THE DISCOVERY OF PRECURSOR CELLS AND NEUROGENESIS IN POSTNATAL DORSAL ROOT SENSORY GANGLIA

By

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A Thesis Presented to the University of Manitoba in Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy in Physiology

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The Discovery of Precursor Cells and Neurogenesis in Postnatal Dorsal Root Sensory Ganglia

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Michael Peter Namaka

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

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THE DISCOVERY OF PRECURSOR CELLS AND NEUROGENESIS IN POSTNATAL DORSAL ROOT SENSORY GANGLIA

BY

MICHAEL PETER NAMAKA

2000
THE UNIVERSITY OF MANITOBA

ABSTRACT

Neurogenesis is known to continue in various regions of the central nervous system (CNS) throughout life. However. it is currently unknown whether the same can be said for the dorsal root ganglia (DRG) of the peripheral nervous system (PNS). The mitogen. basic fibroblast growth factor (bFGF) has been previously demonstrated to induce proliferation of CNS neuronal precursors in culture. Since it has also been shown that bFGF is up-regulated within DRG following axotomy. it is quite possible that the DRG also contains bFGF-responsive neuronal precursors. As a result. *in vitro* and *in vivo* experiments were undertaken in order to explore this possibility.

The addition of bFGF to cell cultures of early postnatal DRG induced a proliferative response that became evident by the formation of spherical aggregates that stained for the CNS stem cell marker nestin. The replacement of bFGF with trophic factors induced similar cell clusters to differentiate into cells that morphologically resembled DRG neurons. stained for neuronal markers. and generated action potentials. Furthermore. bromodeoxyuridine (BrdU). used as a marker of cytogenesis. was detected in neurofilament-160^{+} (NF-160^{+}) and/or microtubule associated protein^{+} (MAP-2)$^{+}$ cells that morphologically resembled neurons. Nerve growth factor (NGF) and sonic hedgehog (Shh) were also capable of producing spherical. process bearing. cell aggregates that stained for neuronal markers. Thus. the DRG appears to contain neuronal precursors that can proliferate in response to several mitogens.

Sciatic nerve lesion experiments in juvenile rats were employed to demonstrate that neurogenesis occurs normally following nerve injury. These studies demonstrated a consistent ~40% relative increase in proliferating cells ($BrdU^+$) on the lesioned side compared to the contralateral unlesioned DRG. This observed proliferative response appeared to be greater at 1 week than 2 weeks post-axotomy. Many $BrdU^+$ cells also appeared to stain for the neuronal marker neuron-specific enolase (NSE) suggesting the presence of neuronal precursors. These double-labeled cells were preferentially upregulated in the ipsilateral ganglia, reaching maximal numbers within the first 48 hours after nerve transection. However, this upregulation was transient, lasting for only 1 week. Similar cells ($BrdU^+/NSE^+$) were also identified in adult DRG post-axotomy. In addition, $BrdU^+$ labeling in neurons previously anterogradely labeled with Flouro-Gold suggest that pre-existing neurons within adult DRG can become mitotic.

These results support the hypothesis that DRG contain neural precursors throughout life that can proliferate and subsequently differentiate into neurons in response to injury. These findings may indicate a greater range of plasticity that is available to somatosensory systems during maturation and following injury, perhaps to replace ineffectual or dying neurons.

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ABBREVIATIONS

BDNF: Brain Derived Neurotrophic Factor
bFGF: Basic Fibroblast Growth Factor
BMP-2: Bone Morphogenic Protein-2
BMP-4: Bone Morphogenic Protein-4
BrdU: Bromodeoxyuridine
CA: Carbonic Anhydrase
CCK: Cholecystokinin
CGRP: Calcitonin Gene Related Peptide
CNS: Central Nervous System
CNTF: Cilliary Neurotrophic Factor
DRG: Dorsal Root Ganglia
EGF: Epidermal Growth Factor
FG: Flouro-Gold
GAL: Galanin
GDNF: Glial Derived Neurotrophic Factor
MAP-2: Microtubule Associated Protein-2\
NCSC: Neural Crest Stem Cells
NEP: Neuroepithelial Cells
NF-160: Neurofilament-160
NGF: Nerve Growth Factor
NOS: Nitric Oxide Synthase
NPY: Neuropeptide Y
NSE: Neuron Specific Enolase
NT-3: Neurotrophin-3
NT-4: Neurotrophin-4
NT-5: Neurotrophin-5
PBS: Phosphate Buffered Saline
PBST: Phosphate Buffered Saline with Triton-X100
PNS: Peripheral Nervous System
RSD: Reflex Sympathetic Dystrophy
Shh: Sonic Hedge Hog
SSC: Saline Sodium Citrate
Sub P: Substance P
TBS: Tris Buffered Saline
TBS-T: Tris Buffered Saline with Triton –X100
Trk: Tyrosine Kinase
TTX: Tetrodotoxin
VIP: Vasoactive Intestinal Protein

OVERVIEW

Peripheral nerve injury is known to cause considerable death of sensory neurons housed within the dorsal root ganglia (DRG). Injury-induced neuronal apoptosis has been implicated in both acute and chronic pain syndromes such as hyperalgesia and reflex sympathetic dystrophy (RSD), respectively. Besides injury, diseases such as acquired immuno-deficiency syndrome (AIDS) or diabetes mellitus are also known to selectively destroy sensory neurons, also producing chronic neuropathic pain syndromes.

The purpose of this research project is to determine if postnatal DRG retain the capacity for neurogenesis. The ability of postnatal animals to generate new sensory neurons results in intriguing possibilities that could be investigated in order to minimize death of sensory neurons that are known to occur following injury or disease. Hence, this research could have significant clinical implications in terms of preventing various types of pain syndromes. The diversity of sensory neurons will be discussed in detail by examining their numerous classifications and various morphological subdivisions. Embyrological development will also be addressed in an attempt to gain knowledge for the purpose of composing plausible mechanisms underlying postnatal neurogenesis. Moreover, a detailed summary of the axotomy-induced changes of sensory neurons will be discussed in order to demonstrate the plasticity of this sensory ganglia. Evidence in support of injury-induced neurogenic repair will also be addressed. In addition, a thorough review of the literature in support of, and against postnatal neurogenesis will be discussed. Finally, hypotheses will be proposed based on the information that will be tested through *in vitro* and *in vivo* experimentation.

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INTRODUCTION

1. BACKGROUND

The dorsal root ganglia (DRG) lie within the vertebral column immediately adjacent to the spinal cord. They contain the cell bodies of primary afferents of the somatosensory system. Although these neurons display a characteristic unipolar morphology, they are sometimes referred to as pseudounipolar since they originated as bipolar cells (Ranson, 1912). Structurally, they can be divided into 3 components: cell body, peripheral and central branches. The peripheral processes receive information from sensory receptor organs and central branch (dorsal root) transmits this afferent information centrally to the spinal cord for higher order processing within the CNS (Lieberman, 1976). The peripheral branch is also responsible for the transfer of trophic factors from the peripheral target. These trophic factors serve to regulate neuron function and survival (Stucky et al, 1998; Ernfors et al, 1995; Crowley et al, 1994; Farinas et al, 1994).

2. EARLY CLASSIFICATION OF DRG

In the past, DRG cells were often described as homogeneous (Scharf, 1958), however despite similarities, they are remarkably diverse. Over the last 100 years, several attempts have been made to separate the DRG into distinct neuronal subtypes. The lack of success has been due to the inability to identify a defined boundary that absolutely separates one subpopulation from the next. In the early 1900's size histograms were used as a means of subdivision (Warrington and Griffith, 1904). Cells were classified as either small (<25 μm), medium (25-50 μm) or large (>50 μm). During the same time, a general relationship was established between the cell body and axonal size. Large cells gave rise

to large axons and small cells to small axons (Dogiel, 1908). Close to the mid 1900's this relationship was further extended to include varying degrees of axonal myelination (Erlanger and Gasser 1937). It was generally accepted that large cells possess large myelinated axons whereas small cells have small diameter unmyelinated axons. By the late 1900's it was also generally accepted that conduction velocity served as an excellent estimate for axon size (Yoshida and Matsuda, 1979; Cameron et al.1986). In general, it was found that a linear relationship exists between axonal diameter and conduction velocity. Hence, the faster the conduction velocity, the larger the size of the axon. Furthermore, the threshold for exciting axons with an electrical stimulus was found to be inversely related to the axonal diameter. Hence, low threshold large myelinated axons have faster conduction velocities than high threshold small unmyelinated axons.

3. SUBDIVISIONS OF SENSORY AFFERENTS

Currently, there are four generally accepted subdivisions of sensory afferents that have been identified: cutaneous, muscle, joint, and visceral. Since our investigative research is primarily directed at cutaneous afferents, they will comprise the principal focus of the discussion. Henceforth, the other subdivisions of sensory afferents will only be briefly addressed.

There are three main types of cutaneous afferents fibers: A β, A and C. The A β class of afferent fibers contains myelinated, large diameter sensory axons that display a rapid conduction velocity of 30-100m/s (Erlanger and Gasser, 1937). The A class contains intermediate sized thinly myelinated sensory axons that possess a conduction

velocity of 4-30 m/sec (Boivie and Perl. 1975). The unmyelinated C fibers represent the smallest diameter sensory axons which have a slow conduction velocity of less than 2.5 m/s (Gasser. 1950). In the skin they are typically present in proportions of 20%. 10% and 70% respectively. though their ratios may vary (Millan. 1999). Although each class encodes sensory information. they are differentially sensitive to noxious and innocuous stimuli. For example. under normal circumstances. all three classes of cutaneous fibers can transmit non-nociceptive information. however. only C and A . but not A β. fibers are able to transmit nociceptive information. In the absence of tissue or nerve injury. cutaneous A β fibers are responsive only to touch. vibration. pressure and other modes of non-noxious. low intensity mechanical stimuli. However. it has been suggested that A β fibers may contribute to nociceptive transmission from muscle (Mense. 1993: Woolf et al. 1994; Wilson et al. 1996b). Conversely. temperature and pain impulses are preferentially relayed through A and C fibers.

There are two main classes of A fibers. The first class known as Type I is activated by high intensity mechanical stimuli in the noxious range (for example. pinch). These high threshold. rapidly-conducting mechanoreceptors are weakly responsive to high intensity heat. cold and chemical stimuli (Handwerker and Kobal. 1993: Meyer et al. 1994: Simone and Kajander. 1997). Although these fibers do not preferentially respond to thermal stimuli. repeated thermal stimulation may eventually sensitize them to become heat-responsive. Type II A fibers are not only less common than Type I but display a much slower conduction velocity. These fibers have a lower threshold to noxious heat

stimuli than their Type I counterparts, and are more responsive to cooling than to mechanical stimuli (Craig et al. 1996; Simone and Kanjander, 1997).

The numerous classes of C-fibers account for their vast diversity. For example, chemospecific nociceptors, thermoreceptors (responsive to warming or cooling) as well as low threshold mechanoreceptors (responsive to pressure) are just a few classes that have been identified (Meyer et al. 1994). Besides these, there are also cold-sensitive C fibers and high threshold receptors responsive to noxious thermal and/or mechanical stimuli. In the latter group there is an abundance of fibers that respond to both mechanical and heat stimuli plus respond to irritant, chemical stimuli. For this reason, these fibers are termed polymodal (Lynn et al, 1996).

The three subtypes of cutaneous afferents can not only be divided by the above-mentioned responsiveness to various sensory stimuli but can also be segregated according to their central projections into the grey matter of the dorsal horn within the spinal cord. The dorsal horn is divided into seven distinct laminae that are organized into five specific regions. The marginal layer is composed of lamina I while lamina II_0 and II represent the substantia gelatinosa. The third region, the nucleus propius is composed of lamina III and IV. Lamina V and VI are considered the deep laminae while the circumcanular region is composed of lamina X.

In the absence of tissue or nerve injury, non-nociceptive stimuli from the periphery are relayed via A β fibers in the dorsal root (central projection) to all regions of the dorsal

horn except for the marginal layer. The mechanical and thermal noxious stimuli relayed by A fibers project to lamina I. II_o .III. IV and V. Finally the C fibers are more restricted in terms of their central projections terminating in laminae I and II_o (Millan. 1999).

Muscle and joint afferents are subdivided in a similar fashion to cutaneous afferents. however a different terminology is used: Group I. II. III and IV (Lloyd and Chang. 1948: Rexed and Therman. 1948) . The myelinated fibers belonging to group I are large diameter and have a rapid conduction velocity of 72-120 m/s. Group II and III are thinly myelinated intermediate sized fibers that conduct at 24-71 m/s and 6-23 m/s respectively (Hunt. 1954). Group IV unmyelinated fibers are small diameter and have a slow conduction velocity of 6-23 m/s (Stacey. 1969). Although each of these groups have further subclasses within them. only a select few that are relevant to this thesis will be discussed.

Muscle spindles are innervated by both group Ia and II afferent fibers. The large myelinated Ia fibers are velocity responsive while the small myelinated group II fibers are length sensitive. Group Ib afferents innervate the golgi tendon organs which are located at the junction between the muscle fibers and the tendon. These myelinated Ib afferents are responsive to force. The majority of terminals which innervate skeletal muscle correspond to small group III myelinated fibers (equivalent to A fibes) and to group IV unmyelinated fibers (equivalent to C fibers). Group III fibers are activated by mechanical stimulation and hence are responsive to force and stretch. They can also be sensitized by thermal and chemical stimuli and about one third may be classified as

nocisponsive (Abrahams et al. 1984; Marchettini et al, 1996). Analogous to group III fibers, group IV fibers are responsive to thermal stimuli: however a higher percentage (50%) are nociceptive (Mense and Meyer. 1988; Mense. 1993). The intense discharge of nociosponsive. group III and IV fibers in response to excessive increases in pressure may be considered as a warning function analogous to the protective role of cutaneous nociceptors inasmuch as the onset of muscular pain leads to the discontinuation of excessive muscle contraction (Marchettini et al. 1996).

Joint afferents resemble muscle afferents except they have only a few group I fibers. Visceral afferents share the same terminology as cutaneous afferent axons. This general classification of sensory afferents described above still remains the most widely accepted method used today.

4. OTHER CLASSIFICATION METHODS OF DRG

Early assessment of DRG cells with a light microscope resulted in the appearance of two cytologic subdivisions (Scharf. 1958; Lieberman. 1976). Based on the appearance of usual basic stains and on the organization of the Nissel material. the cells were classified as either light or dark. The lightly stained clear cells tended to be large while the intensely stained dark cells tended to be small. The main problem with this type of classification is that not all the clear cells seem to be large. This was demonstrated by Lawson et al. (1984) using the antibody RT-97 that recognizes neurofilaments thought to be specific for the large clear cells. They demonstrated that cells of all sizes were labeled

with RT-97. They also demonstrated that the dark cells are always small but the clear cells are of all sizes.

The DRG was also explored to correlate a specific peptide/enzyme to a specific subtype of sensory neuron. Although certain peptides/enzymes are predominantly found in selective subtypes, they are not selectively restricted to them (Hunt and Rossi, 1985; Leah et al, 1985a). Hence, the presence of a peptide does not yet have a clear functional correlate (Hokfelt et al, 1994).

Attempts have also been made to segregate the DRG according to topographic organization. It was thought that certain categories of neurons were located in particular regions within the ganglion. However, the cells seem to be distributed randomly throughout the ganglia without any apparent organization. (Norcio and DeSantis, 1976; McLachlan and Janig, 1983; Peyronnard et al, 1986).

5. MORPHOLOGICAL SUBTYPES OF SENSORY NEURONS

Despite the lack of specific markers to identify specific neuronal subtypes, there has been a uniform consensus that the DRG are composed of distinct subpopulations of functionally heterogenous neurons. These neuronal subtypes signal receptor-transduced stimuli of the diverse sensory modalities described above, that range from touch, temperature and pain to proprioception. There are five known phenotypically-distinct subtypes of DRG neurons that have been identified. Their distinction from each other is partly based on neurotrophin specificity for selective receptor subtypes (Averill et al.

1995). Independent lines of investigation have shown the neurotrophins to protect against neuronal dysfunction and death in animal models of injury and neurologic disease (Yuen and Mobley, 1996).

The neurotrophin, nerve growth factor (NGF) was discovered over 10 years ago as a diffusible substance capable of inducing neurite outgrowth in explants from sympathetic and sensory ganglia (Levi-Montalcini, 1987). It serves as the prototypic neurotrophin that defines the properties and functions of this class of growth factors. NGF is synthesized at a considerable distance from the cell body by peripheral tissues referred to as targets that are contacted by axons of the NGF-sensitive neurons. In the periphery, the tissue sources of NGF (and other neurotrophic factors) are typically nonneuronal cells (Thoenen,1995). NGF is then retrogradely transported from the target into the nerve terminal and up the axon and into the cell body (Thoenen and Barde, 1980). Once the retrograde flow of NGF is established it must continue for the lifetime of the neuron to develop and maintain the functional differentiated state of the neuron (Barde, 1989). In addition to target-derived factor acquisition, autocrine and non-target-derived paracrine modes of factor presentation are likely to be important to neurons within the DRG (Bothwell, 1995; Gill and Windebank, 1998).

Most of the biological effects of nerve growth factor (NGF) are mediated by trkA, the high affinity tyrosine kinase receptor for NGF (Chung et al, 1999). However, in order for maximal effects to occur, NGF must bind to trkA and as well to a low affinity neurotrophin receptor called P75 (Meakin and Shooter, 1992). Thus, the P75 receptor interacts with the trk receptors to increase the affinity of the neurotrophin binding and/or

signaling efficiency through receptor dimerization (Jing et al 1992). The P75 receptor binds all neurotrophins with similar affinities and is not specific to NGF responsive neurons (Choa, 1992).

NGF selectively supports the survival of the majority of small and intermediate-sized murine dorsal root ganglia neurons (<30 microns) that possess predominantly unmyelinated or thinly myelinated nociceptive afferent fibers (Molliver and Snider, 1997; Friedel et al. 1997; Crowley et al 1994; Shu and Mendell, 1999). This NGF responsive population of DRG neurons predominantly display immunoreactivity for calcitonin gene related peptide (CGRP) and Substance P (Sub P) but does not include those cells that display immunoreactivity for somatostatin (Verge et al 1989b; Averill et al 1995). The proportion of NGF/trkA-dependent neurons contained within the ganglia tends to decrease with increasing age up to PND 14. In development, approximately 70-80% of dorsal root ganglion (DRG) cells are dependent on nerve growth factor (NGF) for their survival, while in the adult only some 40% of DRG cells express the high-affinity NGF receptor, trkA (Bennett et al. 1996). On the day of birth, 71% of DRG cells are found to express trkA. However, this percentage gradually falls with age reaching adult levels of ~43% by postnatal day 21(Molliver and Snider, 1997). The expression of p75 does not parallel that of trkA. It remains relatively constant between 45 and 50% of cells from birth to postnatal day 14 (Bennett et al, 1996).

Brain derived neurotrophic factor (BDNF) was the second member of the neurotrophin family discovered. Following its isolation, BDNF was shown to be capable of supporting the survival of sensory but not sympathetic neurons (Barde et al, 1992). Molecular

cloning of the BDNF gene (Leibrock et al, 1989) revealed its structural similarity to NGF, leading to the concept of the neurotrophin family. Using a homology cloning approach, two additional members, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) were later identified (Snider, 1994; Bothwell, 1995). It was later shown that NGF, BDNF, NT-3 and NT-4/5 share approximately 50% sequence identity (Hallbook et al 1991). TrkB is the receptor for both BDNF and NT-4/5 responsive subpopulations of sensory neurons. The fact that NT-4/5 and BDNF both bind trkB raises the question of how neurons discriminate between these two ligands. These neurotrophins preferentially support the survival, development and maintenance of intermediate size (25-40 μm) trkB bearing sensory neurons, which possess predominately myelinated afferent fibers (Stucky et al, 1998; Ernfors et al, 1995). Although BDNF and NT4/5 both signal through the high affinity trkB receptor (IP et al, 1993; Barbacid, 1994), their specific actions are different. BDNF is essential for the survival of vestibular afferents (Ernfors et al, 1995), whereas the action of NT4 is necessary for the survival of D-hair receptors (Stucky et al, 1998; Stucky et al 1997; Airaksinen et al, 1996). Analogous to NGF and NT-3, these neurotrophins must bind to the high affinity trkB receptor and the low affinity P75 receptor in order to exert their maximal biological effects (Jing et al 1992). BDNF and NT-4/5 are transported from peripheral targets through connecting afferent fibers in a retrograde fashion to the cell bodies of the BDNF and NT4/5 responsive sensory neurons (Curtis et al, 1998; Matheson et al 1997). In addition to target-derived factor acquisition BDNF and NT-4/5 can be also supplied in an autocrine and paracrine fashion from neuronal and non-neuronal adjacent cells within the DRG (Bothwell, 1995; Gill and Windebank, 1998). Although NT-4 is the most ubiquitously expressed neurotrophin, it is

the least well understood functionally (Ibanez, 1996). These neuronal subpopulations can be identified by their immunoreactivity for the TrkB receptor (Muragaki et al. 1995). The lack of more specific immunomarkers makes it very difficult to separate these two neuronal subpopulations.

Another subpopulation of sensory neurons consists of those which are preferentially responsive to NT-3. NT-3 supports the survival, development and maintenance of medium (25-40 μm) and large (>40 μm) trk C bearing sensory neurons possessing myelinated afferent fibers (Matheson et al, 1997). Analogous to NGF, it must bind to the high affinity trkC receptor and the low affinity P75 receptor in order to exert its maximal biological effects (Jing et al 1992). NT-3 is transported from peripheral targets through connecting proprioceptive afferent fibers in a retrograde fashion to the cell body of NT-3/trkC responsive sensory neurons (Curtis et al, 1998; Matheson et al 1997). These cells predominantly display immunoreactivity for neuropeptide Y (Sterne et al 1998). RT-97 (a monoclonal antibody that recognizes the 200 kDa subunit of a neurofilament protein characteristically expressed by large diameter sensory neurons) (Boland and Dingledine, 1990; Averill et al 1995), the enzyme carbonic anhydrase (CA) and the calcium binding protein, parvalbumin (Mayeux et al. 1993; Ichikawa et al, 1994).

More specifically, the signaling of NT-3 through the trkC receptor is required for the preferential survival of the muscle proprioceptors such as the Ia muscle spindle afferents and the Ib golgi tendon organ afferents. It also has a survival function for D-Hair Follicle cutaneous mechanoreptor afferents and Merkel cells (Ernfors et al, 1994b; Farinas et al. 1994; Hory-Lee et al, 1993). In addition to target-derived factor acquisition, NT-3 can

also be supplied in an autocrine and paracrine fashion from neuronal and non-neuronal adjacent cells within the DRG (Bothwell, 1995; Gill and Windebank, 1998).

Interestingly, the survival effects of NT-3 are not restricted to NT-3/trkC responsive neurons. NT-3 has also been shown to support the survival of trkA/NGF and trkB/BDNF responsive neurons (White et al, 1996; Barbacid, 1994; Glass and Yancopoulos, 1993; Meakin and Shooter, 1992). The functional implication of NT-3's ability to cross-talk with other neuronal subpopulations is yet to be determined.

The final subpopulation of sensory neurons are those which contain the glial derived neurotrophic factor (GDNF) receptor, tyrosine kinase, *Ret* (Molliver et al, 1997). GDNF was discovered as a potent neurotrophic factor for midbrain dopaminergic neurons (Lin et al, 1993) and was shown to be a member of the transforming growth factor- (TGF-) superfamily and not a member of the neurotrophin family. The biological effect of GDNF on a subgroup of sensory neurons has been well documented (Matheson et al. 1997; Moore et al, 1996; Buj-Bello et al, 1995). GDNF preferentially supports the survival, development and maintenance of small (<25μm) *Ret* receptor bearing sensory neurons (Snider and McMahon, 1998). This population has been shown to constitute a subgroup that is different from the NGF/trkA-dependent neurons (Molliver et al, 1997). However, there is still a small (9%) degree of overlap between the two groups (Kashiba et al, 1998). Similar to the neurotrophins, GDNF must bind to a high affinity *Ret* receptor and a low affinity GDNFR   receptor in order to exert its maximal biological effects. Recent *in vivo* axotomy experiments indicate that this GDNF responsive subpopulation is much less

dependent on the retrograde transport of GDNF through connecting myelinated and unmyelinated, nociceptive afferent fibers from peripheral targets. They appear to rely more on the local production of GDNF from adjacent neuronal and support cells within the DRG (Trupp et al. 1997).

This subpopulation represents the only non-peptide transmitter containing group of neurons (Snider and McMahon. 1998). These cells display immunoreactivity for enzymes such as Flouride Resistant Acid Phosphatase (FRAP) and/or thiamine monophosphatase (TMP) plus the lectin IB4 (Bennett et al. 1998).

Postnatally, the proportion of GDNF/*Ret*-dependent neurons contained within the ganglia tends to increase with increasing age up to PND 14. This corresponds to the identical time frame that the NGF/trkA-responsive neurons decrease. The proportion of cells which bind the lectin IB4 increase from 9% (day of birth) to 40% by day 14. The down-regulation of trkA is unlikely to be directly responsible for the emerging IB4 binding (Bennett et al. 1996).

In summary, although neurotrophin signaling through their respective receptors is highly selective, it is not absolute (Glass and Yancopoulos, 1993) and thus should be used only as a general guideline for subdivision. Similarly, characterization of the various neuronal subtypes according to cell size or specific peptide transmitter expression (or lack of) is also not a definitive means of isolation. However, it merely represents properties predominantly expressed by each subtype. For example, some large diameter sensory neurons express CGRP instead of NPY.

6. DEVELOPMENT OF DRG

It has been suggested that neurons of the peripheral nervous system (PNS) and the central nervous system (CNS) evolve from a common neuroepithelial cell (NEP) (Mujtaba et al. 1998). During development, the NEP cells can take one of two pathways. They can differentiate into neural crest stem cells (NCSC), which eventually form various ganglia of the PNS, such as DRG (Kitchen et al. 1994; Goldstein et al. 1990). Conversely, they can take an alternative pathway, avoiding the formation of NCSC's. This alternative pathway allows them to differentiate directly into P75 immunonegative, CNS progenitors, that are capable of undergoing further differentiation to produce neuronal and nonneuronal cells of the CNS (Mujtaba et al 1998).

Bone Morphogenic Proteins–2/4 (BMP-2/4), have been identified as key factors involved in the differentiation of the NEP cells to NCSC's (Kalyani et al. 1998: Schneider et al. 1999; Sela-Donenfeld and Kalcheim, 1999). These NCSC's, can be distinguished from the NEP parent cells by their positive immunoreactivity for the low affinity receptor, P75 (Mujtaba et al 1998).

In mice, the migration of these P75 positive NCSC's takes place around embryonic day 9 (E9) (Farinas et al, 1998). At this stage these cells possess no trk A, B or C labelling. During this time period, neural precursor cell proliferation and migration are regulated by integrins (Jacques et al, 1998).

Once migration is complete, the onset of neurogenesis begins to take place around E10. The onset of trk C labelling is first detectable between E9 and E10. These trk C immunoreactive cells exhibit a clear bipolar, neuronal morphology. At E10, trk C labelling is characteristically low in caudal DRG and increases progressively in more anterior DRG.

By E11, greater than 60% of the DRG cells are still precursors. Studies conducted by Farinas et al. (1998) reveal a lack of co-localization of Brdu with any trk labeling. This suggests that trk expression is only found in post-mitotic neurons. Distribution of trk labelling at E11 was also conducted by Farinas et al (1998). They show that in L1 DRG, 70% of neurons express trk C, 40 % express trk B and 20 % express trk A. Peak neurogenesis occurs between E12 and E13. By E13 there is a dramatic change in expression pattern of trk receptors. At E13, less than 10% express trk C, 8% express trk B while almost 80 % express trk A. Contact with peripheral targets of newly generated neurons occurs between E13 to E15. From E13.5 to the onset of the postnatal period, 50 % of the generated neurons die.

It has been suggested that during the time of contact with peripheral targets, BDNF or NT-3 is secreted by mature differentiated neurons, thereby promoting cell survival until peripheral targets are innervated. In this manner, the newly generated cells provide a autocrine feedback loop (Acheson et al, 1995). At E15.5 and later stages, trk C labelling reimerges. However, during the same time period, trk A downregulates such that by the first three postnatal weeks, about half of the cells lose trk A, so by adulthood this receptor

is only found in 40-45% of DRG neurons (Molliver et al. 1997; Molliver and Snider. 1997; Bennett et al. 1996). Furthermore, during late embryonic life (E15 onwards) and over the first postnatal week, another receptor tyrosine kinase, *ret*, is upregulated in the population of small cells that down regulates trk A (Bennett et al. 1996). Adult levels of this receptor are reached by postnatal day 7 (PND 7) (Bennett et al, 1998). These neurons are sensitive to GDNF in the postnatal period and this characteristic is maintained in adulthood (Snider and McMahon, 1998).

The different trk's are expressed by distinct populations of DRG neurons from E15 into adulthood. Trk A is expressed predominantly by small neurons, trk B in the intermediate size and trk C in the large diameter. *Ret* expression is predominantly within small to intermediate size. non-peptidergic neurons (Mu et al. 1993). Recent characterization analysis depicts the percentage of trk expression within the DRG, the soma areas of these neurons expressing different trks and the relative distribution of the trk receptors at various developmental stages ranging from E15 to PND21 (Mu et al, 1993).

During development, satellite cells are also present within the ganglia. These small diameter oval shaped cells are usually located within the immediate vicinity of the neurons, often surrounding their circumference. Besides their proposed supportive and nurturing roles, these cells have also been shown to have a paracrine role through secretion of trophic factors such as NGF (Gill and Windebank, 1998). The paracrine release of trophic factors is thought to be responsible for ensuring neuronal survival during the time of peripheral target innervation between E13-E15. This function becomes

less essential after neuronal target innervation because trophic factors are provided in abundance from their target source.

7. RESPONSE TO INJURY

Traumatic lesions of the PNS initiate a highly reproducible and stereotypical sequence of histopathological reactions known as Wallerian degeneration which has been extensively investigated in the past (e.g. Griffin and Hoffman. 1993; Fawcett and Keynes. 1990). Both peripheral lesion paradigms (crush and permanent transection) result in considerable cell death of sensory neurons housed within the DRG. The injury-induced specific cellular responses can be tracked by monitoring the differential expression of specific genes known to be involved in degeneration. regeneration and repair. In a recent report (Gillen et al. 1997), more than 60 genes and their products were identified and shown to display a specific pattern of regulation following peripheral nerve lesion.

Although. injury-induced death is not universal to all sensory neuronal subtypes. it does predominantly occur in the small diameter. NGF/TrkA responsive neurons (Mannion et al. 1996; Shortland and Woolf. 1993). Recent studies have indicated that following axotomy. the neurons expressing trkA decrease by 50% compared to the unchanged values for neurons expressing the *ret* receptor (Kashiba et al. 1998). Moreover. in axotomized animals, GDNFR (a component of the GDNF receptor) was shown to markedly increase. It is speculated that increased GDNFR together with *ret* contribute to the survival of the GDNF responsive neurons. Their ability to maintain functional receptor components. despite injury, is thought to be the reason that allows GDNF

(produced intrinsically as well as by surrounding satellite cells) to functionally sustain this neuronal subtype (Trupp et al. 1997). Conversely, the NGF/TrkA responsive neurons show a consistent decrease in their receptor components following axotomy which corresponds with the decrease in the expression of the low affinity p75 receptor (Krekoski et al. 1996; Verge et al. 1992; Zhou et al. 1996).

Axotomy induced cell death has also been demonstrated in the BDNF and NT-4/5-dependent subtypes (Eriksson et al. 1994). It has been shown that in the abscence of a peripheral target, the administration of BDNF or NT-3 to the proximal stump can rescue 41% of these neurons from undergoing cell death (Eriksson et al, 1994). Contrary to the above, Acheson et al. (1996), indicated that the survival of sensory neurons in the adult is less affected by axotomy or target removal when compared to their response during development. They suggested that BDNF-responsive cells are capable of intrinsically secreting BDNF, thereby serving a paracrine or autocrine role. Their ability to maintain adequate production of trophic support, despite injury, allows BDNF (produced intrinsically as well as by surrounding satellite cells) to functionally sustain this neuronal subtype (Acheson et al, 1996). Under normal circumstances, these two subtypes receive trophic support from their respective targets; however it is possible that following injury, this entire responsibility is transferred to neuronal and non-neuronal cells within the DRG. The gradual upregulation of BDNF and NT-4 in non-neuronal cells, post-transection (Funakoshi et al, 1993), supports this concept of self-preservation. This entire issue still remains open to interpretation.

Neuropeptide changes within the DRG also occur in response to axotomy. Following peripheral axotomy there is a reduced synthesis of normally abundant neuropeptide transmitters such as substance P (Sub P), calcitonin gene related peptide (CGRP) and somatostain. At the same time, peripheral nerve section provokes dramatically increased expression of other peptides not normally abundant in DRG, including vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and galanin (GAL) (Mulderry and Dobson, 1996; Sterne et al. 1998; Nielsch and Keen, 1989). More specifically, in small unmyelinated DRG neurons, CGRP, Sub P and somatostatin as well as NPY-receptor mRNA are downregulated. However, VIP, GAL, cholecystokinin (CCK), NPY and nitric oxide synthase (NOS) are upregulated. In the large myelinated neurons, CGRP is downregulated whereas NPY, VIP, GAL, NOS, as well as NPY-receptor mRNA are upregulated (Hokfelt et al. 1994).

It has been suggested that axotomy transfers the role of excitatory mediator from Sub P and CGRP to VIP. GAL is thought to antagonize VIP instead of its usual antagonistic functions on Sub P and CGRP (Hokfelt et al 1994).

Axotomy may also induce changes in production of known mitogenic substances such as NGF and basic fibroblast growth factor (bFGF) (Geffen and Goldstein, 1996; Murphy et al 1994). Recently, it has been demonstrated that mRNA levels of bFGF are increased in adult DRG cells following nerve injury (Ji et al, 1995). Moreover, the increase production of NGF in non-neuronal cells following axotomy may also be involved in a compensatory repair response (Heumann et al, 1987; Heumann et al 1987). Because

bFGF is a known mitogenic signal, its upregulation may serve to signal quiescent precursors, housed in mature sensory ganglia, to generate new cell populations, including neurons following injury. The bFGF-induced mitogenic signal may be an adaptive regenerative response since many studies have demonstrated that, at least in neonates, axotomy induces considerable neuronal apoptosis (Groves et al. 1997; Oliveira et al. 1997).

8. THE NEUROGENESIS DEBATE

Peripheral nerve injuries that axotomize DRG neurons can induce pronounced phenotypic and physiologic plasticity both within the DRG (Hokfelt et al., 1994;Himes and Tessler, 1989) and spinal cord (Koerber et al., 1994;Mannion et al., 1996;Woolf et al., 1992). These injuries can lead to abnormalities in sensory encoding resulting in neuropathic pain syndromes (Woolf and Doubell, 1994;Bennett, 1994;Devor, 1996). One rather well studied example of nociceptive plasticity correlates allodynia with the sprouting of 'touch sensitive' Aβ fibers to pain encoding neurons within spinal cord laminae I and II. This occurs following nerve damage or the selective destruction of pain transmitting C-fibers and their projections to this region using the neurotoxin capsaicin (Mannion et al., 1996;Shortland and Woolf, 1993). Thus, DRG cell death induces the structural reorganization of spinal sensory pathways resulting in the production of hyperalgesia. Interestingly, this plasticity appears to be temporary (Himes and Tessler, 1989;Woolf et al., 1995) suggesting that compensatory changes can re-establish appropriate sensory encoding. One unexplored possibility is that new neurons are generated.

This issue sparked considerable debate in the mid 1980's to early 1990's between the researchers Devor and Laforte. In 1985, Devor and Govrin-Lippmann proclaimed that they had observed neurogenesis in adult DRG based on soley on age related neuronal counts. This report was later challenged by Aldskogius and Risling, (1989) and La Forte et al. (1991) who conducted similar neuronal counts using the technique devised by Devor, revoking Devor's claims of adult neurogenesis. However, in 1991, Devor and Govrin-Lippmann, rechallenged the criticisms put forth by La Forte and others. They indicated that La Forte incorrectly applied their counting method and did not take into account sex-related differences. It was their belief that neurogenesis occurs only in those animals in which growth continues throughout life (males), but not in those whose body size stabilizes soon after sexual maturity (females) (Devor and Govrin-Lippmann, 1991). As a result, a subsequent age related counting experiments conducted by Devor (1991) once again provided an excellent rebuttle to La Forte by demonstrating injury-induced neurogenesis.

Nearly a decade later, researchers are still disputing the idea of adult neurogenesis. While some experiments suggest that adult DRG are stable and do not change in relation to age or body size (Pover et al, 1994), others have demonstrated increased neuronal numbers with increasing age (Cecchini et al, 1995). This ongoing dispute still remains active simply due to the lack of a reliable defined technique that can conclusively demonstrate neurogenesis.

Although this issue is still unresolved, the published data in support of neurogenesis far outweighs that against it. For example, Ji et al. (1995) reported that following sciatic nerve axotomy, mRNA for the basic fibroblast growth factor (bFGF) is upregulated within DRG neurons. The mitogenic ability of bFGF to induce proliferation of neuronal precursors in the central nervous system (CNS) and peripheral nervous system (PNS) is well documented (Ray et al, 1997; Weiss et al, 1996; Murphy et al. 1994). This novel observation questions the involvement of bFGF in sensory ganglia in response to injury. Thus, it is quite possible that bFGF may be an integral component involved in the proliferation of neuronal precursors during injury-induced neurogenic repair. Furthermore, Ljungberg et al. (1999) observed inexplicable increases in neuronal numbers following spinal nerve axotomy and subsequent infusion of nerve growth factor (NGF) or neurotrophin-3 (NT-3) at the proximal nerve stump of adult rat. Other *in vivo* experiments have also shown axotomy-induced increases of DRG neuron number (Farel and Boyer, 1999; Meeker and Farel, 1997).

The adult mammal, including humans, contains multipotent stem cells in various regions of the CNS (Doetsch et al., 1999; Gould et al., 1999; Eriksson et al., 1998; Gage et al., 1995; Johansson et al., 1999; Ray et al., 1997). While it has been predicted that the PNS also contains stem cells (Anderson, 1989), to our knowledge neither the presence of stems cells nor injury-induced neurogenesis has been conclusively identified in mammalian DRG. Despite the previous debates on this issue, recent careful analysis supports continuing neurogenesis into adulthood (Ciaroni et al, 2000; Popken and Farel 1997). One obvious function of neurogenesis would be to replace sensory neurons that

die following nerve injury (Ljungberg et al., 1999;Oliveira et al., 1997; Cecchini et al 1999; Guillery and Herrup, 1997; St Wecker and Farel 1994). Since this issue remains unresolved and mechanistically undefined, we have undertaken *in vitro* and *in vivo* studies to demonstrate and characterize neurogenesis in postnatal, juvenile and adult DRG.

9. HYPOTHESES AND OBJECTIVES

This thesis will address the following hypotheses with the stated objectives:

A. Mature sensory ganglia house neuroblasts that can be induced to generate new neurons during the lifespan of the organism.

Objective: To provide evidence supporting the existence of neuronal precursors in mature sensory ganglia.

B. Axotomy-induced expression of bFGF results in the proliferation of neuronal precursors.

Objective: To demonstrate that, following axotomy, new sensory neurons are generated.

C. Following mitogen-induced generation of neural precursor cells postnatally, trophic factors induce their differentiation into identifiable neuronal phenotypes, recapitulating their role during development. This occurs in an attempt to replace those that die following axotomy.

Objective: To utilize cell cultures of postnatal sensory ganglia to characterize the cellular mechanisms responsible both for proliferation of neuronal precursors and subsequent differentiation into sensory neurons.

SECTION I.
IN VITRO: NEUROGENESIS IN POSTNATAL MURINE DORSAL ROOT GANGLIA

1. MATERIALS AND METHODS

1a. DORSAL ROOT GANGLIA

DRG from early postnatal (day 1 or 2) male/female CD1 mice were extracted and enzymatically dissociated according the methods outlined by Devon (1995). The cells were plated at a fixed cell density on collagen coated 35 mm Petri dishes (Corning) or collagen coated 8 well plates (Corning) in the presence of culture media consisting of HAMS F14 or DMEM-F12 (Gibco). In many experiments. 10 ng/ml NGF and 10% heat-inactivated horse serum were added to the medium in order to replicate a commonly used culture medium for experiments on DRG neurons (e.g. Crawford et al., 1997). We will refer to this medium as the 'standard' DRG medium. The thymidine analog bromodeoxyuridine (BrdU) was added to most cultures at a concentration of 1 μM to detect DNA synthesis as an assay for cell proliferation. Immunohistochemistry involved detection of BrdU and the neuron-specific markers NF-160 and/or MAP-2. In several experiments bFGF (Upstate Biotechnology) was added to the medium at 10 ng/ml. All primary cultures were fixed in 4% paraformaldehyde (15 minutes) for subsequent immunohistochemical analysis.

1b. QUANTIFICATION ANALYSIS

Prior to counting, criteria were established for neuronal identification. In order to be counted as a "typical" neuron, cells had to display: immunoreactivity for NF-160/MAP-2 and extensive process formation with a spherical cell soma. All cell counts were conducted in 3 randomly selected wells present in each 8 well plate. The 3 randomly chosen wells in the first plate were consistently counted for data analysis in all subsequent plates. The counting procedure was conducted utilizing a 10x fluorescence objective. Based on the field of view provided by this objective, each well was subdivided into 6 equal sections. Sectioning each well into these defined areas prevented the possibility of double counting. During counting in bFGF enriched cultures, a consistent population of cells were noted that did not meet the criteria previously established for identification of "typical" neurons. These cells were immunolabeled for NF-160/MAP-2 and possessed spherical somas but they lacked processes. These cells often appeared in grape-like clusters. Although they were immunopositive for NF-160/MAP-2, the labeling intensity was much lower that that noted for the typical neurons. The cells possessing this unusual morphology were counted as a separate population as displayed in Figure 2e.

1c. ELECTROPHYSIOLOGY

Cultures were perfused with HEPES-buffered recording solution consisting of (in mM): NaCl, 150; KCl, 5; HEPES, 10; $MgCl_2$, 1; $CaCl_2$,2; glucose 10; pH 7.4, 320 mOsm. Whole-cell voltage clamp recordings were obtained using an Axopatch 1D amplifier (Axon Instruments). Microelectrodes were pulled on a two-stage upright puller

(Narishige PP-83) and filled with solution containing (in mM): K-gluconate. 140: ethylene glycol-bis(β-amino ethyl ether) N,N,N',N',-tetraacetic acid (EGTA), 11; KOH. 35; HEPES. 10; $CaCl_2$, 1. pH was adjusted to 7.3. Intracellular support consisting of 2 mM Mg-adenosine 5'-triphosphate and 4 mM guanosine 5'- triphosphate was included in the electrode solution. Resulting tip resistances were between 4 and 5 MΩ. Data was acquired using pCLAMP (v 6.0; Axon Instruments). and stored to computer. Data was analyzed with pCLAMP and SigmaPlot software (v. 4.01; Jandel Scientific).

1d. IMMUNOHISTOCHEMISTRY

During immunohistochemical processing, tissue was fixed in 4% paraformaldehyde for 15 minutes and then washed (6x20 minute) in phosphate buffered saline with triton-X100 (PBST). Primary antibodies were then added in conjunction with horse or sheep serum (1:100) and allowed to incubate for 2 days under refrigeration. then washed (5x20 minute) with PBST before the addition of the secondary antibody. Tissue was then incubated in secondary antibody in conjunction with horse or sheep serum (1:100) for 1.5 hours and then washed (2x20 minute) with PBST. The immunohistochemical processing for BrdU was similar to that described by Tamatani et al (1995): Following 2 washes in PBST. the tissue was incubated in a final mixture containing 50% formamide and (2x) saline sodium citrate (SSC) for ½ hour at 65C. It was then washed for 5 minutes in (2x) SSC at room temperature before incubation (30 minutes, 37C) in 2 N HCL. This was then followed by a 10- minute rinse with 0.1 M sodium borate buffered to a pH of 8.5. It was then washed (5x20 minutes) with Tris buffered saline (TBS) followed by a 30 minute wash in Tris buffered saline with 0.1% triton-X100 (TBS-T) and 3% horse serum. before

being incubated overnight in Sheep anti-BrdU and sheep serum (1:100). After this incubation period, the tissue was washed (3x30minute) with TBST and incubated in the secondary antibody (Anti-sheep IgG FITC) for 1.5 hours. Finally, it was washed (1x20 minute) in TBST and 50 mM Tris HCL (2x20 minute) before being coverslipped with DAPI.

The following primary and secondary antibody combinations were utilized for immunohistochemical processing of *in vitro* tissue: (i) Polyclonal sheep anti-BrdU (Fitzgerald; 1:100) and donkey anti-sheep (H+L) FITC (Jackson;1:50). (ii) Rabbit anti-NSE (Chemicon; 1:300) and donkey anti-rabbit IgG CY3 (Jackson; 1:250). (iii) Mouse anti-NF-160 (Sigma; 1:100) and donkey anti-mouse CY3 (Jackson;1:250). (iv) Mouse anti-MAP-2 (Sternberger;1:1000) and donkey anti-mouse CY3 (Jackson:1:250). (v) Rabbit anti-CGRP (Serotec; 1:8000) and anti-rabbit FITC (Jackson; 1:100). (vi) Mouse anti-nestin (PharMingen; 1:1000) and donkey anti-mouse CY3 (Sigma; 1:250). (vii) Rabbit anti-GFAP (Cedarlane; 1:100) and donkey anti-rabbit IgG FITC (Jackson:1:100). Mouse-anti-pan-axonal (1:1000) (SMI).

1e. SPINAL CORD STEM CELLS

Spinal cords were removed from E16- E18 CD1 albino mouse embryos in a modified Hanks Balanced Salt Solution (HBSS) without Ca^{2+} or Mg^{2+}, containing KCl (4 mM), KH_2PO_4 (0.6 mM), NaCl (80 mM), $NaHCO_3$ (0.35 mM), $NaHPO_4$ (0.048 mM), D-glucose (1 mM), pH 7.3. The cords were minced and incubated in Ca^{2+}/Mg^{2+} free, HBSS with 100ng/ml papain (Sigma), 100ng/ml DNAse, and 1mg/ml protease (Sigma type I)

for 30 minutes. After enzyme incubation, the tissue fragments were gently triturated and then washed in DMEM/F12 medium (1:1) containing DNAse (1mg/ ml) for 15 minutes. The cell suspension was then washed and resuspended in DMEM/F12 medium containing N2 supplement (Sigma). Cells were plated $4x10^4$ in uncoated $75cm^2$ flasks and 20ng/ml each of epidermal growth factor (EGF) and bFGF were added. After 7 days in culture, floating cell clusters were withdrawn, centrifuged at 400 rpm, and resuspended in fresh EGF/bFGF containing medium. Plastic 8-well multiwell microscope slides (Gibco) were pre-coated with poly-D-lysine/ laminin. An aliquot of multipotent neurospheres was washed twice in the growth medium, consisting of DMEM/ F12 or Neurobasal medium with B27 supplement (1:50; Gibco), sodium pyruvate (1mM), and glutamine (2mM). Neurospheres of various sizes were isolated and plated (a few spheres per well) using a micro-pipette in a minimal volume of medium containing a trophic factor. An additional 200 μl volume was added 24 hours later. After 4 days, cultures were fixed in 4% paraformaldehyde and washed (5x) in phosphate-buffered saline containing Triton-X100 (PBST) before immunohistochemical processing for MAP-2 and pan-axonal.

RESULTS

When DRG were cultured in serum-free medium, few cells survived plating in the absence of bFGF after 15 days (Fig. 1a top); however, in the presence of bFGF, cells attached to the substrate and proliferated forming clusters after 15 days (Fig. 1a middle). By day 22, these cells completely covered the bottom of the dish and cell clusters became more evident (Fig. 1a bottom). Some spherical clusters lifted off the substrate and were suspended in the medium while others remained adherent to the culture dish substrate. By day 39, cell proliferation was even more extensive, and immunostaining for nestin, a known marker for CNS stem cells (Lendahl et al., 1990) in conjunction with the nuclear marker DAPI, distinguished nestin$^+$ from nestin$^-$ cells, to reveal many nestin$^+$ cell clusters (Fig. 1b). Figure 2 illustrates examples of bFGF-derived cell aggregates similarly cultured in serum-free medium for 1 and 10 weeks respectively. In both instances, bFGF was withdrawn and the trophic factors neurotrophin-3 (NT-3) (Fig. 2a) or glial-derived neurotrophic factor (GDNF) (Fig. 2b) were subsequently added for 4 days. In both cases, immunostaining with NF-160/MAP-2$^+$ revealed putative neurons with axonal processes emanating from the sphere, suggesting that new neurons can be generated, even after 10 weeks in serum-free culture.

These observations prompted a more detailed examination of the proliferative actions of bFGF on neural precursors, and on the effects of trophic factors on neural differentiation. To study the differentiation of bFGF-derived putative neural precursors, DRG were first cultured for 14 days on collagen coated 8 well plates in the presence of serum-free medium enriched with bFGF (1600 cells/well). This was then followed by further culture

for 4 days in the absence of bFGF but in the presence of various individual trophic factors (NGF, CNTF, BDNF, GDNF, and NT3). Control neurons received no trophic support, and afterwards, no NF-160/MAP-2$^+$ cells were detected. However, many NF-160/MAP-2$^+$ cells were observed in wells with trophic factors added. These cells had a characteristic DRG neuron morphology and extensive process formation (Fig. 3a-e, PNS). Though not quantified, the different trophic factors appeared to promote the differentiation of neurons with qualitatively different morphological phenotypes. These results suggest that bFGF promotes the propagation of neural precursors that then differentiate into neurons in the presence of trophic factors. In addition, DRG progenitor-derived neurons are clearly different than spinal cord progenitor-derived neurons induced to differentiate using the same trophic factors (Fig. 3a-e, CNS). CNS neurons were multipolar.

Whole cell recordings were performed to determine whether the bFGF-proliferated cells induced to differentiate into NF-160/MAP-2$^+$ cells with DRG morphology also displayed electrical properties characteristic of neurons. Dissociated DRG were cultured for 14 days in serum-free medium containing bFGF. As shown above, this procedure eliminates DRG-derived neurons but induces neural precursors to proliferate. Then, bFGF was removed and NGF added for four days to induce differentiation. Cells displaying morphology typical of DRG neurons were targeted (Fig. 4a). All 17 cells recorded were spherical and possessed fine processes leaving the soma. The mean soma diameter of this group of neurons was 29.6 ±4.0 μm, and mean resting membrane potential was -55mV. In response to depolarizing current steps, twelve cells fired action potentials; 4 displayed repetitive firing (Fig. 4b). In response to depolarizing voltage steps, 16/17 cells displayed

inward currents while all 17 generated outward currents. Addition of TTX at high concentrations (5-7 μM) completely blocked the inward currents in only one of the two cells tested (Fig 4c) suggesting the possible identification of TTX sensitive and insensitive subtypes of sensory neurons.

We next studied the actions of bFGF on neurogenesis in a serum- and NGF-containing culture medium. This was undertaken for two reasons. First, in vivo, DRG are exposed to blood-borne elements (see (Devor, 1999)) and also experience increases in NGF and bFGF following axtomy (Carnahan et al., 1991;Ji et al., 1995). Secondly, NGF and serum containing media are commonly used for DRG culture (Crawford et al., 1997). BrdU was added to the culture medium to identify the proliferating cells. In these experiments, primary DRG cultures were divided into two groups and grown at a fixed density (15,000 cells/dish) on collagen-coated 35 mm dishes for 2, 7, 14, 21 and 28 days in a DRG culture medium containing serum and NGF with and without bFGF. Cells co-labeled for BrdU and neuron-specific markers (NF-160/BrdU$^+$ or MAP-2/BrdU$^+$) were observed at all time periods sampled, suggesting that neurons were being generated in a 'standard' DRG medium (Fig. 5a), as well as in the bFGF-enriched treatment group (Fig. 5b). Figure 5a depicts an example of a double-labeled cell (NF-160/BrdU$^+$) that was cultured for 4 weeks in standard DRG media. Morphologically, newly generated neurons were indistinguishable from pre-existing, BrdU$^-$ neurons.

In order to quantify this neurogenic effect, a similar procedure was undertaken on collagen-coated, 8 well plates (2,200 cells/well) in three separate experiments. Figure 5c

presents the mean values. In the standard DRG medium containing serum and NGF. the number of neurons that survive 48 hours after being plated decrease significantly to a given baseline value (~50 neurons) and then remain at a similar or increased level after 4 weeks in culture (Fig. 5c top histogram). These neurons displayed characteristic DRG morphology with extensive process formation (Fig. 5d). In comparison, neurons cultured in standard medium enriched with bFGF had significantly increased number of NF-160/MAP-2^+ cells by week 3 ($p < .05$; Mann-Whitney Rank Sum Test), and a similar quadrupling of putative neuron number was observed at week 4. This increase was due to the appearance of a new population of weakly staining NF-160/MAP-2^+ cells that were spherical, loosely-attached to the substrate, without obvious processes, and that often appeared in clusters (Fig. 5c, bottom histogram and Fig. 5e).

While the above data demonstrated that neurogenesis can be induced from bFGF-proliferated neural progenitors in the presence of trophic factors, the observation that neurogenesis occurred in the presence of NGF with serum was unexpected (Fig. 5a). Evidence of mitosis in putative neurons in NGF and serum-containing medium is demonstrated in Figure 6. The neuron double labeled for MAP-2/BrdU in Figure 6a appears to be in metaphase. A clear example of a neuron with two $BrdU^+$ nuclei is shown in Figure $6b_1$, and another example of double nuclei is provided by using the nuclear label DAPI (Fig $6b_2$). These neurons appear to be in telophase ready to undergo cytokinesis. Figure $6c_1$ depicts two $BrdU^+$ neurons that appeared to have just completed cytokinesis, and the neurons in Figure $6c_2$ completely separated and were migrating along axon tracts. These mitotic neurons are small diameter; consistent with the notion that

NGF supports not only survival (Crowley et al., 1994;Ruit et al.. 1992) but mitosis of small diameter neurons via trkA receptor activation (Cordon-Cardo et al.. 1991).

If NGF is acting as a mitogen. then. like bFGF. cell proliferation should be inducible in a serum-free medium. Thus. we tested the actions of NGF and other putative mitogens. DRG were cultured for 6 weeks in serum-free medium alone or with either NGF. sonic hedge hog (Shh) or EGF at 10 ng/ml. In two separate experiments. NGF and Shh. but not EGF or controls. produced spherical cell clusters containing many MAP-2/NF-160^{+} cells bearing processes (Fig. 7). Thus. NGF and Shh can not only induce neural precursors to proliferate but are also capable of inducing their differentiation into neurons.

Figure 1

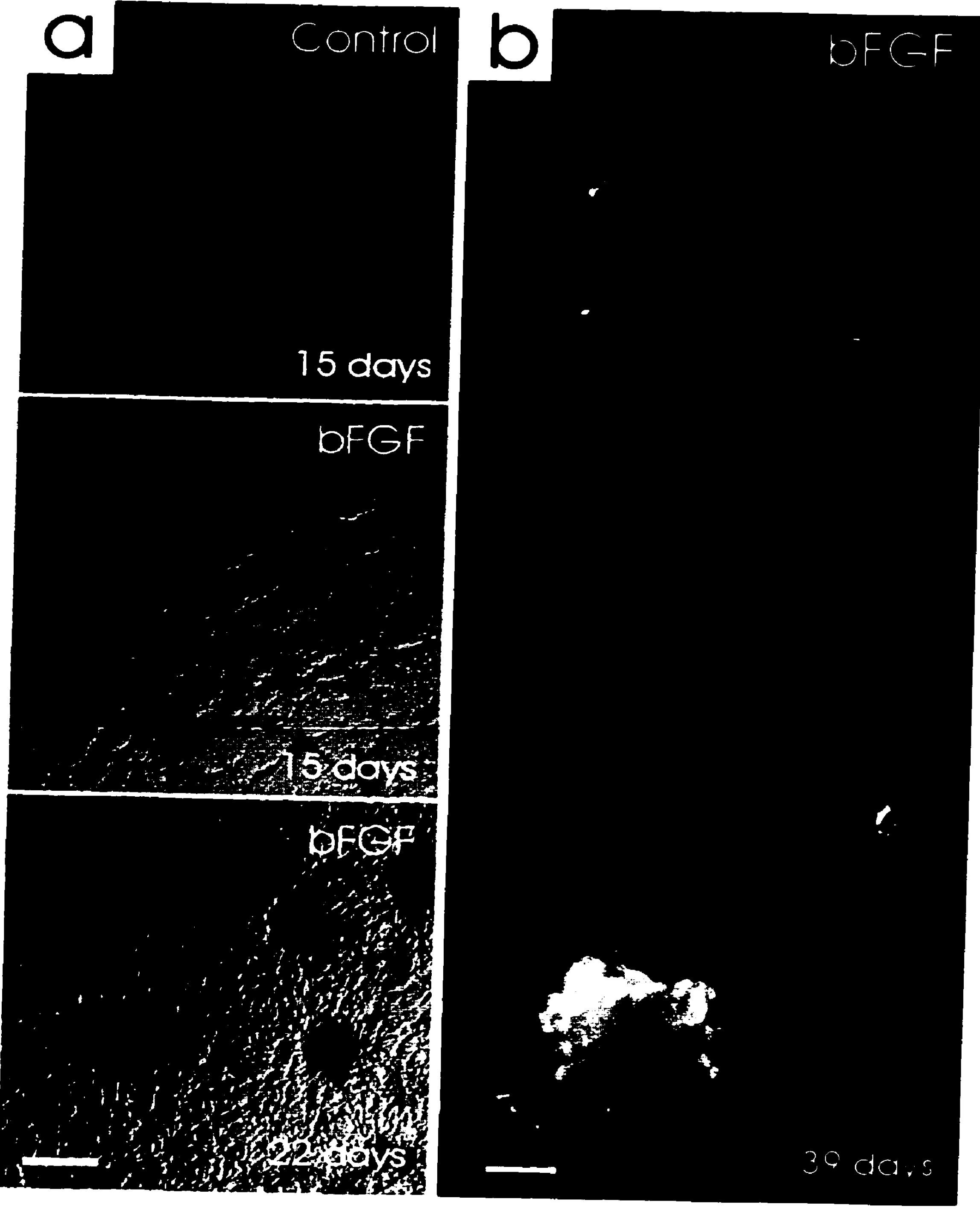
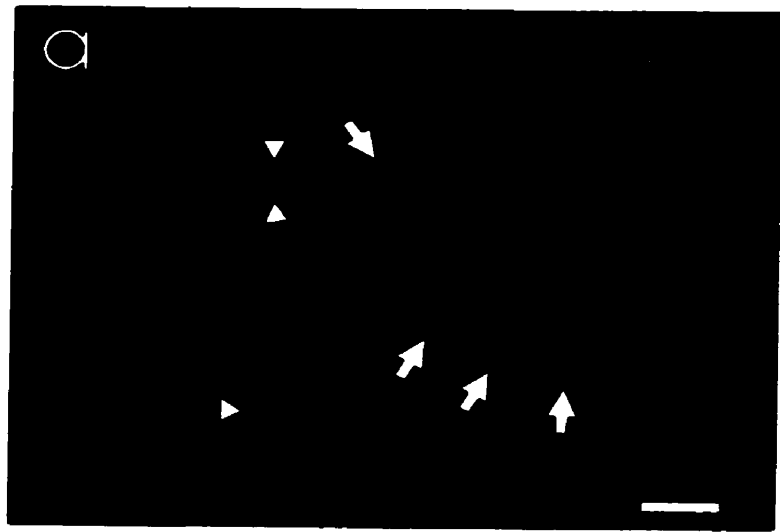


Figure 2



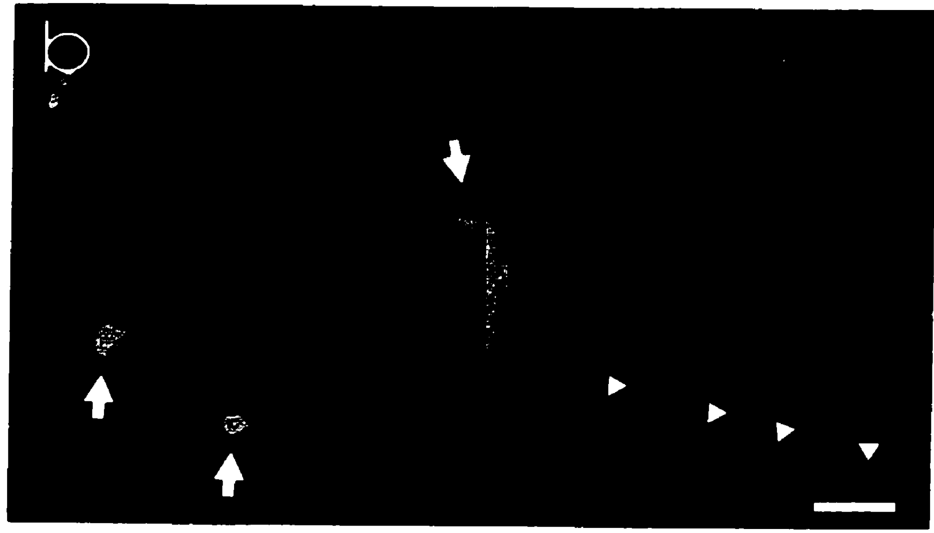


Figure 3

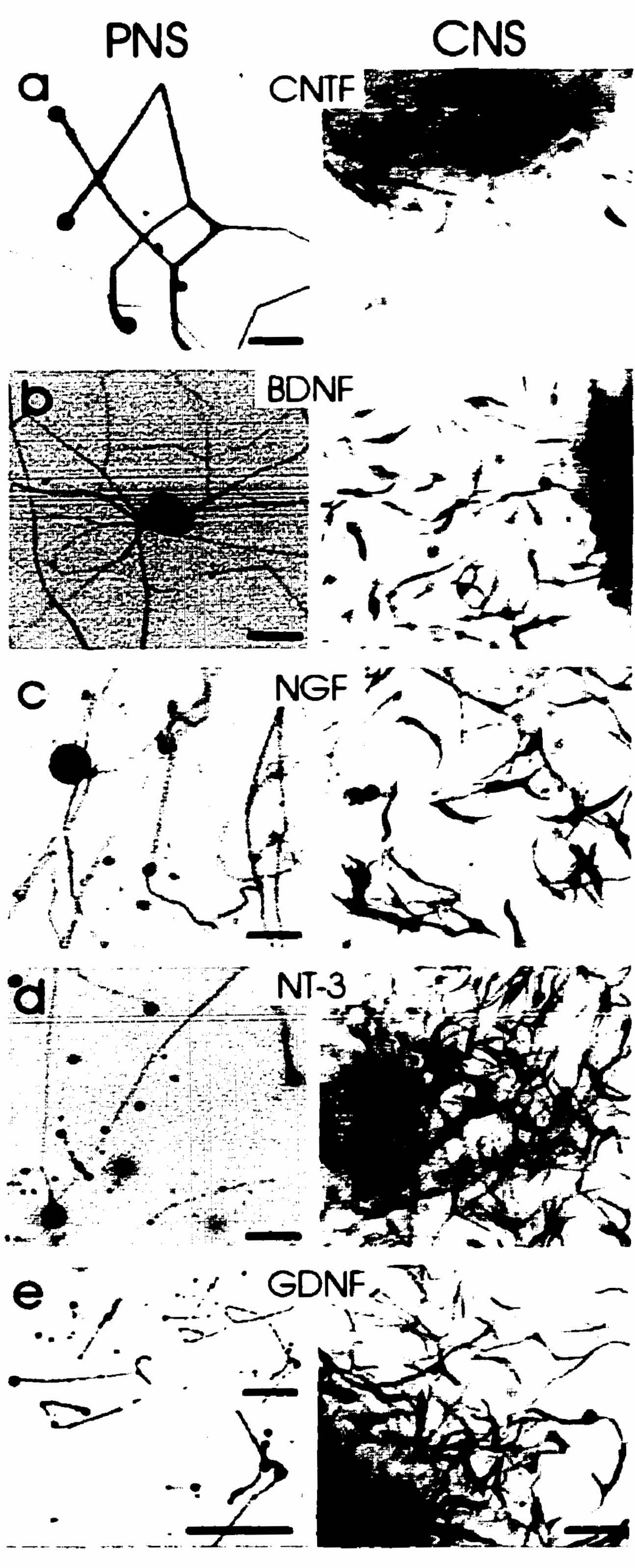


Figure 4

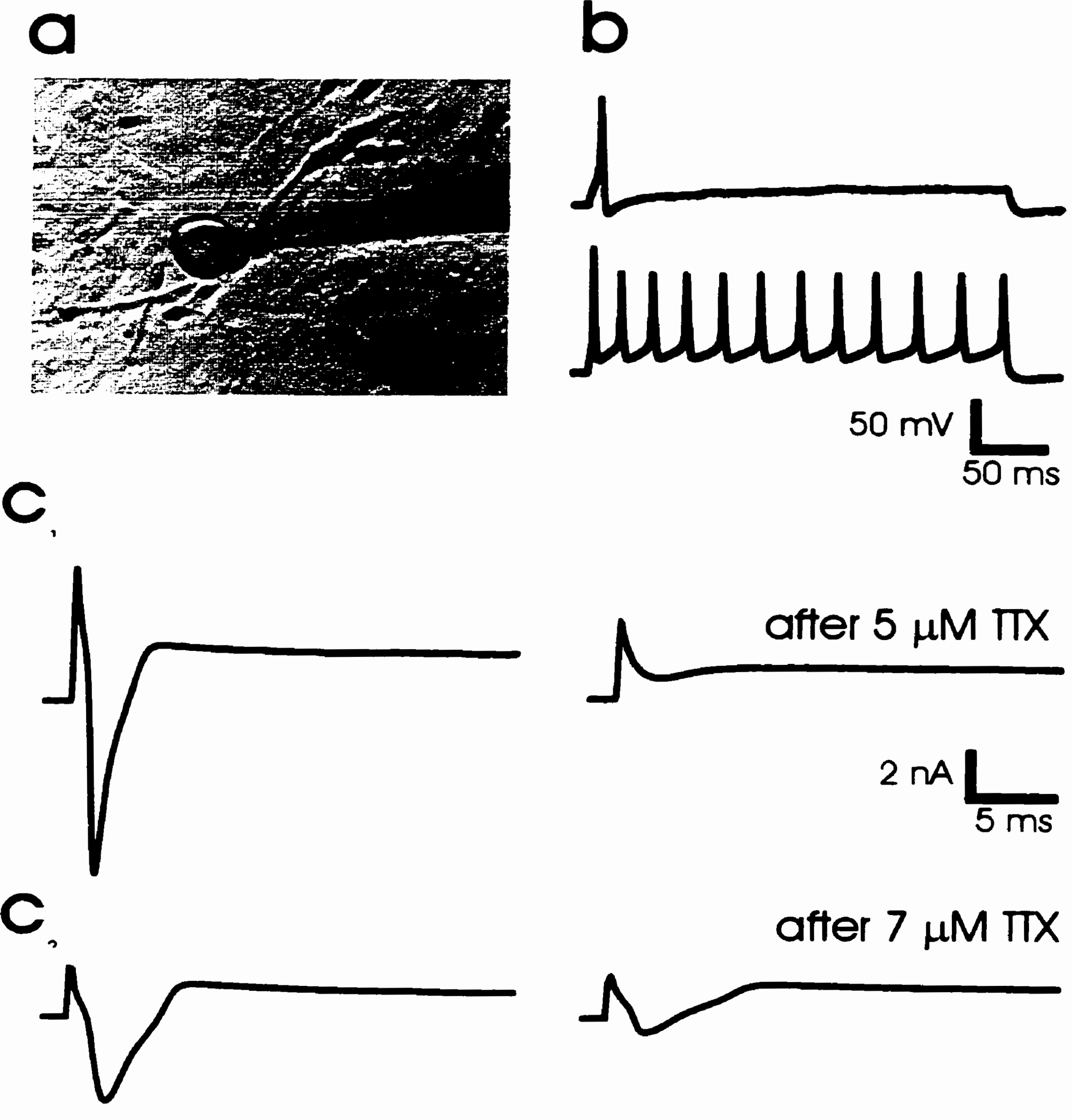


Figure 5

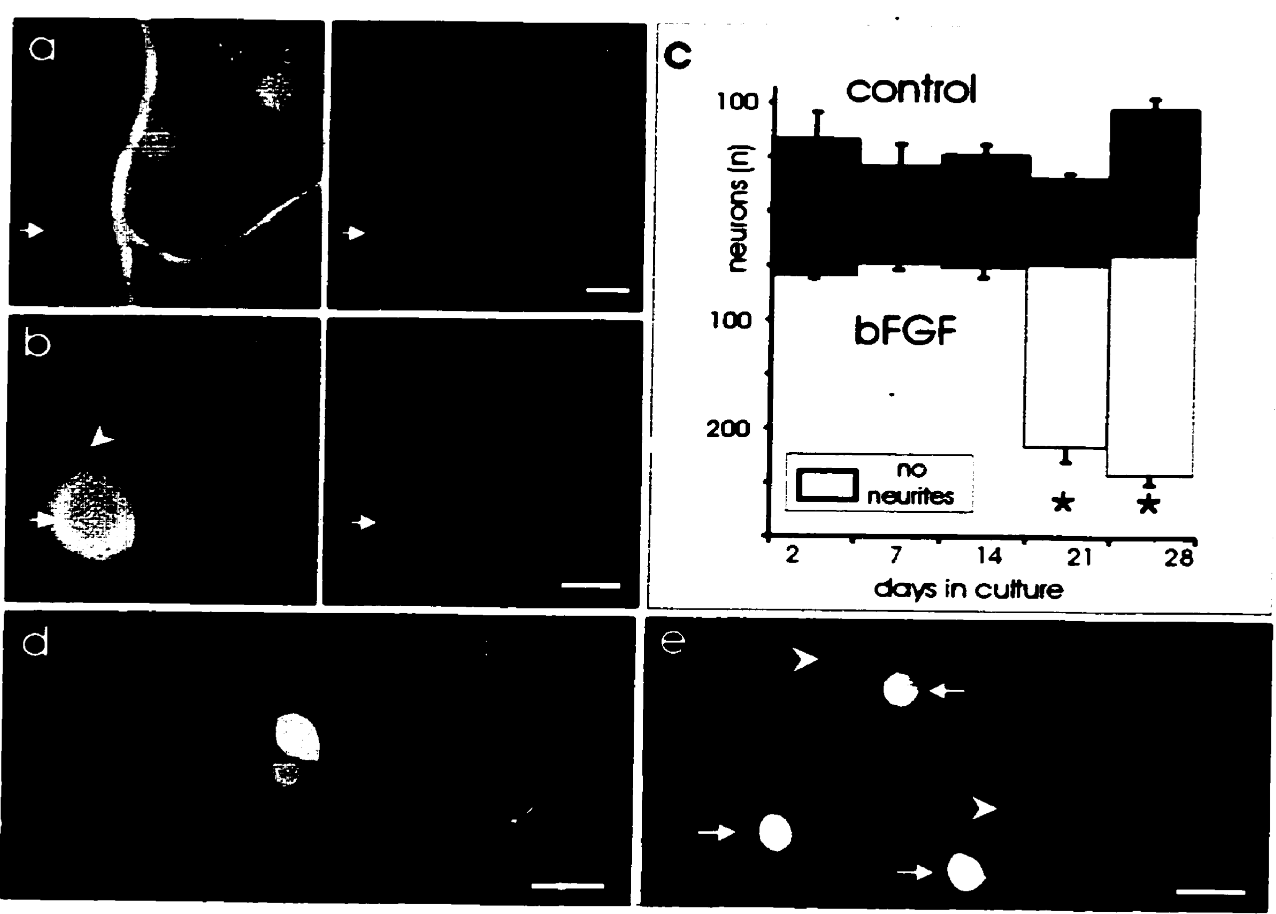


Figure 6

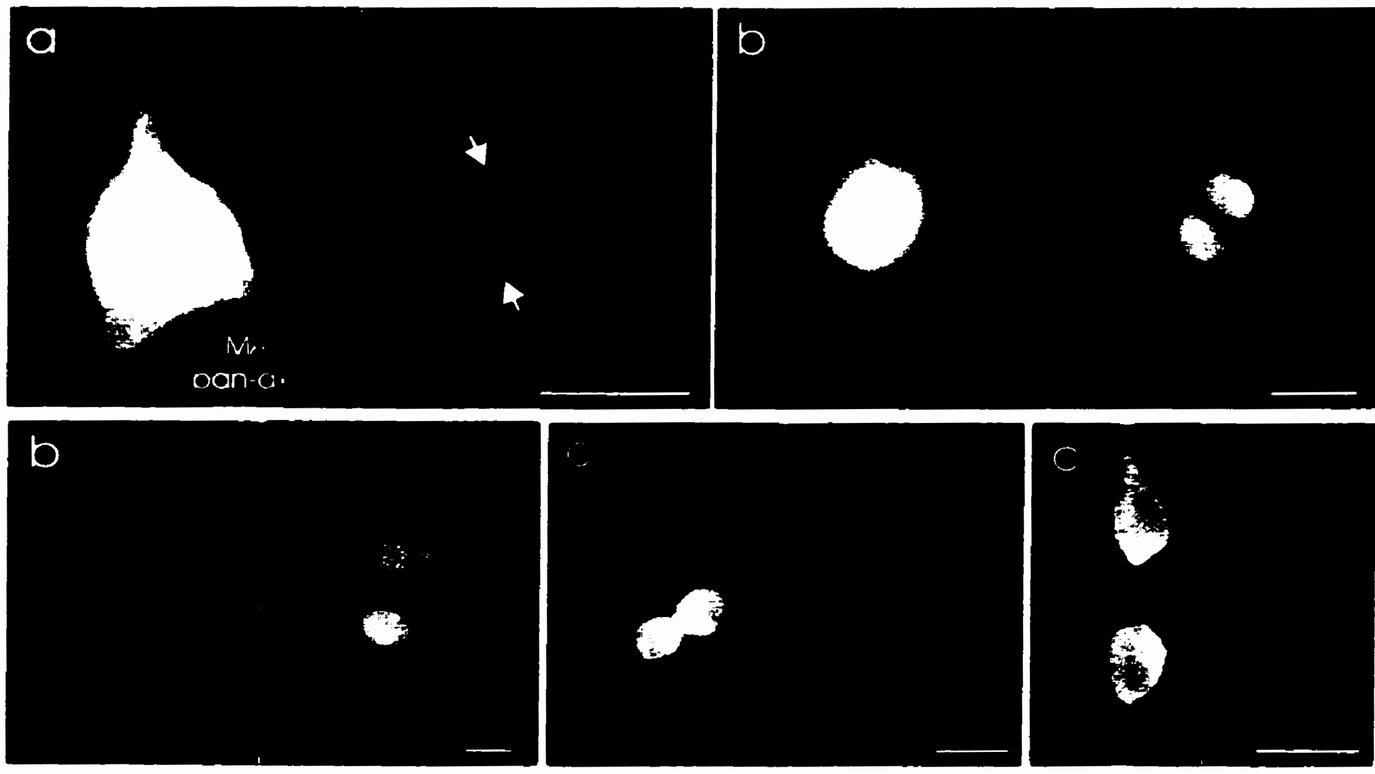
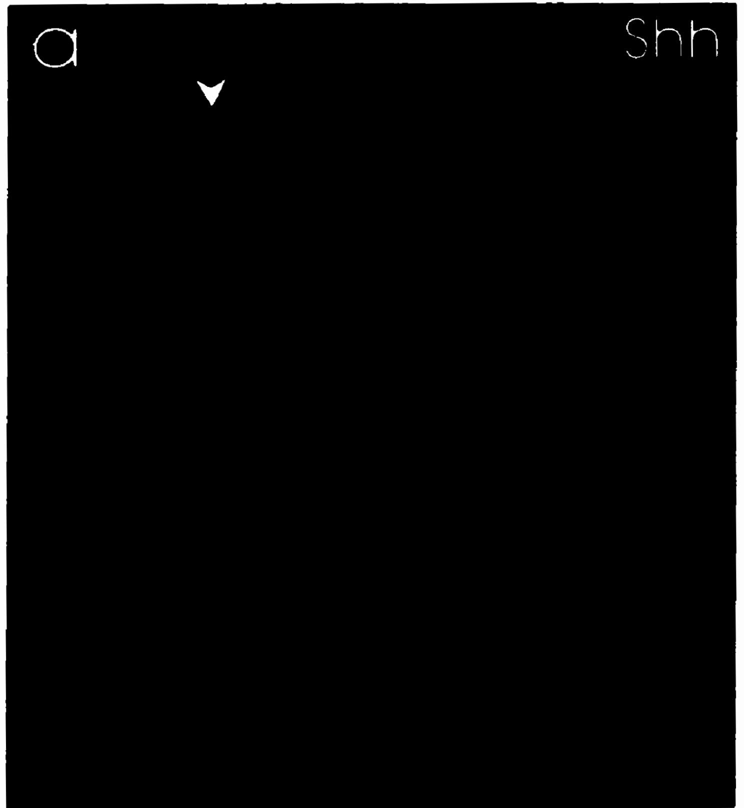


Figure 7



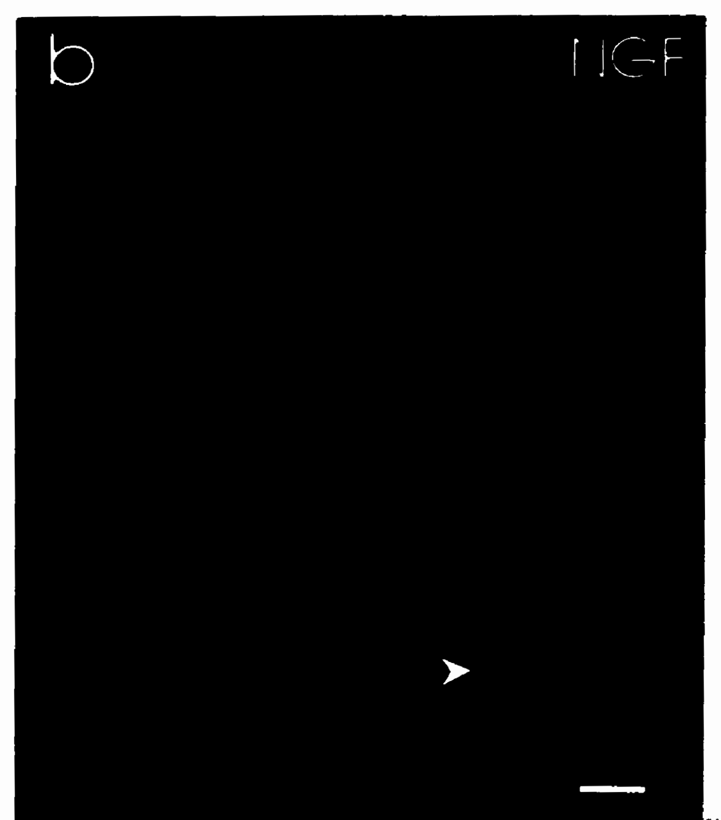


FIGURE LEGENDS

Figure 1. bFGF supports robust cell proliferation. **a.** DRG cultured in serum free medium in the absence (top) and in the presence of bFGF (middle and bottom). After 15 days in culture, few cells survived without the addition of bFGF (top). In comparison, the bFGF enriched group displayed robust cell proliferation with evidence of cluster formation over the same time period (middle). Many cell clusters are observed in the bFGF-enriched group after 22 days in culture (bottom). **b.** Example of a nestin^{+} cell cluster (red). Staining of cell nuclei with DAPI (blue) shows that the majority of cells outside the cell cluster are not nestin^{+}. Scale bars are 250 μm (a) and 50 μm (b).

Figure 2. Neurogenesis in postnatal DRG. **a & b.** Dissociated DRG were cultured for 1 (**a**) or 10 weeks (**b**) in a serum-free bFGF-enriched medium followed by a 4 day incubation in NT-3 or GDNF, respectively. In both instances NF-160/MAP-2^{+} cells were observed (arrows), some of which extended axonal processes (arrowheads). In **b**, two images are montaged at different focal planes to show NF-160/MAP-2^{+} neurons within the sphere (left side) and an axonal process emanating from the sphere (right side).

Figure 3. Trophic factors induce precursors to differentiate into neurons. Comparison of effects of trophic factors on bFGF proliferated precursors from isolated DRG (PNS – left) or bFGF/EGF proliferated precursors from isolated spinal cord (CNS – right). Neurons were identified by their immunolabeling to MAP-2/NF-160 in DRG (left) or MAP-2/panaxonal in spinal cord (right). When DRG cells are cultured in serum-free medium enriched with bFGF for 2 weeks, no neurons were observed (not illustrated). However,

following removal of bFGF and addition of a trophic factor to the medium for 4 days, neurons with morphology characteristic of DRG neurons appear (left column). Different trophic factors support the differentiation of distinct morphological phenotypes as shown for CNTF (a) BDNF (b) NGF (c) NT-3 (d) and GDNF (e). In the presence of GDNF, cell bodies appear to be too small to be neurons (inset). In contrast to the effects of trophic factors on DRG precursors, removal of mitogens and addition of the same trophic factors for 4 days to spinal cord precursors results in the differentiation of neurons with characteristic multipolar CNS morphology (right). Scale bars are 50 μm.

Figure 4. bFGF proliferated spherical cells with DRG neuron morphology are electroresponsive. **a.** Cells targeted for whole cell recordings were spherical in shape. **b.** In response to depolarizing current steps most neurons fired a single spike (top) but repetitive firing was also observed (bottom). **c.** In voltage clamp, the inward currents were TTX-sensitive confirming that voltage-gated Na^+ channels contributed to the generation of action potentials. Scale bars: **b** = 50 mV, 50 ms; **c** = 2 nA, 5 ms.

Figure 5. a. A group of NF-160$^+$ DRG neurons after 4 weeks in a 'standard' culture medium containing horse serum and NGF. Only one of these NF-160$^+$ cells are BrdU$^+$ identifying it as a neuron or NF-160$^+$ neural precursor. Arrows denote the Brdu$^+$/NF-160$^+$ cell in both panels. **b.** MAP-2$^+$/BrdU$^+$ cell cultured for 1 week as above but also enriched with bFGF. Arrows denote same cell in both panels. Note the short neurite emanating from the cell soma (arrowhead). **c.** Histogram of 'neuron' number over time in standard medium (top) or medium enriched with bFGF (bottom). Neuron counts were conducted

according to the methods outlined for quantification analysis. Lighter-shaded histogram bars observed in bFGF represent a weakly-stained atypical population of NF-160/MAP-2^- precursors characterized by: an absence of processes and weak attachment to the substrate. **d.** Example of two neurons grown for 4 weeks in standard DRG media. **e.** Example of cells grown for 4 weeks in standard DRG media enriched with bFGF. NF-160/MAP-2^- cells are seen, often in clusters (arrowheads) interspersed among strongly stained NF-160/MAP-2^+ neurons with processes (arrows). These small spherical cells do not contain processes and hence, may be neuronal precursors. Scale bars are 10 μm in a & b and 50 μm in d & e.

Figure 6. Evidence that differentiated DRG neurons can undergo mitosis. **a.** While DRG neurons are usually spherical, this neuron is spindle-shaped and its DNA, while blurred, appears to be in metaphase (arrows denote furrow). **b_1 & b_2.** Further examples of neuronal precursors that display axons. Each contains 2 nuclei. These precursors are therefore in the telophase stage of cell division. **b_1** shows strong BrdU nuclear labeling, suggesting that mitosis occurs in neurons in culture. **b_2** uses the DNA marker DAPI to demonstrate a single neuron with 2 nuclei. **c_1 & c_2.** Examples of pairs of neurons in close proximity that stain for BrdU. It is likely that these cells recently completed cytokinesis. Scale bars are 10 μm.

Figure 7. NGF and Shh appear to support cell proliferation and neurogenesis. After 6 weeks in serum-free medium enriched with NGF (**a**) or Shh (**b**), spherical cell aggregates were found that contained many MAP-2/NF-160^{+} cells with axonal processes (arrowheads). Scale bar = 50 μm.

SECTION II.
IN VIVO: CYTOGENESIS IN MATURE RAT DORSAL ROOT GANGLIA

1. Materials and METHODS

1a. IN VIVO DRG EXPERIMENTS ON JUVENILES

Thirteen post-natal day (PND) 11 Sprague-Dawley (SD) rat littermates were anesthetized with methoxyfluorane by inhalation and separated into the five experimental groups illustrated in Fig.8. Eight animals were operated on and received a complete unilateral sciatic nerve transection. A 1.5 cm incision was made rostro-caudally and a blunt separation of the muscles was performed to expose the sciatic nerve. Before a complete transection was made, a suture of 6-0 silk was tied onto the nerve proximally. Each rat received the analgesic banamine (0.025 ml/30g) subcutaneously (s.c) and penicillin (22 000 units/kg) intramuscularly (i.m). Two animals were designated as sham controls and underwent the entire surgery excluding any manipulation of the exposed nerve. Three other rats served as additional controls and underwent no surgery. Nine of the 13 animals were euthanized 7 days following axotomy. while the remaining four animals were sacrificed on day 14 post-axotomy.

Animals were divided into three different groups according to their experimental procedure. In the active lesion and sham control group, half of the animals received intraperitoneal (I.P.) injections of BrdU (50 μg/g) on days 1 and 2 post-surgery and half of the animals were injected on days 5 and 6. Animals were perfusion fixed with 20 ml of

heparinized saline (20 I.U. heparin and 20 mg $NaNO_3$ in 9% saline) followed by 80 ml of 4% paraformaldehyde. DRG were extracted and immersion fixated for 1 hr. The tissue was subsequently stored in 10% sucrose with 50mM $NaPO_4$ and 0.1% sodium azide. L4 DRG were cryostat sectioned at 6 μm. A double blind study was conducted and included scanning sections into the Neurolucida image analysis system (MicroBrightfield Inc.). Following this, the numbers of BrdU, neuron specific enolase (NSE) and BrdU/NSE positive cells were tabulated. In total, 10 sections per DRG were analyzed.

1b. IN VIVO DRG EXPERIMENTS ON ADULTS

One adult male underwent axotomy of the right sciatic nerve. The animal received I.P injections of BrdU (50μg/g) on days 1 and day 2 post-axotomy. At 72 hours post-axotomy the animal was sacrificed and perfused with 4% paraforaldehyde. The L4 DRG were cryostat sectioned at 6 μm and immunostained for BrdU and NSE.

Two additional adult rats underwent bilateral sciatic nerve injections of 1% Fluoro-Gold (Fluorochrome Inc.) on day 0 to label existing DRG neurons via anterograde axonal transport. On day 7, both rats received a unilateral sciatic nerve transection and were pulsed with BrdU (50μg/g) immediately before surgery and 5 hrs post axotomy. At 27 hrs post-axotomy, both rats were perfused with 4% paraformaldehyde. The L4 DRG were cryostat sectioned at 6μm and immunostained as above.

1c. IMMUNOHISTOCHEMISTRY

During immunohistochemical processing, tissue was fixed in 4% paraformaldehyde for 15 minutes and then washed (6x20 minute) in phosphate buffered saline with triton-X100 (PBST). Primary antibodies were then added in conjunction with horse or sheep serum (1:100) and allowed to incubate for 2 days under refrigeration, then washed (5x20 minute) with PBST before the addition of the secondary antibody. Tissue was then incubated in secondary antibody in conjunction with horse or sheep serum (1:100) for 1.5 hours and then washed (2x20 minute) with PBST. The immunohistochemical processing for BrdU was similar to that described by Tamatani et al (1995)(Tamatani et al., 1995): Following 2 washes in PBST, the tissue was incubated in a final mixture containing 50% formamide and (2x) saline sodium citrate (SSC) for ½ hour at 65C. It was then washed for 5 minutes in (2x) SSC at room temperature before incubation (30 minutes, 37C) in 2 N HCL. This was then followed by a 10- minute rinse with 0.1 M sodium borate buffered to a pH of 8.5. It was then washed (5x20 minutes) with Tris buffered saline (TBS) followed by a 30 minute wash in Tris buffered saline with 0.1% triton-X100 (TBS-T) and 3% horse serum, before being incubated overnight in Sheep anti-BrdU and sheep serum (1:100). After this incubation period, the tissue was washed (3x30minute) with TBST and incubated in the secondary antibody (Anti-sheep IgG FITC) for 1.5 hours. Finally, it was washed (1x20 minute) in TBST and 50 mM Tris HCL (2x20 minute) before being coverslipped with DAPI.

The following primary and secondary antibody combinations were utilized for immunohistochemical processing of either *in vivo* tissue: (i) Polyclonal sheep anti-BrdU

(Fitzgerald; 1:100) and donkey anti-sheep (H+L) FITC (Jackson;1:50).(ii) Rabbit anti-NSE (Chemicon; 1:300) and donkey anti-rabbit IgG CY3 (Jackson; 1:250).

RESULTS

Staining juvenile and adult DRG for the stem cell marker nestin revealed a population of small cells that were nestin^{+} and therefore candidate stem cells (Fig. 9a & b). Nestin expression was preferentially observed on the lesioned side typical of an injury evoked response. The cells displaying nestin immunoreactivity appeared to be predominantly located around the circumference of the medium and large diameter neurons. These oval shaped, nestin^{+} cells, appeared to be the smallest cells in the ganglia. Nesin staining did not appear to be located within the cells that displayed typical neuronal morphology. However, some smaller, spherical shaped cells did appear to be labeled but not to any convincing degree. The population of nestin^{+} cells that were observed in our experimentation appear to be identical to the nestin^{+} satellite cells identified in embryonic DRG (Hockfield and McKay, 1985). Although our results identify a population of nestin^{+} cells in mature DRG, we cannot determine if they are in fact stem cells because of the lack of specificity of nestin as a stem cell marker for the PNS (Hockfield and McKay, 1985).

Due to the ambiguity of nestin as a stem cell marker for the PNS, it was decided to combine BrdU immunostaining with the neuron specific marker NSE in an attempt to obtain evidence of neurogenesis. Many double-labeled cells were identified in all treatment groups previously established for the juvenile lesion experiments (Fig. 10 & 11). The active lesion group injected with BrdU on days 1 & 2, and sacrificed on day 7 post-axotomy demonstrated the largest elevation in the number of BrdU^{+}/NSE^{+} cells on the ipsilateral, lesioned side when compared to the contralateral control

(Fig. 12a). Unfortunately, this change was not statistically significant due to the small sample size. Our results suggest that maximal proliferation of the putative neuronal precursors appears to occur in the first 48 hours after injury (Fig 12a). However, the axotomy-induced proliferative response appears to be transient, lasting about 1 week post-injury (Fig. 12a). Comparing the cell soma diameters of these BrdU$^+$/NSE$^+$ cells (11.1 +/- 2.6 μm) to other DRG neurons (21 +/- 6.8 μm) indicates that these cells overlap in diameter with the smallest diameter neurons housed within the ganglia (Fig 12b). However, these cells also appear to overlap in diameter with the surrounding satellite cells (Cecchini et al, 1999) thereby further confusing the issue of their true identity and origin.

We also conducted data analysis to quantify cytogenesis using the nuclear label of cellular proliferation, BrdU. Our results indicate that axotomy induces a increase in the number of BrdU$^+$ cells in the ipsilateral ganglia compared to the contralateral ganglia (Fig. 13a & b). Using a double-blinded analysis, we found that sciatic nerve lesions in juveniles causes an approximate 40% relative increase in the number of BrdU$^+$ cells (cytogenesis) in the ipsilateral ganglia compared to the contralateral side regardless of the time of BrdU injection and age of sacrifice after axotomy (Fig. 13a & b). This cytogenic response appears to be transient as there were fewer BrdU$^+$ cells observed at 14 days as compared to 7 days post-axotomy (Fig. 13b).

One of the outstanding issues that still needed to be addressed was whether neurons become mitotically active in response to injury. As a result, DRG cryostat cross sections

were examined for evidence in support of this idea. Although Figure 14 depicts NSE$^+$ regions that appear to overlap with BrdU$^+$ labeling, a closer look at higher magnification reveals a BrdU$^+$/NSE$^+$ cell with apparent fissure formation between nuclei in a dividing cell (Fig. 14b). In further pursuit of this issue, the sciatic nerves of adult animals were injected with Flouro-Gold in an attempt to label pre-existing neurons within the adult DRG. Remarkably, following axotomy, some of these pre-labeled neurons were also BrdU$^+$ (Fig. 15) suggesting that some adult neurons are mitotically active and retain this ability into adulthood.

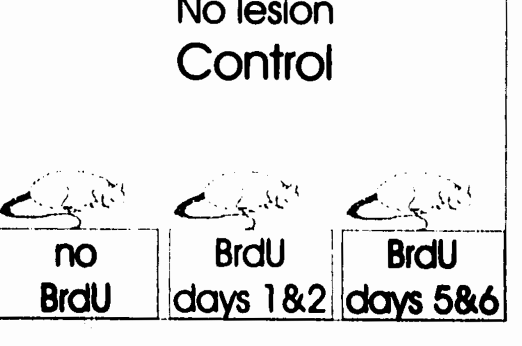
Adult lesion experiments also revealed the presence of BrdU$^+$/NSE$^+$ cells (Fig. 16). Although a quantitative analysis was not conducted on these adult specimens, there appeared to be much fewer BrdU$^+$/NSE$^+$ cells than previously identified in the juvenile animals, demonstrating that the response to injury is more restricted than in juveniles. Another interesting observation of the lesion experiments in adults as compared to juveniles was that the contralateral side was almost completely devoid of BrdU labeling. Thus, adult animals appear to have an age restricted decrease in potential for injury-induced neurogenic/cytogenic repair.

Experimental Protocol

PD11 RATS

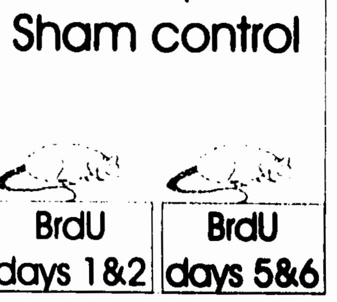
Day 0 = Surgery

No lesion
Control



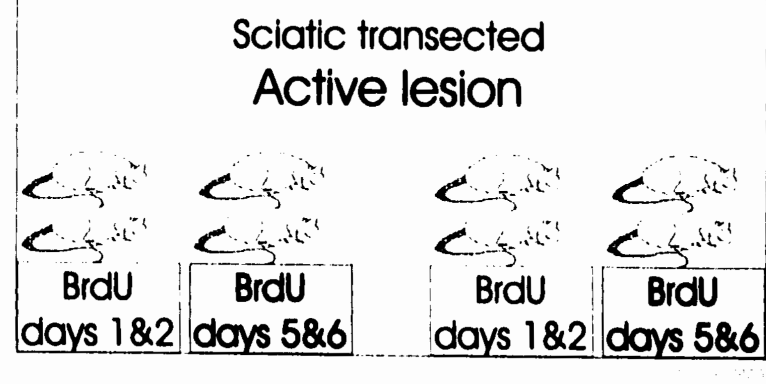
no BrdU	BrdU days 1&2	BrdU days 5&6

Sciatic exposed
Sham control



BrdU days 1&2	BrdU days 5&6

Sciatic transected
Active lesion



BrdU days 1&2	BrdU days 5&6	BrdU days 1&2	BrdU days 5&6

Figure 9

Figure 10

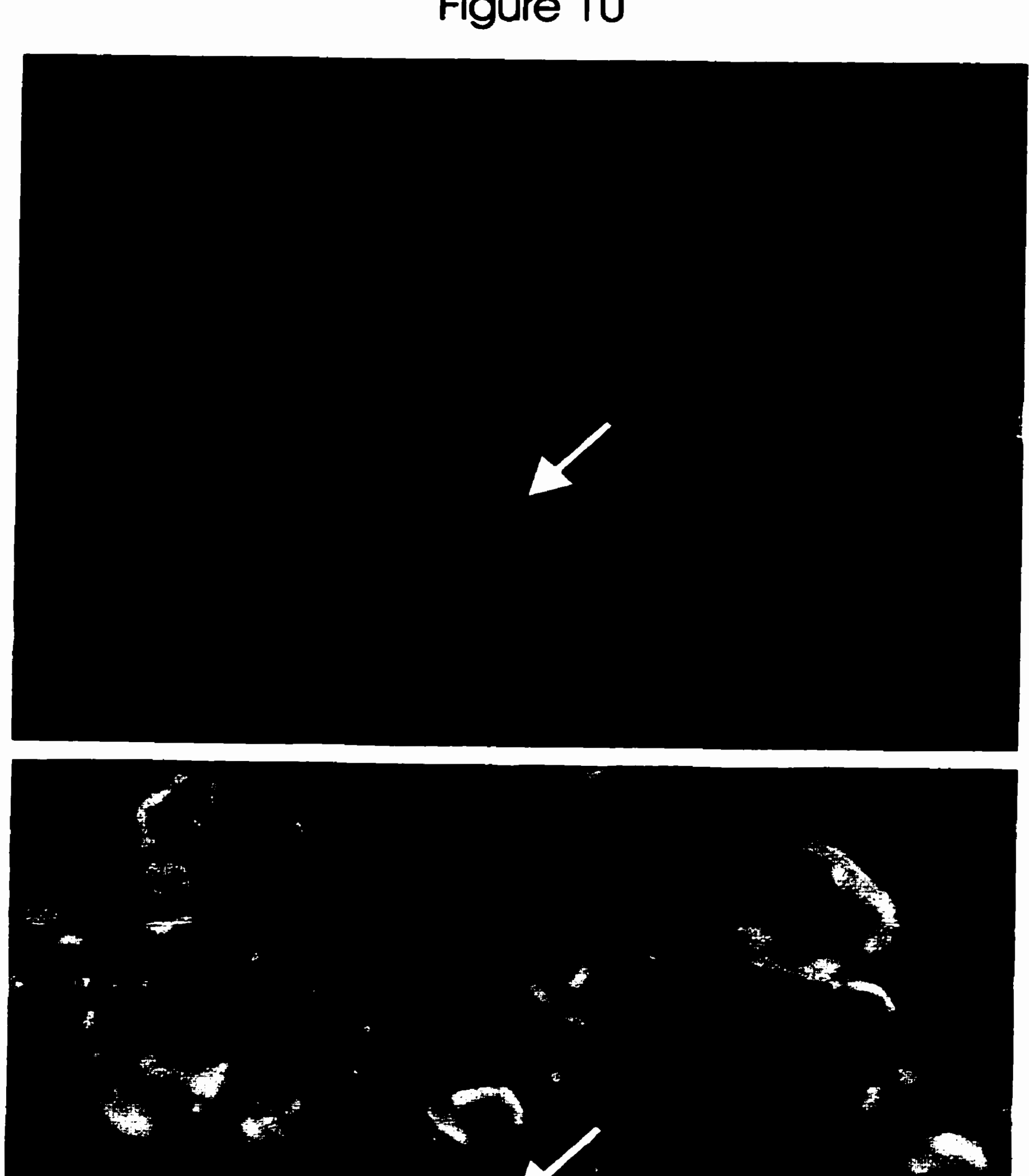


Figure 11

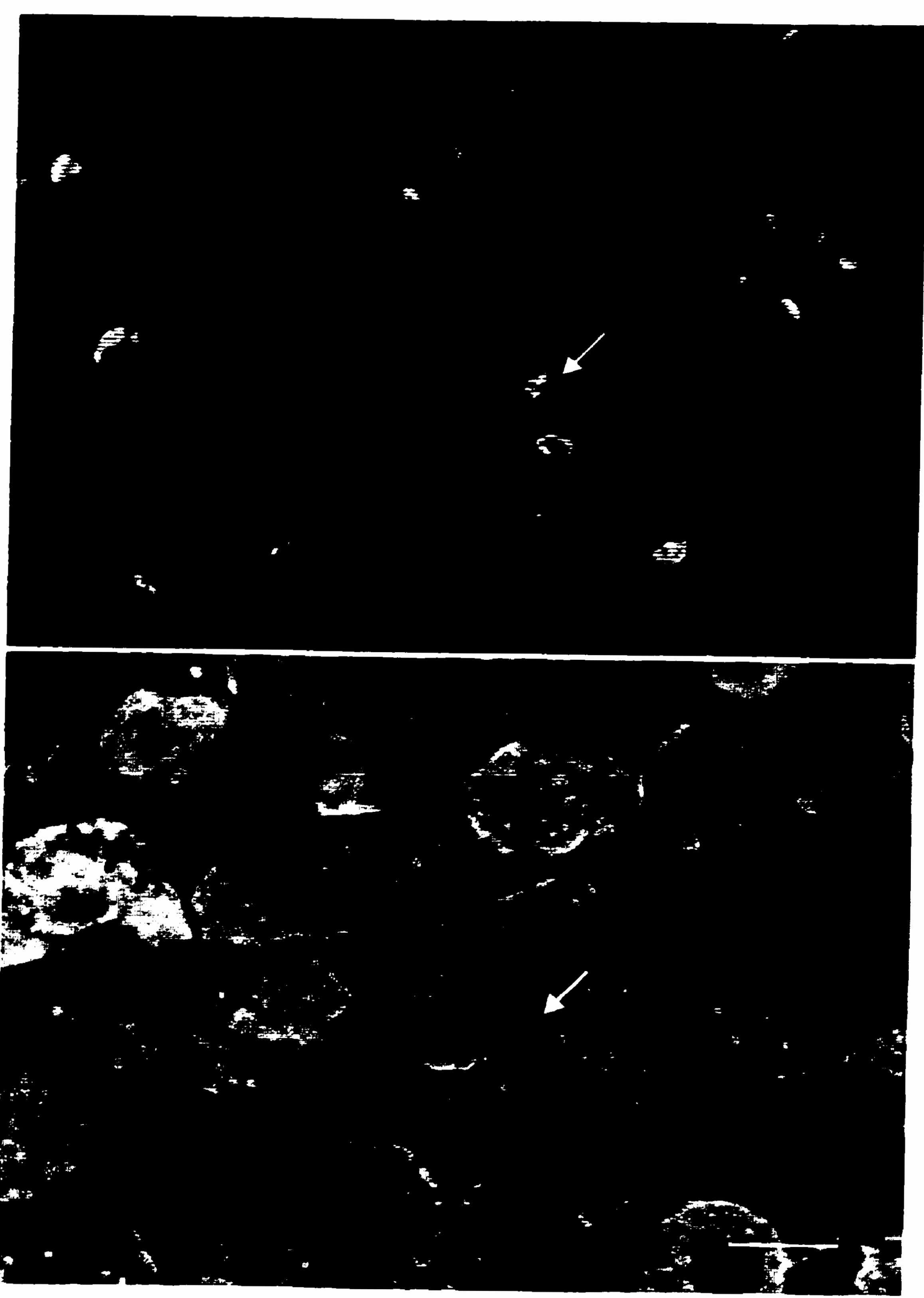


Figure 12

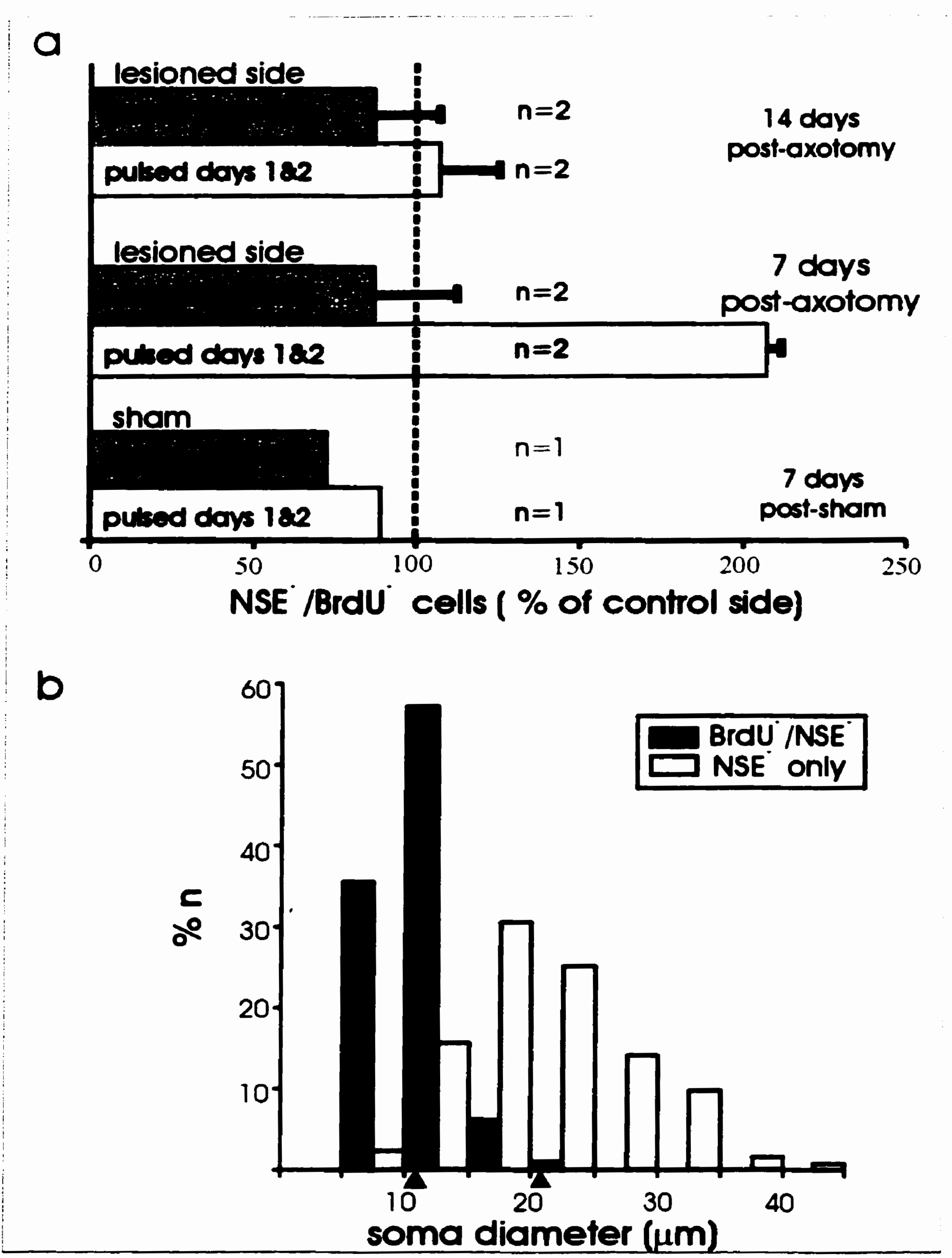


Figure 13

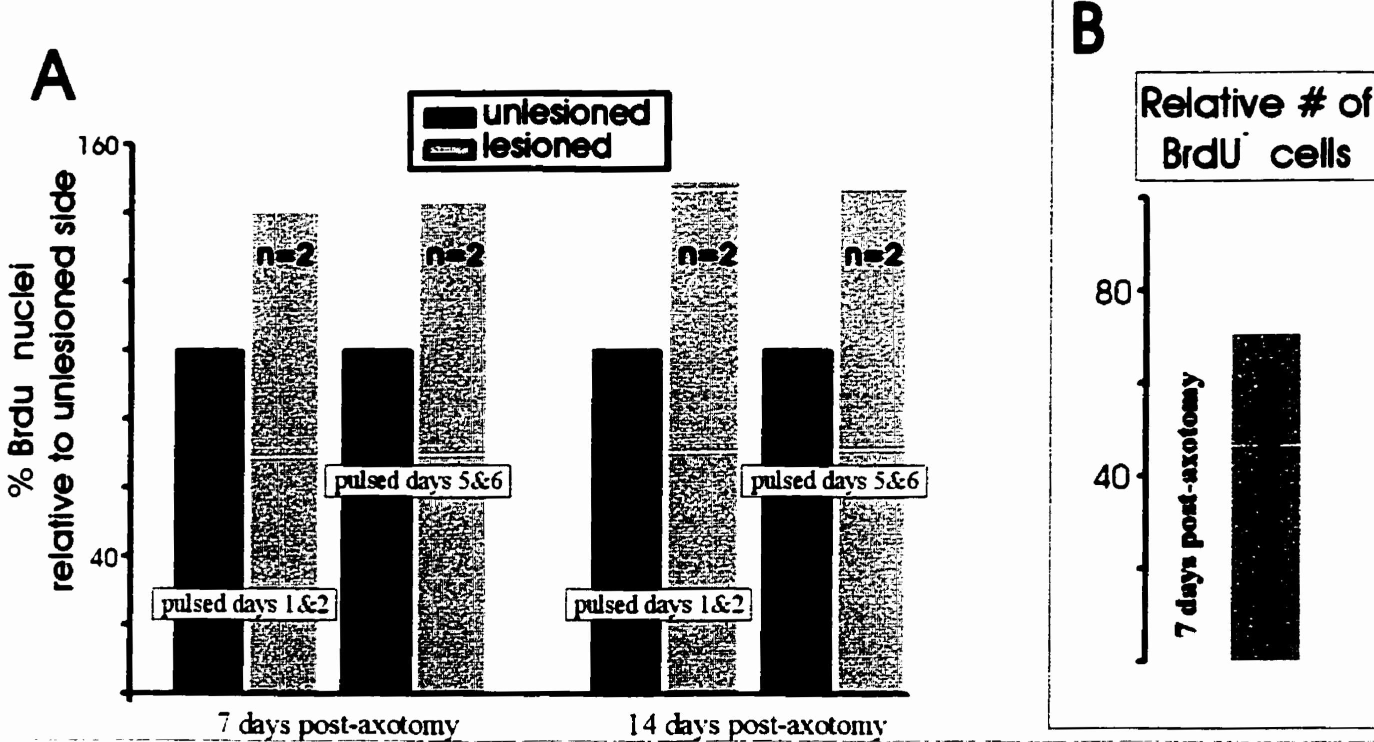


Figure 14

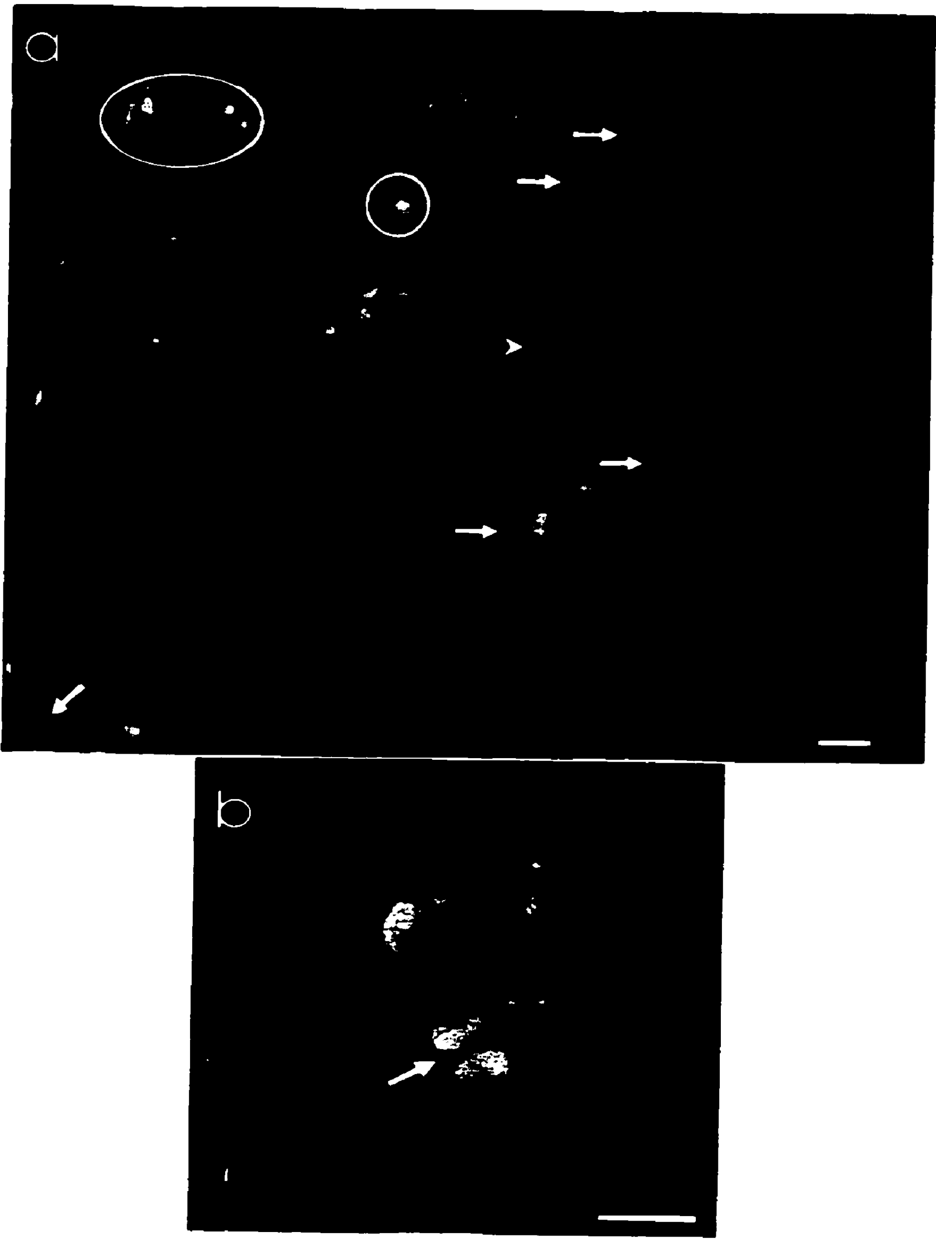


Figure 15

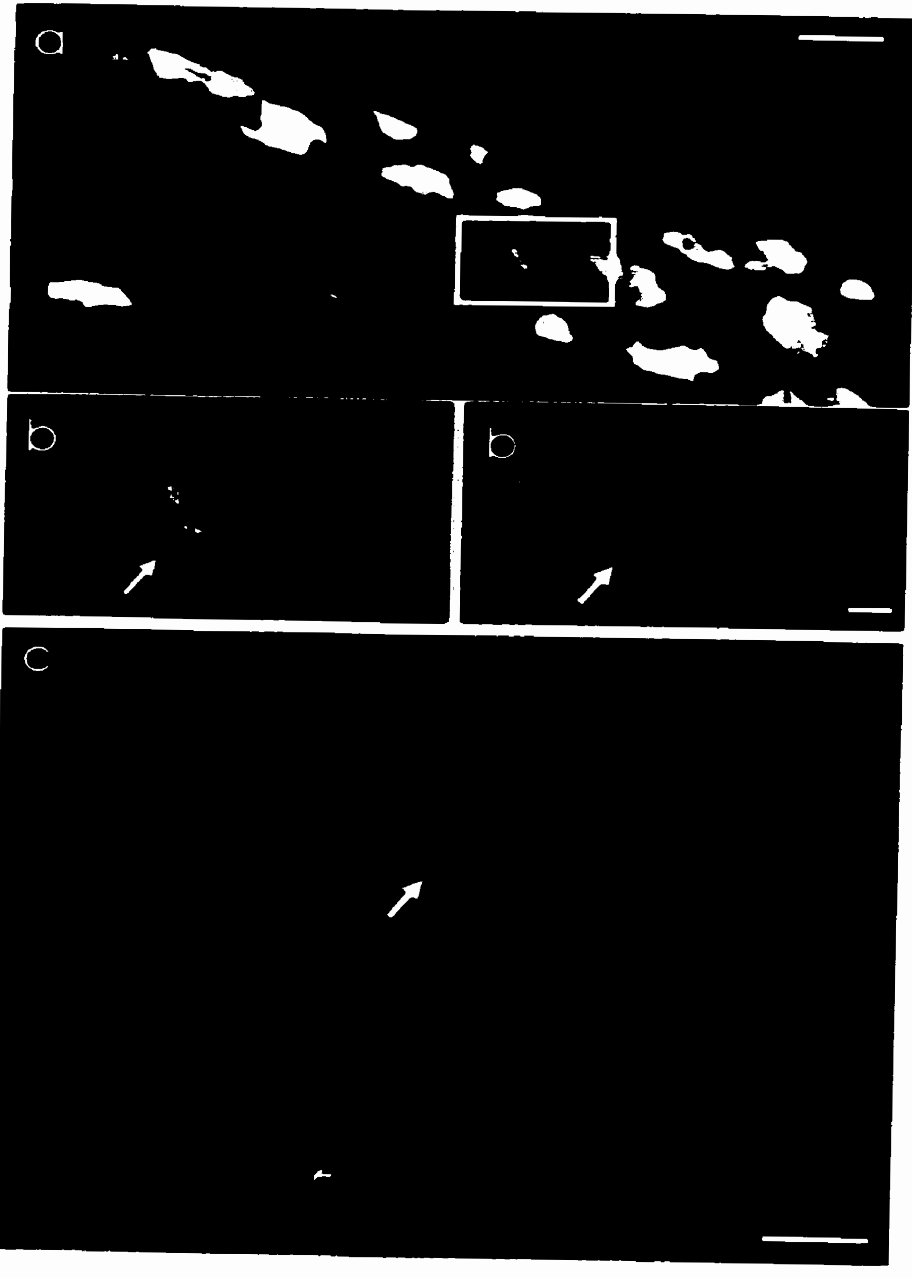


Figure 16

FIGURE LEGENDS

Figure 9. Axotomy induced cytogenesis *in vivo*. **a & b.** Sciatic nerve lesion induces nestin staining in satellite cells surrounding DRG neurons only on the lesioned side in both juveniles (**a**) and adults (**b**). Scale bars are 50 μm.

Figure 10. Neurogenesis in mature DRG. All images were taken of L4 DRG obtained from a PND19 sham control animal, BrdU-pulsed on days 1 and 2 post-axotomy and sacrificed at day 7. **Top and bottom** figures are photomicrographs from an identical region. **Top.** Arrow depicts a $BrdU^{+}$ cell. **Bottom.** Arrow pointing to the identical cell that is also NSE^{+}. Scales bar is 25 μm.

Figure 11. Axotomy-induced neurogenesis in mature DRG. The image was taken from the L4 DRG obtained from a PND 19 active lesion animal, with BrdU-pulsed on days 1 and 2 post-axotomy and sacrificed at day 7. **Top and bottom** figures are photomicrographs from an identical region. **Top.** Arrow depicts a $BrdU^{+}$ cell. **Bottom.** Arrow pointing to the identical cell that is also NSE^{+}. Scales bar is 25 μm.

Figure 12. Axotomy appears to induce neurogenesis *in vivo*. a. Axotomy induces a proliferation of $NSE^{+}/BrdU^{+}$ cells in week 1 that disappears after 2 weeks. Histogram comparing the relative numbers of $NSE^{+}/BrdU^{+}$ double labeled cells on lesion verses un-operated side. Animals pulsed with BrdU on days 1 & 2 after sciatic nerve lesion and sacrificed 7 days post-axotomy showed a marked increase in double labeled cells. b. Double-labeled putative neurons are very small. Histogram comparing the distribution of

cell soma diameters from a sample population of NSE$^+$ neurons (gray) verses cells thought to be both NSE and BrdU immunostained (black). Mean soma diameters are presented as shaded arrowheads on the abscissa.

Figure 13. Axotomy induced cytogenesis. **a.** Histogram comparing the number of BrdU$^+$ nuclei between lesioned and unlesioned L4 DRG in all experimental groups. Note the consistent ~40% relative increase in BrdU$^+$ nuclei on the lesioned side. **b.** There are fewer BrdU$^+$ cells 14 days after axotomy than 7 days post-axotomy.

Figure 14. BrdU staining compared to NSE$^+$ neurons. All images were taken of the ipsilateral L4 DRG BrdU-pulsed on days 1 & 2 and sacrificed at day 7 post-axotomy. BrdU (green) and NSE (red) labeling are superimposed. **a.** Arrows point to neurons with apparent BrdU staining. Arrowhead points to a small neuron with weak BrdU staining. Other neurons appear to be BrdU$^+$ but the yellow colored nuclei (circled) may suggest that these are nuclei from non-neural cells that overlap neurons on the z-axis. **b.** High-power image showing BrdU overlapping the NSE staining. Arrow points to apparent fissure between nuclei in a dividing cell. Scale bars are 20 (a) and 10 μm (b) respectively.

Figure 15. Evidence suggesting that adult neurons become mitotic. Sciatic nerve injection of Fluoro-Gold in adult 1 week prior to axotomy labels pre-existing DRG neurons with peripheral projections. Neurons are irregularly-shaped due to fixation problems. Fluoro-Gold labeled neurons appear yellow/white in color. **b.** Blow-up of boxed area in **a** to demonstrate a Fluoro-Gold labeled neuron ($\mathbf{b_1}$) whose nucleus stains

for BrdU (**b**$_2$). Arrows point to same position in **b**$_1$ and **b**$_2$. **c.** Superimposed images show that a Fluoro-Gold labeled neuron in another animal is clearly BrdU$^+$ (arrow). Note adjacent Fluoro-gold labeled neurons do not label for BrdU. Scale bar for **a** is 50 μm and 10 μm for **b & c**.

Figure 16. Axotomy-induced neurogenesis in adult DRG. All images were taken of L4 DRG obtained from an adult male animal. BrdU-pulsed on days 1 and 2 post-axotomy and sacrificed at day 3. High power image showing a BrdU$^+$ nuclei overlapping in an identical NSE$^+$ cell. Scale bar is 20 μm.

DISCUSSION

1. SUMMARY

In vitro studies conducted on early postnatal DRG demonstrated that neurogenesis does extend beyond embryological development. Cellular proliferation became evident by the development of spherical cell clusters following the addition of bFGF to DRG culture. The addition of different trophic factors induced these bFGF-responsive spheres to differentiate into neurons that appeared to be of different phenotypes. These newly generated neurons were identified as such by their positive immunoreactivity for neuronal markers such as NF-160 and/or MAP-2 and by their characteristic electrophysiological recordings. Although neuronal precursors appear to be involved in this neurogenic response, there is also supportive evidence that neurons themselves become mitotically active and hence are capable of neurogenesis. Moreover, the possibility that postnatal DRG contain stem cells can not be ruled out nor can it be proven due to the lack of specificity of nestin as a stem cell marker for the PNS.

In vivo sciatic nerve lesion studies conducted in juveniles and adults also support the findings of neurogenesis demonstrated by our *in vitro* studies. Putative neuronal precursors were identified by their dual immunoreactivity for NF-160/MAP-2 and BrdU. However, due to their relative small size and positioning within the ganglia, these cells may be proliferating satellite cells. Hence, a conservative interpretation of our results is that of axotomy-induced cytogenesis that may include neurogenesis. Furthermore, the results of these studies identified the time interval of maximal cytogenic/neurogenic repair to within the first 48 hours following injury. Similar double labeled cells were also

identified in the adult animals, suggestive that neurogenesis or at least cytogenesis is possible even into adulthood. Finally, Flouro-Gold experiments conducted in adults support our *in vitro* findings of the involvement of mitotically active neurons in a possible neurogenic repair response.

2. POSSIBLE MECHANISMS FOR NEUROGENESIS

Recent reports in DRG have provided supportive evidence to suggest that neurogenesis within sensory ganglia is a life-long event (St Wecker and Farel, 1994; Ciaroni et al, 2000; Farel and Boyer, 1999; Poken and Farel, 1997). However, the mechanism for the induction of neurogenesis from either stem cells, neuronal precursors and/or neurons still remain unknown. It is my belief that the key to identifying the underlying mechanisms behind the neurogenic response lies in the function of the many factors intrinsically produced within the ganglia and how they strategically interact with various DRG cell types. *In situ* hybidization studies have identified an altered expression following injury of the mRNA of several factors produced within the ganglia. For example, *in vivo* experiments have shown that following axotomy, mRNA for bFGF is upregulated in DRG neurons (Ji et al., 1995). Similarly, following spinal nerve ligation, mRNA for NGF increases 4-fold peaking after one day and lasting for at least three weeks (Shen et al. 1999). Moreover, mRNA for BDNF, GDNF, NT-3 and NT-4 have also been shown to be upregulated in neuronal/non-neuronal cells post-transection (Zhou et al, 1999; Trupp et al, 1997; Acheson et al, 1996; Funakoshi et al, 1993). Although axotomy has been shown to increase DRG neuron number in postmetamorphic bullfrog (Farel and Boyer, 1999) and possibly in adult rat following infusion of NGF or NT-3 (Ljungberg et al, 1999).

nobody has demonstrated a connection between injury-induced upregulation of endogenous factors like the aforementioned neurogenesis.

As a result, there are several possible explanations that could be used to identify the underlying mechanisms of neurogenesis. For example, because bFGF and NGF are known mitogenic signals (Geffen and Goldstein, 1996; Murphy et al 1994), their upregulation following injury may serve to signal quiescent precursors, housed in mature sensory ganglia, to generate new cell populations including neurons. These injury-induced mitogenic signals may serve to provide a compensatory regenerative response since many studies have demonstrated that axotomy can induce considerable neuronal apoptosis (Ljungberg et al,1999; Groves et al, 1997; Oliveira et al. 1997).

Another possibility is that since NGF supports the survival of nociceptors (Crowley et al. 1994), it could also selectively support neurogenesis of nociceptive neurons. Recent experiments support this possibility of an NGF-mediated neurogenic response (Ljungberg et al, 1999). This would explain the need for an upregulation of NGF mRNA post-axotomy (Shen et al, 1999).

While NGF and bFGF may mediate the proliferative response of neuronal precursors, the simultaneous upregulation of other factors such as NT-3, GDNF, NT-4 and BDNF may be required to sustain the survival and phenotypic differentiation of any newly generated neurons. This function is quite plausible since the survival and differentiating effects of neurotrophic factors are well documented (Stucky et al, 1998; Molliver et al, 1997;

Acheson et al, 1995; Ernfors et al, 1995; Ernfors et al, 1995b; Crowley et al 1994: Farinas et al, 1994). Clearly, the involvement of the neurotrophins in neurogenic repair must be further explored in order to clarify this issue.

3. RATIONALE FOR *IN VITRO* AND *IN VIVO* EXPERIMENTATION

Although *in vivo* experimentation provides a realistic representation of injury-induced events, *in vitro* studies allows for a more precise control of the cellular environment, for the purpose of identifying critical factors on assessing cellular responsivity. Thus, for this thesis both methods were conducted in order to interrelate the individual advantages of each. It is important to recognize that the culturing of DRG neurons can mimic the phenotypic changes observed following axotomy *in vivo* (McMahon et al, 1999; Hokfelt et al, 1994). For example, Substance P and CGRP levels are reduced *in vivo* following axotomy, and *in vitro* following cell culture. As a result, the *in vitro* method may well serve as a model to study several features of axotomy-induced changes that occur *in vivo*. The close interrelationship that exists between these two experimental designs provides an overall model conducive to exploring the underlying mechanisms involved in neurogenesis.

4. STEM CELLS: DO THEY EXIST?

Researchers have previously described precursor cells derived from the neural crest (Morrison et al., 1999;Greenwood et al., 1999;Lo and Anderson, 1995). However, to the best of our knowledge, similar cells have not been identified within DRG. As a result, this prompted our interest to explore this intriguing possibility. When early postnatal DRG were cultured in the presence of bFGF, spherical aggregates of cells began to form.

This observation provided the first real evidence of bFGF-induced cytogenesis. Since our initial goal was to identify the presence of stem cells, we decided to stain the spheres for nestin, a known stem cell marker for the CNS (Lendahl et al 1990). Nestin$^+$ spheres were shown to form in the presence of bFGF.

These interesting results, obtained *in vitro* prompted us to explore the possibility that the axotomy-induced increase in bFGF observed *in vivo* (Ji et al. 1995) could also generate nestin$^+$ cells. Hence, sections of control and axotomized DRG obtained from juvenile and adult animals were examined for nestin immunoreactivity. A subpopulation of cells only in axotomized DRG were identified that appeared to be nestin$^+$. These oval shaped cells appeared to be the smallest cells of the ganglia and were often found surrounding the circumference of medium to large diameter neurons.

However, the use of nestin-immunoreactivity to demonstrate DRG stem cells was equivocal as nestin may lack the specificity of a stem cell marker for the PNS (Hockfield and McKay, 1985). Based on their apparent small size and positioning within the ganglia, these cells are likely to be satellite cells (Cecchini et al. 1999). Interestingly, similarly located nestin$^+$ satellite cells have been previously identified in embryonic rat DRG (Hockfield and McKay, 1985). However, similarly located cells also became immunopositive for GFAP. This observation is consistent with the characteristics described for reactive astrocytes of the CNS (Pekney et al, 1999). Hence, the emergence of nestin$^+$ staining may reflect an injury-induced response and not one of stem cell proliferation. Conversely, it is entirely possible that these nestin$^+$ cells are in fact stem

cells. As a result, this issue still remains unresolved until a specific stem cell marker for the PNS is identified.

Based on the above findings, it remained uncertain that DRG contain stem cells. Fortunately, our *in vitro* data provided strong supportive evidence for the existence of neuronal precursors. bFGF-responsive spheres similar to those previously identified as nestin$^+$ differentiated into neurons following the removal of bFGF and the addition of trophic factors. This ability of bFGF-responsive, nestin$^+$ spheres to differentiate into neurons is a strong indication for the existence of neuronal precursors. Moreover, the additional observation that different trophic factors could induce bFGF precursors to differentiate rather selectively into DRG neurons with multiple morphological phenotypes and characteristic electrophysiological properties provides the first direct evidence in support of mitogen proliferated and trophic factor induced neurogenesis postnatally.

During development, the DRG are derived from precursors in the neural crest (Morrison et al., 1999; Greenwood et al., 1999), and neurogenesis ends several days before birth in both rat and mouse DRG (Kitao et al., 1996; Fariñas et al., 1996). However, in our cultures of early postnatal DRG, a population of spherical 'grape-like' cell clusters that were weakly immunopositive for neuronal markers but devoid of neurites were observed in standard medium (containing NGF and horse serum) enriched with bFGF. As these cell types were not observed when bFGF was applied in a serum-free medium, unknown factors in addition to bFGF appear necessary for their emergence. These atypical cells

were thought to be either undifferentiated neurons derived from neuronal precursors or neuronal progenitors. Since the ability of neuronal precursors to express neuronal markers and be able to actively proliferate is not a novel concept (Blagrund et al. 1996), we decided to apply BrdU to our cultures, thereby allowing it to be incorportated into the DNA of dividing cells for the purpose of immunohistochemical detection. Surprisingly, $BrdU^+/NF\text{-}160^+$ and/or $BrdU^+/MAP\text{-}2^+$ cells were identified in a select subpopulation of cells that were grown in bFGF-enriched standard DRG media. This novel observation provided supportive evidence that neuronal precursors do exist in postnatal DRG.

Recent studies in juvenile bullfrog DRG have identified a dormant population of immature, incompletely differentiated neurons that can be induced to differentiate into a mature form in response to injury (Farel and Boyer, 1999). These cells are different in appearance to typical mature neurons yet express immunoreactivity for a neuron-specific marker (Farel and Boyer, 1999; Meeker and Farel, 1997). Our *in vitro* results suggest that a similar population of neuronal precursors exist within the mammalian DRG. It is possible that these cells remain in an immature precursor state because the mitogenic signal elicited by bFGF is greater than the differentiation signal elicited by trophic factors (e.g. NGF) and other unknown substances present in the serum. It is my belief that this mitogenic signal prevents the cells from exiting the cell cycle to take on a mature typical neuronal phenotype and may therefore account for their weak labeling pattern and lack of process formation.

The existence of $BrdU^+/NF\text{-}160^+$ and/or $MAP\text{-}2^+$ cells were also noted when DRG were cultured in standard DRG media that was not enriched with bFGF. However, these neuronal precursors displayed a vastly different morphology. Repeated experiments conducted in standard DRG media not enriched with bFGF reveal a consistent population of process bearing cells that display the identical morphological phenotype of "typical" DRG neurons in culture. The ability of these $BrdU^+$ neuronal precursors to intermix and camouflage with surrounding neurons makes them difficult to identify if not for their positive immunoreactivity to BrdU. Recent studies support our identification of this novel type of neuronal precursor that display process formation, express neuronal markers and actively proliferate (Blaugrund et al, 1996).

Although both aforementioned phenotypes of neuronal precursors are vastly different, they both not only possess the capability of dividing to ensure self renewal but also possess the capability to exit the cell cycle and enter a differentiated state. These observations demonstrated the effect of environmental conditions on cellular responsiveness.

The unambiguous observation of mitotically-active neurons in culture supports the intriguing possibility that some neurons retain the capacity for mitosis. Because NGF supports the survival of nociceptors (Crowley et al., 1994), it is possible that NGF in the culture medium selectively supports neurogenesis of these cells, particularly since NGF activation of the trkA receptor can induce a mitogenic response (Cordon-Cardo et al., 1991). This possibility is further supported by the observed formation of 'neurospheres'

after 6 weeks in NGF-containing serum-free medium. Moreover, Ljunberg et al (1999) also provided evidence supporting a NGF mediated neurogenic response in DRG. They reported unexplainable increases in DRG neuron numbers following spinal nerve transection with immediate application of NGF or NT-3 to the proximal stump, suggesting a possible role for neurotrophins in axotomy-induced neurogenic repair. It is also conceivable that up-regulated neurotrophins work together with increased bFGF expression following axotomy (Ji et al., 1995) to activate cellular mitogenic signaling pathways. Furthermore, the unexpected ability of Shh to produce neurospheres after 6 weeks in culture in serum free medium suggests that numerous other factors may be involved in progenitor proliferation.

Our novel observations that differentiated mammalian DRG neurons appear to retain the capacity to divide is consistent with other recent *in vitro* observations of bFGF-induced mitosis in hippocampal neurons (Brewer, 1999) as well as *in vivo* observations in the anterior subventricular zone of mitotic migrating TuJ1^{+} 'neurons' destined to become olfactory bulb interneurons (Menezes et al., 1995).

C-fibers are small diameter sensory neurons that signal tissue damage from the periphery. These nociceptors possess features suggestive of a developmentally immature phenotype. For example, polymodal nociceptors are unmyelinated, terminate in unspecialized free nerve endings, and retain long-duration action potentials (Djouhri et al., 1998; Gee et al., 1999). Interestingly, we observed only neurons with small diameter axons undergoing mitosis, which is consistent with recent findings that only the small diameter neurons

expressed bFGF mRNA in control adult (Ji et al, 1995). For these above reasons, C-fibers are good candidate neurons to retain an ability to divide postnatally.

6. TROPHIC EFFECTS ON AXON MORPHOLOGY

Neurotrophic factors are essential for the survival and phenotypic differentiation of several subtypes of sensory neurons (Crowley et al., 1994; Molliver et al., 1997; Lentz et al., 1999; Stucky et al., 1998; Acheson et al., 1995; Ernfors et al., 1995; Ernfors et al., 1994; Farinas et al., 1994; Molliver et al., 1997). In addition, neurotrophic factors have also been shown to support the development of diverse sensory axon morphologies (Lentz et al, 1999). For example, NGF increases axon length with minimal branching while NT-3 or BDNF responsive neurons produce extensive branching (Lentz et al., 1999). While not quantified, we have also observed a neurotrophic effect on axon morphology in bFGF proliferated precursors. These results support our previous observations that suggested that trophic factors differentiate bFGF-responsive precursors back into the subpopulations of neurons that normally comprise the ganglia.

7. NEURONAL PRECURSORS AND MITOTICALLY ACTIVE NEURONS

Our observations suggest that early postnatal DRG house a population of mitogen-responsive neuronal precursors/neurons that retain their capacity for neurogenesis beyond embryological development. Because our cell culture procedures were capable of generating new neurons, it is also possible that neurogenesis occurs *in vivo* following sciatic nerve injuries that functionally axotomize DRG neurons. As there is suggestive evidence that mature DRG house neural precursors (St.Wecker and Farel, 1994; Ciaroni

et al., 2000; Farel and Boyer, 1999; Popken and Farel, 1997), our above findings also support the possibility that adult animals retain or contain cells that can proliferate and give rise to more neuronal precursors by increased expression of bFGF and NGF. Hence, *in vivo* sciatic nerve lesion experiments involving juvenile and adult animals were undertaken in order to assess the capacity for neurogenesis into adulthood. Several observations obtained from our *in vivo* data support this connection.

First, the existence of a subpopulation of cells in juveniles and adults that were $BrdU^+/NSE^+$ suggests that DRG do retain the capacity for neurogenesis into adulthood. Although neuronal immunomarkers exist for DRG neurons, there are currently no specific markers to identify DRG neuronal precursors. Identifying the cells responsible for this neurogenic response becomes further complicated by the inability to distinguish proliferating neuronal precursors from the possibility of mitotically active neurons.

Preliminary *in vivo* adult lesion experiments provided additional support of neuron-induced neurogenesis. Neurons previously anterogradely labeled with Flouro Gold were found to be $BrdU^+$, suggesting that a small population of pre-existing neurons with axonal projections to the periphery retain the capacity to become mitotic. However, the use of a confocal microscope would have been beneficial in providing stronger evidence of double labeled cells. This concept of mitotically active neurons *in vivo* has been substantiated not only by our *in vitro* findings of neuron-induced neurogenesis but also by other recent reports of neonatal and adult neurogenesis in other areas of the nervous system (Brewer, 1999; Gould et al, 1999; Menezes et al, 1995).

Despite the exiciting possibility of mitotically active neurons in the adult, these results lack the convincing clarity illustrated by our *in vitro* data. For example, although the BrdU$^+$ labeling appears to be in a region of Flouro Gold$^+$ labeling, it does not seem to be soley restricted to the characteristic nuclear labeling pattern normally displayed by BrdU. As a result, depending on the cut of the DRG section examined, the possibility remains that it could be a BrdU$^+$ nucleus of an overlying satellite cell.

These suggested *in vivo* findings of mitotically active neurons lead back to the question of whether neuronal precursors, neurons or both are responsible for the neurogenic response. Methods that could separate and conclusively identify neuronal precursors from mitotically active neurons could be very helpful in determining the individual contribution of each to the overall neurogenic response.

Although a conclusive identity for these neurogenic cells still remains unknown, it is quite possible that stem cells, neuronal precursors and neurons are all involved in the neurogenic response. Whether these cells work alone or in conjunction to each other still remains to be determined. The ability of these cells to proliferate *in vitro* and characterize them through *in situ* hybridization techniques could prove to be beneficial in terms of separating out these cell types involved in the neurogenic repair response.

In an attempt to identify and characterize the specific cells involved in neurogenesis. *in vivo* experiments involving juvenile animals were conducted. Cells displaying double

labeling for $BrdU^+/NSE^+$ were found to have an average cell diameter of 11.1 μm. warranting their classification as the smallest cells within the ganglia. They often appeared in pairs which appeared to be predominantly distributed in close proximity to the circumference of both medium and large diameter neurons dispersed throughout the ganglia. Their unusually small diameter suggests (as previously discussed) that they may be derived from the small, immature, unmyelinated C fibers.

The observation that many $BrdU^+$ cells also appeared to stain for the neuronal marker NSE suggests that injury-induced neurogenesis and cytogenesis may be simultaneously taking place. Double blind studies demonstrated that at 1 week following sciatic nerve lesion, there was a marked increase in the number of $BrdU^+/NSE^+$ cells in the ganglia ipsilateral ot the lesion, compared to contralateral control. These differences disappeared at 2 weeks post-axotomy. Interestingly, these observation parallel the time course of axotomy-induced changes in bFGF (Ji et al, 1995) and NGF mRNA levels (Shen et al. 1999). Hence, these results suggest that the optimal time window for neurogenic repair appears to be within the first week post-axotomy which parallels the time window previously stated for cytogenesis. Furthermore, because the most dramatic increase in the number of $BrdU^+/NSE^+$ cells occurred in the 7 day active lesion group injected with BrdU days 1 and 2 post-axotomy, we were able to further narrow the time window for optimal neurogenic repair to the first 48 hours post-injury. Hence, in juveniles, 'neurogenesis' may occur early after axotomy and newly generated neurons may die in the absence of additional trophic support from the periphery. For this reason, robust increases in neuronal numbers did not coincide with the robust axotomy-induced

proliferative response displayed by the neuronal precursors within the first 48 hours after injury. This time dependence of lesion induced neurogenesis may recapitulate the neurogenesis and programmed cell death that occurs during development. where the neurons that fail to receive trophic support from their peripheral targets undergo apoptosis (Oliveira et al. 1997; Groves et al. 1997; Gillardon et al 1996). These results suggest that sensory neuron replacement may be a naturally occurring process throughout life to accommodate changes in sensory demand such as growth. development and maturation. Addition of new neurons during maturation or replacement of old neurons during the natural aging process is quite possible because of autocrine and paracrine production of trophic factors from neuronal and non-neuronal cells. This alternative source of trophic factor generation could sustain the growth and development of a small number of precursor derived neurons in the uninjured state. Conversely, a dramatic insult. that separates the DRG from its peripheral target. completely removes the target-derived trophic support, creating an highly competitive environment for remaining trophic factors. The net result is considerable cell death of a large portion of sensory neurons. The body's natural response to such an insult could be to upregulate proliferative activity of neuronal precursors housed within the ganglia in an effort to replace those neurons lost due to injury. Our results demonstrate a critical 48 hour window post-axotomy of robust cytogenesis. perhaps to replace dying neurons. However. this response is transient. Thus. a critical 48 hour period post-axotomy may be a clinically relevant period in terms of preventing destructive changes that are known to be directly associated with injury induced death of sensory neurons.

7. DRG SATELLITE CELLS

Besides the possibility that these newly-generated cells could be stem cells, neuronal precursors or mitotically active neurons, there is also a real possibility that they could be just proliferating satellite cells. Each neuronal body housed within the DRG is completely surrounded by a satellite cell sheath that is in direct contact with the perikarion. The satellite cell sheath is in turn enveloped by connective tissue (Pannese, 1981). Satellite cells play a trophic role towards the enveloped neurons. These supportive cells deliver trophic factors such as NGF and NT-3 in a paracrine fashion to adjacent neurons (Zhou et al, 1999). Satellite cells retain their mitotic ability after birth and can be induced to proliferate in response to injury (Lu and Richardson, 1991; Pannese, 1981). The mean soma area of satellite cell nuclei in juvenile and adult animals has been reported to be 44 μm^2 and 40.3 μm^2 respectively. These values translate into mean nuclei diameters of 7.48 and 7.16 μm respectively (Cecchini et al, 1999). Satellite cells display a characteristic oval shape morphology and to the best of our knowledge do not express positive immunoreactivity for neuronal immunomarkers such as NSE. However, the small diameter of these cells corresponds quite well to the mean soma diameter of the population of cells that we previously suggested were neuronal precursors/stem cells. Furthermore, although the $BrdU^+/NSE^+$ cells we previously identified were fairly spherical in shape, it is not impossible for the satellite cells to obtain similar morphology, simply due to the cryostat sectioning procedure itself. Moreover, it is also possible that the $BrdU^+/NSE^+$ cells that we previously describe in our *in vivo* experiments, may not be truly double labeled. In fact, depending on the section of tissue examined as well the

close proximity that satellite cells have to neurons, a $BrdU^+$ nuclei from an overlying satellite cell could be overlapped with a NSE^+ cell thereby creating a false impression of what appears to be a $BrdU^+/NSE^+$ cell. Furthermore, if these are in fact satellite cells and they are NSE^+ then the reliability of NSE as a specific neuronal marker must be called into question. Future studies may benefit from the use more specific neuronal markers such as TuJ1 or Neun. The use of these markers in conjuction with BrdU labeling may provide the additional support for neurogenesis arising from pre-existing neurons. Nevertheless, the results obtained from our juvenile *in vivo* sciatic lesion experiments clearly demonstrate an injury-induced cytogenic response within the DRG. Recently, other researchers examining adult rat DRG have also identified virtually identical $BrdU^+/NSE^+$ cells to what we have described and have boldly proclaimed the discovery of proliferating neuronal precursors without addressing the real possibility that they may in fact be only proliferating satellite cells (Ciaroni et al, 2000). The possibility that these cells may be satellite cells creates uncertainty in interpretation of our *in vivo* data in terms of our ultimate question of whether neurcgenesis is carried into adulthood. Hence, we have interpreted our data more conservatively to conclude that axotomy clearly demonstrates cytogenesis, in conjunction with the possibility of neurogenesis. These results raise the question as to the involvement, if any, of satellite cells in the neurogenic response. A better understanding of the intricate relationship that exists between neurons and satellite cells may help clarify this issue.

The results obtained from juvenile animals demonstrate that axotomy induces cytogenesis. Following sciatic nerve lesion, there is a consistent ~40% relative increase in

proliferating cells ($BrdU^+$) on the lesioned side compared to the contralateral unlesioned DRG. Although BrdU is a marker for cellular proliferation, it does not indicate the type of cells involved in this proliferative response. Hence, axotomy-induced cytogenic repair may be representative of a proliferative response displayed by satellite cells, neurons, neuronal precursors or even stem cells. One possible function of this repair mechanism would be to replenish the continuous supply of trophic factors that no longer exists due to the injury-induced separation from the peripheral target. This injury-induced compensatory response appears to be a logical possibility since both neuronal and non-neuronal cells have been demonstrated to upregulate production of trophic factors in response to injury (Shen et al, 1999; Zhou et al, 1999; Lee et al, 1998). Further characterization of this cytogenic response indicates that maximal proliferation occurs at 1 week rather than 2 weeks post-axotomy. This suggest that the optimal time window for injury induced cytogenic repair appears to be limited to the first week following injury. These findings become very important when trying to explore possible ways to minimize neuronal loss following nerve injury.

8. PITFALLS OF BrdU LABELING

One potential criticism of our findings is that we used BrdU as a sole indicator for proliferating cells. In a recent review, the use of BrdU may not be the best choice for dectection of proliferating cells (Nowakowski and Hayes, 2000). Firstly, they indicate that since BrdU is incorporated into dividing cells predominantly during the S phase of the cell cycle, one may actually under estimate the number of proliferating cells that may have missed detection simply because they may have been in a different stage of the cell

cycle. Furthermore, because the kinetics for BrdU cellular incorporation are not stoichiometric, the amount of BrdU exposed to the cell membrane may be vastly different then that which actually gets incorporated into the actively dividing cell. Hence, our results would have been more convincing if we would have also used thymidine, which is at least is known to have nonlinear stoichiometry. However, even with the use of thymidine, we would still restrict our identification of proliferating cells to those only in S phase. The use of an agent, Ki-67 may have overcome this pitfall because this agent is capable of identifying proliferating cells in all stages of the cell cycle (Sasaki et al, 1997) thereby producing more reflective results.

Another potential criticism of BrdU labeling is that it has been shown to identify cells undergoing DNA repair instead of cells undergoing active proliferation (Selden et al, 1993). However, because we were able to demonstrate an increase in the number of $NSE^{+}/BrdU^{+}$ cells with increased time following injury, analagous to that described by Gould et al, 1999, plus the fact that we demonstrated the presence of mitotic figures, it would appear very unlikely for this to be soley a DNA repair response. Other possible factors that can influence BrdU labeling resulting in variablity of results are: the species of animal, age, dose of BrdU used, fixation procedure and methods of BrdU detection (Nowakowski and Hayes, 2000).

Although there are pitfalls that can be encountered when using BrdU for detecting proliferating cells, the BrdU labeling displayed in our *in vitro* model was used as additional proof of cellular proliferation. Hence we were able to directly apply our results

obtained *in vitro* for the purpose of BrdU detection *in vivo* with a higher degree of confidence in the labeling of mitotically active cells.

9. AGE AND GENDER ISSUES

Adult lesion experiments also displayed a preferential upregulation of BrdU$^+$ cells in the ipsilateral ganglia, indicative that injury-induced cytogenesis does continue into adulthood. However, there appeared to be a lower overall number of identifiable BrdU$^+$/NSE$^+$ cells in the adults when compared to the juvenile animals. Although not quantified in the adult, the cyto/neurogenesis was clearly less than in the juveniles, consistent with age-related reduction in nervous sytem plasticity referred to as the infant lesion effect (Bregman and Goldberger, 1983). Interestingly, these findings of neurogenesis/cytogenesis in the adult appear to be dependent on the sex of the animal. It has been suggested that adult neurogenesis occurs in animals in which growth continues throughout life (males) but not in those whose body size stabilizes soon after sexual maturity (Devor et al, 1991). Because these apparent sex-related differences are relevant only in adults, they were not addressed in the previously described juvenile experiments where the animals of either sex have not reached full sexual maturity.

9. CLINICAL CONSIDERATIONS

Peripheral nerve axotomy can result in the death of many neurons (Ljungberg et al. 1999; Groves et al, 1997; Oliveira et al, 1997; Himes and Tessler, 1989), which can lead to abnormalities in sensory encoding resulting in the development of neuropathic pain syndromes (Devor, 1996; Woolf and Doubell 1994; Bennett, 1994). However, the

development of chronic pain syndromes is not restricted soley to axotomy-induced death of sensory neurons. For example, clinically diagnosed diseases such as diabetes mellitus have been shown to mimic axotomy-induced death of sensory neurons (Delcroix et al. 1997). This disease appears to selectively target the destruction of sensory neurons through a sequence of events that activates apoptotic pathways such as jun NH2-terminal kinase (JNK) or stress activated protein kinase (SAPK1) (Fernyhough et al, 1999; Sasaki et al 1997). Current studies have demonstrated that diabetes mellitus decreases the retrograde axonal transport of NGF thereby selectively destroying NGF responsive sensory neurons (Delcroix et al, 1997). As a result, if neurogenesis does in fact exist and can be selectively induced, it is quite apparent the impact it could have on possible treatments of this neurodegenerative disease.

Over the past few years, there has been tremendous enthusiasm for the application of knowledge concerning neurotrophins and other growth factors to human neurological diseases (Zochodne, 1996). The use of *in vitro* experimental models provides an ideal testing ground for these substances (Sotelo et al, 1991). In fact, it is possible that specific neurotrophins and growth factors could theoretically prevent loss of diabetic dorsal root ganglion cells or enhance regeneration of diabetic nerves. Several of the neurotrophins support DRG cells in culture or prevent their loss during neonatal development and few help prevent retrograde loss of adult DRG cells after axotomy (Zochodne, 1996). As a result, Phase I clinical trials involving administration of NGF, BDNF and NT-3 are currently being conducted in humans for this purpose. Recently, it has also been suggested that sequential cocktails of neurotrophins are required to support and rescue

neurons and that the use of single agents may not be sufficient (Zochodne. 1996). However, it is still uncertain whether neurotrophins might act as a general cocktail for DRG cells to prevent diabetes related injury or whether they are actually deficient in human diabetic patients to begin with.

Sensory peripheral neuropathy is also common with human immunodefiency virus (HIV) infection. Analogous to diabetes, this disease also targets the destruction of sensory neurons, however, the cause still remains unknown (Brannagan et al, 1997). It has been suggested that the presence of glycoprotein 120 (gp120) bound to the surface of DRG neurons contributes to the development of sensory neuropathy in AIDS (Apostolski et al. 1994).

One major drawback in the use of chemotherapeutic agents has been their ability to produce drug-induced states of neuropathic pain (Tanner et al. 1998). Chemotherapy-induced neuropathic pain, by agents such as taxol and vincristine, occurs in cancer patients who receive these drugs as antineoplastic agents. The absence of knowledge concerning mechanisms of chemotherapy induced neuropathic pain has hindered the development of treatment strategies. However, the selective neurotoxic activity against DRG neurons has been suggested as a major contributing factor in development of various pain syndromes. Recent *in vitro* studies have demonstrated that the addition of trophic factors reduces the neurotoxic effects of these agents which in turn eludes to the possibility of preventing neuropathic pain syndromes (Schmidt et al, 1995).

The death of sensory neurons, regardless of the causes stated above, is a common feature shared in the development of chronic pain syndromes (Devor, 1996; Mannion et al, 1996; Koerber et al, 1994; Woolf et al, 1992; Devor, 1991). As a result, the possibility that neurogenesis has been retained into adulthood, could have significant clinical implications in terms of preventing chronic pain syndromes. Morever, the ability to expand neuronal precursors in culture and then differentiate them back into their respective neuronal subpopulations could open up intriguing possibilities for future experiments. For example, specialized testing of a variety of analgesics on a pure population of nociceptive neurons could optimize drug development for the pharmaceutical management of pain. Furthermore, *in vivo* transplantation of *in vitro* generated precursors could minimize and/or restore the damage caused by injury, disease (HIV, diabetes) or drugs (antineoplastics) as previously addressed above. Finally, the delivery of trophic factors to the proximal stump via a mini-osmotic pump, immediately after injury could also reduce the destruction of sensory neurons thereby minimizing long term negative consequences.

CONCLUSION

We conclude that postnatal DRG contain cells capable of proliferating and giving rise to neurons. Multiple factors support neurogenesis as bFGF, NGF, and Shh are implicated in precursor proliferation whilst Shh, GDNF, CNTF and the neurotrophins appear to support neuronal differentiation. However, the issue of neurogenesis being a retained feature through to adulthood requires further investigation. Although our results support the idea of axotomy-induced neurogenic repair, we can only make a definitive statement with regard to axotomy-induced cytogenesis. This conservative approach is taken because of the possibility that the proliferating $BrdU^+/NSE^+$ cells observed may be satellite cells. However, we have identified a time period of 48 hours post-axotomy where maximal cellular proliferation takes place. The identification of this critical time period of injury-induced cytogenic repair may provide the crucial insight needed to minimize axotomy-induced neuronal apoptosis in the clinical situation.

The findings of this research project may provide evidence for a greater range of plasticity available to somatosensory systems experientially and following growth, injury and repair. Further studies are required to elucidate the cellular mechanisms that activate neurogenesis in DRG and to explore the possible neurogenic repair responses of potential clinical importance.

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