheral nerve segments grafted into lesioned areas of the brain or spinal cord (e.g., David and Aguayo, 1981), illustrating the inherent capability of CNS neurons for outgrowth and regeneration in a suitable environment. A variety of components are likely to contribute to this situation; factors present in the CNS that restrict regeneration may be absent from the PNS, factors present in the PNS that promote regeneration are absent in the CNS, or most likely, a combination of these.

The extracellular matrix (ECM) that surrounds and supports neurons and their processes appears to play an important role in this process. Following nerve crush, the continuity of the Schwann cell columns remains relatively intact, directing regenerating axons toward their original targets. Although there is no predetermined path after nerve transection, axons have been shown to grow preferentially along the inner surface of Schwann cell basal laminae (Ide and Kato, 1990; Ide *et al.*, 1983).

Thrombospondin (TSP) is a 420 kDa trimeric glycoprotein component of extracellular matrices (Dixit *et al.*, 1984) which has been shown to be involved in cell attachment (Varani *et al.*, 1986; Roberts *et al.*, 1987), migration (O'Shea *et al.*, 1990; Majack *et al.*, 1986), neurite outgrowth (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992), and haptotaxis and chemotaxis (Taraboletti *et al.*, 1987) of a variety of cell types. TSP also binds other ECM components including type I collagen (Lahav *et al.*, 1982), type V collagen (Mumby *et al.*, 1984), laminin (Mumby *et al.*, 1984), fibronectin (Lahav *et al.*, 1982, 1984), heparin (Lawler *et al.*, 1978), glycosaminoglycans (Dixit *et al.*, 1984; Lawler *et al.*, 1985), plasminogen (Silverstein *et al.*, 1984), tissue- and urokinase-type plasminogen activators (Silverstein *et al.*, 1986). The distribution of TSP is highly developmentally regulated, appearing with cell migrations and tissue rearrangements (O'Shea and Dixit, 1988); the distribution of TSP is highly restricted in the adult (Wight *et al.*, 1985). In addition, it is densely present in the developing nervous system, becoming largely restricted to the adult PNS; the adult CNS contains considerably less TSP. Its striking pattern of deposition, its association with cell migration in the nervous system, as well as its ability to support neurite outgrowth from a variety of neurons (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992), led us to examine its distribution in the sciatic nerve following various forms of nerve damage.

Materials and Methods

Tissue: Adult female CD-1 mice were anesthesized with Metofane (Pitman-Moore, Washington Crossing, NJ), and the sciatic nerve at the mid-thigh level was exposed, then crushed or transected. Nerves were crushed for 10 seconds using a

#5 forceps, and the crush site examined to ensure that the epineurium was not damaged. Transections were performed by cutting the sciatic nerve with fine iris scissors, and the proximal and distal segments were returned to their normal position. Long term denervation was achieved by ligating 6-0 suture around the nerve proximal and distal to the transection point, and the proximal nerve segment was diverted into the gluteus muscle. In 4 animals, the sutures preventing reinnervation were removed, and the proximal nerve stump was reanastomosed to the distal nerve stump with 7-0 suture after 60 days denervation. The reanastomosed nerves were allowed to regenerate for 10-17 days. Unoperated and sham operated nerves served as controls. Animals were sacrificed by cervical dislocation 1-90 days post-operatively, nerves were removed and rapidly frozen in Optimal Cutting Temperature embedding compound (OCT; Miles Laboratories Inc., Elkhart, IN) in hexane cooled over an acetone-dry ice slurry. Ten micron frozen sections were collected on 0.1% poly-L-lysine coated slides, and stored at 4° C until processing for immunocytochemistry.

Immunocytochemistry: Adjacent sections were washed in PBS, and tissue exposed to normal goat serum (1:20), followed by primary antibody for 2 hours at room temperature. The primary antibodies used were rabbit anti-human TSP (1:40), rabbit anti-neurofilament 200 (NF; 1:100; Sigma Chemicals Co., St. Louis, MO), rabbit anti-laminin (Sigma Chemical Co., St. Louis, MO), rabbit anti-bovine glial fibrillary acidic protein (GFAP; 1:100; Dako Corp., Carpinteria, CA), anti-S100 (1:50; Sigma Chemical Co., St. Louis, MO). Monoclonal antibodies against specific macrophage markers were obtained from hybridoma cell supernatants M3/38 (anti-MAC-2; ATCC TIB 166; Rockville, MD), M3/84 (anti-MAC-3; ATCC TIB 168; Rockville MD), and M1/70 (anti-MAC-1 a-unit; ATCC TIB 128; Rockville MD). Control sections were exposed to PBS, to rabbit serum, or anti-

TSP antibody preabsorbed with TSP bound to sepharose beads which were removed by centrifugation, in place of the primary antibody. The chromosomal dye Hoechst 33342 (0.001% bisBenzimide, Sigma Chemical Co., St. Louis, MO) served as a counter-stain when added to the primary antibody (Bainbridge and Macey, 1983). The specificity of the anti-TSP antibody was previously characterized (O'Shea and Dixit, 1988).

After exposure to the primary antibody, sections were washed in PBS, exposed to the secondary antibody, goat anti-rabbit IgG conjugated to FITC (Cappel, Elkhart, IN; St. Louis, MO) or rabbit anti-rat IgG congugated to FITC (Cappel, Elkhart, IN) for use with M3/38, M3/84, and M1/70, for 30 minutes at room temperature and then washed thoroughly in PBS. Slides were coverslipped with glycerol containing 0.1% phenylenediamine, then were viewed and photographed in a Leitz Aristoplan microscope using a L-1 fluorescence cube for FITC, A1 for Hoechst. Relative levels of TSP immunoreactivity were compared by observing the constant immunoreactivity of TSP-positive endothelial cells of blood vessels in the same tissue section.

Results

Control adult sciatic nerve: At the mid-thigh level, the mouse sciatic nerve runs as two closely related but separate bundles. In sections of control nerve, anti-neurofilament immunoreactivity was present as thick wavy bands. The anti-GFAP immunoreactivity had a similar wavy pattern, but was less intense, except for occasional brightly staining regions associated with Schwann cell processes. Anti-TSP staining also followed the wavy pattern; at nodes of Ranvier TSP followed the

outline of the node (Figure 3.1). Intense anti-TSP immunoreactivity was present in the axon, in the Schwann cell basement membrane/endoneurium, but was absent in the myelin sheath. TSP was also present in the perineurium, epineurium, and surrounding the endothelial cells of the artery accompanying the nerve.

Nerve crush: During the first few days following injury, myelin sheaths begin to break down, Schwann cell tubes collapse, and Schwann cells dedifferentiate and proliferate (Daniloff *et al.*, 1986; Blumcke *et al.*, 1966). This proliferation of Schwann cells was reflected by the increase in anti-S100 staining and increased concentration of Hoechst stained nuclei near the trauma site following all forms of nerve trauma. The number of macrophages also increased in the trauma region, indicated by staining with M3/38 and M3/84. As reported by others (e.g., Neuberger and Cornbrooks, 1989), anti-NF staining appeared similar to controls for 3-7 days following nerve crush injury. After this time, the anti-NF staining pattern distal to the crush site began to fragment and decline. However, thin strands of anti-NF immunoreactivity often ran along the nerve distal to the crush site, possibly indicating rapidly regenerating nerve fibers; or more likely, the presence of nerves that were not damaged by the crush injury. Anti-GFAP immunoreactivity distal to the crush site appeared to increase from the first day following nerve injury, as the bands of high anti-GFAP staining became more numerous. By three days post crush, the nerve was slightly edematous and TSP deposition was irregular. Regions of increased TSP deposition seemed to be correlated with local concentrations of Hoechst stained nuclei. In many instances the levels of TSP reflected the degree of local Wallerian degeneration, with relatively intact regions having less TSP than highly degenerated regions. TSP distal to the crush site increased in a fine reticular pattern associated with the endoneurium. Laminin immunoreactivity was not obviously altered in the endoneurium proximal or distal to the trauma site.

By 7-14 days following crush injury, axons were regenerating past the crush site. Proximal to the crush, NF immunoreactivity was near control levels with bright thick bands, but distally only a few densely staining NF axons had progressed past the crush site. GFAP, which had been localized in a seemingly random pattern distal to the crush, was present at very high levels at the crush site and distally slightly ahead of the thick bands of NF staining. Groups of axons containing dense TSP immunoreactivity at their leading edge crossed the crush site, while distal to the crush site, levels of TSP immunofluorescence in the connective tissue were higher than control levels. The TSP staining appeared to be correlated with both macrophages (M3/38 and M3/84) and Schwann cells (anti-S100). This overall pattern of TSP staining was maintained in the area of axonal outgrowth, as reinnervation proceeded distally. The pattern established during regeneration following nerve crush is nearly identical to that following nerve transection, which is described below.

Nerve Transection: One day after transection, proximal to the transection site the nerve appeared normal, with an occasional empty Schwann cell sheath. However, 3 days following nerve section, in the endoneurium the levels of TSP were lower than controls, with TSP levels near control in the epineurium. A centripetal gradient of TSP immunoreactivity developed, as more central (deeper) regions had slightly higher levels of TSP than those located more peripherally (superficial). By 7 days, TSP levels had greatly increased above control in the endoneurium distal to the transection site as well as with axonal outgrowth. In the same region, there were small deposits of NF, while increased GFAP immunoreactivity was present in randomly located clumps.

Regenerating axons were entering the distal stump by 14 days. TSP staining was increased proximal to the region of nerve regeneration and also just in front of the wave of ingrowing axons (Figure 3.2A). Neurites grew preferentially along the inside of the epineurium, which exhibited slightly higher levels of TSP staining than the endoneurium. A locally high concentration of Hoechst stained nuclei (Figure 3.2B) was associated with high levels of TSP and axonal regeneration as indicated by anti-neurofilament staining (Figure 3.2C). In regions of neuronal outgrowth, increased GFAP levels were observed slightly behind the NF staining (Figure 3.2D). The only major differences between nerve crush and nerve transection were the increased rate and success of regeneration following nerve crush and the formation of an intensely TSP positive fibroblastic cap following nerve transection.

By 30 days post nerve section, the axons from the proximal nerve stump had reinnervated the distal nerve segment. TSP levels remained elevated in the scar formed at the cut site and in the distal nerve segment; TSP levels in the proximal nerve segment were beginning to decline toward normal levels. By 60 days after nerve transection, TSP levels (Figure 3.3A) had returned to near control levels in re-innervated regions, as identified by the presence of axoplasmic staining (Figure 3.3C) and GFAP staining (Figure 3.3D). It is interesting to note that TSP (Figure 3.4A) remains high in close proximity to an incompletely innervated region while having returned to normal in the innervated portion (Figure 3.4C), GFAP staining is found primarily in the innervated region (Figure 3.4D). This observation suggests that an interaction between axons and Schwann cells may keep TSP levels elevated in the incompletely innervated region.

Prolonged Denervated Nerve: The method of suturing the proximal and distal ends of the nerve, and diverting the proximal nerve segment into the gluteus muscle was

successful in maintaining an axon-free environment. Anti-NF staining gradually declined following nerve transection, so that 14-21 days post-transection no neurofilament staining was observed in the distal segment. Anti-GFAP staining appeared to increase gradually following transection, as spots of high immunofluorescence became more common, peaking between 14-21 days, after which the frequency of spots with high activity declined. Three days following transection, TSP immunoreactivity, which had a very crisp thin pattern in control nerve (Figure 3.5A), increased in clumps of Schwann cells which appeared to produce very high levels of TSP (Figure 3.5B). These patches of increased TSP in the endoneurium became more common up to 7-10 days when increased levels of TSP were found uniformly throughout the distal nerve environment (Figure 3.5C). TSP levels remained high up to 30 days when the entire cross-section of the nerve, and the endoneurium continued to thicken (Figure 3.5D).

After 60 days denervation, the distal nerve stump atrophied. TSP immunoreactivity had decreased below control levels in the connective tissue with slight, persistent TSP immunoreactivity in the central-most region of the nerve. TSP immunoreactivity remained at near control levels in the perineurium (Figure 3.5E). This pattern of immunostaining had no discernible difference at 60 or 90 days. Substitution of PBS, normal rabbit serum (1:500), or TSP pre-absorbed antibody for the primary antibody resulted in virtually no immunostaining on control or experimental sections (Figure 3.5F).

Reanastomosis of long term denervated nerve: Following reanastomosis, regenerating axons from the proximal nerve stump were able to enter the 60 day long term denervated distal stump. In regions of close proximity to regenerating neurites, levels of TSP were dramatically increased to levels comparable to

uninterrupted reinnervation (Figures 3.6A, 3.6C). However in areas of the distal stump away from regenerating neurites, TSP levels remain low. Anti-GFAP staining remains closely correlated with the regenerating axons (Figure 3.6D).

Discussion

Thrombospondin, one of a group of extracellular matrix molecules present in the adult sciatic nerve, promotes neurite outgrowth *in vitro* (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992). The current report demonstrates the presence of TSP in the axoplasm, axolemma, Schwann cells, endoneurium, and perineurium of the adult mouse sciatic nerve. Following nerve crush or transection, TSP deposition by Schwann cells and macrophages rapidly increased distal to the site of injury, returning to normal levels following successful nerve regeneration. However, if reinnervation was prevented, TSP levels remained high in excess of 30 days, but gradually decline to very low levels by 60-90 days. Reinnervation of the distal nerve stump after 60 days resulted in a re-expression of TSP at levels comparable with normal nerve regeneration, but only in a localized region within several hundred microns of regenerating axons.

These dramatic changes in its distribution indicate that TSP may play an important role in nerve regeneration. Although laminin (Cornbrooks and Neuberger, 1987; Neuberger and Cornbrooks, 1989; Kuecherer-Ehret *et al.*, 1990) and heparan sulfate proteoglycan (Neuberger and Cornbrooks, 1989) are present in the peripheral nerve and promote neurite outgrowth *in vitro*, they do not undergo the significant changes in expression levels observed for TSP. Similar to TSP, N-CAM (Daniloff *et al.*, 1986), and Ng-CAM (Daniloff *et al.*, 1986) have been observed to accumulate following nerve injury. Tenascin/cytotactin, unlike TSP, is

normally not observed in the adult sciatic nerve; however, tenascin deposition increases following nerve injury. TSP, like tenascin (Martini *et al.*, 1990), is found at high concentrations in the fibroblast-rich fibronectin-positive, L1-negative, and often N-CAM-positive (Martini and Schachner, 1988) caps that form over the cut ends of the nerve.

The pattern of TSP expression following nerve injury in the regeneration paradigms examined indicated that TSP may play a crucial role in peripheral nerve regeneration. In the simplest model of nerve trauma and spontaneous reinnervation, TSP deposition increased in association with and distal to axonal regeneration and returned to control levels when reinnervation was completed. TSP is produced in great quantities by both Schwann cells (unpublished data) and macrophages (Jaffe *et al.*, 1985), which become activated following peripheral nerve truama and are crucial for successful regeneration (Brown *et al.*, 1991; Muller and Minwegen, 1987). Using the long-term denervation model, we show that when reinnervation was blocked for greater than 60 days, levels of TSP declined to below control. However, reinnervation of the distal nerve segment after reanastomosis resulted in a re-expression of TSP at very high levels. Since TSP levels increase in advance of NF-positive axons and away from the site of reanastomosis, it is likely that regenerating axons signal Schwann cells to produce TSP.

Consistent with the findings that TSP is involved in neuron migration in the embryo and neurite outgrowth *in vitro*, TSP appears to also play a role in axonal regeneration *in vivo*. The increased deposition of TSP following loss of axonal contact may create an environment to encourage axonal outgrowth across the

damaged zone. Further work on the role of this important ECM molecule in axonal guidance should begin to elucidate that role.

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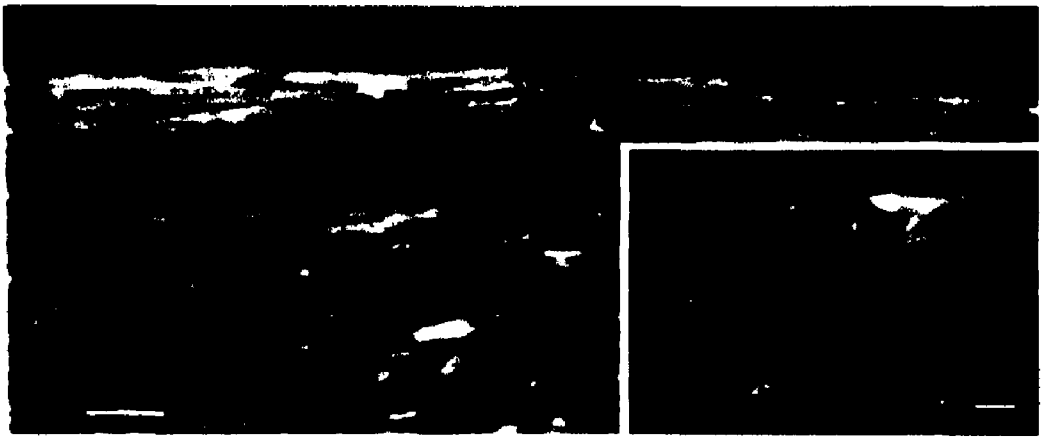

Figure 3.1. In sagittal section of the control sciatic nerve, thrombospondin is present in axons, in the endoneurium, perineurium, and at nodes of Ranvier (arrowheads in inset). Scale bar = 50 um, inset scale bar = 10 um.

Figure 3.2. Immunocytochemical localization of thrombospondin (A), Hoechst 33342 stained nuclei (B), neurofilament protein (C), and glial fibrillary acidic protein (D) in a region of axonal regeneration (arrowheads in C) in sagittal sections of a sciatic nerve 14 days after transection. In addition to staining immediately in front of the wave of regenerating axons, TSP is present at high levels in muscle (m), the perineurium (p) and endoneurial sheaths (arrows). Scale bar = 100 um.

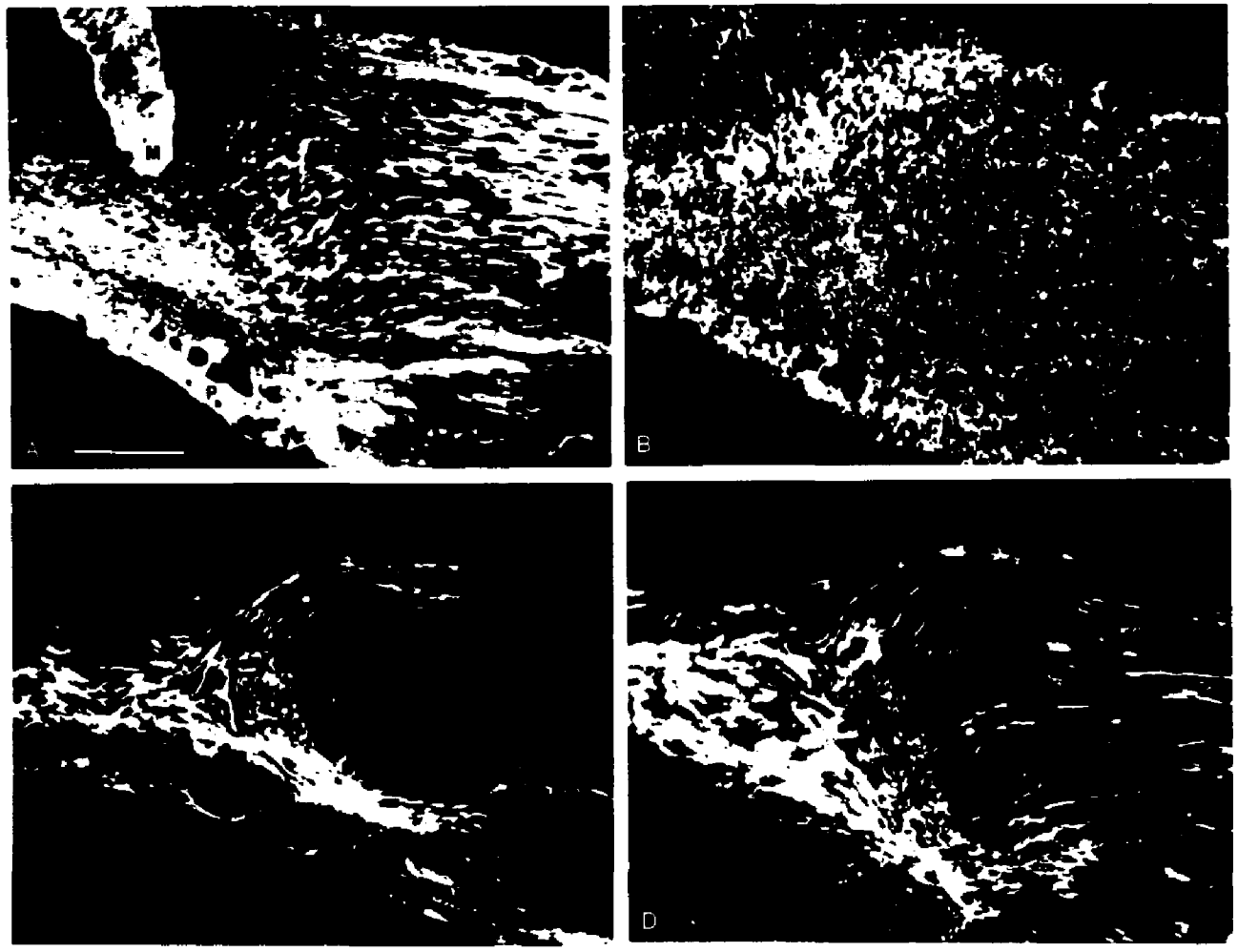

Figure 3.2.

Figure 3.3. Longitudinal section illustrating successful regeneration sixty days after nerve transection. Thrombospondin (A), neurofilament protein (C), and glial fibrillary acidic protein (D) levels are returning to near control. There was a persistant increase in the number of Hoechst stained nuclei (B), but this was decreased considerably compared to that observed early in the phases of regeneration. Scale bar = 100 um.

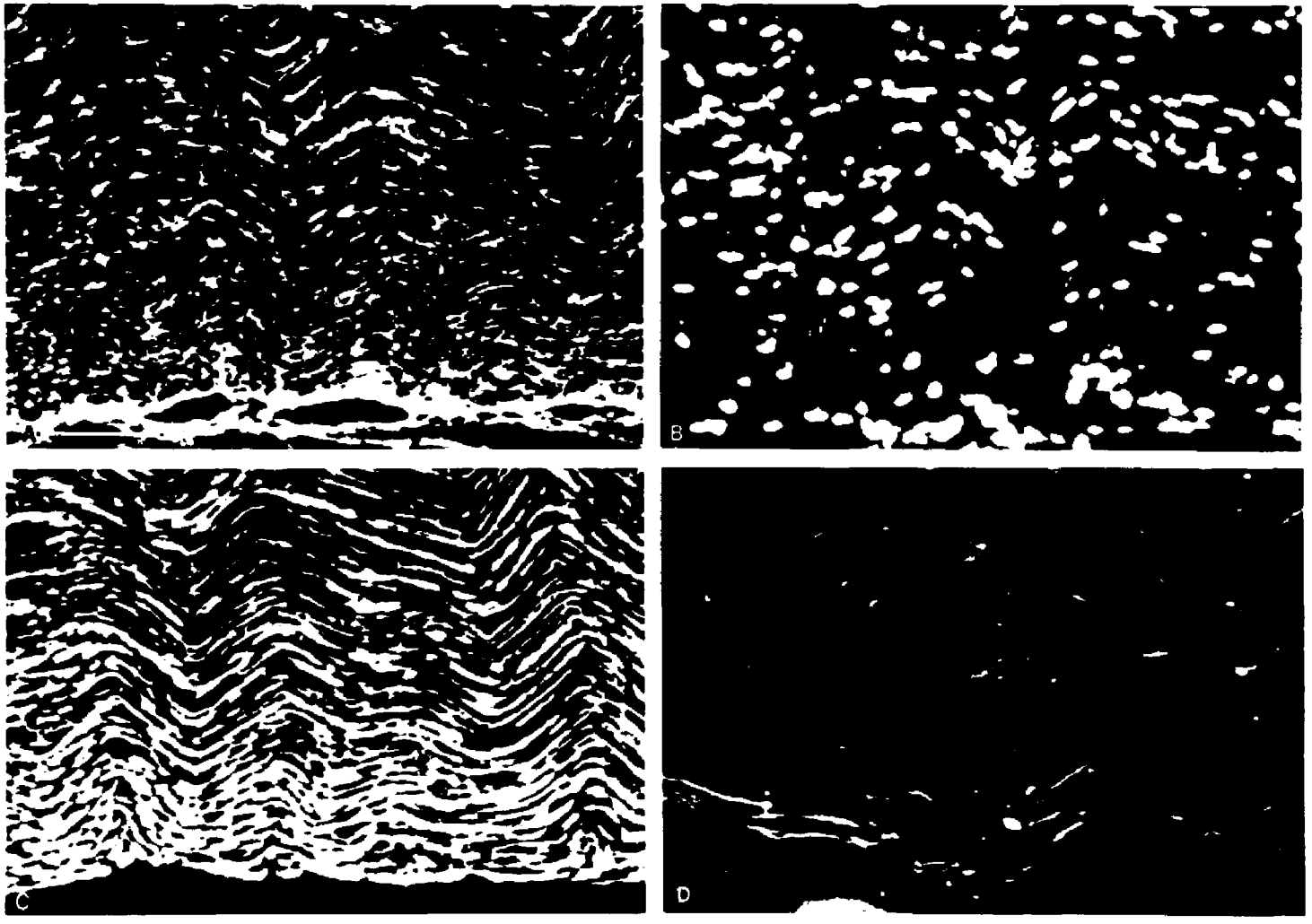

Figure 3.3.

Figure 3.4. Longitudinal section of a sciatic nerve 60 days after nerve transection. Levels of TSP (A), NF (C), and GFAP (D), as well as concentration of nuclei (B) are returning to normal indicating completion of reinnervation (see upper left of each micrograph). However reinnervation in this field is not complete, as indicated by the disorganized pattern of NF (C, lower right). In this highly cellular region, TSP remains high and largely GFAP-negative. Scale bar = 100 um.

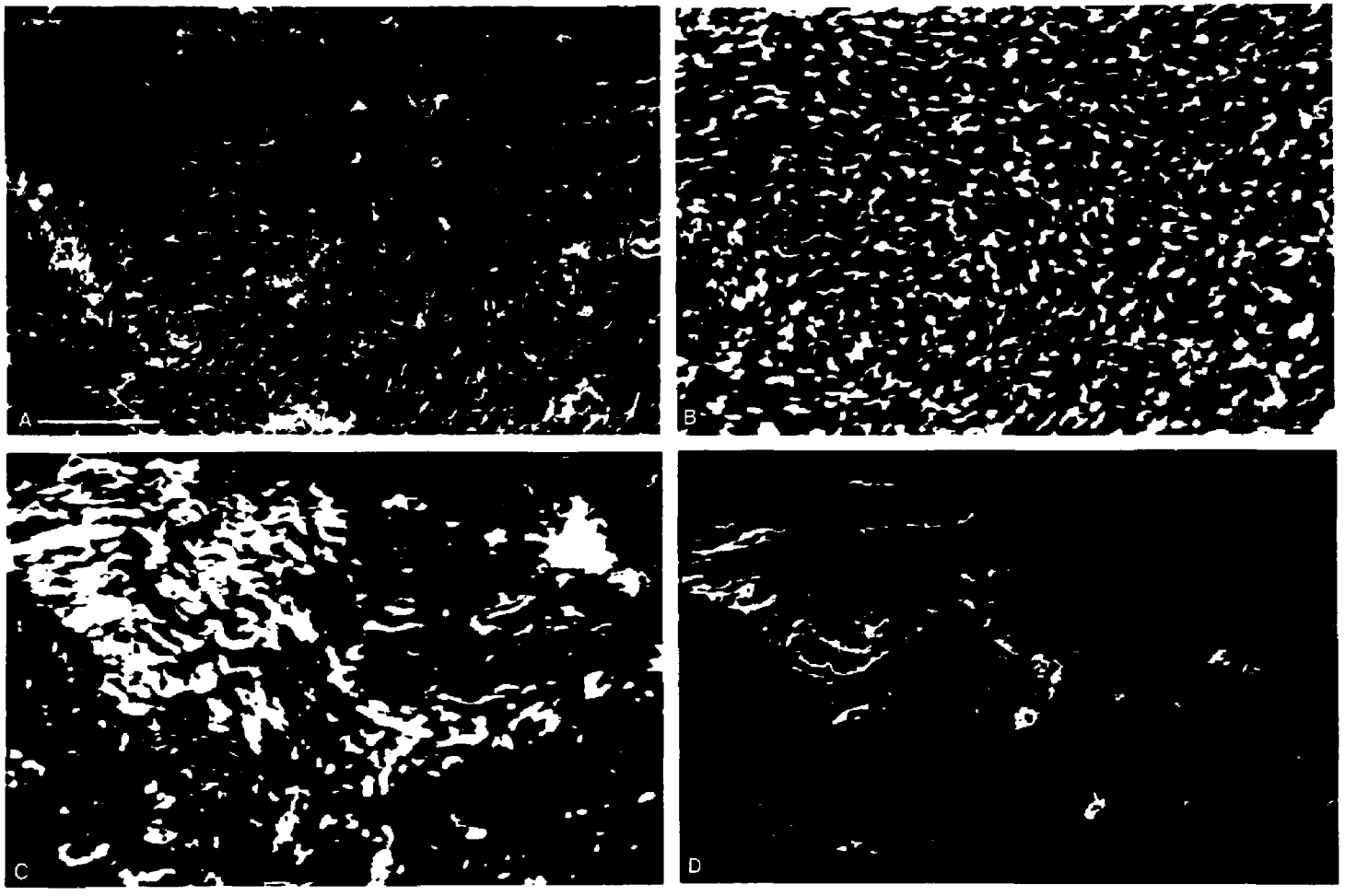

Figure 3.4.

Figure 3.5. Immunocytochemical localization of thrombospondin in cross-sections of control sciatic nerve (A), distal nerve stump after 1 day (B), 7 days (C), 28 days (D), and 60 days (E) following nerve section and blocked reinnervation, and a control section in which the primary antibody was replaced by preimmune serum (F). Inset A illustrates the presence of TSP in the axolemma (arrowhead) and lack of TSP within the myelin (arrow). Arrowheads in B and C indicate local regions of increased TSP; p = perineurium. Scale bar = 50 um.

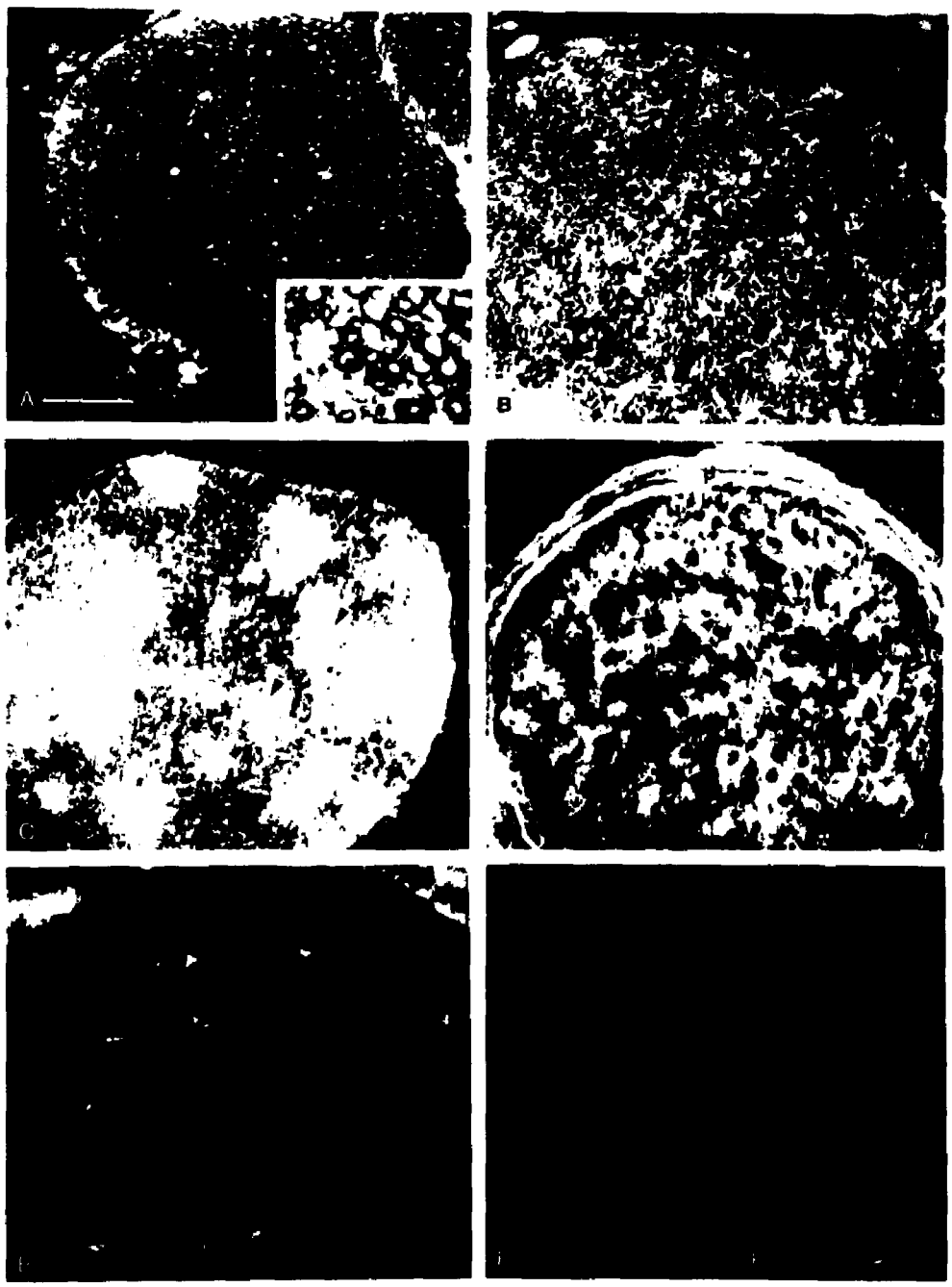

Figure 3.5.

Figure 3.6. Ten days following reanastomosis, TSP levels (A) are observed to be increased distal to the anastomosis site. This increase is highly correlated with the presence of regenerating neurofilament-positive axons (C) and GFAP expression (D). The number of Hoechst stained nuclei (B) remains high at this early stage of reinnervation. Scale bar = 100 um.

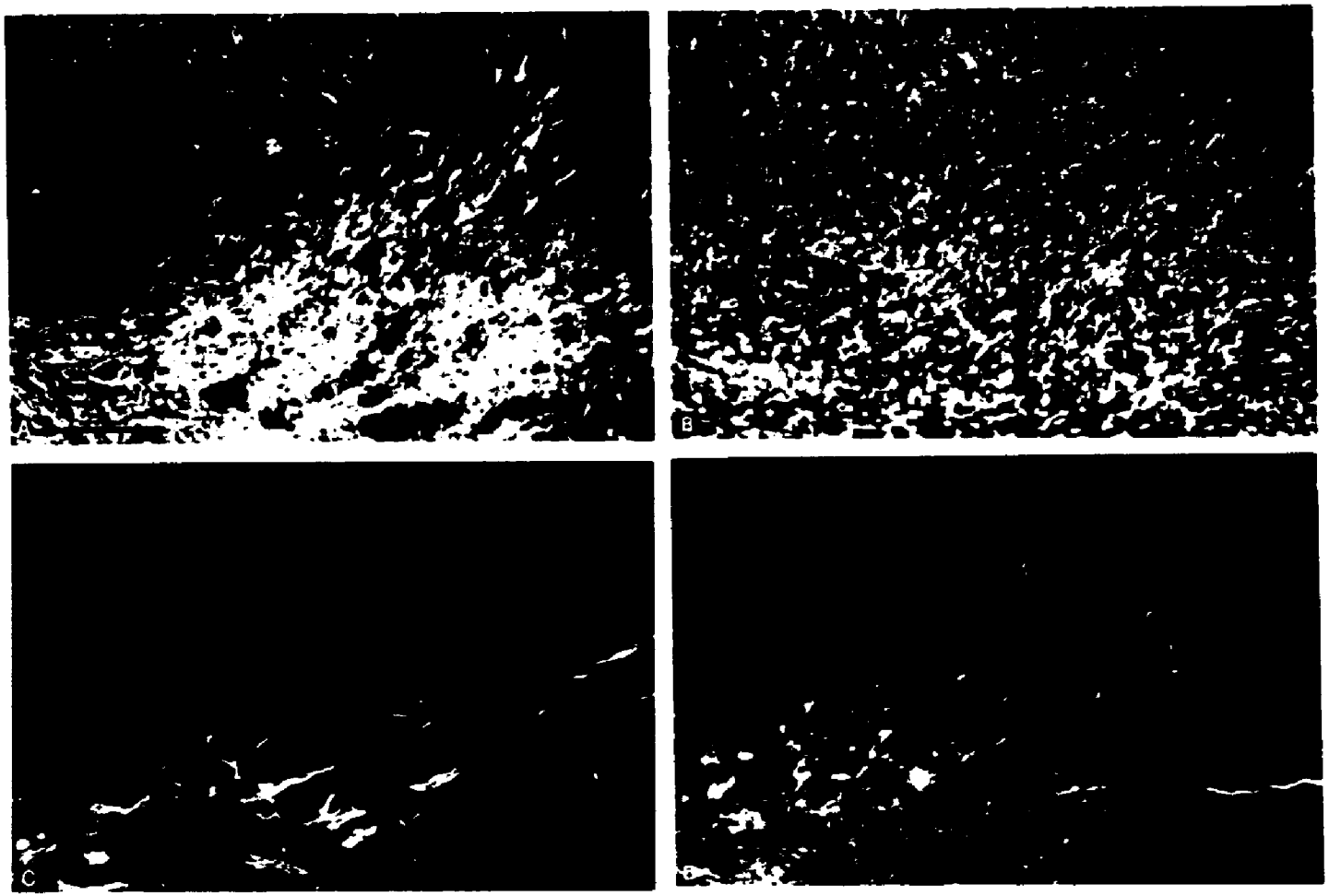

Figure 3.6.

CHAPTER IV

INCREASED THROMBOSPONDIN EXPRESSION FOLLOWING OPTIC NERVE CRUSH IN THE GOLDFISH, BUT NOT IN THE MOUSE.

Introduction

Unlike the goldfish optic nerve, the mammalian optic nerve does not regenerate following injury (Cankovic, 1968; Murray, 1976), but mouse retinal ganglion cells can regenerate if a suitable environment is available. However, the neurons whose axons form the mouse optic nerve (the retinal ganglion cells) extend neurites through peripheral nerve segments grafted into the optic nerve (So and Aguayo, 1985; Fukuda *et al.*, 1990). Conversely, peripheral nerve axons which show robust regeneration are unable to regenerate through optic nerve segments grafted into the sciatic nerve (Politis and Spencer, 1986; Hall and Kent, 1987). In addition, sections of goldfish optic nerve are capable of supporting neurite outgrowth *in vitro*, unlike those from mouse optic nerve (Bastmeyer *et al.*, 1991; Savio and Schwab, 1989; Carbonetto *et al.*, 1987). Similarly, oligodendrocytes (Caroni and Schwab, 1988a,b; Bastemeyer *et al.*, 1991), myelin (Bastemeyer *et al.*, 1991), and ECM molecules (Liesi, 1985; Hopkins *et al.*, 1985) support neurite outgrowth in the goldfish, but not in the mouse. This suggests that some factor(s) necessary to support regeneration is/are present in the goldfish but are absent in the mouse optic nerve; or that factor(s) which inhibit regeneration are present in the mouse and absent in the goldfish optic nerve; or, most likely a combination of factors interact to determine the suitability of an environment for regeneration.

Thrombospondin (TSP) is a 420 kDa trimeric glycoprotein component of extracellular matrices (Dixit *et al.*, 1984) and is involved in cell attachment (Varani *et al.*, 1986, Roberts *et al.*, 1987), cell migration (O'Shea *et al.*, 1990; Majack *et al.*, 1986), neurite outgrowth (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992), and haptotaxis and chemotaxis (Taraboletti *et al.*, 1987) of a variety of cell types. TSP binds other ECM components including type I collagen (Lahav *et al.*, 1982), type V collagen (Mumby *et al.*, 1984), laminin (Mumby *et al.*, 1984), fibronectin (Lahav *et al.*, 1984; Lawler *et al.*, 1985), plasminogen (Silverstein *et al.*, 1984), and tissue- and urokinase-type plasminogen activators (Silverstein *et al.*, 1986). Therefore TSP potentially has the ability to organize the extracellular matrix. The pattern of TSP expression in the embryonic nervous system is highly developmentally regulated, appearing in association with neural crest cell migration (Boyne *et al.*, 1989), axonal outgrowth, and in the migration of cerebellar granule cells (O'Shea *et al.*, 1990). Based on evidence that TSP appears to be involved in regeneration of the mouse sciatic nerve (Hoffman *et al.*, 1990, submitted), this study examines the deposition of TSP in the varying degrees of regeneration observed following injury to mouse and goldfish optic nerves.

Materials and Methods

Tissue: Adult male CD-1 strain mice (Charles River, Portage, MI) were anesthetized with methoxyfluorane (Metofane; Pitman-Moore, Washington Crossing, NJ), 5-10 cm goldfish were anesthetized using MS-222. In both animals, one eye was retracted and the optic nerve was crushed within 3 mm of the back of the eye for 20 seconds with curved #7 forceps. Following surgery, the goldfish were maintained in water with 10g NaCl/gallon at 19°C. Sham operations, in which the eye was retracted but the nerve was not crushed, were also performed. At

least 5 animals per group were examined at specific time points from 1 to 60 days. Mice were sacrificed by cervical dislocation, goldfish were anesthetized in ice water and the cervical spinal cord was cut. The optic nerve was cut in the region of the optic chiasma, the eye and optic nerve were removed and frozen intact in Optimal Cutting Temperature embedding media (OCT; Miles Laboratories, Elkhart IN), in hexane cooled over an acetone-dry ice slurry. Ten micron frozen sections were collected on 0.1% poly-lysine coated slides, and stored at $4^{o}C$ until processing for immunocytochemistry.

Immunocytochemistry: Adjacent sections were washed in phosphate buffered saline, exposed to normal goat serum (1:20), followed by: rabbit anti-human TSP (O'Shea and Dixit, 1988), rabbit anti-neurofilament 200 (1:100, NF; Sigma Chemical Co., St. Louis, MO), rabbit anti-laminin (1:50, LN; Sigma Chemical Co., St. Louis, MO), rabbit anti-cow glial fibrillary acidic protein (1:100, GFAP; Dako Corp., Carpinteria, CA), rabbit anti-galactocerebroside (1:20, GALC; Chemicon Int'l., Temecula, CA). Monoclonal antibodies against specific macrophage markers were obtained from from hybridoma cell supernatants M3/38 (anti-MAC-2; ATCC TIB 166; Rockville, MD), M3/84 (anti-MAC-3; ATCC TIB 168; Rockville, MD), M1/70 (anti-MAC-1 a-unit; ATCC TIB 128; Rockville, MD), or commercially MRC OX-42 (anti-rat macrophages; Bioproducts for Science, Indianapolis, IN). Sections were exposed to primary antibodies for 2 hours at room temperature. In many cases the nuclear stain Hoechst dye (0.001% bisBenzimide, Sigma Chemical Co., St. Louis, MO) was added to the primary antibody. In place of primary antibody control sections were exposed to PBS, pre-immune rabbit serum, or anti-TSP antibody preabsorbed with TSP bound to sepharose beads, which were removed by centrifugation. Sections were washed in PBS, exposed to FITC conjugated goat anti-rabbit IgG (Sigma Chemicals, St. Louis, MO) for 30 minutes at room

temperature, and then rinsed with PBS. Slides were coverslipped with glycerol containing 0.1% phenylenediamine, then viewed and photographed in a Leitz Aristoplan microscope using a L-1 fluorescence cube to visualize FITC and an A1 cube for Hoechst staining of the same section.

Results

Thrombospondin immunoreactivity was virtually absent in the normal adult mouse optic nerve, but present in the adult goldfish optic nerve. Slight TSP immunoreactivity was found in association with a small population of glial cells (Figure 4.1A); however, TSP levels were high in the pia-arachnoid layer surrounding the optic nerve. Anti-GFAP and anti-NF immunofluorescence was present throughout the optic nerve (Figure 4.1E,G), while anti-laminin staining was restricted to the pia-arachnoid layer and vascular endothelium (not shown). In contrast, TSP was present diffusely throughout the goldfish optic nerve (4.1B), and concentrated in connective tissue septa surrounding nerve fascicles and in the pia-arachnoid layer. Anti-GFAP and anti-NF staining of the goldfish optic nerve (Figures 4.1F,H) exhibited a pattern of staining that was similar to that of the mouse optic nerve. The anti-GFAP staining observed in the goldfish is likely due to non-specific binding of intermediate filaments, since the pattern observed was identical to that observed with anti-vimentin antibodies.

Seven days following optic nerve crush, in both systems, the expression of immunostaining had changed drastically. Throughout the mouse optic nerve, TSP levels had increased slightly. Very high levels of TSP (Figure 4.2A) were also found in regions of increased density of cell nuclei near the site of trauma (Figure 4.2C). Often these areas of high TSP expression were GFAP negative (Figure

4.2E). At this stage, neurofilament protein distal to the nerve crush was being degraded, while NF staining proximal to the crush site remained intense (Figure 4.2G). In contrast, in the goldfish optic nerve, TSP deposition had increased dramatically, and was present in a very distinct fasicular pattern (Figure 4.2B). There was an obvious increase in the number of Hoechst stained nuclei throughout the goldfish optic nerve, but there were no clumps of cells producing TSP as observed in the mouse (Figure 4.2D). Anti-GFAP staining appeared to be increased throughout the goldfish optic nerve (Figure 4.2F), while high levels of anti-NF staining appeared to proceed in a proximal to distal pattern, indicating the axonal regeneration occurring in the goldfish optic nerve.

After seven days, in the non-regenerating mouse optic nerve, increased levels of TSP were present in primarily GFAP-negative regions, which also had increased numbers of nuclei. Increased levels of GFAP were found throughout the optic nerve except in the TSP-positive regions, whereas NF staining was only present proximal to the crush site. In contrast, TSP was present in a fascicular pattern in the regenerating goldfish optic nerve. Overall, GFAP stained throughout the nerve, and increased NF staining proceeded in a proximal to distal gradient of axonal regeneration.

In the mouse, the nuclei-dense, TSP-positive, GFAP-negative region (Figures 4.3A,B,D), was strikingly correlated with the presence of macrophages as indicated by staining with M3/84 or M3/38 (Figure 4.3C). Anti-galactocerebroside-staining oligodendrocytes were present uniformly throughout the optic nerve and the crush region. Unfortunately, we were unable to obtain cell type-specific antibodies that would cross-react with goldfish tissue for similar identifications.

Sixty days following nerve crush, the response of both the mouse and goldfish optic nerve to crush trauma was essentially complete. TSP levels remained high (Figure 4.4A) in regions of increased numbers of nuclei (Figure 4.4C) and in the meningeal layers, but was absent in other regions of the mouse optic nerve. GFAP immunostaining remained high throughout the nerve, except in regions of increased TSP deposition, which remained GFAP-negative (Figure 4.4E). Anti-NF staining distal to the crush site was completely absent, though NF staining of peripheral nerves innervating the extraocular muscles is apparent (Figure 4.4G). In the goldfish, levels of TSP, GFAP, and NF returned to near normal levels (Figures 4.4B,F,H), although the overall morphology was more disorganized than controls, with a dense accumulation of nuclei remaining throughout the nerve (Figure 4.4D).

Discussion

This work demonstrates that the presence of the extracellular matrix molecule thrombospondin correlates with successful optic nerve regeneration. Thrombospondin is absent from control adult mouse optic nerves, but is present at low levels in control goldfish optic nerves. Following optic nerve crush in the mouse, TSP was present in GFAP-negative patches associated with macrophages near the crush site. In contrast, following goldfish optic nerve crush, TSP was dramatically increased in a very organized pattern, associated with the basement membranes along which axons regenerate.

The patches of TSP activity observed following optic nerve crush in the mouse present an interesting dilemma. Thrombospondin present within the mouse optic nerve is associated with regions of dense accumulations of nuclei, which normally are associated with gliosis; however, the thrombospondin enriched regions

are GFAP-negative, indicating that the cells are not astrocytes. In addition, these patches of TSP were strongly correlated with the presence of macrophages.

In agreement with previous studies, macrophages were found almost exclusively at the site of injury (Perry *et al.*, 1987). In contrast, macrophages are found throughout the peripheral sciatic nerve following injury (Perry *et al.*, 1987), and it appears that macrophage activity plays an important role in peripheral nerve regeneration (Brown *et al.*, 1991; Perry and Gordon, 1988). Surprisingly, macrophages are able to modify the normally nonpermissive rat optic nerve, which then will support neurite outgrowth *in vitro* (David *et al.*, 1990). However, the permissive region observed following rat optic nerve transection, while being ED1-positive (a rat macrophage marker), was also GFAP-positive, indicating the presence of astrocytes; in contrast, following optic nerve crush in the mouse, the macrophage region was GFAP-negative. When sections of normal adult rat optic nerve were cultured with mononuclear phagocytes or medium conditioned by activated peritoneal macrophages, they became capable of supporting neurite outgrowth *in vitro* (David *et al.*, 1990). This could be a result of removal or inactivation by macrophages of an inhibitory factor, or alternatively, deposition of a neurite promoting factor. Since TSP is among the products of activated, but not inactivated macrophages (Jaffe *et al.*, 1985) it might interact with the tissue sections to provide a permissive environment for neurite outgrowth. The ability of TSP to support neurite outgrowth *in vivo* after mouse optic nerve crush is unknown, as following degeneration, the axons do not reach the TSP-positive region.

A variety of cellular and extracellular factors in the goldfish support nerve regeneration or neurite outgrowth *in vitro*. The initial regenerating axons are found in association with astrocytic processes along the collagenous connective tissue

septae (Wolpurg, 1981), where laminin has been localized (Hopkins *et al.*, 1985). The presence of TSP in this same region, suggests that TSP is available as a substrate for regenerating axons. Following crush injury, mature oligodendrocytes are prevalent in the rat, while immature oligodendrocytes are present in the goldfish optic nerve (Sivron *et al.*, 1990). *In vitro* assays have shown that rat oligodendrocytes are non-permissive for neurite outgrowth, but goldfish oligodendrocytes support some neurite outgrowth (Caroni and Schwab, 1988a,b; Bastemeyer *et al.*, 1991). The presence of multiple neurite promoting factors, such as thrombospondin, laminin, and immature oligodendrocytes, in the goldfish, and their absence in the mammalian optic nerve suggests that these factors are important in regeneration.

While TSP appears to be associated with nerve regeneration, it has not been determined which of several possible roles TSP may play in this critical process. The heparin-binding domain of TSP may act as a chemotrophic factor as it is cleaved when TSP is inserted into the extracellular matrix (Majack *et al.*, 1985). In addition, TSP has cell binding activity for a variety of cell types in discrete domains (Prater *et al.*, 1991; Lawler *et al.*, 1988; Roberts *et al.*, 1987; Murphy-Ullrich and Mosher, 1987). Thrombospondin is also a potent substrate for neurite outgrowth *in vitro* (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992), as having the potential to organize the matrix by binding other extracellular matrix molecules such as laminin (Mumby *et al.*, 1984) and fibronectin (Lawler *et al.*, 1985; Lahav *et al.*, 1984) which support neurite outgrowth *in vitro*. TSP also binds fibrinogen, plasminogen (Silverstein *et al.*, 1984), tissue- and urokinase-type plasminogen activators (Silverstein *et al.*, 1986), and binding by TSP increases the synthesis of plasmin by 40-fold (Silverstein and Nachman, 1987). The ability to act

as both a substrate and a center of localized proteolysis may allow TSP to direct neurite outgrowth through a cell and extracellular matrix filled environment.

Consistent with the observation that TSP deposition increases during regeneration of mouse peripheral nerve (Hoffman *et al.*, 1990), TSP expression increased throughout the regenerating goldfish optic nerve following crush injury. Conversely, in the non-regenerating mouse optic nerve, TSP was present only in regions of concentrated macrophages. The presence of TSP along the path of regenerating goldfish retinal ganglion cell axons indicates that TSP may participate with other ECM molecules to direct goldfish optic nerve regeneration. The lack of regeneration following optic nerve crush in the mouse may be the result of the absence of organized tracts of TSP (and other ECM molecules) and the presence of possible inhibitory factors.

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Figure 4.1. Longitudinal sections of control adult mouse (A, C, E, G) and goldfish (B, D, F, H) optic nerves, in all photomicrographs the retina is to the left. In the mouse optic nerve, TSP (A) is present only in the pia-arachnoid layer (p), nuclei are regularly spaced (C), and GFAP (E) and NF (G) are present throughout the optic nerve. In the goldfish optic nerve, TSP (B) is present in the pia-arachnoid layer (p) as well as throughout the optic nerve, including the connective tissue septae. The goldfish nuclear pattern (D), GFAP (F), and NF (G) expression are similar to those of the mouse optic nerve. Scale bar = 100 um.

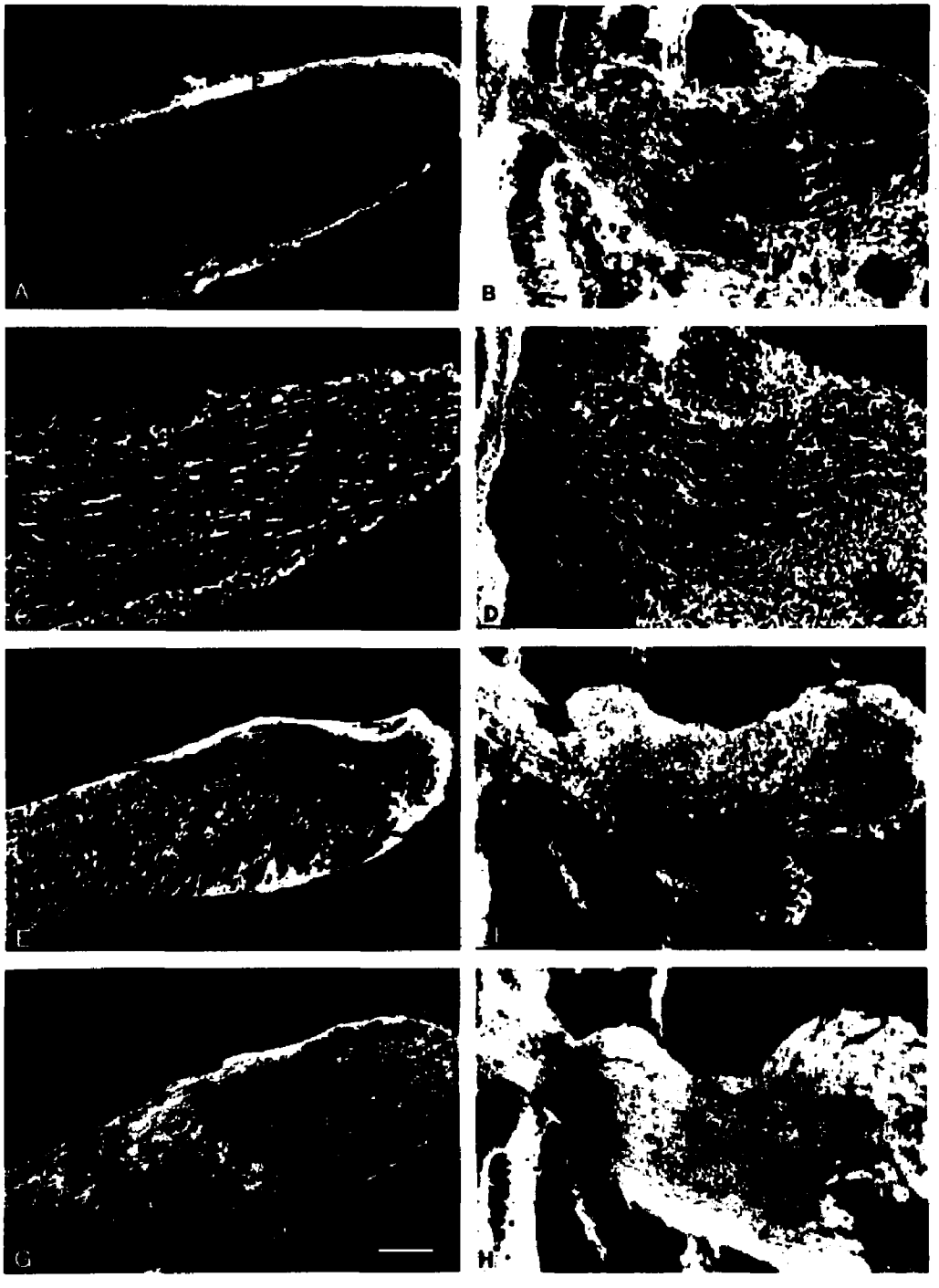

Figure 4.1.

Figure 4.2. Mouse (A,C,E,G) and goldfish (B,D,F,H) optic nerves seven days after crush injury, illustrating the localization of thrombospondin (A,B), Hoechst stained nuclei (C,D), glial fibrillary acidic protein (E,F), and neurofilament protein (G,H). In the mouse optic nerve, TSP is present in a region of accumulation of nuclei (A,C; arrowheads), which is GFAP-negative (E; arrowhead). Anti-NF staining is decreased distal to, but persists proximal to the crush site (G). In the goldfish optic nerve, TSP is increased to very high levels along connective tissue septae (double arrowhead in B). There is a slight overall increase in number of nuclei with no apparent clumping (D). GFAP immunoreactivity (F), reflecting intermediate filaments, appears to be increasing, while neurofilament staining (H) is increasing in the region of neurite outgrowth. Scale bar = 100 um.

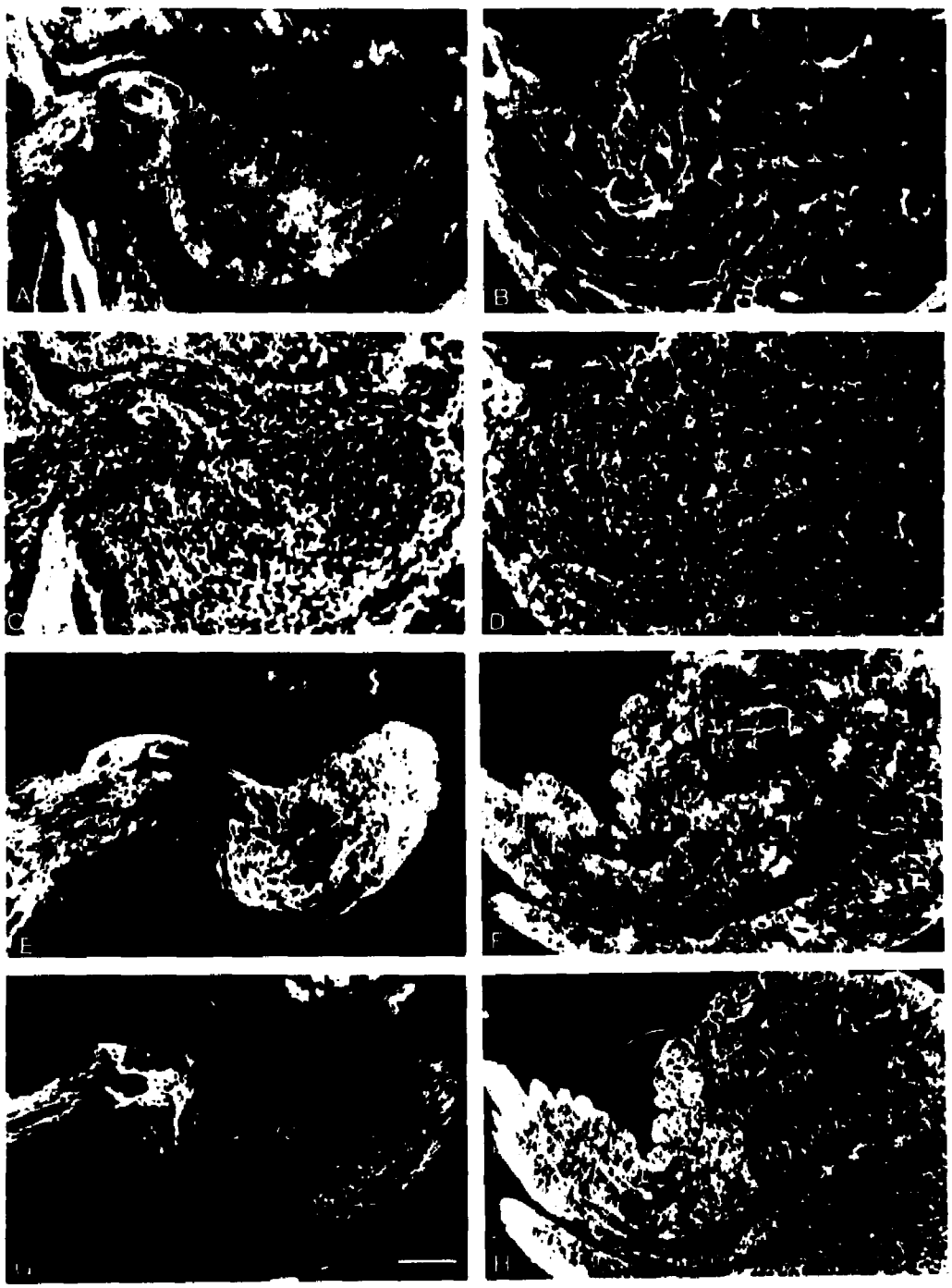

Figure 4.2.

Figure 4.3. In the mouse optic nerve 10 days after crush injury, TSP (A) is associated with regions of concentrated nuclei (B). The pattern of TSP immunoreactivity corresponds to regions containing macrophages as indicated using the macrophage marker M3/38 (C), and is negatively correlated with the expression of GFAP (D). Scale bar = 100um.

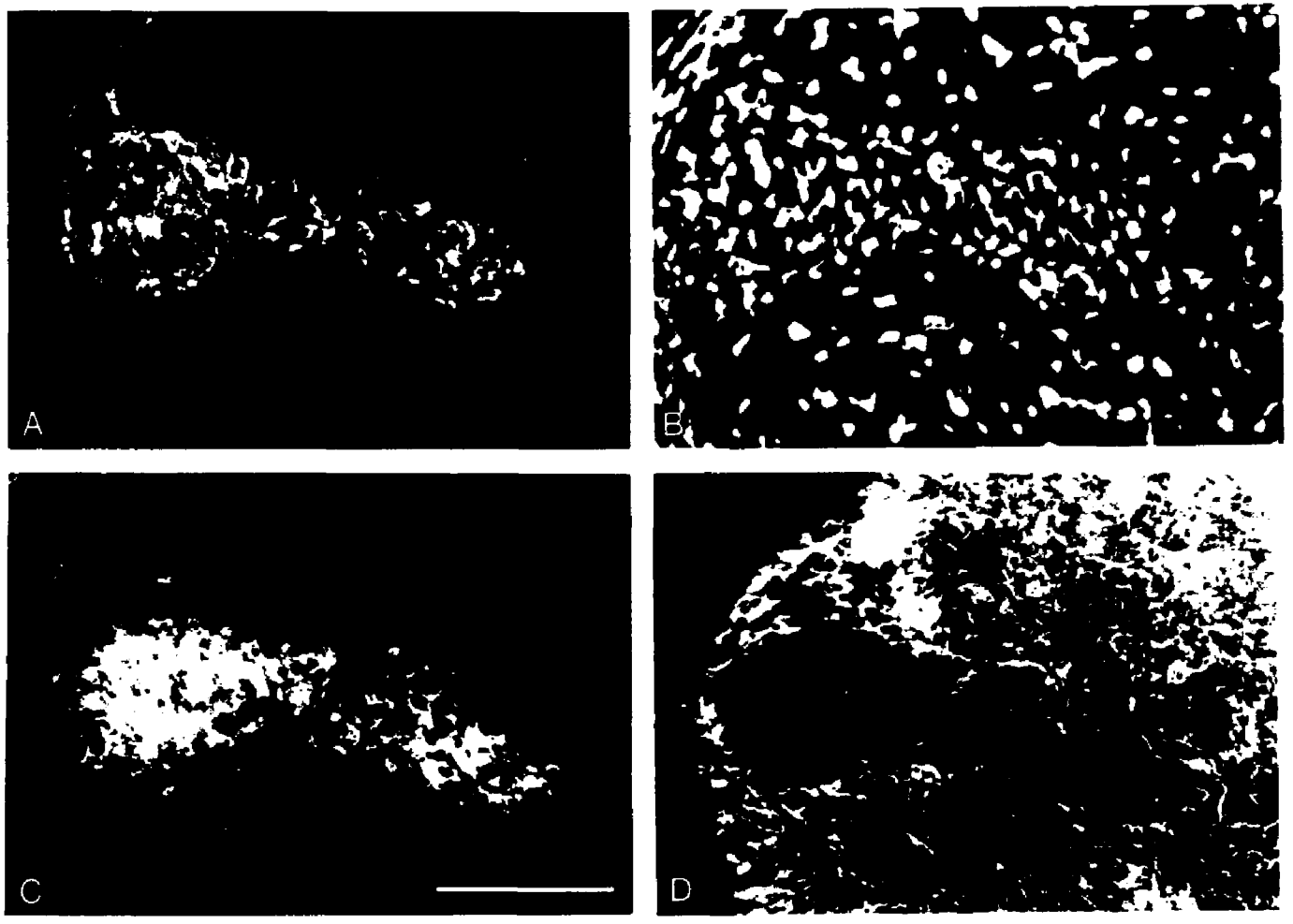

Figure 4.3.

Figure 4.4. In the mouse (A,C,E,G) sixty days after optic nerve crush, there are still regions of TSP activity (A) associated with accumulations of nuclei (C), which remain GFAP-negative (E). There is no neurofilament protein (G) found within the mouse optic nerve, although some staining is observed in nerves innervating extraocular muscles outside the optic nerve (arrowhead). In contrast, in the goldfish optic nerve (B,D,F,H) sixty days after nerve crush, levels of TSP (B), GFAP (F) and NF (H) are returning to normal, even though the overall structure of the nerve is more disorganized than control and there is an overall increase in the number of nuclei in the nerve (D). Scale bar = 100 um.

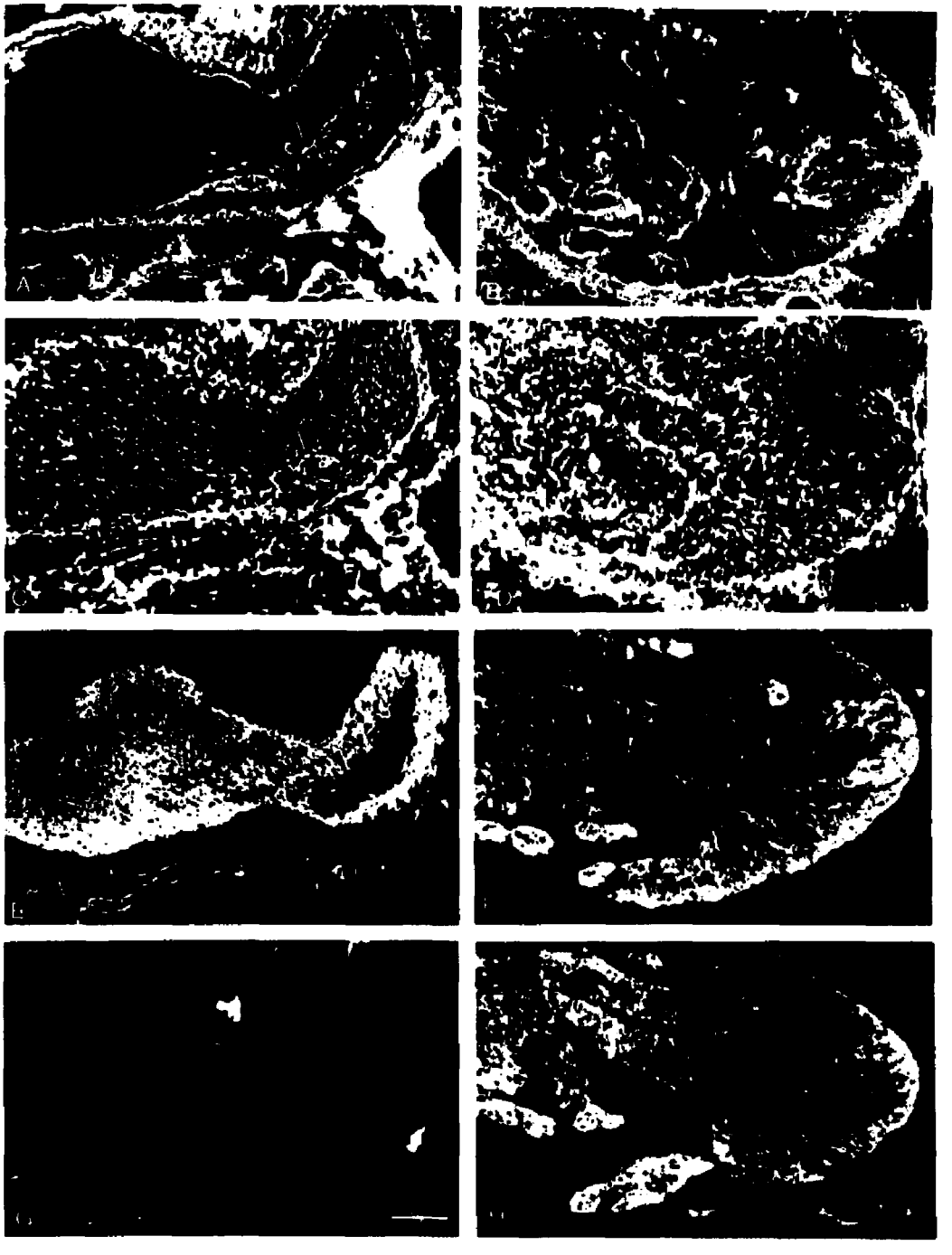

Figure 4.4.

CHAPTER V

THROMBOSPONDIN PROMOTES SCIATIC NERVE REGENERATION IN POLYETHYLENE TUBE GRAFTS.

Introduction

Surgical repair following peripheral nerve trauma is possible, but often not feasible when gaps are formed in the nerve because of a lack of suitable graft materials. Axons will regenerate through grafted peripheral nerve segments, and grow less successfully through skeletal muscle grafts (Keynes *et al.*, 1984; Glasby *et al.*, 1986). Currently, there is no clinically accepted prosthetic nerve graft available. Development of possible nerve grafts has been attempted for over 50 years; the first grafts tested were blood vessels (Weiss and Taylor, 1946). Presently two main types of grafts are being tested, tubes formed of either synthetic polymers or bioresorpable materials, such as collagens and carboxylic acids. These tube grafts are filled with factors thought to promote nerve regeneration, and grafted into a nerve. Early studies demonstrated that whole blood, but not Ringer's solution or blood plasma, would support moderate regeneration through short grafts (Weiss and Taylor, 1946). Recently, factors known to promote neurite outgrowth *in vitro* have been tested in nerve grafts, including laminin (LN; Madison *et al*, 1985, 1987; Muller, 1988; Toyota *et al.*, 1990), nerve growth factor (NGF; Rich *et al.*, 1989; Hollowell *et al.*, 1990), acidic fibroblast growth factor (Cordeiro *et al.*, 1989), basic fibroblast growth factor (Aebischer *et al.*, 1989), collagen (Archibald *et al.*, 1991),

fibrin-fibronectin matrix (Knoops *et al.*, 1991), cultured peripheral nervous system cells (Shine *et al.*, 1985), and in the current investigation, thrombospondin.

Thrombospondin (TSP) is a recently identified extracellular matrix molecule associated with neural development (O'Shea and Dixit, 1988). TSP was originally identified as a 420 kDa glycoprotein released by blood platelets exposed to thrombin (Baenzinger *et al.*, 1971), but has been observed in a variety of extracellular matrices in adult (Wight *et al.*, 1985) and embryonic tissues (O'Shea and Dixit, 1988). The presence of TSP is temporally and spatially correlated with neural crest migration (Boyne *et al.*, 1989), cerebellar granule cell migration (O'Shea *et al.*, 1990), and peripheral nerve outgrowth (O'Shea and Dixit, 1988). In addition, TSP supports neurite outgrowth *in vitro* from DRG explants, PC12 cells, isolated sensory neurons, and chick ciliary ganglionic neurons (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992). Furthermore, the expression of TSP is correlated with the regenerative potential of nervous systems across species, and appears to be associated with successful nerve regeneration in the mouse sciatic and goldfish optic nerve (Hoffman *et al.* 1990, 1991, submitted a,b,c). These data suggest that TSP, which supports neurite outgrowth *in vitro* and is present in regenerating nervous systems *in vivo*, may support nerve regeneration *in vivo*. This study directly examines possible roles TSP may play in nerve regeneration, through the use of specific anti-TSP antibodies which block neurite outgrowth *in vitro* (O'Shea *et al.*, 1991).

Materials and Methods

Grafts: Adult male CD-1 strain mice (Charles River, Portage, MI) were anesthesized with Metofane (Pitman-Moore, Washington Crossing, NJ), and the

sciatic nerve at the mid-thigh level was exposed. A small piece of the nerve (about 3 mm) was removed, and the cut ends of the nerve were placed into a 5 mm polyethylene tube (I.D. = .76 mm, O.D. = 1.22 mm; Clay Adams, Parsippany, NJ), leaving a 4 mm gap. The tubes were filled with approximately 2 uL of either 10 ug/ml purified platelet TSP, 20 ug/ml laminin, 100 ug/ml anti-TSP polyclonal antibodies, or phosphate buffered saline (PBS). Then the nerve stumps were sutured into the tube using 7-0 suture, with care taken that the suture did not compress the nerve. Four animals per group were examined for extent of nerve regeneration.

Histology: Four weeks after grafting, the animals were sacrificed by cervical dislocation. The grafts were exposed, and the nerve on either side was cut, and the entire piece was placed in 4% formaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 hours. The polyethylene tube was carefully removed while the nerve was in fixative, then the nerve was postfixed for 2 hours in 1% osmium tetroxide. Nerves were dehydrated through a series of graded alcohols, followed by propylene oxide, and were then embedded in Araldite (Ted Pella, Redding, CA). Transverse 1 um sections were cut on an Ultracut microtome (Reichardt-Jung, Vienna, Austria) through the nerve at the mid-tube level, stained with toluidine blue, and photographed. Photographs were printed at a standard magnification (440x), areas of nerve, blood vessels, and total area were calculated using a digitizing tablet interfaced with a microcomputer. T-tests were carried out to compare area and cross-sectional proportion between the experimental groups; level of significance was set at $p \leq 0.05$. For the purposes of calculating the area filled with Schwann cells, the axonal component, estimated by multiplying the number of axons by a cross-sectional area of 10 um^2, was subtracted from the area of digitized Schwann cell fascicles.

Results

Differences in the thickness of nerve regenerates between experimental groups was apparent visually even prior to removal of the polyethylene tube. In overall thickness of the nerve cable running through the graft, TSP appeared slightly larger than LN. Regenerates through both TSP and LN were larger than PBS, and anti-TSP were the smallest. Grafts containing PBS were thicker at the proximal end than at the distal end (Figure 1A). Other graft groups were of roughly equivalent thickness at proximal and distal ends. LN regenerates tended to be very thick at the ends, and narrower through the midpoint of the graft (Figure 1B). Grafts containing anti-TSP antibody were more variable than the other groups, ranging from a nerve cable slightly smaller than PBS to a very thin, barely complete cable (Figure 1C). TSP regenerates appeared thicker, and were more uniform along the length of the cable. (Figure 1D).

The 1 um sections supported the initial observation that TSP regenerates were thicker than the other groups, low power photomicrographs of representative grafts are illustrated in figure 2A,C,E,G. Axons and Schwann cells were concentrated toward the center of the regenerate, with varying amounts of connective tissue around the perimeter. Axons in grafts through PBS (Figure 2B), LN (Figure 2D), and TSP (Figure 2H) were usually present in well-defined fasicles, compared with those growing through anti-TSP antibody filled grafts which were less defined (Figure 2F). Nerve cables through PBS, LN, and anti-TSP routinely had an increased number of and increased size of blood vessels than in TSP (Figure 3B). The proportion of total regenerate area filled by Schwann cells was significantly lower in grafts containing anti-TSP (15.2%) than those containing TSP (35.8%), there was no difference in the proportion of area filled by axons (anti-TSP

14.4%, TSP 14.3%; Figure 3B). Grafts through anti-TSP filled chambers had slightly more connective tissue than the other groups (Figure 3B).

The number of myelinated axons present within the grafts was greatest in TSP (1264 $\pm$ 97, n=4), followed by LN (780 $\pm$ 194, n=4), anti-TSP (593 $\pm$ 172, n=4), and PBS (472 $\pm$ 313, n=2; Figure 4). There was a significant difference between TSP and the other groups. The difference between axon number in PBS, LN, anti-TSP was not significant.

Discussion

These data suggest that TSP supports regeneration of a nerve cable through the initially acellular environment of a polyethylene tube. Regenerates through TSP were thicker and contained more axons than all other groups, including LN. However, regenerates through TSP were significantly less vascularized than all other groups, and nerve cables through anti-TSP filled chambers had slightly more connective tissue than other groups.

Although regenerates through TSP were vascularized, the vessels present were fewer in number and routinely smaller than in the other experimental groups. The proportion of cross-sectional area of the PBS, LN, and anti-TSP grafts composed of blood vessels was significantly higher than that of TSP. This observation supports the suggested antiangiogenic role for thrombospondin first described in blood vessel formation *in vitro* (Iruela-Arispe *et al.*, 1991). In both cases, presence of anti-TSP antibodies resulted in a stimulation of angiogenesis.

Often, addition of factors to either promote or disrupt nerve regeneration affects the rate of, but does not alter the success of, regeneration in the long term. Antibodies against neural cell adhesion molecule (NCAM; Remsen *et al.*, 1990) and alpha-1, beta-1 integrin (Toyota *et al.*, 1990) disrupt nerve regeneration through the grafts. Antibodies against NCAM, a cell-surface associated glycoprotein which supports neurite outgrowth (Sunshine *et al.*, 1987), delays functional recovery at 20 days, but not at 30 days (Remsen *et al.*, 1990). Similarly, addition of NGF to tubes resulted in enhanced regeneration at 4 weeks (Rich *et al.*, 1989), but no significant difference at 10 weeks (Hollowell *et al.*, 1990).

Consistent with the observation that anti-TSP antibodies block neurite ougrowth *in vitro* (O'Shea *et al.*, 1991), presence of anti-TSP antibodies in the grafts decreased axon ingrowth. Surprisingly, the proportion of the cross-sectional area composed of axons is equal in TSP and anti-TSP grafts (Figure 3B); however, the proportion of the cross-sectional area containing Schwann cells is significantly less in the anti-TSP filled grafts. These data suggest that the anti-TSP antibody may interfere with the proliferation or migration of Schwann cells into the tubes.

The environment of the polyethylene grafts is initially acellular and must be modified before regeneration is possible (Toyota *et al.*, 1990). Prior to the onset of axonal growth a matrix is established in the fluid filled tube (Williams *et al.*, 1988; Seckel, 1990). In addition, non-NGF neuronotrophic factors accumulate within the tube grafts (Longo *et al.*, 1983a,b) as well as soluble factors which promote Schwann cell adhesion, migration, and proliferation *in vitro* (LeBeau *et al.*, 1988a). As the matrix is laid down, Schwann cells, endothelial cells, and fibroblasts migrate from both nerve stumps (LeBeau *et al.*, 1988a; Williams *et al.*, 1988). Following the matrix stage is a period of perineurial sheath development, axonal sprouting

from the proximal stump, and blood vessel growth into the graft from both ends (LeBeau *et al.*, 1988a; Williams *et al.*, 1988).

The presence of Schwann cells is a limiting factor in nerve regeneration. While axons will regenerate, albeit less successfully, through acellular basal lamina tubes formed by repeated freeze-thawing of nerve, the distance of successful axonal regeneration was limited by the extent of Schwann cells (Hall, 1986; Sketelj *et al.*, 1989; Nadim *et al.*, 1990). In addition, if the integrity of the acellular basal lamina tubes was destroyed, regenerating axons require proximity to viable Schwann cells (Sketelj *et al.*, 1989). However, a small percentage of axons were present in slight advance of Schwann cells (Hall, 1986; LeBeau *et al.*, 1988b), and it appears as if Schwann cells migrate along axons from the proximal stump (Ide *et al.*, 1983). Following peripheral nerve trauma, Schwann cells normally proliferate, but only migrate into regions devoid of Schwann cells (Hall, 1978; Langford and Owens, 1990; Daniloff, 1991). Interestingly, if reinnervation is blocked, Schwann cells will migrate a limited distance into acellular grafts (Anderson *et al.*, 1991) It becomes apparant that the extent of both axonal outgrowth and Schwann cell migration into an acellular graft are interrelated, as the maximal distance is achieved mutually.

TSP plays an important role in nerve regeneration, likely through its interaction with other factors of the extracellular environment. Previous studies have shown that a combination of laminin, testosterone, ganglioside GM 1, and catalase promote regeneration to a much greater extent than any of the components alone (Muller, 1988). TSP is capable of controlling the extracellular environment through binding of other matrix components such as type I collagen (Lahav *et al.*, 1982), type V collagen (Mumby *et al.*, 1984), laminin (Mumby *et al.*, 1984), fibronectin (Lahav *et al.*, 1982, 1984), heparin (Lawler *et al.*, 1978),

glycosaminoglycans (Dixit *et al.*, 1984; Lawler *et al.*, 1985), plasminogen (Silverstein *et al.*, 1984), and tissue- and urokinase-type plasminogen activators (Silverstein *et al.*, 1986).

The current investigation work suggests that TSP may promote Schwann cell infiltration of the acellular graft. The establishment of Schwann cells within the regenerate is essential for more complete axonal regeneration through the graft and into the distal nerve stump, this migration of Schwann cells is restricted in anti-TSP filled grafts. Therefore, TSP appears to play a crucial role in determining the success of peripheral nerve regeneration through acellular polyethylene grafts and may also be involved in other types neural repair.

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Figure 5.1. Photomicrograph of polyethylene tube grafts immediately following removal. Nerve cables growing through PBS filled tubes (A) were slightly smaller than those through LN (B), but larger than those growing through anti-TSP (C). Nerves growing through TSP filled grafts (D) were routinely thicker than the other groups. p = proximal edge of graft, d = distal edge of graft, scale bar = 1 mm.

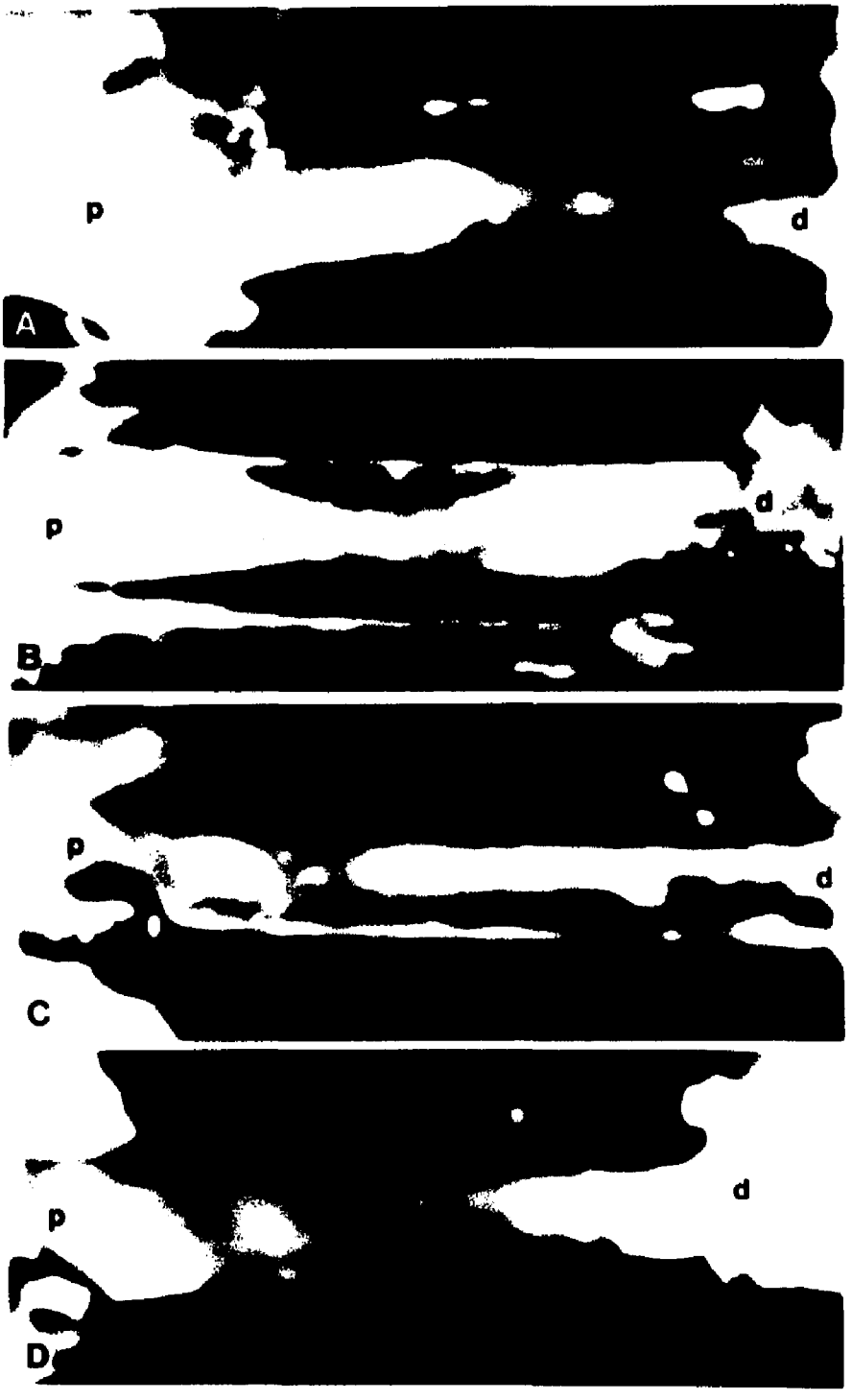

Figure 5.1

Figure 5.2. Representative photomicrographs of cross-sections through the midpoint of regenerates through PBS (A,B), LN (C,D), anti-TSP (E,F), and TSP (G,H). At low power, nerves growing through TSP (G) and LN (C) had consistently greater cross-sectional areas than through PBS (A) or anti-TSP antibody (E) filled grafts. Large blood vessels (arrows) are apparent in grafts through PBS (B), LN (D), and anti-TSP (F), but not TSP (H). Axons in PBS, LN, and TSP, but not anti-TSP grafts were typically surrounded by well defined Schwann cell fascicles. scale bar in G = 100 um, in H = 50 um.

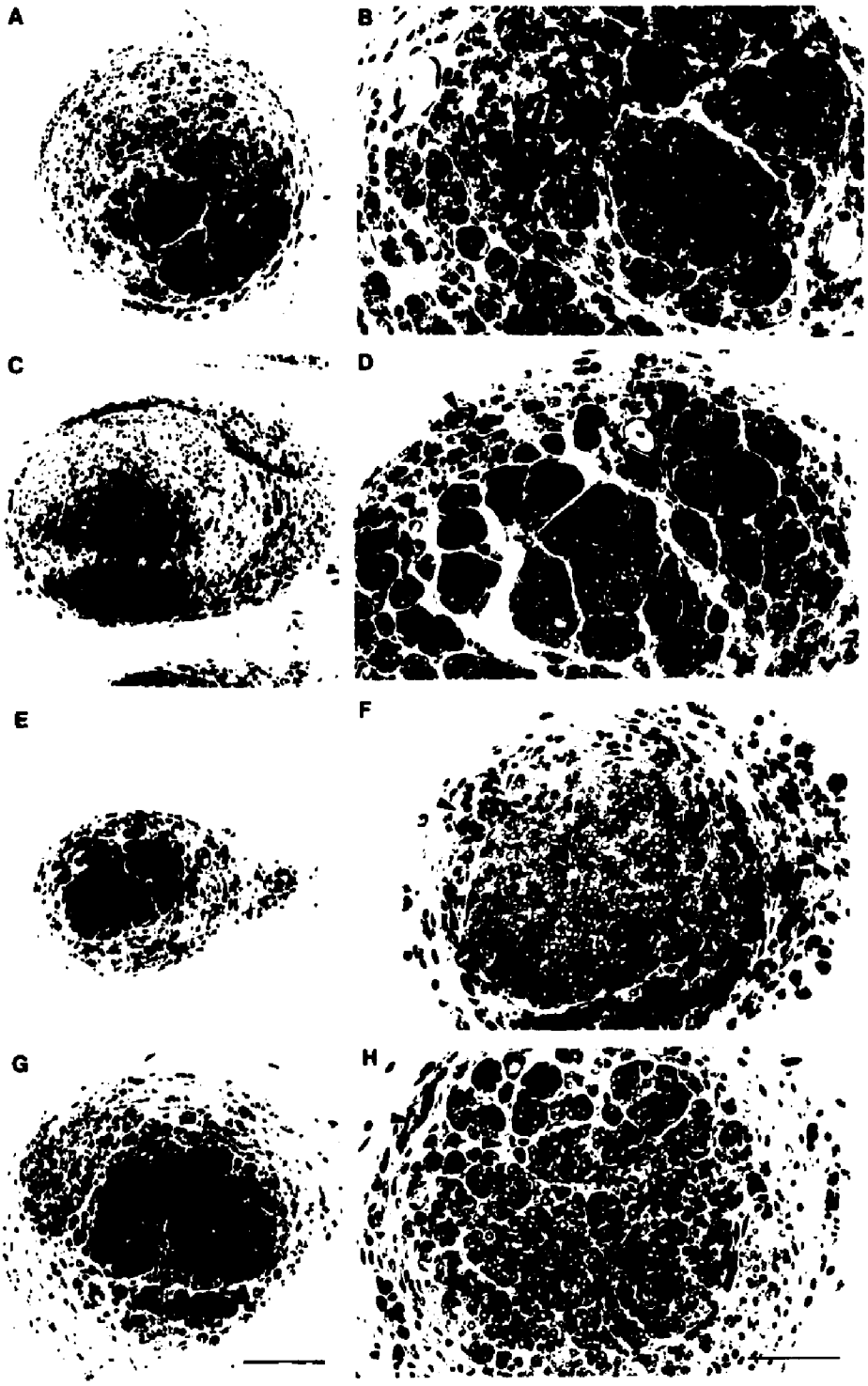

Figure 5.2.

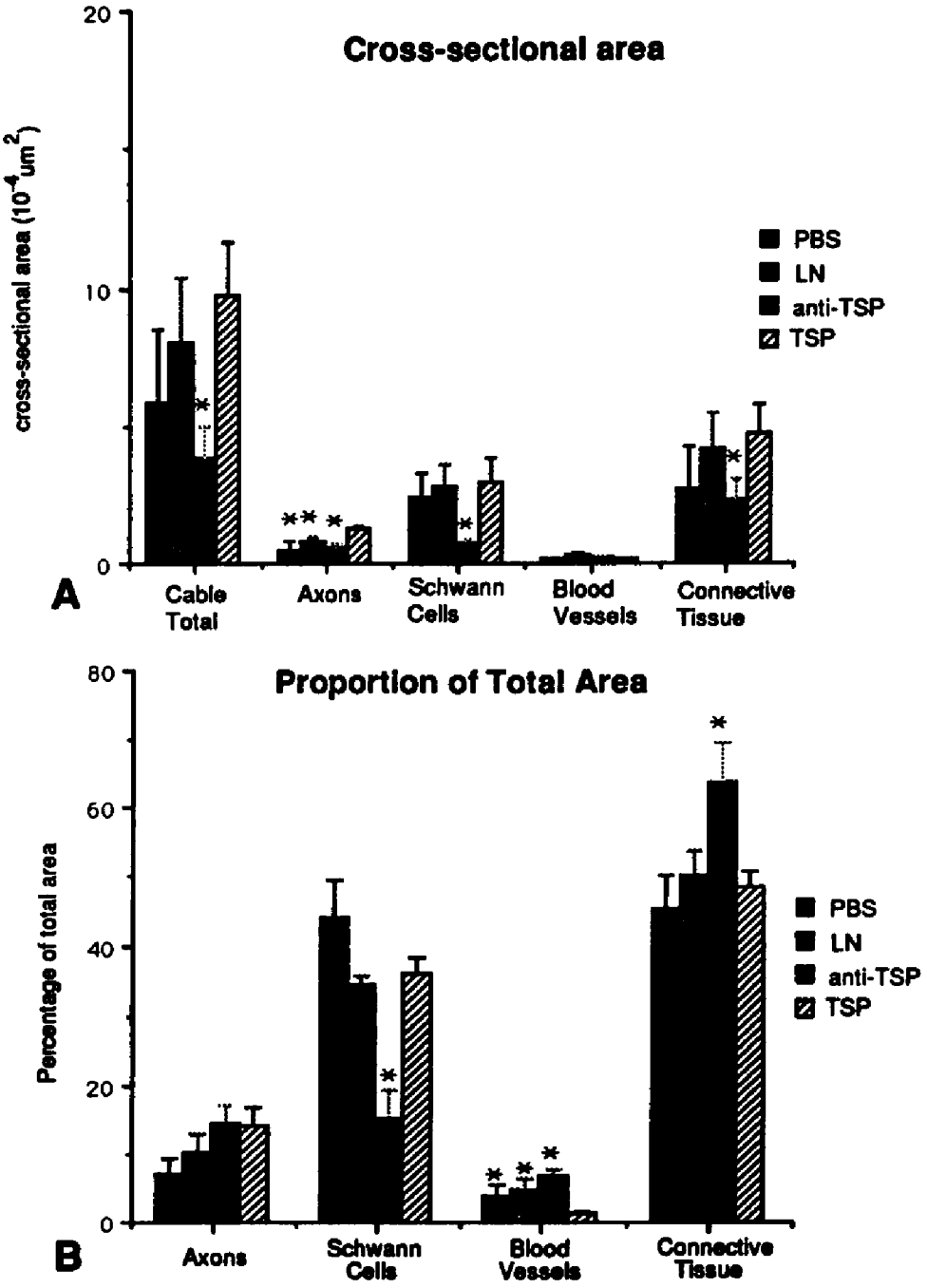

Figure 5.3. Area of nerve cable, axons, Schwann cells, blood vessels, and connective tissue measured at the midpoint of the graft (A). Percentage of area of individual cable components in relation to total cable area (B). * < 0.05 when compared with TSP.

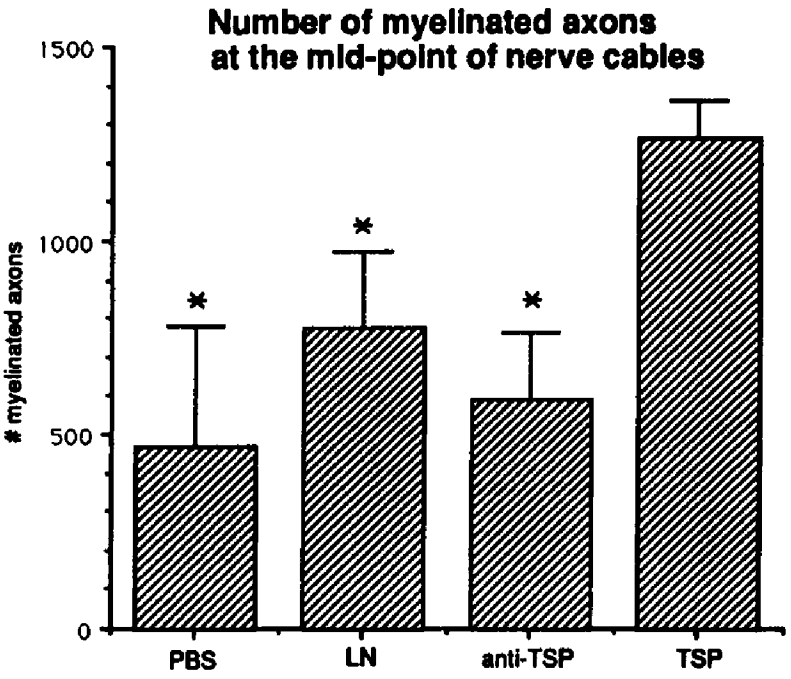

Figure 5.4. Mean number of myelinated axons at the midpoint of the nerve cable. * < 0.05 when compared with TSP.

CHAPTER VI
CONCLUSIONS

Summary of Findings

Recent studies have shown that the extracellular environment is crucial in determining the success or failure of nerve regeneration. The neurons of the adult mammalian CNS are incapable of functional recovery following trauma (Ramon y Cajal, 1928), yet, these same neurons are capable of sending neurites into peripheral nerve segments grafted into their proximity (David and Aguayo, 1981). In comparable experiments, it has been demonstrated that CNS neurons will send neurites into embryonic spinal cord tissue, but not into adult spinal cord tissue (Tessler *et al.*, 1988). These observations form an interesting paradox in that the extracellular environments of the adult peripheral or embryonic central nervous tissue will support neurite outgrowth, while adult central nervous tissue will not.

The present work has investigated an extracellular molecule that fulfills the requirements of the regeneration paradox. Thrombospondin (TSP) is an extracellular matrix molecule that is present in the developing peripheral and central nervous systems (O'Shea and Dixit, 1988), but becomes primarily restricted to the peripheral nervous system in the adult (Wight *et al.*, 1985). This temporal expression pattern of TSP closely parallels the ability of nervous system tissue to support neurite outgrowth and potentially nerve regeneration. In addition, TSP is a

potent substrate for neurite outgrowth *in vitro* (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Osterhout *et al.*, 1992).

While TSP has been studied extensively in the developing murine nervous system (O'Shea and Dixit, 1988; O'Shea *et al.*, 1990), little was known about the expression of TSP in the adult nervous systems of any species. Different vertebrate classes have varied regenerative potential, lower vertebrates such as goldfish and newts are capable of both central and peripheral nervous system regeneration, while mammalian regeneration is restricted to the peripheral nervous system (Windle, 1956). The data support the initial hypothesis that TSP is expressed in nervous tissues having a high regenerative potential. TSP was present in goldfish, frog, and newt optic nerves, but not in the non-regenerative mouse optic nerve. Similarly, TSP was present in adult goldfish and newt, but was absent in the mouse and frog spinal cords. This correlation was strengthened by the presence of TSP in juvenile frog spinal cords. These observations parallel previous work in the mouse that demonstrated that TSP was found in embryonic CNS tissues, but declined in the adult (O'Shea and Dixit, 1988). The decrease in levels of TSP in the adult frog CNS is not complete as TSP persists in the adult optic nerve, which remains capable of regeneration. As expected, TSP was found in tracts within the CNS of goldfish, but not mouse. This study is the first phylogenetic study of expression patterns of TSP, which has a high degree of homology across species (Urry *et al.*, 1991; Laherty *et al.*, 1992). The expression of TSP was correlated with the regenerative potential of nervous system tissues, indicating that TSP was localized in a position to be involved in nerve regeneration.

After establishing that TSP was present in regions of adult nervous systems with the potential for regeneration, it was necessary to examine TSP expression

patterns following trauma. The first system examined was the mouse sciatic nerve following either crush or transection injury. In non-injured nerve, TSP was present in the axoplasm, axolemma, Schwann cells, endoneurium, and perineurium. Following nerve crush or nerve transection, levels of TSP rapidly increased distal to the trauma site. Particularly elevated levels of TSP were just in front of the wave of regenerating axons, gradually returning to normal if reinnervation proceeded. When reinnervation was blocked, TSP remained high in the endoneurium in excess of 30 days, but gradually declined to below control levels by 60-90 days. TSP was re-expressed in the distal nerve stump, following reanastomosis of the proximal and distal segments. These results indicate that TSP may play a role in the adult peripheral nervous system in the mouse.

Since TSP levels were increased following trauma to the peripheral nervous system, it was necessary to examine expression patterns of TSP in a non-regenerating central nerve. The optic nerve was chosen because it allowed the comparison of a non-regenerating (mouse) with a regenerating (goldfish) nerve. TSP was present in the pia-arachnoid layer surrounding both the control mouse and goldfish optic nerves, but was present throughout only in the goldfish optic nerve and was absent from the mouse optic nerve. Following crush injury of the mouse optic nerve, TSP was increased only in patches associated with glial fibrillary acidic protein-negative, macrophage-rich regions. However, following crush injury of the goldfish optic nerve, TSP was dramatically increased along the connective tissue septae along which regenerating axons grow (Wolpurg, 1971). By 60 days following crush injury of the goldfish optic nerve, TSP was returning to control levels; in contrast, TSP was still present in GFAP-negative patches in the mouse optic nerve. These results indicate that organized tracts of TSP may be involved in successful goldfish optic nerve regeneration, and the lack of an organized pattern of

TSP may be a factor in limiting regeneration of the mouse optic nerve. The correlation of TSP and macrophages in the mouse optic nerve is particularly interesting since macrophages have been reported to modify mouse optic nerve sections to support neurite outgrowth *in vitro* (David *et al.*, 1990).

After having established that TSP is correlated with nerve regeneration *in vivo*, and knowing that TSP promotes neurite outgrowth *in vitro* (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991), investigations were begun to determine the precise role TSP plays in nerve regeneration *in vivo*. Polyethylene tubes, filled with TSP, anti-TSP, laminin, or PBS, were grafted into mouse sciatic nerves leaving a 4 mm gap. Nerve cables which had regenerated through grafts filled with TSP were consistently thicker and composed of more axons than all other groups. In addition, it was apparent that TSP had an antiangiogenic effect, as all other groups had significantly higher numbers and increased area of blood vessels. By comparing the proportion of the graft filled with either axons or Schwann cells, it became evident that the anti-TSP had a much greater inhibitory effect on Schwann cell migration into the graft than on axonal regeneration. These results suggest that TSP promotes Schwann cell infiltration into the grafts, which in turn supports axonal growth and nerve regeneration.

Implications

These results indicate that TSP may play a crucial role in nerve regeneration. TSP fits the theoretical criteria of a factor present in regenerative systems, while being absent in non-regenerative nervous systems. Thus, TSP joins a small group of extracellular molecules, such as laminin (Liesi, 1985; Sandrock and Matthew, 1987), which are capable of supporting neurite outgrowth and have expression

patterns which correspond with active nerve regeneration. Unlike laminin, TSP undergoes significant changes in expression level following peripheral nerve trauma (Cornbrooks and Neuberger, 1987; Neuberger and Cornbrooks 1989; Kuecherer-Ehret *et al.*, 1990), and therefore may play a more important role in determining the ability of the environment to support axonal outgrowth.

It has long been established that neurite outgrowth is a contact mediated process requiring an adhesive substrate (Letourneau, 1975). TSP supports neurite ourgrowth *in vitro* (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991), and has several distinct domains involved in cell adhesion (Murphy-Ullrich and Mosher, 1987; Roberts *et al.*, 1987; Lawler *et al.*, 1988; Prater *et al.*, 1991). Similar to other adhesive glycoproteins such as laminin (Kleinman *et al.*, 1985), fibronectin (Ruoslahti *et al.*, 1981), and tenascin (Erickson and Bourdon, 1989) which bind other ECM components, TSP binds type I collagen (Lahav *et al.*, 1982), type V collagen (Mumby *et al.*, 1984), laminin (Mumby *et al.*, 1984), fibronectin (Lahav *et al.*, 1982, 1984), and glycosaminoglycans (Dixit *et al.*, 1984; Lawler *et al.*, 1985). Thus, the presence of TSP may serve as a substrate or organize other adhesive glycoproteins in the ECM for axonal regeneration.

Recent studies suggest that controlled protease activity is also required for neurite outgrowth (Moonen *et al.*, 1982; Pittman *et al.*, 1989b). Plasminogen activator is released by growth cones (Krystosek and Seeds, 1984) and has been implicated in neurite outgrowth from sensory neurons (Krystosek and Seeds, 1984; Pittman, 1985; McGuire and Seeds, 1990), sympathetic neurons (Pittman, 1985; Pittman *et al.*, 1989a), PC12 cells (Pittman *et al.*, 1989a; Saito *et al.*, 1990), and neuroblastoma cells (Shea *et al.*, 1991). In addition, proliferating Schwann cells produce plasminogen activators (Kalderon, 1984; Krystosek and Seeds, 1984). TSP

is unique in that binding of protease Nexin I (Browne *et al.*, 1988), plasminogen (Silverstein *et al.*, 1984) and tissue- and urokinase-type plasminogen activators (Silverstein *et al.*, 1986; Silverstein *et al.*, 1990) results in an increase in the synthesis of plasmin by 40-fold (Silverstein and Nachman, 1987). The plasmin activity generated by these plasminogen activators appears to be required for neurite penetration and growth into a 3-dimensional extracellular matrix (Pittman and Williams, 1988). The protease activity generated by plasminogen activators and protease inhibitors must be closely regulated, as too much activity by either results in inhibition of neurite outgrowth (Hawkins and Seeds, 1986, 1989).

Many of the properties important in supporting neurite outgrowth, are potentially important for glial migration. TSP supports migration of a wide range of cell types (Majack *et al.*, 1986; O'Shea *et al.*, 1990) through cell binding domains and organization of other ECM components. TSP also acts as a chemoattractant for human carcinoma and melanoma cell lines (Taraboletti *et al.*, 1987) and neutrophils (Mansfield *et al.*, 1990). Indirect evidence presented here suggests that TSP is crucial for Schwann cell migration into polyethylene grafts, where Schwann cells support nerve regeneration (Hall, 1986).

TSP has the unique ability to precisely regulate the permissiveness of an environment for nerve regeneration. TSP may act as an adhesive substrate, an organizer of other extracellular matrix molecules, and as a director of localized proteolytic activity, potentially directing the rapid adhesion/deadhesions necessary for neurite outgrowth (Lindner *et al.*, 1986). TSP appears to promote Schwann cell migration and axonal outgrowth and inhibition of TSP results in the diminished nerve regeneration observed in polyethylene tubes filled with anti-TSP antibodies.

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