Fibroblast Growth Factor-1 Modulates Peripheral Nerve Injury Response and Regeneration

by

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List of Abbreviations

BDNF	Brain-derived neurotrophic factor
ChAT	Choline acetyltransferase
CM	Nerve collection medium
CNTF	Ciliary neurotrophic factor
ECM	Extracellular matrix
ERK	Extracellular-signal regulated kinase (MAPK)
FGF1	Fibroblast growth factor 1 (acidic FGF)
FGF2	Fibroblast growth factor 2 (basic FGF)
GDNF	Glial-cell line derived neurotrophic factor
HSPG	Heparan-sulfate proteoglycan
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
LDH	Lactate dehydrogenase
LIF	Leukemia inhibitory factor
MCP-1	Monocyte chemotactic protein-1
MGSA	Melanoma growth stimulating activity
NGF	Nerve growth factor
RPA	Ribonuclease protection assay
SAPK	Stress activated protein kinase

Introduction

Summary: The objective of this work is to define the role of acidic fibroblast growth factor (FGF1) in peripheral nerve injury and regeneration. FGF1 has been shown to have trophic effects on a wide range of neurons and mitogenic effects on many cell types of both central and peripheral nervous systems. Interestingly, unlike other growth factors, FGF1 lacks the N-terminal sequence required for secretion through the ER-Golgi pathway. In addition, FGF1 expression in the nervous systems is limited to adults, suggesting that it does not play a role in neuronal development. Finally, FGF1 is stored in high concentrations within the axonal cytoplasm of motor and sensory neurons, suggesting that the peripheral nerve might be the location of FGF1's actions. Together, these characteristics suggest that FGF1 does not act according to the traditional model of target derived trophic factors; instead, FGF1 might function as an injury factor that regulates injury responses and provides temporary trophic support to the damaged neurons. The work described here will study FGF1's role in peripheral axon regeneration by testing the following hypothesis: *upon axonal damage, FGF1 present in the axonal cytoplasm leaks out from the damaged axons to act on neighboring axons and non-neuronal cells to provide trophic support and promote regeneration.*

Introduction

In our every day actions--as we talk softly to appease a crying baby, run to catch a fleeing bus, or swing a hammer at a nail--peripheral nerves in our bodies relay messages produced in the central nervous system (CNS) to command the execution of an action. At the same time, these nerves also relay signals produced by the various sensory organs back to the CNS--as we hear that the baby has stopped crying, see that the bus as stopped to wait for us, or as we feel the throbbing pain of a finger crushed by an improperly wielded hammer. As such, peripheral nerves form the essential link between our central nervous system and the environment that surrounds us.

When the peripheral nerve is severed by damage, the flow of information stops, presenting a possible life-threatening situation for the organism. One can imagine the fate of a rabbit that has sustained sciatic nerve damage after narrowly escaping the claws of a pursuing bobcat. If the nerve damage were not repaired, the decreased mobility of the injured hind leg would mean a certain doom for the rabbit if it were to encounter another predator. Perhaps because of an evolutionary advantage conferred to an animal that can repair damages to their peripheral nerves, the peripheral nerves in a wide range of animals can regenerate following injury. Although the repair does not achieve the complexity and refinement of the connections established during early development, under the right circumstances, axons in injured peripheral nerves can regain contact with their targets and bring back function. As such, peripheral nerves are ideal structures for us to explore the permissive and promotive mechanisms that allow injured axons to regenerate. While many questions still remain about what makes peripheral nerves supportive of axonal regeneration after injury, neuroscientists have made enormous progress to understand the process of regeneration.

I. Peripheral Nerve Structure and Cell Types

In order to understand how axons regenerate in the peripheral nerve, one needs to understand its normal structure and function. Several types of neurons project processes through peripheral nerves. These include motor neurons (such as facial motor neurons), sensory neurons (such as DRG sensory neurons), and autonomic neurons (such as sympathetic neurons). The cell bodies of these neurons can either be enclosed in the CNS, as the spinal motor neurons are, or they can be part of the peripheral nervous system, residing in groupings of cell bodies packed together in structures called ganglia (**Figure**

A). Therefore, while some neurons reside in the central nervous system, their processes extend out to the PNS where they are governed by injury responses that are different from those in the CNS (Lundborg, 1987).

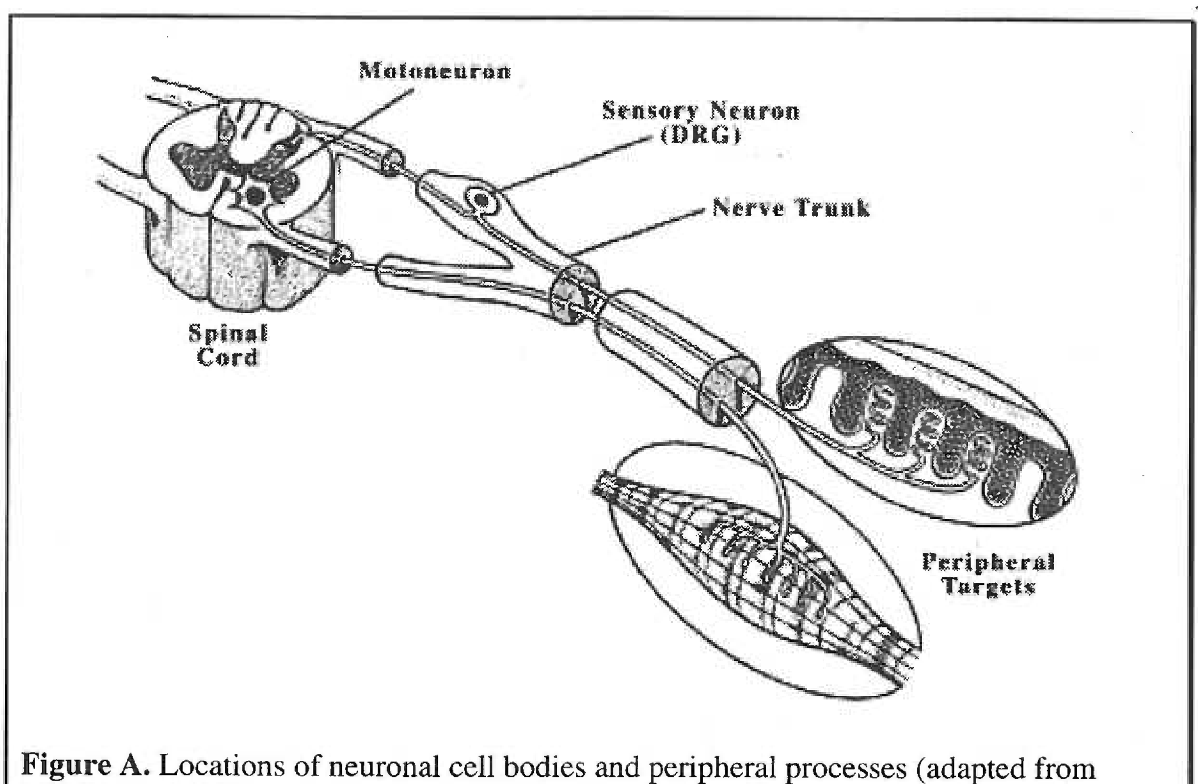

Figure A. Locations of neuronal cell bodies and peripheral processes (adapted from Lundborg, 1987).

The axons are the main functional structure of the peripheral nerves, relaying information through transient electrical currents. Other cells play important roles in the maintenance of peripheral nerve functions. Most obviously, the Schwann cells wrap around fast conducting axons to provide myelination (for review, see Thomas et al., 1993). In addition, Schwann cells also tightly associate with slow conducting axons, without myelinating them, suggesting that Schwann cells play supportive roles other than speeding up signal propagation. Indeed, studies of development and injury have shown that Schwann cells are important in maintaining axons and neuronal survival (Riethmacher et al., 1997). Other cell types in peripheral nerves include the fibroblasts, which produce the extracellular matrix (Thomas et al., 1993), mast cells which participate in injury responses, and endothelial cells which form the vascular network to bring nutrients to the cells of the nerve.

Peripheral nerves are nourished by blood vessels that course through the nerve in a longitudinal fashion. These blood vessels, which enter at several points along the nerve, originate from different branches of the vasculature, such that an entire nerve is supplied by different blood vessels. This prevents the whole nerve from relying on single blood vessels; if a particular vessel is blocked, parts of the nerve are still nourished by other blood vessels.

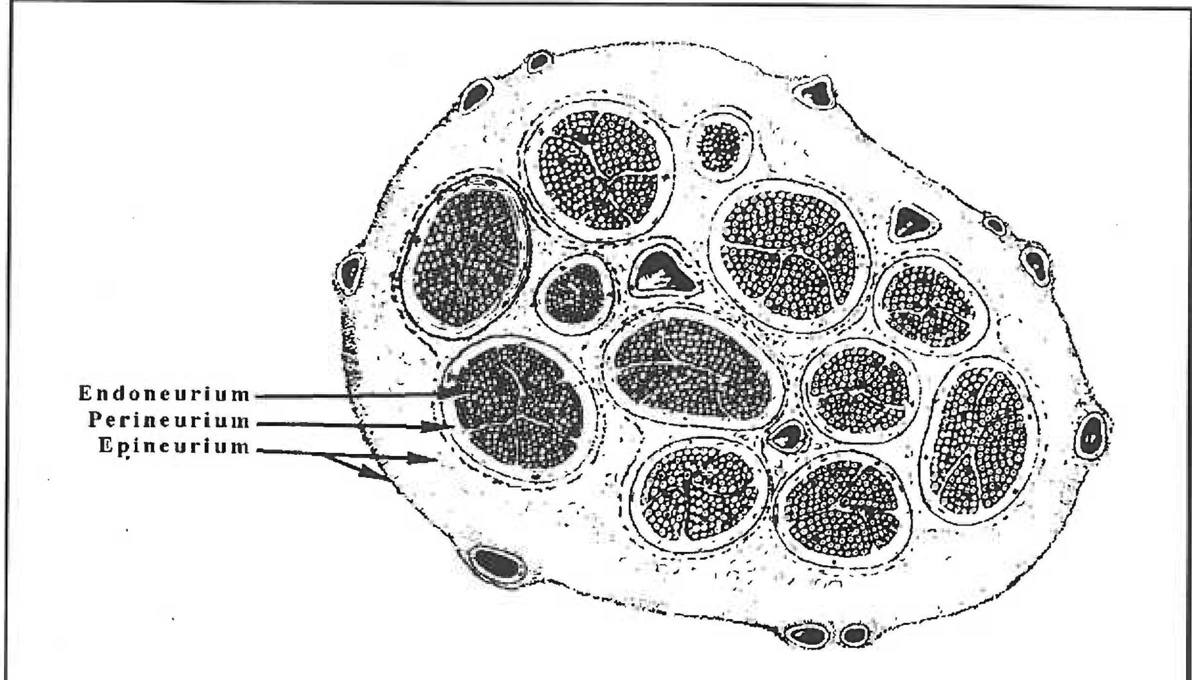

Figure B. Layers of the peripheral nerve. A schematic diagram of a nerve cross section is shown, with the three layers indicated: epineurium, the outermost layer, perineurium, the intermediate layer forming the nerve fascicles, and endoneurium, the innermost layer containing axons and Schwann cells (Adapted from Lundborg, 1987).

Given the vulnerability of the peripheral axons as they exist beyond the protection of the skull and the spinal vertebrae, peripheral nerves are structured to protect the axons from physical and chemical damage. The nerves are organized into three different layers: epineurium, the perineurium, and the endoneurium (Thomas et al., 1993; see **Figure B**). The epineurium is the outermost layer of peripheral nerves, and it is composed of fibroblasts and collagen fibers arranged longitudinally. It provides cushioning protection for the nerve from physical damages, and this function is aided by the presence of fatty tissue in this layer. At locations where the nerve crosses joints, the epineurial layer is generally thicker to provide more physical protection for the nerve. The perineurium, the intermediate layer, is made up of layers of flattened cells separated by layers of collagenous

connective tissues. At points where contiguous cells contact each other, these flattened cells are joined with tight junctions; these tight junctions are responsible for maintaining the blood-nerve-barrier. When blood vessels traverse the perineurium to link the vascular network in the epineurium with the capillary network in the inner most layer (the endoneurium), the blood vessels are accompanied with perineurial "sleeves" for some distance. The endoneurium, the inner most layer, is composed of units of axon-Schwann cell and is the functional core of the peripheral nerve where electrical signal propagation and molecule transport is carried out.

II. Peripheral nerve injury response

Injury to the peripheral nerve initiates a well defined-pattern of cellular changes distal to the site of damage (**Figure C**; for review, please refer to Lundborg, 1987; Hall, 1989; Fawcett and Keynes, 1990; Griffin & Hoffman, 1993). Immediately following injury to the nerve, the most prominent change is the breach of axonal and other plasma membranes. In addition, the blood-nerve-barrier, normally maintained by the perineurium, becomes disrupted, allowing a number of molecules previously foreign to the endoneurium to invade the nerve's inner most layer. This might immediately change the pH, signaling molecules, precursor molecules, and presence of foreign cells in the nerve environment.

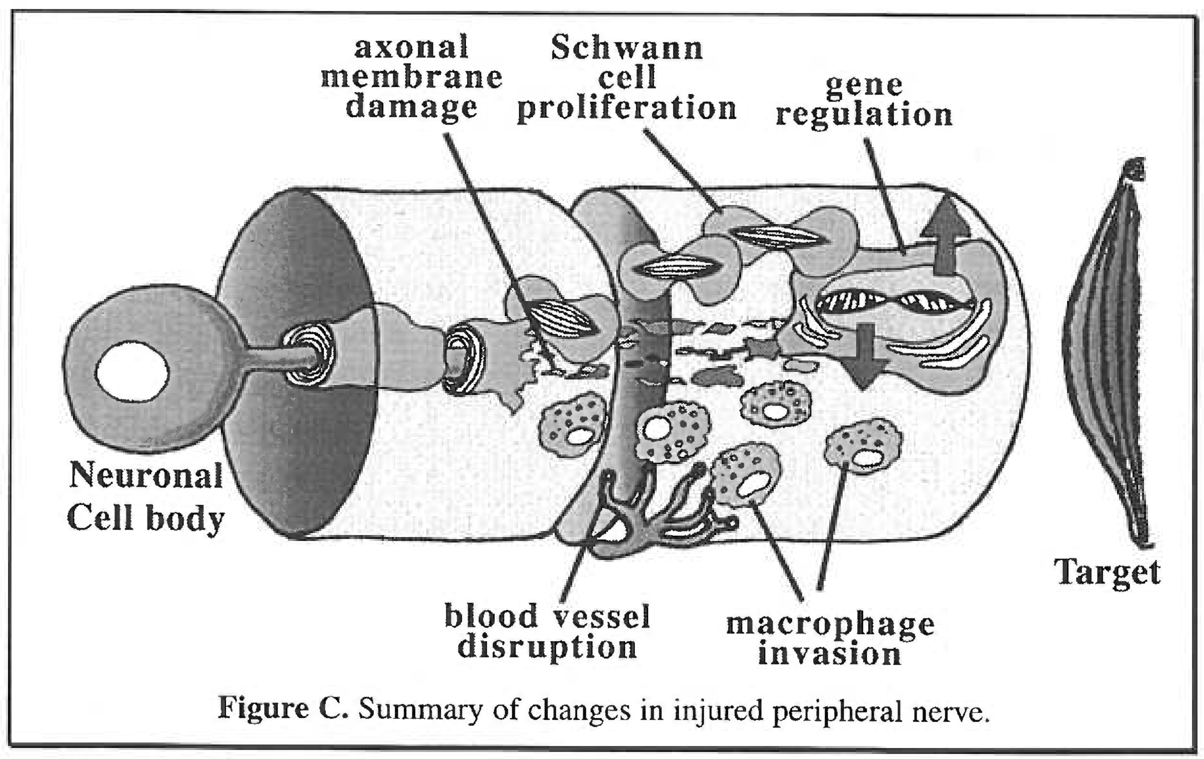

Figure C. Summary of changes in injured peripheral nerve.

In the first few days following injury, axonal degeneration proceeds proximo-distally from the site of injury and the myelin sheaths that surround the degenerating axons are broken up into ovoids (Griffin & Hoffman, 1993). Macrophages, which are initially virtually absent in the nerve, invade the nerve soon after injury and phagocytose the axonal and myelin debris (Perry et al., 1987; Perry and Brown, 1992; Avellino et al., 1995). The number of macrophages increases first within the sites immediately adjacent to the site of injury, and then a dozen hours later spreads throughout the distal portions of the nerve. In addition to the phagocytosis of debris, macrophages might also regulate gene expression. For example, it has been shown that macrophages might upregulate NGF in the injured nerve via the secretion of IL-1β (Lindholm et al., 1987). Schwann cells shed their myelin, dedifferentiate, and divide while staying within their basal laminae, producing cordons of Schwann cells known as bands of Bungner (Salzer and Bunge, 1980; Pellegrino et al., 1986; De Vries, 1993).

The series of changes that occurs in the peripheral nerve--especially in the distal portions--is very important to the regeneration of the damaged axons. This is illustrated by many studies showing that pre-degenerated nerves (injured nerves that have been given time to undergo the changes described above) are better substrates for process outgrowth than freshly injured nerves (Kerns et al., 1993; Danielsen et al., 1994; Danielsen et al., 1995). This suggests that some growth promoting molecules are absent in the normal nerves, and that expression of growth promoting molecules is upregulated only after the nerve becomes injured. Apparently, the growth promoting activities are quite powerful because peripheral grafts have been shown to promote growth of central neuronal processes both in the brain and in the spinal cord (David & Aguayo, 1981). This suggests that, following injury, the peripheral nerve is stimulated to undergo regeneration-supportive changes, such as producing more adhesion molecules, growth factors, or chemotropic factors, or reducing inhibitory molecules.

Indeed, it is now known that nerve injury induces the expression of neurotrophic factors such as NGF (Heumann et al., 1987; Lindholm et al., 1987), BDNF (Meyer et al., 1992; Funakoshi et al., 1993), and GDNF (Springer et al., 1994; Trupp et al., 1995); of Schwann cell mitogens and survival factors such as neuregulins (Carroll et al., 1997); and of pro-inflammatory cytokines such as IL-1 (Rotshenker et al., 1992), IL-6 (Bolin et al., 1995; Bourde et al., 1996; Reichert et al., 1996; Ito et al., 1998), and LIF (Curtis et al.,

1994; Ito et al., 1998). Most of these factors are not expressed in normal nerves, but, after injury, they begin to be expressed in the non-neuronal cells of the nerve.

Many of the upregulated factors are expressed by the immature Schwann cells during early development (Buck et al., 1987; Ernfors et al., 1989), as well as after injury. Indeed, during the process of regeneration, many of the neurons and the non-neuronal cells that surround them revert to an early developmental phenotype (Heumann et al., 1987).

III. Peripheral nerve development and maintenance

The peripheral nerve is made up of cells derived from different sources. Schwann cells, sensory neurons, and sympathetic neurons are all derived from the neural crest, while motor neurons are derived from neural tube and other cells such as the fibroblasts have a mesodermal origin.

In the developing PNS, Schwann cell precursors proliferate and migrate along established axons beginning at E10. During this time, Schwann cells begin to lay down basal lamina that organizes the peripheral nerve (Jessen and Mirsky, 1991; Bunge, 1993). As the Schwann cell precursors mature and become bona fide Schwann cells, their pattern of gene expression changes; at this point the Schwann cells begin expressing several molecules that reappear following injury. For example, while the early Schwann cells express the low affinity NGF receptor p75, as they mature and begin to myelinate axons, they abolish the expression of this receptor. However, these Schwann cells re-express these receptors following injury in the adult (Buck et al., 1987; Ernfors et al., 1989). The final event that marks the full maturation of Schwann cells is their myelination of axons, a process that continues into the first postnatal week in rodents (Bunge, 1993).

In rodents, motor neurons undergo their final mitosis between embryonic days 10 and 11 (Phelps et al., 1988). Soon afterwards, these neurons send their processes out toward their peripheral targets. It is during this time that the neurons begin expressing choline acetyltransferase (Phelps et al., 1991; Chen and Chiu, 1992), the enzyme that produces the neurotransmitter acetylcholine (ACh). The processes from the motor neurons arrive at their target muscles between E13 and E17 (Dennis et al., 1981; Phelps et al., 1991). It is at this time that normal cell death occurs, eliminating more than 50% of the original neurons produced (Harris and McCaig, 1984; Sheard et al., 1984). During the first postnatal

weeks, polysynaptic connections at the motor end plates are eliminated, resulting in single innervations of the motor end plates (Sheard et al., 1984).

Because the cells that make up the peripheral nerves come from different tissue sources and have to span vast distances, signals are likely passed between the various cells to bring them together to form the nerve. In addition to these signals, the neurons and Schwann cells are also mutually dependent for survival. This is illustrated by the phenotype of the mice lacking the receptor for the Schwann cell survival factor neuregulin. In the knockouts of the neuregulin receptor erbB3, Schwann cell numbers are greatly reduced because Schwann cells and their precursors were not able to receive trophic signals (Riethmacher et al., 1997). While motor neurons in these knockout animals initially appear in normal numbers, a few days later most of the motor neurons die, even after some of them have reached their peripheral targets (Riethmacher et al., 1997). This demonstrates that during early development, neurons are dependent on Schwann cells for survival. This is an important concept that will recapitulate in the adult following injury. As the Schwann cell precursors mature, their pattern of gene expression drastically changes (Jessen and Mirsky, 1991; Bunge, 1993), and it is during this maturation that the neurons start to lose their dependence on Schwann cells for survival and begin to be dependent on the targets for trophic support.

When neurons establish a connection with their targets, they begin to depend on their targets for trophic support (Johnson et al., 1980; Crews and Wigston, 1990). This dependence on targets likely serves several purposes for both the development and maintenance of the nervous system. According to the neurotrophic hypothesis of neuronal axonal pathfinding and synapse formation, neurons which do not reach their correct targets or arrive late are eliminated due to the lack of trophic support. In this fashion, the dependence of neurons on target trophic support is used to ensure the correct matching of neurons to their targets and to match neuron number to target size.

Several classes of molecules have been proposed to act as neurotrophic molecules: the neurotrophins, which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT3), ciliary neurotrophic factor (CNTF), and glial cell line-derived neurotrophic factor (GDNF) all have been shown to promote neuronal survival. These distinct classes of trophic factors act through several different families of receptors. This enables each class of neurons to express different repertoires of receptors,

creating mechanisms for neurons to be selectively stimulated by specific sets of trophic factors produced by their corresponding target tissues.

For the peripheral axons, this means that their source of trophic factors is quite a distance away--sometimes up to 2 meters, as in the case of the sciatic nerve in the larger animals such as the giraffe. While some groups of neurons lose this dependence in the adulthood, others, such as the sympathetic neurons, retain their dependence on their peripheral targets (Gorin and Johnson, 1980; Johnson et al., 1982). This dependence on distal targets for trophic support presents a problem for the neurons after injury. When axons are severed, the neuronal cell body can no longer be provided with the trophic signal derived from the target because the conduit that usually transmits these signals is severed by the injury.

Even though many neuronal populations are dependent on trophic factors during adulthood, many of these same neurons do not die when they experience injury. This suggests that, after injury, the source of trophic support might be changed from the target to another area, such as the nerve itself. It is likely that when axons are severed in the adult animal, the cells that line the injured axons become trophic surrogates by providing the needed trophic molecules. There is evidence suggesting that this switch of trophic source occurs only in the adult. In rodents less than a week old, transection of the facial nerve causes a large portion of the facial motor neurons to die while the same injury in the adult animal causes relatively few neuronal deaths (Kuzis et al., 1999). A lack of alternative trophic source might explain why the juvenile motor neurons are susceptible to the injury.

What is the cause of this lack of alternative trophic source in the juvenile nerve? It might be that the Schwann cells in the juvenile nerve are too immature to produce trophic factors in response to injury signals, or that these Schwann cells lack the receptors to receive the injury signal. Alternatively, young axons might lack the injury signals present in adult nerves. Any of these possibilities could account for the increased death of juvenile neurons following peripheral nerve injury. Interestingly, several growth factors, including FGF1, have been found to be expressed with time courses that have an inverse relationship with the decline of juvenile neurons' susceptibility to injury (Kuzis et al., 1999). It is possible that these factors are responsible for this increased resistance to injury. One such factor is fibroblast growth factor 1 (FGF1). Its characteristics suggest that it plays an important role in peripheral nerve injury response and regeneration.

IV. Characteristics of FGF1

Acidic fibroblast growth factor (FGF1) is a member of the fibroblast growth factor family (Burgess and Maciag, 1989; Eckenstein, 1994). FGF1 is present at high concentrations in neurons of several motor nuclei and sensory ganglia; these include the facial motor nucleus, the spinal motor neurons, and the primary sensory neurons of the dorsal root ganglia (Elde et al., 1991; Stock et al., 1992; Oellig et al., 1995). In these neurons, FGF1 is observed both in the cytoplasm of the cell bodies and in their peripheral processes. In the sciatic nerve, which contains the peripheral processes of sensory and motor neuron populations, FGF1 expression is high, accounting for 0.1% of all soluble nerve proteins (Eckenstein et al., 1991).

FGF1 acts by binding to the extracellular domains of transmembrane FGF receptors, which are encoded by four genes and produced in various splice variants (Johnson and Williams, 1993). The binding of the FGF family members to the FGF receptors requires heparan sulfate proteoglycans (Yayon et al., 1991; Ornitz et al., 1992; Rapraeger, 1995), which are present as abundant components of the extracellular environment and on cell surfaces. The binding of FGF1 to the FGF receptors causes receptor dimerization and autophosphorylation. The activation of the FGF receptors leads to the activation of downstream effectors which include the extracellular signal-regulated kinases (Vainikka et al., 1994; Wang et al., 1994), and phospholipase-Cγ (Burgess et al., 1990). The activation of these molecules leads to further downstream events that result in different types of cellular activity such as proliferation, differentiation, or survival.

Although FGF1 acts by binding the extracellular domains of the FGF receptors, it appears not to become available to the extracellular environment via secretion. This is because FGF1 does not have the N-terminal signal peptide required to allow FGF1 to go through the ER-Golgi secretory pathway. It has been suggested that FGF1 is only released from its cytoplasmic stores after an injury to the membrane. In studies using cultured endothelial cells, McNeil and colleagues demonstrated that basic FGF (FGF2), a family member closely resembling FGF1 (55% homology), is released from cytoplasmic stores following mechanical disruption of the cell membranes (McNeil et al., 1989; Muthukrishnan et al., 1991). These observations suggest that FGF1 can be released through breaches in the membrane and that its biological role might be related to injury to the neurons.

In vitro studies demonstrated that FGF1 has a wide variety of actions on many cells of mesodermal and neuroectodermal origin (Burgess & Maciag, 1989). In the central and peripheral nervous system, FGF1 stimulates the proliferation of astrocytes (Pettmann et al., 1985), oligodendrocytes (Eccleston and Silberberg, 1985), and Schwann cells (Davis & Stroobant, 1990). In addition to the mitogenic effects on non-neuronal cells, FGF1 also functions as a neurotrophic factor to promote the survival of various neuronal populations in culture. For example, it promotes the survival of peripheral sympathetic, sensory, and spinal cord motor neurons (Unsicker et al., 1987; Eckenstein et al., 1990; Sendtner et al., 1991). These are actions shared by many growth factors, such as NGF and BDNF, that are proposed to play a role in nervous system development.

Although FGF1 is present at high levels in the adult nervous system, FGF1 is not expressed until late in development (Kuzis et al., 1995; Oellig et al, 1995). The earliest detection of FGF1 expression in the brain stem motor neurons, for example, is on the day of birth, and the adult level of FGF1 in these neurons is not reached until postnatal day 21 in the rat (Kuzis et al., 1995). The expression of FGF1 in the sensory neurons in the dorsal root ganglia follows a similar time course (Oellig et al., 1995). This time course of FGF1 increase closely parallels the time course of increase in neonatal neurons' ability to survive injury (Kuzis et al, 1999). Unlike most adult motor neurons, which successfully survive and re-innervate their target, large percentages of neonatal motor neurons die after a crush injury (Kuzis et al., 1999). This vulnerability to injury continues until about 2 weeks after birth, when FGF1 expression approaches adult levels. Because of its late onset of expression, FGF1 is not likely to play a role in regulating neuronal development. Instead, its correlation with the adult neuron's ability to survive crush injuries suggests that it plays a role in the maintenance of mature nervous systems after injury.

V. FGF1 in Peripheral Nerve Injury

To test whether FGF1 plays a role in restoring function after injury, several studies examined the effects of exogenous FGF1 on injured peripheral nerves. These *in vivo* studies of exogenously applied FGF1 showed that FGF1 can prevent neuronal death and promote axonal regeneration following injury. In the newborn rat, FGF1 applied locally to the nerve transection site can prevent a large percentage of the neuronal death observed in the non-treated lesioned side. FGF1 treatment reduced neuronal loss from 82% to approximately 30% (Cuevas et al., 1995). In the adult rat, where most neurons can survive axonal injury, FGF1 treatment increased the rate of regeneration. Using different

approaches to measure the rate of axonal regeneration, several research groups observed that FGF1 increased the number of regenerating axons crossing the injury gap (Cordeiro et al., 1989) and increased the rate of axonal regeneration (Walter et al., 1993; Laird et al., 1995). In addition, FGF1 has been shown to aid the regeneration of injured axons across peripheral nerve bridges into severed spinal cords (Cheng et al., 1996).

Although *in vivo* studies have demonstrated that exogenous FGF1 helps the recovery of injured peripheral nerve axons, the exact nature of FGF1 action is still unclear. FGF1 can have several effects in the injured peripheral nerve. For example, because it is neurotrophic for many of the neurons that send their processes out to the periphery, FGF1 release following injury can provide the injured neurons an initial source of trophic factor to replace the source originally derived from the neurons' targets. This would keep the injured neurons alive until their regenerated axons regain contact with their target.

The level of FGF1 in the injured nerve actually *diminishes* following injury (Eckenstein et al., 1991; Ishikawa et al., 1992). This suggests that the actions of FGF1 are not long term; rather, the short time of FGF1 availability following injury suggests that it might play an important role in *initiating* responses rather than maintaining a sustained response. In support of this speculation, several studies have demonstrated that FGF1 is able to induce the production of another trophic factor, nerve growth factor (NGF), in cultured astrocytes and fibroblasts (Ono et al., 1991; Yoshida & Gage, 1992; Yoshida et al., 1992). These observations suggest that, in addition to providing initial direct neurotrophic support for injured neurons, FGF1 can indirectly influence neuronal survival and axonal regeneration by triggering the production of trophic factors.

In the peripheral nerve, mRNA and protein levels of several neurotrophic factors are upregulated following injury (see above). In addition, factors that might mediate immune responses also have been shown to be upregulated in the injured nerve (Carroll & Frohnert, 1998). The non-neuronal cells in the lesion site are responsible for producing these trophic factors. Many of these trophic factors are upregulated at regions adjacent to the site of the injury (both proximally and distally) where axonal membrane damage has occurred. These are possible sites where FGF1 is released from cytoplasmic stores through breaches in the membrane. In addition, the upregulation of trophic factor expression occurs very rapidly, in some cases in less than 6 hours after injury (Heuman et al., 1987; Reichert et al., 1996; Carroll & Frohnert, 1998), suggesting that the molecules that trigger these changes originate from the vicinity of the injury site. At later time points (e.g. one week post

injury), these same trophic factors are upregulated throughout the distal stump, where the axons have degenerated. The correlation with the degeneration of the axonal membrane once again supports the hypothesis that the FGF1 released through damaged membrane initiates these changes in trophic factor expression to promote regeneration. This suggests that FGF1 is one of the first molecules to arrive in the nerve to trigger injury responses, and is an important component of the injury repair system in the peripheral nerve.

VI. Goals of the study

The goals of this study are to test whether FGF1 is released after nerve injury, and whether FGF1 initiates injury responses in the peripheral nerve and promotes axonal regeneration. The characteristics of FGF suggest the following model of FGF1 action in the injured nerve: upon axonal damage, FGF1 present in the axonal cytoplasm leaks out from damaged axons to act on neighboring cells to initiate injury responses and to promote nerve regeneration. We address several hypotheses generated by the above model: 1) that FGF1 is made available to the extracellular environment after injury, 2) that FGF1's downstream signaling components are activated in the nerve following injury, and 3) that the absence of FGF1 leads to reduced downstream signaling after injury and results in impaired regeneration.

Chapter 1

FGF1 is Released From Injured Sciatic Nerve in a Rapid Time Course

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Abstract

Fibroblast growth factor 1 (FGF1) is a neurotrophic and mitogenic factor for a wide variety of cells in the nervous system (Burgess & Maciag, 1990; Eckenstein, 1994). Despite of its known potency, it has been difficult to assign a biological role for FGF1 because, unlike traditional growth factors, FGF1 lacks the N-terminal signal peptide required for secretion through the ER-Golgi pathway, and studies in cell culture have not detected release of FGF1 into the media. FGF1 expression is abundant in neurons, where it is localized to both the cell body and peripheral processes (Elde et al., 1991; Stock et al., 1992). Because of its high concentration in the periphery and its lack of signal peptide, we hypothesized that FGF1 is released through breaches in axonal membrane following axon damage. We studied the release of FGF1 from injured axons in an *in vitro* model of nerve injury. Analysis of media conditioned by injured axons (CM) demonstrated the release of substantial mitogenic activity in the early hours after injury. This mitogenic activity was neutralized by a FGF1 selective antibody, suggesting that FGF1 composed the majority of the mitogenic activity observed. In addition, the collection of FGF1 from the damaged nerve was significantly facilitated by the inclusion of soluble heparin in the collection media. This suggests that in the nerve, FGF1 released from damaged axons is sequestered by heparan sulfate proteoglycans in the extracellular matrix, and that this sequestration might be significant in FGF1's function. Together, the data suggests that FGF1 is an injury factor that might play a role in peripheral nerve injury repair.

Introduction

After injury the distal stump of the peripheral nerve undergoes dramatic changes to prepare for the regeneration of the damaged axons (Hall, 1989; Fawcett & Keyes, 1990). While these changes are well-documented, the signals that trigger these changes still remain elusive. What sort of signal would truly reflect the fact that the nerve, and its axons and Schwann cells, are injured? One potential way for the axons to signal their injury is to expose their internal contents to their neighboring cells. The neighboring cells could either recognize specific "spilled" components of the axons, or could just recognize the generalized cytoplasmic protein. Because the axons share many internal components with their neighbors such as Schwann cells and fibroblasts, these common internal components might not be useful to signal the injury of the axons. Many of the axons' internal components, such as neurofilaments and neurotransmitters, are unique to the axons, and therefore might serve as specific signals to the Schwann cells when the axons are damaged. However, many of these axon-specific molecules are probably not present in high enough concentration to be effectively presented as signaling molecules; instead, specific axonal factors might be assigned to act as injury factors.

An ideal molecule that might serve the purpose of an axonal injury signal would be one that: 1) is present abundantly such that when there are breaches in the membrane sufficient amounts could be released into the extracellular space, 2) is known to act as a signaling ligand on the cells present in the peripheral nerve, 3) can provide beneficial effect when it signals the cells in the peripheral nerve, 4) is normally not available to the extracellular space.

One molecule that fits several of the above criteria is fibroblast growth factor 1 (FGF1). FGF1 is a prototypic factor of the FGF family of growth factors. It can stimulate a wide variety of reactions upon its interaction with target cells, and these responses range from cell division to cell survival (Burgess & Maciag, 1990; Eckenstein, 1994). FGF1 is present in the axons in high concentrations (Eckenstein et al., 1991). It has been estimated that approximately 0.1% of total protein in the sciatic nerve is FGF1. FGF1 has the striking feature of lacking the N-terminal signal peptide that is required for it to go through the traditional ER-Golgi secretory pathway. This is unlike most growth factors such as NGF and EGF which have signal peptides that allows them to be secreted. However, like other growth factors, FGF's have receptors that are transmembrane tyrosine receptor kinases and FGFs are required to bind to the extracellular side of these receptors to activate

them (Johnson & Williams, 1993). This suggests that FGF1 must exit the cell in order to stimulate its receptors.

Because FGF1 is sequestered in large amounts near the membrane along neuronal cell bodies and processes (Elde et al., 1991; Stock et al., 1992) it is highly likely that FGF1 might take a simple route out of the cytoplasmic store after the plasma membrane is breached by physical injury. This idea of FGF leaking through breaches in the membrane has been demonstrated in studies in which cultured endothelial cells are scraped and the surrounding media is collected and tested for the presence of FGF's. Indeed, following scraping, FGF2 appears in the collection medium (McNeil et al., 1989; Muthukrishnan et al., 1991). Thus it is likely that FGF1, a close relative of FGF2, can also be released through breaches in the membrane.

In the following study we test whether FGF1 is released from axons following peripheral nerve injury. Injured nerve segments were placed into media and proteins that leaked from the nerves were collected and tested for the presence of FGF1. To test whether FGF1 present in the culture medium is leaked out through passive diffusion through the breaches in the membrane (and not through mechanisms specifically tailored for FGF1) the levels of two other cytoplasmic proteins, LDH and ChAT, in the culture medium were also measured. The time course of the appearance of these proteins will be compared to those of FGF1 to allow us to discern the mechanism by which FGF1 is released from the axonal cytoplasm.

While *in vitro* studies have shown that FGF1 has multiple effects and can be very important in a number of events, the physiological functions of FGF1 remains unclear. Here, with the demonstration of FGF1 release from injured axons, we are providing evidence that FGF1 can serve as an injury signal.

Results

To test whether FGF1 is released from injured axons, rat sciatic nerves from adult rats were cut into 0.5 cm fragments and placed in collection media (CM). Dulbeco's minimum essential medium (DMEM) was used as the culture media, and the preparation was kept in a 37°C incubator. At different time points, the CM was removed and replaced with fresh equilibrated DMEM. In this fashion, CM was obtained hourly for the first 6 hours following nerve injury. The CM from the various hours was then tested in the AKR2B bioassay for the presence of mitogenic activities and to establish a time course of FGF1 release.

Biological activity is released from injured nerve with a rapid time course
In the fractions collected from the first two hours after injury, significant amount of mitogenic activity was present (**Figure 1.1**, open bars). The mitogenic activity was the most abundant in the first two hours, and declined thereafter until it reached undetectable levels at the sixth hour after injury. Combined, the amount of mitogenic activity released in these first six hours represented approximately 20 % of the total mitogenic activity originally present in the nerve (total mitogenic activity is the sum of mitogenic activity present in all the time points and the mitogenic activity left over in the nerve itself).

Because the AKR2B bioassay can respond to a wide range of growth factors (Shipley, 1986), the identity of the mitogenic activity had to be further defined. Because FGF1's activity in the AKR2B assay is highly dependent on the presence of soluble heparin (Eckenstein et al., 1991), we tested whether the removal of soluble heparin in the test media would abolish the mitogenic activity. Indeed, when heparin was absent from the AKR2B assay, the mitogenic activity that we observed previously was drastically reduced (Data not shown). This suggests that the mitogenic activity collected in the CM is dependent on heparin for it to carry out its effect on the AKR2B cells.

As a more stringent test for the presence of FGF1, we added FGF1 neutralizing antibodies into the AKR2B assay together with the CM. The anti-FGF1 antibody selectively neutralizes the mitogenic activities of FGF1 without greatly inhibiting the activities of FGF2 in the AKR2B bioassays (**Figure 1.2A**). At the dilution that we used, the antibody reduced 50% of the [^{3}H]-thymidine incorporation caused by 5ng/ml FGF1, and at lower FGF1 concentrations, the antibody is even more effective, reducing up to 85% of the factor's effectiveness (e.g. 0.2ng/ml). The presence of the FGF1-neutralizing antibody

abolished most of the mitogenic activity collected from the injured nerve (**Figure 1.2B**). Together, the data demonstrate that FGF1 constitutes the majority of the mitogenic activity released from the injured nerve into the collection media.

Heparin facilitates the appearance of more FGF1 in the collection media

Because the extracellular matrix surrounding the axons in the peripheral nerve contains high levels of heparan sulfate proteoglycans (HSPG), it is possible that a large portion of the sequestered in the extracellular matrix (ECM) and not collected in the CM. Therefore, we tested whether the nerve contained more FGF1 than we had eluted in our previous experiment. Soluble heparin (10μg/ml) was added to the collection media to elute any FGF1 bound to HSPGs in the ECM. With the presence of the soluble heparin in the CM, more FGF1 activity was detected at each of the time points than the collections performed in the absence of heparin, and the release time course was more prolonged (**Figure 1.1**, black bars). FGF1 was still present in the 6th hour fractions, and the amount of FGF1 collected over the six hours was approximately 36% of the total FGF1 present in the nerve. This is almost double the amount of FGF1 collected in the absence of heparin in the CM. This suggests that in the nerve, the FGF1 released from damaged axons is bound to the HSPGs in the extracellular matrix.

We further tested the possibility that the FGF1 is sequestered in the ECM by initially incubating the injured nerves in the heparin minus (-) collection media for the first six hours and then transferring the nerve into collection media that contained heparin on the seventh hour. For the first six hours, the time course of release appeared the same as that seen in **Figure 1.1** (open bars), where after the first few hours, FGF1 release subsided to very low levels. However, when these nerves were transferred to after 6 hours without heparin to heparin-containing media, abundant FGF1--approximately 10% of total FGF1 in the nerve--appeared in the CM (data not shown). This demonstrates that while FGF1 was released from injured axons and a portion was released into the collection media, a large fraction of this factor remained bound to the extracellular matrix in the nerve. The sequestration of FGF1 to the extracellular matrix might be important to the biology of FGF1.

FGF1 release time course is similar to LDH and ChAT release

If FGF1 is released through breaches in the membrane via simple diffusion, then the time course of its release should be relatively similar to the release of other molecules that are stored within the axonal cytoplasm. Therefore, we chose to study the appearance of two

cytoplasmic enzymes, lactate dehydrogenase (LDH) and choline acetyltransferase (ChAT), into the collection media. LDH is a ubiquitous metabolic enzyme and has been used in many different assays to estimate cell number or the amount of cell death in cultures. ChAT is an enzyme that produces the neurotransmitter acetylcholine and is present in the motor neurons. With the measurement of the release of these two proteins, we can better understand how other cytoplasmic proteins act after a nerve injury.

Collection media were obtained every hour for the first six hours after injury, and the samples were tested for the presence of LDH and ChAT. Both LDH and ChAT appeared in the CM mostly during the first two hours following injury (**Figure 1.3**). In these two hours, approximately 20% and 35% of the total LDH and ChAT, respectively, were released. Unlike FGF1, the release of LDH and ChAT were not significantly affected by the presence of soluble heparin, demonstrating that heparin sulfate proteoglycans in the ECM do not have an affinity for these molecules. In comparison to the appearance of FGF1 in the collection medium, LDH and ChAT time courses showed similar time courses but were much more short-lived. The time course of LDH, ChAT, and FGF1 release in the absence of soluble heparin in the collection media were very similar, suggesting that under that condition, these molecules were released from the injured axons through common mechanisms. However, when soluble heparin was included in the collection media, FGF1 appearance in the collection media became more prolonged and were greater in magnitude while LDH and ChAT releases remained the same as in the heparin-minus collection media. This suggests that heparin did not have a general effect on the nerve such as delaying the resealing time of the damaged axonal membranes or reducing the clotting of blood vessels.

Time Course of Axon Resealing

To better interpret our FGF1 release data, we wanted to know how the time course of FGF1 release compared to the time course of axon resealing in our in vitro injury system. We studied the time course of diffusion of rhodamine-conjugated dextran into injured axons. Nerve fragments were placed into 2% solution of rhodamine-conjugated 10kD dextran for five minutes at different hours after injury. The nerve fragments were then immersion fixed, sectioned, and observed for the presence of rhodamine staining within the axons. Of all the time points studied--ranging from immediately after injury (control) to the sixth hour after injury--only the axons in the freshly cut nerves showed substantial incorporation of the rhodamine stain (**Figure 1.4**, top panels). Axons from the other time points did not show staining above background and the stainings were mostly confined to the extracellular space (**Figure 1.4**, lower panels). This observation agrees with previous

studies of in vitro axon resealing rates (Xie & Barrett, 1991). This suggests that the majority of the axons reseal their damaged membranes by the end of the first hour after injury, and that intra-axonal FGF1 can only be released within this first hour.

FGF1 release in vivo

To bring our FGF1 release experiments a step closer to the understanding of *in vivo* functions, we performed *in vivo* experiments where cut stumps of the sciatic nerve were immersed *in situ* in collection media, and the resulting CM was analyzed for the presence of FGF1. We found that mitogenic activity was released from the injured nerve and a large portion of this activity was abolished by the FGF1 neutralizing antibody (**Figure 1.5**). This result correlates well with the *in vitro* experiments, and suggests that the *in vitro* experiments are relevant physiologically.

Discussion

FGF1 is thought to be released through breaches in the membrane because it lacks the signal peptide required for secretion through the ER-Golgi pathway. Its high concentration in the axonal cytoplasm (estimated to be 0.1% of total soluble protein in the nerve) suggests that it can readily be made available after membrane breach (Eckenstein et al., 1991). Here, we tested whether FGF1 is released from damaged peripheral nerve axons using a simple *in vitro* injury system. In this system, freshly collected sciatic nerve fragments were placed in culture media and factors released from these nerve segments were analyzed with the AKR2B mitogenic assay for the presence of FGF1.

Because AKR2B cells respond to a range of growth factors in addition to FGF1, we needed strategies to identify the mitogenic activity as FGF1. In the bioassay, the presence of FGF1 neutralizing antibody abolished nearly all of the mitogenic activities present in the CM, suggesting that the [^{3}H]-thymidine incorporation is caused by FGF1. The neutralization of the mitogenic activity by anti-FGF1 antibody, and the dependence of the mitogenic activity on soluble heparin, demonstrate that the activity present in the CM is FGF1.

The collection of FGF1 in the CM was greatly facilitated by the presence of soluble heparin in the media. The difference in the total amount of FGF1 collected under the two different conditions (+ or - heparin) probably does not reflect the different amounts of FGF1 release from axons; instead, this might reflect the fate of released FGF1 in the extracellular space. It is possible that upon its release from axons, FGF1 became sequestered by heparin sulfate proteoglycans in the ECM. When the nerves were transferred from heparin (-) media to heparin (+) media 6 hours after their initial culture, a robust pulse of FGF1 re-appeared in the CM , suggesting that the source of extracellular FGF1 have not been depleted, even though no FGF1 was detected in CMs from the previous hour. This strongly suggests that even though FGF1 was released from injured axons, a significant fraction bound to the ECM via heparan sulfate proteoglycans.

The above data suggests that equal amounts of FGF1 were released from the axons in either of the collection conditions (+ or - heparin), but that their collection, or elution, into the CM were determined by whether soluble heparin was present in the collection media. Indeed, the ECM contains high level of heparan sulfate proteoglycan (HSPG) which has been shown to bind FGFs with high affinity. This binding is so strong that when R. Elde

and colleagues were probing sections of sciatic nerve with a mixture of FGF1 antibody and excess FGF1 as a control to show antibody specificity, the complex of FGF1/FGF1 antibody bound and stained all of the extracellular spaces heavily (Elde et al., 1991). Our studies of FGF1 release from nerve fragments support this previous observation, and suggest that the released FGF1 is bound to the ECM, most likely to HSPGs. This sequestration of FGF1 released from injured axons might have important implications for the biological function of FGF1.

It has been suggested that heparan sulfate proteoglycans (HSPGs) can stabilize FGF1 and extend its half life by preventing degradation (Yayon et al., 1991; Ornitz et al., 1992). This can extend the time with which FGF1 can exert its action. This is supported by previous studies in our laboratory where we observed that FGF1 remains in the distal stump for up to four days after injury (Eckenstein et al., 1991), even though the axons have degenerated by day two and can no longer provide FGF1. This suggests that FGF1 was being sequestered and protected from degradation. In addition to increasing its stability, FGF1's binding to HSPGs can increase the binding of FGF1 to its receptors (Yayon et al., 1991; Ornitz et al., 1992). Extracellular HSPGs might also maintain a FGF1 gradient in the extracellular space, providing a directional cue.

While we observed the appearance of FGF1 into the collection media, it is unclear how these molecules were released from their cytoplasmic stores within the axons. Other studies have speculated that FGF1 might be released via coupling with molecules involved in neurotransmitter release (Tarantini et al., 1998). However, these observations required the overexpression of FGF1 in a cell line and might not represent physiological situations. Instead, we hypothesized that the release of FGF1 was simply via diffusion through breaches in the axonal membrane. If this is the case, then other cytoplasmic proteins should also be released with similar time courses as FGF1. To test this possibility, we measured the amount of ChAT and LDH released into the CM at different hours after nerve injury, and compared their time course of release to that of FGF1. The release of ChAT and LDH followed a rapid time course similar to that of FGF1. While the collection of FGF1 into the CM was influenced by the presence of heparin in the collection media, the release of LDH and ChAT did not appear to be affected by the presence of heparin--similar amounts of LDH and ChAT appeared in the CM regardless of heparin's presence. This suggests that the effects of heparin on FGF1 collection was based on specific interactions between HSPGs and FGF1 and not on alterations of membrane resealing or blood vessel coagulation.

The release of cytoplasmic FGFs through breaches in the plasma membrane had been previously tested by McNeil et al. (McNeil et al., 1989; Muthukrishnan et al, 1991) who showed that FGF2 was released through breaches in endothelial cell membranes. In their study, cultured endothelial cells were scraped with a rubber policeman and the culture media was collected at different time points. Analysis of the media showed that FGF2 appeared only after the cells were damaged. While their experimental system differs from ours, their results provide additional support for the direct release of FGF1 via diffusion through membrane breaches.

Because the nerve segments used in our study were placed in an artificial culture, one might argue that this might not be a valid model for the study of FGF1 release following nerve injury. An alternative model is to grow neurons *in vitro*, damage them, and collect the media around them. However, this model would lack the 3 dimensional relationship between the axons and their surrounding cells, and the culture situation might introduce additional variables as the level of FGF1 expressed by neurons in culture might not truly reflect those in physiological situations (Oellig et al, 1995). Also, in such systems, FGF1's interactions with HSPGs in the ECM might be difficult to detect. With the experiments that we performed we couldn't rule out that the FGF1 present in the CM was already in the extracellular space prior to injury. To test this, the most telling experiment would be to perform micro-dialysis sampling the extracellular environment in the nerve before and after injury--an experiment that would be very technically challenging. Nevertheless, numerous previous immunocytochemical studies have shown that in the intact nerve FGF1 is clearly within the axonal cytoplasm and not present in the extracellular space (Elde et al, 1991; Stock et al., 1992). Based on this evidence we can confidently conclude that the FGF1 that appears in the CM most likely were released from their axonal stores after injury.

Our observation that FGF1 is quickly released from axons suggests that after an injury, FGF1 might be one of the first factors available on the scene, and that it might be responsible for ERK activation. It would be of definite interest to see in systems that lack FGF1--such as FGF1 knockout mice--whether FGF1's downstream signaling components such as ERK activation following injury are affected. Because Schwann cells have FGF receptors and are known to respond to FGFs (Davis & Stroobat, 1990; Grothe et al, 1997), they are the likely target of FGF1's action. FGF1 might induce changes in Schwann cell gene expression or help to stimulate proliferation. In doing so, FGF1 becomes the injury signal to convey axon damages to nearby Schwann cells and contributes to the process of

regeneration by promoting changes in Schwann cell gene expression and proliferation, which have been shown to be necessary for regeneration.

In summary, the data presented here demonstrated that FGF1 is quickly released from breaches through the axonal membrane, with passive diffusion as a likely mechanism, and that the released FGF1 is tightly associated with the extracellular matrix. These conclusions suggest that FGF1 might trigger injury induced responses in the nerve, and that FGF1 might play a critical role in the regeneration of damaged axons.

Acknowledgement:

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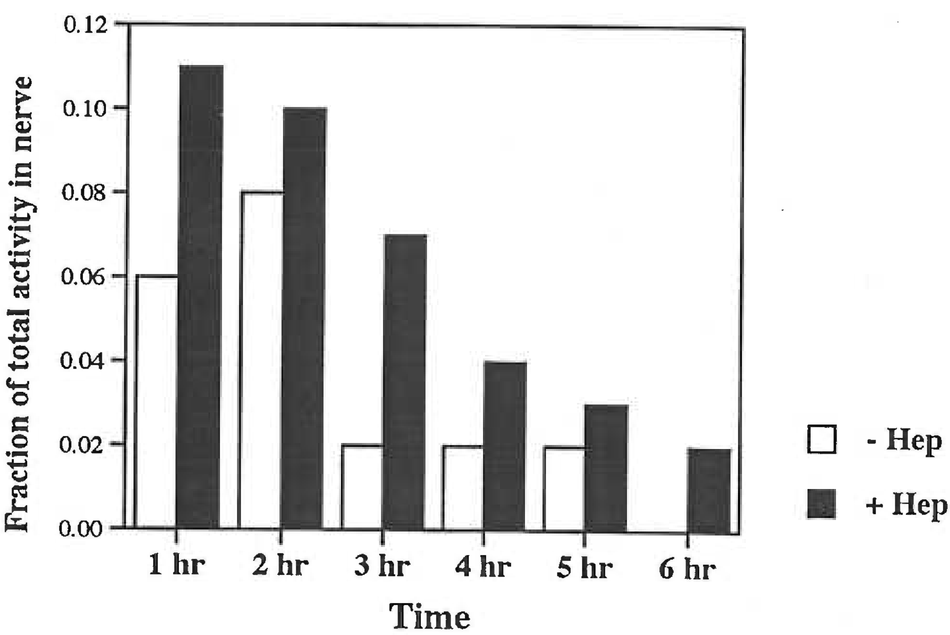
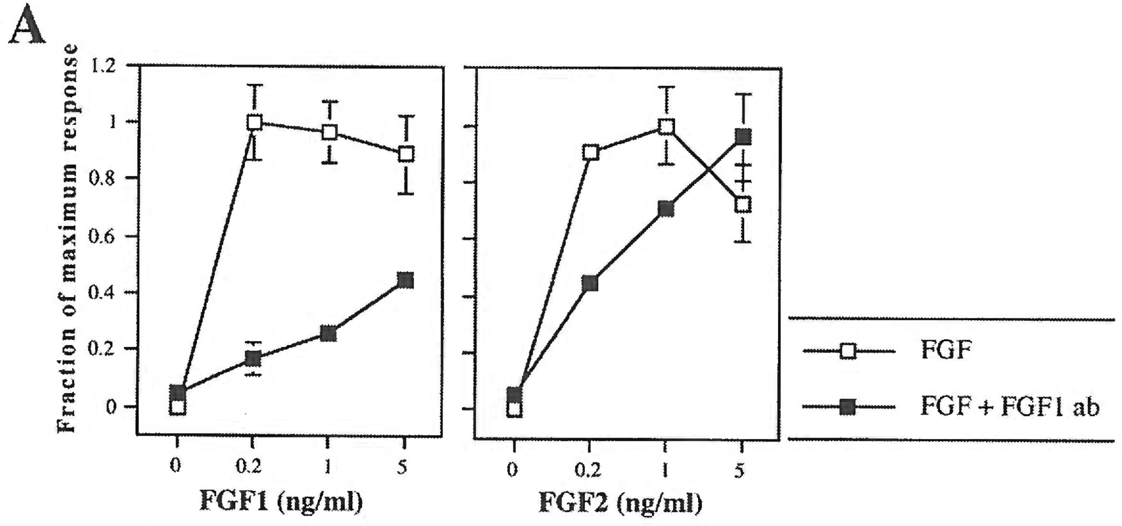


Figure 1.1 Release of mitogenic activity from sciatic nerve fragments in culture.

Sciatic nerve fragments were cultured either in media alone (open bars) or in media containing 10µg/ml heparin (black bars). The media (CM) were collected at every hour for 6 hours, and tested in the AKR2B assay. Note that in the absence of heparin, the release of the mitogenic activity was robust in the first two hours, followed by substantial decrease thereafter. At the sixth hour, no mitogenic activity was detected in the CM. When heparin is present in the collection media, more mitogenic activity appeared at each time point, and the duration of their collection is increased. The Y-axis represents the fraction of mitogenic activity compared to the sum of activities in all of the hours combined with the mitogenic activity found remaining in the nerve fragments. The graph shows results from a representative experiment. Two independent follow-up studies showed similar results.

Figure 1.2 Mitogenic activity in conditioned media is blocked by FGF1 neutralizing antibody.

(A) Characterization of anti-FGF1 antibody in the AKR2B bioassay. Chicken anti-FGF1 (1:100) is tested against various concentrations of either FGF1 (left panel) or FGF2 (right panel). Note that the antibody significantly reduced FGF1 activity at all of the concentrations tested (error bars: SEM, n =3). The antibody partially inhibited of mitogenic activity stimulated by low concentrations of FGF2. All assays were performed in the presence of 2μg/ml heparin. **(B)** The nerve collection media was tested in the AKR2B assay in the presence of the FGF1 antibody. The mitogenic activity released from nerve fragments were significantly blocked by the FGF1 antibody (Stippled bars). At all the later time points (3 hours and beyond), the FGF1 antibody reduced ^{3}H-thymidine incorporation in the CM down to basal levels represented in the graph as zero percent. The graph shows results from a representative experiment. Two independent follow-up studies showed similar results.


A
Fraction of maximum response
1.2
1
0.8
0.6
0.4
0.2
0
0
0.2
1
5
FGF1 (ng/ml)
FGF2 (ng/ml)
FGF
FGF + FGF1 ab

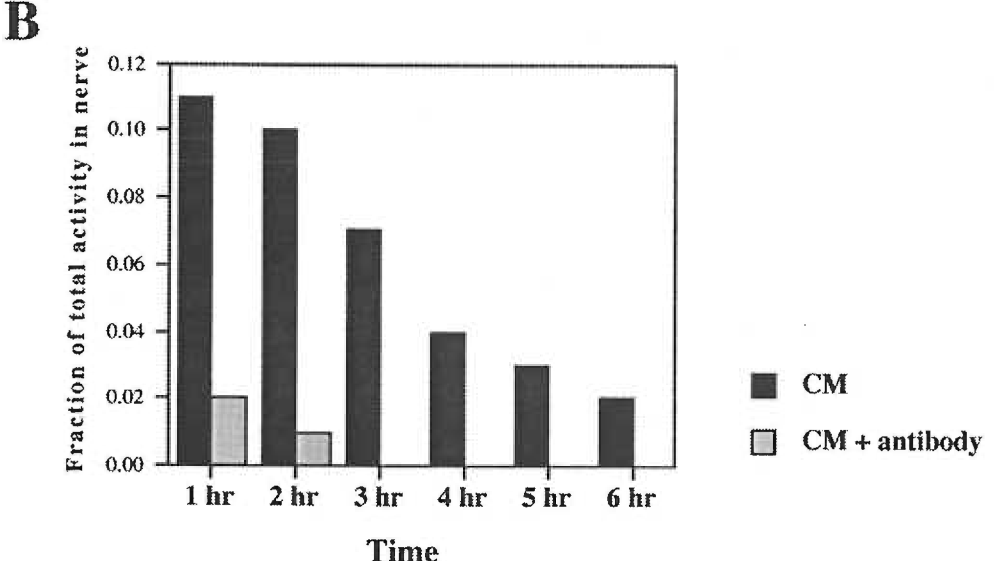
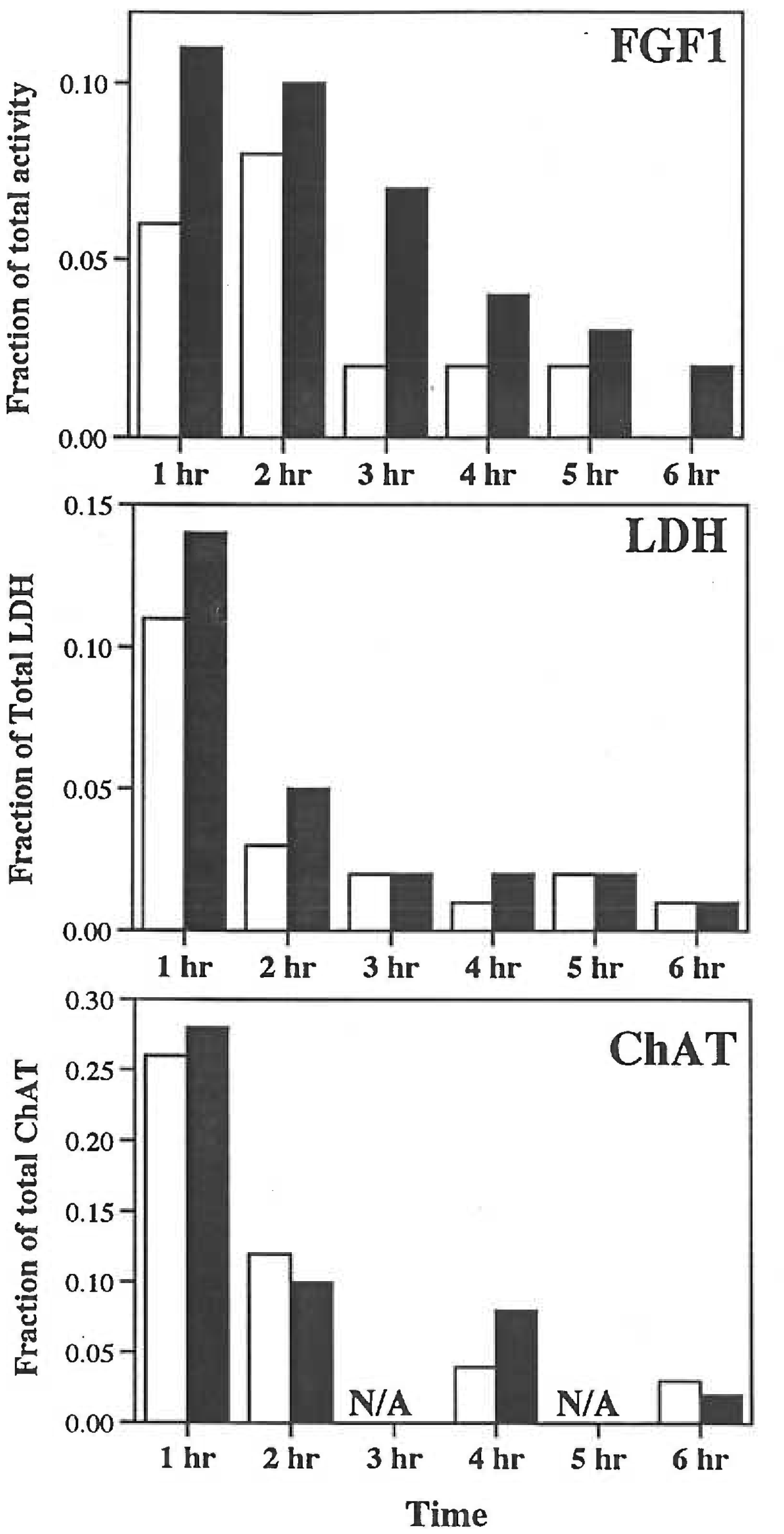

B
Fraction of total activity in nerve
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1 hr
2 hr
3 hr
4 hr
5 hr
6 hr
Time
CM
CM + antibody

Figure 1.3 Time course of cytoplasmic protein release from injured sciatic nerve.

Collection media (CM) from cultures of sciatic nerve fragments were collected every hour for six hours. The CM were then tested in the AKR2B bioassay to detect the presence of FGF1 (top graph), and in the LDH (middle graph) and ChAT (bottom graph) assays for the presence of these molecules. The same CM was tested in these three assays. Note that like FGF1, both LDH and ChAT had rapid release time courses. However, unlike FGF1, the release of these enzymes is not significantly altered by the presence of heparin in the collection media. Once again, the Y-axis represents the fraction of activity (FGF1, LDH, or ChAT) compared to the sum of activities in all of the hours combined with the activity found remaining in the nerve fragments. The graph shows results from a representative experiment. Two independent follow-up studies showed similar results.


FGF1
Fraction of total activity
0.10
0.05
0.00
1 hr
2 hr
3 hr
4 hr
5 hr
6 hr
LDH
Fraction of Total LDH
0.15
0.10
0.05
0.00
ChAT
Fraction of total ChAT
0.30
0.25
0.20
0.15
0.10
0.05
0.00
N/A
N/A
Time
No Heparin in CM
10μg/ml Heparin in CM

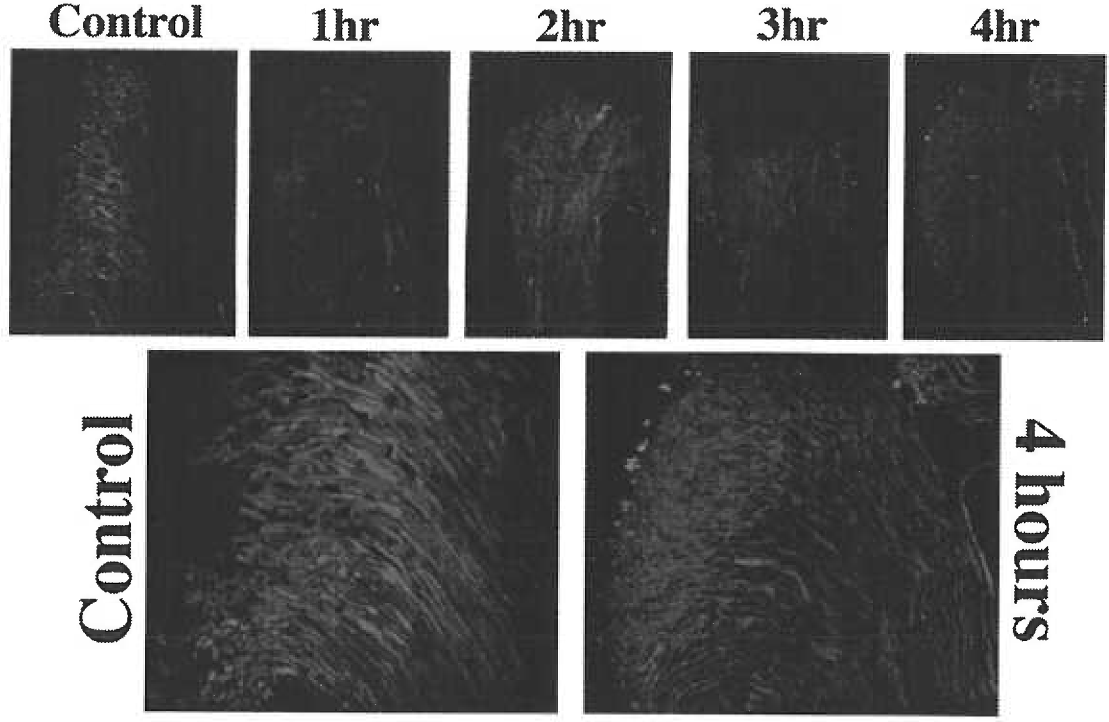


Figure 1.4 Rhodamine dextran is absorbed only by freshly damaged axons. Nerve fragments were incubated in 1% rhodamine dextran at different time points following injury, and the uptake of rhodamine dextran into axons is observed. **Top row:** Note that only the control nerve, which has just been freshly transected, has significant uptake of rhodamine dextran into the axons. In the other time points, only faint staining of the nerve ends are seen, and the stainings were limited to extracellular spaces. **Bottom row:** Magnified view of control and 4 hour nerve stumps. The incorporation of rhodamine dextran into the axons of the freshly transected nerve (control) can be easily seen. In the 4 hour nerve, on the other hand, staining is faint and limited to the extracellular space.

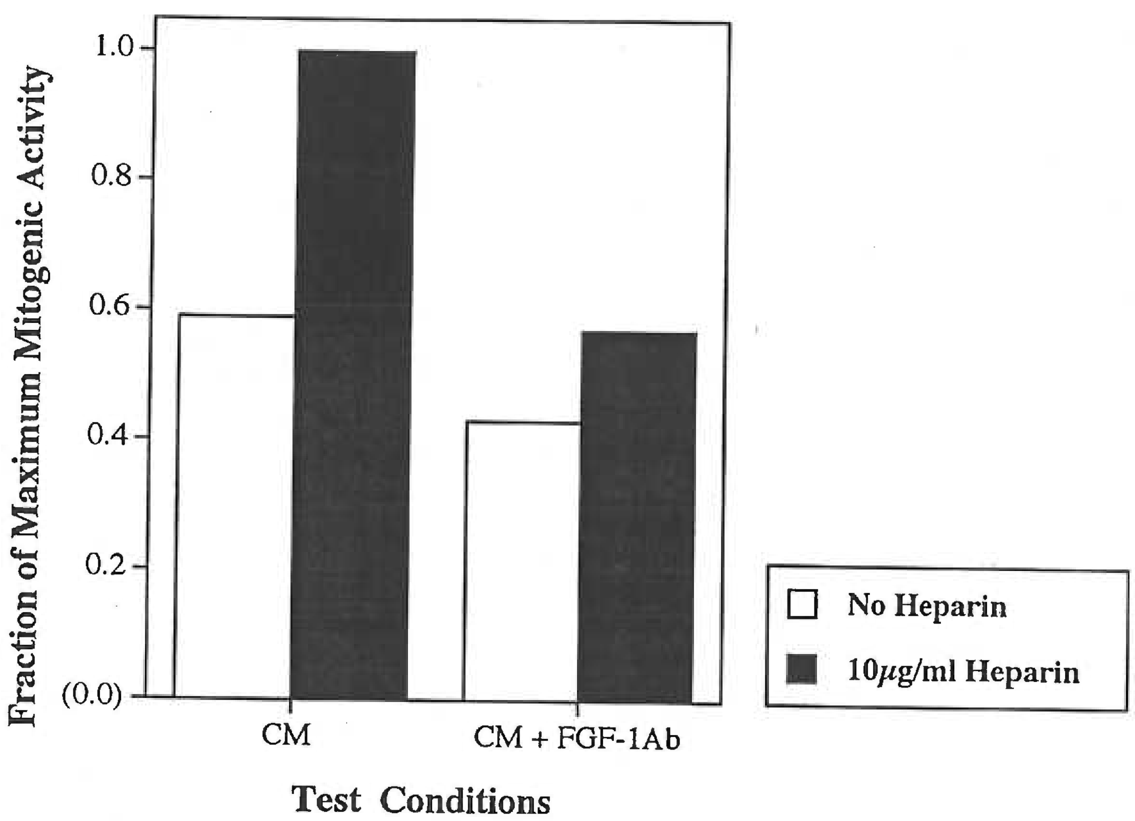


Figure 1.5 In vivo collection of FGF1 from transected sciatic nerve. In situ freshly-cut nerve stumps were immersed into collection media (CM) alone (open bars) or into CM with 10μg/ml heparin (black bars) for 1 hour. The CM was then tested for the presence of mitogenic activities in the AKR2B bioassay. The CM contained substantial mitogenic activity, with more mitogenic activity collected when heparin was included in the CM. Anti-FGF1 antibody was able to abolish a significant portion of the mitogenic activity. In the CM containing heparin, approximately 40% of the mitogenic activity was abolished by the antibody. In the CM without heparin, approximately 30% of the activity was abolished.

Chapter 2

Patterns of ERK and STAT3 Phosphorylation after Sciatic Nerve Transection Indicate Distinct Roles in Nerve Regeneration.

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Abstract

Peripheral nerve injury induces a specific pattern of expression of growth factors and cytokines, which regulate injury responses and regeneration. Distinct classes of growth factors and cytokines signal through specific intracellular phosphorylation cascades. For example, the ERK phosphorylation cascade mediates signaling through transmembrane tyrosine kinase receptors and the JAK/STAT cascade mediates signaling through the gp130 receptor complex. We tested whether specific phosphorylation patterns of ERK and STAT3 result from nerve injury and whether such phosphorylation correlates with the expression of specific growth factors and cytokines. At sites adjacent to a nerve transection, we observed that ERK phosphorylation peaked early, persisted throughout 16 days, and was equally intense at proximal and distal sites. In contrast, STAT3 phosphorylation peaked later than ERK but did not persist as long, and was stronger in the proximal than in the distal segment adjacent to the injury. In addition, in distal segments further away from the injury site, ERK became phosphorylated with a delayed time course, while STAT3 remained unphosphorylated. These patterns of phosphorylation correlated well with the expression of neurotrophin and Interleukin-6 mRNAs in the distal stump, and with the release of FGF1 and CNTF from injured axons and Schwann cells, respectively. Together, these observations suggest that ERK activation is more important than the activation of STAT3 in the establishment of a regeneration promoting extracellular environment in the far distal stump of transected nerves, and that STAT3 activation is important in the control of cellular responses close to the site of injury.

Introduction

Injury to the peripheral nerve initiates a well defined pattern of cellular changes distal to the site of damage (for reviews, see Hall, 1989 and Fawcett & Keynes, 19990). In the first few days following injury, axonal degeneration proceeds distally from the site of injury and the myelin sheaths that surround the degenerating axons are broken up into ovoids. Macrophages invade the nerve soon after injury and phagocytose the debris (Perry et al., 1987; Perry & Brown, 1992; Avellino et al., 1995). Schwann cells dedifferentiate and divide while staying within their basal laminae, producing cordons of Schwann cells known as bands of Bungner (Salzer & Bunge, 1980; Pellegrino et al., 1986; De Vries, 1993). These changes modify the nerve into an environment suitable for the regeneration of injured axons from areas just proximal to the damage. Specific sets of growth factors and cytokines are thought to regulate these cellular changes and to promote axonal regeneration.

Nerve injury induces the expression of neurotrophic factors such as NGF (Heumann et al., 1987; Lindholm et al., 1987), BDNF (Meyer et al., 1992; Funakoshi et al., 1993), and GDNF (Springer et al., 1994; Trupp et al., 1995), of Schwann cell mitogens and survival factors such as neuregulins (Carroll et al., 1997), and of pro-inflammatory cytokines such as IL-1 (Rotshenker et al., 1992), IL-6 (Bolin et al, 1995; Bourde et al, 1996; Reichert et al., 1996; Ito et al., 1998), and LIF (Curtis et al, 1994; Ito et al., 1998). In addition to the factors induced by injury, normal peripheral nerves already contain cytoplasmic stores of neurotrophic factors and Schwann cell mitogens such as FGF-1 (Eckenstein et al., 1991; Elde et al., 1991; Oellig et al., 1995) and CNTF (Stockli et al., 1991; Rende et al., 1992) that are poised to act when the nerve becomes injured. How this complex array of existing and upregulated factors interact to regulate axonal regeneration and other injury responses is not entirely clear, especially as there appears to be considerable overlap between the biological activities of some of the factors .

Specific intracellular protein kinase phosphorylation cascades mediate how cells respond to distinct growth factors and cytokines (Hill & Treisman, 1995). Although a particular kinase cascade is not used exclusively by a single growth factor, factors that stimulate related responses preferentially activate similar cascades. For example, the extracellular signal regulated kinase (ERK) members of the MAP kinase cascade are activated by growth factors that bind to transmembrane protein kinase receptors. This class of factors includes the neurotrophins, FGFs, and GDNF (Wang et al., 1994; Creuzet et al., 1995; Segal &

Greenberg, 1996; Worby et al., 1996; Creedon et al., 1997; Soler et al., 1999). Factors that bind the GP130 receptor complex—such as IL-6, LIF, and CNTF--activate the JAK/STAT pathway (Darnell et al., 1994; Kishimoto et al., 1994; Ihle et al., 1998). In addition, pro-inflammatory cytokines and cellular stress (such as heat shock or UV irradiation) transduce their signals through the stress activated protein kinase (SAPK) pathway (Kyriakis et al., 1994; Paul et al., 1997). The observation of the phosphorylation state of these distinct cascades in the injured nerve likely will reveal the integrated action of different classes of factors as they shape the cellular response to nerve injury.

Peripheral nerve transection represents an ideal tool to determine whether the injury results in distinct spatio-temporal activation of different phosphorylation cascades, and whether such patterns correlate with known cellular changes or the expression of specific sets of growth factors and cytokines. In order to address these questions, the present study quantified the extent of phosphorylation of ERKs, STAT3, and SAPK at the different sites along transected rat sciatic nerve at post-injury times ranging from 30 minutes to 16 days. The number of activated macrophages present and the levels of multiple growth factor (the neurotrophin family and GDNF) and cytokine (CNTF, IL-1 β and IL-6) mRNAs were measured in parallel samples to allow a comparison of intracellular signaling, regulation of gene expression, and cellular responses. The results demonstrate that injury induces distinct spatial and temporal patterns of activation of the individual signaling cascades. In the distal stump, these patterns of activation correlated well with the regulation of expression of the growth factors and cytokines assayed, but no such correlation was observed in the proximal stump.

Results

The goal of the present study was to determine whether specific patterns of activation of distinct intracellular kinase cascades are induced by nerve injury and whether such patterns correlate with changes in the expression of growth factors and cytokines. Antibodies that specifically recognize the phosphorylated forms of ERK, STAT3 and SAPK were used to quantify by western blot the spatial and temporal pattern of activation of these three distinct kinase cascades after sciatic nerve injury. For this, sciatic nerves of adult rats were transected, and four 3 mm long segments of nerve were harvested at different post-injury times. The segments are (see **Figure 2.1**): proximal segments 3-6 mm from the injury (segment A) the proximal stump (B), the distal stump (C), and the distal segment 3-6 mm from injury (D). In addition, multiprobe ribonuclease protection assays (RPA) analysis were conducted to measure the levels of neurotrophin, CNTF, GDNF, IL-1 and IL-6 mRNA levels in the injured nerve.

Distribution of phosphorylated ERK, STAT3, and SAPK

Rapid and pronounced phosphorylation of ERK, STAT3, and SAPK was observed in nerve segments immediately adjacent to the site of injury (segments B and C, **Figure 2.1**). All three molecules showed significant phosphorylation within 30 minutes after the lesion, but the subsequent time course of phosphorylation revealed some clear differences between the three cascades. For example, ERK phosphorylation reached near-maximum levels at 30 minutes, while STAT3 and SAPK phosphorylation increased more slowly and did not reach peak level until after 6 hours. Also, ERK phosphorylation remained high for up to 16 days post-injury, while STAT3 and SAPK phosphorylation began to decrease 8 days after injury. In addition, the time course and extent of ERK phosphorylation was very similar between segments B and C, while STAT3 and SAPK phosphorylation was more pronounced and prolonged in the proximal (segment B) than in the distal stump (segment C).

Only slight, statistically non-significant, increases in phosphorylation of STAT3 and SAPK were observed at sites further away from the injury (between 3 mm and 6 mm proximal or distal to the injury site, segments A and D). The time course of these small increases paralleled the changes observed in segments adjacent to the injury. In contrast, significant increases in ERK phosphorylation was observed in the far distal segment D, and this increase was delayed by at least 24 hours when compared to adjacent segment C. No significant change in ERK phosphorylation was observed in the far proximal segment A.

The increases in phosphorylation observed, especially at later time points, could either be due to an increase in the total amount of the signaling proteins (in which the fraction of the phosphorylated species remain constant while the overall level would increase), or due to an increase in the specific phosphorylation state of the proteins regardless of whether the amount of total proteins changes. To distinguish between these two possibilities, antibodies that recognized ERK, STAT3, and SAPK, regardless of their phosphorylation state, were used to observe the expression of these signaling molecules. In each of the four segments ERK, STAT3, and SAPK levels remained fairly constant throughout the time course studied (**Figures 2.1** and **2.2**). There were slight increases in the levels of STAT3 and SAPK at the later time points (e.g. 4 days in segment C), but these increases did not correlate with the increases in the phosphorylation level. This demonstrates that the changes reported above reflect changes of the phosphorylation state of ERK, STAT3 and SAPK.

In order to check the success of the transection injury, the amount of neurofilament, an axon marker, was measured on western blots (**Figure 2.1**). The level of neurofilament remained relatively constant throughout proximal segments A and B. In contrast, in the distal segments C and D, neurofilament levels began to diminish after 24 hours, were not detectable after 48 hours post-injury and did not reappear at the later time points, demonstrating that the transection was complete and that no axonal regeneration occurred in the distal stump.

Correlation of signaling activation with changes in gene expression

The expression patterns of a number of neurotrophic factors and cytokines in the injured nerve were studied in order to correlate the expression pattern of potential intercellular signals with the activation of intracellular signaling pathways . Messenger RNA levels for IL-1 beta, IL-6, CNTF, NGF, BDNF, and GDNF were measured using multi-probe ribonuclease protection assays (RPA). The amounts of RNA isolated from nerves were relatively small, thus segments A and B (the 6 mm of nerve proximal to the injury) and segments C and D (the distal 6 mm) were combined for analysis. All of the mRNAs species studied were upregulated following nerve injury, except CNTF mRNA, which decreased after the injury. Like the signaling molecule activations, these changes in mRNA showed distinct time courses and spatial distributions, with changes in the distal stump being markedly larger than those seen in the proximal stump (**Figure 2.3**).

Three temporally distinct waves of gene regulation were observed in the distal stump. The mRNAs coding for IL-1 beta, IL-6, and NGF were upregulated very rapidly following injury with a shared time course reaching maximum expression at 6 hours. This peak was short-lived and began to decrease by 24 hours. In contrast, GDNF mRNA levels increased with an intermediate time course starting at 24 hours, peaked at 2 days, and began to decline at 4 days. BDNF mRNA levels increased late (8 days after the injury) and remained high for at least another week (the last time point studied, **Figure 2.3**).

An interesting correlation emerged when the pattern of ERK phosphorylation was compared with the time course of induction of trophic factors known to activate the ERK cascade (NGF, GDNF and BDNF). Although none of the upregulated factors individually matched the prolonged activation of the ERK signaling cascade, together, the sum of the peaks of mRNA upregulation seem to fit the persistent ERK phosphorylation curve (**Figure 2.4**). Similarly, an examination of STAT3 phosphorylation and CNTF and IL-6 regulation revealed a striking correlation (**Figure 2.4**, distal stump): STAT3 phosphorylation increased just when IL-6 mRNA was induced (starting at 60 minutes), and STAT3 phosphorylation decreased when both CNTF and IL-6 mRNA levels diminished (beginning at 24 hours and completely by 2 days).

The proximal stump showed limited changes in the expression of the mRNAs studied. Only NGF, IL-1β, and IL-6 mRNAs were upregulated. These mRNAs still retained the rapid upregulation time course seen in the distal stump, however, the magnitude of expression in the proximal stump was reduced to about 50% of those seen in the distal stump (**Figure 2.3**). The mRNAs coding for GDNF and BDNF were not significantly induced in the proximal stump. This limited upregulation of trophic factor and cytokine mRNAs did not correlate with the robust and persistent phosphorylation of relevant signaling molecules. For example, when the pattern of ERK activation in the proximal segment was compared to the patterns of trophic factor regulation, ERK phosphorylation persisted throughout the 16 days while the trophic factor and cytokine mRNAs were only upregulated during the first day (**Figure 2.4**). Similarly, STAT3 phosphorylation in the proximal stump was more persistent than the duration of IL-6 expression and remained high while both IL-6 and CNTF decreased at the later time points. It is thus likely that growth factors and cytokines not included in the study (such as neuregulin and LIF) may be responsible for ERK and STAT3 activation in the proximal stump.

Correlation of Signaling Activation with Macrophage Invasion.
The distribution and time course of macrophage invasion was observed in the injured nerve in order to see if macrophage invasion correlated with the phosphorylation of ERK, STAT3 or SAPK. Macrophages were identified by immunofluorescence, using the macrophage specific antibody ED-1, and counted at 4 distinct locations along the nerve, at 1 and 4 mm away from the injury site in both the distal and proximal direction (**Figure 2.5**).

The number of macrophages did not increase in the nerve at 30 minutes after injury , which contrasts with the robust ERK, STAT3, and SAPK phosphorylation by this early time (**Figures 2.1** and **2.2**). The number of macrophages had begun to increase at 6 hours at all four of the locations studied, and generally continued to increase throughout 8 days after injury.

The distal segments demonstrated a greater increase in macrophage number than the proximal segments; this is in contrast to STAT3 and SAPK phosphorylation, which is more pronounced in the proximal stump. Overall, the pattern of increase in macrophage number correlated better with the upregulation of trophic factor and cytokine mRNAs, than with the time course of signaling molecule phosphorylation.

Discussion

Specific intracellular phosphorylation cascades mediate cellular responses to distinct extracellular stimuli, such as the response to growth factors and cytokines after nervous system injury. Ultimately, an injured cell's decision to grow, survive, differentiate, or die is controlled by the integrated action of all the different phosphorylation cascades. The present study demonstrates that sciatic nerve transection induces distinct spatial and temporal patterns of phosphorylation of ERK, STAT3 and SAPK, as demonstrated on western blots using phospho-ERK, phospho-STAT3 and phospho-SAPK specific antibodies. These antibodies could not be used successfully for immunohistochemical detection, thus the present study can not identify the specific cell types (Schwann cells, macrophages, endothelial cells, fibroblasts, or neuronal axons) that contain the phosphorylated signaling molecules.

Overall, STAT3 and SAPK showed a similar pattern of phosphorylation that was distinct from the pattern of ERK phosphorylation. An important difference between these signaling cascades is that at sites at least 3 mm distal from the injury, ERK alone showed significant phosphorylation, and that the delayed timing of ERK phosphorylation at distal sites correlated with axonal degeneration. In addition, ERK phosphorylation remained high both at the site of injury and at distal sites throughout the time course studied, while STAT3 and SAPK phosphorylation levels returned to almost baseline. Together, these observations suggest that the extracellular signals that activate the ERK cascade are of importance for inducing the cellular changes that transform the distal nerve tube into an excellent environment for regeneration. On the other hand, signals that activate the STAT3 and SAPK cascades likely are important regulators of cellular responses at the site of injury. These local responses include inflammatory mechanisms and the removal of debris. Interestingly, the level of STAT3 and SAPK phosphorylation was significantly higher and longer lasting in proximal than in distal areas adjacent to the injury. The main difference between these two areas is that the proximal stump contains a large number of axons that attempt, but fail, to regenerate, while all axons in the distal stump degenerate within 48 hours after transection. Therefore, the long-lasting high level of STAT3 and SAPK activation likely is due to the presence of the tips of these axons.

Previous work has identified a significant number of growth factors and cytokines that are involved in regulating the response of peripheral nerves to injury (Heumann et al., 1987; Rotshenker et al., 1992; Funakoshi et al., 1993; Curtis et al., 1994; Bolin et al., 1995;

Trupp et al., 1995; Bourde et al., 1996; Reichert et al., 1996; Carroll et al., 1997; Ito et al., 1998). It is important to define which classes of factors may activate the signaling pathways described above. Phosphorylation that occurs within 30 minutes after injury likely is due to non-protein stress signals or to growth factors and cytokines that are already present in the nerve prior to the lesion and do not require induction of gene expression. Normal adult peripheral nerves contain high levels of FGF1 and CNTF, which appear to be stored in cytoplasmic compartments of axons and Schwann cells respectively (Eckenstein et al., 1991; Elde et al., 1991; Stockli et al., 1991; Rende et al., 1992). Therefore, the rapid activation of ERK and STAT3 pathways at the site of lesion might be due to action of these factors which may be released after breach of the plasma membrane. In addition, the initial phase of ERK activation in far distal segments might be due to the release of FGF1 from degenerating axons. However, within a short time, disrupted plasma membranes will seal (Spira et al., 1993) or degeneration will proceed to completion, thus neither FGF1 nor CNTF are expected to be available in the extracellular space for prolonged time periods. On the other hand, growth factors and cytokines induced by nerve injury are more likely to provide such sustained signals. In this study, the mRNA levels coding for a few of these factors (NGF, BDNF, NT3, GDNF, CNTF, IL-6 and IL-1β) were quantified in order to test whether the regulation of expression of these potential signals correlated with the activation of corresponding intracellular signaling cascades. In the distal stump, the transient induction of IL-6 expression correlated well with the pattern of STAT3 phosphorylation, and the successive and overlapping induction of NGF, GDNF and BDNF expression correlated well with the overall sustained level of ERK activation. Clearly, a number of additional factors, including LIF (Curtis et al., 1994) may be involved in STAT3 activation, and neuregulin may be involved in ERK activation in the distal stump (Carroll et al., 1997; Kwon et al., 1997). In the proximal stump in contrast, the very moderate and transient induction of expression of the cytokines and growth factors assayed showed a dramatic lack of correlation with the intense phosphorylation of STAT3, SAPK and ERK. One possible explanation is that the precise factors and cytokines responsible for the phosphorylation of the signaling molecules (for example LIF or neuregulin) were not included in the multiprobe RPA used here. It is also possible that the cytokines and growth factors responsible for the sustained intense activation of intracellular signaling pathways in the proximal stump are released by the tips of axons that attempt to regenerate. The mRNA coding for these axonal factors would be present only in the neuronal cell bodies and not in the nerve, thus the present study would not have detected them.

None of the phospho-specific antibodies used for the present study allowed the unambiguous immunohistochemical localization of phosphorylated intracellular signaling molecules. This prevents any firm identification of the cell type containing the activated molecules. Nevertheless, the absence of axons from the distal stump 48 hours after the injury indicates that the high and sustained levels of ERK phosphorylation in the distal stump are not present within neuronal elements. In addition, the invasion of macrophages 4 mm distal to the injury is already significant at 6 hours after the injury, when ERK phosphorylation is still low at this site. This suggests that the delayed phosphorylation of ERK in the distal stump occurs in Schwann cells where activation of the ERK pathway likely controls the expression of genes important for regeneration.

In summary, the present study suggests that a sustained activation of the ERK cascade is important for inducing and maintaining a regeneration promoting environment in injured peripheral nerve. The sequential induction of expression and action of distinct growth factors may represent the mechanism that results in sustained ERK-activation in this tissue. The precise molecular identity of these growth factors remains to be established, but we hypothesize that FGF1 acts immediately after the injury, while neurotrophins, GDNF and neuregulins provide intermediate and sustained signals. In addition, growing axons themselves likely release or induce the expression of cytokines that activate the JAK/STAT or SAPK pathways. These cytokines might function to signal the arrival of regenerating axons in an injured nerve fragment.

Acknowledgment:

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Figure 2.1 Western blot analysis of ERK, STAT3; and SAPK phosphorylation along transected sciatic nerve

Top Drawing: Schematic diagram of transected nerve showing the segments collected for analysis. The asterix (*) denotes the location of the transection.

Lower panels: Result of Western blot analysis using antibodies that specifically detect phosphorylated ERK (P-ERK upper row, first set), phosphorylated STAT3 (P-STAT3, upper row, second set), and phosphorylated SAPK (P-SAPK, upper row, third set). Each row demonstrates the changes in phosphorylation levels at different time points (0-60 days) following the injury at the four locations analyzed. The lower rows (labeled ERK, STAT3, and SAPK) in each set show western blots probed with antibodies that recognize the three signaling molecules irrespective of their phosphorylation status. Each lane on the western blot represents a single animal. Note that the total levels of ERK, STAT3, and SAPK remain relatively constant after injury, while specific patterns of signaling molecule phosphorylation are induced. Overall, the patterns of STAT3 and SAPK phosphorylation are similar to each other and distinct from the pattern of ERK phosphorylation. The row of western blots shown at the bottom was probed with antibodies for the axonal marker neurofilament (NF), and results demonstrate that axonal degeneration in the distal stump is complete after 48 hours and that axonal regeneration is not detectable at later time points.

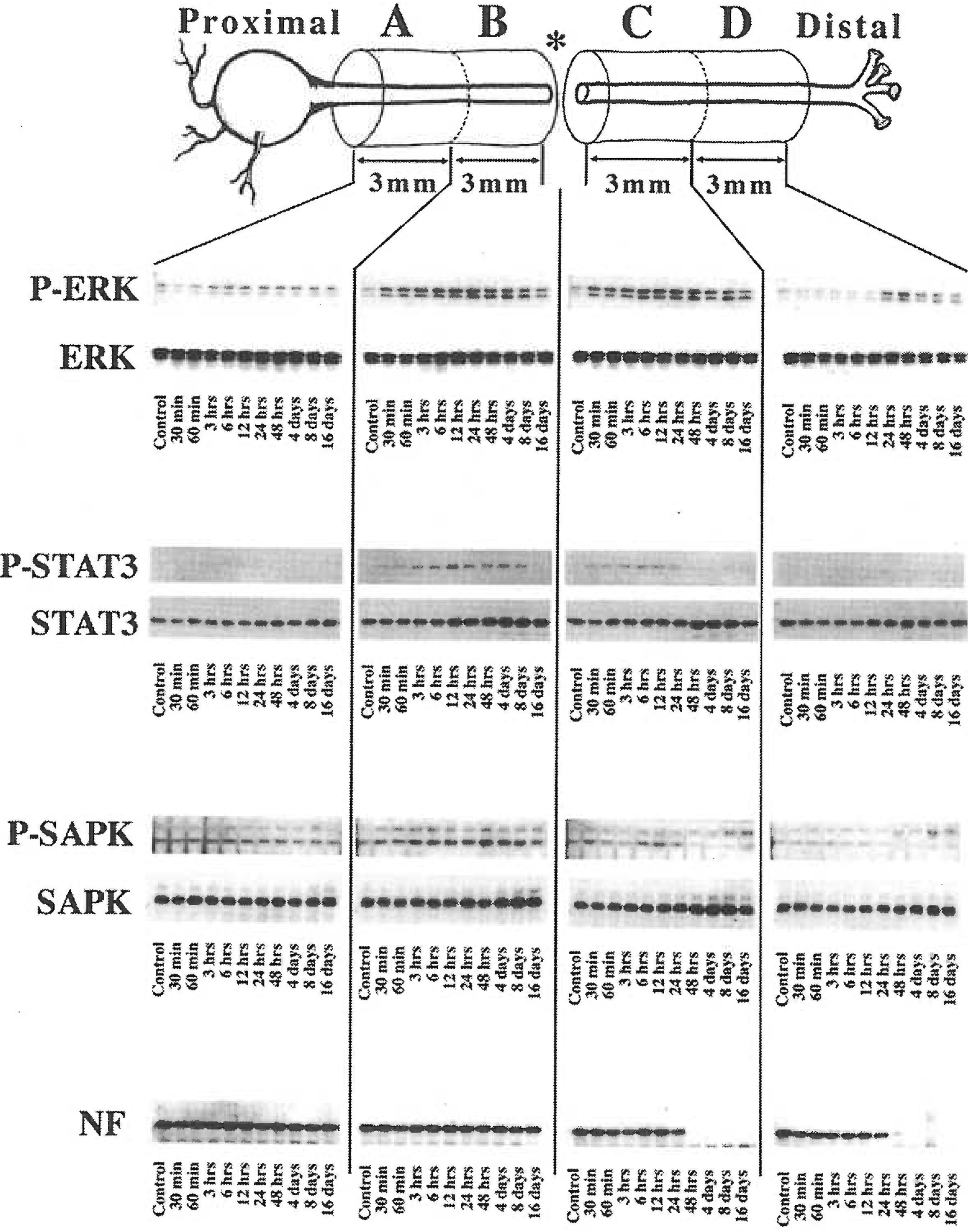

Proximal
A
B
*
C
D
Distal
3mm
3mm
3mm
3mm
P-ERK
ERK
P-STAT3
STAT3
P-SAPK
SAPK
NF
Control
30 min
60 min
3 hrs
6 hrs
12 hrs
24 hrs
48 hrs
4 days
8 days
16 days

Figure 2.2 Quantitative analysis of ERK, STAT3, and SAPK phosphorylation after nerve injury.

Top Drawing: Schematic diagram of transected nerve showing the segments collected for analysis. The asterix (*) denotes the location of the transection.

Lower Panels: intensity of signaling molecule phosphorylation at different time points (0-16 days) following the injury at the four locations indicated. The intensity of phosphorylation was measured by densitometry of western blots (see Figure 1) as described in the methods section. Values shown are the means (+/- SEM) of independent analyses of multiple animals (n = 4 animals (p-ERK, p-STAT3) or 2 animals (p-SAPK)). For each molecule analyzed, the mean data points with the highest densitometric measurement was assigned a value of 100% in order to present data in a unified format. Note that nerve transection initiates a rapid phosphorylation of ERK, STAT3, and SAPK in nerve segments adjacent to the injury (segments B and C), which is sustained for several days. At later time points, however, STAT3 and SAPK phosphorylation decline, while ERK phosphorylation remains near maximal for at least 16 days. Both STAT3 and SAPK are phosphorylated to a greater extent and more prolonged in the proximal segment (B) than the distal segment (C) adjacent to the injury. In addition, only ERK shows a delayed and sustained phosphorylation in the far distal segment (D).

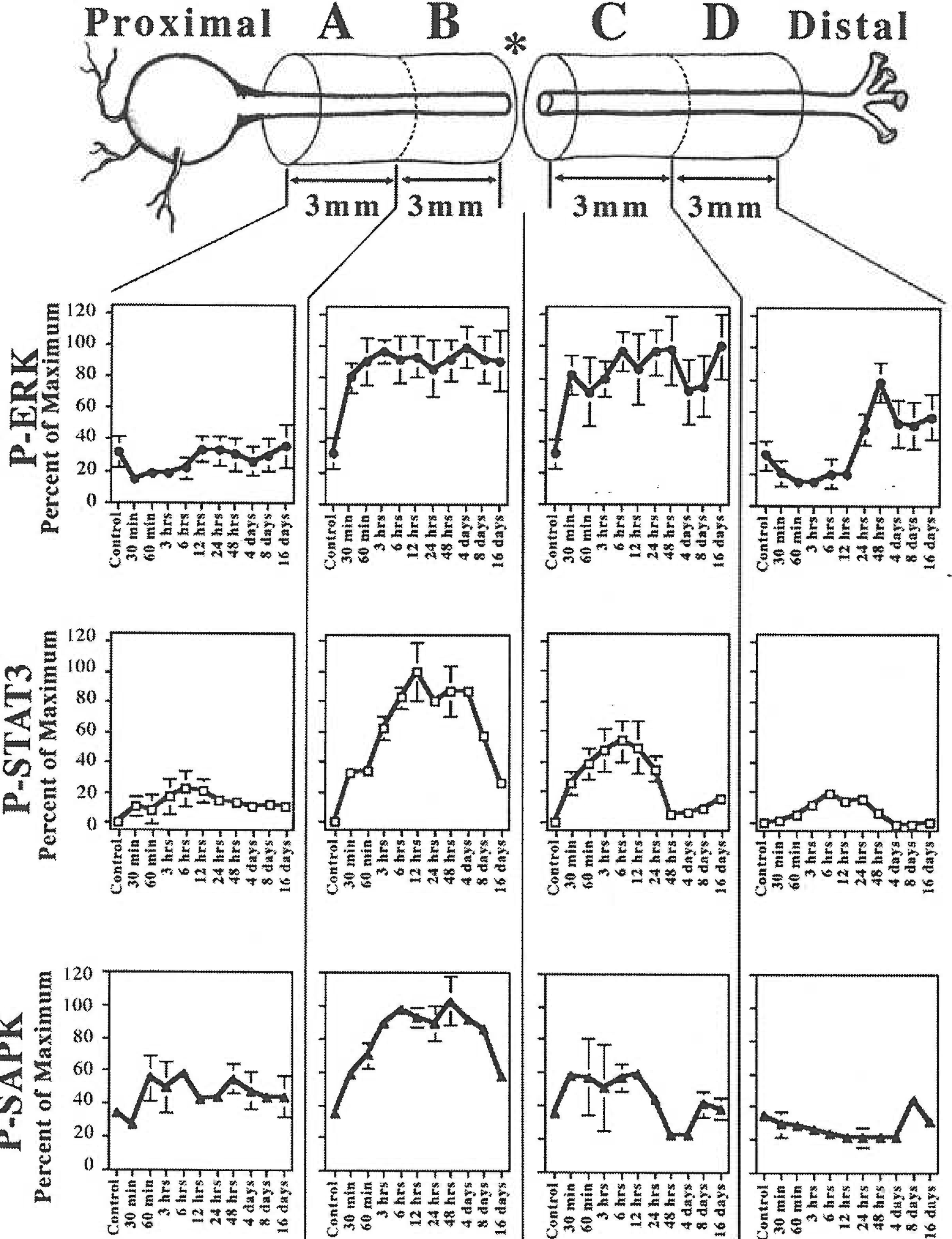

Proximal
A
B
*
C
D
Distal
3mm
3mm
3mm
3mm
P-ERK
Percent of Maximum
P-STAT3
Percent of Maximum
P-SAPK
Percent of Maximum
0
20
40
60
80
100
120
Control
30 min
60 min
3 hrs
6 hrs
12 hrs
24 hrs
48 hrs
4 days
8 days
16 days

Figure 2.3 Expression of neurotrophic factor and cytokine mRNAs in transected sciatic nerve.

Total RNA was isolated from 6 mm long nerve segments distal and 6 mm proximal to the transection, and expression patterns of the mRNAs indicated was analyzed by multiprobe ribonuclease protection assay (RPA). RNA isolated from the spleen of an animal injected with lipopolysaccharide was used as a positive control.

Top Panel: Multi-probe RPA showing bands corresponding to protected neurotrophic factor (NT) mRNAs. Note that only NGF expression is induced rapidly in both distal and proximal segments and that expression is sustained for at least 16 days. GDNF and BDNF expression is induced at intermediate or late time points, respectively, only in the distal stump. CNTF expression is significantly downregulated in the distal stump, and to a lesser degree in the proximal stump.

Bottom Panel: Multi-probe RPA showing bands corresponding to protected cytokine mRNAs. Note that of all the cytokines analyzed, IL-1β and IL-6 mRNAs show the most marked induction after injury. Both mRNAs are expressed rapidly after transection, in both distal and proximal stumps, and show significant downregulation one day later. The expression of mRNAs used for loading controls (L32 and GAPDH) was not affected by the injury.

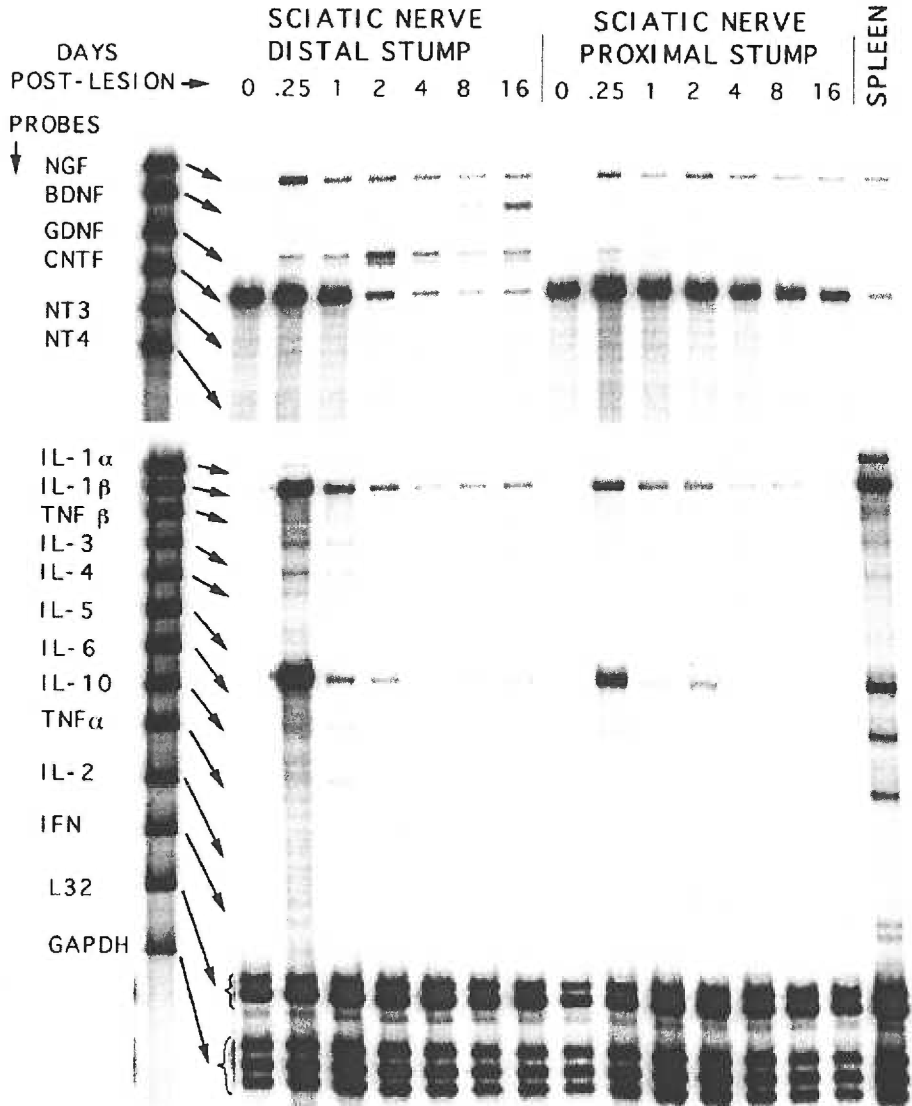
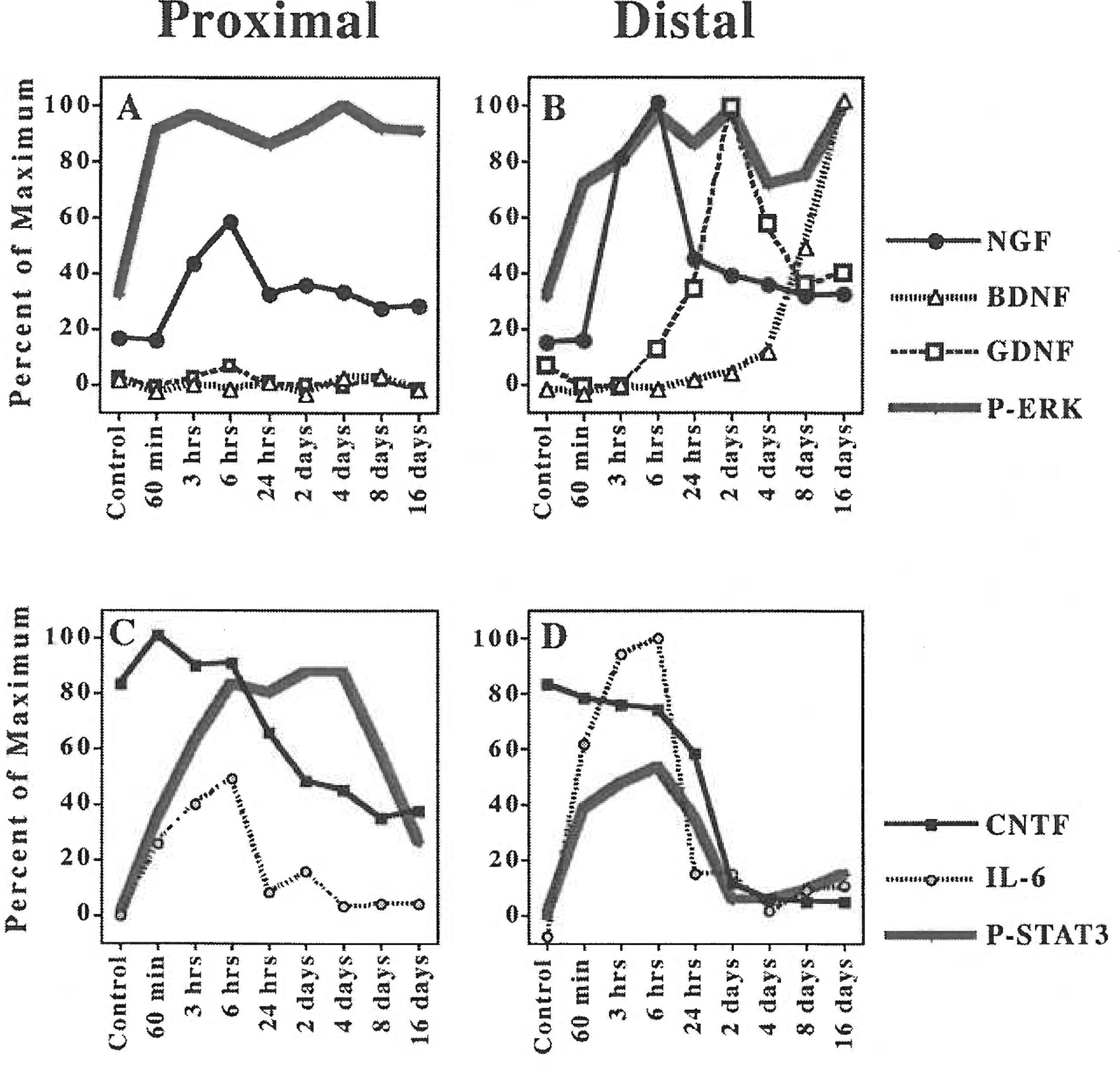

SCIATIC NERVE
DISTAL STUMP
SCIATIC NERVE
PROXIMAL STUMP
SPLEEN
DAYS
POST-LESION
0
.25
1
2
4
8
16
PROBES
NGF
BDNF
GDNF
CNTF
NT3
NT4
IL-1α
IL-1β
TNF β
IL-3
IL-4
IL-5
IL-6
IL-10
TNFα
IL-2
IFN
L32
GAPDH

Figure 2.4 Quantitative analysis of the expression patterns of neurotrophic factor and cytokine mRNAs, and correlation with ERK or STAT3 phosphorylation.

Multiprobe RPA data (such as those shown in Figure 1.3) were quantified using a phosphorimager and the NIH-image software package. For each mRNA analyzed, the data point with the highest densitometric measurement was assigned a value of 100% in order to present data in a unified format.

Top Panels: The expression patterns of that signal through transmembrane tyrosine kinase receptors and the ERK pathway are shown and the phosphorylation pattern of ERK is superimposed. Note that the sequential induction of expression of neurotrophic factors correlates well with the pattern of ERK phosphorylation in the distal stump (B), while no such correlation can be observed in the proximal stump (A).

Bottom Panels: The expression patterns of cytokines that signal through the gp130 receptor complex and the STAT3 pathway are shown and the phosphorylation pattern of STAT3 is superimposed. Note that the pattern of IL-6 expression correlates well with the pattern of STAT3 phosphorylation in the distal stump (D), while STAT3 phosphorylation is more prolonged than IL-6 expression in the proximal stump (C). In addition, CNTF expression is high in uninjured nerve, but STAT3 is not phosphorylated, demonstrating that CNTF does not signal in normal nerve.


Proximal
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Percent of Maximum
Percent of Maximum
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Control
60 min
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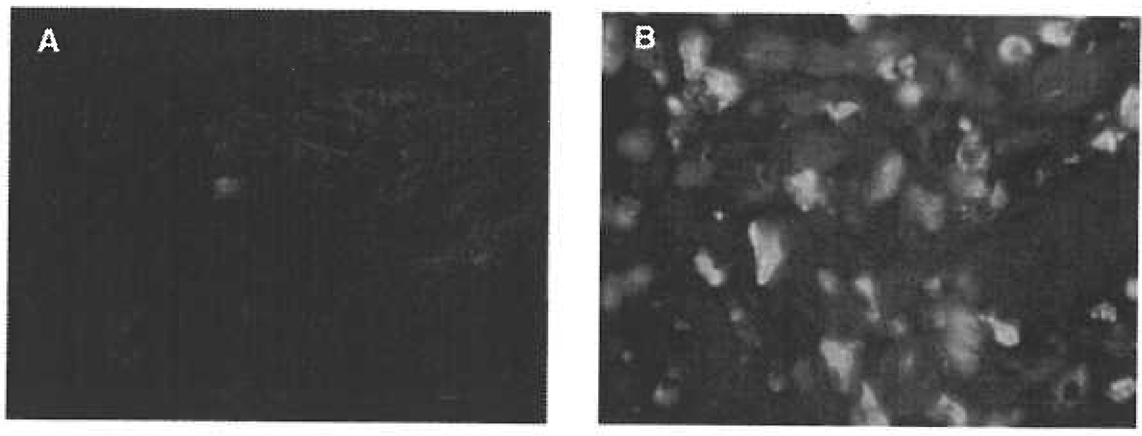
2 days
4 days
8 days
16 days
NGF
BDNF
GDNF
P-ERK
CNTF
IL-6
P-STAT3



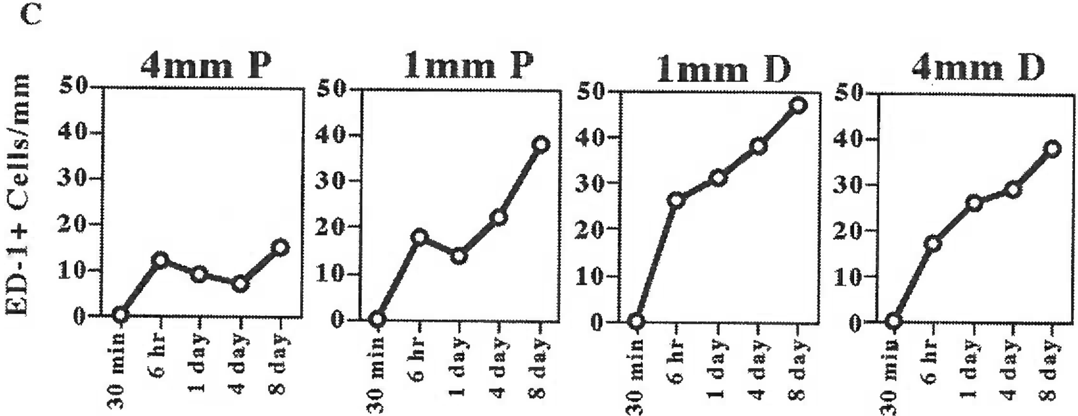


Figure 2.5 Macrophage invasion into transected sciatic nerve

Top Panels (A and B): Examples of ED-1 staining in sections of sciatic nerve. Panel A, non-injured nerve showing no detectable ED-1 staining. Panel B, section from 1 mm distal to the injury, taken 6 hours after transection, shows increased presence of ED-1 labeled cells.

Bottom Panes (C): The number of ED-1 positive cells was estimated by counting labeled cells in three fields of view each (160x final magnification) at sites 1 mm and 4 mm proximal (P) and distal (D) to the lesion. Note that no ED-1 positive cells are seen at any location 30 minutes after injury, but that significant numbers of labeled cells are present at all sites 6 hours after injury. In addition, the number of labeled cells continues to increase over the next week at all sites, except for the most proximal site analyzed.

Chapter 3

FGF1 Knockout Mice Demonstrate Reduced ERK Phosphorylation and Delayed Regeneration Following Sciatic Nerve Injury

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Abstract

Our previous observations showed that FGF1 is quickly released from damaged axons. In addition, we showed that ERK, a signaling pathway downstream of FGF1 action, is activated in the nerve following injury at locations and time points where axonal membrane damage occurs. Together, the data suggests that FGF1 is likely to play a role in peripheral nerve injury responses, and here we test this possibility by studying peripheral nerve regeneration in mice that lack a functional FGF1 gene. The FGF1 knockout mice developed, bred, and behaved identically to their wild type counterparts. While FGF1 knockout mice were identical to their wild type littermates under normal conditions, injury of the peripheral nerve demonstrated differences between the two groups of mice. At two time points following sciatic nerve transection, ERK activation in the FGF1 knockout mice was reduced at the distal segment adjacent to injury in comparison to the wild type. In addition, the functional recovery of hind limb function in FGF1 knockout mice following sciatic nerve crush was delayed. These data demonstrate that FGF1 functions as a injury factor in the peripheral nerve to regulate injury responses and to promote regeneration.

Introduction

Fibroblast growth factor 1 (FGF1) has a wide range of cellular targets in the nervous system. Central cells such as astrocytes, oligodendrocytes, and neurons, and peripheral cells such as Schwann cells and fibroblasts, all have been shown to express receptors and respond to FGF1 stimulation. However, FGF1 elicits different responses in different cells. In general, with non-neuronal cells--oligodendrocytes, Schwann cells, astrocytes--FGF1 induces proliferation and growth (Eccleston & Silberberg, 1985; Pettmann et al., 1985; Davis & Stroobant, 1990), while with neurons--motor, sensory, or sympathetic neurons--FGF1 has neurotrophic effects (Unsicker et al., 1987; Eckenstein et al., 1990; Sendtner et al., 1991). While these in vitro actions are confirmed and well-known, it is difficult to assign a physiological role for FGF1. The following question arises considering the biology of FGF1: What could be the role of a factor that can act on all the cells of the nervous system? Under what condition would a molecule that has these multiple effects be needed? It is possible that FGF1 has an injury response function, and by testing FGF1's fit into this role, we might be able to answer some of these questions.

Because FGF1 is not expressed in early nervous system development, it most likely does not play a role in nervous system development (Kuzis et al.,1995). Parallel studies of the vulnerability of neonatal neurons to peripheral injuries demonstrated a correlation between increases in FGF1 levels in these maturing neurons and their decreased susceptibility to neuronal death (Kuzis et al., 1999). These studies suggests that FGF1 might play a neuroprotective role following peripheral nerve injury.

It has been well established that FGF1 is present in peripheral axons (Eckenstein et al.,1991; Elde et al., 1991; Oellig et al., 1995). This localization of FGF1 suggests that the peripheral nerve is FGF1's location of action. We have performed two sets of studies that provided supporting evidence. First, we showed that following nerve injury, FGF1 was released from damaged axons and that passive diffusion through breaches in the membrane is a likely mechanism. We also showed that following injury, signaling cascades downstream of FGF are activated in temporal and spatial patterns that correlate with breaches in axonal membrane. These observations suggest the possibility that the release of FGF1 following injury might have important functions for the repair of the system.

Because FGF1 has neurotrophic effects on the neurons of the peripheral nervous system (Eckenstein et al., 1990), FGF1 could act as a temporary replacement neurotrophic factor

for neurons that have lost their contact with their peripheral targets. If this is the case, the removal of FGF1 from this system will result in lack of trophic support for injured neurons, and many of these neurons might die. Alternatively, FGF1 might be an injury signal that stimulates non-neuronal cells along the pathway of the regenerating axons to produce growth promoting substances. For example, FGF1 has been shown in several studies to stimulate NGF production in astrocytes, establishing a precedence of trophic factor upregulation by FGF1 (Ono et al., 1991; Yoshida & Gage, 1991; Yoshida & Gage 1992; Figueiredo et al., 1995). FGF1 might signal through ERK to induce the production of molecules necessary for axonal regeneration.

If FGF1 is required for peripheral axon regeneration, then the removal of FGF1 from this system will result in the lack of the necessary molecules for axonal regeneration, and axonal regeneration might be impaired, or even fail. To test the role of FGF1 in nerve regeneration, we studied peripheral nerve responses to injury in FGF1 knockout mice. First, we tested whether FGF1's downstream signaling is affected in the knockouts. To achieve this, we studied the time course and magnitude of ERK activation in the injured nerves of the FGF1 knockout mice. Second, as the ultimate test of the FGF1 hypothesis, we studied the biological consequences of FGF1's absence in nerve injury by measuring the rates of functional regeneration in the crushed sciatic nerve of the FGF1 knockout animals.

Results

Generation and characterization of FGF1 knockout mice

The FGF1 knockout mice were created by the laboratory of Dr. Claudio Basilico (Miller et al., 2000). The knockout genotype was generated by replacing a segment of the FGF1 gene with a neomycin-containing DNA fragment via homologous recombination (**Figure 3.1A**). Details of the generation of the knockout mice are described fully in Miller et al. (2000).

In their initial characterization of the FGF1 knockout mice, C. Basilico and colleagues confirmed the absence of FGF1 protein in the knockout animals through Western analysis of various tissues (Miller et al., 2000). Analysis of whole-cell extracts from brain and heart showed that FGF1 was present in wild type and FGF1+/- animals but absent in the FGF1 knockout animals. Heparin-sepharose was used to concentrate FGF present in various tissues, and in these concentrates, FGF1 was undetectable in up to 5 mg of concentrate from the FGF1 null mice. This absence of FGF1 was observed in the FGF1 null mice in a range of tissues, including skull, long bone, liver, spleen, lung, skeletal muscle, kidney, testis, and eye (Miller et al., 2000). Histopathological examination of a range of tissues by Miller et al. also showed that FGF1 knockout animals did not have any defects. In addition, there were no disorders in elderly FGF1 knockout animals, suggesting that there are no long-term effects of FGF1 absence (Miller et al., 2000).

In our own laboratory, Southern blot analysis was performed to confirm the FGF knockout genotype (**Figure 3.1B**), and the knockout genotype was further confirmed with Western blotting of brain tissue extract using FGF1 antibodies (**Figure 3.1C**). The crossing of FGF1 heterozygotes produced wild type, heterozygous, and FGF1 knockout pups in frequencies that corresponded with the predicted Mendelian ratios, suggesting that the FGF1 null genotype did not cause embryonic lethality. In agreement with the observations made by Miller et al. (2000), the gross appearance--such as body weight and brain size--and behavior of FGF-1 knockout mice did not differ from their wild type littermates.

FGF2 is not upregulated to compensate for the absence of FGF1

It is possible that the lack of FGF1 was compensated by the upregulation of other FGF members. To test this possibility, we studied the level of FGF2--the FGF member with the highest homology to FGF1--in the FGF1 knockouts to detect any compensatory increases. Using western blotting, we observed no significant alteration of FGF2 level in brain

extracts of the FGF1 knockout mice (**Figure 3.1C**). These observation supports the observation by Miller et al. (2000) that FGF2 is not upregulated in the absence of FGF1. Additional studies showed that the expression of another FGF family member (FGF9) and the FGF receptor 1 also were not upregulated in the knockout mice. These results suggest that the lack of abnormal phenotypes in the knockout is not due to the upregulation of FGF2 and the FGF receptor.

ERK phosphorylation in FGF1 -/- injured sciatic nerve is reduced

The observation of normal phenotypes in FGF1 knockout mice matched well with our model of FGF1 action which predicts that FGF1 is important only following injury to the nervous system. Because FGF1 expression is high in the peripheral nerves, we sought to test the effect of its absence in the regeneration of peripheral axons.

Our previous observation of signaling cascades suggests that ERK activation after nerve injury is likely due to FGF1. ERK is quickly activated at the site of injury and after a delay of two days in the far distal portions where axons were undergoing degeneration. The time and location of ERK activation suggest that ERK activation is coupled closely with the breakdown of axons, where FGF1 is released. To test this hypothesis, we studied the pattern of signaling activation in the injured sciatic nerves of the FGF1 knockout mice.

Sciatic nerves of FGF1 knockout mice and their wild type littermates were transected, and at three time points (60 min, 2 days, and 8 days) various segments of the injured nerve were collected, lysed, and analyzed on Western blots. The Western blots were probed with antibodies against phospho-specific ERK antibody. The same blots were subsequently re-probed with ERK antibody to allow the analysis of the results as a P-ERK/ERK ratio.

The results from this set of studies were similar to the results obtained from our earlier studies in the rat (see **Figure 2.1**). The wild type nerve segments duplicated the typical phospho-ERK stainings that we had obtained from rat tissues, with rapid activation of the ERK in the segments adjacent to the injury site, and delayed ERK activation in the far distal segment (segment D). Nerve segments from the FGF1 knockout mice also demonstrated these typical phospho-ERK patterns (**Figure 3.2**). While the general pattern of ERK activation in the knockout nerves looked similar to those of the wild type, there were notable and statistically significant differences in the magnitude of ERK activation. Data points from three independent experiments were analyzed using un-paired t-tests. The results revealed two data points in which the wild type and knockout ERK activation were

significantly different. On both the 60 minute and the 8 day time points, ERK activation in segment C was significantly lower in the knockout nerve, with nearly 50% reduction and 35% reduction at 60 minutes and 8 days, respectively (based on densitometric measurement; **Figure 3.3**). While at other time points ERK activation also appeared to be lower in the knockout nerve, such as segment A at 60 minutes, statistical analysis did not reveal any significant differences.

Control nerves from the knockout mice showed basal ERK activation similar to those of the wild type mice (Data not shown), suggesting that there were no differences in uninjured nerves, and that the differences were truly due to events following injury.

Functional regeneration in FGF1 knockout mice is delayed

The ultimate measure of axonal regeneration is the successful return of function after injury. Therefore, the functional recovery of injured sciatic nerve in the knockout mice was studied using a functional assay for motor function recovery, the Sciatic Functional Index (SFI). The SFI measures the rate of motor function recovery following a sciatic nerve crush injury (de Medinaceli et al., 1982; Lowdon et al., 1988; Bain et al., 1989; Inserra et al., 1998). Because the epineurial sheath that surrounds the nerve remains intact after a crush injury, the pathway for the transected axons to regenerate is maintained, allowing the transected axons to grow and re-establish contact with their peripheral targets. To study functional regeneration using the SFI, the sciatic nerve in one side of the animal was crushed, and on different post-injury days, prints of the hind paws were recorded as the animals walked along an enclosed walk way. The foot prints were recorded on pre-stained paper that turned dark blue upon exposure to moisture on the animals' hind feet (**Figure 3.4A**). The recorded footprints clearly showed the recovery of the hind paw function through time (**Figure 3.4A**). To obtain a reliable interpretation of the rates of recovery, for each animal, footprints from the injured side were compared to the footprints on the contralateral (control) side using the following parameters: paw length, total toe spread, and intermediate toe spread (**Figure 3.3A**). Analysis was completed by entering these measurements into SFI equations to arrive at a numerical representation of functional recovery (with control scores at zero and lesioned scores in the range of -100; de Medinaceli et al., 1982; Bain et al., 1989).

The SFI analysis demonstrated that regeneration of the motor axons in the sciatic nerve were delayed in the FGF1 knockout mice (**Figure 3.3B**). Both the FGF1 knockouts and their wild type littermates showed drastic reduction in their SFI score typical of a crush

injury in the first few days. The SFI score remained low for the next 10 days. After that point, the SFI scores for the wild type animals quickly increased towards control levels such that by day 18, the SFI for the wild type animals returned to their pre-injury level. Throughout the course of this recovery, the FGF1 knockout mice lagged behind the recovery of the wild type mice. For example on day 11 where the SFI score for the wild types were in the range of -70 to -80, the SFI score for the knockouts were still in the range of -110. On day 12, where the SFI score for the wild types were in the range of -50, the score for the knockouts were still in the -80 to -90. The differences between the wild type and knockout SFIs on days 11, 12, and 13 were statistically significant. On later days the average SFI scores for the knockouts also appeared lower their wild type counter parts'. However, these apparent differences were not statistically significant, and can only be considered a trend that might warrant future, more refined investigations.

Discussion

Considerations of FGF1's characteristics lead us to hypothesize that FGF1 plays a role in injury response and regeneration. To test this hypothesis, we characterized the phenotype of the FGF1 knockout mouse following peripheral nerve injury. These observations demonstrate that in FGF1 knockout mice nerve injury response is altered and functional regeneration is impaired.

Observations made by Claudio Basilico and colleagues showed that under normal conditions, the FGF1 knockout mice develop, grow, behave, and breed normally (Miller et al., 2000). This is unlike the FGF2 knockout mice which showed abnormalities in blood pressure regulation and wound healing (Ortega et al., 1998). As we established our own FGF1 knockout colony and began to breed them, we have also come to the same conclusion that phenotypically, there were no detectable differences between the FGF1 knockouts and their wild type littermates.

The fact that FGF1 knockouts have normal phenotype was consistent with our hypothesis that FGF1 only plays a role after nervous system injury. Previous studies from our laboratory had shown that FGF1 is not expressed in the rodent until the day of birth (Kuzis et al., 1995), and that the greatest level of FGF1 expression in the neurons is not obtained until 3 weeks later. This temporal expression pattern precludes FGF1 from playing a role during the development of the nervous system, and suggests that FGF1's actions are limited to the adult. In addition, unlike growth factors that are secreted, FGF1 lacks signal peptide and appears to not be secreted under normal conditions. In a previous chapter, we showed that FGF1 is released from injured axons, demonstrating that mechanical injury is a way to release it from its cytoplasmic stores. In addition, our studies of injury response suggest that a rapidly released factor was responsible for activating the ERK cascade in the nerve. Together, these facts suggest that FGF1 plays a role after nervous system injury, and therefore, FGF1 knockouts are not likely to have any aberrant phenotype under normal conditions. We would, however, expect the FGF1 knockouts to be different in its injury response and axonal regeneration in comparison to their wild type counter parts.

To test the effect of FGF1's absence on the nerve's response to injury, we studied the pattern of ERK activation in FGF1-/- nerves. In the previous chapter, we showed that ERK is quickly activated at locations where axonal membranes were either damaged or undergoing disintegration (**Figures 2.1** and **2.2**). The location and timing of ERK

activation suggest that FGF1 is responsible for these activations. Therefore, we performed an analysis of ERK phosphorylation in the injured sciatic nerves of the FGF1 knockout mice and their wild type littermates. Our initial observation of ERK phosphorylation in the knockout nerves demonstrated that these nerves have ERK activation patterns similar to the wild type nerves. However, when we analyzed the magnitude of ERK phosphorylation for the different time points and locations, we found that at two distinct time points the distal stump (segment C) demonstrated decreased ERK signaling.

At both 60 minutes and 8 days after injury, segment C in FGF1 -/- nerves showed reduced ERK phosphorylation (**Figure 3.2** and **3.3**). This suggests that in this segment, the ERK phosphorylation observed in wild type injured nerves are either directly or indirectly due to FGF1. However, these results give rise to several major questions. Segment C is directly adjacent to injury, and contains axonal membrane damaged by the transection through which FGF1 can leak out. This could explain the ERK phosphorylation at the 60 minute time point, where FGF1 is spilling out from freshly transected axons. The puzzling issue is why is ERK signaling affected only in segment C and not in segment B? Like segment C, segment B is also adjacent to the injury. However, unlike segment C, segment B contains axons that are still connected to the neuronal cell bodies. The kinetics of axon resealing--a parameter that might be important in the release of FGF1--could be different between these two stumps. Studies of axon resealing in Aplysia neurons have shown that the rate of axon resealing is identical in the proximal and distal cut ends (Spira et al., 1993). However, subsequent studies of Ca^{++} distribution showed that Ca^{++} diffusion is more extensive in the distal stumps (Ziv & Spira, 1993). Therefore, even though the rates of axon resealing is the same for both proximal and distal cut sites, the difference in Ca^{++} diffusion could provide a mechanism for the preferential release of FGF1 in the distal. A study by Maciag and colleagues suggests that FGF1 is associated with the Ca^{++} binding protein S100A13 in their in vitro release assay (Carreira et al., 1998), providing a hint that the difference in Ca^{++} diffusion into the cut ends might provide a mechanism for the preferential release of FGF1 at the distal tip. The findings of reduced ERK activation not only suggests that FGF1 is directly involved in the early injury responses in the nerve, they also suggest that FGF1 is preferentially released from the distal stump at the injury site.

Another interesting observation from the ERK activation study is that FGF1 appears to be responsible for the activation of ERK in segment C on day 8. This is a late time for FGF1 to exert its influence because as our laboratory had shown previously, FGF1 levels in transected distal stumps are nearly abolished by 3 days after injury (Eckenstein et al.,

1991). Therefore, FGF1 could not be present to stimulate the ERK phosphorylation. However, as we have suggested above, FGF1 might upregulate the expression of several growth factors in the injured nerve (Ono et al., 1991; Yoshida & Gage, 1991; Figueiredo et al., 1995). In fact, as we have shown in chapter 2 of this document, several trophic factors are upregulated in the distal stump of the nerve, and these seem to correlate with the persistent ERK activation. Taking these facts into account, it is possible that the reduced ERK activation seen in the knockout nerve could have been caused by factors upregulated by FGF1.

Despite FGF1's potential role in causing the ERK activation in the nerve, ERK phosphorylation is still significantly increased after injury in the FGF1 knockout mice. This suggests that even in the absence of FGF1, many other molecules are still activating ERKs and possibly proceeding with the repair of the damaged nerve. Growth factors from the vasculature, such as platelet-derived growth factor (PDGF), could potentially increase ERK phosphorylation at these sites. To understand the impact of FGF1's absence on nerve regeneration, we proceeded to study functional regeneration in the knockout mice.

We used sciatic functional index (SFI) as an assessment of functional recovery following a crush injury to the sciatic nerve (de Medinaceli et al, 1982). In the first 10 days after injury, both the wild type and knockout mice showed the same level of functional deficiencies (**Figure 3.4**). However, starting on day 11, the wild type mice began showing improvements in their hind limb functions that were significantly better than those of the knockout mice; these statistically significant differences persisted through days 12 and 13. On these 3 days, the SFI for the knockout mice also improved but at a slower rate. By 15 days post injury, the wild type SFI were indistinguishable from control SFIs. The knockout animals also showed SFI scores that were close to normal levels, but there is a trend of lower average scores than the wild types at all time points. Because these differences were not statistically significant, we only viewed it as a trend and not as a bona fide difference. In all, the SFI results showed that FGF1 -/- nerves have delayed regeneration but eventually regain their function to almost the same degree as their wild type counterparts.

The SFI data suggests that while the FGF1 might affect the regeneration process in some respect, its absence does not preclude functional regeneration from taking place. The process of regeneration takes the coordination of several different processes--such as Schwann cell division, axonal outgrowth, pathfinding, myelination--and when one of these

processes is adversely affected by the lack of FGF1, regeneration can still take place to a degree that will still confer the functions measured by the SFI. While the SFI is an excellent measurement for the gross return of motor function in the hind paws, it's conceivable that it might not detect the subtle defects. For example, there could be a difference in the total number of motor axons that regrow to the target. This reduced number of regenerated axons could be masked by each axon's increased innervation of muscle fibers to provide in the end a relatively normal level of contractile force. Subtleties of reinnervation differences might not be detected by the gross measurements of how far apart are the toes spread, or how the calf muscles raise the heel to only allow the ball of the hind foot to reach the ground. It will be necessary to further dissect these observed impairments using histological methods in order to understand the mechanisms of FGF1's contribution to regeneration. The data from SFI provides good clues about the time points most pertinent for further study.

To observe these subtle differences we could perform histological analysis of both the nerve and the spinal cord neuronal cell bodies. These analysis, such as counting the number of motor axons in the distal stump or the retrograde labeling of motor neuron cell bodies could reveal whether relatively similar number of motor axons successfully regenerate in the FGF1 -/- and the wild type nerve. In addition, electrophysiological measurement of nerve conduction velocities could detect a difference in conduction velocity or amplitude. These further studies should give more insights into our observations of the deficiencies in regeneration in the FGF1 knockout mice.

Acknowledgement.

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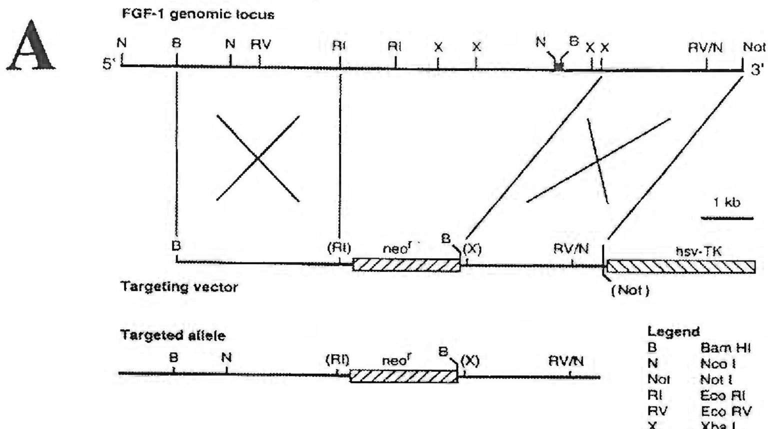
Figure 3.1 Generation and characterization of FGF1 knockout mice.

(A) Generation and targeting of the replacement vector. The pPNT replacement vector was used to construct a targeting plasmid to delete approximately 5 kb of genomic DNA which includes the entire first coding exon of FGF1.

(B) Genotyping of FGF1 knockout animals by Southern blot. DNA was isolated from the tails of 8 wild type and 8 FGF1 knockout littermates (animals were pre-screened by PCR). Five μg of DNA was digested with BamH1 and analyzed by Southern blot using an FGF1 probe. Bands of the predicted size (7.0 kb for wild type and 4.4 kb for knockout animals) were observed. The lane on the far right contains DNA isolated from the heterozygous mother of some of the offspring.

(C) Western blot characterization of FGF1, FGF2, FGF9, and FGFR1 expression in control, FGF1, and FGF2 knockout animals. Four independent panels of Western blots are shown. Protein was extracted from the brain of control (lane 1), FGF1 knockout (lane 2), and FGF2 knockout (lane 3) animals. Extracts were enriched for FGFs by heparin chromatography or for FGFRs by wheat germ agglutinin chromatography. Equal amounts of protein were loaded and probed with antibodies specific for FGF1, FGF2, FGF9, or FGFR1. Note that FGF1 was absent in FGF1 knockouts. Also note that FGF2, FGF9, and FGFR1 levels were unchanged in the FGF1 knockout animals.

(D) Ribonuclease protection assay of FGF1 mRNA expression in control and FGF1 knockout mice. The protected band indicating the level of FGF1 mRNA is 118 bases long. The results shown here demonstrates the abundant expression of FGF1 mRNA in heart (lane 1), midbrain (3), and spinal cord (4), while cerebral cortex (2) and spleen (5) contain only small amounts of the message. No FGF1 mRNA is detected in the same tissues of the FGF1 knockout mice (lanes 6 - 10).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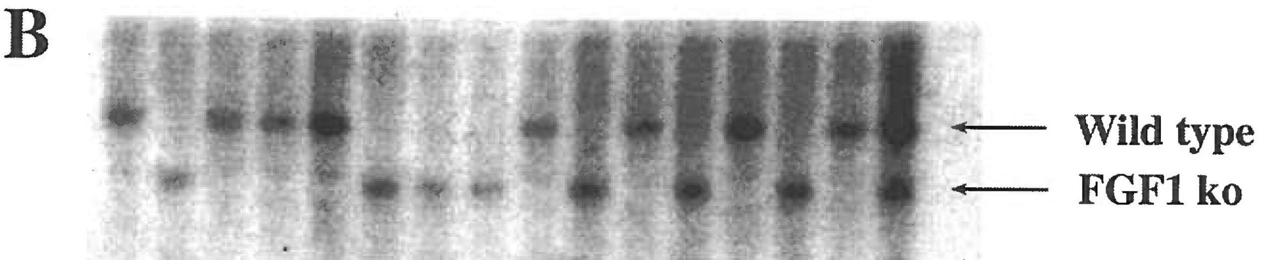
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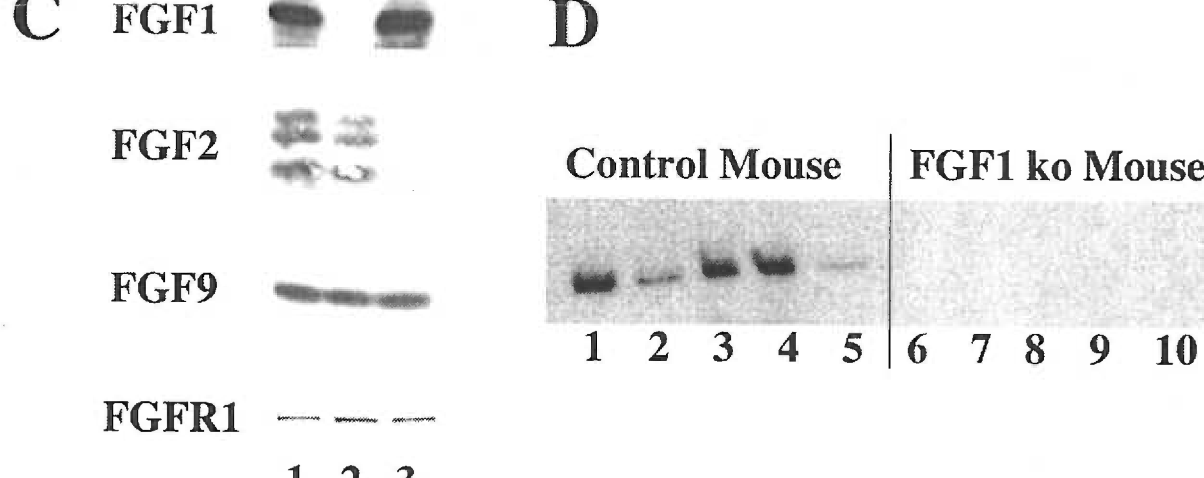
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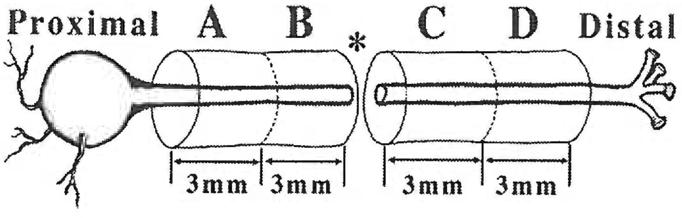


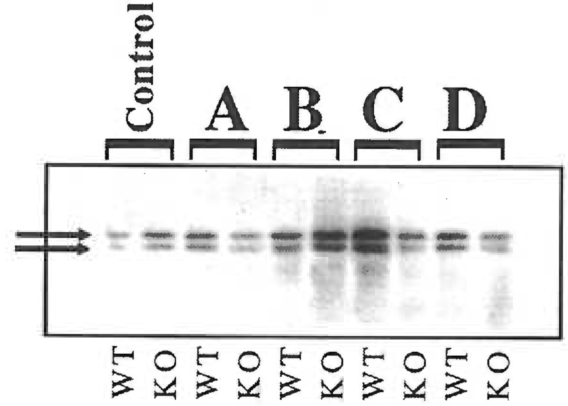


Figure 3.2 Western blot of phospho-specific ERK at various locations in the injured sciatic nerve

Transected sciatic nerve from wild type (WT) and FGF1 knockout (KO) animals were collected 60 minutes after transection injury. One single animal for each genotype is analysed on this blot. Locations of the different segments collected are shown above the blot. Lysates of nerve tissue from the four different segments were analyzed on Western blots for the presence of phosphorylated ERK. Non-injured nerves were used also analyzed (control). Note that in segments A, C, and D, the knockout tissues showed slight reductions in P-ERK intensity.

Figure 3.3 Characterization of ERK signaling in injured sciatic nerve of the FGF1 knockout mice.

Top Drawing: Schematic diagram of transected nerve showing the segments collected for analysis. The asterix (*) denotes the location of the transection.

Lower Panels: Quantitative analysis of ERK phosphorylation at different time points (60 minutes, 2 days, and 8 days) after injury in wild type (open bars) and FGF1 knockout (stippled bars) sciatic nerves. The same blots were first probed with phosphorylate ERK specific antibodies, stripped, and re-probed with ERK antibodies. The intensity of P-ERK and ERK labeling was measured by densitometry of western blots. For each data point, the resulting measurement of P-ERK was divided by ERK measurements to obtain the P-ERK/ERK ratio (Y-axis). Values shown are the means (+/- SEM) of independent analyses of multiple animals (n = 3). Note that FGF1 knockout nerves showed statistically significant differences in ERK phosphorylation at segment C, at 60 minutes and 8 days after injury (P = 0.0116 and 0.0195 respectively).

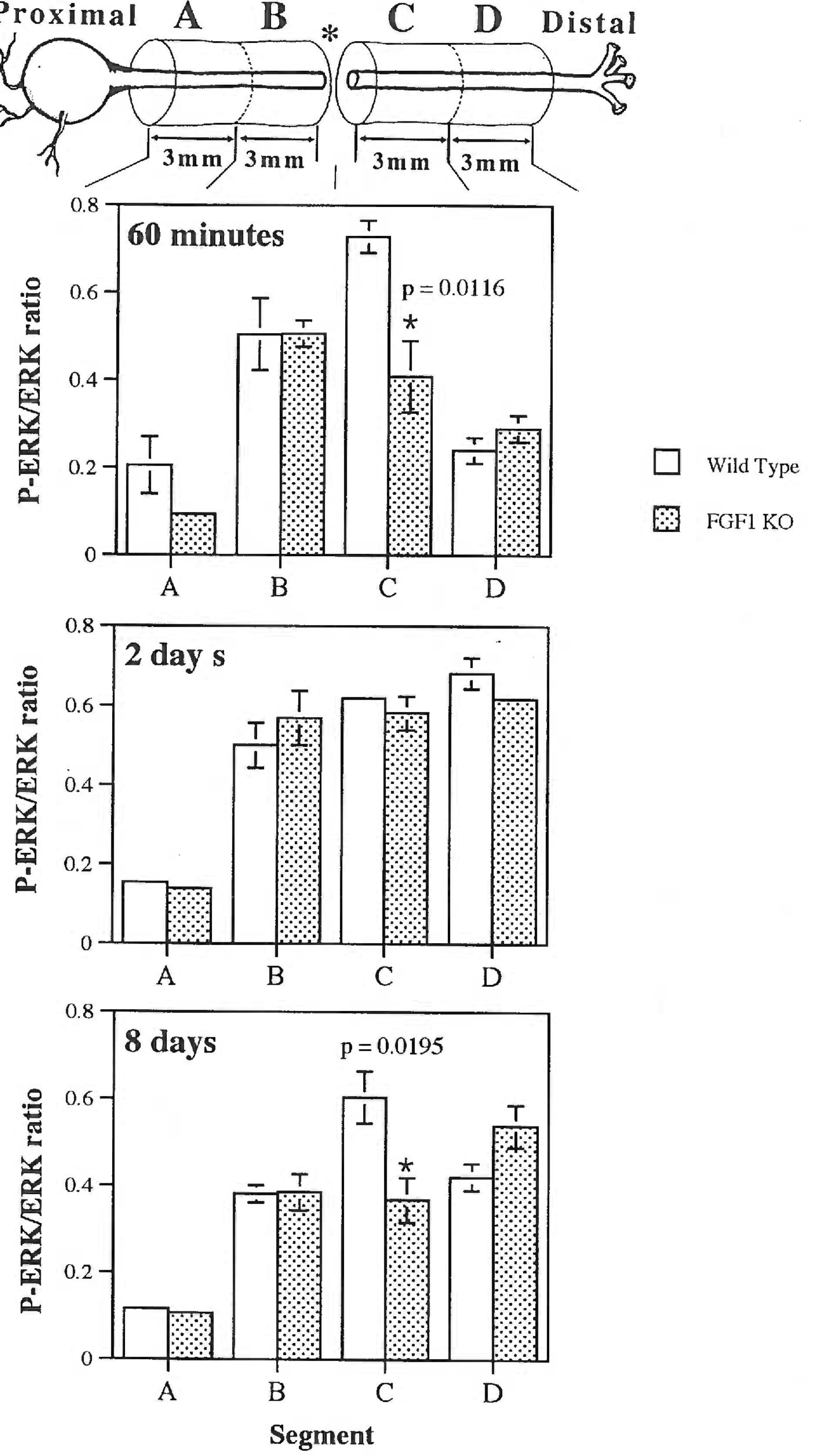
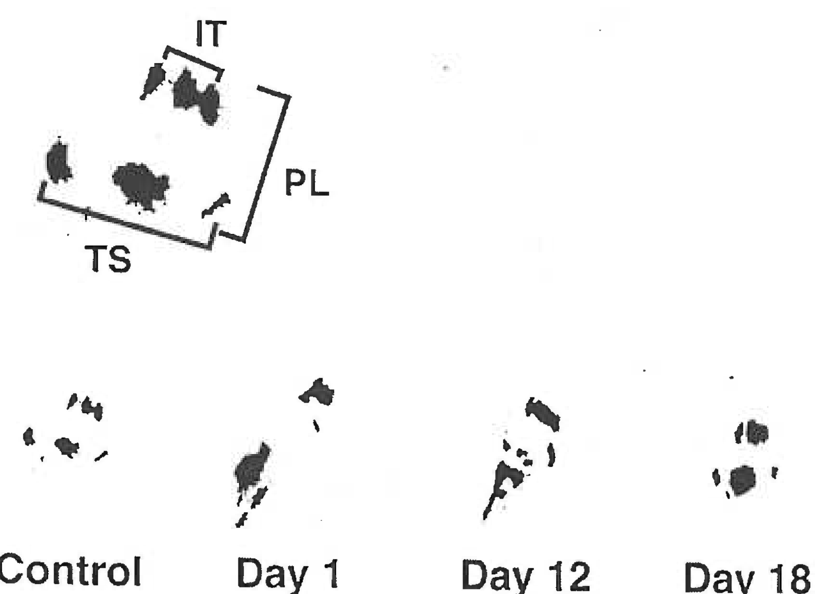

Proximal
A
B
*
C
D
Distal
3mm
3mm
3mm
3mm
60 minutes
p = 0.0116
*
P-ERK/ERK ratio
0.8
0.6
0.4
0.2
0
A
B
C
D
Wild Type
FGF1 KO
2 day s
8 days
p = 0.0195
Segment

Figure 3.4 Characterization of functional regeneration in the sciatic nerve of the FGF1 knockout mice using the Sciatic Functional Index (SFI).
Top Panel (A): Sample of footprints obtained from a FGF1 knockout animal on different days following sciatic nerve crush. The SFI uses several parameters obtained from these footprints to calculate the degree of recovery. These parameters include the print length (PL), total toe spread (TS, the distance between the first and fifth toe), and the intermediate toe spread (IT, the distance between the second and fourth toes). The footprints were obtained by dipping the animals' hind feet in water, and having the animals walk in an enclosed track lined with bromophenol blue-treated paper. The treated paper, which has a light yellowish green appearance, turns dark blue upon contact with moisture. Thus, when the animals walk with their wet hind feet on the paper, dark footprints appear. These footprints were then digitized and the parameters were measured using the NIH-image software package.
Bottom Panel (B): Results from SFI analysis of wild type and FGF1 knockout animals. Footprint measurements from the control side (N) and injured side (E) of each animal were entered into the SFI formula described by Bain et al. (1989) to generate a SFI index for each animal at each time point. The equation is shown right above the bar graph. The resulting SFI were averaged for the wild type (n = 6) and FGF1 knockout (n = 7) groups, and the results are shown here (+/- SEM). Note that at days 11, 12, and 13 the SFI for the wild type (filled bars) and the FGF1 knockouts (open bars) are significantly different (*; p values are shown).


A
IT
PL
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Day 1
Day 12
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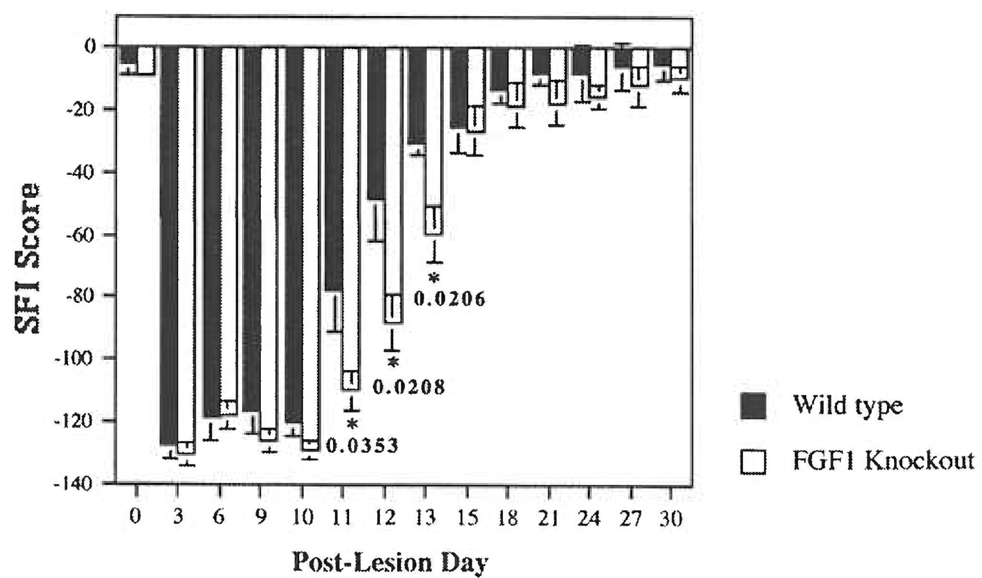
B
SFI = -38.3[(EPL-NPL)/NPL] + 109.5 [(ETS-NTS)/NTS] + 13.3 [(EIT-NIT)/NIT] - 8.8
SFI Score
Post-Lesion Day
0
-20
-40
-60
-80
-100
-120
-140
0 3 6 9 10 11 12 13 15 18 21 24 27 30
0.0353
0.0208
0.0206
Wild type
FGF1 Knockout

Supplementary Chapter

Facial Motor neurons from FGF1 Knockout Mice Have Increased Vulnerability to Peripheral Nerve Injury

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Abstract

Fibroblast growth factor 1 (FGF1) has been shown to be a potent trophic factor for a variety of neurons in culture. In addition, it has been demonstrated to rescue injured neurons when applied exogenously. Together, the data suggests that FGF1 plays a role in providing trophic support for neurons following an injury to their peripheral processes. To test this possibility, we studied the survival of injured facial motor neurons in the FGF1 knockout mice. At two months following injury, there is substantial decrease of facial motor neuron number in both the wild type and FGF1 knockout animals. However, the knockout animals demonstrated greater loss in neuron number in comparison to their wild type littermates. This result suggest that FGF1 might provide trophic support for injured neurons.

Introduction

When axons are injured--disconnecting the neuronal cell body from the target--the neurons' usual source of neurotrophic factors becomes inaccessible. Despite of this, most adult neurons can survive an injury for quite some time. It is likely that an alternate(s) source(s) of trophic factors might come into play.

During the early postnatal weeks, motor neurons are actually vulnerable to injury and a large number of them will die (Crews & Wigston, 1990; Kuzis et al., 1999). This susceptibility to injury lasts until two weeks after birth, following which the neurons gain the survivability of adult motor neurons. Because axons contain large concentrations of FGF1, it could serve as a replacement source of trophic factors for the first few days after injury, until either the distal stump upregulates their trophic factors, or until the axons re-establish contact with their distal targets. Indeed, studies of FGF1 during development showed that FGF1 is not expressed in neurons until the first postnatal week (Kuzis et al., 1995), and that the juvenile neurons do not contain adult FGF1 levels until 3 weeks after birth. The studies of early motor neuron vulnerability and FGF1 expression pattern suggest that FGF1 might play a role in protecting the adult neurons from injury. In vitro, FGF1 has been shown to promote the survival of almost all neuron types, including motor neurons, sensory neurons of the DRG, and sympathetic neurons (Unsicker et al., 1987; Eckenstein et al., 1990; Sendtner et al., 1991). Therefore, when FGF1 becomes accessible after injury, as we have shown in chapter 1, it can act on these varied neuron types to promote survival.

In the previous chapters we have hypothesized that FGF1 acts as an injury factor to regulate gene expression in non-neuronal cells in the injured nerve, and promote axonal regeneration indirectly, it is also possible that FGF1 has a direct neurotrophic action on the neurons. While these two hypothesis state two different roles for FGF1, they are not mutually exclusive. It is conceivable that as an injury factor, FGF1 promotes neuronal survival and triggers peripheral changes in gene expression to prepare for the growth of the axons. Here we tested the hypothesis that FGF1 serves as a temporary survival factor for injured neurons.

Application of exogenous FGF1 at the proximal end of the transected facial nerve can rescue many juvenile neurons that would have otherwise died (Cuevas et al., 1995). FGF1 is expressed by the adult facial motor neurons, which are less vulnerable to peripheral

injury than neonatal facial motor neurons (Kuzis et al., 1995; Kuzis et al., 1999). This suggests that in this system, FGF1 provides trophic support for injured neurons, and that the facial motor neurons might be a good system to study FGF1's neurotrophic function. However, despite the ability of the adult neuronal populations to survive injury in the short term, prolonged axotomy can still lead to the death of a large percentage of adult neurons. To test the role of FGF1 in maintaining neuronal survival after injury, and to see if FGF1's absence increases the vulnerability of permanently axotomized motor neurons, we analyzed the facial motor nucleus of both FGF1 knockout mice and their wild type littermates two months following transection injuries to the facial nerve. The results suggest that FGF1 plays a role in promoting the survival of axotomized facial motor neurons.

Results

Although the FGF1 knockout mice did not demonstrate any gross defects in phenotype and behavior, we tested whether facial motor neurons in the knockout animals developed in normal numbers. Using stereological methods, the number of facial motor neurons in the non-injured facial motor nuclei in both the knockouts and their wild type counterparts was determined. FGF1 knockout mice appeared to have slightly fewer neurons than the wild type mice, however, this difference was small and not statistically significant (**Table 4.1**). This showed that the generation of facial motor neurons was not severely disturbed in the FGF1 knockout mice. This observation agrees with our hypothesis that FGF1 is not involved in the development of the nervous system. We then proceeded to analyze the vulnerability of the FGF1 knockout facial motor neurons to injury.

To perform the facial nerve transection, facial nerves were transected after they exited the skull through the stylomastoid foramen. To perform the transection, a 3mm segment of the main nerve trunk was removed, thus eliminating the paths for the damaged axons to regenerate. The transection was performed only on one side of the animals, leaving the other side intact. Two months after the facial nerve transection, the animals were sacrificed, and their brainstems were collected for analysis of facial motor neuron survival. Serial sections of 60μm thickness were analyzed using stereology to estimate the number of motor neurons in each facial nuclei.

At 2 months after injury, motor neuron number on the lesioned side decreased significantly in both the wild type and FGF1 -/- brain stems. In comparison to the uninjured contralateral nuclei, only 30 to 50 % of the motor neurons remained in the injured nuclei. While these decreases were observed in both the wild type and FGF1 -/- mice, we observed a statistically significant difference in the number of surviving neurons between these two groups. The FGF1 -/- mice had lower number of facial motor neurons remaining than the wild type animals (**Figure 4.1** and **Table 4.2**).

Discussion

This study demonstrates that facial nerve transection leads to a decrease of motor neuron number in both the wild type and FGF1 knockout mice, but that the knockout animals demonstrated a greater loss in neuronal number. This suggests that FGF1 is important for the trophic support of these motor neurons after injury. However, several questions arise about the mechanisms by which FGF1 can provide this trophic support.

Our laboratory has shown that in other peripheral nerves, such as the sciatic nerve, FGF1 expression diminishes in the proximal stump after injury (Eckenstein et al., 1991). In addition, we have also shown that FGF1 is likely to be released only after injury (Chapter 1 of this document). These facts suggest that after injury FGF1 is available for only a short period of time. This presents the question of how a trophic factor present only for a short time after injury influences the long term survival of axotomized neurons. It is possible that upon the axotomy, FGF1 provides the initial trophic support for the injured neurons. Therefore, when neurons in the FGF1 knockout mice are damaged, they do not receive the initial replacement support. This makes them more vulnerable through the period of attempted regeneration. As both wild type and knockout neurons suffer through prolonged lack of contact with their targets, the knockout neurons, weakened by the lack of FGF1 in the early moments of injury, have a significantly higher chance of dying.

How would the fate of the neurons be different if the injury did not cause a permanent separation between the neurons and their targets? We did not address the effect of a crush injury on FGF1 -/- facial motor neurons; however, in our observation of the crushed spinal motor neurons, motor neuron numbers in neither the wild type nor the knockouts seemed to have decreased. The difference could be that axons in the crush injury can reach the distal stump and receive alternate trophic support while transected axons do not have this opportunity. Therefore, although FGF1-/- neurons do not receive the initial FGF1, neurons in a crush injury can eventually find alternative trophic support as they reach the distal stump. While we've seen that *crush injuries* do not have devastating effects on neurons in the spinal cord, this possibility should still be tested in the facial motor system where the system is more amenable to numeric analysis of neurons.

Additionally, to better understand the timing of neuronal vulnerability to chronic axotomy and how FGF1 influences this vulnerability, different time points need to be analyzed to define possible differences between the wild type and FGF1 -/- mice in the rate at which the

motor neurons die after a permanent transection. Perhaps by two months even neurons in the wild types are also unhealthy such that any difference between the knockout and the wild types are reduced. An analysis of shorter survival time points might reveal a more significant difference and gain insights into the time course in which the FGF1 alters the fate of neurons after injury.

Acknowledgement:

The authors would like to acknowledge Doris Kulhanek for the maintenance and genotyping of the FGF1 knockout mice colony. This research was supported by NIH Grant AG70424.

	Wild Type	FGF1 knockout
Mean Motor neuron Number	3270	3027
SEM	100.6	179.3

Table 4.1 Facial motor neuron numbers in wild type and FGF1 knockout mice. The number of facial motor neurons in non-injured the facial motor nuclei of wild type and FGF1 knockout animals were estimated by stereological methods. The mean of the counts from each group (n = 3) are shown with the SEM. The results show that the number of facial motor neurons in the two groups of mice are similar.

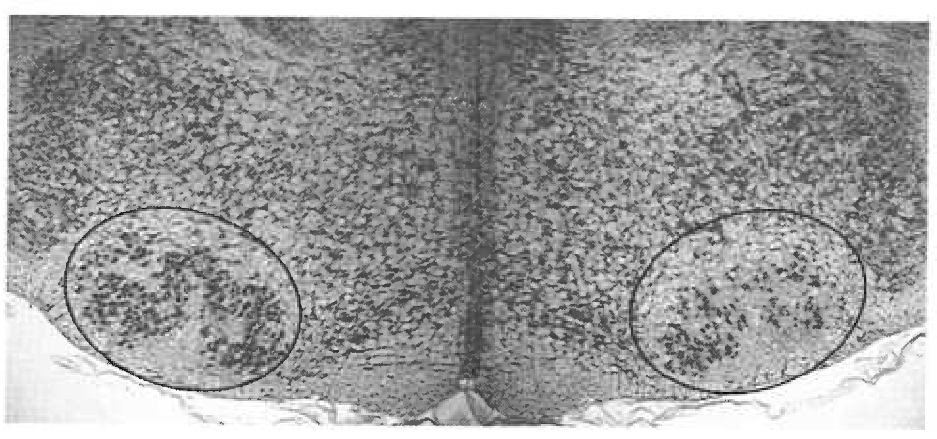
Figure 4.1 Reduction of facial motor neuron numbers following peripheral nerve injury.

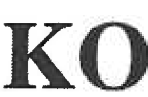
Top Figures (A): Shown are facial nuclei from wild type (top) and FGF1 knockout (bottom) animals 2 months after unilateral facial nerve transection. In each of these figures, the control nuclei are shown on the left, and the injured nuclei are shown on the right. Notice that the injured nuclei in both the wild type and FGF1 knockout animals show drastic reductions in neuron numbers. Also note that the FGF1 knockout injured nuclei showed a greater loss of motor neurons in comparison to the wild type.

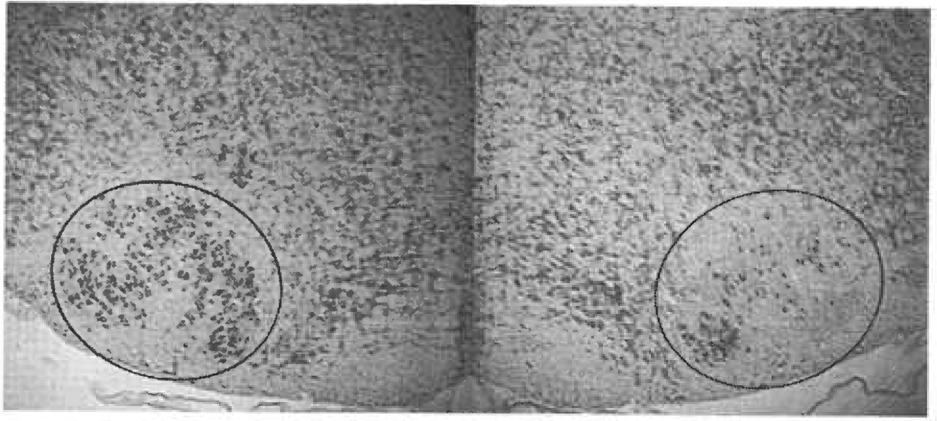
Table 4.2 (B) Comparison of neuron loss in wild type and FGF1 knockout facial nuclei. The number of motor neurons in the injured facial motor nuclei of wild type and FGF1 knockout animals are shown here (FMN number columns). In each group the values represent means from the counting of nuclei from 3 animals. The injured/control ratios are obtained by dividing the neuron number from the injured side over the number from the control side from the same animal. The ratios shown in the table are means from within each group 9n = 3). The difference in the injured/control ratio between the wild type and the FGF1 knockout groups is statistically significant ($p = 0.0359$; one-tailed t-test).

A

Control **Lesioned**







B

	Wild type FMN number	Wild type injured/control	FGF1 ko FMN number	FGF1 ko injured/control
Mean	1339	0.4106	1007	0.3358
SEM	27.9	0.01725	22.81	0.02550

Discussion

I. Summary of results and conclusions

In the studies described above, we demonstrated that FGF1 was released from injured sciatic nerve, and that some of the released FGF1 remained bound to heparan sulfate proteoglycans (HSPG) in the extracellular matrix. This result suggests that FGF1 is made available to the extracellular space through damages in the axonal membrane, and that FGF1's binding to local HSPGs may stabilize these factors for prolonged activity in the injured nerve. We also demonstrated that signaling cascades downstream of FGF1 were activated in the injured nerve. ERK was phosphorylated at sites where axons were either damaged or undergoing degeneration. The correlation of ERK phosphorylation with locations of axon degeneration suggest that FGF1 is responsible for the activation of ERK. After nerve transection, ERK was activated in the FGF1 knockout mice in patterns similar to the wild type's, suggesting that FGF1 is not the main factor responsible for these ERK activations. However, at the 60 minute and 8 day time points, ERK phosphorylation was significantly reduced in the FGF1 knockout at the distal segment directly adjacent to injury. This suggests that the tip of the distal stump is a major site of FGF1 action, where FGF1 is the main inducer of ERK activation, and where FGF1 might regulate non-neuronal cell gene expression. In addition, the FGF1 knockout mice demonstrated impaired sciatic nerve regeneration as measured by the Sciatic Functional Index. On several time points, the SFI scores for the FGF1 knockout mice lagged behind those of the wild type. However, despite of the delayed recovery, the FGF1 knockout mice eventually obtained control level SFI scores. This demonstrates that FGF1 facilitates part of the regeneration process, but that under the tested conditions, FGF1 was not necessary for the eventual regeneration of the damaged axons. Finally, observation of neuronal numbers in the facial motor nuclei two months following peripheral facial nerve transection showed that the absence of FGF1 increases neuronal susceptibility to injury. Fewer neurons remained in the FGF1 -/- motor nuclei two months after injury compared to the wild type nuclei. This suggests that following injury, FGF1 might provide trophic support for damaged neurons. Together, these results suggest that FGF1 functions as an injury signal in the peripheral nerve and present a refined model of FGF1 function in the injured peripheral nerve.

II. Refined model of FGF1 action

FGF1's involvement in peripheral nerve injury begins at the moment when axonal membranes are damaged and FGF1 is released through breaches in the axonal membrane. The released FGF1 functions as an injury factor that coordinates the actions of several

distinct cellular populations to restore function to the damaged nerve. FGF1 accomplishes this through two distinct mechanisms: 1) by providing temporary trophic support directly to injured neurons, and 2) by regulating gene expression in non-neuronal cells. The changes in gene expression, in turn, will support axon outgrowth, immune cell response, and Schwann cell survival. These actions are summarized below and in the accompanying diagram (**Figure 5.1**).

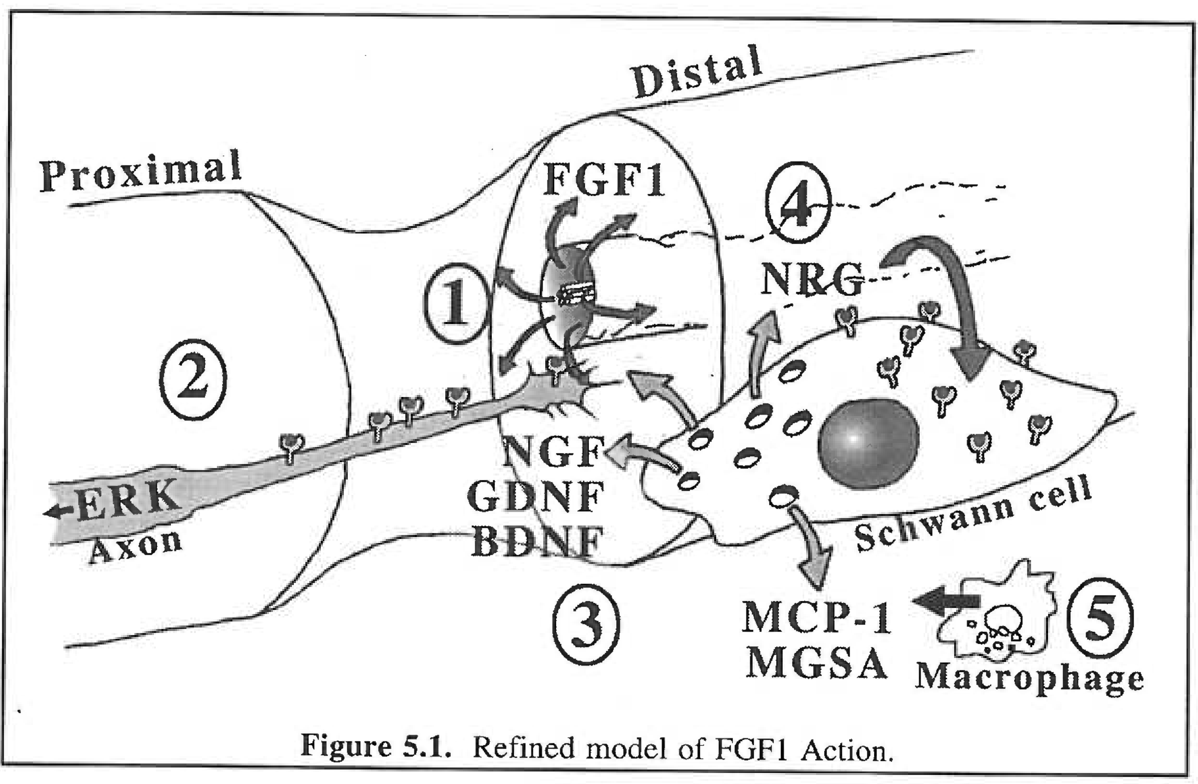


Figure 5.1. Refined model of FGF1 Action.

In the early hours of injury, FGF1 released from the ends of cut axons binds to FGF receptors on these same axons in an autocrine fashion (**1** in **Figure 5.1**). The binding of FGF1 to its receptor activates molecules of the ERK pathway which are retrogradely transported to the cell body (**2** in **Figure 5.1**). The retrograde transport of ERK, in combination with retrograde transport of signals downstream of LIF and CNTF, suppresses cell death machinery that would otherwise be activated by the disruption of target-derived trophic signals. In addition, these retrograde signals upregulate growth related genes in the neurons to increase axon growth rate. Because FGF1 levels in the injured nerve decreases after injury (Eckenstein et al., 1991), FGF1's trophic action is short lived and must be replaced by other trophic factors that are upregulated in the injured nerve. Neurons obtain these replacement trophic factors when their axons regenerate

across the injury site and reach the distal stump where trophic factors such as NGF, BDNF, and GDNF are upregulated.

In the injured nerve, FGF1 is preferentially released in the tip of the distal stump to exert effects on non-neuronal cells (**1** in **Figure 5.1**). After its release, FGF1 is sequestered in the extracellular matrix (ECM) by heparan-sulfate proteoglycans (HSPGs). While FGF1 release only lasts for a short time (less than 2 hours), the sequestration of FGF1 by HSPGs stabilizes FGF1 and increases the duration of FGF1 presence in the ECM. The HSPGs facilitate FGF1 binding to FGF receptors on nearby Schwann cells, and this binding triggers the upregulation of the neurotrophic factor NGF with a rapid time course (**3** in **Figure 5.1**). The early upregulation of NGF at the tip of the distal stump attracts growth cones to grow distally past the injury gap, providing the growing axons with a directional cue and preventing unproductive misdirected sprouting. Because FGF1 is stabilized by HSPGs in the ECM, it continues to regulate Schwann cell gene expression a few days after injury. In this fashion, FGF1 induces the delayed upregulation of trophic factors--GDNF and BDNF--in a sequential manner to provide trophic support for the regenerating axons as they grow within the distal stump. Through regulation of Schwann cell gene expression, FGF1 indirectly provides trophic support for regenerating axons.

In addition to neurotrophic factors, FGF1 also induces the upregulation of factors involved in Schwann cell maintenance and immune response. FGF1 induces the upregulation of neuregulin, a Schwann cell survival factor normally expressed by axons, in Schwann cells of the distal stump (**4** in **Figure 5.1**). The expression of neuregulin in Schwann cells allows the survival of Schwann cells in the absence of axon support. FGF1 also induces the upregulation of chemotactic factors for macrophages and neutrophils (**5** in **Figure 5.1**). FGF1 stimulates the upregulation of neutrophil recruitment factor melanoma growth stimulating activity (MGSA) to recruit neutrophils to the injury site to destroy bacterial parasites that might invade the nerve. FGF1 also induces the upregulation of macrophage recruitment factor monocyte chemotactic protein-1 (MCP-1) to recruit macrophages to the injury site. These macrophages clear a path for the regenerating axons by removing axon and myelin debris, and provide sources of cytokines that further regulate gene expression in Schwann cells.

Finally, FGF1 induces formation of new blood vessels and increases blood flow by stimulating endothelial cell proliferation at the injury site. The migration and proliferation of endothelial cells results in the formation of new blood vessels which grow in a proximo-

distal fashion to bridge the injury gap. This increased circulation provides the nutrients needed for the growing axon tips and for the Schwann cells undergoing mitosis and gene regulation changes.

In sum, our refined model states that FGF1 facilitates axonal regeneration by its initial direct actions on neurons, followed by its regulations of distinct cellular processes to upregulate neurotrophic factors, generate immune responses, rebuild damaged blood vessels, and sustain Schwann cell survival.

III. FGF1's direct actions on neurons

Results from previous studies of FGF1 function suggest that FGF1 has direct neurotrophic actions on neurons. Motor and sensory neurons have been shown to express FGF receptors (Kato et al., 1992; Oellig et al., 1995). In culture, FGF1 rescues these neurons from cell death (Unsicker et al., 1987; Walicke, 1988; Eckenstein et al., 1990). Correlative data of FGF1 expression and neuronal development have shown that FGF1's increased expression in the neonatal nerve coincides with the neonatal neurons' decreased vulnerability to injury (Kuzis et al., 1999), suggesting that FGF1 has a protective role for injured neurons. Exogenous applications of FGF1 to transected stumps of facial nerves rescue neonatal motor neurons from cell death (Cuevas et al., 1995). Together, these results suggest that FGF1 is a neurotrophic factor for neurons after nerve damage. If FGF1 is a neurotrophic factor for damaged neurons, then injury to nerves in FGF1 knockout mice should result in the death of more neurons. Our results showed that in the facial motor nuclei of FGF1 knockout mice, a fewer number of motor neurons remained at two months following peripheral nerve transection than in wild type mice. Approximately 10% more neurons died in the FGF1-/- motor neurons than in the wild type (a statistically significant difference, $p = 0.0359$). Although this is not a drastic reduction, the reduced number of surviving neurons does demonstrate that FGF1 has a protective role.

The magnitude of increased cell death caused by the lack of FGF1 might be masked by other effects. In wild type animals, a large number of motor neurons also die after the prolonged axotomy, suggesting a deficiency in trophic support for the neurons. However, the wild type and the FGF1 knockout neurons might die from different causes. The knockout neurons likely died from the absence of temporary neurotrophic support (FGF1) after injury. On the other hand, the wild type neurons, which survive the initial trauma supported by FGF1, die at a later time due to the disappearance of secondary neurotrophic

factors. We would predict that a more detailed time course study of the facial motor nuclei response to peripheral transection should reveal that FGF1-/- neurons begin to die earlier than the wild type neurons, and this might provide a more definitive support for the direct trophic actions of FGF1 on damaged neurons.

In pure neuronal cultures, it is possible to assign a direct neurotrophic role for FGF1 (Walicke & Baird, 1988; Unsicker et al., 1992); however, in the nerve, where axons are accompanied by non-neuronal cells, the designation of FGF1 as a neurotrophic factor is more difficult. In the presence of non-neuronal cells, FGF1's effects on neurons might be mediated through gene expressions in non-neuronal cells. The distinction of whether FGF1's effects on neurons are direct or indirect could come from histological observations of FGF1's downstream signaling cascades. For example, one can observe whether the retrograde axonal transport of phosphorylated ERK is decreased in injured FGF1-/- nerves. If retrograde transport is due to the direct actions of FGF1, then ERK transport should be present in the wild type nerve, and be absent in the knockouts. In a set of Western blot experiments we attempted to address this questions; however, we did not see a statistically significant difference between the wild type and the knockouts. Possibly, the amount of retrogradely transported ERK in the axons is minimal compared to the amount of basal phosphorylated ERK in the rest of the nerve, and the Western blotting method is not sensitive enough to discern these differences. Immunohistochemical identification of ERK phosphorylation in axonal processes, and comparison between wild type and FGF1 -/- axons, should be essential in identifying the presence of ERK retrograde transport and the contribution of FGF1. The increased ERK signaling in axons could be due to factors upregulated by FGF1 and not due to direct stimulation by FGF1. As such, a more telling and specific set of experiments will be studies of phosphorylated FGF receptor localization in axons. This should provide further evidence for FGF1's direct actions on neurons. Our laboratory is currently undertaking the production of antibodies that specifically recognize the phosphorylated versions of the FGF receptors, and these tools will be essential in our identification of cellular populations and components that are responsive to FGF1 following injury.

In addition to providing trophic support, the retrograde signaling induced by FGF1 could regulate neuronal gene expression and alter axonal growth rate. Nerve injury induces the retrograde transport of signals originating from the injury site (Moix et al., 1991; Greeson et al., 1992; Murphy et al., 1999). This retrograde injury signal has important implications for gene expression in the neurons. For example, the upregulation of IL-6 and the p75

NGF receptor mRNA in injured motor or sensory neurons requires positive retrograde signals from injured nerves (Moix et al., 1991; Greeson et al., 1992; Murphy et al., 1999). While these studies did not identify the nature of these signals, it is possible that the retrograde transport of FGF1 activated ERK, combined with the retrograde transport of STAT3 (downstream signal of CNTF and LIF) could represent such positive signals.

Following injury, genes involved in axonal growth such as GAP-43 are upregulated in spinal motor neurons (Piehl et al., 1993). The upregulation of these genes might be responsible for increases in the rate of axon growth after conditioning lesions. In these studies, the rate of axon growth in a second lesion is increased significantly compared to axon growth from a single lesion (McQuarrie, 1978), suggesting that when the second lesion takes place, the axon outgrowth machinery is already running at high gears and therefore axons grow at a faster rate (McQuarrie et al., 1991). The delayed rate of functional recovery in FGF1 knockout animals (shown by the SFI) suggests that axons in the these mice might have a slower rate of axonal outgrowth. It is likely that in the FGF1 knockout mice, the axon outgrowth machinery are not activated to the same degree as the wild types, and thus axons would re-grow at a slower rate. Indeed, several studies have shown that FGF1 regulates the expression of the growth promoting protein GAP-43 in neurons after injury and in culture (Costello et al., 1990; Mohiuddin et al., 1996; Meiri et al., 1998; Piehl et al., 1998; Kawamata et al, 1999). Conditioning lesions in FGF1 knockout mice might reveal that regeneration rate does not increase after a priming lesion because components of the regeneration machinery such as GAP-43 are not upregulated by FGF1. As a result, FGF1 -/- axons would grow slower than wild type axons. Further measurement of axon growth using histology and expression of growth associated proteins such as GAP-43 should confirm the differences in growth rate and suggest downstream mechanisms responsible for this difference.

IV. FGF1 upregulates trophic factors in the distal stump (NGF, GDNF, BDNF)

Injury induces the expression of a number of neurotrophic factors in the distal stump, and they might provide the trophic support for the regenerating axons before they reach their targets. FGF1 is likely to play a role in inducing the upregulation of these trophic factors. FGF1 has been shown to upregulate the expression of NGF in cultured astrocytes (Yoshida & Gage, 1991), and following injury in the CNS (Figueiredo et al., 1995). It is possible that in the injured nerve, FGF1 similarly induces the expression of NGF in the Schwann cells near the injury site. Because of FGF1's proposed early action, we would

expect the early upregulated factors--such as NGF and IL-6--to be targets of FGF1 induction. In the nerve, NGF is rapidly upregulated following injury (with an initial peak at 6 hours), at a time prior to the infiltration of macrophages. In fact, when macrophage invasion into the injured nerve is prevented, the initial early peak of NGF upregulation still remains, suggesting that macrophages (which have been speculated to be responsible for NGF upregulation through their secretion of IL-1β) are not responsible for this initial peak of NGF. Because FGF1 can be sequestered and stabilized by HSPGs in the ECM, FGF1 can have a longer lasting influence and induce the upregulation of the factors, such as GDNF and BDNF, which are increased in a more delayed fashion. Through the upregulation of trophic factors in the distal stump of the injured nerve, FGF1 indirectly provides trophic support for damaged neurons as their axons grow through the distal stump. This hypothesis could be tested by measuring trophic factor upregulation in the injured nerves of the FGF1 -/- mice. If FGF1 is responsible for inducing the upregulation of the trophic factors, then we would expect trophic factor upregulation to be reduced or abolished.

In our studies of ERK activation following injury, we observed that ERK phosphorylation was significantly reduced in the distal tip of the FGF1 -/- nerve. Although we cannot rule out that FGF1 has actions in other locations, the data suggest that the distal tip is a major site of FGF1 action. It is unclear what might account for the preferential actions of FGF1 in the distal tip. Axons in both the distal and proximal tip have breaches in their membrane caused by the injury, and studies of axon resealing in cultured neurons have shown that the rate of axon resealing is identical in the proximal and distal cut ends (Spira et al., 1993), suggesting that the observed preferential action of FGF1 in the distal tip is not due to the prolonged release of FGF1 . However, similar studies of axon membrane resealing demonstrated that Ca^{++} influx is more extensive in the distal stump (Ziv & Spira, 1993). This difference in Ca^{++} diffusion could provide a mechanism for the preferential release of FGF1 in the distal stump. A study by Maciag and colleagues suggests that FGF1 is associated with the Ca^{++} binding protein S100A13 in their *in vitro* release assay (Carreira et al., 1998), providing a possible mechanism on how differences in Ca^{++} diffusion might account for the preferential release of FGF1 at the distal tip.

The possible preferential action of FGF1 at the distal tip can have significant functional implications. The early upregulation of NGF by FGF1 in the distal stump may provide a directional cue for regenerating axons. Classic experiments by Gundersen and Barrett (1979) showed that NGF directs the growth of axons in culture. The upregulation of NGF

in the distal tip of the transected nerve could function similarly to direct the distal growth of regenerating axons. In the absence of NGF, such as in the FGF1 knockouts where NGF is not upregulated, the initial growth of the regenerating axons might be misrouted. Only axons that have serendipitously grown in the right direction will receive other neurotrophic factors expressed in the further distal portion of the stump and successfully reach their targets. Because motor neurons are not known to respond to NGF, other similarly upregulated factors might be responsible for providing the directional cue for the regenerating motor neurons. These might include GDNF and BDNF, although they are upregulated at a much delayed time course compared to NGF. The lack of trophic factor might delay the regeneration of motor axons or reduce the number of axons that correctly grow distally towards their original targets. This might be another mechanism by which the functional recovery of motor function in the FGF1-/- mice was delayed.

Experiments measuring the expression of NGF, GDNF, BDNF mRNAs in the damaged nerves of wild type and FGF1 knockout mice should reveal whether neurotrophin mRNA expression is decreased in the FGF1 knockout nerves. Furthermore, we can test the anatomical consequences of the reduced neurotrophin expression by using Y-chamber studies. Previous Y-chamber studies--where the distal two arms of the Y-chamber contain two distinct types of targets--reveal that distal stumps release soluble factors that attract growing axons (Politis et al., 1982; Lundborg, 1987; Nachemson et al., 1988). Wild type or FGF1 -/- distal stumps can be placed in the ends of the Y-chambers and the ability of FGF1 -/- distal stumps to attract regenerating axons can be tested. This type of study should provide a measure of the consequences of any differences in trophic factor expression.

V. FGF1 regulates Schwann cell physiology

Schwann cell/axon interactions are important for maintaining Schwann cell survival and physiology. After injury, FGF1 signaling might upregulate the Schwann cell survival factor, neuregulin, to produce an autocrine regulation of Schwann cell survival. Following nerve injury, axons in the distal stump degenerate and Schwann cells lose their axonal partners. In the newborn mouse, this loss of axons following injury results in the death of a larger portion of the Schwann cells, demonstrating the dependence of Schwann cells on the axons (Grinspan et al., 1996). In the adult nerve, however, Schwann cells can survive prolonged periods in the absence of axon contact (Grinspan et al., 1996). One possible mechanism of the adult Schwann cells' ability to survive axotomy is that following injury

they begin producing the Schwann cell survival factor, neuregulin (Carroll et al., 1997). The mRNA and protein of the various neuregulin splice forms, normally expressed by neurons (Marchionni et al., 1993; Dong et al., 1995), are upregulated in Schwann cells a few days after nerve injury (Cohen et al., 1992; Carroll et al., 1997). This upregulation is maintained for at least a month and accompanied by an increased phosphorylation of the neuregulin receptor ErbB2 in the nerve (Cohen et al., 1992; Carroll et al., 1997; Kwon et al., 1997). Although the identity of the signal that triggers this upregulation of neuregulin is unknown, it is possible that FGF1 stimulates the upregulation of neuregulin. Cultured Schwann cells have been shown to proliferate in response to FGFs (Davis & Stroobant, 1990); however, this response can be abolished by anti-neuregulin antibodies, suggesting that the actions of FGF is indirect and that FGF's actions on Schwann cells might be mediated by upregulating neuregulins in Schwann cells (Rosembaum et al., 1997).

FGF1's upregulation of neuregulin in Schwann cells allows the Schwann cells to survive in the absence of axons until regenerating axons reach them again. If this hypothesis is correct, then Schwann cells in FGF1 knockout mice would not able to upregulate neuregulins after injury and would die as a result. This hypothesis could be tested by measuring neuregulin expression and the level of Schwann cell apoptosis in FGF1 knockout nerves following a transection injury where axons are prevented from regenerating into the distal stump.

VI. FGF1 at the interface of nervous and immune system.

Following nerve injury, several mediators of immune responses are also upregulated rapidly at the injury site. These responses are critical in recruiting cells to the injury site, not only to fight off possible infections, but to also clean up debris that might stand in the way of regenerating axons (Perry & Brown, 1992). Several studies have suggested that the breakdown of axonal membranes after injury is crucial in the production of cytokines and in the recruitment of macrophages (Bruck et al., 1995). In the Ola mice, where distal axons do not degenerate after injury, nerve transection fails to induce the upregulation of monocyte chemotactic protein-1 (MCP-1) and melanoma growth stimulatory activity (MGSA), chemoattractants for macrophages and neutrophils, respectively (Carroll & Frohnert, 1999). As a consequence, in these mice, macrophage invasion is drastically reduced following nerve injury (Bruck et al., 1995), and the regeneration of sensory axons is impaired (Bisby & Chen, 1990). The results from these studies suggest that the release of an axonal component is essential in initiating these immune responses. Because FGF1 is

released through axonal membrane breaches, and because the recruitment of macrophages is highly dependent on the breakdown of axonal membranes, FGF1 is a likely candidate to regulate the responses that lead to recruitment of immune cells.

VI. Future directions

Results from our study, together with data presented by other researchers, suggest that FGF1 coordinates various aspects of the peripheral nerve injury response to promote functional regeneration (**Figure 5.2**). FGF1 can directly stimulate neuronal survival in the early hours after injury, and at later times, indirectly promote neuronal survival by upregulating neurotrophic factors in the vincity of injury. These upregulated factors also serve as directional cues to direct axon outgrowth. In addition, FGF1 can regulate Schwann cell survival and maintenance by upregulating the Schwann cell survival factor neuregulin in the distal stumps of injured nerves. FGF1 might also function to recruit immune cells to the nerve by upregulating chemotactive factors for macrophages and neutrophils. Finally, because of FGF1's well-known role as an angiogenesis factor, FGF1 can also influence the formation of new blood vessels at the injury site to restore blood flow (Thompson et al., 1988).

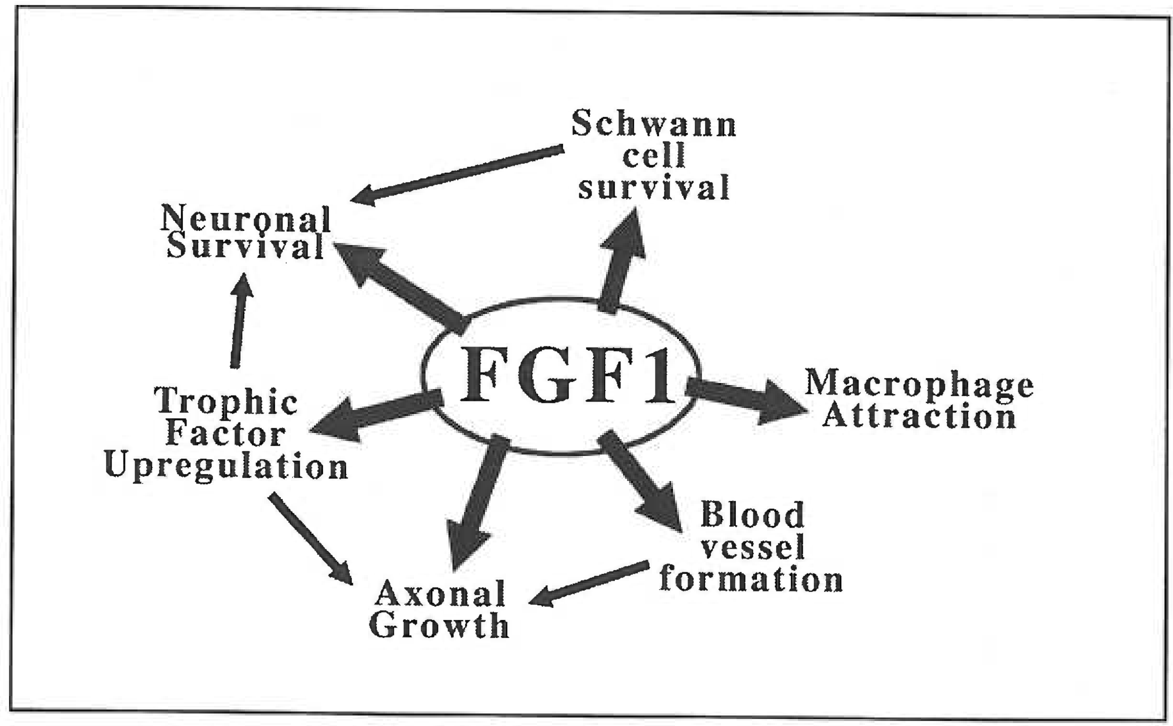


Figure 5.2. FGF1 coordinates various cellular responses to promote regeneration.

Considering that these are seemingly important events in the process of nerve repair, it is surprising that in the FGF1 knockout mice crushed sciatic nerves can still regain functional regeneration, albeit with a delayed rate of recovery. The functional restoration of damaged nerves requires that several processes be completed: 1) that neurons survive the initial trauma and continue to survive throughout the period of regeneration; 2) that transected axons grow successfully through the distal stump; 3) that functional synapses are established once axons reach their targets; and 4) that Schwann cells provide myelination to appropriate axons. While our model of FGF1 action predicts that FGF1 has either direct or indirect regulation of some of these processes, our data argues that other injury factors are also involved, and thus in the absence of FGF1, these processes can still be accomplished. For example, in the absence of FGF1, CNTF--a neurotrophic factor that might also function as an injury signal--can still rescue the survival of a number of neurons, minimizing neuronal death. A double knockout deleting both of these injury signals might reveal an even greater effect on neuronal survival after injury. Alternatively, crush injury--in contrast to transection injury--might not be severe enough to reveal the full extent of deficit due to the lack of FGF1. For example, while Schwann cells might be initially sick due to the lack of neuregulin in FGF1 knockout nerves, regenerating axons should soon reach these Schwann cells and provide neuregulin. The effects on Schwann cells in the FGF1 knockout mice should be more severe following a transection where Schwann cells do not come in contact with axons. In this case, we would predict that a large number of Schwann cells should die in the knockout nerves, similar to the death of Schwann cells in neonatal nerves (Grinspan et al., 1996). The molecular consequences of FGF1 absence--and their effects on functional recovery--can be better understood when we further analyze the histology and gene regulation of injured nerves in FGF1 knockout mice.

Histological analysis of nerve regeneration in FGF1 knockout mice should be performed to identify the nature of defects that caused the observed delay in nerve regeneration. The impairment in FGF1-/- regeneration could be due to either reduced axon growth rate or reduced number of axons reaching target. Histological analysis of nerve sections can easily distinguish these two possibilities. The reduced axon growth rate could be due to the reduced intrinsic rate of axon growth or reduced environmental cues. Examination of growth associated genes in neurons, such as GAP-43, could reveal potential deficits in gene regulation in FGF1 knockout neurons. Alternatively, examination of gene regulation in the nerve (see below) could reveal defects in the environmental cues. A reduction in the axons reaching the target could be due to several problems. An initial misdirected growth of axons (lack of directional growth), reduced intrinsic rate of axon growth, reduced

environmental cues, or increased neuronal death could all contribute to reduced number of axons reaching the target. Once again, these potential deficits can be measured with histological analysis of the nerve and the cell bodies. Throughout these studies, special attention should be paid to differences in sensory and motor axon responses, as these distinct neuronal populations might have different needs after injury. Because FGF1 also might regulate the response of non-neuronal cells to nerve injury, the responses of these cells in FGF1 knockout mice should also be observed. These include the formation of new blood vessels at the injury site, the invasion of macrophages and neutrophils, and the physiology of the Schwann cells (e.g. mitosis vs. apoptosis). The histological analysis of axons and their neighboring non-neuronal cells in FGF1 knockout mice should reveal FGF1's influence following injury.

Finally, because FGF1 is thought to regulate gene expression in the injured nerve, the measurement of changes in gene expression in FGF1 knockout nerves is an essential study. It will be important to measure the levels of trophic factors, such as NGF, GDNF, BDNF, and LIF, in the FGF1 knockout nerve. In addition, the regulation of chemokines, such as MCP-1 and MGSA, and the Schwann cell survival factor and mitogen, neuregulin, should also be observed. Because of the molecular tools available, such as commercial ribonucleotide probes, the measurement of these gene regulations are very feasible and should provide important tests for our model of FGF1 action in regulating injury responses.

VII. Concluding remarks

These proposed studies of the FGF1 knockout mice present an exciting opportunity to test our model of FGF1 action. The knowledge gained might have important implications for the treatment of both peripheral and central nervous system damages. As such, these studies should be performed with much diligence and thoughtfulness. It is my hope that the efforts projected into these studies will eventually reduce the suffering of our fellow human beings afflicted with disabling injuries.

Materials and Methods

Materials and Methods 1

1. Studies of FGF1 Release Following Sciatic Nerve Injury

1.1 Sciatic Nerve Injury & Tissue Collection

For *in vitro* release experiments, adult female long evans rats (180 - 200g) were anesthetized with a cocktail mixture of ketamine (55.6 mg/ml), xylazine (5.6 mg/ml), and acepromazine (1.1 mg/ml) at a dosage of 1 ml cocktail per kilogram body weight. An incision (approximately 3 cm long) was made at the upper thigh to expose the sciaic nerve, and sciatic nerve fragments (approx. 2.5cm) were collected. The nerves were cleaned in cold EBSS and further cut into 0.5 cm segments. Three 0.5 cm segments were then placed into 0.5 ml of collection media (CM: 1 mg/ml BSA and +/- 10μg/ml low molecular weight heparin in DMEM) and incubated at 37°C. Conditioned media were collected every hour and immediately frozen on dry ice. At the end of the collection period, the nerve fragments were also collected and homogenized in 0.5 ml PBS pH 8.2.

1.2 Rhodamine dextran incorporation into cut axons

Sciatic nerves from adult female long evans rats were collected into EBSS, cleaned, cut into 0.5 cm fragments. Fragments were incubated in DMEM + 1mg/ml BSA + 10 μg/ml heparin at 37°C. At different time points ranging from 1 to 6 hours, sciatic nerve fragments were placed in 1% rhodamine dextran in PBS (dextran tetramethylrhodamine, 10,000 MW, lysine fixable (fluororuby) Molecular probes, cat.# D-1817) for 2 minutes. The nerve fragments were then rinsed in PBS for 2 minutes. The fragments were then fixed overnight in 4% formaldehyde at 4°C. The segments were then washed overnight in PBS, followed by sequential incubations in 15% and 30% sucrose. The nerve fragments were then cut into 25 μm longitudinal sections with a cryostat, and observed under a fluorescent microscope.

1.3 AKR2B Mitogenic Assay

The AKR2B cells belong to a mouse embryonic fibroblast cell line that proliferate in response to a number of growth factors including FGF1 (Shipley, 1986). The AKR2B cells were grown in 48-well plates to confluency in McCoy's 5A (0.5 ml per well; Sigma, Cat# M-4892) supplemented with 5 % fetal calf serum. The cells were then serum-deprived for 48 hours in by replacing the media with Medium 202 (Cascade Biologics, Cat# M-200-500) supplemented with penicillin and streptomycin. The cells were then fed fresh Medium 202, pen/stp, supplemented with 2μg/ml insulin, and treated with either

nerve-conditioned media (CM), nerve lysate, or FGF1 in the presence of 2 μg/ml heparin for 22 hours. The cells were then pulsed with ^{3}H-Thymidine to a final concentration of 2 μCi/ml (NEN; Cat # NET 027Z) for 1 hour and washed in cold 10% trichloroacidic acid 3 times for 10 minutes each. DNA was precipitated with 400 μl of 0.2 M NaOH containing 50μg/ml Herring sperm DNA for 30 minutes. ^{3}H-Thymidine incorporation was measured after mixing 200 μl of dissolved DNA with 4 ml of acidified Ecoscint H (300 μl glacial acidic acid in 1 liter of Ecoscint H) fluorophor.

1.4 ChAT Assay.

To detect the presence of Choline-Acetyl Transferase, 20 μl of nerve-conditioned media (1/25 of total CM) was mixed with 10 μl of 10 mM choline chloride (1 mg/ml BSA, 0.2 mM eserine sulfate, 0.5% triton X-100, 10 mM EDTA, 50 mM sodium phosphate pH 7.3 - 7.4) and 5μl ^{3}H-acetyl CoA (in 10 mM sodium phosphate, pH 6.0). The mixture was then incubated in 37°C for 30 minutes. After stopping the reaction with 1 ml of cold PBS, 0.2 ml of kalignost (5 mg/ml tetra-phenyl boron in acetylnitrile) was added to the mixture. The reaction mixture was added to 5 ml of toluene-based scintillation fluid and the [^{3}H] that had been incorporated into Acetyl Choline was counted.

1.5 Lactate Dehydrogenase Assay

Sciatic nerve conditioned media (CM) was diluted 1:1 with homogenate buffer (0.5% triton X, 10mM EDTA, and 1mg/ml BSA). 20 μl of the CM mix was added to wells in 96-well plate. 100 μl of a LDH reaction mixture (60mg lactic acid, 167 mg INT (2-[4-iodophenyl]-3-(4-nitrophenyl)-5 phenyltetrazolium chloride), 43 mg phenazine methosulphate, and 431 mg NAD in 12 ml of 0.2M Tris buffer pH 8.2) was added into each well to start the LDH reaction. The 96-well plate was immediately placed into a kinetic microplate reader to measure the rate of LDH reaction.

1.6 FGF1 Antibody Generation

Recombinant FGF1 was injected into hens to produce anti-FGF1 antibody. 1mg/ml stocks of recombinant FGF1 expressed in our laboratory was provided to Aves International Inc. (Oregon) which performed the immunizations and the subsequent isolation of chicken IgY from egg yolk. This chicken anti-FGF1 (1091) was characterized in our AKR2B bioassays, and we found that at dilution of 1:100 it block 50% of activity stimulated by 5ng/ml FGF1, and has significant selectivity for FGF1 over FGF2.

Materials and Methods 2

Studies of Kinase Signaling Activation in Injured Sciatic Nerve

2.1 Sciatic Nerve Injury

Female Long Evan rats (180 to 200g body weight) were anesthetized with a cocktail of ketamine (55.6 mg/ml), xylazine (5.6 mg/ml), and acepromazine (1.1 mg/ml), at a dosage of 1 ml cocktail per kilogram body weight. Sciatic nerves on both sides of the body were exposed at the upper thigh level, approximately 3 mm below the sciatic notch, and a 3mm segment of the sciatic nerve fragment was removed to complete the transection. Sham operations were performed by exposing the sciatic nerves and stripping away the connective tissues surrounding the nerve. Following surgery, wound sites were closed with wound clips (Fine Science Instruments) and the animals were returned to animal care facility until sacrifice.

2.2 Sciatic Nerve Collection & Homogenization

At appropriate post-lesion times, the rats were deeply anesthetized with the anesthesia cocktail. The sciatic nerves were exposed and 3 mm fragments of the sciatic nerve were collected from various points along the nerve (see **Figure 2.1**). For control tissue, nerves were collected from non-operated rats. Nerve fragments collected from the two sides of the same animal were pooled and immediately frozen on dry ice. The nerve fragments were then stored at -80°C until further processing. Nerve fragments from each animal were homogenized in 400 µl of SDS sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% w/v bromophenol blue). The homogenized tissue was divided into aliquots and stored at -80°C until use.

2.3 Western Blotting Detection of Phosphorylated Kinases

Homogenized samples (20 µg of protein per lane) were run on 10% SDS acrylamide gels and electroblotted at room temperature for 1 hour to polyvinylidene fluoride (PVDF) blotting membranes (BioTrace PVDF, Gelman Sciences). Sets of four gels representing the four segments were processed simultaneously in order to analyze all time points and nerve fragments under identical conditions. The same control samples were loaded on all gels within an individual experiment in order to normalize band intensities across different gels. The membranes were briefly washed in PBS, and then blocked for 2 hours at room temperature with 5% (w/v) dry skim milk (Carnation) in PBS (pH 7.6) + 0.1 % Tween-20 (Blocking Buffer) with gentle shaking. The membranes were then

incubated overnight at $4^{o}C$ in primary antibody [rabbit anti-phospho-ERK, rabbit anti-phospho-Stat3, and rabbit anti-phospho-SAPK (all from New England Biolab, diluted 1:1000 in 0.05% Tween-20, 5% BSA, and 0.02% sodium azide in PBS)]. The membranes were then washed 3 times for 10 minutes each with Blocking Buffer, and then incubated in alkaline phosphatase-conjugated secondary antibodies (goat anti-rabbit; New England Biolab; diluted 1:1000 in Blocking Buffer) for 1 hour at room temperature. The membranes were washed as above, followed by washing for two times with 10 mM Tris-HCl, 10 mM NaCl, 1 mM $MgCl_2$, pH 9.5. and incubation in CDP-Star (1:500 dilution, New England Biolab) (0.1 M diethanolamine, 1.0 mM $MgCl_2$, pH 9.5) for 5 minutes at room temperature. The membranes were placed in a sheet protector and exposed to Kodak autoradiography films for various lengths of time (1 - 15 minutes). Three independent sets of animals and western blot experiments were analyzed.

2.4 Immunohistochemisty detection of macrophages in injured nerve.

Sciatic nerves were immersion fixed in 5% formalin for 24 hours at room temperature. Following fixation, tissues were washed in PBS overnight, and subsequently equilibrated in 15% sucrose-PBS, and then in 30% sucrose-PBS. Ten μm sections were cut in a cryostat and collected onto gelatin-coated slides. The sections were washed in PBS for 10 minutes, incubated in blocking solution (10% horse serum, 0.5% Triton-X in PBS) for 1 hour at room temperature, and then incubated overnight at room temperature in mouse anti-macrophage antibody (ED-1 from Accurate, diluted 1:1000 in blocking solution). Sections were washed 4 X 10 minutes with PBS, and incubated with fluorescein-conjugated secondary antibodies (from Cappel, diluted 1:200) for one hour at room temperature, washed and coverslipped. The number of ED-1 positive cells was estimated by counting labeled cells in three fields of view each (160X final magnification) at sites 1mm and 4mm proximal and distal to the lesion.

2.5 Ribonuclease Protection Assay (RPA)

Total RNA from rat sciatic nerves was isolated using an acidified phenol guanidinium method (Tri Reagent, from Molecular Research Center). Riboprobes were transcribed from multiprobe template sets using the Riboquant In Vitro Transcription Kit (Pharmingen). All enzymes and buffers used in the probe synthesis and in the Ribonuclease protection assay (RPA) were from Pharmingen and the RPA was conducted according to the manufacturers specifications. After hybridization an dRNAse digestion, samples were run on a 5% acrylamide/urea sequencing gel. The gel was then dried and exposed to a Kodak phosphorimager screen for quantification.

2.6 Quantification of Western blot and RPA Results

Digital image files were obtained by scanning the exposed films (Western blots) or acquiring the phosphoimager outputs (RPA), and analyzed using the NIH Image software package. Care was taken to analyze only signals within the linear response range of the films. The mean labeling intensity of specific bands was measured after subtraction of background density measurements.

Materials and Methods 3

Studies of Injury Response and Regeneration in FGF1 Knockout Mice

3.1 Generation of FGF-1 Deficient Mouse

Mice with targeted disruption of the FGF1 gene were produced by homologous recombination. The mice were kindly provided by C. Basilico and colleagues, and their generation was first described in Miller et al. (2000). A clone containing FGF1's first exon was isolated from a 129SVJ mouse genomic library (Stratagene). The library was screened with the human FGF1 cDNA followed by a 120-bp NcoI-BamHI fragment containing the first exon. Two independent clones spanning approximately 12 kb were isolated. The targeting plasmid was constructed by the use of the pPNT replacement vector. The XhoI and EcoRI sites of pPNT were digested, blunted, and used to insert the 2.8-kb BamHI-EcoRI 5' arm and the 2.4-kb XbaI-NotI 3' arm, respectively. Upon homologous recombination, a deletion of approximately 4.7 kb that includes the entire first exon results. The plasmid was linearized with Not I and purified, and 30 μg was electrophorated into E14 embryonic stem (ES) cells. Transfected cells were selected in G418 (400μg/ml) and FIAU [1-(2'-deoxy-2'-fluoro-1-b-D-arabinofuranosyl)-5-iodouracil] (0.25μM). Clones were screened by Southern analysis prior to injection into C57Bl/6 blastocysts.

3.2 Sciatic nerve injury

Adult wild type and FGF-1 -/- mice (ranging from 2 to 5 months old) were anesthetized with a continuous flow of 2% isoflurorane and 1% oxygen. An incision of approximately 1.5 cm was made at the upper thigh to expose the sciatic nerve. The nerve is loosened from surrounding connective tissue through gentle dissection. The nerve was then crushed at the sciatic notch using a pair of jeweler's forceps (Dumont PP/45, Fine Science Tools) by closing the tips of the forceps perpendicularly on the nerve for two 10 second periods, after which the crush injury was observed for completeness, focusing on the clear separation of the two stumps and retention of the overlying epineurium. For sciatic nerve transection, similar procedures were performed except that the nerve was completely cut and the distal stump was tucked under the muscles, away from the proximal stump. The wound was then closed with cyanoacrylate adhesive reinforced by 6-0 silk sutures.

3.3 Sciatic Nerve Functional Index

The sciatic nerve functional index developed by DeMedinaceli (De Medinaceli et al., 1982) is a functional assessment of sciatic nerve regeneration following injury. In this

assay, footprints of mice were recorded in a 11 x 2 inch runway lined with paper pre-treated with bromophenol blue (5% bromophenol blue, sulfone form, Sigma, in absolute acetone; Lowdon et al.,) The treated paper turns from light yellowish-green to dark blue upon contact with moisture. When the hind paws of the mice were dipped in TBS (with 0.2% Tween-20) and allowed to walk on the stained paper, the prints of the hind feet are revealed on the treated paper and serve as permanent records the footprints. Before the sciatic nerve injury, the mice were trained to walk on the track several times (0 day time point). After the crush surgery, the footprints of the mice were measures in 3 day intervals from day 3 to day 30. The footprints on the papers were then digitized, and 3 footprint parameters --outer toe spread, inner toe spread, and paw length (**see Figure 3.3**)--were then measured on both the lesioned and control sides with NIH image software. The recovery of the sciatic nerve function was then quantified by inputting the measurements into formulas (Bain et al., **see Figure 3.3**).

3.4 Western blotting

Similar procedures for western blotting were described above. Please refer back to sections 1.2 (Sciatic nerve collection & homogenization), 1.3 (Western blotting detection of phosphorylate kinases). The changes to the above procedure are described below.

For this set of studies, the P-ERK blots were stripped by incubating the membranes in a stripping buffer (0.98g Tris-HCL, 2g SDS, 0.7ml β-Mercaptoethanol per 100ml) for 30 minutes at 50°C. The stripped blots were subsequently washed 2X in TBS-Tween for 10 minutes each, followed by either a two-hour blocking at room temperature or overnight blocking at 4°C. (Blocking solution: 5% non-fat dry milk in TBS-Tween). After blocking, the blots were probed with rabbit anti-ERK antibodies (New England Biolab; 1:1000 dilution). The re-probing of the P-ERK blots with ERK antibodies allowed us to correct our quantification of P-ERK by taking into account potential loading differences. For quantification of western blots, digital image files were obtained by scanning the exposed films and analyzed using the NIH Image software package. Care was taken to analyze only signals within the linear response range of the films. The mean labeling intensity of specific bands was measured after subtraction of background density measurements. The measurements of P-ERK bands from individual experiments were then divided by ERK measurements from the same sample to obtain the P-ERK/ERK ratio. This ratio was averaged for the three independent sets of experiments without any further modification.

Material and Methods 4

Studies of Facial Motor neuron Survival in FGF1 Knockout Mice

4.1 Facial nerve transection

Adult wild type and FGF-1 -/- mice (ranging from 2 to 5 months old) were anesthetized with a continuous flow of 2% isoflurorane and 1% oxygen. The mice were shaved behind the right ear, and an incision of approximately 5 mm was made just dorsal and caudal to the external ear. After several layers of fatty tissue were dissected away, the facial nerve was exposed. The facial nerve trunk was released from surrounding connective tissue through gentle dissection, following which the nerve trunk was transected by removing a 3 mm section of the nerve. This injury transects all axons in the facial nerve except for a small population which projects through the retroauricular branch (which leaves the main trunk at the Stylomastoid foramen prior to the injury site). After the transection is completed, the skin incision was closed with cyanoacrylate adhesive (VetBond).

4.2 Tissue Collection, Processing, and Embedding

At 60 days after injury, the mice were anesthetized with a cocktail of ketamine, xylazine, and acepromazine. The animals were then perfused with PBS for 2 minutes until the outflow from the right ventricle is clear of blood, followed by perfusion with 10% formalin in PBS for 10 minutes. Following perfusion, whole brains were collected and fixed further with immersion fixation with 10 % formalin in PBS overnight at room temperature. The tissues were then washed in PBS for at least overnight at 4°C until further processing. At this point, the brain was further dissected into the forebrain and brainstem, which contains the facial nucleus, to facilitate the penetration of solutions. The tissues were dehydrated with sequential overnight washes in 75%, 90%, and 99% ethanol at 4°C. The tissues were then immersed in room temperature Technovit 7100 plastic embedding solution for 14 days with changes of the Technovit solution every other day to allow complete penetration of the plastic solution into the tissue blocks. After the last incubation, the tissues were embedded in polymerized Technovit plastic. The tissues were then cut on a microtome, where 60 μm-thick serial sections were collected into Giemsa stain (diluted 1:500 in dH_2O) and allowed to shake overnight on a rotating shaker. After an overnight incubation in the Giemsa stain, the sections were slightly destained in PBS for 2 hours followed by a 1 hour wash in dH2O. The sections were then mounted on gelatin-coated slides, dried overnight, and coverslipped with DPX mounting medium.

4.3 Estimation of motor neuron number using stereology

To perform stereological measurement of neuron numbers, we used a motorized xyz-stage, controlled by the Stereology Investigator software run on a MS Windows computer. For each section to be counted, the outline of the nucleus was drawn at low magnification (4X). When appropriate grid size and sampling sites were set, counting was done a high magnification (60X). The grid size and sampling sites were previously determined in preliminary experiments to obtain the parameters needed to achieve accurate estimates. For the counting, a couple of criteria were set to prevent counting errors. On the XY plane, the location and shape of the neuron would determine the inclusion or the exclusion of a neuron. To be counted, any portion of the cell must not cross any exclusion line. On the Z axis, a buffer zone was set to span 10 μm from the top and 10μm from the bottom of each section, to prevent the double counting of large cells that might span multiple sections. Only nucleoli that is clearly in focus in the counting zone is included. Another sampling criteria is to include only large cells with nuclear diameter greater than 12μm . These strict criteria facilitated the accurate estimate of neuron numbers using the stereological sampling method. All consecutive sections containing visible facial motor neurons were counted.

References

Avellino, A. M., Hart, D., Dailey, A. T., MacKinnon, M., Ellegala, D. and Kliot, M. (1995) Differential macrophage responses in the peripheral and central nervous system during wallerian degeneration of axons. Exp Neurol 136: 183-98.

Bain, J. R., Mackinnon, S. E. and Hunter, D. A. (1989) Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. Plast Reconstr Surg 83: 129-38.

Bisby, M. A. and Chen, S. (1990) Delayed wallerian degeneration in sciatic nerves of C57BL/Ola mice is associated with impaired regeneration of sensory axons. Brain Research 530: 117-20.

Bolin, L. M., Verity, A. N., Silver, J. E., Shooter, E. M. and Abrams, J. S. (1995) Interleukin-6 production by Schwann cells and induction in sciatic nerve injury. Journal of Neurochemistry 64: 850-8.

Bourde, O., Kiefer, R., Toyka, K. V. and Hartung, H. P. (1996) Quantification of interleukin-6 mRNA in wallerian degeneration by competitive reverse transcription polymerase chain reaction. J Neuroimmunol 69: 135-40.

Brown, M. C., Lunn, E. R. and Perry, V. H. (1992) Consequences of slow Wallerian degeneration for regenerating motor and sensory axons. Journal of Neurobiology 23: 521-36.

Bruck, W., Bruck, Y., Maruschak, B. and Friede, R. L. (1995) Mechanisms of macrophage recruitment in Wallerian degeneration. Acta Neuropathol (Berl) 89: 363-7.

Buck, C. R., Martinez, H. J., Black, I. B. and Chao, M. V. (1987) Developmentally regulated expression of the nerve growth factor receptor gene in the periphery and brain. Proc Natl Acad Sci U S A 84: 3060-3.

Bunge, R. P. (1993) Expanding roles for the Schwann cell: ensheathment, myelination, trophism and regeneration. Curr Opin Neurobiol 3: 805-9.

Burgess, W. H., Dionne, C. A., Kaplow, J., Mudd, R., Friesel, R., Zilberstein, A., Schlessinger, J. and Jaye, M. (1990) Characterization and cDNA cloning of phospholipase C-gamma, a major substrate for heparin-binding growth factor 1 (acidic fibroblast growth factor)-activated tyrosine kinase. Mol Cell Biol 10: 4770-7.

Burgess, W. H. and Maciag, T. (1989) The heparin-binding (fibroblast) growth factor family of proteins. Annu Rev Biochem 58: 575 - 606.

Carreira, C. M., LaVallee, T. M., Tarantini, F., Jackson, A., Lathrop, J. T., Hampton, B., Burgess, W. H. and Maciag, T. (1998) S100A13 is involved in the regulation of fibroblast growth factor-1 and p40 synaptotagmin-1 release in vitro. J Biol Chem 273: 22224-31.

Carroll, S. L. and Frohnert, P. W. (1998) Expression of JE (monocyte chemoattractant protein-1) is induced by sciatic axotomy in wild type rodents but not in C57BL/Wld(s) mice. Journal of Neuropathology & Experimental Neurology 57: 915-30.

Carroll, S. L., Miller, M. L., Frohnert, P. W., Kim, S. S. and Corbett, J. A. (1997) Expression of neuregulins and their putative receptors, erbb2 and erbb3, is induced during wallerian degeneration. Journal of Neuroscience 17: 1642-1659.

Chen, E. W. and Chiu, A. Y. (1992) Early stages in the development of spinal motor neurons. J Comp Neurol 320: 291-303.

Chen, S. and Bisby, M. A. (1993) Impaired motor axon regeneration in the C57BL/Ola mouse. Journal of Comparative Neurology 333: 449-54.

Cheng, H., Cao, Y. and Olson, L. (1996) Spinal cord repair in adult paraplegic rats: partial restoration of hind limb function [see comments]. Science 273: 510-3.

Cohen, J. A., Yachnis, A. T., Arai, M., Davis, J. G. and Scherer, S. S. (1992) Expression of the neu proto-oncogene by Schwann cells during peripheral nerve development and Wallerian degeneration. J Neurosci Res 31: 622-34.

Cordeiro, P. G., Seckel, B. R., Lipton, S. A., D'Amore, P. A., Wagner, J. and Madison, R. (1989) Acidic fibroblast growth factor enhances peripheral nerve regeneration in vivo. Plast Reconstr Surg 83: 1013-9; discussion 1020-1.

Costello, B., Meymandi, A. and Freeman, J. A. (1990) Factors influencing GAP-43 gene expression in PC12 pheochromocytoma cells. J Neurosci 10: 1398-406.

Creedon, D. J., Tansey, M. G., Baloh, R. H., Osborne, P. A., Lampe, P. A., Fahrner, T. J., Heuckeroth, R. O., Milbrandt, J. and Johnson, E. M., Jr. (1997) Neurturin shares receptors and signal transduction pathways with glial cell line-derived neurotrophic factor in sympathetic neurons. Proc Natl Acad Sci U S A 94: 7018-23.

Creuzet, C., Loeb, J. and Barbin, G. (1995) Fibroblast Growth Factors Stimulate Protein Tyrosine Phosphorylation and Mitogen-Activated Protein Kinase Activity In Primary Cultures Of Hippocampal Neurons. Journal of Neurochemistry 64: 1541-1547.

Crews, L. L. and Wigston, D. J. (1990) The dependence of motor neurons on their target muscle during postnatal development of the mouse. J Neurosci 10: 1643-53.

Cuevas, P., Carceller, F. and Gimenezgallego, G. (1995) Acidic Fibroblast Growth Factor Prevents Post-Axotomy Neuronal Death Of the Newborn Rat Facial Nerve. Neuroscience Letters 197: 183-186.

Curtis, R., Scherer, S. S., Somogyi, R., Adryan, K. M., Ip, N. Y., Zhu, Y., Lindsay, R. M. and DiStefano, P. S. (1994) Retrograde axonal transport of LIF is increased by peripheral nerve injury: correlation with increased LIF expression in distal nerve. Neuron 12: 191-204.

Danielsen, N., Kerns, J. M., Holmquist, B., Zhao, Q., Lundborg, G. and Kanje, M. (1994) Pre-degenerated nerve grafts enhance regeneration by shortening the initial delay period. Brain Res 666: 250-4.

Danielsen, N., Kerns, J. M., Holmquist, B., Zhao, Q., Lundborg, G. and Kanje, M. (1995) Predegeneration enhances regeneration into acellular nerve grafts. Brain Res 681: 105-8.

Darnell, J. E., Jr., Kerr, I. M. and Stark, G. R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264: 1415-21.

David, S. and Aguayo, A. J. (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214: 931-3.

Davis, J. B. and Stroobant, P. (1990) Platelet-derived growth factors and fibroblast growth factors are mitogens for rat Schwann cells. J Cell Biol 110: 1353-60.

Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y. and Yancopoulos, G. D. (1993) LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. Science 260: 1805-8.

de Medinaceli, L., Freed, W. J. and Wyatt, R. J. (1982) An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. Exp Neurol 77: 634-43.

De Vries, G. (1993). Schwann cell proliferation. Peripheral Neuropathy. Philadelphia, W.B. Saunders. 3rd, ed. 290-298.

Dennis, M. J., Ziskind-Conhaim, L. and Harris, A. J. (1981) Development of neuromuscular junctions in rat embryos. Dev Biol 81: 266-79.

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R. and Jessen, K. R. (1995) Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. Neuron 15: 585-96.

Eccleston, P. A. and Silberberg, D. H. (1985) Fibroblast growth factor is a mitogen for oligodendrocytes in vitro. Brain Res 353: 315-8.

Eckenstein, F. P. (1994) Fibroblast growth factors in the nervous system. J Neurobiol 25: 1467-80.

Eckenstein, F. P., Andersson, C., Kuzis, K. and Woodward, W. R. (1994) Distribution of acidic and basic fibroblast growth factors in the mature, injured and developing rat nervous system. Prog Brain Res 103: 55 - 64.

Eckenstein, F. P., Esch, F., Holbert, T., Blacher, R. W. and Nishi, R. (1990) Purification and characterization of a trophic factor for embryonic peripheral neurons: comparison with fibroblast growth factors. Neuron 4: 623-31.

Eckenstein, F. P., Kuzis, K., Nishi, R., Woodward, W. R., Meshul, C., Sherman, L. and Ciment, G. (1994) Cellular distribution, subcellular localization and possible functions of basic and acidic fibroblast growth factors. Biochem Pharmacol 47: 103-10.

Eckenstein, F. P., Shipley, G. D. and Nishi, R. (1991) Acidic and basic fibroblast growth factors in the nervous system: distribution and differential alteration of levels after injury of central versus peripheral nerve. J Neurosci 11: 412-9.

Elde, R., Cao, Y. H., Cintra, A., Brelje, T. C., Pelto-Huikko, M., Junttila, T., Fuxe, K., Pettersson, R. F. and Hokfelt, T. (1991) Prominent expression of acidic fibroblast growth factor in motor and sensory neurons. Neuron 7: 349-64.

Ernfors, P., Henschen, A., Olson, L. and Persson, H. (1989) Expression of nerve growth factor receptor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motor neurons. Neuron 2: 1605-13.

Fawcett, J. W. and Keynes, R. J. (1990) Peripheral nerve regeneration. Annu Rev Neurosci 1990;13:43-60 :

Figueiredo, B. C., Pluss, K., Skup, M., Otten, U. and Cuello, A. C. (1995) Acidic FGF induces NGF and its mRNA in the injured neocortex of adult animals. Brain Res Mol Brain Res 33: 1-6.

Funakoshi, H., Frisen, J., Barbany, G., Timmusk, T., Zachrisson, O., Verge, V. M. and Persson, H. (1993) Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. J Cell Biol 123: 455-65.

Gorin, P. D. and Johnson, E. M., Jr. (1980) Effects of long-term nerve growth factor deprivation on the nervous system of the adult rat: an experimental autoimmune approach. Brain Res 198: 27-42.

Greeson, D. M., Moix, L., Meier, M., Armstrong, D. M. and Wiley, R. G. (1992) A continuing signal maintains NGF receptor expression in hypoglossal motor neurons after crush injury. Brain Res 594: 351-5.

Griffin, J. W. and Hoffman, P. N. (1993). Degeneration and regeneration in the peripheral nervous system. Peripheral Neuropathy. Philadelphia, Saunders. 361-372.

Grinspan, J. B., Marchionni, M. A., Reeves, M., Coulaloglou, M. and Scherer, S. S. (1996) Axonal interactions regulate Schwann cell apoptosis in developing peripheral nerve: neuregulin receptors and the role of neuregulins. J Neurosci 16: 6107-18.

Grothe, C., Meisinger, C., Hertenstein, A., Kurz, H. and Wewetzer, K. (1997) Expression of fibroblast growth factor-2 and fibroblast growth factor receptor 1 messenger RNAs in spinal ganglia and sciatic nerve: regulation after peripheral nerve lesion. Neuroscience 76: 123-35.

Gundersen, R. W. and Barrett, J. N. (1979) Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor. Science 206: 1079-80.

Hall, S. M. (1989) Regeneration in the peripheral nervous system. Neuropathol Appl Neurobiol 15: 513-29.

Harris, A. J. and McCaig, C. D. (1984) Motor neuron death and motor unit size during embryonic development of the rat. J Neurosci 4: 13-24.

Heumann, R., Korsching, S., Bandtlow, C. and Thoenen, H. (1987) Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection. Journal of Cell Biology 104: 1623-31.

Hill, C. S. and Treisman, R. (1995) Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 80: 199-211.

Hu, B. R. and Wieloch, T. (1994) Tyrosine phosphorylation and activation of mitogen-activated protein kinase in the rat brain following transient cerebral ischemia. J Neurochem 62: 1357-67.

Humpel, C., Chadi, G., Lippoldt, A., Ganten, D., Fuxe, K. and Olson, L. (1994) Increase of basic fibroblast growth factor (bFGF, FGF-2) messenger RNA and protein

following implantation of a microdialysis probe into rat hippocampus. Exp Brain Res 98: 229-37.

Ihle, J. N., Thierfelder, W., Teglund, S., Stravapodis, D., Wang, D., Feng, J. and Parganas, E. (1998) Signaling by the cytokine receptor superfamily. Ann N Y Acad Sci 1998 Dec 11;865:1-9 :

Inserra, M. M., Bloch, D. A. and Terris, D. J. (1998) Functional indices for sciatic, peroneal, and posterior tibial nerve lesions in the mouse. Microsurgery 18: 119-24.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J. and et al. (1992) CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. Cell 69: 1121-32.

Ishikawa, R., Nishikori, K., Furukawa, Y., Hayashi, K. and Furukawa, S. (1992) Injury-induced reduction of acidic fibroblast growth factor levels in the distal parts of rat sciatic nerve. Neurosci Lett 135: 113-6.

Ito, Y., Yamamoto, M., Li, M., Doyu, M., Tanaka, F., Mitsuma, T. and Sobue, G. (1998) Differential temporal expression of mRNAs for ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and their receptors (CNTFRalpha, LIFRbeta, IL-6Ralpha and gp130) in injured peripheral nerves [In Process Citation]. Brain Res 793: 321-7.

Jessen, K. R. and Mirsky, R. (1991) Schwann cell precursors and their development. Glia 4: 185-94.

Johnson, D. E. and Williams, L. T. (1993) Structural and functional diversity in the FGF receptor multigene family. Adv Cancer Res 60: 1 - 41.

Johnson, E. M., Jr., Gorin, P. D., Brandeis, L. D. and Pearson, J. (1980) Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. Science 210: 916-8.

Johnson, E. M., Jr., Gorin, P. D., Osborne, P. A., Rydel, R. E. and Pearson, J. (1982) Effects of autoimmune NGF deprivation in the adult rabbit and offspring. Brain Res 240: 131-40.

Kato, H., Wanaka, A. and Tohyama, M. (1992) Co-localization of basic fibroblast growth factor-like immunoreactivity and its receptor mRNA in the rat spinal cord and the dorsal root ganglion. Brain Res 576: 351-4.

Kawamata, T., Ren, J., Cha, J. H. and Finklestein, S. P. (1999) Intracisternal antisense oligonucleotide to growth associated protein- 43 blocks the recovery-promoting effects of basic fibroblast growth factor after focal stroke. Exp Neurol 158: 89-96.

Kerns, J. M., Danielsen, N., Holmquist, B., Kanje, M. and Lundborg, G. (1993) The influence of predegeneration on regeneration through peripheral nerve grafts in the rat. Exp Neurol 122: 28-36.

Kishimoto, T., Taga, T. and Akira, S. (1994) Cytokine signal transduction. Cell 76: 253-62.

Kuzis, K., Coffin, J. D. and Eckenstein, F. P. (1999) Time course and age dependence of motor neuron death following facial nerve crush injury: role of fibroblast growth factor. Exp Neurol 157: 77-87.

Kuzis, K., Reed, S., Cherry, N. J., Woodward, W. R. and Eckenstein, F. P. (1995) Developmental time course of acidic and basic fibroblast growth factors' expression in distinct cellular populations of the rat central nervous system. J Comp Neurol 358: 142-53.

Kwon, Y. K., Bhattacharyya, A., Alberta, J. A., Giannobile, W. V., Cheon, K., Stiles, C. D. and Pomeroy, S. L. (1997) Activation of ErbB2 during wallerian degeneration of sciatic nerve. J Neurosci 17: 8293-9.

Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. and Woodgett, J. R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. Nature 369: 156-60.

Laird, J. M., Mason, G. S., Thomas, K. A., Hargreaves, R. J. and Hill, R. G. (1995) Acidic fibroblast growth factor stimulates motor and sensory axon regeneration after sciatic nerve crush in the rat. Neuroscience 65: 209-16.

LaVallee, T. M., Tarantini, F., Gamble, S., Carreira, C. M., Jackson, A. and Maciag, T. (1998) Synaptotagmin-1 is required for fibroblast growth factor-1 release. J Biol Chem 273: 22217-23.

Lindholm, D., Heumann, R., Meyer, M. and Thoenen, H. (1987) Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. Nature 330: 658-9.

Lowdon, I. M., Seaber, A. V. and Urbaniak, J. R. (1988) An improved method of recording rat tracks for measurement of the sciatic functional index of de Medinaceli. J Neurosci Methods 24: 279-81.

Lundborg, G. (1987) Nerve regeneration and repair. A review. Acta Orthop Scand 58: 145-69.

Marchionni, M. A., Goodearl, A. D., Chen, M. S., Bermingham, M. O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K. and et, a. l. (1993) Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system [see comments]. Nature 362: 312-8.

McNeil, P. L., Muthukrishnan, L., Warder, E. and D'Amore, P. A. (1989) Growth factors are released by mechanically wounded endothelial cells. J Cell Biol 109: 811-22.

McQuarrie, I. G. (1978) The effect of a conditioning lesion on the regeneration of motor axons. Brain Res 152: 597-602.

McQuarrie, I. G. and Jacob, J. M. (1991) Conditioning nerve crush accelerates cytoskeletal protein transport in sprouts that form after a subsequent crush. J Comp Neurol 305: 139-47.

Meiri, K. F., Saffell, J. L., Walsh, F. S. and Doherty, P. (1998) Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-

43) function and is associated with GAP-43 phosphorylation in growth cones. J Neurosci 18: 10429-37.

Meyer, M., Matsuoka, I., Wetmore, C., Olson, L. and Thoenen, H. (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol 119: 45-54.

Miller, D. L., Ortega, S., Bashayan, O., Basch, R. and Basilico, C. (2000) Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. Mol Cell Biol 20: 2260-8.

Mohiuddin, L., Fernyhough, P. and Tomlinson, D. R. (1996) Acidic fibroblast growth factor enhances neurite outgrowth and stimulates expression of GAP-43 and T alpha 1 alpha-tubulin in cultured neurones from adult rat dorsal root ganglia. Neurosci Lett 215: 111-4.

Moix, L. J., Greeson, D. M., Armstrong, D. M. and Wiley, R. G. (1991) Separate signals mediate hypoglossal motor neuron response to axonal injury. Brain Res 564: 176-80.

Murakami, M., Hibi, M., Nakagawa, N., Nakagawa, T., Yasukawa, K., Yamanishi, K., Taga, T. and Kishimoto, T. (1993) IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science 260: 1808-10.

Murphy, P. G., Borthwick, L. S., Johnston, R. S., Kuchel, G. and Richardson, P. M. (1999) Nature of the retrograde signal from injured nerves that induces interleukin-6 mRNA in neurons. J Neurosci 19: 3791-800.

Muthukrishnan, L., Warder, E. and McNeil, P. L. (1991) Basic fibroblast growth factor is efficiently released from a cytolsolic storage site through plasma membrane disruptions of endothelial cells. J Cell Physiol 148: 1-16.

Nachemson, A. K., Hansson, H. A. and Lundborg, G. (1988) Neurotropism in nerve regeneration: an immunohistochemical study. Acta Physiol Scand 133: 139-48.

Oellig, C., Pirvola, U., Taylor, L., Elde, R., Hokfelt, T. and Pettersson, R. F. (1995) Acidic FGF and FGF receptors are specifically expressed in neurons of developing and adult rat dorsal root ganglia. Eur J Neurosci 7: 863-74.

Ono, T., Saito, H., Kishimoto, T., Okumoto, T. and Miyamoto, K. (1991) Stimulation of biosynthesis of nerve growth factor by acidic fibroblast growth factor in cultured mouse astrocytes. Neurosci Lett 126: 18-20.

Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E. and Leder, P. (1992) Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. Mol Cell Biol 12: 240-7.

Ortega, S., Ittmann, M., Tsang, S. H., Ehrlich, M. and Basilico, C. (1998) Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. Proc Natl Acad Sci U S A 95: 5672-7.

Paul, A., Wilson, S., Belham, C. M., Robinson, C. J., Scott, P. H., Gould, G. W. and Plevin, R. (1997) Stress-activated protein kinases: activation, regulation and function. Cell Signal 9: 403-10.

Pellegrino, R. G., Politis, M. J., Ritchie, J. M. and Spencer, P. S. (1986) Events in degenerating cat peripheral nerve: induction of Schwann cell S phase and its relation to nerve fibre degeneration. J Neurocytol 15: 17-28.

Perry, V. H. and Brown, M. C. (1992) Role of macrophages in peripheral nerve degeneration and repair. Bioessays 14: 401-6.

Perry, V. H., Brown, M. C. and Gordon, S. (1987) The macrophage response to central and peripheral nerve injury. A possible role for macrophages in regeneration. J Exp Med 165: 1218-23.

Pettmann, B., Weibel, M., Sensenbrenner, M. and Labourdette, G. (1985) Purification of two astroglial growth factors from bovine brain. FEBS Lett 189: 102-8.

Phelps, P. E., Barber, R. P., Brennan, L. A., Maines, V. M., Salvaterra, P. M. and Vaughn, J. E. (1990) Embryonic development of four different subsets of cholinergic neurons in rat cervical spinal cord. J Comp Neurol 291: 9-26.

Phelps, P. E., Barber, R. P. and Vaughn, J. E. (1988) Generation patterns of four groups of cholinergic neurons in rat cervical spinal cord: a combined tritiated thymidine autoradiographic and choline acetyltransferase immunocytochemical study. J Comp Neurol 273: 459-72.

Phelps, P. E., Barber, R. P. and Vaughn, J. E. (1991) Embryonic development of choline acetyltransferase in thoracic spinal motor neurons: somatic and autonomic neurons may be derived from a common cellular group. J Comp Neurol 307: 77-86.

Piehl, F., Arvidsson, U., Johnson, H., Cullheim, S., Dagerlind, A., Ulfhake, B., Cao, Y., Elde, R., Pettersson, R. F., Terenius, L. and et al. (1993) GAP-43, aFGF, CCK and alpha- and beta-CGRP in rat spinal motor neurons subjected to axotomy and/or dorsal root severance. Eur J Neurosci 5: 1321-33.

Piehl, F., Hammarberg, H., Hokfelt, T. and Cullheim, S. (1998) Regulatory effects of trophic factors on expression and distribution of CGRP and GAP-43 in rat motor neurons. J Neurosci Res 51: 1-14.

Planas, A. M., Soriano, M. A., Berruezo, M., Justicia, C., Estrada, A., Pitarch, S. and Ferrer, I. (1996) Induction of Stat3, a signal transducer and transcription factor, in reactive microglia following transient focal cerebral ischaemia. Eur J Neurosci 8: 2612-8.

Politis, M. J., Ederle, K. and Spencer, P. S. (1982) Tropism in nerve regeneration in vivo. Attraction of regenerating axons by diffusible factors derived from cells in distal nerve stumps of transected peripheral nerves. Brain Res 253: 1-12.

Rapraeger, A. C. (1995) In the Clutches Of Proteoglycans - How Does Heparan Sulfate Regulate Fgf Binding. Chemistry & Biology 2: 645-649.

Reichert, F., Levitzky, R. and Rotshenker, S. (1996) Interleukin 6 in intact and injured mouse peripheral nerves. Eur J Neurosci 8: 530-5.

Rende, M., Muir, D., Ruoslahti, E., Hagg, T., Varon, S. and Manthorpe, M. (1992) Immunolocalization of ciliary neuronotrophic factor in adult rat sciatic nerve. Glia 5: 25-32.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R. and Birchmeier, C. (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature 389: 725-30.

Rosenbaum, C., Karyala, S., Marchionni, M. A., Kim, H. A., Krasnoselsky, A. L., Happel, B., Isaacs, I., Brackenbury, R. and Ratner, N. (1997) Schwann cells express NDF and SMDF/n-ARIA mRNAs, secrete neuregulin, and show constitutive activation of erbB3 receptors: evidence for a neuregulin autocrine loop. Exp Neurol 148: 604-15.

Rotshenker, S., Aamar, S. and Barak, V. (1992) Interleukin-1 activity in lesioned peripheral nerve. J Neuroimmunol 39: 75-80.

Salzer, J. L. and Bunge, R. P. (1980) Studies of Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development, Wallerian degeneration, and direct injury. J Cell Biol 84: 739-52.

Schlessinger, J. and Ullrich, A. (1992) Growth factor signaling by receptor tyrosine kinases. Neuron 9: 383-91.

Segal, R. A. and Greenberg, M. E. (1996) Intracellular signaling pathways activated by neurotrophic factors. Annu Rev Neurosci 19: 463-489.

Seger, R. and Krebs, E. G. (1995) The MAPK signaling cascade. Faseb J 9: 726-35.

Sendtner, M., Arakawa, Y., Stockli, K. A., Kreutzberg, G. W. and Thoenen, H. (1991) Effect of ciliary neurotrophic factor (CNTF) on motor neuron survival. J Cell Sci Suppl 15: 103-9.

Sheard, P., McCaig, C. D. and Harris, A. J. (1984) Critical periods in rat motor neuron development. Dev Biol 102: 21-31.

Shipley, G. (1986) A serum-free [3H]thymidine incorporation assay for the detection of transforming growth factors. Journal of Tissue Culture Methods 10: 117 - 123.

Soler, R. M., Dolcet, X., Encinas, M., Egea, J., Bayascas, J. R. and Comella, J. X. (1999) Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motor neurons [In Process Citation]. J Neurosci 19: 9160-9.

Spira, M. E., Benbassat, D. and Dormann, A. (1993) Resealing of the proximal and distal cut ends of transected axons: electrophysiological and ultrastructural analysis. J Neurobiol 24: 300-16.

Springer, J. E., Mu, X., Bergmann, L. W. and Trojanowski, J. Q. (1994) Expression of GDNF mRNA in rat and human nervous tissue. Exp Neurol 127: 167-70.

Stock, A., Kuzis, K., Woodward, W. R., Nishi, R. and Eckenstein, F. P. (1992) Localization of acidic fibroblast growth factor in specific subcortical neuronal populations. J Neurosci 12: 4688-700.

Stockli, K. A., Lillien, L. E., Naher-Noe, M., Breitfeld, G., Hughes, R. A., Raff, M. C., Thoenen, H. and Sendtner, M. (1991) Regional distribution, developmental changes, and cellular localization of CNTF-mRNA and protein in the rat brain. J Cell Biol 115: 447-59.

Tarantini, F., LaVallee, T., Jackson, A., Gamble, S., Carreira, C. M., Garfinkel, S., Burgess, W. H. and Maciag, T. (1998) The extravesicular domain of synaptotagmin-1 is released with the latent fibroblast growth factor-1 homodimer in response to heat shock. J Biol Chem 273: 22209-16.

Thomas, P. K., Berthold, C. H. and Ochoa, J. (1993). Microscopic anatomy of the peripheral nervous system. Peripheral Neuropathy. Philadelphia, Sauder. 28-73.

Thompson, J. A., Anderson, K. D., DiPietro, J. M., Zwiebel, J. A., Zametta, M., Anderson, W. F. and Maciag, T. (1988) Site-directed neovessel formation in vivo. Science 241: 1349-52.

Trupp, M., Ryden, M., Jornvall, H., Funakoshi, H., Timmusk, T., Arenas, E. and Ibanez, C. F. (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J Cell Biol 130: 137-48.

Unsicker, K., Reichert, P. H., Schmidt, R., Pettmann, B., Labourdette, G. and Sensenbrenner, M. (1987) Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central nervous system neurons. Proc Natl Acad Sci U S A 84: 5459-63.

Vainikka, S., Joukov, V., Wennstrom, S., Bergman, M., Pelicci, P. G. and Alitalo, K. (1994) Signal transduction by fibroblast growth factor receptor-4 (FGFR-4). Comparison with FGFR-1. J Biol Chem 269: 18320-6.

Walicke, P. A. (1988) Basic and acidic fibroblast growth factors have trophic effects on neurons from multiple CNS regions. J Neurosci 8: 2618-27.

Walicke, P. A. and Baird, A. (1988) Neurotrophic effects of basic and acidic fibroblast growth factors are not mediated through glial cells. Brain Res 468: 71-9.

Walter, M. A., Kurouglu, R., Caulfield, J. B., Vasconez, L. O. and Thompson, J. A. (1993) Enhanced peripheral nerve regeneration by acidic fibroblast growth factor. Lymphokine Cytokine Res 12: 135-41.

Wang, J. K., Gao, G. and Goldfarb, M. (1994) Fibroblast growth factor receptors have different signaling and mitogenic potentials. Mol Cell Biol 14: 181-8.

Worby, C. A., Vega, Q. C., Zhao, Y., Chao, H. H. J., Seasholtz, A. F. and Dixon, J. E. (1996) Glial cell line-derived neurotrophic factor signals through the RET receptor and activates mitogen-activated protein kinase. J Biol Chem 271: 23619-22.

Xie, X. Y. and Barrett, J. N. (1991) Membrane resealing in cultured rat septal neurons after neurite transection: evidence for enhancement by Ca(2+)-triggered protease activity and cytoskeletal disassembly. J Neurosci 11: 3257-67.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991) Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64: 841-8.

Yoshida, K. and Gage, F. H. (1991) Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. Brain Res 538: 118-26.

Yoshida, K. and Gage, F. H. (1992) Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. Brain Res 569: 14-25.

Ziv, N. E. and Spira, M. E. (1993) Spatiotemporal distribution of Ca2+ following axotomy and throughout the recovery process of cultured Aplysia neurons. Eur J Neurosci 5: 657-68.