ROLES OF NERVE GROWTH FACTOR IN DEVELOPING AND MATURE DORSAL ROOT GANGLION NEURONS

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ROLES OF NGF IN DEVELOPING AND MATURE DRG NEURONS

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ABSTRACT

This thesis examines the roles of nerve growth factor (NGF) in developing and mature dorsal root ganglion neurons, especially in the context of reparative nerve growth mechanisms in the adult peripheral nervous system (PNS). Peripheral nerve injury evokes two types of growths in the periphery, regeneration and/or collateral sprouting, However, these growth processes differ in a number of respects. Axonal regeneration is triggered by the injury and occurs independently of NGF whereas collateral sprouting is evoked and sustained by an increase in a target-derived signal, NGF. Indeed, cutaneous denervation is shown to result in a significant and prolonged increase in the level of NGF mRNA compared to the level in innervated skin. In addition, NGF mRNA is demonstrated to be expressed in not only the distal nerve pathways but also in non-nerve associated epidermally- and dermally-located cells. These findings indicate that an increase in NGF synthesis is associated with the increased availability of NGF in denervated skin, and that cutaneous nerves play a role in regulating NGF synthesis.

Other findings in this thesis serve to strengthen the distinction between the injury-induced nerve growth responses. mRNA expression of the two NGF receptors, $p75^{NGFR}$ and *trk*A, are shown to increase in undamaged DRG neurons whose axons are sprouting into denervated skin, a proposed response that is most-likely related to the increased availability of target-derived NGF based on the findings that i) NGF mRNA is increased in denervated skin, and ii) polyclonal anti-NGF antiserum blocks the increase in the mRNA level of at least

$p75^{NGFR}$. In contrast to these findings, there was little or no change in receptor mRNA levels in regenerating neurons, consistent with the observations that NGF does not play a role in this process.

Finally, here it is demonstrated that the NGF-driven collateral sprouting is severely impaired in adult rats that had received daily injections of anti-NGF serum during the first 2 weeks of postnatal life. However, after nerve crush the same nociceptive axons regenerated normally. Since NGF expression levels was found to be normal in innervated and denervated skin, NGF insufficiency was not responsible for the impairment in sprouting. Moreover, the possibility of a permanently defective axoplasmic transport was eliminated since sensory thresholds and axon calibers were also normal. Lastly, the finding that systemically-injected NGF promoted nociceptive sprouting, implies that the neurons had not become NGF-insensitive, nor had they reached a sprouting "ceiling". Indeed, the difference between the effects of exogenous and endogenous NGF is understandable if the latter accesses only the nerve terminals, and if the terminals' ability to take up NGF is defective. Thus, the exogenous NGF may work entirely through regions of the neuron that are outside this functional "compartment". To this end, although NGF uptake must have been adequate to account for the healthy state of the neurons, the terminals apparently failed to take up enough of the increased NGF produced in the collateral sprouting paradigm either to evoke sprouting, or to cause the usual upregulation of $p75^{NGFR}$ in the neurons of the dorsal root ganglion (DRG); there was, however, enough increased uptake to bring about the usual upregulation of trkA. This last finding suggests that neurotrophin deficiencies induced by adverse conditions during

development might permanently compromise the organism's ability to mount neurotrophin-based reparative nerve growth in response to neuronal loss or peripheral nerve injury.

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FORMAT AND ORGANIZATION OF THE THESIS

The present thesis was prepared in the "open face" format as outlined in the School of Graduate Studies Calendar; section 2, sub-section 2.7, "Theses". This thesis is comprised of three original published research papers (Chapters 2, 3 and 4) and a fourth paper (Chapter 5) that has been submitted for publication.

CONTRIBUTION TO MULTI-AUTHORED PAPERS

Chapter 2

Publication

Mearow KM, **Kril Y**, Gloster A, Diamond J (1994) Expression of NGF receptor and GAP-43 mRNA in DRG neurons during collateral sprouting and regeneration of dorsal cutaneous nerves. *Journal of Neurobiology* 25:127-142

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Contributions

Operative procedures and sample collections were performed by Y. Kril. The data contained in this paper were generated through physiological, molecular and immunohistochemical analyses that were equally completed by Y. Kril and Dr. K.M. Mearow. The data were analyzed and the paper was written by Y. Kril and Dr. K.M. Mearow. Participation of Dr. A. Gloster was limited to a preliminary experiment. The primary supervisor for this study was Dr. K.M. Mearow.

Chapter 3

Publication

Mearow KM, **Kril Y** (1995) Anti-NGF treatment blocks the upregulation of NGF receptor mRNA expression associated with collateral sprouting of rat dorsal root ganglion neurons. *Neuroscience Letters* 184:55-58.

Contributions

Operative procedures and sample collections were performed by Y. Kril. The data contained in this paper were generated through molecular analysis that was equally completed by Y. Kril and Dr. K.M. Mearow. The NGF antisera was prepared by Dr. J. Stanicz. The data were analyzed and the paper was written by Y. Kril and Dr. K.M. Mearow. The primary supervisor for this study was Dr. K.M. Mearow.

Chapter 4

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Contributions

Operative procedures and sample collections were performed by Y. Kril. The data contained in this paper were generated through molecular analyses that were equally completed by Y. Kril and Dr. K.M. Mearow. The data were analyzed and the paper was written by Y. Kril and Dr. K.M. Mearow. The primary supervisor for this study was Dr. K.M. Mearow.

Chapter 5

Publication

Kril Y, Diamond J (1997) NGF-dependent nerve growth responses are permanently compromised in adult rats deprived of NGF during early postnatal life. Submitted to *Journal of Neuroscience*.

Contributions

Operative procedures and sample collections were performed by Y. Kril. The data contained in this paper were generated through physiological, molecular and electron microscopic analyses that were completed by Y. Kril with the assistance of Ms. E. Pertens, who performed the cutting and photographing of the electron microscopic tissue sections, and Dr. J. Stanicz, who prepared the NGF antisera. The NGF used in this study was prepared by Y. Kril. The data were analyzed and the paper was written by Y. Kril. The primary supervisor for this study was Dr. J. Diamond.

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CHAPTER 5: 4th PAPER

OVERALL OBJECTIVES OF THE THESIS

The objectives of this study are twofold as follows: (1) to elucidate specific molecular correlates associated with axonal regeneration and collateral sprouting of adult primary afferent neurons, particularly in the context of their differing growth factor dependency; (2) to establish that the ability of adult cutaneous primary afferent neurons to reinnervate their target tissue following nerve injury is severely compromised by a transient neurotrophic insufficiency induced during early life, and to seek molecular correlates of this compromise.

RATIONALE FOR THE STUDY

It is well established that peripheral nerve injury, and even disorders of the axons themselves, may hinder essential physiological functions such as muscle contraction, blood pressure regulation and tissue healing processes. Thus, the difficult task of repairing these functional disabilities clearly rests on the capacity of the peripheral nervous system to re-establish functional synaptic connections with its target tissues.

In the peripheral nervous system (PNS) there are two types of nerve growth processes by which a nerve-deprived target tissue can become reinnervated: the regeneration of the injured axons, and the collateral sprouting of axons that survived the initial traumatic event. In adult rodent skin, only certain populations of intact sensory neurons undergo collateral sprouting - the nociceptive neurons giving rise to Aδ- and C-fibres (Devor et al., 1979;

Jackson and Diamond, 1984; Doucette and Diamond, 1987; Diamond et al., 1987, 1992a). In direct contrast, all classes of sensory axons (Aα/β, Aδ and C) regenerate after nerve damage (Diamond et al., 1992b).

Studies examining the reinnervation of denervated cutaneous tissue in adult rats have revealed that the collateral sprouting of nociceptive axons is under the obligatory influence of NGF (Diamond et al., 1987, 1992a) whereas the regeneration of the axons proceeds quite independently of NGF. It has been shown that collateral sprouting is completely prevented by the administration of anti-NGF serum, and rapidly resumes following termination of this treatment, and that its rate is enhanced by the administration of exogenous NGF. Remarkably, neither administration of NGF nor anti-NGF affects the regeneration of these axons after injury (Diamond et al., 1987, 1992b). In addition to the significant difference in NGF-responsiveness following nerve injury, these two nerve growth processes also differ in relation to the gross morphology of their neuritic outgrowth patterns. Collateral sprouting is the arborizing growth of intact nerve terminals within target tissue and leads to the expansion of the pre-existing innervation field into neighbouring foreign territory. In contrast, regeneration involves the regrowth of damaged axons, initially along the longitudinally-oriented degenerating nerve pathways towards the target field. However once in the target tissue, the expansion of regenerating nerve terminals appears to be indistinguishable morphologically from collateral sprouting. The distinction between the two nerve growth processes at the arborizing stage is made even more apparent with the use of anti-NGF; within the target tissue the arborizing growth of collateral sprouting is completely prevented

but there is no effect on the growth of regenerating neurites. Based on these findings **the first major aim of this thesis is to further examine the hypothesis that the mechanism governing the initiation and maintenance of neuritic extension in DRG neurons undergoing collateral sprouting is different from that involved in the regulation of axonal regeneration**. This hypothesis is tested by examining the expression of NGF receptor and a growth-associated protein, GAP-43, in: a) dorsal root ganglia (DRG) whose axons were undergoing either regeneration, following a crush injury to a selected peripheral nerve branch, or collateral sprouting of intact axons following the elimination of nerves supplying adjacent regions of skin in the adult rat, and b) in DRG neurons whose axons were prevented from undergoing collateral sprouting, through the administration of anti-NGF antiserum. These studies are detailed in Chapters 2 and 3, respectively.

It has been proposed (Diamond et al., 1987, 1992a) that collateral sprouting is evoked by the increase in NGF availability that results from the elimination of nerves that normally remove it. Moreover, it is hypothesized that the sprouting would continue until NGF levels returned to normal, the consequence of which is the restored uptake of NGF by the newly-sprouted nerve terminals. Indeed, this proposal is in line with the observation that NGF-responsive axons compete for limited amounts of NGF, and when one population is removed from a shared target tissue, NGF uptake by remaining nerves is increased (Korsching and Thoenen, 1985). However, an earlier proposal suggested that the increased level of "sprouting factors" in denervated effector tissues may also have resulted from an increase in their rate of synthesis consequent to the removal of a neural regulatory influence (Diamond

et al., 1976). However, evidence from *in vivo* studies is not clear regarding this possibility (Shelton and Reichardt, 1986; Clegg et al., 1989). Thus, **the second major aim of this thesis is to test the hypothesis that NGF production in adult rat skin is under neural control.** This hypothesis is tested by measuring the effects of denervation on the level of NGF mRNA in adult rat skin, and is detailed in Chapter 4.

Numerous studies have shown that in the developing animal, alterations in NGF levels through the administration of anti-NGF antisera leads to a profound reduction in the population of small neurons in DRG (Levi-Montalcini and Angeletti, 1966; Johnson et al., 1980; Yip et al., 1984; Ruit et al., 1992), to an irreversible alteration in the morphological phenotype of sympathetic neurons (Ruit and Snyder, 1991), reportedly, to a respecification of the electrophysiological phenotype of DRG nociceptive neurons (Ritter et al., 1991; Lewin et al., 1992; Lewin and Mendell, 1994). What remains unknown however, is whether periods of NGF deprivation will have long term effects on the function of the surviving sensory neurons in the adult PNS. A serendipitous observation made by the author on adult animals which had received anti-NGF treatment during the first postnatal week suggested the existence of an apparently continuing defective collateral sprouting of their undamaged peripheral nociceptive nerves. Given that collateral sprouting functions as a compensatory mechanism following neuronal damage, this observation led to **the final major aim of this thesis which is to test the hypothesis that a brief period of NGF deprivation during early postnatal development permanently compromises the ability of cutaneous nociceptive neurons to undergo functional collateral sprouting but has no effect on their**

regenerative capacity. This hypothesis is investigated using a well-established experimental paradigm for the study of collateral sprouting and regeneration of sensory fibres (Jackson and Diamond, 1984; Nixon et al., 1984; Doucette and Diamond, 1987; Diamond et al., 1987, 1992a,b), in adult rats whose NGF levels were experimentally reduced for a two week period during various perinatal periods or during maturity. This study is detailed in Chapter 5.

In summary, the information obtained from this thesis contributes to the understanding of cellular mechanism(s) underlying the regulation of neuronal growth processes evoked by nerve injury, and thus may facilitate the development of intervention strategies useful for the treatment of various neuropathological conditions.

CHAPTER 1

LITERATURE REVIEW

A. PREAMBLE

Investigations pertaining to the role of nerve growth factor (NGF) in the peripheral nervous system (PNS), indicate quite clearly that NGF is critically important for the normal development of subsets of sensory neurons, particularly the nociceptive primary afferent neurons. However, the physiological role of NGF is dependent on the maturational stage of the sensory neuron. In the developmental period, NGF regulates the survival of nociceptive neurons. As the neuron matures, the role of NGF changes from one of a survival factor, to one that influences and maintains the phenotypic specification of nociceptors, and one that provides a basis for plasticity in the normal uninjured, as well as the injured peripheral nervous system.

This review will detail the development of DRG neurons, the establishment of cutaneous innervation, and the mechanisms of nerve growth. In addition, this review will examine the relevance of NGF in sensory neuron development and in the regulation of functional plasticity of sensory neurons.

B. DEVELOPMENT OF DORSAL ROOT GANGLIA

B.1. CELL TYPES

The cell bodies of sensory neurons reside within dorsal root ganglia (DRG). The neurons are divided into two main morphological populations based on the staining patterns

of cytoplasmic Nissl substance (aggregates of ribosomes and rough endoplasmic reticulum) and neurofilament content (Lawson, 1992; Lawson et al., 1993). These two populations, whose sizes are normally distributed, with some overlap, are the "large light" (L) neurons and the "small dark" (SD) neurons. While L-type neurons are distributed over the entire size range of the neurons and give rise to A-fibres, SD neurons are limited to the lower end of the size distribution and give rise to C-fibres (Harper and Lawson, 1985; Lee et al., 1986; Lawson et al., 1993). In addition, DRG neurons have been further characterized on the basis of somal size, modality responsiveness, central termination patterns, cytochemistry, and more recently, according to their sensitivity to neurotrophins (Lawson, 1992; Carr and Nagy, 1993; Klein et al., 1993; Lawson et al., 1993; Crowley et al., 1994; Ernfors et al., 1994; Farinas et al., 1994; Jones et al., 1994; Klein et al., 1994; Smeyne et al., 1994); specific characteristics of sensory neurons that are relevant to the thesis subject are presented below.

B.2. ORIGIN OF NEURONS

Numerous studies have contributed to our knowledge of underlying molecular mechanisms that are involved in the derivation of the peripheral nervous system (PNS) during avian embryogenesis (LeDouarin al., 1992). DRG neurons are derived from trunk neural crest cells opposite somites 6-28. These precursor cells migrate dorsoventrally along a pathway between the neural tube and somites, their migration through the somitic mesenchyme being limited to the rostral rather than caudal regions (Teillet et al., 1987). The differential expression of permissive and/or instructive (Layer et al., 1988) and non-permissive and/or inhibitory (Stern et al., 1986) extracellular matrix molecules (ECM) in the rostral and

caudal regions, respectively, of the somitic mesoderm (Norris et al., 1989) has been proposed to function as directional guideposts during the migration of neural crest cells (Kalcheim and Teillet, 1989). It has also been suggested that these specialized regions provide a molecular mechanism by which the environment influences cellular proliferation and differentiation, and the consolidation of sensory neurons into segmentally-located ganglia (Kalcheim et al., 1987; Davies et al., 1990; LeDouarin et al., 1992; reviewed by Kalcheim, 1996) (see below).

Available evidence suggests that developing DRG remain in close apposition to the neural tube (LeDouarin et al., 1992), and that the consolidation of DRG proceeds in a rostrocaudal direction, with DRG at more rostral levels forming in advance of those at more caudal levels (Wessels et al., 1990). Lumbar sensory ganglia in rodents become evident between embryonic days 12 and 13 (E12 and E13) (Lawson et al., 1974; Kitao et al., 1996), whereas cervical ganglia are distinguishable about one day earlier (Lawson et al., 1974; Lawson and Biscoe, 1979; Lawson, 1992). Cell size histograms (Lawson, 1979), and cytochemical analyses based on neurofilament immunoreactivity (Lawson and Waddell, 1991; Kitao et al., 1996), demonstrate L-type DRG neurons are evident about one day earlier than SD neurons (E12-15 and E13-16, respectively). Although both types of DRG neurons are fully distinguishable by postnatal day 1 (P1) (Lawson, 1992), maximal distinctions between the cell types appear only after P10 (Coggeshall et al., 1994).

B.3. AXON PROFILE

In adult mammals, the primary function of sensory neurons is to transmit sensory information to the CNS regarding the location, intensity and nature of peripheral stimuli, and

to date, more than 40 types of functional primary afferents have been established by electrophysiological recording from single axons in filaments from peripheral nerves or dorsal roots (reviewed by Perl, 1992). Primary afferent fibres are divided into two main groups, comprising the myelinated and unmyelinated fibre populations. These two groups are further subdivided by conduction velocity into subsets of cutaneous axons which include A-beta (Aβ; large diameter, thickly myelinated), A-delta (Aδ; small diameter, thinly myelinated), and C (small diameter, unmyelinated) afferent fibres. In addition, cutaneous sensory units are further characterized by the most effective peripheral stimulus required to initiate impulse activity in the primary afferent fibre. The mechanically excitable Aβ primary afferent units of the skin are responsive to low intensity movement distortions of the skin surface such as brushing, touching, pressure and vibration (Lynn and Carpenter, 1982; Perl, 1992). These sensory afferents are further subclassified as either rapidly-adapting or slowly-adapting sensory units according to their responsivity to other displacement parameters (e.g., duration and velocity of skin/hair displacement). Of the Aδ fibres, three distinct types exist. The most abundant type is the high threshold mechanoreceptive afferent in both hairy and non-hairy (glabrous) skin which mediates noxious mechanical stimuli such as pinching and pricking, as well as noxious cold (Simone and Kajander, 1996). The second group in rat cutaneous nerves are the high threshold mechanoreceptors, and which require marked mechanical distortion for their activation (Lynn and Carpenter, 1982). Finally, the third group of Aδ fibres comprises the low threshold mechanoreceptor (D-hair) afferents that are responsive to slow movement and distortion of hair follicles, and adapt rapidly to maintained stimuli. To date, at least six

functionally different kinds of unmyelinated cutaneous primary afferents have been identified including three nociceptive afferents (polymodal, high threshold mechanical and noxious cold nociceptors), two kinds of thermoreceptors (warming and cooling), and one type of low threshold mechanoreceptor that are activated by gentle mechanical stimulation. More recently, another group of cutaneous Aδ and C afferents have been demonstrated to remain unresponsive to various forms of acute physical stimuli under normal physiological conditions, however, some of these afferents are activated when exposed to prolonged high threshold stimulation or in the presence of chronic inflammation (Coggeshall et al., 1983; Handwerker et al., 1991). These "silent" afferents may play a crucial role in the maintenance of pain states in chronic pathological disorders (reviewed by McMahon and Koltzenburg, 1990).

C. CUTANEOUS INNERVATION

C.1. INTRODUCTION

Rat skin is divided into hairy or non-hairy (glabrous skin) skin, both of which are richly innervated by two different populations of neurons. One group of neurons comprises the heterogenous population of DRG sensory neurons, and for the most part exhibit immunoreactivity for substance P (SP) and calcitonin gene-related peptide (CGRP), markers that are expressed in small to medium DRG neurons (Lawson, 1992; Carr and Nagy, 1993). The sensory endings of these neurons are distributed throughout the dermis and epidermis with some fibres terminating near and in the epidermis as free-nerve endings, whilst others are observed to be associated with specialized target structures or cells, and blood vessels (Rice et al., 1986; Rice and Munger, 1986; Schotzinger and Landis, 1990). The second group of

innervating neurons comprises a homogenous group of neural crest-derived postganglionic, catecholamine containing cells whose axons innervate dermally-located blood vessels and pilomotor muscles within the dermis as well as some hair follicles (Schotzinger and Landis, 1990). The major function of sensory neurons is to provide information from the periphery to the central nervous system (CNS) regarding the location, type and intensity of peripheral stimuli whereas the sympathetic neurons transmit signals from the CNS to the target tissues. Since the focus of this thesis is directed toward issues dealing with sensory afferents, discussion regarding the sympathetic component of the peripheral nervous system will be limited; however, it is important to keep in mind that various aspects of sensory neuron function and cutaneous innervation may be greatly influenced by the presence or even absence of postganglionic sympathetic fibres.

C.2. PERIPHERAL PROJECTIONS

C.2.1. Prenatal Development

Following the consolidation of DRG, peripherally-directed axons exit the ganglion via spinal nerves. Neurite outgrowth of DRG neurons proceeds in a rostrocaudal direction with the outgrowth at more rostral levels occurring in advance of those at more caudal levels (Smith, 1983; Reynolds et al., 1991; Mirnics and Koerber, 1995a). In cervical and lumbar but not thoracic regions, the sensory nerves come together to form plexuses where axons from different spinal nerves mix, and eventually sort out to project in individual peripheral nerve trunks to their target region (Reynolds et al., 1991; Mirnics and Koerber, 1995a). In the thoracic region the situation is simpler because such mixed spinal nerve mixtures do not occur

(Smith, 1983). Nonetheless, at all levels the axons of the individual DRG's project by way of the individual nerve trunks to innervate specific regions of the skin, known as dermatomes. Cutaneous nerve fibres begin to innervate axial and proximal hindlimb skin between E14-15, with axial and hindlimb dermatomes being established about half a day later (Mirnics and Koerber, 1995a). Various anatomical labelling and physiological mapping techniques have shown that while the dermatomes of adjacent DRG overlap extensively, neighbouring innervation fields of individual cutaneous nerves overlap very little (Scott, 1992). The ramifying axonal terminals that derive from single axons establish the terminal neuritic arbors; the skin area that is supplied by individual axons is known as the receptive field of that axon, and these overlap extensively depending on the specific axonal modality concerned. By E18, embryonic dermatome patterns are consistent with that seen in the adult rat (Brown and Koerber, 1978), suggesting that adult patterns of innervation are established early in development.

C.2.2. Postnatal Development

Sensory afferents undergo substantial growth, reorganization and physiological maturation in the early postnatal period. At birth, although sensory innervation of skin is relatively well-established, specialized receptors such as hair follicles and tylotrich-touch dome complexes may be totally or partially uninnervated (Nurse and Diamond, 1984; Payne et al., 1991; Casserly et al., 1994). Large axon bundles located within the deep layers of the dermis, and which are often associated with axon networks around blood vessels, send smaller bundles through the dermis to end at the subepidermal border where they begin to form an

elaborate plexus; fine fibres project from the plexus, and end as free nerve endings in the lower epidermis. Soon after birth the dermal and subepidermal plexuses become more complex, and vacated surrounding areas become innervated as resident and newly ingrowing thin myelinated axons undergo a vigorous collateral sprouting (Schotzinger and Landis, 1990; Payne et al., 1991; Fitzgerald et al., 1991; Reynolds et al., 1991). Innervation of specialized target cells by myelinated A fibres begins at about P7, and continues into the third week of life at which point an adult-like appearance is achieved (Payne et al., 1991; Casserly et al., 1994). Postnatal development of cutaneous innervation is also accompanied by changes in skin structure related to the wave of hair follicle growth and the progressive thinning of the dermis and epidermis. By P21, all tylotrich-touch dome complexes have received their nerve supply but only 80% of guard hairs, and less than 10% of vellus hair follicles are innervated (Rice et al., 1986; Rice and Munger, 1986). Using selective axonal markers, Reynolds and Fitzgerald (1995) demonstrated that large myelinated fibres remain within axon bundles in the dermis, and in the subepidermal plexus from birth. However, while the thinly myelinated Aδ- and unmyelinated C-fibres, which comprise primarily the nociceptive afferent population (Kruger et al., 1981; Kruger et al., 1985), also primarily remain within bundles in the dermis and around the perivascular network, some of these axons penetrate into the epidermis.

Myelination of small- and large-diameter A axons begins shortly after birth (Friede and Samorajski, 1968), an event that coincides with the maturation of soma membrane properties of A-fibres (reviewed by Koerber and Mendell, 1992); however afferent receptor properties (thresholds and firing patterns) of A-fibres are not fully established until approximately P14

(Fitzgerald, 1987). In contrast, although receptor properties of C-fibre afferents are fully mature at birth (Fitzgerald, 1987), their ability to cause neurogenic plasma extravasation (Kenins, 1981) cannot be evoked until the second postnatal week (Fitzgerald and Gibson, 1984). This delay in functional maturation is attributed to the slow development of neuronal transmitters and receptors (Fitzgerald and Gibson, 1984; Mulderry, 1994) as well as to the delayed establishment of proper neural connectivity in the CNS (Fitzgerald, 1988; Pignatelli et al., 1989; Williams et al., 1990). Thus, when taken together, these findings indicate that the postnatal period is a critical time in the physiological development of cutaneous primary afferent neurons.

D. MECHANISMS OF NERVE GROWTH

D.1. NEURITE GROWTH

Various studies have investigated mechanisms that are involved in axonal growth and guidance during development, and in the regenerative and compensatory (collateral sprouting) nerve growth processes evoked by peripheral nerve injury. It is now widely accepted that regulation of axon growth is mediated by two seemingly different molecular mechanisms involving the recognition of diffusible neurotrophic factors (discussed below) or extracellular matrix (ECM) molecules, by specific receptors on axonal terminals (Dodd and Jessell, 1988; Tannahill et al., 1995; Tuttle and Matthew, 1995; reviewed by Goodman, 1996). For example, in the developing organism, extracellular matrix constituents such as laminin and fibronectin, promote sensory neurite growth (Ackers et al., 1981; Rogers et al., 1983; Lander et al., 1985; Calof et al., 1994) through the binding of integrin receptors (Hynes, 1987;

Reichardt and Tomaselli, 1991) on neuronal growth cones (Bentley and Caudy, 1988; Goodman, 1996). In contrast, the expression of peanut-lectin-binding glycoprotein on the posterior somite causes sensory growth cones to collapse thus restricting nerve growth to the permissive anterior (rostral) half of each somite (Rickman et al., 1985; Davies et al., 1990). It is unlikely, however, that the ECM constituents by themselves are sufficient to guide axon growth since ECM components are expressed in both permissive and non-permissive environments (Rogers et al., 1983). Therefore, it is more plausible that the specificity in axon guidance results from the relative amounts of instructive and inhibitory molecules that are present in the environment and/or the number of functional receptors (e.g., integrin receptors for ECM molecules) that are expressed on subpopulations of neurons.

Taken together, these findings suggest that the growth cone is capable of integrating both permissive and inhibitory environmental cues, and therefore, regulation of axon growth could be achieved by regulating growth cone competency. At the molecular level, however, the findings that nerve growth coincides with the expression of various "growth-associated" proteins in the corresponding cell bodies of neurons, has led to the hypothesis that nerve growth is regulated, at least in part, by the cellular expression of specific proteins essential for continued axon elongation and terminal arborization within target tissue (Skene, 1989). An example involving regulation of axon growth at the level of the growth cone and the cell body is best demonstrated by studies using rat pheochromocytoma (PC12) cells following their exposure to the neurotrophic substance NGF (Green and Tischler, 1976). Signalling by NGF is mediated by high-affinity transmembrane receptors with intrinsic tyrosine kinase

activity (reviewed by Segal and Greenberg, 1996), and is addressed below. An initial brief exposure of these cells to NGF, a diffusible trophic factor, leads to the initiation of transcriptionally-mediated neurite extension, an event that requires the activation of signalling programs involving immediate early gene and delayed response gene expression (reviewed by Greene, 1984). However, subsequent exposure to NGF, a couple of days later, promotes in a transcriptionally-independent manner, neurite extension at the level of the growth cone. Interestingly, NGF induces the expression of the neural cell recognition molecules, L1/Ng-CAM and N-CAM on PC12 cells, substance that mediate cell body adhesion, and neurite outgrowth (reviewed by Reichardt, 1992). This finding indicates that interactions between neurotrophins and recognition molecules may play an important role in supporting neurite outgrowth either during development or following nerve injury. Supportive evidence for the involvement of extrinsic factors in the regulation of nerve growth during reparative nerve processes is addressed below.

D.2. COLLATERAL SPROUTING

D.2.1. Introduction

Collateral sprouting refers to the process of multiple branching of an uninjured axon to form a neuritic arbor, and serves at least two primary functions: 1) to establish nerve innervation fields in the developmental period, and to maintain them during changes in target field size and shape during bodily growth (Purves et al., 1988), and 2) to provide a basis for plasticity in the intact and injured nervous system (reviewed by Diamond et al., 1992c). In the event that collateral sprouting is increased for any reason (see below), three possible

morphological outcomes exist. Firstly, the terminal innervation *density* of individual axonal fields can increase but the fields remain confined to the normal boundaries of the "parent" dermatome. Secondly, sprouting could cause the *size* of the peripheral nerve field to expand. Finally, the third outcome comprises both an *increased innervation density and an expansion of the normal innervation territory* of the parent nerve. Experimentally, collateral sprouting is examined by partial denervation of target tissue which induces neurite outgrowth from remaining uninjured fibres into the neighbouring regions. Since the earliest observation of sensory fibre collateral sprouting in frog tadpole skin (Speidel, 1941), this phenomenon has been extensively examined in the sensory systems (Diamond et al., 1992c). Collateral sprouting of sensory axons has been investigated using behavioural (Devor et al., 1979; Nixon et al., 1984; Doucette and Diamond, 1987; Diamond et al., 1987, 1992a), as well as histological and molecular (Nixon et al., 1984; Kinnman and Aldskogius, 1986; Hill et al., 1988; Reynolds and Fitzgerald, 1992; Mearow et al., 1994; Mearow and Kril, 1995; Reynolds and Fitzgerald, 1995) techniques.

D2.2. During Development

Collateral sprouting during the developmental period is evoked using experimental paradigms based on peripheral nerve section (Kinnman and Aldskogius, 1986; Fitzgerald, 1987), selective axonal denervation through the administration of specific neurotoxins (Aberdeen et al., 1991; Holzer, 1991), or by tissue damage (Reynolds and Fitzgerald, 1995). These studies have demonstrated that sensory nerve terminals sprout extensively within the dermis and epidermis, and interestingly, the resulting hyperinnervation is evident several

months following the neonatal treatment (Reynolds and Fitzgerald, 1995). The significant collateral sprouting of large myelinated A afferents that is evoked by partial denervation (Jackson and Diamond, 1984; Kinnman and Aldskogius, 1986) or skin wounding (Reynolds and Fitzgerald, 1995) in the *postnatal period* contrasts with that observed in adult animals (see below), which is restricted to primarily the small-diameter myelinated and unmyelinated fibres (Devor et al., 1979; Horsch, 1981; Jackson and Diamond, 1984). While the exact reason accounting for the enhanced collateral sprouting of both A- and C-fibres following neonatal denervations is not known, it is most easily explained by the fact that the sensory neurons remain in an "active growth mode" in the postnatal period (see above), and therefore, may be more responsive to changes in extrinsic factors *in vivo* (the regulation of collateral sprouting is discussed below). Furthermore, although it is not known why only small diameter sensory axons retain the capacity to undergo collateral sprouting in adulthood, one possibility may involve alterations in mechanisms regulating neuronal responsivity to extrinsic factors (e.g., the type or level of receptor expressed by the neuron). Nonetheless, when taken together, these findings indicate that the final pattern of terminal arbors is determined during a critical period in postnatal life, and that extrinsic factors play an important role in modifying peripheral innervation density *in vivo*.

D.2.3. In Adult Skin

The collateral sprouting of sensory fibres in adult mammals was first demonstrated by Weddell and colleagues (1941) who showed that the innervation of skin in adult rabbits is re-established by neuritic outgrowth of uninjured axons supplying the neighbouring territory.

Since then, the phenomenon of collateral sprouting has been extensively examined in adult rats, studies which have revealed the existence of significant differences in the ability of different axon modalities to undergo collateral sprouting (Diamond et al., 1992c). For example, following partial denervation of skin in the adult rat, although the large myelinated fibres mediating light touch (Horsch, 1981; Jackson and Diamond, 1984; Diamond et al., 1987, 1992a) fail to undergo collateral sprouting, the intact nociceptive Aδ- and C-fibres innervating the same target tissues sprout robustly (Nixon et al., 1984; Doucette and Diamond, 1987; Kinnmann and Aldskogius, 1986, 1988). The collateral sprouting of cutaneous thermal and mechano-nociceptive sprouting begins about 10-12 days following partial denervation of dorsal skin in adult rats, as demonstrated by behavioural analysis (Diamond et al., 1992c). Interestingly, impulse activity evoked either by application of noxious mechanical or thermal stimuli to the skin at the time of surgical denervation, or by electrical stimulation of the nerve itself, advances the onset of the collateral sprouting of Aδ or C-fibres, respectively, by about 5-6 days, a phenomenon designated as "precocious sprouting" (Nixon et al., 1984; Doucette and Diamond, 1987; Diamond et al., 1992a). These studies also showed that the reduced latency associated with the precocious sprouting paradigm was negated if the impulse activity to the cell body was blocked by TTX, a result indicating that the acceleration of collateral sprouting involves cellular responses.

D.3. REGENERATION

D.3.1 Introduction

Axonal regeneration refers to a process that is evoked by nerve injury (crush or

transection), and involves a number of sequential steps involving both the cell body and axon, e.g., changes in gene expression; formation of axonal sprouts from the proximal stump, elongation, directional guidance, arborization and synapse formation within target tissue (reviewed by Ide, 1996). Spontaneous axonal regeneration of both myelinated fibre types (Aβ and Aδ) and unmyelinated axons occurs extensively in the PNS (Diamond et al., 1992b). Peripheral nerve injury results in characteristic morphological and metabolic alterations in the cell body, as well as changes in the proximal and distal branches of the injured neuron (reviewed by Lieberman, 1971; Sunderland, 1978; Aldskogius et al., 1992). In addition, nerve injury to the peripheral branch of a primary afferent neuron can also result in profound changes in chemical, electrical and structural properties of the primary afferent terminals in the dorsal horn, a process that is referred to as transganglionic degeneration (Janscó, 1992; Aldskogus et al., 1992; Wilson and Kitchener, 1996).

Injury to the peripheral nerve results in the degeneration, and eventual disappearance of the distal portion of the axon, a process known as Wallerian degeneration (reviewed by Fawcett and Keynes, 1990). As a consequence of nerve injury, the blood-nerve barrier breaks down (Bush et al., 1993), and macrophages infiltrate the nerve (Ide, 1996). During the early phase of Wallerian degeneration, Schwann cells within the proximal and distal stumps undergo a transient state of proliferation, an event most likely triggered by various cytokines, including interleukin-I, that are released by the macrophages (Ide, 1996). The Schwann cells begin to degrade the myelin sheaths, and subsequently transfer this debris to the macrophages. As Wallerian degeneration progresses, Schwann cells cease to proliferate; however, they

begin to extend cellular processes, the result of which is the formation of a cellular column, commonly referred to as the band of Büngner. Multiple regenerating axons emerge from the nodes of Ranvier located on the proximal stump near the lesion, traverse the bands of Büngner into the distal nerve segment, and continue to grow towards their target organs.

It is well-established that the type of injury sustained by the peripheral nerve is a critical factor in determining the success or failure of axonal regeneration. In the intact peripheral nerve, axons reside within endoneurial tubes formed by the basal lamina of Schwann cells. Crushing the nerve leaves the endoneurial tube intact, thus the regenerating axons can grow directly into their original sheaths towards their original targets. However, transection of a peripheral nerve disrupts the continuity of the endoneurial tube, and the regenerating axons may enter their original nerve sheaths or alternatively, they may enter a foreign sheath which would direct them to a foreign target. The accuracy and success of axonal regrowth into native distal components is a critical prerequisite for functional recovery (reviewed by Johnson and Munson, 1992).

D.3.2. Cellular Response to Peripheral Nerve Injury

Peripheral nerve injury results in morphological and metabolic changes in the cell body that appear to alter the role of the cell body from one of maintenance to one which leads to adaptive responses that most-likely limit the consequences of the injury and promote neuron survival and axonal regeneration (Lieberman, 1971; Aldskogius et al., 1992; Hökfelt et al., 1994). The major morphological features of the cell body response to axotomy is the breaking up of Nissl bodies, and the disintegration of rough endoplasmic reticulum, as well

as the movement of the nucleus to an eccentric position within the cell body. In parallel with these morphological changes are profound alterations in neuronal metabolism.

Shortly after axotomy the levels of gene expression and protein synthesis, of substances associated with both neural function and the promotion of axonal growth, are dramatically altered. For example, nerve injury causes a downregulation in the synthesis of mRNA and protein for the neurotransmitter-related substances, substance P (Jessell et al., 1979; Henken et al., 1990; Villar et al., 1989), and CGRP (Noguchi et al., 1989, 1990; Doumoulin et al., 1991) in DRG neurons. In direct contrast, there is an upregulation in galanin (Hökfelt et al., 1987; Villar et al., 1989) and VIP (Noguchi et al., 1989; Villar et al., 1989) peptides and mRNA in DRG ganglia, proteins that are believed to be involved in the reparative process. A reduction in the synthesis and transport of the neurofilament triplet proteins (cytoskeletal components) also occurs following nerve injury, and is believed to be the reason for the reduced diameter of axons in the proximal stump (reviewed by Cleveland, 1996). At the same time, axonal injury resulting in the regrowth of axons is accompanied by elevations in mRNA level of other several structural proteins such as Tα1-α-tubulin and βII- and βIII-tubulins (Hoffman and Cleveland, 1988; Miller et al., 1989; Wong and Oblinger, 1990; Jiang et al., 1994; Jones and Oblinger, 1994), while mRNA levels encoding other structural proteins, including Tα26-α tubulin and β-IV tubulin remain unaltered (Hoffman and Cleveland, 1988; Miller et al., 1989). In addition to these changes, significantly increased levels of GAP-43 mRNA and protein have also been demonstrated in regenerating axons (Skene, 1989; Van der Zee et al., 1989; Tetzlaff et al., 1991; Schreyer and Skene, 1991).

However, while GAP-43 expression is not an absolute requirement for neurite growth *in vivo* (Strittmatter et al., 1995), it does influence neurite adhesion, and persistent growth cone spreading and branching, at least *in vitro* (Yanker et al., 1990; Aigner and Caroni, 1993, 1995; Strittmatter et al., 1994), thereby suggesting that the presence of GAP-43 in growth cones and nerve terminals may potentiate their responses to local growth signals. In the rat, approximately 40-50% of adult rat DRG neurons express appreciable levels of both GAP-43 protein and mRNA, with expression being most pronounced in the smaller neurons (Verge et al., 1990; Schreyer and Skene, 1991). Findings such as these have led to the suggestion that GAP-43 is associated with neuronal plasticity, perhaps being involved in structural or synaptic re-modelling (Skene, 1989).

The mechanism(s) by which these perikaryal responses to axon injury are initiated are still not well understood. However, the disruption in axonal contact between the neuron and its effector target, and therefore the subsequent loss of a target-derived signals, such as NGF, has been strongly suggested to be responsible for the decrease in the functional, morphological, and neurochemical differentiated state of axotomized neurons (reviewed by Verge et al., 1996). However, since NGF administration does not completely mitigate all of these axotomy-induced alterations, the mechanisms involved in the regulation of these cellular responses most likely involves a process consisting of complex interactions with trophic factors and local environmental influences (Aldskogius et al., 1992; Ide, 1996).

E. NERVE GROWTH FACTOR

E.1. INTRODUCTION

Although Ramón y Cajal (1919) was the first to propose a mechanism that would promote and maintain appropriate neural connections, such a regulatory mechanism was revealed only after the discovery of NGF (reviewed by Levi-Montalcini, 1987). Indeed, the discovery of NGF subsequently led to the genesis of the "Neurotrophic Hypothesis", a concept underlying most experimental analyses of the developmental role of neurotrophins (Thoenen and Barde, 1980; Purves, 1988; Davies, 1996a). This hypothesis states that neurotrophins located in the target tissue of a neuron are bound by the neuronal membrane, internalized and then retrogradely transported along the axon to the cell body where they affect cellular functions. The fundamental principle of this theory is that neurotrophins in the periphery are synthesized in low amounts, and that their limited availability regulates both the number of surviving neurons and the innervation density. Since then, NGF has been shown to have three distinct physiological roles: 1) to regulate the survival of developing neural crest-derived peripheral, and subsets of central, neurons; 2) to establish and maintain neuronal phenotype, and 3) to influence innervation density by the regulation of collateral sprouting of nerve fibres during development, and following nerve or tissue injury.

E.2. THE NEUROTROPHIN FAMILY

NGF is the first in the family of neuron growth- and survival- promoting factors that has come to include the highly related brain-derived neurotrophic factor (Barde et al., 1982; Lindsay et al., 1985; Hofer and Barde, 1988), neurotrophin-3 (Maisonpierre et al., 1990b;

Hohn et al., 1990a,b), neurotrophin-4/5 (Hallböök et al., 1991; Berkemeier et al., 1991), and neurotrophin-6 (Gotz et al., 1994). These neurotrophins play important roles in the development of central and peripheral neurons during development. For example, cellular responses triggered by neurotrophins include proliferation, differentiation and survival of neuroblasts (reviewed by Kalcheim, 1996), as well as adaptive changes (reviewed by Diamond et al., 1992c; Verge et al., 1996), and quite possibly, the survival responses of mature neurons following their injury (reviewed by Acheson and Lindsay, 1996).

E.3. NGF FORMS

Although the "nerve growth-promoting protein" was initially isolated from mouse salivary glands, subsequent studies showed the existence of two forms of salivary gland-derived NGF with different molecular weights (reviewed by Maness et al., 1994). The higher molecular weight form, which was termed the 7S NGF complex, is composed of three differentially charged subunits, beta, alpha and gamma, whereby the overall composition consists of two alpha and two gamma subunits and a single beta (β) subunit. The β subunit of NGF (β-NGF), which is the sole unit responsible for the bioactivity associated with NGF, is synthesized as a precursor protein and is enzymatically processed to its final form. Physiologically mature β-NGF is composed of two identical, noncovalently bound 118 amino acid monomers. Also isolated from mouse submaxillary glands is the secondary form of the β-subunit, termed 2.5S NGF, and with the exception of N- and C-terminal modifications, exhibits substantial sequence homology with β-NGF. It is suggested that 2.5S NGF is generated by the proteolysis of the dissociated β subunit from the 7S complex.

E.4. NGF SYNTHESIS DURING DEVELOPMENT AND IN THE ADULT

The study of the initiation of NGF synthesis in skin has been accomplished through descriptive studies of the development of the trigeminal ganglion and its innervation of the periphery in the embryonic period. NGF synthesis begins just prior to the arrival of the first sensory fibres to the skin (Davies et al., 1987). The onset of NGF synthesis is not evoked by the innervating fibres since normal NGF mRNA expression patterns is observed in developing heart or skin tissue when innervation is either reduced or completely eliminated (Rohrer et al., 1988). The early stage of innervation correlates with increases in NGF mRNA and protein in the presumptive dermis and epidermis of the mouse whisker pad (Harper and Davies, 1990). However, as innervation progresses NGF protein and mRNA levels begin to decrease (Davies et al., 1987); presumably, the decrease in NGF levels results from its transport by the innervating fibres to their somata (Davies et al., 1987). Furthermore, although the decrease in the concentration of NGF mRNA suggests the possibility that its synthesis is down-regulated by the maturing innervation, the finding that NGF mRNA levels decrease even in the absence of peripheral innervation (Rohrer et al., 1988) argues against the existence of a feedback mechanism between the innervation and target in the regulation of NGF synthesis. To date, the mechanism involved in NGF synthesis during development is not known, however, soluble factors produced by target tissues may be involved (Shörnig et al., 1993). Nevertheless, a second, and relatively prolonged increase in NGF protein occurs during the latter stage (about P14-30) of postnatal development (Constantinou et al., 1994). While the significance of the postnatal rise in NGF remains unknown, the authors have

suggested that it differentiates the period where sensory neurons switch their dependence on NGF from survival and growth to phenotypic maintenance. An alternative function for the increased amounts of NGF, or at least a portion of it, might be that the NGF is utilized to support ongoing adjustments of axonal growth (e.g., elongation, and branching and arborization within target tissues) that are required to maintain a normal level of innervation density while the target size increases during bodily growth.

In adult skin, NGF mRNA and protein continues to be produced, albeit at very low levels, by a variety of cells including epidermally-derived keratinocytes (Tron et al., 1989; English et al., 1994), and dermally-derived fibroblasts (Acheson et al., 1991). The correlation between the levels of endogenous NGF and its mRNA in a number of target tissues and the density of sympathetic (Korshing and Thoenen, 1983; Heumann et al., 1984; Shelton and Reichardt, 1984) or sensory (Harper and Davies, 1990) innervation suggests a direct relationship between NGF synthesis and the utilization of NGF by the innervating nerves. Until the studies described later in this thesis (Mearow et al., 1993), there was still no conclusive evidence that the synthesis of NGF is influenced by innervating fibres. For example, chemical sympathectomy leads to an increased amount of NGF in various tissues (Ebendal et al., 1980; Korshing and Thoenen, 1985) in the absence of significant changes in NGF mRNA (Shelton and Reichardt, 1986). This finding suggests that the increased levels of accumulated NGF do not result from changes in NGF synthesis but rather from interruptions in the retrograde transport mechanism that normally removes NGF from the target tissue (Korsching and Thoenen, 1985). However, since most of the target areas

examined in these studies are innervated by both sympathetic and sensory fibres, NGF synthesis may very well be regulated by the sensory fibres remaining within the target tissue following removal of sympathetic fibres. The possibility that NGF production in skin is under neural control is examined in this thesis, and is discussed in Chapter 4.

Taken together, these findings provide strong evidence that the spatial and temporal patterns of NGF expression in peripheral target tissues play a crucial role in the establishment of innervation and neuronal phenotype, and in the maintenance of mature neurons *in vivo* (see below).

E.5. RETROGRADE TRANSPORT

The fact that nerve terminals, the sites of neurotrophin/receptor interaction *in vivo*, are often located many centimeters away from the cell body, implies that endogenous NGF-evoked biological responses must be accomplished through retrograde axonal transport (cf. Campenot, 1994). Indeed, exogenous ^{125}I-NGF accumulates in cell bodies of sensory neurons after its uptake by endocytosis at peripheral axon terminals (Stockel et al., 1975a,b; Thoenen and Barde, 1980; Richardson and Riopelle, 1984; Hendry, 1992). Although retrograde transport of endogenous NGF has been difficult to examine (reviewed by Rush et al., 1995), its transport has been demonstrated by the accumulation of NGF distal to a peripheral nerve ligature (Palmatier et al., 1984; Abrahamson et al., 1986, 1987a,b). However, it must be noted that a portion of the NGF may have resulted from the production of NGF by Schwann cells at the site of the ligature (Abrahamson, 1987b; Bandtlow et al., 1987; Lindholm et al., 1987).

Although endogenous NGF is retrogradely transported to the cell body (Korsching and Thoenen, 1983; Heumann et al., 1984), it is unlikely that NGF by itself mediates biological responses, since introduction of NGF directly into the cytoplasm, which circumvents its uptake by receptors, does not mimic the receptor-mediated effect (Heumann et al., 1981; Rohrer et al., 1982). This result suggests the possibility that NGF by itself is not the retrograde signal, and therefore NGF signalling must involve a receptor-generated messenger system that is retrogradely transported (Hendry, 1992; Campenot, 1994). The current concept is that it is retrogradely transported following the internalization of NGF by receptor-mediated endocytosis, to the cell body where it exerts its effects (Palmatier et al., 1984; Johnson et al., 1987; Raivich and Kreutzberg, 1987; Raivich et al., 1991). Furthermore, since exogenous NGF, when injected into hindpaw skin of adult rats, leads to an increase in the retrograde transport of tyrosine phosphorylated TrkA, it is quite possible that endogenous NGF binding at the nerve terminal results in the transport of an "active" NGF-receptor complex (Ehlers et al., 1995). Therefore, the rapid effects of NGF (see below) such as the initiation of neurite sprouting might be mediated at the nerve terminals by NGF-induced activation of TrkA signalling, while the long-term effects requiring alterations in protein synthesis would require that the activated receptor-NGF complex be transported retrogradely to the cell body. An alternative possibility is that a second messenger (other than the NGF-receptor complex) may be generated at the terminal, and then transported retrogradely to the cell body (Hendry, 1992). In the latter case, the transport of the NGF-receptor complex may be merely a mechanism by which NGF is removed from the terminal

and relocated to the cell body for degradation.

E.6. NGF RECEPTORS

E.6.1. Binding Characteristics

As is found with many protein growth factors, NGF transmits its neurotrophic action by binding to specific receptors on responsive cells. The first detailed characterization of NGF receptors focused on the binding characteristics of ^{125}I-NGF on chick embryo sensory ganglion cells (Sutter et al., 1979). Radioligand binding studies revealed the existence of two distinct NGF binding sites that differed in their affinity for NGF, a major type with a low-affinity and a less abundant form with a high-affinity (reviewed by Barker and Murphy, 1992). While the rates of NGF association are fast for both of these sites, the rates of NGF dissociation from these binding sites is markedly different, being fast for the low affinity site, and slow for the high affinity site. In addition, dose-response analysis of NGF-dependent survival and differentiation in chick sensory neurons suggested that only the higher affinity, slow binding site mediates biological activity (Sutter et al., 1979). Subsequent investigations have demonstrated that NGF binds to two different receptors, the high-affinity TrkA and the low-affinity p75^{NGFR} receptor, on NGF-responsive neurons (Chao, 1994; Meakin and Shooter, 1992; Barbacid, 1994; Snider, 1994).

E.6.2. Types of Neurotrophin Receptors

The low affinity NGF receptor (p75^{NGFR}) is a glycosylated protein consisting of an extracellular ligand-binding domain, a single transmembrane region and a cytoplasmic domain that is highly conserved among species (Johnson et al., 1986; Radeke et al., 1987; Chao,

1994). $p75^{NGFR}$ been demonstrated to bind all neurotrophins with equal affinity; however the kinetics of this binding are somewhat different between the neurotrophins (Rodriguez-Tébar et al., 1990, 1992; Squinto et al., 1991; Hallböök et al., 1991). The four extracellularly-located cysteine-rich domains of $p75^{NGFR}$ appear to play a crucial role in the formation of the NGF binding pocket since disruption of these domains, especially the third and fourth domains, completely abolishes NGF binding in PC12 cells (Barker and Murphy, 1992). In addition to intact $p75^{NGFR}$, this receptor also exists in a truncated and soluble form that is detected *in vivo* in urine and plasma (Barker and Murphy, 1992). While this form is expressed at relatively high levels in neonatal rats, truncated $p75^{NGFR}$ levels are reduced in the intact adult rat, but increase substantially after sciatic nerve lesion. Based on these observations and the fact that truncated $p75^{NGFR}$ also binds NGF, BDNF and NT-3, several physiological roles for this receptor have been proposed, including roles that would have both positive and negative outcomes on TrkA signalling (see below).

TrkA was the first high affinity neurotrophin receptor to be identified of the Trk protein family which includes TrkB and TrkC (Barbacid, 1994). These proteins have intrinsic tyrosine kinase activity, and have been shown to be products of *trk*-related proto-oncogene (Kaplan et al., 1991 a,b; Klein et al., 1991). Trk proteins consist of an extracellular region responsible for ligand binding, a single transmembrane domain and a cytoplasmic region containing a tyrosine kinase catalytic domain. Individual Trk receptor subtypes bind preferentially to different neurotrophins. TrkA binds to, and is activated by NGF, NT-3 and NT-4, however both the binding and biological response to the two latter trophic factors are

significantly reduced relative to NGF (Cordon-Cardo et al., 1991; Berkemeier et al., 1991; Ip et al., 1992). The primary function of the Trk family of tyrosine kinases is to transmit the effects of the neurotrophic factors within cells, the signals of which lead to either transient and/or long-term changes in gene expression that alters overall cellular responses (reviewed by Chao, 1992)(see below).

E.6.3. NGF receptor expression *in vivo*

$p75^{NGFR}$ mRNA is expressed by sensory neurons during the differentiation phase (Ernfors et al., 1988; Heurer et al., 1990). Although $p75^{NGFR}$ is expressed on developing axonal processes, albeit at very low levels, its expression is dramatically increased 5-10 fold in the trigeminal ganglion, soon after the axons reach their targets (Wyatt et al., 1990). Despite the tight temporal correlation between the appearance of $p75^{NGFR}$ mRNA and target innervation, its initial expression and subsequent increase does not appear to be under the influence of retrogradely transported NGF since $p75^{NGFR}$ mRNA expression in trigeminal (sensory) ganglia occurs normally in mice homozygous for a targeted disruption in the NGF gene (Davies et al., 1995); identification of the regulatory factor/mechanism has not yet been identified. While levels of $p75^{NGFR}$ mRNA begin to decrease by P7, adult levels are not reached until the third postnatal month (Ehrhard and Otten, 1994). $p75^{NGFR}$ mRNA is expressed in approximately 50% of developing rat DRG neurons whose sizes range across the entire spectrum (Carroll et al., 1992; Schecterson and Bothwell, 1992), an expression pattern that appears to be maintained into adulthood (Ernfors et al., 1993; Mu et al., 1993; Wetmore and Olson, 1995).

Developmental expression of *trk*A is observed by E15 in DRG rat neurons (Ernfors et al., 1993; Mu et al., 1993), and like $p75^{NGFR}$ mRNA expression, its initial expression and subsequent increase do not appear to be under the influence of target-derived NGF (see above) (Davies et al., 1995). Although absolute cell counts of TrkA-expressing DRG neurons during development in the rat have not been performed, correlations with soma size have been described, with these receptors being expressed in primarily small-diameter DRG neurons (reviewed by Philips and Armanini, 1996). As with $p75^{NGFR}$ mRNA, *trk*A levels begin to decrease at about P7 but unlike $p75^{NGFR}$ expression, *trk*A expression reaches adult-like appearance at a much earlier timepoint, that being by P15 (Ehrhard and Otten, 1994).

The distribution of NGF receptors on adult rat DRG has been extensively examined, and has demonstrated that Trk expression varies amongst the subpopulations of neurons according to size, peripheral projections and expression of neurochemical markers (Philips and Armanini, 1996). *trk*A mRNA and protein is expressed by 40-50% of lumbar and thoracic DRG neurons in adult rats, and is localized in small- to medium-sized neurons (Ruit et al., 1992; Verge et al., 1992; Mu et al., 1993; McMahon et al., 1994; Averill et al., 1995; Molliver et al., 1995). Retrograde- and double-labelling studies demonstrate that 50% of lumbar sensory neurons retrogradely labelled from skin express *trk*A, while 40% do not express any known *trk*s or peptides (McMahon et al., 1994; Bennett et al., 1996). Of the large DRG neurons, 20% express *trk*A (Averill et al., 1995; Wright and Snider, 1995), and about 20% coexpress *trk*C and *trk*A (Wright and Snider, 1995). Moreover, *trk*A is extensively colocalized with $p75^{NGFR}$ positive neurons in adult DRG neurons (Kashiba et al.,

1995; Wright and Snider, 1995).

Treatment with exogenous NGF leads to an upregulation in the expression of NGF binding sites (Bernd and Greene, 1984; Verge et al., 1989), as well as an increase in $p75^{NGFR}$ mRNA expression in embryonic, postnatal and adult sensory neurons (Lindsay et al., 1990; Verge et al., 1992; Wyatt and Davies, 1993; Gutman et al., 1994). In addition, while systemic NGF treatment leads to an increase in *trk*A mRNA levels in adult (K.M. Mearow, personal communication) it does not influence *trk*A gene expression in DRG neurons when administered during the first two weeks of life (Gutman et al., 1994). These findings suggest the existence of a positive feedback mechanism that may be important in the survival and differentiation of NGF-responsive neurons during different developmental periods, as well as in the adult PNS.

E.7. NGF SIGNALLING

Typical neuronal responsiveness to NGF is correlated with the presence of high affinity receptors for NGF (Greene et al., 1986; Weskamp and Reichardt, 1991). Furthermore, biological activity appears to be governed by the expression of the two membrane-bound receptors $p75^{NGFR}$ and TrkA, described in the previous sections. NGF binding to TrkA results in receptor dimerization and the stimulation of TrkA tyrosine phosphorylation, as well as the phosphorylation and activation of other proteins, including the enzymes phospholipase C-γ1, phosphatidyl-inositol 3 kinase, and the adapter protein, Shc (Kaplan and Stephens, 1994; Segal and Greenberg, 1996). These proteins eventually induce the activation of a number of signalling molecules such as ras, several serine/threonine kinases

including Raf-1, B-Raf, the Erks (Map kinases), which leads to characteristic biological effects (e.g., neuron survival and neurite outgrowth) of NGF through the activation of immediate early and delayed response genes (c.f., Campenot, 1994; Kaplan and Stephens, 1994; Segal and Greenberg, 1996).

However, despite substantial evidence suggesting that the presence of TrkA is required to generate high affinity binding sites and mediate cellular responses to NGF, the role of $p75^{NGFR}$ in neurotrophin high affinity binding and neuronal responsiveness, and in NGF signalling, remains a subject of controversy. Some binding studies suggest that coexpression of $p75^{NGFR}$ and Trk proteins is required to produce both high and low affinity binding sites (Chao, 1994; Chao and Hempstead, 1995). Survival assays show enhanced responses to neurotrophins in fibroblasts and MAH cells (a sympathoadrenal progenitor cell) expressing both $p75^{NGFR}$ and Trk proteins (Hantzopoulos et al., 1994; Verdi et al., 1994), and $p75^{NGFR}$ interaction with TrkA increases the rate of association of NGF to TrkA (Mahedeo et al., 1994), thus increasing TrkA affinity for NGF (Benedetti et al., 1993). Furthermore, $p75^{NGFR}$ expression was necessary for NGF responsiveness, as assayed by tyrosine phosphorylation and c-fos transcription (Berg et al., 1991). Assessment of these findings led to a model proposing that a receptor complex comprising both $p75^{NGFR}$ and TrkA is responsible for the biological effects of NGF (Chao and Hempstead, 1995). However, other proposals suggest that $p75^{NGFR}$ acts as a transferring mechanism leading to an increase in the association rate of NGF for TrkA (Jiang et al., 1992), or that $p75^{NGFR}$ modifies the specificity of TrkA for NGF (Benedetti et al., 1993; Barker and Shooter, 1994; Hantzopoulos et al., 1994). For example,

inhibition of NGF binding to $p75^{NGFR}$ through pharmacological manipulation, leads to a reduction in the binding and phosphorylation of NGF to TrkA, and the activation of immediate early gene responses to NGF (Barker and Shooter, 1994). Indeed, studies of embryonic and postnatal sensory neurons derived from mice with a null mutation in the $p75^{NGFR}$ gene (Lee et al., 1992) show that $p75^{NGFR}$ enhances neuronal sensitivity to NGF, and neuron survival *in vitro* (Davies et al., 1993; Lee et al., 1994). This modulatory effect of $p75^{NGFR}$ is consistent with the milder forms of sensory neuropathies in $p75^{NGFR}$ mutant mice (Lee et al., 1992a) compared to those sensory disturbances in TrkA mutant mice (Smeyne et al., 1994). In contrast to these findings are results from other studies which suggest Trk proteins are quite capable of mediating cellular responses independent of $p75^{NGFR}$. For example, antibodies that block NGF binding to $p75^{NGFR}$ do not block NGF-evoked neurite outgrowth in PC12 cells (Weskamp and Reichardt, 1991). In fibroblast cells transfected with Trk, Trk binds and is activated by NGF in the absence of $p75^{NGFR}$ (Glass and Yancopoulos, 1993). Finally, exposure of mutant NGF (able to bind Trk but not $p75^{NGFR}$) to sympathetic neurons increases their survival and differentiation (Ibáñez et al., 1992).

Regardless of receptor involvement, NGF signalling evokes two types of effects leading to rapidly and slowly generated events (Greene, 1984). Firstly, NGF interaction with its receptor can evoke rapid local responses, such as the initiation of neurite outgrowth. An example of such an NGF-induced local fast effect is seen from experiments using compartmented cultures of neonatal rat superior cervical ganglion neurons (Campenot, 1994). In order to stimulate neurite outgrowth from nerve terminals located in one compartment,

NGF must be delivered directly to those nerve terminals; exposure of NGF to the cell body or nerve terminals which are located in a second compartment does not induce sprouting of nerve terminals located in the first compartment. These effects occur within minutes, and while they do not require the synthesis of new proteins (Greene, 1984) they most likely involve alterations in local calcium ion concentrations generated by the activation of second messenger systems (Goodman, 1996; Segal and Greenberg, 1996). The slow response induced by NGF includes the prevention of natural cell death and the maintenance of nerve growth (see below), and involves retrograde signalling and synthesis of specific proteins at the transcriptional and post-transcriptional level (see below).

F. EVIDENCE FOR NGF EFFECTS IN THE RAT

F.1. NEURONAL SURVIVAL

One significant aspect in the development of the nervous system is that the initial overproduction of neurons is followed by a period of neuron loss, the purpose of which is believed to "refine" original course patterns of neural connectivity. This process of cell death is distinct from other types in that it coincides with the maturation of synapses; at this stage, typically more than 50% of the total neuronal population is lost (reviewed by Oppenheim, 1981; Cowan et al., 1984). The dependence on the periphery for the survival of neurons has been demonstrated in two ways. Firstly, natural neuron loss coincides with the arrival of fibres in the peripheral innervation field (Davies et al., 1987). Secondly, natural neuron loss is enhanced following the reduction or removal of the target field (Oppenheim, 1981). Therefore, in developing mammals the period of neuronal death appears to be under the

control of extrinsic factors rather than an intrinsic genetic program. The prevailing paradigm regarding the formation of neural connections during development is that following the final mitotic cycle, differentiated DRG neurons extend axonal processes to peripheral targets, and once in close apposition with the target, superfluous and inappropriately connected neurons degenerate and die. Moreover, it is believed that those neurons reaching maturity had successfully competed for a limited amount of a target-derived neurotrophic factor, a belief that is the basis of the neurotrophic hypothesis.

Two major approaches have been used to examine neuronal dependency on NGF for survival. First, numerous investigations have relied on the removal of endogenous NGF either by administering high doses of anti-NGF antiserum during various perinatal periods (Levi-Montalcini, 1965) or through the autoimmunization of pregnant females which leads to the transfer of maternal anti-NGF antibodies to the neonate (Johnson et al., 1986). The second, and more recent approach to examine the role of NGF in neuronal survival has been through the generation of homologous recombinant mice lacking NGF or either of the NGF receptors (Klein, 1994; Snider, 1994; Snider and Silos-Santiago, 1996). Removal of endogenous NGF *in utero* results in a 70-85% loss of sensory neurons (Johnson et al., 1980, 1986; Ruit et al., 1992; Crowley et al., 1994; Smeyne et al., 1994), with the loss being restricted to small DRG neurons most likely expressing TrkA (Carroll et al., 1992). Initial studies first reported no loss of sensory neurons following NGF removal in the early postnatal period (Johnson et al., 1980; Kessler and Black, 1980; Schwartz et al., 1982); subsequent studies indicated a 20-40% loss of primarily small-diameter sensory neurons (Yip et al., 1984; Hulsebosch et al., 1987).

These studies revealed that the loss of neurons from thoracic ganglia was proportionately greater than that from the lumbar regions, the result of which most likely derives from the fact that in thoracic ganglia a greater proportion of neurons are those from which originate NGF-sensitive primary afferents that innervate cutaneous targets (Lewin and Mendell, 1993; McMahon et al., 1994). It has been reported that anti-NGF given after P2 fails to produce any loss of DRG neurons (Lewin et al., 1992). These results suggest that the dependency of sensory neurons on NGF for their survival is greater during embryonic development, and that the NGF-dependency diminishes in the early postnatal period. In support of this conclusion, chronic NGF deprivation of adult rats, accomplished through autoimmunization, has no effect on the survival of DRG neurons (Gorin and Johnson, 1980). However, findings in the present studies (Chapter 5) show that a substantial number of DRG neurons are lost when anti-NGF is administered between P13-27.

Although the mechanism by which NGF deprivation produces neuronal death is not completely understood, *in vivo* studies have demonstrated that both naturally-occurring and axotomy-evoked cell death requires active RNA and protein synthesis during the embryonic period. In addition, *in vitro* studies have shown that the neuronal loss induced by NGF-deprivation is prevented by inhibitors of RNA and protein synthesis (Hendry, 1992). These results indicate neuronal death during development is an active process requiring ongoing metabolic activity. Thus, it would appear that NGF not only stimulates appropriate genes required for survival and growth, but it also suppresses those genes involved in promoting cell death.

F.2. COLLATERAL SPROUTING

Based on the observation that the elimination of peripheral nerves or the inhibition of axonal transport mechanisms (Aguilar et al., 1973; reviewed by Diamond, 1982) evoked a vigorous outgrowth of the neighbouring undamaged nerve terminals into the denervated regions, it was hypothesized that the sprouting stimulus was the increased amounts of NGF in the target tissues. Such increases have been indicated by results of "competition" experiments involving the removal of some of the nerves which normally take-up NGF from the target tissue (Korsching and Thoenen, 1985; Shelton and Reichardt, 1986; Diamond et al., 1992c). Moreover, it was further proposed that the collateral sprouting of cutaneous afferents would continue until the NGF levels returned to normal, as a consequence of NGF uptake by the newly sprouted terminals (Diamond et al., 1992c). Indeed, by examining the effects NGF deprivation on the extent and rate of nociceptive fibre collateral sprouting, Diamond and colleagues (1987, 1992a) provided significant evidence establishing the crucial role played by NGF in the regulation of such sprouting. The results showed that daily subcutaneous injections of anti-NGF, which reduces the availability of endogenous NGF through its sequestration (reviewed by Levi-Montalcini, 1968), reversibly inhibited the collateral sprouting of mechano- and heat-nociceptive axons into adjacent denervated skin. Importantly, morphological examination showed that this sprouting was not simply the result of "silent" receptor activation; that is, the anti-NGF treatment did not simply prevent the recovery of function in axons that had actually sprouted morphologically, but were normally "silent" until the denervation procedures led to their becoming functionally detectable.

Interestingly, in further studies, it was demonstrated that prolonged anti-NGF treatment to the intact adult rodent leads to a reversible shrinkage of cutaneous nociceptive innervation fields (most likely by terminal regression), a finding that strongly suggests that NGF is also required for the maintenance of already established peripheral innervation fields in the intact animal (Diamond et al., 1991). Since the polyclonal sheep antibody to mouse NGF used in these studies was shown to block NGF- and NT-3-evoked neurite sprouting in an *in vitro* system using mouse dorsal root ganglia, the exact neurotrophin responsible for the collateral sprouting of nociceptive axons is not fully established (Diamond et al., 1992a; Van der Zee et al., 1995). Additional evidence providing conclusive evidence regarding the crucial role of NGF in the regulation of injury-evoked collateral sprouting, is the finding that systemic NGF not only enhances the rate of nociceptive fibre sprouting into denervated skin, but it evokes *de novo* collateral sprouting of nociceptive nerves into normally-innervated skin (Diamond et al., 1992a).

Interestingly, Diamond and colleagues (1992a) have also demonstrated that interactions between NGF and impulse activity are important in initiating precocious sprouting (see above); the reduced latency to onset of sprouting reverts to normal when the conditioning stimulus (used to evoke precocious sprouting) is given under an anti-NGF "umbrella". While the mechanism underlying this effect remains unknown, membrane depolarization has been shown to enhance the expression of both immediate early and delayed response genes in embryonic rodent sensory neurons, at least *in vitro* (reviewed by Hughes and Dragunow, 1995). Therefore, a possible explanation for the reduced latency to collateral

sprouting by the conditioning stimulus is that the electrical activity augments the rate or shortens the latency of delivery of some growth-related molecules made in the cell body, to axonal transport mechanisms. Of relevance, membrane depolarization in MAH cells, an immortalized sympathoadrenal progenitor cell line, induces trkA gene expression and equally important, it also enhances biological responsiveness to NGF (Birren et al., 1992). When amalgamated, these findings indicate the existence of an important relationship between electrical activity and neurotrophic factor responsiveness in neurons that may contribute to the regulation of collateral sprouting.

While the mechanism regulating injury-evoked collateral sprouting in the developing rodent is not as well-established, increased levels of NGF following wounding of hindpaw skin of neonatal rats (Constantinou et al., 1994), is correlated to a vigorous collateral sprouting of nociceptive axons in hindpaw skin following similar wounding (Reynolds and Fitzgerald, 1995). However, in this situation the sprouting response in the neonate is not restricted to small-diameter NGF-responsive primary afferents (Smeyne et al., 1994), assumed to be nociceptors (Lewin and Mendell, 1993) but includes a robust sprouting of large-myelinated fibres that typically do not express trkA (Carroll et al., 1992). It had already been shown that Aβ axons sprout into adjacent denervated skin if this was tested during the first two weeks of postnatal life (Jackson and Diamond, 1984). These findings suggest the involvement of trophic factors other than NGF in the regulation of collateral sprouting, at least during development. Indeed, this possibility seems especially relevant given the fact that BDNF, NT-3 and NT-4 mRNA, as well as NGF mRNA and protein are highly expressed in

skin during development (Korsching and Thoenen, 1983; Goedert et al., 1986; Davies et al., 1987; Hohn et al., 1993; Maisonpierre et al., 1990a,b; Ernfors et al., 1992; Costantinou et al., 1994), and may even be upregulated after skin injury (Whitbey and Ferguson, 1991).

Nevertheless, alterations in denervation-induced peripheral innervation patterns of sensory neurons, such as those observed by Diamond and colleagues (1992c), would ultimately require a modification in biosynthetic events. Indeed, many *in vivo* and *in vitro* studies have demonstrated that increases in NGF availability initiates cellular changes that may contribute to changes in neuronal morphology. For example, while NGF overexpression in the epidermis of transgenic mice results in the hypertrophy of peripheral sensory terminals (Albers et al., 1994), administration of exogenous NGF leads to profound changes in terminal and dendritic arborization patterns of developing and mature sympathetic neurons (Thoenen and Barde, 1980; Purves, 1988; Snider, 1988; Campenot, 1994; Ruit et al., 1990; Ruit and Snider, 1991).

While the cellular mechanism(s) by which NGF regulates the collateral sprouting of nociceptive fibres is not well-understood, various investigations have revealed that NGF regulation of neuronal morphology may be governed, at least in part, by its ability to regulate the expression of various genes encoding structural and growth-related proteins. One such example is the increase in Tα1-α tubulin (a major cytoskeletal component of growing neurites) mRNA levels in sympathetic neurons of the superior cervical ganglion, whose axons were undergoing collateral sprouting within their target tissue, the pineal gland (Mathew and Miller, 1990). Presumably, the increase in Tα1-α tubulin mRNA is in response to increased

availability of endogenous NGF (following partial target denervation) since exogenous NGF has been demonstrated to lead to an increase in Tα1-α tubulin mRNA levels in these same neurons both *in vivo* (Mathew and Miller, 1990; Miller et al., 1994) and *in vitro* (Ma et al., 1992). In addition to this change, $p75^{NGFR}$ gene expression has also been shown to increase in sympathetic neurons undergoing sprouting within the pineal gland (Kuchel et al., 1992). This increase is most likely governed by increased availability of endogenous NGF since exogenous NGF, when provided to only the sympathetic nerve terminals, leads to an upregulation in $p75^{NGFR}$ mRNA levels in corresponding neurons (Miller et al., 1994). It appears therefore, that the increase in the NGF receptor and Tα1-α tubulin mRNA levels, in response to NGF, may constitute an important positive feedback mechanism in the regulation of collateral sprouting of postganglionic sympathetic fibres. As is discussed in Chapters 2, 3 and 5, a primary objective of this thesis is to investigate the possibility that an upregulation in NGF receptors may play an important role in the regulation of collateral sprouting of *nociceptive* axons.

F.3. REGENERATION

Based on the finding that NGF (Heuman et al., 1987) and $p75^{NGFR}$ (Taniuchi et al., 1986) synthesis is increased in Schwann cells during Wallerian degeneration, it has been proposed that the $p75^{NGFR}$ on Schwann cells acts to present NGF to the regenerating neurites, thus promoting their survival and continued growth (Taniuchi et al., 1988). Indirect evidence has been taken to suggest that NGF may be important for successful regeneration of sensory axons. For example, in C57/O1a mice, where NGF and $p75^{NGFR}$ expression during Wallerian

degeneration is extremely reduced due to the delayed recruitment of macrophages into the degenerating distal nerve stump, regeneration of sensory axons is impaired (Brown et al., 1991). However, injection of NGF into the degenerating sciatic nerve of the C57/O1a mice produces only a slight enhancement in axonal regeneration (Brown et al., 1991). This result suggests that the low levels of NGF are not responsible for the impaired regenerative response. Together, these findings argue against the hypothesis put forward by Taniuchi and colleagues (1988) that the production of NGF and $p75^{NGFR}$ by Schwann cells in the degenerating stump are required for successful regeneration. Importantly, although Schwann cells are not required for successful axonal regeneration following nerve lesion, the basal lamina of Schwann cells does play a critical role (Ide, 1996). Regenerating axons are quite capable of growing along nerves containing only Schwann cell basal lamina; however, regeneration fails to occur when the integrity of basal lamina is destroyed.

Numerous other studies have provided the strongest evidence for the conclustion that NGF does not contribute to the regenerative capacity of injured sensory neurons. For example, daily subcutaneous injections of anti-NGF did not alter the regeneration of cutaneous primary afferents, as assessed by the time to functional reinnervation of denervated skin in adult rats; regeneration proceeded uneventfully even though the anti-NGF regime was about 5 times that which totally prevented the collateral sprouting of nociceptive axons (Diamond et al., 1987, 1992 a,b,c). In yet another study, sensory axons regenerated quite normally following sciatic nerve lesion in guinea pigs deprived of endogenous NGF through autoimmunization (Rich et al., 1984). In addition, peripheral nerve section has been

demonstrated to lead to significant decreases in high affinity NGF binding in primarily small-diameter DRG neurons (Verge et al., 1989), as well as in the concentration of $p75^{NGFR}$ and trkA mRNA in lumbar DRG neurons (Verge et al., 1989, 1992; Krekoski et al., 1996). However, other investigations have shown either slight, although significant increases, or no change in NGF receptor mRNA levels following nerve injury (Ernfors et al., 1993; Sebert and Shooter, 1993). The axotomy-induced difference in expression levels that are observed in these studies may be derived from variations in the type of lesions (e.g., crush vs transection) and the experimental paradigms (e.g., concomitant ipsi- and contralateral nerve lesions) used in the respective studies, to examine the expression level of these molecules. Other studies have shown that the expression and retrograde transport of NGF to the somata is also substantially decreased following peripheral nerve transection, and during axonal regeneration (Raivich et al., 1991). Thus, the decrease in NGF high affinity binding and retrograde transport, in conjunction with the reduction in NGF receptor expression suggests a limited role for NGF, at least during the initial period of sensory axon regeneration (but see also Campenot, 1994). Once axons re-establish contact with their target tissue both $p75^{NGFR}$ and trkA mRNA levels increase (Krekoski et al., 1996). This finding indicates the possibility that the upregulation of NGF receptors by NGF may play a role during target reinnervation by regenerating axons. The finding that coincident administration of anti-NGF to adult rats does not alter the extent of target reinnervation by regenerating nociceptive axons (Diamond et al., 1992b) would argue against the possibility that NGF is involved in regulating the extent of terminal arborization. NGF could, however, be important for maintaining other NGF-related

functions of the neuron, such as transmitter levels (Verge et al., 1996).

Although NGF does not appear to be required for the regeneration of sensory axons following their injury, there is supportive evidence for its role in abrogating some axotomy-evoked neuronal responses. Following trauma to the peripheral nerve, exogenous NGF has been shown to reduce neuronal atrophy and cell loss (Rich et al., 1987), and to reverse or at least partially prevent, axotomy-induced alterations in neuropeptide (Fitzgerald et al., 1985), high affinity NGF binding, and $p75^{NGFR}$ and trkA mRNA (Verge et al., 1996) levels in DRG neurons. Since some of these effects of axotomy are also produced by NGF deprivation in the absence of nerve injury, the reversal of axotomy-evoked responses does not indicate an obligatory requirement for NGF by regenerating axons but rather only that NGF reverses the effects of axotomy-induced loss of target-derived NGF.

F.4. SPECIFICATION OF NEURONAL PHENOTYPE

Despite earlier neurotrophin studies emphasizing the crucial role of NGF in developmental events, the finding that a large proportion of small-diameter sensory neurons in the adult continue to express high-affinity NGF receptors and retrogradely transport NGF from the periphery to their somata, points to a physiological role for NGF that persists well beyond the developmental period i.e., beyond the period during which NGF is required for the survival of sensory neurons. Indeed, the current concept is that interactions between an innervating neuron and its effector tissue contribute, at least partially, to the maintenance of the phenotypic specification of that neuron. The important role of retrograde specification by environmental cues has been demonstrated by a series of *in vivo* transplantation studies

which demonstrate at least one population of sympathetic neurons is induced to switch its neurotransmitter phenotype through the exposure of innervating nerve fibres to a "novel" target, presumably under the influence of cholinergic differentiation factor (Schotzinger et al., 1994). Administration of anti-NGF during a critical postnatal period has been reported to lead to a respecification of the electrophysiological phenotype of a proportion of Aδ- and C-high threshold afferents to become low-threshold afferents in the sciatic nerve of adult rats (reviewed by Lewin, 1996). These findings suggest that the phenotype of these particular afferents is not pre-determined at birth, and that for a brief postnatal period the axons require feedback from the periphery in order to stabilize their phenotypical identities. In the adult rat, cross anastomized skin and muscle nerves take on characteristics appropriate for the target tissue they innervate, as assessed by the ability of the C-fibres in the cross anastomized nerves to modulate the flexion reflex response (McMahon and Wall, 1989). Based on experiments which demonstrate a greater expression of NGF in skin relative to muscle, these authors suggest that the retrograde transport of target-derived NGF is responsible for the phenotypic conversion of the re-directed sensory C-fibres. Thus, it is apparent from these findings that peripheral factors, like NGF, play a critical role in the establishment and maintenance of the differentiated phenotype of NGF-responsive neurons.

F.5. IN AGING AND DISEASE

Although cellular investigations regarding mechanisms of neurotrophin action have been fruitful, other neurotrophin-related areas are just beginning to provide insight into mechanisms responsible for, and factors contributing to neurodegenerative events in disease

and normal aging. The observation that a number of diseases in both animals and humans are often associated with abnormal expression patterns of NGF have led to the notion that altered levels of NGF synthesis may be involved in the initiation and/or maintenance of neuronal pathologies. For example, recent studies provide strong support that alterations in the production of neurotrophic factors play an important role in the pathogenesis of experimental diabetic neuropathy (Brewster et al., 1994; Ishii, 1995). While alterations consist of changes in both synthesis (Fernyhough et al., 1995; Brewster et al., 1994) and levels of NGF (Fernyhough et al., 1995; Hellweg and Hartung, 1990), they depend on tissue type, and duration of the diabetes in the animals investigated (Hellweg and Hartung, 1990; Fernyhough et al., 1995). Studies involving various nervous areas such as the basal forebrain cholinergic (Rylett and Williams, 1994), as well as the sympathetic and sensory nervous (Crutcher, 1990; Thrasivoulou and Cowen, 1995; Gavazzi et al., 1996) systems have also supported the idea that changes in the availability of neurotrophins plays an important role in the observed reduction in neuronal plasticity in ageing-related neuronal disorders (Cowen, 1993; Finch, 1993).

While the mechanisms regulating NGF synthesis remain unknown, it is clear that alterations in NGF production may be involved in the pathogenesis or may partially account for some functional deficits of disease and ageing. Thus, these findings raise the possibility of new therapeutic concepts for the treatment of neuronal dysfunctions in the peripheral nervous system.

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CHAPTER 2

1st Paper: ***Expression of NGF receptor and GAP-43 mRNA in DRG neurons during collateral sprouting and regeneration of dorsal cutaneous nerves***

2.1. ABSTRACT

The collateral sprouting of intact sensory axons and the regeneration of damaged ones differ in a number of respects. Regeneration is triggered by axotomy-induced damage, probably involves the loss of a peripheral signal and appears to occur independently of NGF, while collateral sprouting is evoked and sustained by an increase in a target-derived signal, namely NGF. New findings strengthen the distinction between these two phenomena. Nerve growth factor receptor (NGFR) mRNA is increased in undamaged DRG neurons whose axons are sprouting into denervated skin. This response is related to an increased availability of target-derived NGF, a proposal supported by a number of findings including increased NGF mRNA in the denervated target. In contrast, we observed little or no change in the NGFR mRNA levels in regenerating neurons, consistent with the observations that NGF does not play a role in this process. However, increases in neuronal GAP-43 mRNA are found during both regeneration and collateral sprouting, a result in keeping with the proposal that GAP-43 is primarily associated with nerve growth, and the observation that GAP-43 expression is not especially influenced by NGF.

2.2. INTRODUCTION

The developing nervous system is clearly influenced by feedback from its targets, both in terms of neuron survival and establishment of patterns of connectivity. Numerous experiments have demonstrated that this kind of plasticity persists in the adult nervous system. Especially striking is the evidence that demonstrates the ability of neurons to respond to denervation of their target tissues by regrowths that can lead to a functional re-innervation of the deprived tissues. The best studied examples of this are in the peripheral nervous system, where two forms of axonal growth are readily observed in the mature animal following peripheral nerve injury. Undamaged axons will often sprout collaterals into neighbouring denervated areas, while damaged axons will readily regrow to reinnervate their former target tissue, particularly if they can access the now-degenerating nerve pathways (Purves, 1988). In adult mammals only certain populations of intact sensory neurons undergo collateral sprouting - the nociceptive neurons giving rise to the Aδ and C fibres (Devor et al., 1979; Doucette and Diamond, 1987; Diamond et al., 1992a). In contrast, all classes of sensory axons will regenerate after damage (Diamond et al., 1992b).

Recent experiments have shown that collateral sprouting of nociceptive fibres *in vivo* is dependent on the presence of NGF (Diamond et al., 1987; Diamond et al., 1992a). The sprouting was blocked by antiserum to NGF (anti-NGF) and rapidly resumed when the anti-NGF treatment ceased. Anti-NGF treatment also rapidly terminated collateral sprouting that was well under way, indicating its continual requirement for endogenous NGF. Furthermore, it was shown that systemic NGF itself evoked collateral sprouting, even in normally

innervated skin (Diamond et al., 1992a). Remarkably, neither NGF nor anti-NGF affected the regeneration of these same sensory axons after damage (Diamond et al., 1987; Diamond et al., 1992b).

These two types of nerve growth also differ in relation to their growth patterns. Collateral sprouting is essentially an arborising growth of additional terminals within the target tissue which leads to expansion of the pre-existing field into foreign territory. In this form of growth the new neurites bud off from the undamaged axons. Regeneration, on the other hand, involves a regrowth of damaged axons, involving the production of a number of neurites, most of which will regress, as one or more persisting "leaders" elongate towards the target area. Within that area, however, a terminal expansion can occur which may appear essentially indistinguishable morphologically from collateral sprouting. However, in the example of sensory nerves, the distinction becomes clear with the use of anti-NGF, which rapidly halts collateral sprouting, but has no effect on the arborising growth achieved by regenerating axons (Diamond et al., 1992a, b). Thus, although it seems unlikely that different cellular processes are involved in the two types of nerve growth, it is in the mechanisms that trigger and maintain the growths that the major differences occur.

As an approach to investigating these mechanisms, we have been examining the molecular correlates that are associated with nerve regeneration and collateral sprouting, particularly in the context of their differing growth factor dependency. Our focus has been on the expression of NGF receptor and a growth associated protein (GAP-43) in dorsal root ganglia (DRG) neurons whose axons are undergoing either (1) regeneration, following a

crush injury, or (2) collateral sprouting of intact axons following the elimination of nerves supplying adjacent regions of the skin. The results indicate that both $p75^{NGFR}$ and *trk*A mRNA levels are increased in collaterally sprouting DRG neurons, and that this increase is more pronounced in small neurons; such neurons give rise to the Aδ and C fibres which are known to undergo NGF-dependent sprouting. In contrast, in the regenerating neurons there are no significant alterations in either $p75^{NGFR}$ or *trk*A mRNA levels over the time course examined. GAP-43 mRNA, on the other hand, increases in both the sprouting and regenerating DRG neurons, consistent with observations that elevated GAP-43 is associated with axonal growth.

2.3. MATERIALS AND METHODS

2.3.1. Animals and Operative Procedures to Produce Isolated Sensory Fields

The procedures are similar to those described in Diamond et al. (1992 a). Female Wistar rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg). The fur was clipped from the skin of the thoracolumbar region of the back and a 4 to 5 cm incision was made to the right of the midline. In 30 animals, the dorsal cutaneous nerves (DCNs) were exposed, and on the left side DCNs L1-L5, T11 and T12, T8 and T9 were crushed about 1 cm from the skin. The skin was sutured using continuous catgut thread. This procedure leaves the DCNs T13, T10 and T7 with intact sensory fields, surrounded by areas of denervated skin. The animal was protected against infection by an intramuscular injection of a penicillin/streptomycin-based antibiotic. Sham operated animals were used to provide control DRGs; the offset incision precludes the use of contralateral

DRGs as controls.

2.3.2. DRG Isolation

At a number of time points following the denervation procedure, the animals were anesthetized and transcardially perfused with ice-cold phosphate-buffered saline (PBS). The appropriate DRGs (the left T13 and T10 for "sprouting" DRGs; T12 and T11 for "regenerating" DRGs) were quickly removed and frozen in Tissue-tek on dry ice in preparation for sectioning. The samples were kept at -70°C until use.

2.3.3. In Situ Hybridization (ISH)

ISH was carried out on frozen (10μm) sections of DRGs according to our established procedure (Mearow et al., 1989). Briefly, frozen sections were thaw-mounted onto gelatin-coated slides and quickly dried on a slide warmer; control and experimental sections were mounted on the same slides. Slides were subsequently fixed with 4% paraformaldehyde in PBS for 5 min, washed with PBS for 5 min, acetylated in 0.1 M triethanolamine for 10 min, washed with 2X SSC for 10 min, and subsequently dehydrated in ethanol and airdried. The ^{35}S-labelled cRNA probe (see below, 0.5-1 X 10^6 dpm) was added to the hybridization buffer (4X SSC, 50% formamide, 1X Denhardt's solution, 100 μg/ml sonicated salmon sperm DNA, 250 μg/ml yeast tRNA, 5% dextran sulfate, 100 mM DTT) and 100 μl of this mix applied to the slides. Sections were covered with coverslips and incubated in a humidified chamber at 42°C for 16-20 hrs. Coverslips were removed in 4X SSC containing 1 mM b-mercaptoethanol, and then washed 2 x 15 min in 2X SSC/50% formamide, 30 min at 37°C in 20 μg/ml RNase A (in 100 mM Tris, pH 7.4, 50 mM NaCl, 10 mM EDTA), 30 min at 37°C

with the same buffer minus the RNase, and final wash in 0.5X SSC at 40°C for 30 min. Slides were briefly rinsed in distilled water and 70% ethanol and air dried.

For autoradiographic visualization, slides were initially apposed to Kodak X-OMAT film for 1-2 days, and then dipped in Kodak NTB2 emulsion, dried and placed in light-tight plastic slide boxes and exposed at 4°C for 2-3 weeks. Slides were developed with Dektol, coverslipped and analysed using an image analysis system (MCID, St Catharines, Ont.)

2.3.4. Probe Preparation

A 2 kb Hinc fragment of the rat NGF-R cDNA (Radeke et al., 1987) was subcloned into the riboprobe vector pBluescript KS (Stratagene, La Jolla). The radiolabelled antisense RNA probe was prepared by first linearizing the DNA template with Hind III, followed by transcription of the RNA with T3 RNA polymerase and ^{35}S-CTP (800 Ci/mmole; Amersham). Specific activity was in the range of 1-2 X 10^9 dpm/μg. The probe for *trk* (pDM97) (provided by Drs. J. Maragos and L. Parada) contains a 464 bp insert of the mouse *trk* cDNA encoding the extracellular domain. Antisense strand transcription was carried out using T7 RNA polymerase and Sac I linearized template. The cDNA encoding GAP-43 (pG43.1, Basi et al., 1987) was linearized with Ava II and antisense strand transcription carried out using Sp6 RNA polymerase.

2.3.5. Immunocytochemistry

Frozen sections of DRGs were brought to room temperature and fixed in 4% paraformaldehyde in PBS. Sections were then blocked with 2% horse serum, 0.1% Triton-X100 in PBS for 1-2 hours, and subsequently exposed to the primary antibodies for 5-20 h.

Primary antibodies were as follows. For $p75^{NGFR}$, the monoclonal antibody 192 (Chandler et al., 1984; Boehringer-Mannheim) was used at 10 µg/ml. For GAP-43, the monoclonal antibody 9-1E12 was provided by D. Schreyer (Schreyer and Skene, 1991) and used at a 1:20,000 dilution. Sections were washed for 30 min to 1 h with PBS, and subsequently incubated with biotinylated secondary antibody (anti-mouse IgG). For TrkA, a rabbit polyclonal antibody raised against a peptide corresponding to residues 763-777 within the carboxy terminal domain of the human $gp140^{trk}$ sequence was utilized (Santa Cruz Biotechnology, Inc.). Visualization of the antibody pattern was carried out using avidin/biotin/peroxidase histochemistry (ABC Kit, Vector Laboratories) according to the manufacturer's instructions, using 3,3'-diaminobenzidine (DAB) as the chromagen.

2.3.6. Data Analysis

Developed slides were analysed using the MCID image analysis system (Imaging Research Inc., St. Catharines, Ont.). A grain counting software option was used to count grains over 100 cells from three sections from each DRG at each time point. The slides carried at least five sections (most often eight) representative of areas throughout the DRG; control and experimental sections were processed on the same slides. Sampling for analysis was carried out on a random basis using a linked channel mode; that is, cells to be analysed were chosen in one channel displaying only a phase contrast image of the unstained ganglion, whereas the calculations of grain number and area were computed from a second channel in which the focal plane was restricted to the autoradiographic silver grains. We analyzed 100 cells (chosen from at least three sections of each ganglion) at each time point in each ISH

experiment; only neurons in which the nucleus was visible under phase contrast were analysed. Subsequent to the image analysis the data was statistically analysed, first, as a function of the total cell population sampled and secondly, following classification of the samples into 3 groups based upon neuronal size. Neurons were classified as small (<30 μm diam.), medium (30-40 μm diam.) and large (>40 μm diam.). The grain density (number of grains per μm^2 neuronal area) was calculated and statistical analysis (ANOVA, student's t test) was performed using the Minitab statistical software.

2.4. RESULTS

2.4.1. Data Analysis

In situ hybridization was carried out on sections of DRGs taken from control animals, and analysis of the hybridized sections was subsequently performed using an image analysis system (MCID, Imaging Research Inc.). For each mRNA to be analyzed, three ISH experiments were run using DRGs from three different animals at each time point. In most cases, a total of 300 neurons was sampled at each time point. The autoradiographic signal was analyzed using grain-counting software associated with the MCID system. Because cRNA probes were used (rather than oligonucleotide probes), there was , in certain cases, a relatively high background. This was compensated for by the thresholding function of the software; in these cases all sections and slides in the series were analyzed at the same threshold parameters.

The data obtained from these experiments were analyzed in two ways. First, the mean values of grain density (no. of grains/neuron area) were calculated for each experiment (n =

100 neurons/time point); these values were then expressed relative to the d0 values so that the results of the different experiments could be compared. These data are presented in Figures 1, 2, 3.

Subsequently, in an effort to learn more about the responses of the different populations of DRG neurons, we then analyzed the data as a function of neuronal size. The neuronal populations were characterized as small (<30 μm diam.), medium (30-40 μm diam.), or large (>40 μm diam.), and the grain densities were then plotted for each population. Data from representative ISH experiments are pesented in Figures 4, 5, and 6; in these plots, the data from 100 neurons/time point are presented.

The characterization of the neuronal populations analyzed in the ISH experiments is presented in Table 1. Here the mean area values, as well as the proportion of the total represented by each group are tabulated for the experimental groups used. It can be seen that small neurons accounted for 20%-30%, medium for 55%-65%, and large for 15%-25% of the cells analyzed.

2.4.2. NGFR Expression in Normal DRG Neurons

Expression of both the low ($p75^{NGFR}$) and high (*trk*A) affinity NGF receptor RNAs was initially examined in normal DRGs. We analyzed 300 cells; these neurons were characterized with respect to size as follows. Neurons with a calculated diameter of < 30 μm accounted for about 20% of the population analyzed (mean area = 645 ± 20 μm^2); those between 30 - 40 um diam made up approximately 55% (mean area = 955 ± 27 μm^2); 25% of the neurons of > 40 μm diam. (mean area = 1588 ± 60 μm^2), (see Table 1).

In the control DRGs, the small (<30 μm diam.) and medium (30-40 μm diam.) sized neurons tended to have higher grain densities (grain number/μm^2 neuronal area) for $p75^{NGFR}$ than the large (>40 μm diam) neurons analyzed (Figures 1, 4, 7). However, it should be pointed out that a proportion of the larger neurons had little or no labeling, while some had substantial labeling; the result being that the standard deviation in this population was always much higher than that in the other two populations. Any grain density represents an increased signal from the hybridized probe and thus a relatively higher level of mRNA present in the cells.

In addition to ISH, immunocytochemistry (ICC) was carried out using a monoclonal antibody to the low affinity receptor (IgG 192, Chandler et al., 1984), to examine the distribution of the receptor protein. In contrast to the ISH results, the ICC findings showed a clear distinction between the small and medium-sized neurons, and the large neurons [Figure 8(A)]. The immunochemical data indicates that approximately 50% of the neurons in the normal DRG expressed relatively high levels of $p75^{NGFR}$ protein.

With respect to *trk*A mRNA expression, labeling was more heterogenous and was found in all neuronal size groups, and not primarily expressed in the smaller neurons (Figures 2, 5, 9). The proportion of neurons in each of the size groups was similar to that indicated above. Small neurons accounted for 20% (mean area = 624 $\pm$ 15 μm^2) of the neurons analyzed; medium sized for 65% (mean area = 939 $\pm$ 35 μm^2) and larger for 15% (mean area = 1549 $\pm$ 61 μm^2) (Table 1). ICC was performed using a polyclonal antibody specific for TrkA (Santa Cruz Biotechnology), and indicated that the small and medium sized neurons

were more intensely stained than the large neurons [Figure (8B)]; approximately 60% of the neurons were TrkA immunopositive.

2.4.3. $p75^{NGFR}$ Expression in Collaterally Sprouting DRG Neurons

The collateral sprouting paradigm was established in a series of animals as described in Methods; the approach here was to "isolate" selected nerve fields within areas of denervated skin. Following this procedure, the intact sensory axons supplying the isolated field sprout into the adjacent denervated territory. Only the thin myelinated (Aδ) and the unmyelinated (C) fibres, arising from the small and medium-sized neurons in the DRG have been shown to be capable of undergoing functional collateral sprouting (Doucette and Diamond, 1987; Diamond et al., 1992a). The large Aα axons of the largest DRG neurons do not show this growth response in the adult animal, although they regenerate normally after axon crush or cut. At 1, 4, 6, 8, 10 and 12 days after the initial sensory field isolation, the animals were sacrificed and relevant DRGs were removed and frozen on dry ice for subsequent analysis. Image and statistical analysis of the hybridized sections was performed as indicated in the Material and Methods.

The ISH results (Figures 1, 4) indicate that $p75^{NGFR}$ mRNA began to increase by 4 days post isolation and the levels remained elevated for the duration of the experiment (12 days). In Figure 3, results from three different experimental series (three animals per time point, 300 cell total) are averaged and presented relative to the day 0 (d0) values for grain density.

The data were then analyzed with respect to the three neuronal populations, and the results presented in Figure 4(A). The data presented are the actual grain density values from one ISH series (n = 100 neurons at each time point). The proportions of neurons in each category were similar to the control values at each of the times points, and there was little change in the average neuronal area analyzed (Table 1). It was evident that the increase was most pronounced in the small (<30 μm diam.) neurons, although there was a significant elevation in the medium neurons by day 8. In contrast, with the exception of an increased value at day 4 in this experiment, there was little alteration in the $p75^{NGFR}$ mRNA in the large neuronal population, known to give rise to the Aα axons.

2.4.4. *trk*A Expression in Collaterally Sprouting DRG Neurons

The corresponding data for *trk*A mRNA expression is presented in Figures 2 and 5. Here, the results for the average of three ISH experiments are presented relative to the d0 values for grain density. They indicate that *trk*A mRNA was increased significantly by day 6 post isolation (Figure 2). The data were then analyzed with respect to the three cell size categories; the data for an individual ISH experiment (n = 100 neurons at each time point) are presented in Figure 5(A). The proportions of neurons in each category were similar to the control values at each of the times points, and there was little change in the average neuronal area analyzed (Table 1). Although the small neurons tend to have higher levels of *trk*A mRNA, there was not a consistent significant difference between the three populations of neurons[(Figure 5(A)].

2.4.5. $p75^{NGFR}$ Expression in Regeneration

Following crush of the DCNs, all the injured DRG neurons will regenerate to reinnervate the denervated skin. Nevertheless, and in contrast to the neurons which undergo collateral sprouting, there was little change in $p75^{NGFR}$ mRNA levels in the regenerating neurons. In Figure 1 the results of three ISH experiments (three animals/ time point, n = 300 neurons/ point) are presented relative to the d0 values, and it is clear that there is little change when the results are analyzed in this way. When the data were analyzed with respect to neuronal size [Figure 4(B)], the small neurons exhibited an initial decrease in $p75^{NGFR}$ mRNA followed by a gradual rise towards the control levels. There was no significant alteration in the mRNA in the medium sized neurons, while a small decrease in the larger neurons was observed at day 6 and 8. At no time (up to 12d, when the experiment was terminated) did $p75^{NGFR}$ mRNA increase significantly above control levels in any of the neuronal populations. Nor was there any shift in the sizes of the neurons analyzed, suggesting that there was no shrinking of cells accompanying the crush injury (Table 1).

2.4.6. *trk*A Expression in Regeneration

The results for *trk*A expression in regenerating DRG neurons are presented in Figure 2; again these are the results of three ISH experiments plotted relative to the d0 values. Similar to expression of $p75^{NGFR}$ mRNA, little significant difference from the d0 values was observed. When the data were analyzed with respect to neuronal size [Figure 5(B)], there was no change in *trk*A expression in any of the neuronal populations over the time course examined, nor was there any change in the population of neurons analyzed with respect to

proportion of neurons in each category and cell area.

2.4.7. GAP-43 in Normal DRG Neurons

GAP-43 expression was examined initially in control DRGs by both ISH and ICC, the latter carried out using a monoclonal antibody, 9-1E12, (Schreyer and Skene, 1991). In control DRGs about 40% of the neurons were stained positively with the antibody, and of these about half were intensely stained small neurons [Figure 8(C)]. Large neurons did not display any significant staining.

The ISH results (similar to the ICC results) indicate that GAP-43 mRNA expression was significantly higher in small and medium sized neurons than in the large neurons (Figure 10). The neuronal population analyzed was categorized into 3 size groups as follows. Small neurons accounted for 30% (mean area = 622 ± 35 μm^2), medium for 55% (mean area = 941 ± 22 μm^2) and large for 15% (mean area = 1520 ± 98 μm^2) of the cells analyzed (Table 1).

2.4.8. GAP-43 Expression in Collateral Sprouting

GAP-43 mRNA expression was then analyzed in the sprouting neurons. In Figure 3 the data from three experiments are presented relative to the d0 values for grain density and indicated that there was a clear increase in GAP-43 mRNA expression by day 4 post-isolation. Analysis was then carried out with respect to the three cell size categories; the data for an individual ISH experiment (n = 100 neurons at each time point) are presented in Figure 6A. It was observed that the significant elevation in GAP-43 mRNA expression occurred primarily in the small and medium sized neurons; expression of GAP-43 in the large neurons did not differ significantly over the early time points examined (Figure 6A); in this particular

experiment, a significant difference from the d0 value was observed at d10, although it was not significantly different from the d8 or d12 point. The proportions of neurons in each category were similar to the control values at each of the times points, and there was little change in the average neuronal area analyzed (Table 1).

2.4.9. GAP-43 Expression in Regeneration

In the damaged neurons able to undergo regeneration, GAP-43 mRNA was increased significantly between day 1 and 4 (Figure 3). The data were then analyzed with reference to the cell size categories and the results of one ISH experiment are presented in Figure 6(B). It was observed that the increase occurred in all neuronal populations by 4-5 days and remained elevated for the course of the experiment (12 days). Again, the proportions of neurons in each category were similar to the control values at each of the times points, and there was little change in the average neuronal area for each category (Table 1).

2.5. DISCUSSION

2.5.1. Overview

In the present study we have examined changes in mRNA for the NGF receptors ($p75^{NGFR}$ and *trk*A), as well as GAP-43, in both collaterally sprouting and regenerating neurons, as an approach to further understand the regulatory mechanisms involved in the two types of axonal growth. Although the cellular mechanisms underlying the neuritic outgrowths that occur in the collateral sprouting of undamaged axons, and the regeneration of damaged ones, are likely to be similar, the two types of growth response have some important differences (Diamond et al., 1987). The most significant of these differences relate to the

mechanisms that initiate and sustain the two growths as they appear in both sensory and sympathetic nerves; collateral sprouting is evoked in the undamaged axons by NGF, and remains as an NGF-dependent growth, while regeneration is triggered by axotomy, and occurs independently of NGF (Diamond et al., 1987, 1992a,b; Gloster and Diamond, 1992).

In sensory neurons, whose intact axons have been stimulated to undergo collateral sprouting, we have shown that there is a progressive increase in both $p75^{NGFR}$ and *trk*A mRNAs. These increases are observed at least 4 days prior to the calculated onset of normal functional collateral sprouting (Diamond et al., 1992a). In contrast, between 1 and 4 days following nerve injury by crush or section, a slight decrease in $p75^{NGFR}$ is observed in DRG neurons, but little change is seen in *trk*A expression. As regeneration proceeds the $p75^{NGFR}$ levels return to control values, and these persist for the 2 weeks that the observations were continued. GAP-43 is a growth associated protein, whose expression has been positively correlated with neurite growth (Skene, 1989); our results indicate that GAP-43 mRNA levels increased in DRG neurons both preceeding and during sprouting, and during regeneration of sensory axons (we cannot say from our data by how much the increase might have preceded the onset of the latter).

In addition, the results demonstrate that there was no significant shift in proportions of neurons falling into any of the cell size categories under any of the experimental conditions; there was no atrophy of neurons that sometimes accompanies axotomy injuries, nor was there any hypertrophy of the neurons exposed to the increased levels of target-derived NGF.

2.5.2. Alterations in NGFR Expression During Collateral Sprouting and Regeneration

The increase in $p75^{NGFR}$ and *trk*A mRNAs during collateral sprouting, but not regeneration, is supportive of the previous results demonstrating the NGF dependence of the former and independence of the latter (Diamond et al., 1992a,b). The time course of the changes in message is also consistent with these physiological findings, which indicated that functional sprouting probably begins 6-8 d after surrounding denervation, and that approximately 2 days of this period are required for endogenous NGF levels in the denervated skin to reach growth-stimulating values. We now know (Mearow et al., 1993) that 2-4 days after its denervation, NGF mRNA levels rise in the skin. If it is assumed that an increased production of NGF follows these increased mRNA levels after a lag time of 12-24 h (cf., Heumann and Thoenen, 1986), then increased NGF should certainly be available in the target by 3-4 days after denervation. It also seems likely that the increased NGF levels would lead to upregulation of the $p75^{NGFR}$ in the DRG neurons whose axon terminals are located in the affected skin. NGF has been shown to regulate expression of $p75^{NGFR}$ both *in vitro* (Lindsay et al. 1990; Miller et al., 1991) and *in vivo* (Higgins et al., 1989; Cavicchioli et al., 1989; Miller et al., 1991; Verge et al., 1992). Furthermore, a recent report indicates that upregulation of $p75^{NGFR}$ mRNA also occurs in sprouting sympathetic neurons (Kuchel et al., 1992). NGF also appears to regulate expression of *trk*A mRNA; although an early report indicated no change in *trk*A expression in PC12 cells following NGF treatment (Kaplan et al., 1991b), more recent observations demonstrate up-regulation of *trk*A mRNA in PC12 cells (Meakin et al., 1992; Holtzman et al., 1992) and in CNS neurons (Holtzman et al., 1992) by

NGF. Interestingly, the time course of the effect of NGF on these mRNAs differs significantly, with elevated expression of $p75^{NGFR}$ occuring within 24 h of exposure to NGF, while *trk*A mRNA increases at 2-3 days following NGF treatment (Meakin et al., 1992; Holtzman et al., 1992).

Thus the time course we observed in the increases in NGFR mRNA expression in sprouting DRG neurons are consistent with their being evoked by the increases of NGF in the denervated target. In support of this interpretation are our observations (Mearow et al., 1992), which indicate that an anti-NGF treatment that blocks collateral sprouting, also blocks this sprouting-associated increase in NGFR mRNA. Our conclusions, based upon a population analysis of these DRG neurons at various times after the sprouting stimulus is provided, assume that it is the smaller sized neurons which undergo sprouting, a valid assumption given the facts that the only two modalities capable of sprouting are the high threshold Aδ and C nociceptive fibres (Diamond et al., 1987; Doucette and Diamond, 1987), which arise from primarily small neurons (Lieberman, 1976); some of the Aδ fibres might however arise from some larger neurons, i.e., the medium sized ones in the present study, (Lawson et al., 1985).

Regeneration, on the other hand, did not appear to be accompanied by significant alterations in either $p75^{NGFR}$ or *trk*A mRNA levels. After nerve crush we do see an initial, but not maintained, slight decrease in $p75^{NGFR}$ mRNA, but no change at all in *trk*A message. Our results seem to be in conflict with previous studies that found a decrease in $p75^{NGFR}$ and *trk*A mRNAs, as well as a decrease in the number of high affinity binding sites, subsequent

to complete transection of the sciatic nerve (Verge et al., 1989, 1992). However, Ernfors et al. (1993) also find no change in *trk*A mRNA levels after a sciatic nerve crush, and in fact see an increase in $p75^{NGFR}$ mRNA. The differences in the results likely relate to the differential effect of a crush injury as compared to a complete transection of the nerve. In both cases, retrograde transport has been disrupted, but in the case of the crush, the axons are able to regenerate, and probably reach the skin by 13- 15 d (at the earliest). Thus they are not deprived of NGF for prolonged periods of time, as they would be in the transection model. Although it might be predicted that if NGF positively regulates NGF receptor mRNA levels, a lack of NGF should result in a down-regulation of the message, our results suggest that this is not the case, at least over the relatively short term. Analagous observations have been made by Lindsay et al. (1990) who demonstrated that while added NGF resulted in increased expression of $p75^{NGFR}$ in DRG neurons in vitro, expression of $p75^{NGFR}$ was not significantly altered compared to control when NGF was absent.

Although our findings support the hypothesis that NGF is not required for sensory nerve regeneration, they do not rule out NGF having other influences on the fibres, particularly when they arrive at the target, e.g., in relation to expression of neuronal phenotype (Ritter et al., 1991). Nor can we rule out the involvement of other trophic factors such as the IGFs (Kanje et al., 1989; Glazner et al., 1993; Vergara et al., 1993).

2.5.3. Alterations in GAP-43 mRNA During Neurite Growth

Our results demonstrate that GAP-43 mRNA expression increases in both the sprouting and regenerating neurons. GAP-43 levels are correlated with nerve growth in the

nervous system, during both development and regeneration (reviewed in Skene, 1989); the increased synthesis appears to be mediated by changes in mRNA levels (Costello et al., 1991; Benowitz and Perrone-Bizzozero, 1991). In DRG neurons after peripheral axotomy, increases in GAP-43 have been seen as early as two days after damage (Sommervaille et al., 1991). A hypothesis to account for the increase in GAP-43 expression following peripheral nerve damage was that the lack of an inhibitory influence leads to an upregulation of GAP-43 (e.g., Bisby, 1988; Skene, 1989; Shreyer and Skene, 1991). Clearly, however, this view cannot apply to the increased expression seen in the undamaged DRG neurons undergoing collateral sprouting. Moreover, it is becoming increasingly clear that persistent high levels of GAP-43 are found in the intact adult nervous system (Stewart et al., 1992; Benowitz et al., 1988). Approximately 40-50% of adult rat DRG neurons express appreciable levels of both GAP-43 protein and mRNA, with expression being most pronounced in the smaller neurons (Shreyer and Skene, 1991; Verge et al., 1990; Sommervaille et al., 1991). Findings such as these have led to the suggestion that GAP-43 is associated with neuronal plasticity, perhaps being involved in structural or synaptic remodelling (Benowitz and Perrone-Bizzozero, 1991; Masliah et al., 1991; Lin et al., 1992; see also Skene, 1989).

In sensory neurons, expression of GAP-43 mRNA has been correlated with the presence of NGF high-affinity binding sites, with most GAP-43 positive neurons bearing NGF receptors and virtually all neurons with NGF receptors having high basal GAP-43 mRNA (Verge et al., 1990). These authors suggest that these high levels of GAP-43 may be maintained by endogenous NGF. This interpretation also supports the speculation that the

relatively high basal concentration of GAP-43 in some neurons may relate to their ability to undergo collateral sprouting. Certainly, our findings suggest that NGF-sensitive collateral sprouting results in increased expression of both NGFRs and GAP-43 mRNAs.

Following injury (and during subsequent axonal regeneration) we see GAP-43 mRNA expression is increased in all DRG neurons, some of which do not possess NGFRs; these results are similar to those found by Verge et al. (1990), who also observed this increase even with continuous infusion of NGF. Using immunohistochemistry, GAP-43 protein has also been shown to be increased in both small and large DRG neurons after sciatic nerve transection (Shreyer and Skene, 1991; Sommervaille et al., 1991). However, differences between these two studies were observed. While the study of Sommervaille and colleagues (1991) demonstrated an increase in GAP-43 immunoreactivity in both the small and large neurons, the increase in GAP-43 levels in large neurons was delayed by some time (between 4 d and 2 wk). This difference may relate to the different GAP-43 antibodies used by the two groups (Shreyer and Skene, 1991). Nevertheless, our results suggest that GAP-43 mRNA increased in all neuronal populations, with little or no difference in the onset of the increase between the different neuronal groups; however the increase seen in the small DRG neurons was more pronounced at earlier time points compared to the large DRG neurons.

The fact that GAP-43 mRNA expression can be increased in two very different situations (axonal damage with loss of peripherally supplied NGF and collateral sprouting evoked by an increased supply of NGF) argues that there is certainly not a single mechanism responsible for its regulation. This view is supported by our preliminary findings that GAP-43

expression is not reduced in DRG neurons during an anti-NGF treatment that fails to prevent their axonal regeneration (though it does block their collateral sprouting).

Table 1. Characterization of the neuronal populations analyzed in ISH experiments.

Note. Neurons were classified as small (< 30 μm diam.), medium (30-40 μm diam.), or large (> 40 μm diam.). Area is in μm^2; values are presented as mean ± S.E.M. n = 200-300 neurons.

		Small		Medium		Large	
mRNA	Condition	Mean Area	% Total	Mean Area	% Total	Mean Area	% Total
$p75^{NGFR}$	Control	645 ± 20	19 ± 2	955 ± 27	54 ± 7	1588 ± 60	27 ± 5
	Sprouting	623 ± 32	20 ± 5	927 ± 33	57 ± 4	1596 ± 103	23 ± 5
	Regenerating	654 ± 25	19 ± 4	985 ± 25	56 ± 3	1755 ± 106	24 ± 4
*trk*A	Control	624 ± 15	20 ± 6	939 ± 35	65 ± 4	1549 ± 61	15 ± 6
	Sprouting	625 ± 38	20 ± 3	938 ± 26	64 ± 5	1566 ± 100	15 ± 5
	Regenerating	629 ± 28	28 ± 5	940 ± 24	56 ± 5	1497 ± 113	16 ± 5
GAP-43	Control	622 ± 35	30 ± 5	941 ± 22	55 ± 2	1520 ± 98	15 ± 3
	Sprouting	605 ± 20	29 ± 5	929 ± 24	57 ± 4	1631 ± 130	14 ± 2
	Regenerating	617 ± 30	26 ± 6	945 ± 35	54 ± 4	1682 ± 85	20 ± 4

Figure 1. Expression of $p75^{NGFR}$ mRNA in sprouting and regenerating neurons. Following ISH, tissue sections were analyzed as described in the text. The results of three experimental groups (three animals per time point, n = 300 cells) are averaged and expressed relative to the control (d0) values of grain density for sprouting (solid bars) and regenerating (hatched bars) neurons. *Significantly different than control (d0), $p < 0.05$.

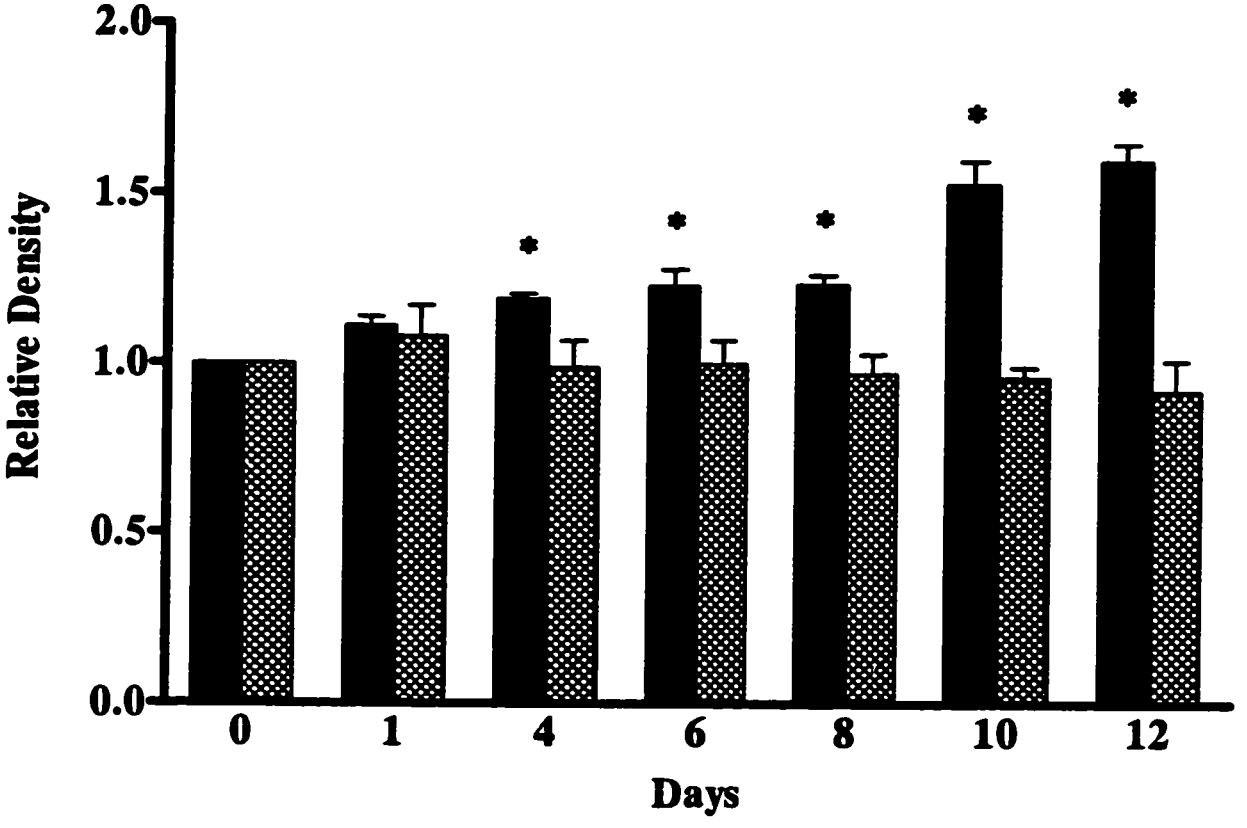
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Figure 2. Expression of *trk*A mRNA in sprouting and regenerating neurons. Following ISH, tissue sections were analyzed as described in the text. The results of three experimental groups (three animals per time point, n = 300 cells) are averaged and expressed (mean + S.E.M.) relative to the control (d0) values of grain density for sprouting (solid bars) and regenerating (hatched bars) neurons. *Significantly different from control (d0), $p< 0.05$.

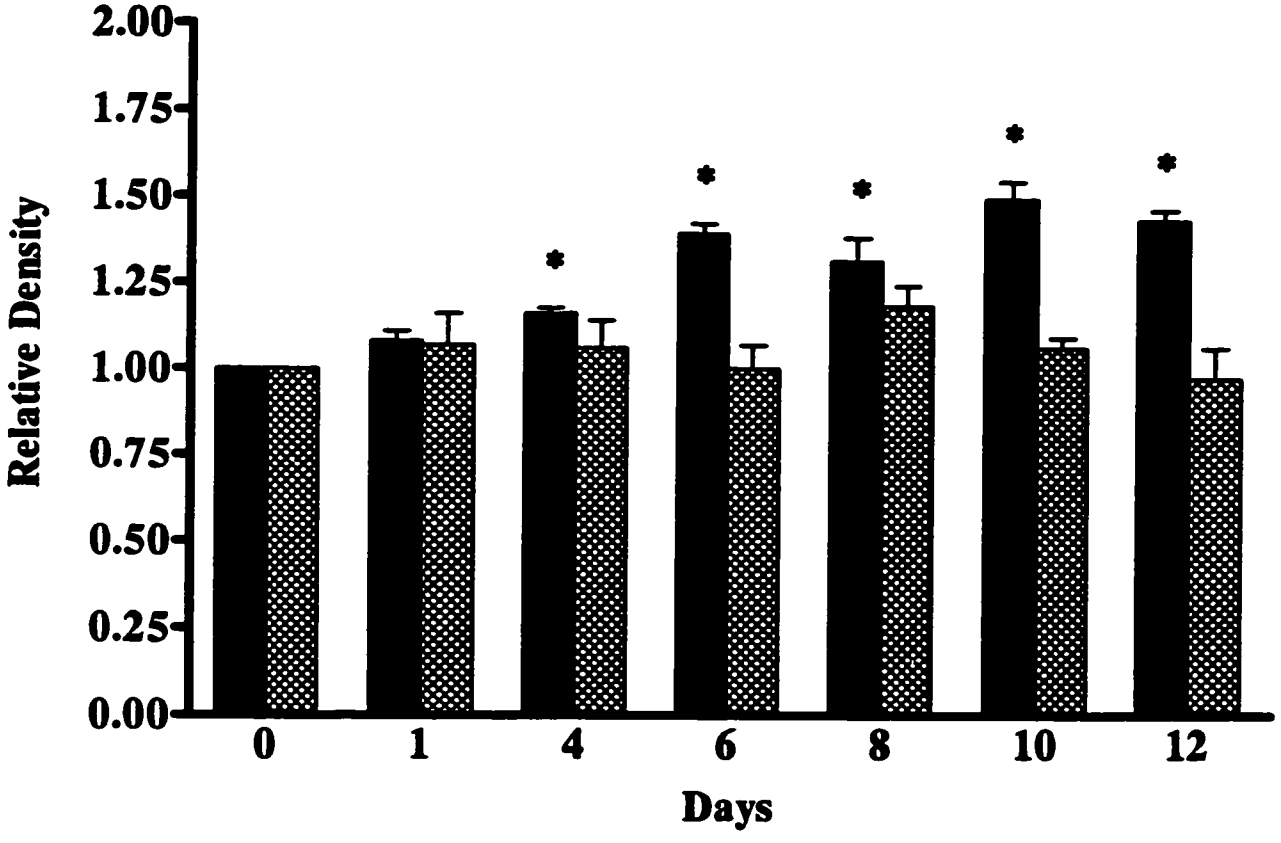
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Figure 3. Expression of GAP-43 mRNA in sprouting and regenerating neurons. Following ISH, tissue sections were analyzed as described in the text. The results of three experimental groups (three animals per time point, n = 300 cells) are averaged and expressed (mean + S.E.M.) relative to the control (d0) values of grain density for sprouting (solid bars) and regenerating (hatched bars) neurons. *Significantly different from control (d0) values, $p<0.05$.

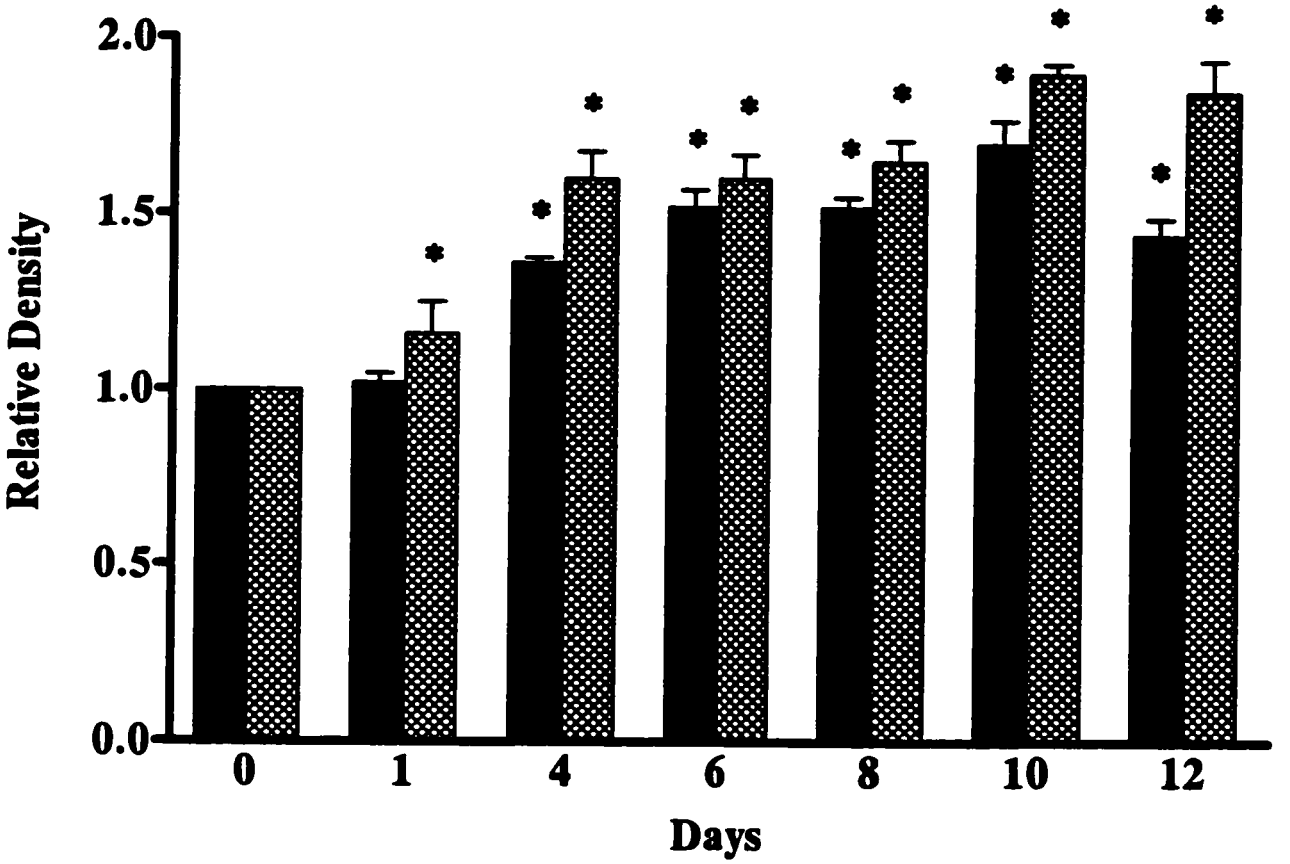
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Figure 4. Expression of $p75^{NGFR}$ mRNA related to neuronal size. The data are presented as grain density (grains per μm^2 neuronal area, mean + S.E.M., n = 100 cells) at each of the experimental time points, with d0 representing the control sections. The sampled neurons are classified as small, < 30 μm (solid bars), medium 30-40 μm (diagonal bars) or large, > 40 μm (cross hatched bars). (A) Sprouting DRGs. (B) Regenerating DRGs. *Significantly different from control (d0), $p< 0.05$.

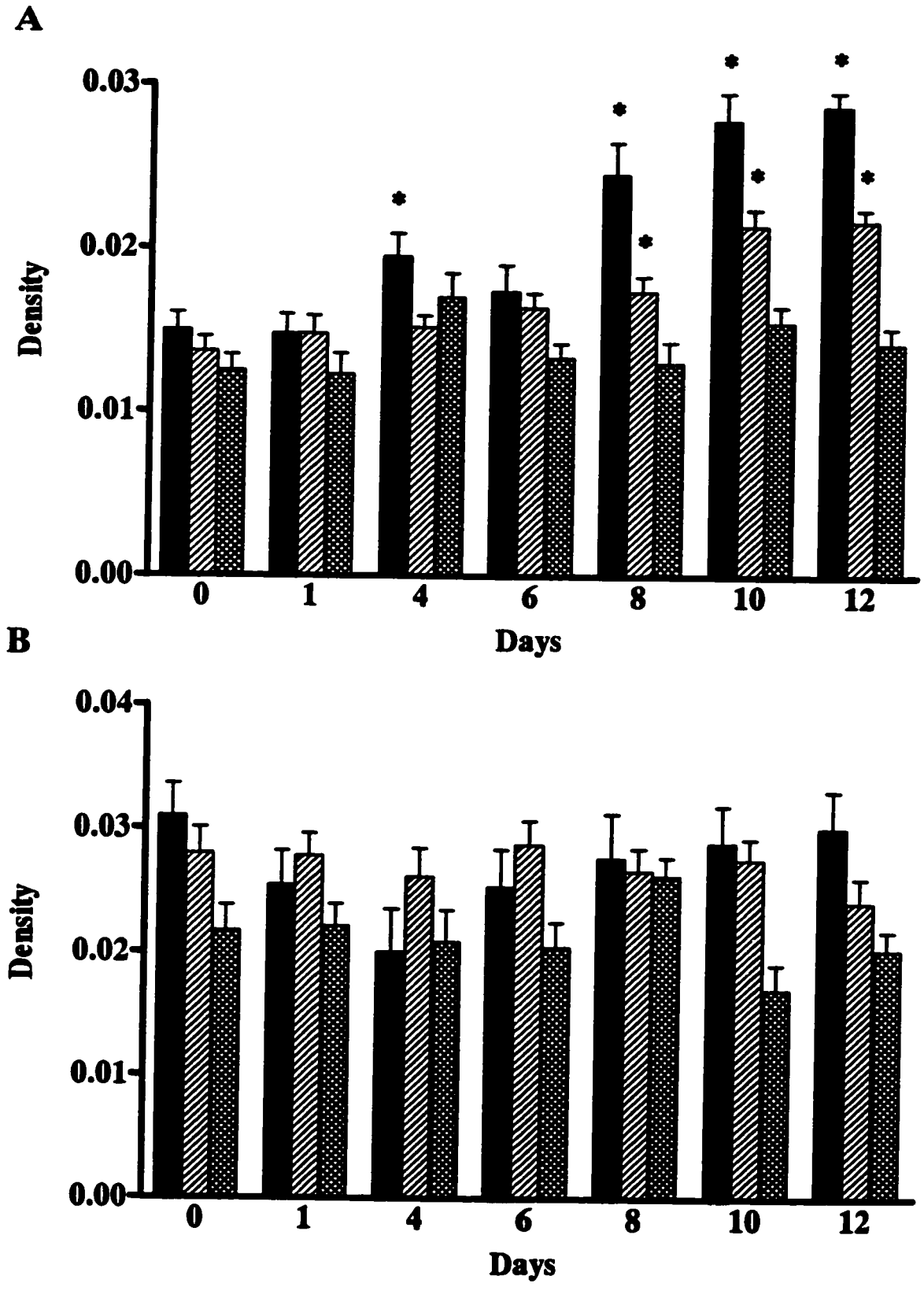
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Figure 5. Expression of *trk*A mRNA related to neuronal size. The data are presented as grain density (grains per μm^2 neuronal area, mean + S.E.M., n = 100 cells) at each of the experimental time points, with d0 representing the control sections. The sampled neurons are classified as small, < 30 μm (solid bars), medium 30-40 μm (diagonal bars) or large, > 40 μm (cross hatched bars). (A) Sprouting DRGs. (B) Regenerating DRGs. *Significantly different from control (d0), $p<0.05$.

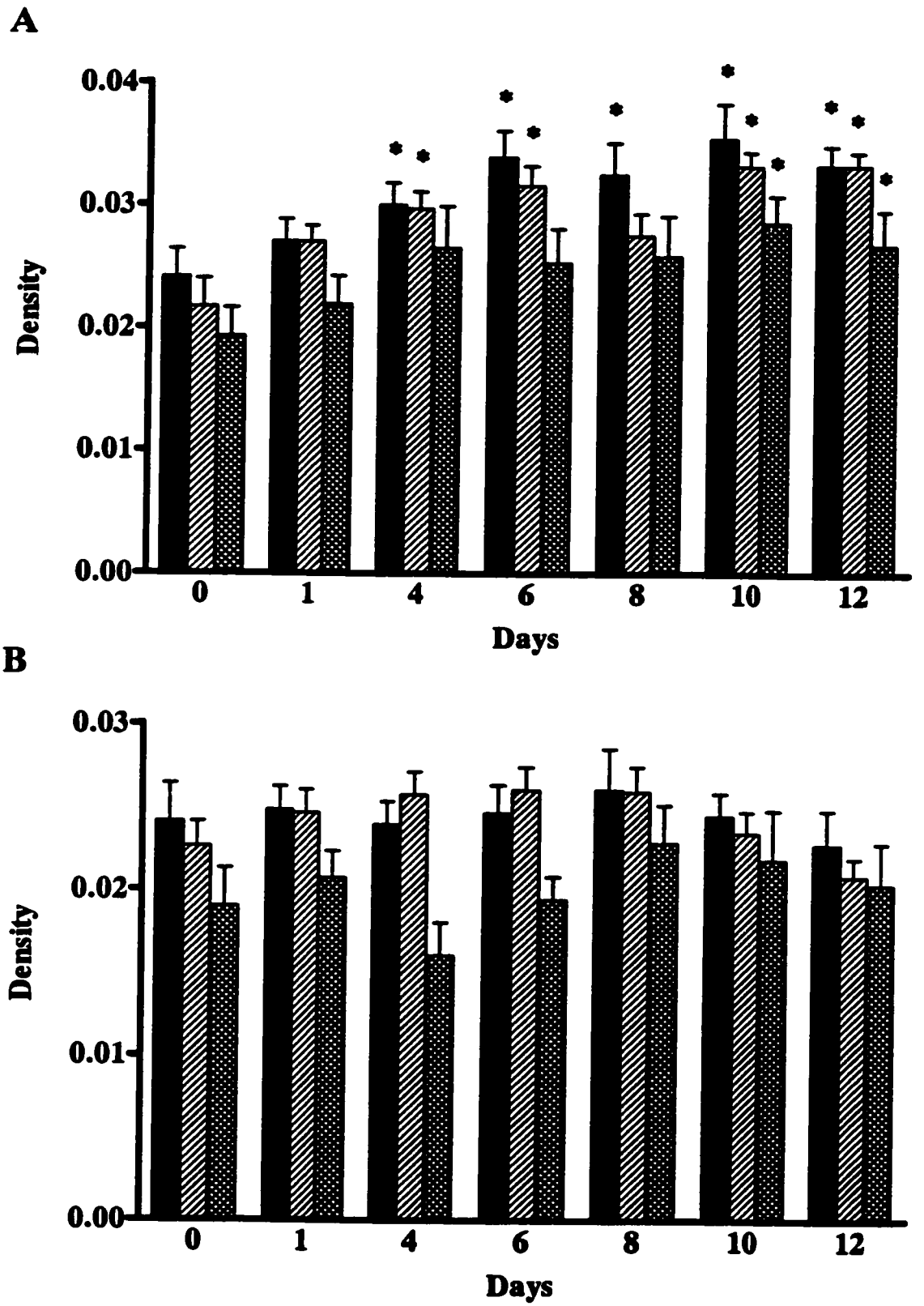
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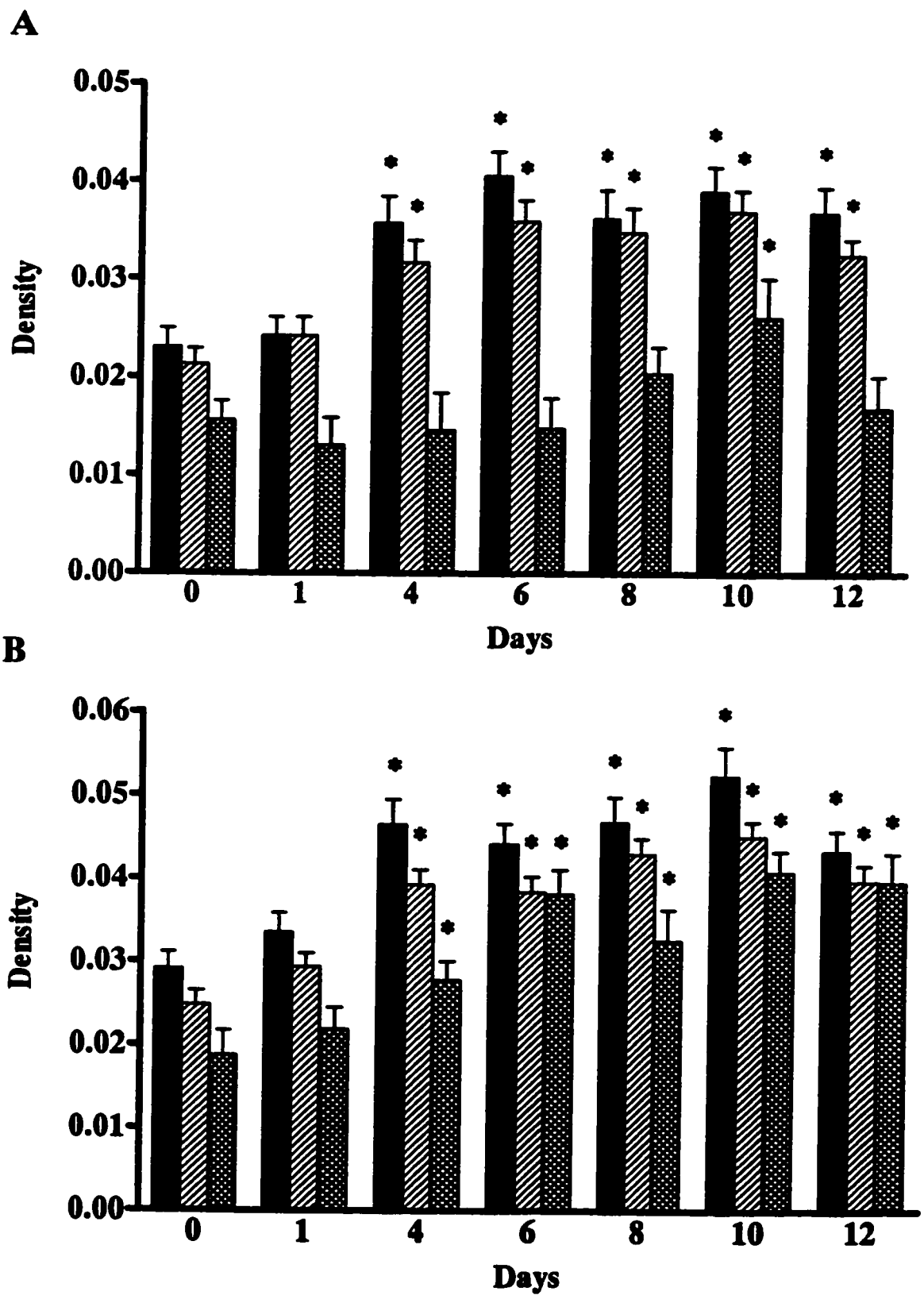
Figure 6. Expression of GAP-43 mRNA related to neuronal size. The data are presented as grain density (grains per μm^2 neuronal area, mean + S.E.M., n = 100 cells) at each of the experimental time points, with d0 representing the control sections. The sampled neurons are classified as small, < 30 μm (solid bars), medium 30-40 μm (diagonal bars) or large, > 40 μm (cross hatched bars). (A) sprouting DRGs. (B) regenerating DRGs. *Significantly different from control (d0) values, $p<0.05$.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Days

Figure 7. $p75^{NGFR}$ expression in DRG neurons. *In situ* hybridization (ISH) was carried out as described in the text. (A-C) Bright-field photomicrographs of $p75^{NGFR}$ ISH. Note labeling (silver grains) in primarily small cells (arrows), although some larger neurons display positive signal. (A) Normal DRG. (B) 4d Sprouting DRG. (C) 4d Regenerating DRG; scale bar = 25 μm. (D-G) Dark-field micrographs of $p75^{NGFR}$ ISH in sprouting (D, 1d; E, 8d) and regenerating (F, 1d; G, 8d) DRGs; scale bar = 75 μm.

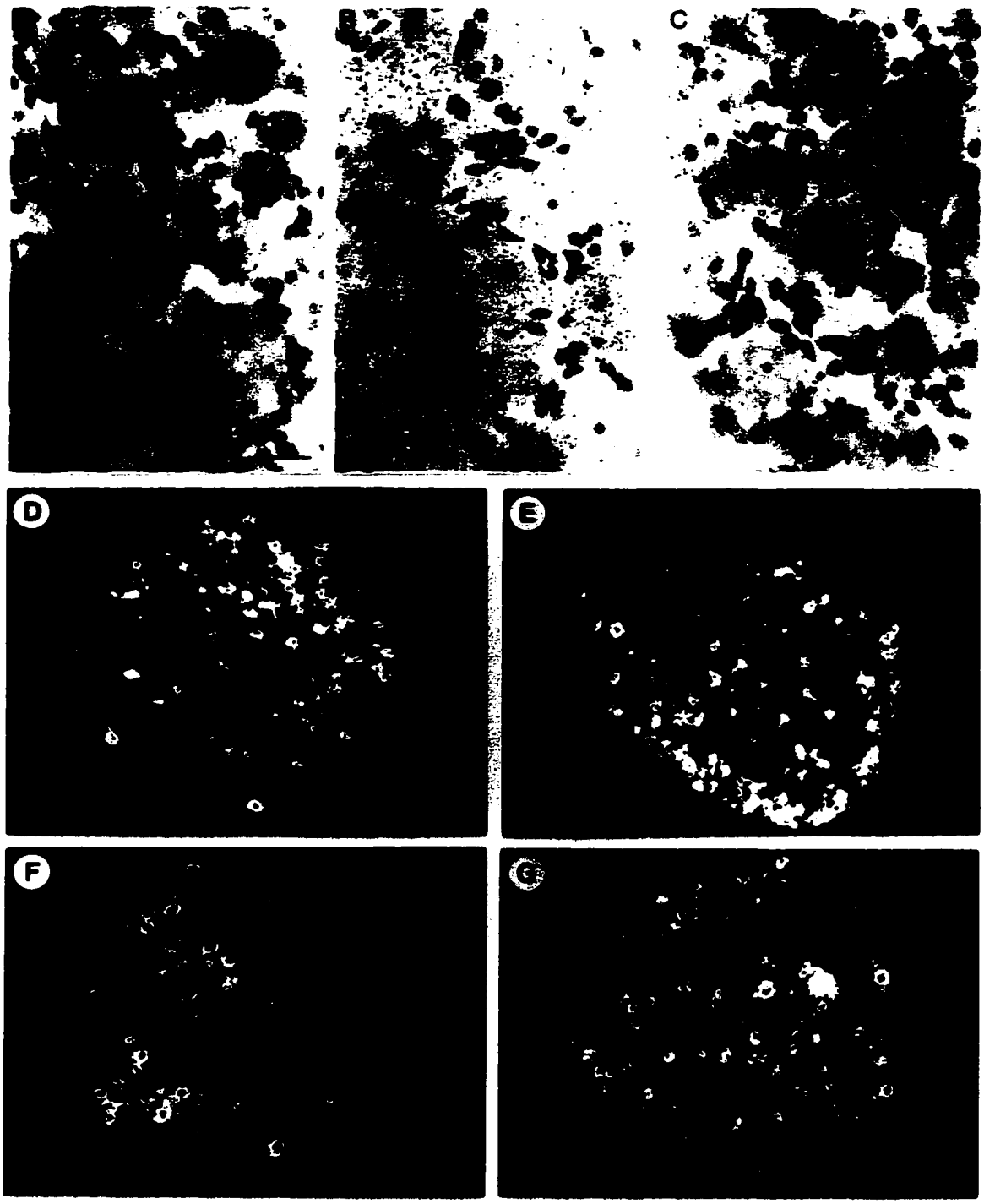
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Figure 8. Immunocytochemical labelling of DRG neurons. Immunocytochemistry (ICC) was carried out as described in the text. (A) ICC for $p75^{NGFR}$ using the monoclonal antibody 192. Primarily small neurons are heavily labeled, while large neurons are unstained. (B) Trk A immunolabeled cells; the pattern of staining is more heterogeneous than for the $p75^{NGFR}$, with smaller neurons being more heavily labeled than the larger neurons. (C) GAP43-immunolabeled cells (labeled with the monoclonal antibody 9-1E12). Again, primarily the small neurons are heavily labeled, while the larger neurons are unstained; scale bar = 100 μm.


A
B

Figure 9. *Trk*A mRNA expression in DRG neurons. *In situ* hybridization was carried out as described in the text. (A-C), Bright-field photomicrographs of *trk*A ISH. Silver grains are found over both small and larger neurons (arrows), although there are some large neurons that are unlabelled (asteriks). (A) Normal DRG. (B) 4d Sprouting DRG. (C) 8d Regenerating DRG; scale bar = 25 μm. (D-G), Dark-field micrographs of *trkA* ISH in sprouting (D, 1d; E, 12d) and regenerating (F, 1d; G, 8d) DRG neurons; scale bar = 75 μm.

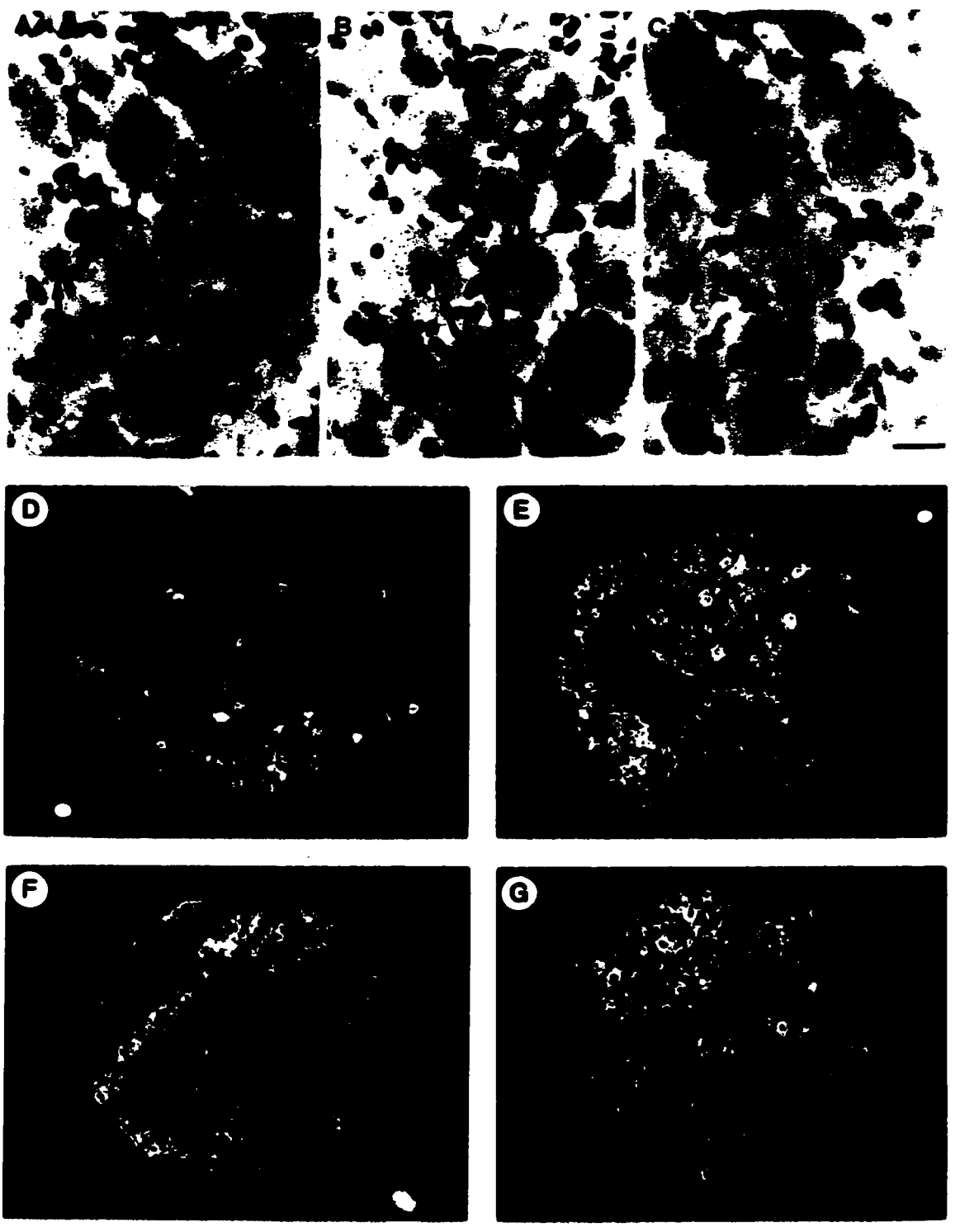
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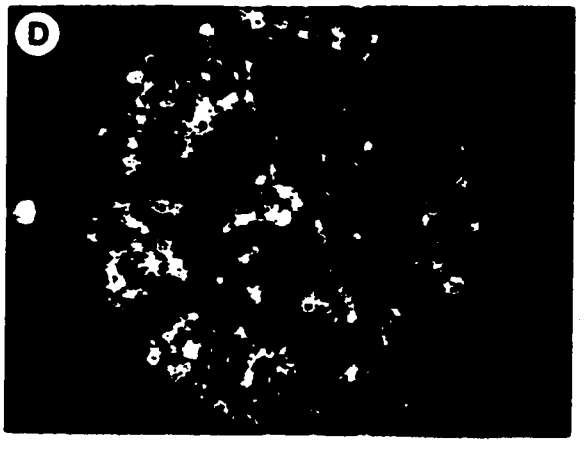
Figure 10. GAP-43 mRNA expression in DRG neurons. ISH was carried out as described in the text. (A-C), Bright-field photomicrographs of ISH of GAP-43 in DRG sections. Note labeling (silver grains) in primarily small cells (arrows), while larger neurons display little positive signal (asterisks). During regeneration, GAP-43 expression is increased in almost all neurons (C). (A) Normal DRG. (B) 4d Sprouting DRG. (C) 4d Regenerating DRG; scale bar = 25 μm. (D-G), Dark-field micrographs show signal for GAP-43 mRNA in sprouting DRGs (A, 1d; B, 8d) and regenerating (C, 1d; D, 8d) DRGs. Expression of GAP-43 is increased during both the sprouting and regeneration; scale bar = 75 μm.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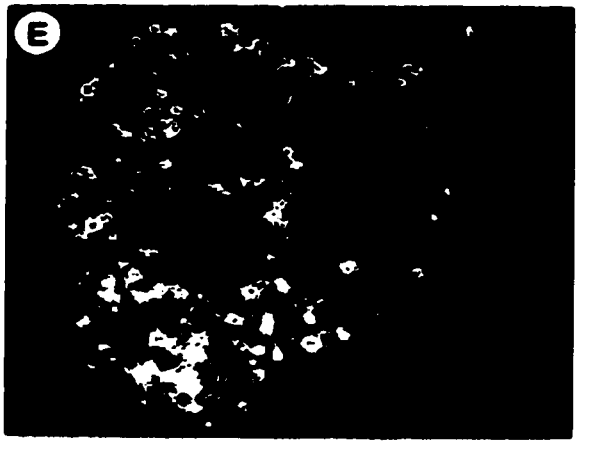
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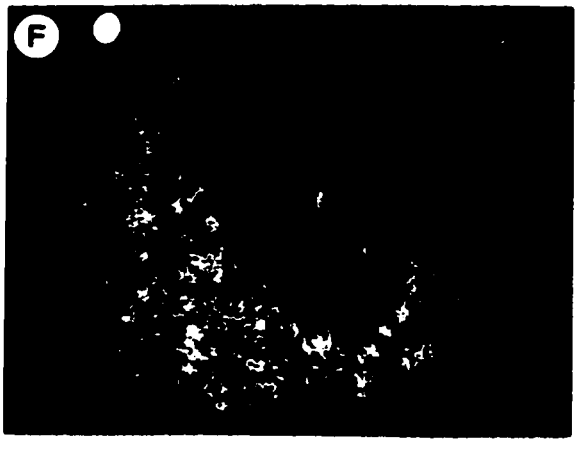
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CHAPTER 3

2nd Paper: ***Anti-NGF treatment blocks the upregulation of NGF receptor mRNA expression associated with collateral sprouting of rat dorsal root ganglion neurons***

3.1. ABSTRACT

The collateral sprouting of intact cutaneous sensory neurons has been shown to be dependent upon the presence of nerve growth factor (NGF). We have examined NGF receptor (NGFR) mRNA expression in DRG neurons undergoing sprouting and in DRG neurons whose sprouting had been prevented through exposure to anti-NGF antiserum during the course of the experiment. The results indicate that the low affinity $p75^{NGFR}$ mRNA is increased by 4 days post-operatively in DRGs from control serum-treated animals, and that this increase is most pronounced in smaller neurons. In contrast, the expression of $p75^{NGFR}$ mRNA was maintained at control levels in DRGs from anti-NGF-treated animals. Results for *trk*A expression indicate an increase in expression at days 4-6 post-operatively in both groups of animals, with the anti-NGF treatment having a delayed influence on mRNA levels. Examination of GAP-43 mRNA levels revealed an increased expression in sprouting DRG neurons, whereas this increase was not observed in DRGs from anti-NGF treated animals. Taken together, these results provide further evidence of NGF's role in the collateral sprouting of nociceptive neurons.

3.2. INTRODUCTION

NGF has been demonstrated to be essential for the survival and phenotypic differentiation of certain populations of developing sensory neurons (Lindsay, 1992). Although mature sensory neurons no longer display this dependency for survival, they continue to express both the low ($p75^{NGFR}$) and high (*trk*A) affinity NGF receptors (Mearow et al., 1994; Verge et al., 1992). As such, it is apparent that NGF continues to play an important role in the functioning of the mature peripheral nervous system (Lewin and Mendell, 1993).

Recently, Diamond and colleagues (Diamond et al., 1992 a,b) have provided behavioural evidence that the collateral sprouting of DRG sensory neurons is an NGF-dependent growth process while their regeneration occurs independently of NGF. In these experiments, nociceptive fibre sprouting into denervated adult rat skin was blocked by systemically administered antibodies to NGF (anti-NGF) and rapidly resumed following the termination of the anti-NGF treatment. This same treatment, however, did not affect the rate or extent of nociceptive fibre regeneration (Diamond et al., 1992b). Interestingly, we have recently shown that NGF mRNA is elevated in denervated adult rat skin (Mearow et al., 1993), thereby suggesting the regulation of NGF availability by cutaneous nerve fibres.

We have previously examined molecular correlates in DRG neurons undergoing either collateral sprouting or regeneration (Mearow et al., 1994). Prior to and during collateral sprouting, the expression of mRNAs for both $p75^{NGFR}$ and *trk*A are increased, as is GAP-43 mRNA. In contrast however, prior to and during regeneration of these sensory axons, only

GAP-43 message is upregulated. Thus increased GAP-43 mRNA is associated with growth per se (sprouting or regeneration), whereas increases in the NGF receptor mRNAs occur only during sprouting. Here, we have extended our investigations to examine gene expression in DRG neurons from animals treated either with anti-NGF antiserum or control serum. Our results indicate that anti-NGF treatment does prevent the increases normally seen in collaterally sprouting of not only $p75^{NGFR}$ and *trk*A, but also of GAP-43 mRNA. These results are consistent with and support further the proposed role of NGF in collateral sprouting of DRG sensory neurons.

3.3. MATERIALS AND METHODS

3.3.1. Animals and Operative Procedures to Produce Isolated Sensory Fields

The procedures used are similar to those described previously (Mearow et al., 1993). Briefly, an incision was made in the dorsal skin of anaesthetized female rats (100-150 g), and the cutaneous nerves (CNs) were exposed. On the left side, CNs L1-L5, T11 and T12, T8 and T9 were cut about 1 cm from the skin and the skin sutured. This procedure leaves the CNs T13 and T10 with intact sensory fields, surrounded by areas of denervated skin. The contralateral intact DRGs were used as internal controls.

3.3.2. Administration of Anti-NGF Antiserum

The anti-NGF antiserum was prepared in sheep; serum titres of each batch were determined in cell culture assay by the ability to completely block the activity of added NGF. Animals in one experimental group were injected subcutaneously in the nape of the neck with 2 µl/g of body weight (approx. 0.2 to 0.5 ml per animal per day). Injections were done daily

for the course of the experiment. Control sheep serum (preimmune serum) was injected in similar amounts into a second group of animals.

3.3.3. DRG Isolation

At a number of time points following the isolation procedure the animals were anesthetized and perfused with phosphate-buffered saline (PBS), and the appropriate DRGs (the left intact T13 and T10 for "sprouting" DRGs; the right T10 and T13 DRGs for control comparison) were removed and frozen in preparation for sectioning.

3.3.4. In Situ Hybridization (ISH)

ISH was carried out on frozen (10 μm) sections of DRGs according to our established procedure (Mearow et al., 1989; Mearow et al., 1994). Fixed sections were hybridized with ^{35}S-labeled cRNA probes (see below) at 42°C for 16 hrs; subsequently slides were washed to high stringency. Slides were then dipped in Kodak NTB2 emulsion and exposed in light-tight plastic slide boxes for 2-3 wks.

3.3.5. Probe Preparation

A 2 kb fragment of the rat p75^{NGFR} cDNA (Radeke et al., 1987) was subcloned into the riboprobe vector pBluescript KS (Stratagene), and the radiolabeled antisense RNA probe was prepared by first linearizing the template followed by transcription with T3 RNA polymerase and ^{35}S-CTP. The probe for *trk*A (pDM97, provided by Drs. J. Maragos and L. Parada) contains a 464 bp insert of the mouse trk cDNA encoding the extracellular domain; antisense strand transcription was carried out using T7 RNA polymerase and linearized template. cDNA encoding GAP-43 (pG43.1) was linearized and transcribed with Sp6 RNA

Sp6 RNA polymerase (Basiet al., 1987). To test for specificity, in some experiments sense RNA probes were transcribed and used in the ISH protocol (see Figure. 1).

3.3.6. Data Analysis

Developed slides were analysed using the MCID image analysis system (Imaging Research Inc., St. Catharines, Ont.). A grain counting software option was used to count grains over 100 cells from 3 sections from each DRG at each time point; control and experimental sections were processed on the same slides. Subsequent to the image analysis the data was statistically analyzed; the grain density (number of grains per μm^2 neuronal area) was calculated and statistical analysis (ANOVA, student's *t*-test) was performed using the Minitab statistical software.

3.4. RESULTS

3.4.1. $p75^{NGFR}$ Expression in Sprouting-Inhibited DRG Neurons

Expression of $p75^{NGFR}$ mRNA was examined using in DRG neurons undergoing sprouting in the presence of control serum and in DRG neurons where sprouting was blocked through the use of anti-NGF treatment. Analysis was carried out on DRG sections from 3 animals at each time point (neuron number = 300); the data are presented relative to the d0 level for each treatment. The results of such analyses are presented in Figure. 2a. $p75^{NGFR}$ mRNA expression is significantly increased in the control serum-treated animals by 4d post-isolation and continues to rise for the duration of the experiment (12d). In contrast, in the anti-NGF treated animals, there is no significant alteration in $p75^{NGFR}$ expression.

3.4.2. *trk*A Expression in Sprouting-Inhibited DRG Neurons

The results of analysis of *trk*A mRNA expression in control-serum treated sprouting DRG neurons are presented in Figure. 2b. There is a gradual increase in *trk*A levels, with the differences being significant by 6d post-isolation. Unlike $p75^{NGFR}$, the anti-NGF treatment seems to have a more delayed influence on *trk*A, there being no difference between the control and anti-NGF groups until 8d post-isolation.

3.4.3. GAP-43 Expression in Sprouting-Inhibited DRG Neurons

Because the analyses were carried out at times prior to, and up to, the time that functional recovery was initially observed, a marker associated with growing neurites was used to ensure that the neuronal population was indeed sprouting. Analysis of GAP-43 expression in these experiments was carried out to provide an internal control for nerve growth. The results are presented in Figure 2c, where it can be seen that GAP-43 expression increases in the sprouting DRG neurons, but is not significantly different from the d0 controls in the anti-NGF group.

3.5. DISCUSSION

3.5.1. Role of NGF in the Expression of NGFRs During Collateral Sprouting

Anti-NGF antiserum treatment has been used as a method of depleting available levels of endogenous NGF, presumably by preventing binding of NGF to the receptors and internalization (Ennis et al., 1979; Yip et al., 1984). In earlier studies, anti-NGF treatment was shown to prevent the collateral sprouting of sensory and sympathetic neurons, but had little effect on their regeneration (Diamond et al., 1992 a,b; Gloster and Diamond, 1992). The

present investigation has examined the expression of NGF receptor and GAP-43 mRNAs in animals subjected to an anti-NGF treatment protocol known to block collateral sprouting. We have also tested for sensory field expansion in these animals and found that there was none, i.e., no sprouting, in the anti-NGF treated animals, while the control serum treated groups did display increased field sizes, i.e., sprouting (data not shown).

In the control-serum group, sprouting proceeded and increases in both $p75^{NGFR}$ and *trk*A mRNAs were found. In the same neuronal population, GAP-43 mRNA was also upregulated supporting the view that the neurons were indeed sprouting. However, when the DRGs from the anti-NGF treated animals were examined, there were no alterations in the expression of $p75^{NGFR}$ or GAP-43, indicating (as the behavioural mapping studies showed) that collateral sprouting was blocked as a result of the anti-NGF treatment. However, *trk*A expression increased up to d6 in both situations, with the anti-NGF seemingly not having any effect until after 1 wk (see below).

The anti-NGF treatment blocks sprouting presumably by blocking the availability of NGF to the intact axons. The anti-NGF treatment alone did not influence significantly the expression of $p75^{NGFR}$, *trk*A or GAP-43 mRNAs in otherwise intact DRG neurons; in other words it is not acting to directly downregulate the neuronal mRNAs, but rather at the level of neutralizing NGF levels that may be increasing in the denervated skin. The increases in NGFR mRNA expression in sprouting neurons are consistent with their being evoked by increases in available NGF in the denervated target area (Mearow et al., 1994); NGF has been shown to upregulate receptor expression both in vitro and in vivo (Lindsay et al., 1990; Miller

et al., 1994). Furthermore, increased expression of NGF receptor mRNAs has also been observed in collaterally sprouting sympathetic neurons (Kuchel et al., 1992; Miller et al., 1994).

It is generally accepted that TrkA mediates the functional responses to NGF; however, recent studies demonstrate that $p75^{NGFR}$ can act to modulate the responses of Trk to NGF, perhaps by increasing the local concentrations of NGF available to Trk receptors (Barker and Shooter, 1994; Hantzopoulos et al., 1994). With respect to sprouting, the nature of the contribution of the different receptors to the growth response is unclear; one interpretation is that both receptors are involved in sprouting. While our results demonstrate upregulation of both in the sprouting DRGs, the changes are more pronounced in $p75^{NGFR}$ expression, particularly in the present study where the anti-NGF treatment that blocks collateral sprouting appears to affect $p75^{NGFR}$ mRNA more than *trk*A expression. One likely possibility to account for the differences relates to the fact that trk responds to a different concentration range that the lower affinity $p75^{NGFR}$ receptor (Barker and Shooter, 1994; Hantzopoulos et al., 1994; Miller et al., 1994). Although the anti-NGF treatment is given daily, low levels of NGF may still be present. These small amounts are not sufficient to activate $p75^{NGFR}$, but may be within the concentration range required to activate Trk and result in increased mRNA expression for the first week or so. The cumulative effect of the anti-NGF would eventually result in NGF concentrations too low to activate either of the receptors. Relevant are recent results that indicate that TrkA phophorylation can be maintained for prolonged periods following NGF application, suggesting that the influence of a trophic factor can persist for some time after

being made available to the receptors (Yamada et al., 1994).

The results of this study provide evidence to support the key role of NGF in the collateral sprouting of nociceptive sensory neurons, but also suggest a requirement for both $p75^{NGFR}$ and *trk* in the growth response.

Figure 1. Darkfield micrographs of in situ hybridization (ISH) for sense (A) and antisense cRNA probes (B) for the low affinity $p75^{NGFR}$ receptor. Note that when the sense probe is used there is no specific signal over the DRG section. Use of the antisense probe results in specific labeling over a population of neurons in the DRG. Calibration bar - 100 μm.


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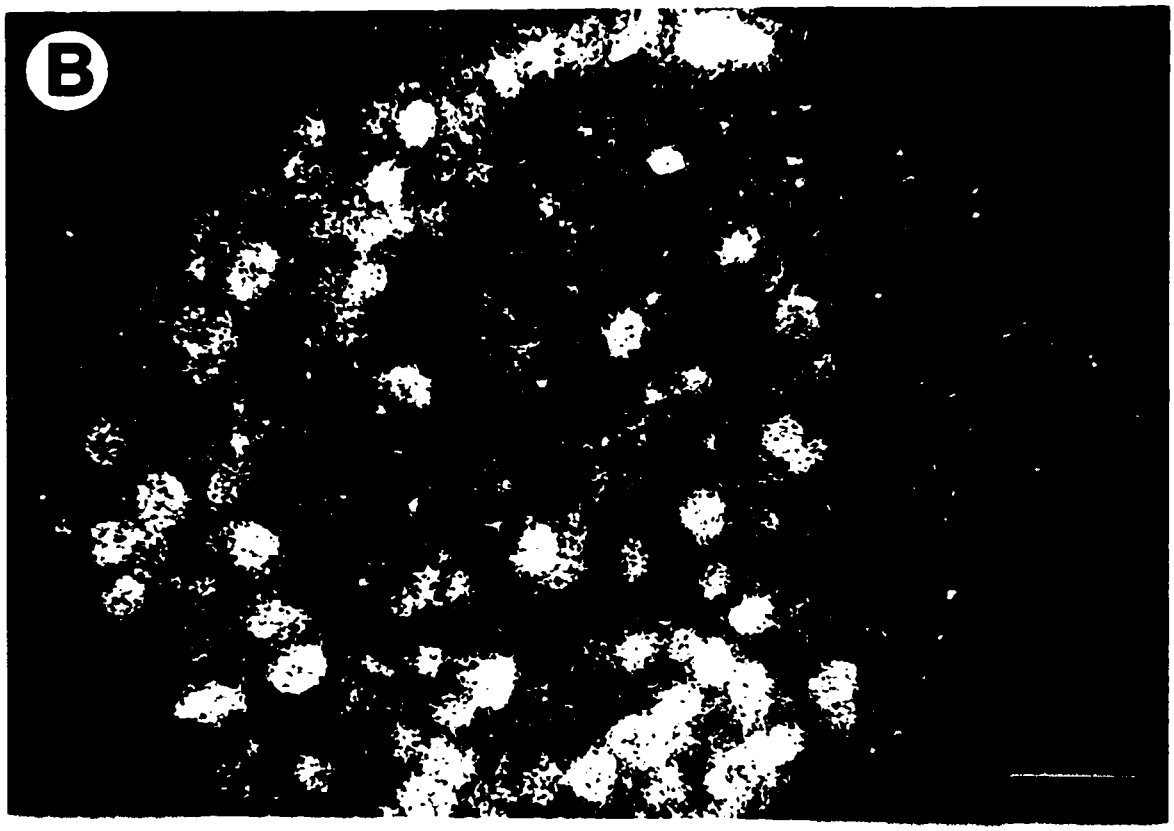
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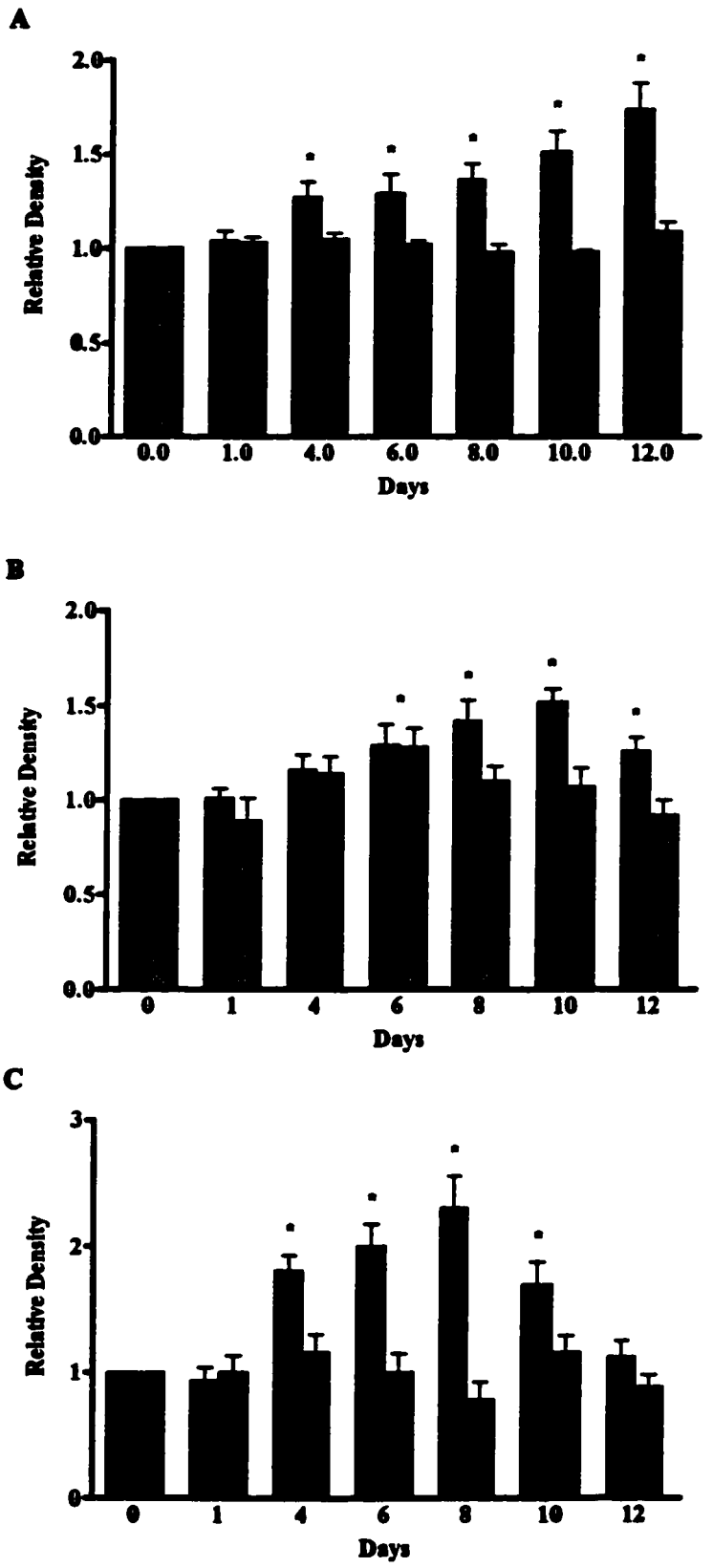
Figure 2. Expression of $p75^{NGFR}$ (A), trkA (B) and GAP43 (C) mRNA in DRG neurons. Following ISH, tissue sections were analyzed as described in the text. The results of three experimental groups (3 animals per time point, n= 300 cells) area averaged and expressed relative to the control (d0) values of grain density for DRG neurons undergoing sprouting (solid bars) and those in which sprouting has been prevented by anti-NGF antiserum treatment (hatched bars). * Significantly different from control, $p < 0.05$.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B
Relative Density
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Relative Density
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CHAPTER 4

3rd PAPER: Increased NGF mRNA expression in denervated rat skin

4.1. ABSTRACT

Using a coupled reverse transcription and competitive polymerase chain reaction protocol, we have measured Nerve Growth Factor (NGF) mRNA expression in adult rat skin following its denervation. By 2-4 days, levels of NGF mRNA were increased approximately 5-10 fold over levels in innervated skin, remaining increased for up to at least 2 weeks. *In situ* hybridization carried out on skin samples revealed that the NGF message was expressed not only in the distal nerve pathways but also in non-nerve associated cells, such as dermal fibroblasts, and in the basal epidermal layer. Denervated skin evokes a NGF-dependent collateral sprouting from neighboring intact sensory axons. The new finding indicates that an increased NGF production is associated with the increased availability of NGF in such skin, and that cutaneous nerves have role in regulating this NGF production.

4.2. INTRODUCTION

Denervated target tissues can rapidly become invaded by a vigorous ingrowth of collaterals emanating from neighbouring undamaged nerve fibres. In adult rat skin this collateral sprouting, which involves only the nociceptive nerves (Doucette and Diamond, 1987), is now known to be dependent on the presence of NGF; it is blocked by anti-NGF antiserum, rapidly resumes when the treatment ceases, and can be evoked in normally-

innervated skin by systemic NGF (Diamond et al., 1987, 1992a). In denervated skin, NGF concentrations would be expected to increase because of the absence of the nerve terminals which normally take up NGF (Korsching and Thoenen, 1985; Shelton and Reichardt, 1986). Collateral sprouting would presumably continue to be evoked until the NGF levels returned to normal, the anticipated consequence of a restored uptake of NGF into the newly sprouted terminals. This view, which does not involve an up-regulation of NGF production for its levels to increase in a denervated target tissue is consistent with the reports (i) that when one NGF-utilizing system of nerves is eliminated from a shared target tissue, there is an increased uptake of NGF into a surviving second system (Korsching and Thoenen, 1985), and (ii) that denervation of the iris *in vivo* does not cause a change in the expression of NGF mRNA (Shelton and Reichardt, 1986). However, an earlier hypothesis, based upon findings in the salamander, had proposed that the levels of sprouting factors might rise in a denervated target tissue because of an increase in their rate of synthesis (Diamond et al., 1976). The present study was undertaken in part to test this hypothesis, by measuring the effects of denervation on the level of NGF mRNA in mammalian skin.

4.3. MATERIALS AND METHODS

4.3.1. Operative Procedures to Produce Denervated Skin

Female Wistar rats (150-200g) were anaesthetized with sodium pentobarbital (45 mg/kg), the fur clipped from the thoracodorsal aspect and an incision made in the dorsal skin to the right of the midline. The exposed dorsal cutaneous nerves (DCNs) on the left side were sectioned close to their exit points from the body wall, and their central stumps allowed

to withdraw behind the musculature. The skin was sutured and the animals allowed to recover. Rats were anaesthetized and skin samples were taken at 2, 4, 6, 8, 12, and 14 days after the surgery; a total of 5 control and 25 experimental animals were used. Prior to sample removal, the physiological stutus of the skin (i.e., still denervated or reinnervated by cutaneous nerves other than the DCNs) was evaluated by mapping procedures previously described (Doucette and Diamond, 1987). Only skin that was confirmed as denervated (indicated by lack of a CTM reflex response) was sampled; approximately 1 cm^2 of skin was removed, frozen quickly on dry ice and sliced into smaller pieces. Samples from the same animals were used for RNA isolation and ISH.

4.3.2. Isolation of Total RNA

Total RNA was isolated from the skin samples by the method of Chomzynski and Sacchi (1987); integrity of RNA was evaluated by electrophoresis through formaldehyde-agarose gels; obviously degraded samples were discarded. RNA was also isolated from male mouse salivary glands for use as the competitive template.

4.3.3. Competitive RT-PCR

1-5 μg of total RNA was then reverse transcribed using random hexamers and M-MLV reverse transcriptase (Gibco-BRL). An aliquot of this reaction was used to amplify a 400 bp fragment of NGF using primers corresponding to nts 610-631 (upstream) and complementary to nts 991-1012 (downstream) of the rat NGF sequence (Whittemore et al., 1988). Competitive PCR (Gilliland et al., 1990) was carried out using mouse NGF cDNA as the competitior, and a constant amount of the reverse transcribed cDNA. Using the same

primers, amplification of the mouse cDNA (obtained from the reverse transcription of the mouse salivary gland RNA) was carried out, and the fragment gel purified, isolated and quantified spectrophotometrically. The mouse NGF fragment was then serially diluted (range 10 ng to 1 fg) and known amounts added to constant amounts of the rat cDNA for amplification. Amplification was carried out using *Taq* DNA polymerase and reaction conditions as suggested by the supplier (Perkin-Elmer, Norwald, CT) with the following parameters: an initial cycle of 5 min at 94°C, 1 min at 52°C, and 1 min at 72°C followed by 1 min at each of 94°C, 52°C, and 72°C for 30 cycles. After a final 5 min extension at 72°C, the samples were removed and placed on ice. In order to distinguish the mouse NGF from the rat NGF, aliquots of the PCR reaction were subjected to digestion with the restriction enzyme *Taq*I site. Following the digest, the samples were analysed by agarose gel electrophoresis, photographed and the negatives of the ethidium bromide stained gels were analysed by densitometry (Imaging Research Inc., St. Catharine's). The target (rat NGF) concentration is estimated from the point at which it is amplified equally or in excess of the competitor, given that the concentration of added competitor is known. In addition to the use of a competitor, PCR was also carried out using in addition to the NGF primers, primer pairs for either cyclophilin (Danielson et al., 1988) (primer 1: nts 44-65; primer 2: complementary to nts 393-414) or T26α-tubulin (Miller et al., 1987) (primer 1: nts 268-289; primer 2: complementary to nts 515-536 (Ginzburg et al., 1981) as internal controls. Following agarose gel electrophoresis, the ethidium bromide stained gels were analysed by densitometry; the ratio of the density of the rNGF fragment to that of the internal control was then analyzed.

4.3.4. In Situ Hybridization (ISH)

ISH was carried out using two 48-mer oligonucleotides (compementary to nts 610-658 and nts 893-940) of the rat NGF cDNA (Whittemore et al., 1988) sequence and end labelled with terminal deoxynucleotidyl transferase (Pharmacia) using $^{[\alpha\text{-}35S]}$dATP (1200 Ci mmol^{-1}, DuPont-NEN) to an average specific activity of 1 X 10^6 dpm μg^{-1}. Hybridization and subsequent washings were performed as previously described (Mearow et al., 1989). Slides were then dipped in Kodak NTB2 emulsion, exposed for 2-4 weeks and analysed using an image analysis system (Imaging Research Inc., St. Catharine's).

4.4. RESULTS

4.4.1. NGF mRNA Levels in Innervated and Denervated Rat Skin

The competitive PCR method used to estimate NGF mRNA levels in innervated and denervated rat skin showed that NGF message increased in denervated skin by at least 10 fold; the increase is seen by 2 days post-denervation and persists for up to 2 weeks after denervation, the longest time examined in the present experiments. The density of the rat NGF fragment (top Band) is compared in Figure 1 to the density of the digested mouse NGF fragments (middle and bottom bands). At the point of equivalence (i.e., when the ratio of rat to mouse NGF equals one), the concentration of rat NGF equals that of mouse NGF. Increases in the ratio indicate increased amount of rat NGF relative to that of the competitor. As seen from Table 1, NGF cDNA can be estimated to increase from 10 fg or less in the control innervated skin to values ranging from 10 fg to 1pg. Assuming the amount of NGF mRNA present in the sample is related to the amount of reverse transcribed and amplified

NGF DNA, the results indicate a least a 10 fold increase of the NGF message in the denervated compared with innervated skin samples.

PCR was also performed using the rNGF primers with T26 α-tubulin (Miller et al., 1987; Ginzburg et al., 1981) or cyclophilin (Danielson et al., 1988) primers as an internal reference (Figure 2a,b). The ratio of the density of the NGF fragment to the T26 fragment was determined for each of the samples and the data is presented in Figure 2c. Using this approach, the results similarly indicate that greater amounts of NGF cDNA are present in denervated samples.

4.4.2. Localization of NGF mRNA in Innervated and Denervated Rat Skin

The ISH results are consistent with the above observations. They show that NGF mRNA is present in both dermal and epidermal layers, in what are likely to be Schwann cells as well as numerous other indeterminate dermal cell types (Figure 3a-d). A relatively strong signal was associated with the hair follicles, in particular their epidermal components. No signal was seen in association with the *cutaneus trunci* muscle, which underlies the dermis.

4.5. DISCUSSION

4.5.1. Role of NGF in Adult Rat Skin

Our results demonstrate that denervation of skin leads to an increase in its NGF mRNA levels. The observations obtained from the initial experiments using T26 α-tubulin or cyclophilin as an internal reference (Figure 2) suggested that NGF mRNA was increased in denervated skin relative to normally innervted skin. Competitive PCR was used as an approach to quantitate the observed changes; relative quantitation can be obtained even if

there is substantial variability in the PCRs since it is the relative, not absolute, amounts of target and competitor that are important (Piatek et al., 1993). It should also be pointed out that increases in NGF cDNA reverse transcribe from the intial RNA samples.

The presence of NGF and its mRNA in adult skin has been previoiusly described, in particular in both epidermally-derived keratinocytes (Tron et al., 1989) and dermally-derived fibroblasts (Acheson et al., 1991). During development, NGF mRNA was detected in both epithelial and mesenchymal layers (Davies et al., 1987; Rohrer et al., 1988; Wheeler and Bothwell, 1992), and the timing of its first appearance indicates that NGF mRNA expression in the skin begins independently of the onset of both the sensory and the sympathetic innervation. However, NGF mRNA levels are known to increase in Schwann cells and other non-neuronal cells associated with degenerating nerve pathways (Heumann et al., 1987), and the increases observed in denervated skin could thus derive, at least in part, from such pathways. It seems unlikely though that more than a small proportion of the increased levels of NGF mRNA we measured was attributable to these degeneration-associated cells. The fraction of skin seen to be occupied by nerves in histological preparations is small (unpublished observations), and we consistently noted the presence of NGF mRNA in epidermal cells and areas that did not appear to be associated with degenerating axonal pathways.

What could be the advantages of an increased expression of NGF in these various components of the target tissue? The most obvious suggestion for a role of NGF following peripheral nerve damage is in the promotion of axonal regeneration to the deprived skin, a

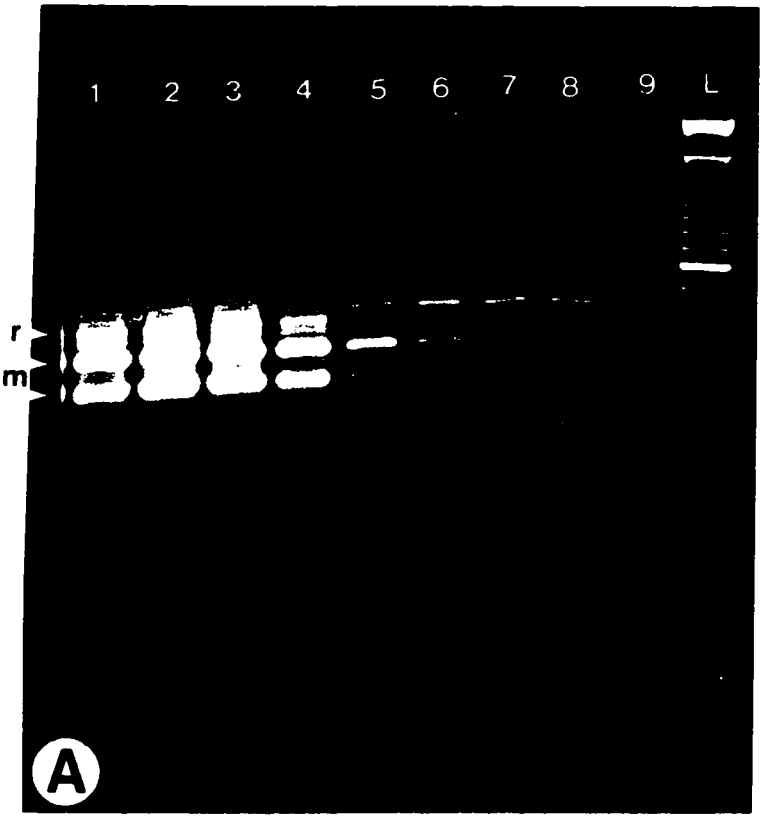
suggestion supported by the increased expression of NGF mRNA in the degenerating peripheral nerve pathway, mentioned above. However, neither sensory (Diamond et al., 1992a) nor sympathetic (Gloster and Diamond, 1992) regeneration seems to depend upon NGF. On the other hand, the *collateral sprouting* of intact axons of both these neural systems into denervated rat skin is totally dependent upon the availability of NGF (Diamond et al., 1992a; Gloster and Diamond, 1992). The time course of the increase we have now found in NGF mRNA levels in skin following its denervation is thus consistent with this increase playing a role in such collateral sprouting. However, increased levels of endogenous NGF would be anticipated because of the absence in denervated skin of the normal uptake of NGF into sensory and sympathetic axons. A simple competition between these two systems for a constant supply of NGF could explain the finding that the selective elimination of one of them leads to an increased retrograde transport of NGF in the other (Korsching and Thoenen, 1985). In keeping with this view, in one target tissue, the iris, denervation was reported not to cause changes in the levels of NGF mRNA (Shelton and Reichardt, 1986). In contrast, the NGF message has been shown to increase 3 fold in skeletal muscle after its paralysis by either denervation or TTX nerve block (Amano et al., 1991), even though motor nerves appear not to utilize NGF in their normal functioning. How do the present finding accord with these various reports? Our view derives from observations made the salamander (Diamond et al., 1976). When axoplasmic transport was reduced by colchicine in one peripheral nerve, which suffered no axonal degeneration or loss of physiological function, adjacent nerves that shared the same target tissue underwent collateral sprouting; the result

was peripheral hyperinnervation. Conceivably, sprouting factors might have accumulated in the tissues as a consequence of the blocking of their retrograde transport in the treated nerve. An alternative explanation, we hypothesized, was that the block of *anterograde* transport led to the reduction in a regulatory feedback control of sprouting factor production (Diamond et al., 1976). As a consequence, more sprouting factor would be synthesized. The findings described in this report are in keeping with this hypothesis. The involvement of non-NGF trophic factors in the *regeneration* of sensory and sympathetic axons to denervated skin was not ruled out by our earlier experiments (Diamond et al., 1992b; Gloster and Diamond, 1992). The mRNA for both BDNF and NT-3 have been shown to be present in skin (Acheson et al., 1991; Maisonpierre et al., 1990a), and the possibility that these may also increase after denervation is being examined.

4.6. CONCLUSIONS

The results of this study demonstrate an increase in NGF mRNA levels in skin following sensory denervation. Such denervated skin evokes collateral sprouting from adjacent intact sensory axons, which has been shown to be NGF-dependent. It had been hypothesized that there must be an elevation in NGF in the denervated territory to evoke this sprouting; the present results provide firm evidence that support this premise and suggest that cutaneous nerves may play a role in regulating trophic factor production.

Figure 1. Total RNA isolated form control and denervated rat skin was converted to cDNA with reverse transcriptase using random hexamer priming. PCR co-amplification of the rat cDNA and known amounts of mouse NGF cDNA was performed as described in the Materials and Methods. The PCR products were digested with *Taq*1, precipitated and then electrophoresed on a 2% agarose gel, stained with ethidium bromide (5μg/ml) and photographed. **(A)** A representative dilution series using known amounts of mouse NGF cDNA (m) and a given amount of the rat total cDNA (r), isolated from control skin. Lane 1: 100 ng mouse NGF, lane 2: 10 ng, lane 3: 1 ng, lane 4: 100 pg, lane 5: 10 pg, lane 6: 1 pg, lane 7: 100 fg, lane 8:10 fg, lane 9: 1 fg. L= 100 bp ladder marker. Note that in Lane 8 the ratio of the top band (r) to the bottom two bands (the restricted mouse NGF, m) is ≥ 1, indicating that the amount of rat NGF cDNA present can be estimated as being ≥ 10 fg. **(B)** PCR amplification of NGF from reverse transcribed RNA isolated from 4 d denervated skin. Lanes 1: 100 pg, 2: 10 pg, 3: 1 pg, 4: 100 fg, 5: 10 fg, 6: 1 fg. In this example, the amount of NGF is estimated as ≥ 1 (or 100 times than in the control shown in A).


1 2 3 4 5 6 7 8 9 L
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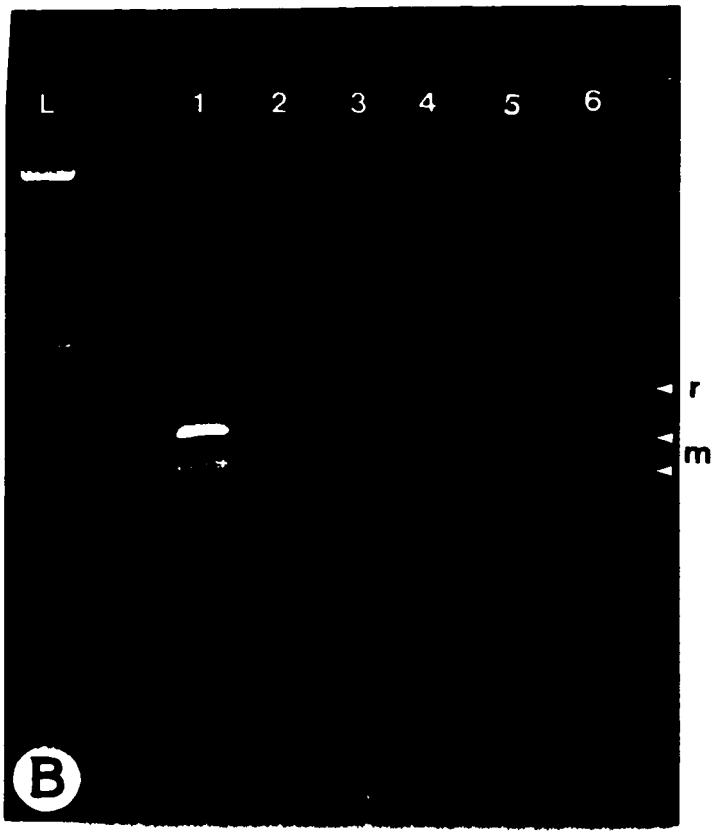
L 1 2 3 4 5 6
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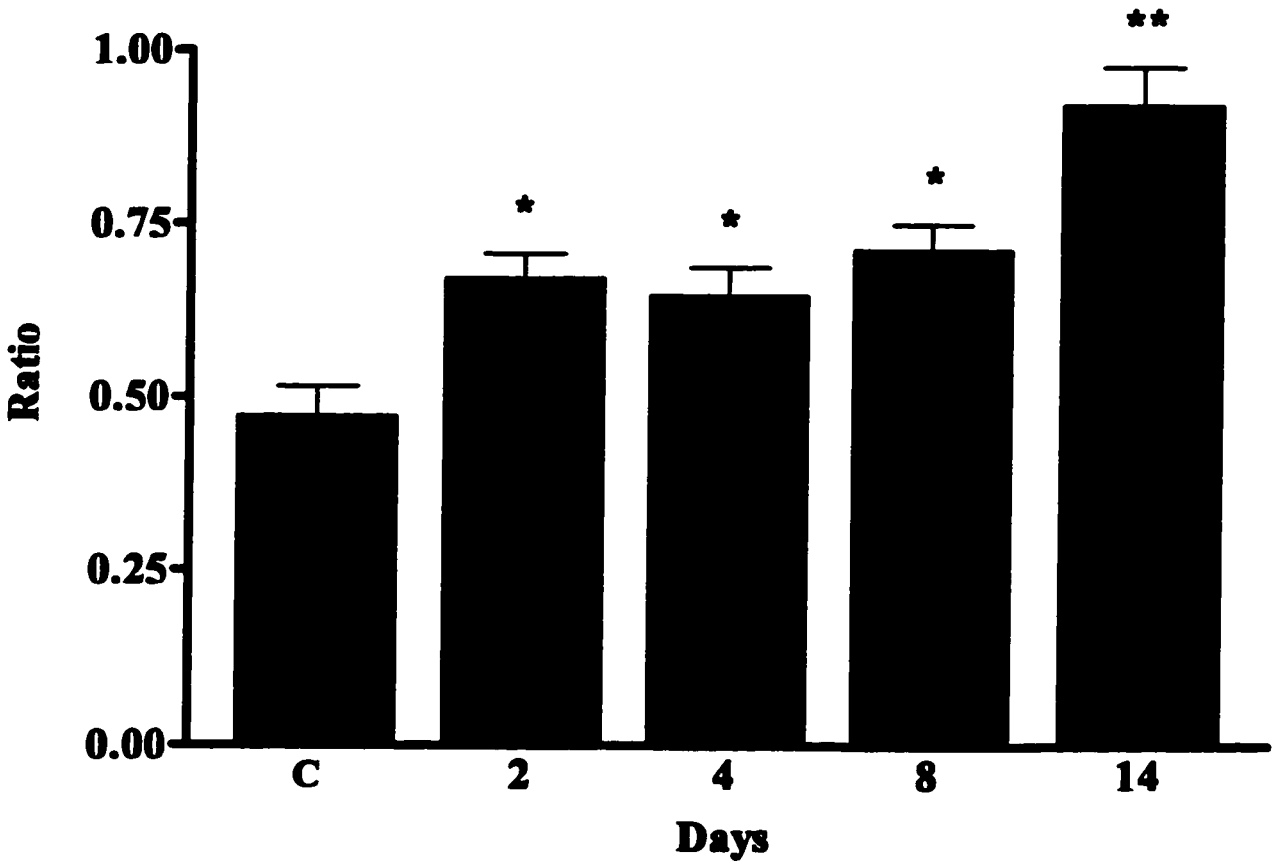
m
B

Table 1. Estimation of NGF cDNA in innervated and denervated rat skin. PCR amplification and analysis was carried out as described in the Methods. The ratios of the ROD of the individual bands were calculated and are presented as mean (S.E.M.). A ratio of $\geq$ 1 indicates the point of equivalence, i.e., rNGF $\geq$ mNGF.

[mNGF]	100 pg	10 pg	1 pg	100 fg	10 fg	1 fg
RATIO	rNGF/ mNGF	rNGF/ mNGF	rNGF/ mNGF	rNGF/ mNGF	rNGF/ mNGF	rNGF/ mNGF
Control	0.0004 (0.0002)	0.05 (0.2)	0.136 (0.03)	0.423 (0.04)	1.09 (0.18)	1.10 (0.02)
day 2	0.04 (0.01)	0.339 (0.11)	1.12 (0.24)	2.28 (0.37)	4.72 (1.21)	2.53 (0.14)
day 4	0.19 (0.01)	0.44 (0.05)	1.31 (0.01)	2.34 (0.26)	2.73 (0.45)	1.89 (0.23)
day 6	0.09 (0.02)	0.19 (0.03)	0.33 (0.08)	1.07 (0.05)	1.51 (0.11)	1.66 (0.11)
day 8	0.18 (0.02)	0.43 (0.05)	1.02 (0.09)	1.66 (0.12)	1.49 (0.14)	2.12 (0.25)
day 14	0.14 (0.05)	0.45 (0.07)	2.48 (0.09)	1.99 (0.34)	3.85 (0.77)	3.6 (0.90)

Figure 2. Plot of the increase in the amplified rat NGF cDNA relative to the amplified internal reference (T26 α-tubulin) cDNA (see Figure 1). Following densitometry of the ethidium bromide stained gels, the ration of the relative optical density (ROD) of the NGF band to the T26 band was calculated and the results (mean ± S.E.M.) from the control and denrvated samples (n=5) are plotted. * significantly different from control, $p < 0.05$; ** significant at $p < 0.001$. Statistical analysis by Student's *t*-test and one way ANOVA.


1.00
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0.50
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C
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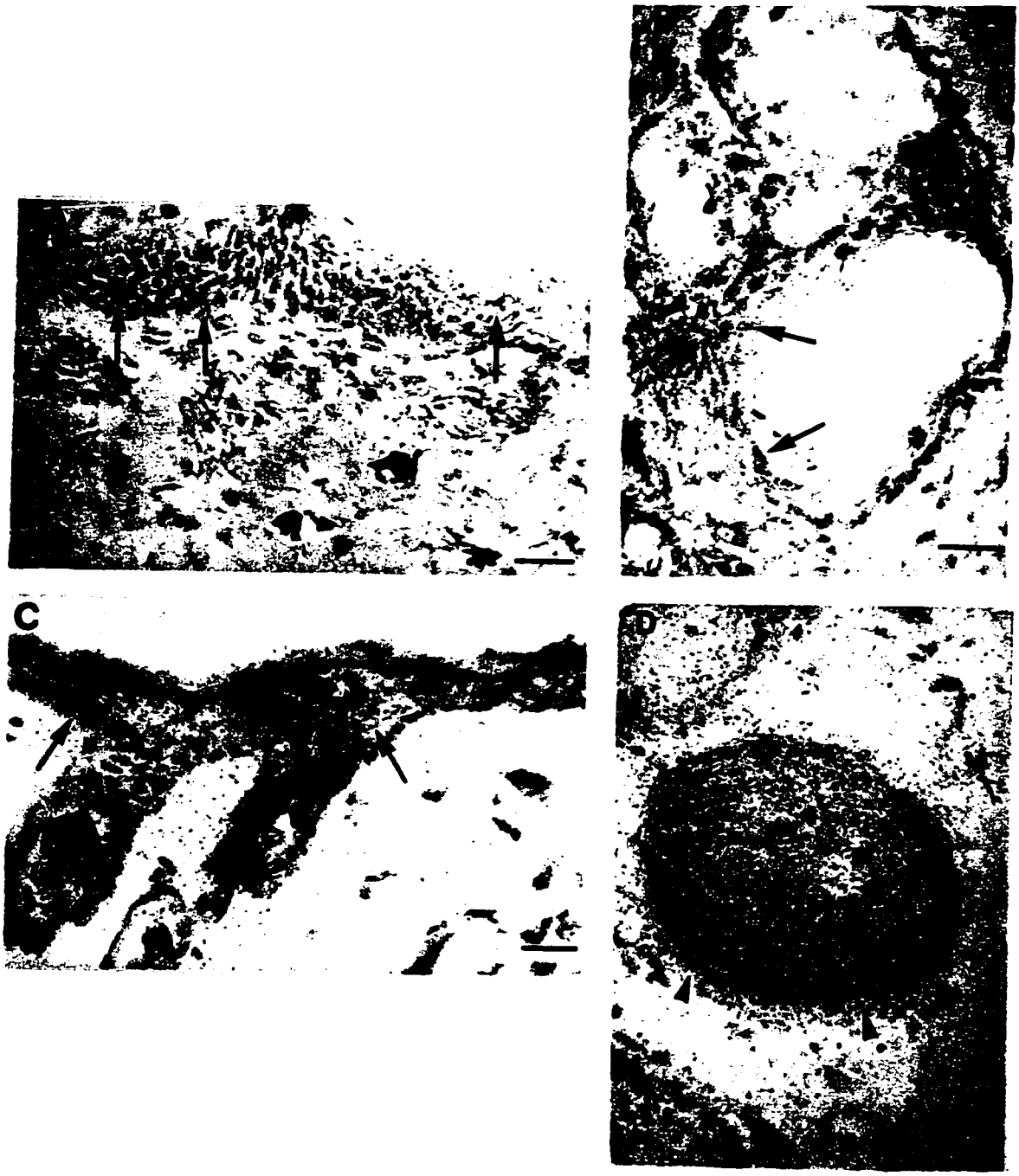
Figure 3. Photomicrographs illustrating relative levels of NGF mRNA in control (A) and denervated (B, C, D) skin. ISH was carried out on sections of skin as described in the Materials and Methods. In A there are silver grains associated with fibroblast-like cells in the dermal layer (arrows). In denervated skin, there appears to be increased labelling associated with fibroblast like cells (B, arrowheads), and with non-neuronal cells associated with the degenerating nerve fibres (D, arrowhead); in these particular examples, the cells and nerve fibres are found in close relation to the base of the hair follicles. In C, increased labelling is seen in the basal epidermal layer (arrowheads). Bar - 50 μm.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CHAPTER 5

4th PAPER: NGF-dependent Nerve Growth Responses are Compromised in Adult Rats Deprived of NGF During Postnatal Life

5.1. ABSTRACT

A serendipitous observation led us to examine the effects of early NGF-deprivation on the subsequent ability of nociceptive neurons to undergo collateral sprouting in skin. This sprouting, which is NGF-driven, was significantly impaired in adult rats which received daily injections of anti-NGF serum during the first 2 weeks of postnatal life, in some animals failing to occur at all over the month of observation. However, after nerve crush the same nociceptive axons regenerated normally (regeneration occurs independently of NGF). This finding, plus the presence of normal sensory thresholds and axon calibers, excluded the possibility of a persistingly defective axoplasmic transport. NGF insufficiency was not responsible for the impairment since NGF expression in skin was normal, and increased normally following denervation. Nor had the neurons become NGF-unresponsive, or reached a sprouting "ceiling" since *injected* NGF promoted nociceptive sprouting at least as effectively as in controls. To explain the difference between the effects of exogenous and endogenous NGF we propose that the latter is restricted to a functional compartment that contains the nerve terminals, and that the ability of these to take up NGF is defective; exogenous NGF is assumed to be most effective on the regions of the neuron that lie outside this peripheral compartment. Although endogenous NGF uptake must be adequate to account for the

healthy state of the neurons, the terminals apparently failed to take up enough of the *increased* NGF produced in the collateral sprouting paradigm either to evoke sprouting or to cause the typical upregulation of $p75^{NGFR}$ mRNA in dorsal root ganglion (DRG) neurons. However, some increased NGF uptake was possible since the normal upregulation of *trk*A occurred. The phenomenon we describe could have wider implications; neurotrophin deficiencies induced by adverse conditions during development might permanently compromise the adult organism's ability to undertake neurotrophin-based compensatory nerve growth after neuronal damage or disease.

5.2. INTRODUCTION

Target tissues exert significant influences on the growth and differentiation of neurons via the provision of specific neurotophic substances such as Nerve Growth Factor (NGF). NGF deprivation in early life brought about by exposure of the developing animal to anti-NGF antibodies leads to a striking reduction in the population of small neurons in dorsal root ganglia (DRG) (Levi-Montalcini and Angeletti, 1966; Johnson et al., 1980; Yip et al., 1984; Ruit et al., 1992), and to an irreversible alteration in the morphological phenotype of sympathetic neurons (Ruit and Snider, 1991). It has also been reported that neonatal anti-NGF treatment leads to a respecification of the electrophysiological phenotype of DRG nociceptive neurons (Ritter et al., 1991; Lewin et al., 1992; Lewin and Mendell, 1994; Lewin and Mendell, 1996). As animals mature the NGF-dependence of their DRG neurons declines; although most adult neurons survive NGF deprivation both in vitro (Grothe and Unsicker, 1987; Lindsay, 1988; but see also Jiang and Smith, 1993) and in vivo (Johnson et al., 1986),

their cell size, transmitter and transmitter enzyme levels are affected (Gorin and Johnson, 1980; Schwartz et al., 1982, Rich et al., 1984; Lindsay and Harmar, 1989), supporting the view that in the adult nervous system neurotrophins are primarily involved in the maintenance of neuronal phenotype.

Neurotrophins can dramatically alter another feature of adult neuronal phenotype, that being the size of the terminal field within the target tissue (Diamond et al., 1992c). For example, nociceptive fields can enlarge 5-fold after surrounding skin is denervated; this expansion is attributable to a collateral sprouting of nociceptive axons (Nixon et al., 1984; Doucette and Diamond, 1987) that is driven by the increased NGF made available in nerve-deficient skin (Diamond et al., 1987; Diamond et al., 1992a; Mearow et al., 1993). Conversely, a reduced availability of NGF can lead to field shrinkage, possibly by terminal regression (Diamond et al., 1988, 1991). In contrast to the total NGF-dependence of their collateral sprouting, the *regeneration* of nociceptive axons after injury occurs independently of NGF (Diamond et al., 1992b). Another feature which distinguishes between these two growth processes is that $p75^{NGFR}$ and *trk*A mRNA levels in DRG neurons are upregulated prior to and during physiologically-defined collateral sprouting but are unaffected in the same neurons when they are regenerating (Mearow et al., 1994), at least until the axons reach their target tissues (Krekoski et al., 1996).

The present study stemmed from an observation that the collateral sprouting of nociceptive nerves apparently failed to occur in some adult rats that had received anti-NGF treatment as neonates. We have now examined this situation in depth. The results show that

after a brief period of neonatal NGF deprivation the ability of nociceptive DRG neurons to respond to adjacent denervated skin by collateral sprouting becomes severely and permanently impaired. Yet NGF is available in the target tissue, the neurons sprout readily in response to *exogenous* NGF, and their axons regenerate normally after nerve crush. These findings suggest that at least one important mechanism of plasticity and repair which resides throughout the entire nervous system of adult mammals could be irreversibly compromised by trophic deprivation in early life.

5.3. MATERIALS AND METHODS

5.3.1. Preparation and Examination of NGF and Its Antiserum

NGF (2.5S) and anti-NGF were prepared as described in Diamond et al. (1992a). Briefly, NGF was purified from male mouse salivary glands according to the method of Mobley et al. (1976) with a further purification according to Darling and Shooter (1984) and Mobley et al. (1985). Neurotrophic biological activity was measured in the dissociated cell assay (Greene, 1977) using neonatal mouse superior cervical ganglion neurons (Coughlin and Collins, 1985); half-maximal response was at 1 ng/ml. 7S NGF was prepared according to a modification (Stach et al., 1977) of the procedure of Varon et al. (1967), and also displayed half-maximal activity in the dissociated cell assay at 1 ng/ml. Anti-NGF antiserum was raised in adult sheep (ewes) against mouse 2.5S NGF, and tested for its ability to block NGF-induced neurite outgrowth by the dissociated cell method mentioned above; a 30,000-fold dilution of antiserum completely blocked the activity of 10 ng/ml 7S NGF. Antibody specificity was determined by the Ouchterlony double-diffusion analysis and by Western blot

as previously described (Diamond et al., 1992a). Although in ELISA assays our polyclonal sheep antibody to mouse NGF recognizes NT-3 and to a lesser extent BDNF (Murphy et al., 1993), in *in vitro* survival assays, the biological activity only of NGF is blocked, whereas that of NT-3 and BDNF is unaffected (R. Kolbeck and Y.-A. Barde, personal communication). In an *in vitro* neuronal system, we find that our antibody blocks the NGF and NT-3 induced sprouting of mouse dorsal root ganglia but not that induced by BDNF (Van der Zee et al., 1995).

5.3.2. Reduction of Endogenous NGF in Rats via Administration of Antiserum

Female and male Wistar rats were injected sub-cutaneously (sc) in the dorsal fat pad during the periods described below and in the Results, with either anti-NGF or with pre-immune serum. The anti-NGF doses to neonatal and adult animals were 40 µl/g, on a weight basis, with the first day of life designated as P0. Neonatal female or male animals, respectively, were treated from either P0-14 ("neonatal" group) or P13-27 ("juvenile") while female adult animals were similarly treated from P60-74 ("adult"). A minimum treatment-free period of approximately 2.5 months was allowed in all animal groups prior to the examination of the various experimental procedures described below.

Because the antiserum dosage regime used here was greater than ten times the minimum dose needed to prevent collateral sprouting of nociceptive fibres in denervated skin of the adult rat (Diamond et al., 1992a), we believe it was supramaximal for the effects seen here.

5.3.3. Procedures to evaluate sizes of cutaneous sensory fields, and the collateral sprouting and regeneration of cutaneous sensory nerves

5.3.3.a. Isolation of a selected nerve field

Nociceptive nerve sprouting was evoked in adult female (180-250 g) or male (300-350 g) rats as previously described (Nixon et al., 1984; Doucette and Diamond, 1987; Diamond et al., 1992a). Briefly, in deeply anaesthetized rats (sodium pentobarbital, 50 mg/kg, intraperitoneal, ip) the entire sensory innervation of most of the back skin was permanently eliminated except for the medial branch of the dorsal cutaneous nerve of thoracic segment 13 (mDCN T13), whose sensory field was thus "isolated" within a vast surround of denervated skin. To examine nerve regeneration (Diamond et al., 1987; 1992b) the same surgical procedures were used but following an initial mapping of the pinch and heat innervation fields (see below) the mDCN T13 was crushed. Specially ground smooth-jawed forceps were applied at two-three sites, approximately 3 cm from the point of nerve entry into the skin, for 3 episodes of 30 sec each. Success of the crush was evaluated by reanesthetising the animal within 2 days and testing the appropriate region of skin for pinch sensitivity; none of the animals used in the study exhibited pinch responses at this time. In our earlier studies we showed that our crushing protocol was followed by near-perfect regeneration of the axons (Diamond et al., 1992b). All animals were protected against infection by an intramuscular injection of long-acting penicillin/streptomycin-based antibiotic. Incidence of overt infection was very low.

5.3.3.b. Field mapping

In lightly anaesthetized animals (sodium pentobarbital 30 mg/kg, ip),

mechanonociceptive ("pinch") fields were mapped using a pair of fine-toothed forceps, and heat-nociceptive ("heat") fields by briefly applying a copper probe whose temperature was maintained at 63-67°C. In order to improve heat transfer, the skin was dampened with a moist cotton swab prior to and during testing. Application of either of these noxious stimuli to the skin of rats results in a reflex contraction of the underlying *cutaneus trunci* muscle (CTM); this reflex is absent in denervated skin and it is *not* activated by stroking of the skin, a stimulus which excites the large myelinated Aβ-fibres within the mDCN T13. The "touch field" subserved by these fibres was mapped directly by recording the afferent impulses evoked in the nerve by brushing the skin with a fine bristle (Jackson and Diamond, 1984). A fine-tipped waterproof fibre pen was used to mark the skin at the border of responsive and unresponsive areas for all three sensory modalities. Following initial sensory field isolation and demarcation, a few spots were tattooed into the skin (about 10 mm from the border) using India ink. These spots provided reference points that allowed for subsequent comparison of field mappings done at later times. A permanent record of the various fields was achieved by transferring the outlined borders and the reference marks onto an acetate sheet using a waterproof marker; the respective areas were measured using an image analysis system (Image Research Inc., St. Catherines, ON., Canada).

5.3.4. Evaluation of "Precocious" Collateral Sprouting and of Axonal Regeneration

To measure collateral sprouting of pinch and heat fibers, nociceptive fields were first mapped on the day following denervation surgery (see above) with subsequent mappings performed every 7 days. Because of the initial mapping, the subsequent collateral sprouting

was "precocious" (Nixon et al., 1984; Doucette and Diamond, 1987); precocious sprouting is an otherwise normal collateral sprouting, but it's onset is reduced in latency by approximately 5d due to the effects of the impulses generated in the nociceptive nerves by the first post-isolation mapping procedure. The precocious sprouting paradigm allows for the identification of changes in field sizes to be directly compared with their initial values.

The same mapping procedure was used to follow the progressive recovery of pinch- and heat-nociceptive fields by regenerating nerves, but the mappings were made every two-three days following nerve crush until signs of recovery were observed, and then every 7 days up to day 35. In contrast to collateral sprouting, impulses evoked in the nociceptive nerves have no effect on their *regeneration* rate (Diamond et al., 1992b).

5.3.5. Testing the Ability of Exogenous NGF to Evoke or Accelerate Collateral Sprouting

The collateral sprouting paradigm was established as described above, in adult rats that had been treated neonatally with either anti-NGF or pre-immune serum from P0-14. The injections were made either in the neck or groin regions, and were previously shown (Diamond et al., 1992a) to act generally and not to have significant local effects except in rare instances; because of this possibility, however, injections were never made in the dorsal skin whose innervation was under study. Beginning on the day of sensory field isolation, and continuing daily for 14 days, the adult animals of each group received an injection (1μg/g in 0.5 ml PBS, ip) of 7S NGF or as a control, cytochrome C (Sigma; CytC). The time course of collateral sprouting (field expansion) was followed by the standard mapping procedure.

5.3.6. Evaluation of Nociceptive Thresholds

Mechanical and thermal nociceptive thresholds were examined in adult rats that had been treated with either anti-NGF or pre-immune serum from P0-14. Thermal thresholds were assessed by applying a copper probe (see above) to the dorsal skin that included the region innervated by the mDCN T13. Immediately prior to testing, the already-heated probe was cooled to about 35°C by transiently dipping it in ice cold water; thereafter the temperature, which was continually displayed, increased uniformly towards its set point of 67°C. The temperature at which the CTM response was evoked (see above) indicated the thermal threshold. To reduce possible heat sensitization influences (Willis and Coggeshall, 1991), this procedure was performed 10 times on alternating sides of the dorsal skin with 30 second intervals between probe applications. Since a satisfactory method was unavailable to determine the threshold for activation of the mechanonociceptive Aδ-fibres innervating thoracic hairy skin (the "pinch" fibres referred to above), we made the assumption that comparable threshold changes would be found in the functionally similar mechanonociceptive Aδ-fibres innervating the skin of the hindpaw. Calibrated Von Frey hairs (manufactured in our laboratory) of increasing force were applied to various regions of the dorsal foot in awake, hand-held animals; the threshold was taken to be the force that elicited a flexion response in about 80% of the trials. Again, to minimize errors due to possible sensitization phenomena the stimulus was applied 10 times at intervals of 30 seconds (for each test period) on alternating hindpaws.

5.3.7. Electron Microscopy and Quantitative Analysis of Axon Populations

At the terminal experiment, animals were deeply anaesthetized with sodium pentobarbital (50 mg/kg, ip) and a 2 cm segment of mDCN T13 was dissected out and processed for electron microscopic examination according to our previously published protocol (Diamond et al., 1992a). Approximately 10-15 electron micrographs were taken from a single section of the nerve; the fields of the micrographs partially overlapped, and included areas from the perineurium to the core of the nerve, so allowing for the entire nerve cross-section to be evaluated. Electron micrographs were taken at 2000X, enlarged 3X, and the enlarged prints were montaged for axon counting. To assist in the differentiation of axon types, fibres were classified into three groups: unmyelinated; small myelinated with an axonal cross-sectional area $\leq 7\mu m^2$, and large myelinated axons $>7\mu m^2$. The designated cutoff of the small myelinated axons at $7\mu m^2$ (Basbaum et al., 1991) ensured that this group included primarily Aδ nociceptors and Aδ low-threshold down hair mechanoreceptors (Burgess and Perl, 1973).

5.3.8. Molecular Studies

5.3.8.a. Operative procedures

All procedures were similar to those described by Mearow and colleagues (1993, 1994). For sprouting studies 36 adult female Wistar rats (18 rats treated from P0-14 with anti-NGF and 18 with control serum) were used; in each animal the cutaneous fields of the two nerves (DCN T10 and DCN T13) were isolated on each side of the midline. To achieve these isolations the left and right DCNs L1-L5, T8, T9, T11 and T12 on both sides were cut

about 1 cm from their point of emergence from the *latissimus dorsi* muscle. The skin was sutured using a continuous catgut thread. Since there is no overlap of the fields of the 2 spared nerves (Jackson and Diamond, 1984; Nixon et al., 1984; Doucette and Diamond, 1987), the nociceptive axons from each nerve were able to sprout into surrounding denervated skin on each side of the midline. At different time points following these procedures animals were deeply anaesthetized and decapitated; selected DRGs were quickly removed, immediately embedded in Tissue-tek and frozen on dry ice in preparation for sectioning. Samples were kept at -70°C until use.

5.3.8.b. *In situ* hybridization (ISH) and Data analysis

Since only thinly myelinated and unmyelinated sensory fibres undergo functional collateral sprouting (Doucette and Diamond, 1987; Diamond et al., 1992a), only small and medium sized DRG neurons were examined.

Tissue sections were processed for ISH of $p75^{NGFR}$ and *trk*A mRNAs using either a "cocktail" consisting of two 48-mer DNA oligonucleotides (^{35}S-labelled oligonucleotide antisense probes) complementary to the cDNAs encoding the highly conserved extracellular and intracellular segments of $p75^{NGFR}$ (Radeke et al., 1987) or TrkA (Meakin et al., 1992), or a 48-mer antisense DNA oligonucleotide that is complementary to the counterpart of base pairs 311-359 of rat GAP-43 (Basi et al., 1987). To assess signal specificity, sense probes were utilized as negative controls; in each case these hybridizations resulted in a loss of specific hybridization patterns that were observed with the antisense probes (results not shown).

Frozen DRGs were sectioned (10μm) and thaw-mounted onto gelatin-coated slides; control and treated sections from a minimum of two time points were mounted on the same slides. On the day of hybridization the slides were brought to room temperature, fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 5 min, washed with PBS (2 x 5 min), acetylated in 0.1*M* triethanolamine for 10 min then dehydrated in ethanol. Subsequently, ISHs were performed according to our established procedure (Mearow et al., 1994; Mearow and Kril, 1995). For cellular resolution, slides were dipped in Kodak NTB2 emulsion, dried, placed in desiccated light-tight plastic slide boxes and exposed at 4°C for 2-3 weeks. Slides were then developed with Dektol, fixed with Kodak fixer, rinsed in distilled water, and coverslipped using Permount.

The number of silver grains per neuron was quantified, in a total of 100 neurons from each DRG, using a computerized image analysis system (MCID; Image Research Inc., St. Catharines, ON. Canada) interphased with a microscope. Only neurons exhibiting a nucleus were used in the analysis. The area covered by autoradiographic silver grains (measured in pixels and converted to μm^2) in each neuron was divided by neuronal area (measured in μm^2) expressed as percentage neuronal area covered by silver grains, and referred to as neuronal grain density. Background grain density was similarly obtained from an adjacent region that had dorsal root axons but no neuronal somata; only signals >4x background were used in the analysis. It is recognized that the DRG neurons whose axonal sprouting or regeneration was studied amounted to only about 25% of the DRG neuron population (Kril and Diamond, unpublished data). Thus possible paracrine interactions in the DRG could lead to changes in

$p75^{NGFR}$, *trk*A or GAP-43 expression in the remaining 75% of the population; such a possibility would indicate a nerve growth-related phenomenon.

5.3.8.c. RT-PCR for NGF Expression

Totally denervated cutaneous tissue was examined in skin taken from three adult female rats that were treated with either anti-NGF or control serum as neonates. The adult denervations were done 7d before skin excision as follows: in the deeply anaesthetized animal a 4-5 cm midline incision was made in the dorsal skin; DCNs T8-L5 on the left side were sectioned approximately 2 cm from their exit points from the body wall; the proximal stump was ligated to prevent axonal regeneration. DCNs on the right side were untouched. Prior to sample removal rats were lightly anaesthetized and the physiological status of the skin was examined using the CTM reflex response (see above) to establish the boundaries between denervated and innervated skin. In deeply anaesthetized rats, approximately 2 cm^2 of denervated and innervated skin were removed, frozen quickly on dry ice, and sliced into smaller pieces for further processing.

Total RNA was isolated from innervated and denervated skin samples with Trizol (BRL), resuspended in diethylpolycarbonate (DEP)-treated water, and treated with DNase (Promega); RNA integrity was evaluated by electrophoresis through agarose gels and degraded samples were discarded. RT-PCR was performed according to our established protocol (Mearow et al., 1993). To detect NGF expression, internal primers to the NGF sequence were utilized [5'-CAT-GGTACAATCTCCTTCAAC-3', corresponding to nts 610-631 (upstream), and 5'-GCCTCTTC TTGCAGCCTTCCT-3' complementary to nts 991-1012

(downstream) of the rat NGF sequence (Whittemore et al., 1988)]. Amplification produced a 400 bp fragment. Primer pairs for cyclophilin [primer 1: complementary to nts 44-65; primer 2: complementary to nts 393-414 (Danielson et al., 1988)] were used as an internal control. Following agarose gel electrophoresis, the negatives of the ethidium bromide stained gels were analysed by densitometry; the ratio of the density of the rNGF fragment to that of the internal control was evaluated.

5.3.9. Statistical Analyses

All results are expressed as mean ± SEM, with the criterion for statistical significance being set at $P<0.05$. Using the SPSS 6.1 programme: 1) nociceptive fibre collateral sprouting was analysed using a three age (neonatal, juvenile and adult) x two treatment (control, anti-NGF) factorial analysis of variance (ANOVA) with repeated measures; 2) axonal regeneration, ISH results, and NGF gene expression were analysed using a 1 age (neonatal) x two treatment ANOVA with repeated measures; 3) axonal counts were analysed using a three age x two treatment ANOVA. Post hoc comparisons were performed using the Bonferroni test.

5.4. RESULTS

Adult rats treated with anti-NGF during the neonatal period had variable degrees of ptosis, attributable to a near-total elimination of tonic sympathetic innervation (Levi-Montalcini and Angeletti, 1966; Zaimis, 1965). The adult animals were otherwise indistinguishable from controls in regard to body weights, appearance, and general activity. However, a more detailed examination showed that the neonatal anti-NGF treatment had

significantly affected certain features of the peripheral sensory nervous system.

5.4.1. Collateral Sprouting of Nociceptive Fibres

Using our standard precocious collateral sprouting paradigm (see Methods), in which appropriate denervations are done to leave a single cutaneous sensory nerve field intact with its then "isolated" within a surround of denervated skin, both the pinch and the heat fields in the control group were found to have expanded significantly by 7 days (by approximately 53% and 45% respectively) (Figures 1A and 1B; cf. Diamond et al., 1987; 1992a). These expansions are attributable to the NGF-dependent collateral sprouting respectively of the Aδ and C fibres (Nixon et al., 1984; Doucette and Diamond, 1987; Diamond et al., 1987). In contrast, the adult animals of the P0-14 anti-NGF group showed no significant expansion of either field during the first 2 weeks, and only a small, though significant, increase by d21 (pinch, 48%; heat, 34%) (Figures 1A and 1B). Collateral sprouting was also reduced, though to a lesser extent (by 15 and 20% respectively for the Aδ and C fibres), in the adult animals treated with anti-NGF during the juvenile period (P13-27; Figures 2A and 2B), while no deficits in sprouting were seen in similarly treated adult rats examined about 60 days later (P60 to P74) (Figures 3A and 3B).

5.4.2. Administration of NGF Rescues Collateral Sprouting in Compromised Animals

To examine whether the impairment in the sprouting of the surviving nociceptive axons in the adult animal was due to loss of responsiveness to NGF, or due to the neurons having already sprouted earlier to a "ceiling" value, NGF injections were given daily for 14 days to adult rats (treated neonatally with either anti-NGF or control serum) throughout the

first 14d of the collateral sprouting paradigm (Diamond et al., 1992a). Surprisingly, there was now a significant expansion of both nociceptive fields in the group of sprouting-impaired adult rats (Figures 4A and 4B, pinch and heat respectively), compared to the control group of control sprouting-impaired animals treated with cytochrome C (Figures 4A and 4B). Moreover, the rate of Aδ and C fibre collateral sprouting was comparable to that in normal adult animals that also received the 14d period of NGF treatment. These findings are summarised in Table I, which shows the *rate* of sprouting in the various animal groups.

5.4.3. Axonal Regeneration

This growth response was totally normal in the sprouting-impaired animals. After a peripheral nerve crush that allowed nerves to regenerate freely along the degenerating distal pathway, responses to pinch were first detected 8-10d postcrush and to heat at about 12-15d postcrush in both sprouting-impaired and control animals, (Figure 5A), and the original pinch and heat field sizes had recovered by d21-28 of the regeneration paradigm (Figures 5B and 5C, respectively); by 35 days of nerve growth both nociceptive fields had approximately doubled their initial sizes, exactly as in control rats. Electrical recordings to test for the first appearance of the touch modality (the large myelinated (Aβ) axons), were not performed, because of the risk of damaging the more slowly regenerating axons within the nerve, but from a few preliminary examinations it was clear that this modality recovered much the same in control animals and in the animals treated neonatally with anti-NGF, and thus the regeneration of Aβ axons appeared also to be completely normal.

5.4.4. Behavioural Mechanical and Thermal Thresholds

Since DRG neuron numbers were reduced in the neonatally anti-NGF treated animals, their impaired collateral sprouting could have resulted from a reduced population of nociceptive terminals in the skin, we were interested in the possibility that behavioral thresholds may have been increased. This was not so. The animals that received anti-NGF from P0-14 had nociceptive thermal thresholds in the dorsal skin, and normal mechano-nociceptive thresholds in the hindpaw test (see Methods) that were indistinguishable from those in the control animals (Figure 6).

5.4.5. NGF Expression in Innervated and Denervated Skin

It is possible that both the reduction in initial nociceptive innervation territories, and the impairment in Aδ- and C-fibre collateral sprouting, could be explained by reductions in the usual NGF levels in the skin of the adult animals. Using RT-PCR we determined NGF expression in normally innervated skin of adult rats that were treated as neonates with anti-NGF; the values were indistinguishable from those of control rats (Figure 7). We also examined the possibility that NGF production in denervated skin is less than normal in the neonatally anti-NGF treated rats, especially given that in normal adult animals NGF expression in skin is significantly increased following its denervation (Mearow et al., 1993). As shown in Figure 7, the level of NGF mRNA was increased in denervated skin (Mearow et al., 1993) of neonatally anti-NGF treated rats to the same extent as that in the control group.

5.4.6. NGFR- and GAP-43 Gene Expression in DRG Neurons

Since NGF-responsivity appears to be modulated by the relative levels of $p75^{NGFR}$ and *trk*A receptors (reviewed by Segal and Greenburg, 1996), another possible explanation for the impaired collateral sprouting could be that these levels were altered by the neonatal anti-NGF treatment. To examine this possiblity we used *in situ* hybridization (ISH) to measure mRNA levels of these receptors both in the resting and sprouting states. We also measured the levels of GAP-43 (a growth-associated protein) mRNA.

The ISH findings of the resting mRNA mRNA levels in DRG from the adult sprouting-impaired rats (P0-14 anti-NGF treatment) were somewhat unexpected. While *resting* $p75^{NGFR}$ and GAP-43 mRNA levels were significantly increased in these animals (between 25- 30% higher than in the controls; Figure 8A), that of *trk*A was slightly, albeit significantly, reduced (by 6%; Figure 8A) compared to controls.

After 14d of collateral sprouting into adjacent denervated skin, mRNA values in control adult animals increased by amounts similar to those already reported (Mearow et al., 1994; Mearow and Kril, 1995): 95% for the low-affinity $p75^{NGFR}$ neurotrophin receptor (Figure 8B); almost 60% for the high affinity *trk*A receptor (which had a 72% peak at 10d; Figure 8C); 65% for the growth-associated protein GAP-43 (Figure 8D). The results from the DRG of the adult sprouting-impaired rats were somewhat unexpected. The mRNA for $p75^{NGFR}$ increased only slightly (by less than 10%) at day 14 (Figure 8B), but in contrast, the *trk*A mRNA levels *increased* during the (largely ineffective) collateral sprouting paradigm in an almost identical manner to that seen in the normally sprouting control animals (Figure 8C).

Perhaps reflecting the small degree of Aδ and C fibre collateral sprouting observed in the impaired animals (see Figures 1A and 1B), GAP-43 mRNA increased be about 25% from its resting level (Figure 8D), a value significantly less than in the control animals above ($p<0.01$).

mRNA measurements were also done in animals in which sprouting was "rescued" by 14 days of daily NGF injections. In the control animals the added sprouting evoked by the exogenous NGF (Diamond et al., 1992a) was associated with additional rises in mRNA for $p75^{NGFR}$, *trk*A and GAP-43 (Figures 8B,C and D). In the impaired animals in which collateral sprouting was "rescued" by the same exogenous NGF treatment, mRNA for $p75^{NGFR}$ now showed an increase of almost 25% (Figure 8B), while *trk*A was affected much as in the non-compromised animals (Figure 8C). mRNA for GAP-43 was also affected to about the same extent as in the non-compromised animals treated with NGF (Figure 8D). Note that in the animals treated with anti-NGF as neonates, $p75^{NGFR}$ and GAP-43 mRNA values were measured relative to resting levels that were already significantly increased (Figure 8A), as described above.

In summary, in sprouting-impaired animals in which mRNA for $p75^{NGFR}$ failed to rise, there was nevertheless a significant increase in mRNA for *trk*A, and a more modest one in mRNA for GAP-43. In these same animals, when collateral sprouting was restored by exogenous NGF, there were essentially normal increases in mRNA for trkA and for GAP-43, and a slightly greater than normal rise in mRNA for $p75^{NGFR}$.

5.4.7. Selective Loss of DRG Projections to the Skin, and Associated Reductions in Sensory Field Areas

Subpopulations of DRG sensory neurons exhibit some axonal branching, as discussed later, hence axon counts do not accurately predict absolute DRG neurons numbers. However, if axonal branching is assumed to be similar in control and experimental animals, then using axon counts to estimate DRG neuron *losses* would introduce negligible errors. The P0-14 anti-NGF treatment led to losses in cutaneous nerves of 17% in the thickly-myelinated (Aβ) fibres, 26% in the thinly-myelinated (Aδ) fibres, and 38% in the unmyelinated (C) fibres (Figures 9A, 9B and 9C, respectively). The P13-27 anti-NGF treatment affected only the C-fibre population (25% loss), while the same treatment of adult animals was without effect on axon numbers. Since we observed no differences in control animals of different treatment groups, axon counts from these animals were pooled and considered as one group. In addition, since preliminary examination showed normal ratios of myelination thickness relative to axon diameter (Willis and Coggeshall, 1991) it seems unlikely that an overall axonal shrinkage had occurred, causing, for example, Aβ fibres to show in the Aδ fibre range.

In adult rats treated of the P0-14 treatment group the sizes of pinch and heat fields were reduced by about 40% and 35% respectively (Figure 10). Despite these field reductions their areas were still large enough to ensure that no skin regions were devoid of nociceptive function; in normal animals the overlap of adjacent fields is substantial (Nixon et al., 1984; Doucette and Diamond, 1987). In contrast, the innervation fields subserved by Aβ (light touch) fibres in the treated rats were essentially normal (Figure 10). Even in the group of rats

treated at P13-27 the pinch and heat fields were reduced, but only by about 20 and 15% respectively (Figures 2A and 2B). The corresponding 2 week treatment of adult animals (Figures 3A and 3B) was without significant effect on any of the sensory modalities, although earlier studies showed that a somewhat longer course of treatment can cause reductions, especially in the heat fields (Diamond et al., 1988, 1991).

5.5. DISCUSSION

5.5.1. Neonatal NGF Deprivation Permanently Compromises Reparative Nerve Growth

In this study we show a vulnerability of peripheral nociceptive neurons to NGF deprivation in early postnatal life; thereafter the ability of the undamaged neuron to undergo the usual vigorous collateral sprouting into a nerve-free region of skin adjacent to their own field of innervation appears to be *permanently* impaired. In contrast, the regeneration of the same nociceptive axons after crush remains unaffected. The phenomenon is highly dependent upon the stage of development and the time of anti-NGF administration. Most affected were animals that had been treated daily in the P0-P14 period. P13-27 treatment resulted in a less profound impairment, whilst the same treatment to adult rats was entirely without effect. Significantly, while the compromised collateral sprouting is an entirely NGF-dependent growth (Diamond et al., 1992a), the unaffected regenerative axonal growth occurs independently of NGF (Diamond et al., 1992b).

5.5.2. Possible Explanations of the Sprouting Defect

The defect in sprouting is not attributable to an absence of NGF. In the affected adult animals, NGF was expressed normally in the skin, and the expression increased in the usual

manner following denervation (Mearow et al., 1993). Moreover, NGF availability in the adult skin would be expected to have increased following the neuronal loss in the affected animals (see below), which should reduce neuronal competition for endogenous neurotrophins (see Diamond, 1982; Korsching and Thoenen, 1985; cf. Woolf et al., 1996). Nor had the neurons become relatively unresponsive to NGF, or reached some limit in their capacity to sprout: when administered *exogenously*, typical doses of NGF continued to evoke sprouting in the compromised animals much as they did in the controls. Although possible impairments of axoplasmic transport were not studied, it is highly improbable that they existed, since all the following parameters were essentially normal in adults of the neonatally treated group: axon calibre (data not shown), nociceptive thresholds, rate of axonal regeneration, and rate of (exogenous) NGF-evoked collateral sprouting (cf. Grafstein and Forman, 1980; Leon and McComas, 1984; Diamond et al., 1992a; Riaz and Tomlinson, 1996).

5.5.3. Defective Uptake Mechanisms in Nociceptive Nerve Terminals?

We propose that the defective collateral sprouting in adult animals treated neonatally with anti-NGF results from an impaired ability of nerve terminals to increase their uptake of endogenous NGF when these rise, as they do in the adjacent denervated skin (Mearow et al., 1993). A degree of ongoing NGF uptake is required to account for the apparently normal health of the neurons, and some increase in its uptake would account for the minor sprouting that did occur in about 30% of animals, and for the unexpected normal increase in *trk*A, relative to $p75^{NGFR}$ mRNA levels in the DRG. The last finding indicates that much smaller increases in NGF are required to upregulate *trk*A gene expression than are needed to evoke

collateral sprouting or to induce an increase in $p75^{NGFR}$ expression; a similar proposal was made on the basis of results obtained in an earlier, different study (Mearow and Kril, 1995).

5.5.4. A Functional Compartmentalisation of the Neuron

We suggest, on the basis of the present and related findings, that the nociceptive terminals effectively occupy a functional compartment of the neuron, and that endogenous NGF is confined to the same compartment. Exogenous NGF, however, must act most effectively on "extracompartmental" regions of the neuron to bring about sprouting in the compromised and control animals. These proposals are supported by other evidence. A single systemic injection of NGF in rats induces hyperalgesia (Lewin et al., 1993) but not sprouting [sprouting requires at least six days of NGF injections (Diamond et al., 1992a, 1996)]; yet the endogenous NGF build-up that induces collateral sprouting into adjacent denervated skin does not cause hyperalgesia (unpublished observations). Further support for our proposal is based on observations made in mice with a targeted mutation in the low affinity NGF receptor ($p75^{NGFR}$): collateral sprouting into adjacent denervated skin fails to occur at all, despite the normal expression of NGF in the skin, and yet *exogenous* NGF readily induces characteristic nociceptive nerve sprouting (Diamond et al., 1995).

5.5.5. What Could Explain a Defective Responsiveness of NGF at Nerve Terminals?

Although the biological activity of NGF is mediated by the high affinity TrkA receptor (Kaplan and Stephens, 1994; Greene and Kaplan, 1995), there is strong evidence suggesting that responsiveness to NGF depends on the relative levels of this receptor and the $p75^{NGFR}$ receptor (Barker and Shooter, 1994; Hantzopulos et al., 1994; Miller et al., 1994; Verdi et

al., 1994). It is therefore conceivable that the NGF-deprivation in the neonatal period might have led to a situation in which the ratio of p75:TrkA at the *nociceptive terminals* assumes a value that does not favour the substantial uptake of NGF required to evoke either the normal sprouting or increase in $p75^{NGFR}$ gene expression. However, more than simply an abnormal differential expression pattern of the two receptors is needed to explain how the terminals could differ from the rest of the neuron in these respects, and a likely candidate is an alteration in intraneuronal trafficking of the receptors; such an alteration would need to have been induced by the neonatal anti-NGF treatment (Ehlers et al., 1995; but see also Laduron, 1995). We are presently examining transport of endogenous NGF in cutaneous nerves of normal and compromised animals subjected to the collateral sprouting paradigm.

5.5.6. Neonatal Anti-NGF Treatment and DRG Neuron Loss

As mentioned earlier, because of axon branching (Langford and Coggeshall, 1981; McCarthy et al., 1995; Nagy et al., 1995, see also Tandrup, 1995), the extrapolation of axon counts to DRG neurons overestimates absolute DRG neuron numbers. However, our interest is in DRG neuron *losses* in the compromised animals; if axon branching can be assumed to be similar in compromised and control animals then *differences* in the highly accurate axon counts can be accepted as good measures of the differences in numbers of their parent neurons in the DRG (cf. Coggeshall et al., 1997). On this basis the P0-14 anti-NGF treatment led to a 26% loss of the smaller neurons that give rise to Aδ fibres, a 38% loss in the small neurons associated with the unmyelinated C fibres and a 17% loss in the large DRG neurons that give rise to the Aβ low threshold mechanosensory fibres (cf. Jenq et al., 1984;

Hulsebosch et al., 1987; Ritter et al., 1991; Urschel et al., 1991). Given the very small proportion of sympathetic fibres in these cutaneous nerves (5-8% of the total unmyelinated population; unpublished observations), and their virtual elimination by the neonatal anti-NGF treatment (Levi-Montalcini, 1987), we use the unmyelinated fibre counts as referring entirely to the nociceptive C fibers (Willis and Coggeshall, 1991; Lawson, 1992). It seems improbable that an overall axonal shrinkage occurred, causing, for example, Aβ fibres to show in the Aδ fibre range, since our preliminary examination shows normal ratios of myelination thickness relative to axon diameter.

Ultimately, the size of segmental nociceptive fields in the adult animal reflect the outcome of three interactive mechanisms during development, namely the existence of developmentally-determined segmental target territories, the neuritic arborization achieved by individual nociceptive fibres under the influence of target-tissue derived neurotrophic factors, and the competition for target sites (ill-defined though these are for nociceptive fibres) among neighbouring segmental nerves (Diamond, 1982; Diamond et al., 1992c). The neonatal anti-NGF treatment could well have affected the second of these mechanisms (cf. Constantinou et al., 1994) thereby accounting for the reduced nociceptive field sizes. Despite these reductions, the fields were still large enough to ensure an overlap between the fields corresponding to adjacent DRGs (Nixon et al., 1984; Doucette and Diamond, 1987) so that no skin regions would be devoid of nociceptive function.

In contrast to reports that anti-NGF treatment fails to cause sensory neuron death after age P2 (Lewin et al., 1992), we found a 25% loss of C fibres in the adult animals of the

P13-27 treatment group. Dosage differences may explain these anomalies; the antiserum regimen used in the present studies was about fifteen times the minimum needed to prevent collateral sprouting of nociceptive fibres in denervated skin of the adult rat (Diamond et al., 1992a).

5.5.7. Neurotrophins Targeted by Neonatal Anti-NGF

The reduction in Aδ and C fibres in the P0-14 anti-NGF group is understandable, given that the majority of the parent neurons of these fibres express *trk*A mRNA (McMahon et al., 1994; Averill et al., 1995). However, since our antibody likely interferes with NT-3 function as well as NGF (Van der Zee et al., 1995; see data referred to in the Methods section), the postnatal loss of Aβ fibres may be explained by the findings that a small proportion of large DRG neurons express TrkA (Averill et al., 1995; Molliver et al., 1995), and that at least 15% of large DRG neurons express *trk*C mRNA (McMahon et al., 1994). Of relevance, NT-3 mRNA (Hohn et al., 1990a,b; Maisonpierre et al., 1990a), NGF and NGF mRNA (Goedert et al., 1986; Davies et al., 1987; Constantinou et al., 1994) are all highly expressed in skin during development.

5.5.8. Neonatal Anti-NGF Treatment and Neuronal Phenotype

Neonatal anti-NGF treatment has been claimed to bring about a phenotypic conversion by which high threshold Aδ and C fibres become low threshold ones (Ritter et al., 1991; Lewin et al., 1992; Lewin and Mendell, 1994). However, if this occurs, it is not the explanation of our findings. A significant collateral sprouting impairment was found in adults of the P13-27 (juvenile) group whose neonatal treatment was well outside the reported

critical period (P4-11) for the claimed phenotype switching (reviewed by Lewin, 1996). As well, in a few preliminary experiments (data not included) we limited the anti-NGF injections to exactly coincide with this critical period, and found subsequent sprouting defects that were, however, considerably less than for the P0-14 group. Of relevance, as in normal rats, light stroking of the back skin in all of our compromised animal groups failed to evoke the CTM reflex, which is normally activated by mechanical stimulation of high threshold Aδ mechano-nociceptive endings; if phenotype switching does indeed occur, the newly acquired low threshold Aδ fibres either must lose their former intraspinal connections (cf. Lewin and Mendell, 1996) or - perhaps less likely - these connections could not have yet developed at the time of the reported switching.

5.5.9. Possible Significance of the Findings

Although the present studies were confined to peripheral nociceptive neurons, they may have a more general significance. Three speculations are pertinent: (i) can the results be extrapolated to other neurons of the nervous system? (ii) would a compromising of collateral sprouting elsewhere in the nervous system have adverse implications for recovery from trauma or disease of the nervous system? (iii) could adverse stressful conditions of the neonatal, infant, or even *prenatal* organism be associated with a coincident trophic inadequacy and subsequent compromising of sprouting of the kind evoked by anti-NGF treatment? If collateral sprouting, which is expressed in the adult central as well as peripheral nervous system, is a potentially beneficial reparative nerve growth, then question (ii) can be answered in the affirmative. For example, motor nerve sprouting in the nerve-deprived

muscles of ALS patients significantly delays the appearance of motor disabilities (reviewed by Dantes and McComas, 1991). Even if collateral sprouting in the CNS can cause adverse consequences such as pain or spasticity (Woolfe et al., 1992; Mannion et al., 1996), to discard the usefulness of sprouting on this basis is akin to dismissing the usefulness of the immune system because of the occurrence of autoimmune disease. Although we have no answers to questions (i) and (iii) above, it is tempting to speculate firstly, that differences in the capacities of adult humans to recover from neurological trauma or disease, or in their susceptibility to disorders such as Alzheimer's or Parkinson's disease, are in part explained by differences in the extent of the compensatory neuronal sprouting that can be achieved in their damaged central nervous systems, and secondly, that these differences are the permanent sequelae of trophic deprivations during early development, possibly associated with short-lived clinical or sub-clinical illnesses that may have even escaped detection at that time.

Figure 1. Expansion of the mDCN T13 pinch (A) and heat (B) fields into surrounding denervated skin at various times of the precocious collateral sprouting paradigm. In this Figure, and in Figures 2, 3, 4 and 10, the ordinate shows the "field area" in mm^2, and the abscissae the times after field isolation when the measurements were made. Solid bars are the results from adult animals treated neonatally with anti-NGF (n=11) and the open bars show the findings from their control littermates (n=10). Data shown as mean ± S.E.M. *=statistically significant ($p<0.01$).

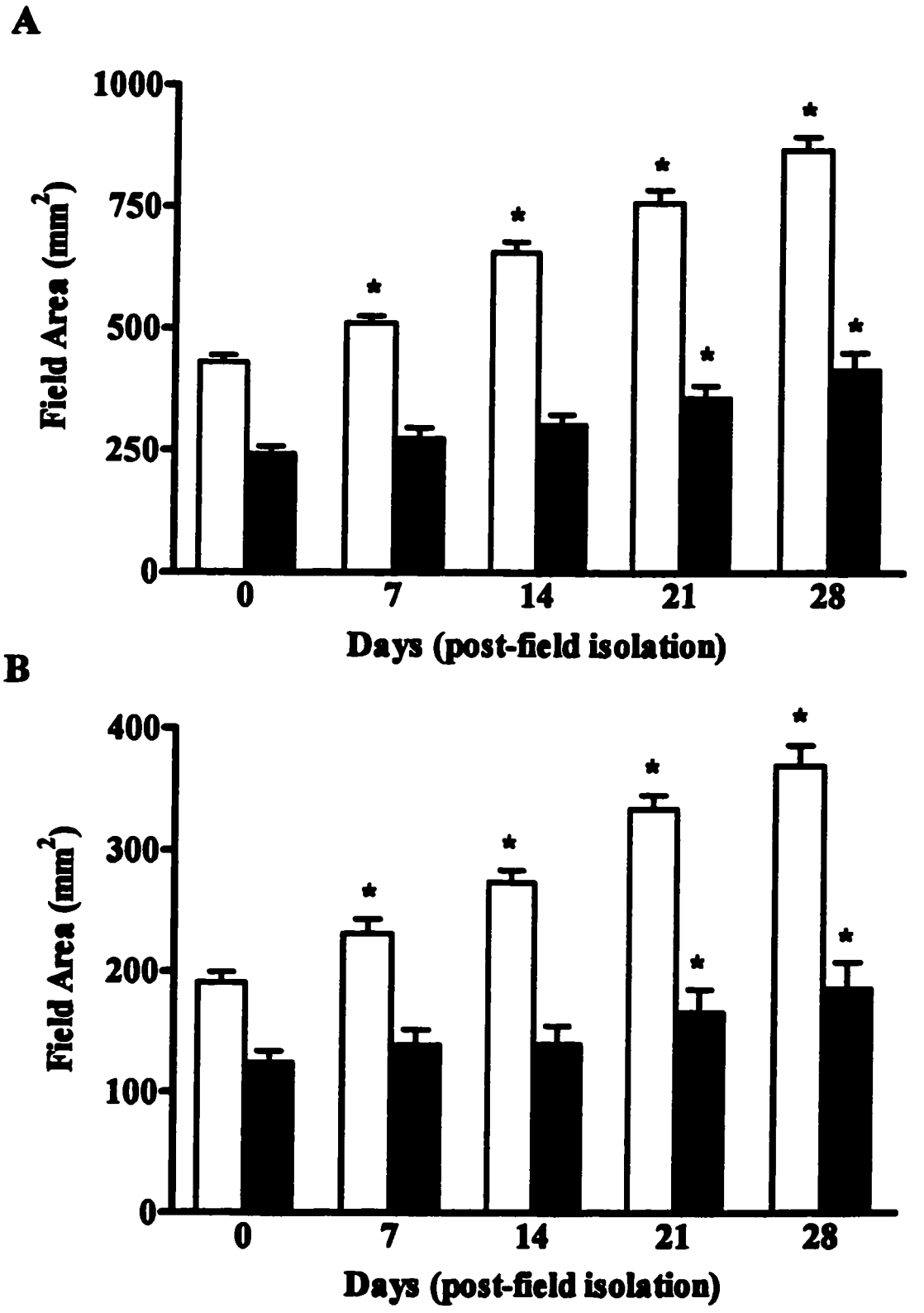
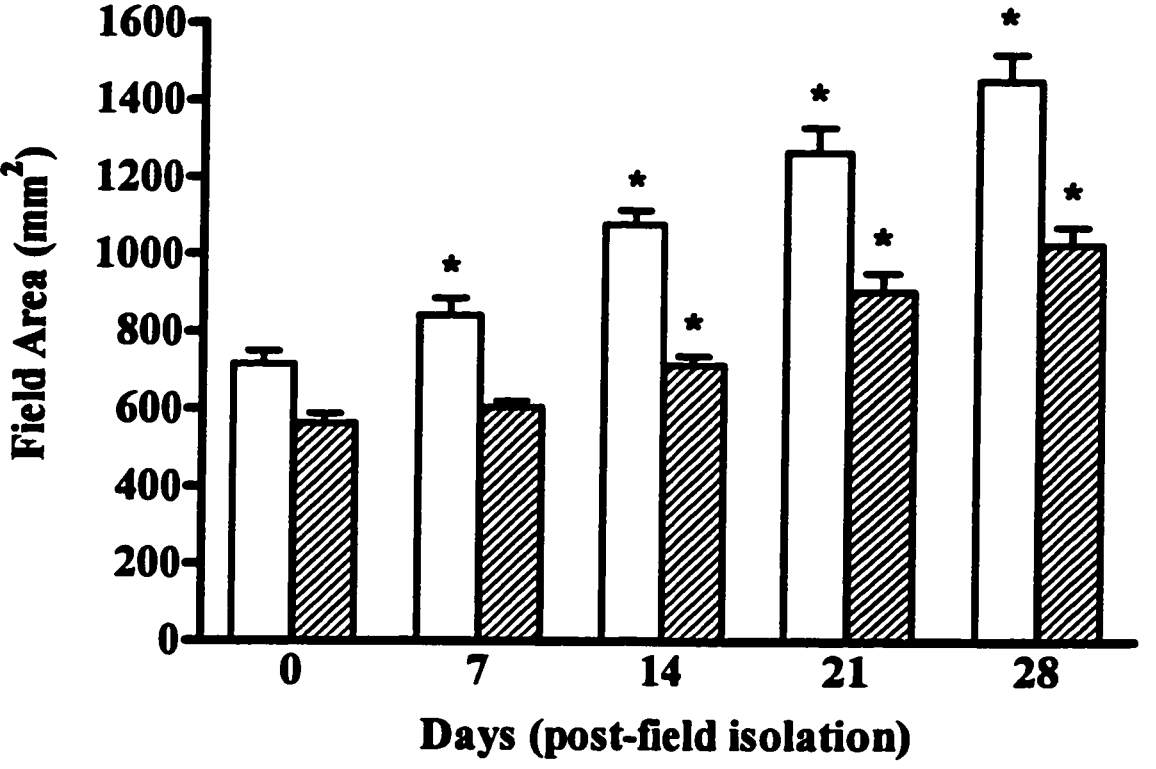

A
1000
750
500
250
0
Field Area (mm²)
0
7
14
21
28
Days (post-field isolation)
B
400
300
200
100
0
Field Area (mm²)
0
7
14
21
28
Days (post-field isolation)

Figure 2. Pinch (A) and heat (B) field expansions following isolation, in adults treated with anti-NGF as juveniles (diagonal bars, n=9), compared to control littermates (open bars, n=5). Note that the reduction in initial nociceptive field sizes, and the still significant impairment in collateral sprouting of Aδ and C fibres (compare with Figure 3). Data shown as mean ± S.E.M. *=statistically significant ($p<0.01$).

A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1400
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800
600
400
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Field Area (mm²)
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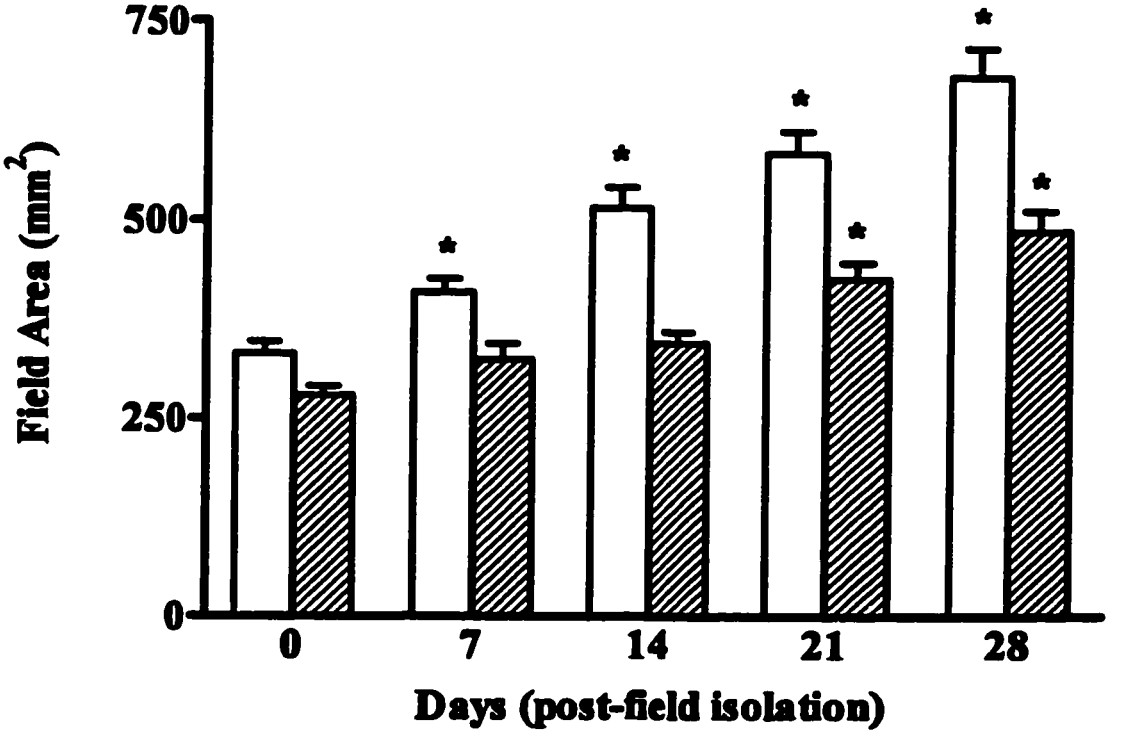
7
14
21
28
Days (post-field isolation)

B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Field Area (mm²)
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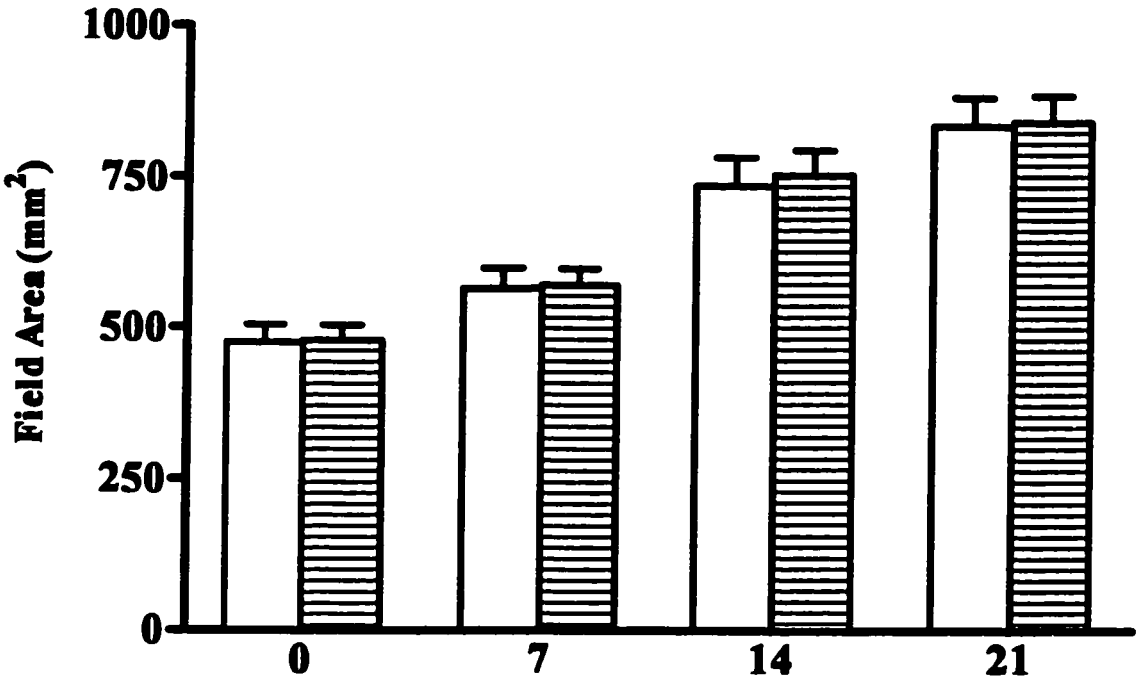
21
28
Days (post-field isolation)

Figure 3. Anti-NGF treatment to adult rats (horizontal bars, n=8) does not alter either initial nociceptive innervation field sizes (but see text) (A, pinch; B, heat) or collateral sprouting as compared with the control group (open bars, n=7). Data shown as mean ± S.E.M. Data shown as mean ± S.E.M. *=statistically significant ($p<0.01$).

A


1000
750
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Field Area (mm²)
0
7
14
21
Days (post-field isolation)

B

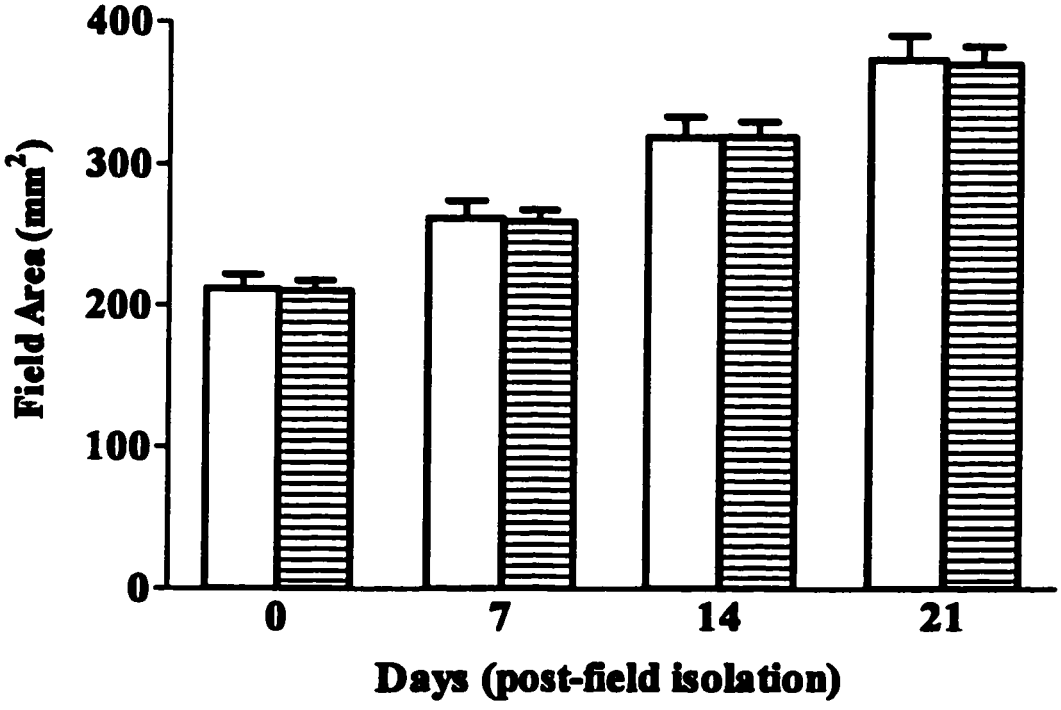

400
300
200
100
0
Field Area (mm²)
0
7
14
21
Days (post-field isolation)

Figure 4. Exogenous NGF "rescues" impaired sprouting. Following field isolation and initial mapping, each group (n=4) of adult animals were administered either NGF or CytC injections for 14 days; subsequent field measurements were made at various postisolation time intervals (see Methods). Note that Aδ (A) and C (B) fibre collateral sprouting in control animals that received daily CytC (open bars) is increased by daily administration of NGF (cross-hatched bars). Importantly, although Aδ (A) and C (B) fibre collateral sprouting is absent in the compromised adult rats treated daily with Cyt C (solid bars), it is clearly increased by daily NGF injections (checkered bars). Data shown as mean ± S.E.M. *=statistically significant ($p<0.01$).

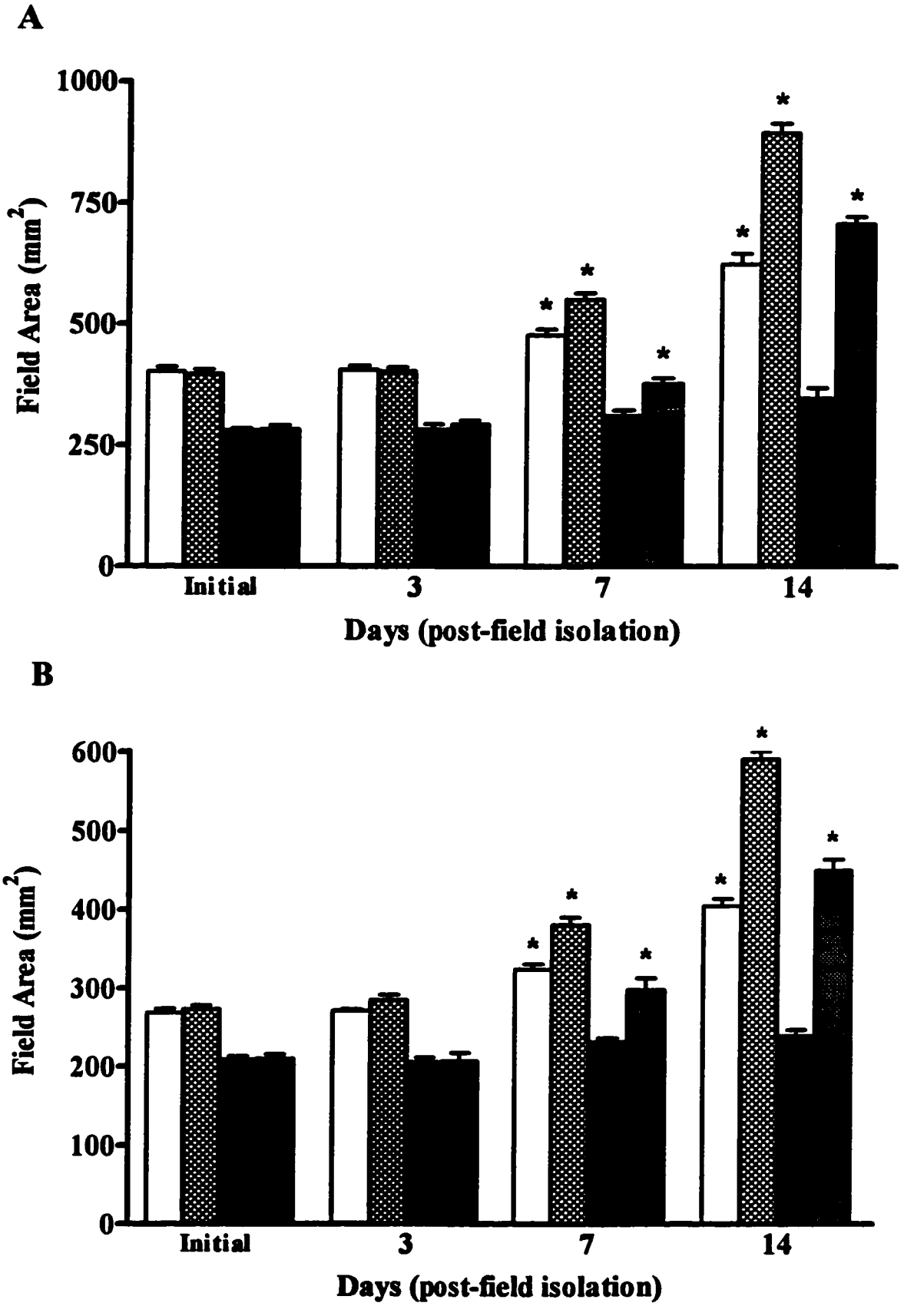
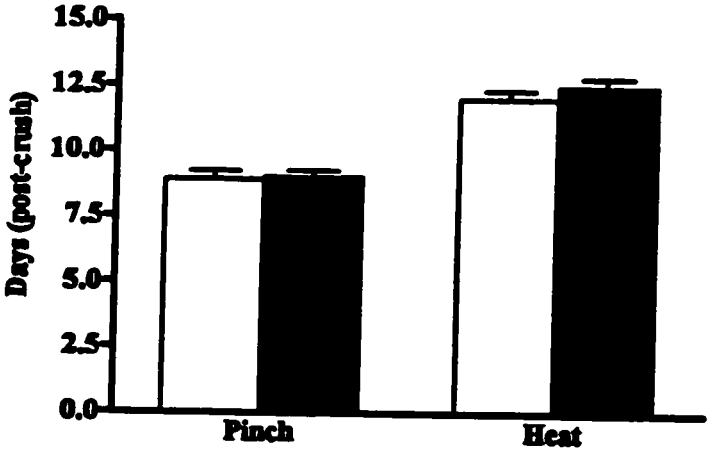

A
1000
750
500
250
0
Field Area (mm2)
Initial
3
7
14
Days (post-field isolation)
B
600
500
400
300
200
100
0
Field Area (mm2)
Initial
3
7
14
Days (post-field isolation)

Table 1. Effect of NGF treatment on the rate of Aδ and C fibre collateral sprouting in sprouting-impaired and control rats. Daily NGF or CytC was administered as described in the Methods. Non-linear regression analysis was used to determine the rate of collateral sprouting in the various treatment groups. Data is presented as $mm^2/day \pm$ S.E.M.

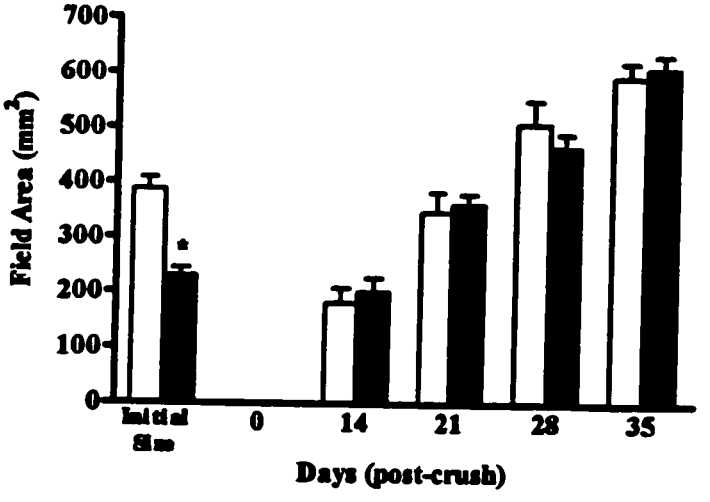
	Sprouting-Impaired Animals		Normal Adult Animals	
	14d daily CytC	*14d daily NGF*	*14d daily Cyt C*	*14d daily NGF*
Aδ Fibres	5.0 ± 0.6	31.4 ± 6.9	16.8 ± 2.6	37.5 ± 6.5
C Fibres	2.5 ± 0.8	18.33 ± 3.0	10.3 ± 1.4	23.7 ± 3.5

Figure 5. Nociceptive reinnervation by regenerating Aδ and C fibres following nerve crush (see Methods). The latency to the first appearance of nociceptive function in the denervated skin from the pinch and heat nociceptive modalities (A), and the subsequent expansion of the pinch (B) and heat (C) fields was not different in adult rats treated with anti-NGF as neonates (solid bars, n=11) compared to their control littermates (open bars, n=9). Data shown as mean ± S.E.M. There was no statistical significance between the groups at any timepoint examined during the regeneration paradigm ($p>0.1$).

A


15.0
12.5
10.0
7.5
5.0
2.5
0.0
Days (post-crush)
Pinch
Heat

B


700
600
500
400
300
200
100
0
Field Area (mm²)
*
Initial Size
0
14
21
28
35
Days (post-crush)

C

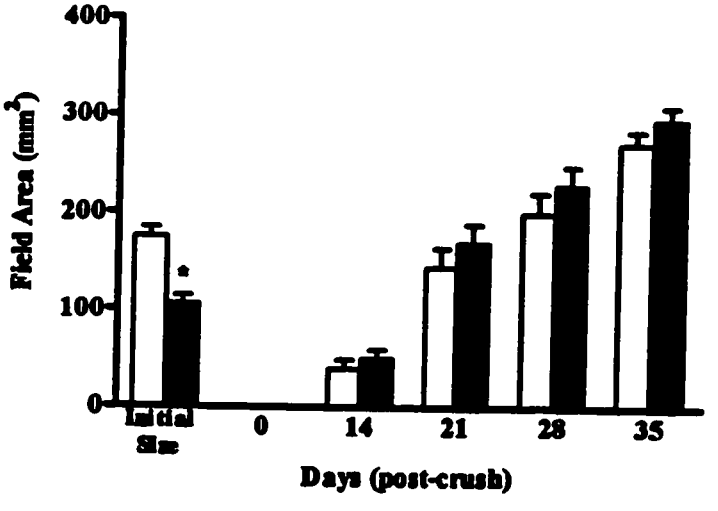

400
300
200
100
0
Field Area (mm²)
*
Initial Size
0
14
21
28
35
Days (post-crush)

Figure 6. Mechanonociceptive thresholds (force that elicits foot withdrawal) and heat-nociceptive thresholds (probe temperature that evokes the CTM response) were not significantly different in adults treated neonatally with anti-NGF (solid bars, n=5) relative to the control group of animals (open bars, n=5). Data shown as mean ± S.E.M.

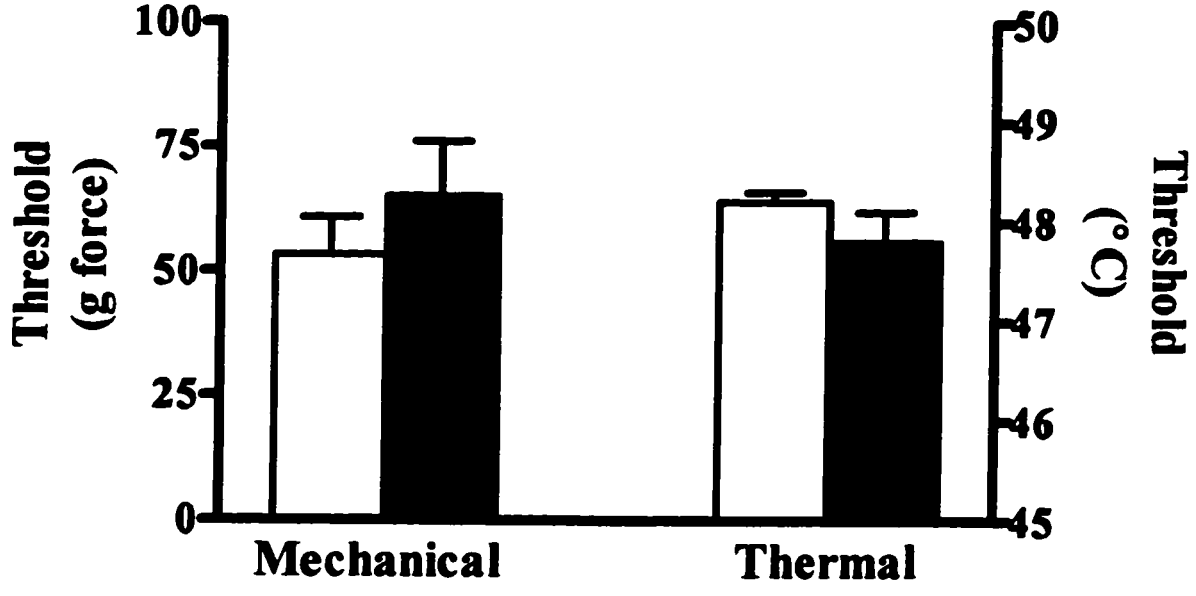

Threshold
(g force)
100
75
50
25
0
Mechanical
Thermal
50
49
48
47
46
45
Threshold
(°C)

Figure 7. PCR of reverse transcribed RNA isolated from skin of adult animals treated as neonates with either control serum (open bars, n=3) or anti-NGF (solid bars, n=3). The results show the ratio of the relative optical density of the NGF band to the internal reference (cyclophilin) band (see Methods) (mean±S.E.M. from each group). Note the *normal increase* in the NGF cDNA in denervated skin taken from both the control (open bars) and the treated (solid bars) animals. Data shown as mean ± S.E.M. *=statistically different from innervated skin ($p<0.01$).

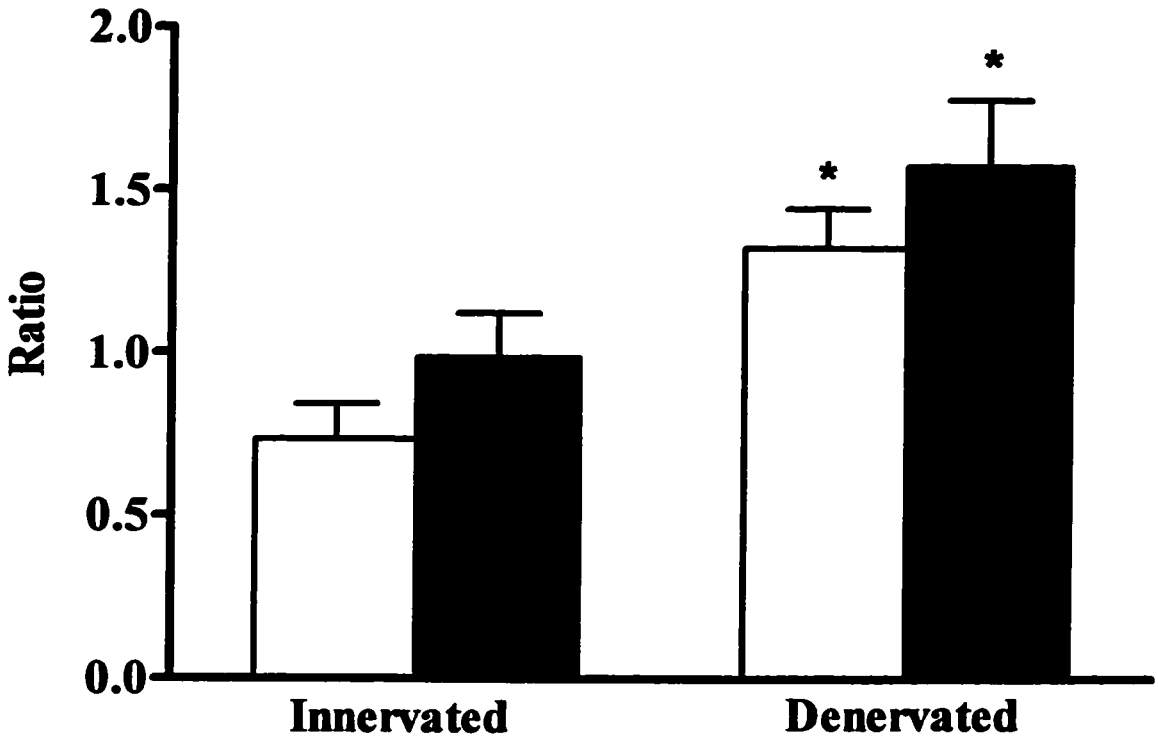

2.0
1.5
1.0
0.5
0.0
Ratio
*
*
Innervated
Denervated

Figure 8. ISH results of expression of p75, *trk*A and GAP-43 mRNA in DRG neurons from adult animals administered either anti-NGF (solid bars) or control serum (open bars) as neonates (see Methods). The results of three experimental groups (three animals per time point, n=300 cells) are averaged and expressed relative to the basal (A) values of grain density for DRG neurons from control (open bars, normal sprouting) and anti-NGF (solid bars, sprouting-impaired) animals. While $p75^{NGFR}$ (B) and GAP-43 (D) mRNA levels remained relatively unaltered throughout the entire timecourse of the sprouting paradigm in the sprouting-impaired animals, the levels of *trk*A (C) mRNA increased normally. mRNA measurements were also made of DRGs taken on the 14^{th} day of the collateral sprouting paradigm but an NGF injection was given every day in these animals (14+NGF). Note that while the exogenous NGF treatment increased the levels of all three markers in both control and treated animals as measured on the 14^{th} day (B,C,D), the *proportional* rise in $p75^{NGFR}$ and GAP-43 expression levels were relative to an already significantly increased resting levels in the treated animals (A). *=Statistically significant from basal value ($p<0.01$). **= Statistically significant from d14 (in the absence of exogenous NGF) ($p<0.01$).

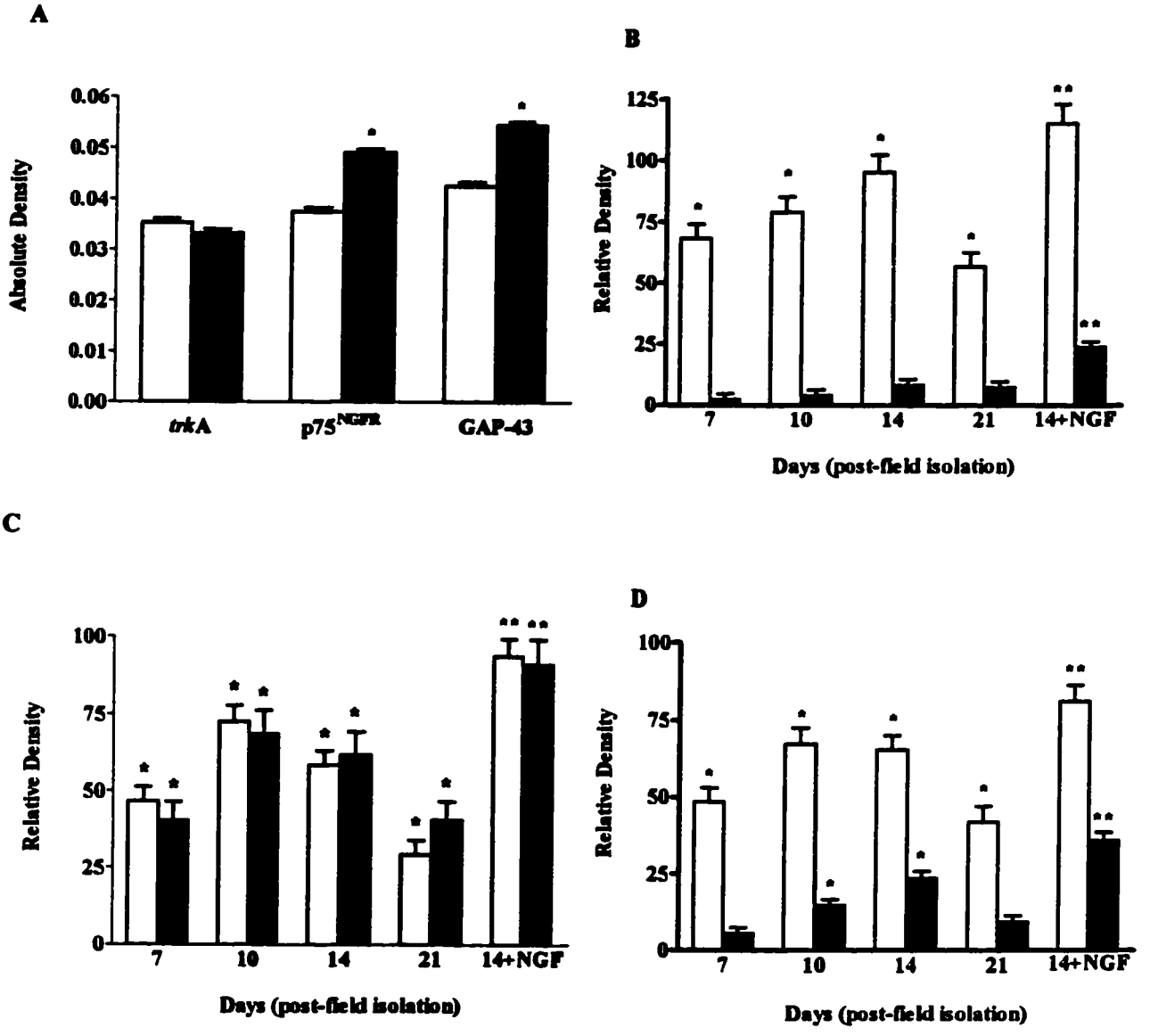
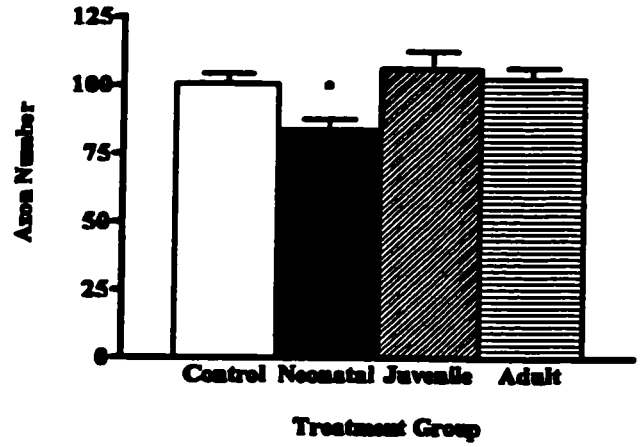
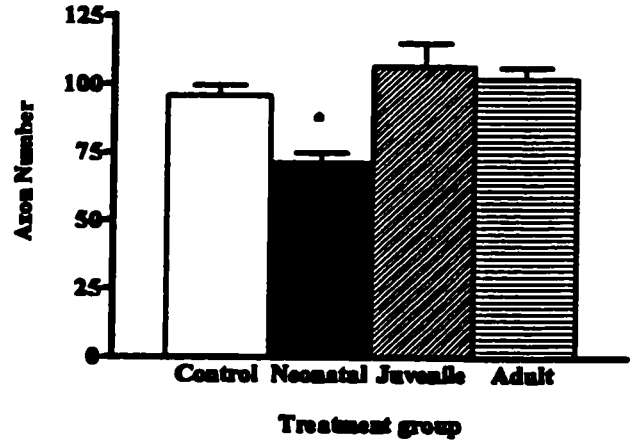

A
Absolute Density
0.06
0.05
0.04
0.03
0.02
0.01
0.00
trkA
p75NGFR
GAP-43
B
Relative Density
125
100
75
50
25
0
7
10
14
21
14+NGF
Days (post-field isolation)
C
Relative Density
100
75
50
25
0
7
10
14
21
14+NGF
Days (post-field isolation)
D
Relative Density
100
75
50
25
0
7
10
14
21
14+NGF
Days (post-field isolation)

Figure 9. Total number of axons in mDCN T13 of *adult* rats from various treatment groups (see Methods). Control group (open bars, n=21); adult animals that received anti-NGF as (i) neonates (solid bars, n=15), (ii) juveniles (diagonal bars, n=4) or (iii) adults (horizontal bars, n=9). A: thickly-myelinated Aβ ("light-touch") fibres; B: thinly-myelinated Aδ ("pinch") fibres; C: unmyelinated C ("heat") axons. Data shown as mean ± S.E.M. *=statistically significant ($p<0.01$).

A


125
100
75
50
25
0
Axon Number
Control
Neonatal
Juvenile
Adult
Treatment Group

B


125
100
75
50
25
0
Axon Number
Control
Neonatal
Juvenile
Adult
Treatment group

C

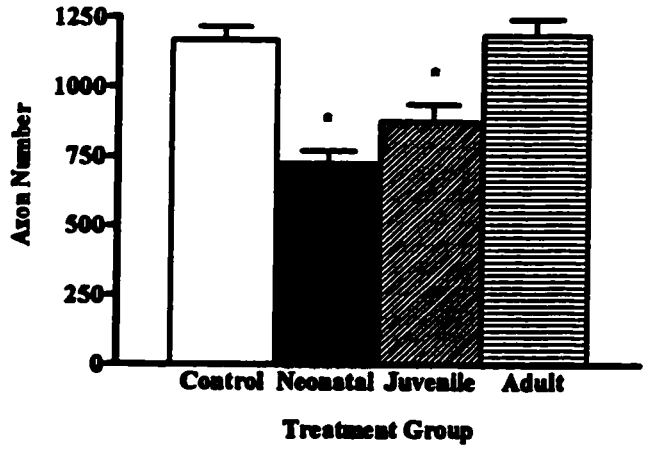
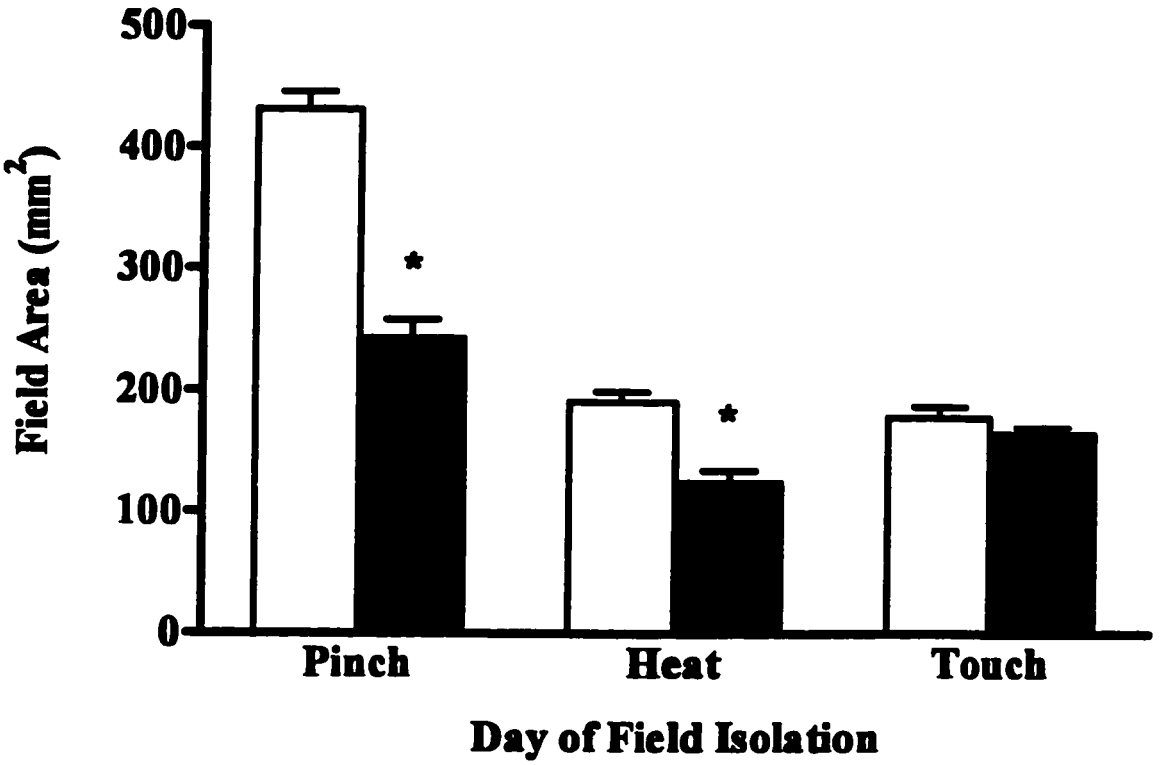

1250
1000
750
500
250
0
Axon Number
Control
Neonatal
Juvenile
Adult
Treatment Group

Figure 10. mDCN T13 innervation fields of pinch, heat and touch modalities examined in adult rats (see Methods). Control group (open bars, n=25); adults treated neonatally (P0-14) with anti-NGF (solid bars, n=32). Data shown as mean ± S.E.M. *=statistically significant ($p<0.01$).


500
400
300
200
100
0
Field Area (mm^2)
*
*
Pinch
Heat
Touch
Day of Field Isolation

CHAPTER 6

UNIFYING DISCUSSION

The results of each chapter have been discussed at length, and as such, will not be repeated here. Instead, this chapter will review the salient findings presented in this thesis and their contribution to the understanding of injury-evoked nerve growth processes, particularly in the context of their differing growth factor dependency.

The primary role attributed to NGF during development is to mediate the role of the peripheral field in promoting neuronal survival and the establishment of proper patterns of neural connectivity. At some point in the early postnatal period, the conventional role of NGF appears to change from one of a survival factor to one that regulates or maintains the phenotype of sensory neurons, including the regulation of the neurons' ability to respond to nerve injury by collateral sprouting or axonal regeneration. Morphological and physiological evidence suggests that both types of axonal growth processes share a common cellular mechanism(s) that is responsible for the laying down of cytoskeletal and membrane constituents of the growing neurites, whereas the mechanisms that *initiate and maintain* the two axonal growth processes appear to be substantially different. Collateral sprouting of intact nociceptive axons is evoked and maintained by NGF (Diamond et al., 1987, 1992a) whereas regeneration of these same axons is triggered by axotomy and continues quite normally in the absence of NGF (Diamond et al., 1992b).

6.1. Role of NGF in the Collateral Sprouting Response in the Adult Rodent

The results of the present study indicate, at the molecular level, that $p75^{NGFR}$ and *trk*A mRNAs increased in DRG neurons whose intact axons had been stimulated to undergo collateral sprouting but were absent when the same axons were made to regenerate by a crush injury. These findings support and extend previous morphological and behavioural results revealing the critical involvement of NGF in mechanisms regulating the collateral sprouting of intact axons but not in those governing the regeneration of injured ones (Diamond et al., 1992a, 1992b). The temporal changes in message observed in this study are also consistent with the physiological findings of Diamond and colleagues (1992a) which indicate that functional collateral sprouting of nociceptive axons begins by 8-10d after cutaneous denervation. It is suggested by these authors, that approximately 2 days of this period are required for the availability of endogenous NGF to increase in denervated skin. The present finding that NGF mRNA levels increase in skin at about 2-4 days after its denervation strongly supports this contention. Furthermore, assuming that an increase in NGF protein follows the increased mRNA levels after a lag time of 12-24 hrs (cf., Heumann and Thoenen, 1986), then increases in target-derived NGF would be made available to the newly growing neurites by 3-4 days following cutaneous denervation. However, the precocious sprouting paradigm indicates that increased NGF levels must be present some 5d earlier than for normal (non-precocious) sprouting. Presumably even before increased NGF *synthesis* occurs, the absence of nerves that normally compete for NGF in skin must lead to NGF protein increases, probably within 24 hours after denervations. That exogenous NGF upregulates the

expression of $p75^{NGFR}$ and *trk*A both *in vitro* and *in vivo* (see Introduction), strongly suggests that the early denervation-induced increases in skin NGF levels are most likely responsible for the upregulation in the levels of mRNAs for $p75^{NGFR}$ and *trk*A in sprouting DRG neurons (whose axon terminals would be located in appropriate areas of skin). Indeed, the finding that NGFR mRNA levels are increased by 4-6 days in sprouting DRG neurons is consistent with their being evoked by increased NGF availability in the denervated target. Further support of this interpretation is derived from the present finding demonstrating that an anti-NGF treatment which effectively blocks collateral sprouting also blocks the sprouting-associated increase in $p75^{NGFR}$ mRNA; however, there was a significant, albeit transient, increase in *trk*A mRNA levels in DRG neurons whose axons were prevented from undergoing sprouting by the anti-NGF treatment. One possibility to explain the increase in *trk*A mRNA is related to the fact that the higher affinity NGF receptor TrkA responds to a lower concentration of endogenous NGF than the lower affinity NGF receptor, $p75^{NGFR}$ (Barker and Shooter, 1994; Miller et al., 1994; Chao and Hempstead, 1995). Thus, the anti-NGF treatment presumably did not completely eliminate all sources of NGF, and the concentration of the residual NGF was sufficiently high to bind and activate TrkA, thereby leading to an increase in its expression level. Of relevance, however, is the fact that this increase was transient (less than one week duration), a result which suggests that the overall effect of the maintained anti-NGF treatment was a reduction in the concentration of endogenous NGF below that which is capable of altering TrkA gene expression. In conclusion, these results when taken together provide strong support for the hypothesis that NGF plays a key role in regulating the

collateral sprouting of intact primary nociceptive afferents (reviewed by Diamond et al., 1992c) but is not involved in their regenerative response following injury. Importantly, these results also support the hypothesis that NGFRs may play an integral role in the regulatory mechanism governing this form of axonal growth.

6.2. Proposed Role of NGFRs in the Collateral Sprouting Response of Adult Rats

The exact contribution of the different NGF receptors to the regulation of collateral sprouting remains to be fully established. However, it seems quite clear that even though $p75^{NGFR}$ by itself is insufficient to mediate neurotrophin-evoked signal transduction it does play a role in establishing neuronal responsiveness to NGF by modulating high affinity neurotrophin binding and NGF-evoked Trk-signalling (see Introduction). In this regard, the contribution of the NGFRs to the collateral sprouting response is made evident from the following findings. Firstly, recent evidence suggests that the collateral sprouting of central neurons evoked by TrkA activation after lesioning the fimbria fornix *in vivo*, requires the presence of $p75^{NGFR}$ (Lucidi-Phillipi et al., 1996). In that study the collateral sprouting of central neurons evoked by the intracerebroventricular infusion of a TrkA agonist was abolished when $p75^{NGFR}$ was prevented from functionally interacting with either itself or TrkA receptors. Secondly, in $p75^{NGFR}$ mutant mice the collateral sprouting of primary afferent nociceptive neurons is completely absent despite the presence of TrkA in DRG neurons (Diamond et al., 1995). These findings, in conjunction with the observations made in this study regarding the temporal expression of NGFRs in sprouting DRG neurons, provide strong support for the hypothesis that $p75^{NGFR}$ and TrkA play crucial roles in the collateral sprouting

response of both central and peripheral neurons. Moreover, these result strongly support the contention that the ratio of $p75^{NGFR}$ to TrkA (see Introduction), rather than the absolute level of either receptor, is a critical factor in determining neuronal responsivity *in vivo*. However, these proposals relate to the nerve *terminals*. Although the determination of the ratio of the NGF receptors on the nerve terminals undergoing collateral sprouting *in vivo* was not examined in the present study, the fact that both $p75^{NGFR}$ and *trk*A mRNAs were upregulated in sprouting DRG neurons suggests that the ratio was maintained, or at the very least was closely approximated during the collateral sprouting paradigm in the rest of the neuron. This also explains why *exogenous* NGF was able to "rescue" collateral sprouting, while the endogenous NGF was relatively ineffective.

6.3. Role of NGF in Axonal Regeneration in the Adult Rodent

In this study it was shown that the regeneration of nociceptive axons after nerve crush is not accompanied by significant alterations in either $p75^{NGFR}$ or *trk*A mRNA levels in DRG neurons; there was an initial, but not maintained, slight decrease in $p75^{NGFR}$ mRNA, but no change in *trk*A mRNA. While these findings are entirely consistent with those of Sebert and Shooter (1993), they differ from those observed by Ernfors et al. (1993) who noted an increase in $p75^{NGFR}$ mRNA but no change in *trk*A mRNA levels after a sciatic nerve crush. Moreover, the results of the present study are in direct contrast with other reports in which both $p75^{NGFR}$ and *trk*A mRNA levels in adult DRG neurons were shown to be downregulated for prolonged time periods subsequent to either a crush injury or a complete transection of the sciatic nerve (Verge et al., 1992; Krekoski et al., 1996). The observed differences in the

severity of effects produced likely relate to the variations in the type of nerve examined in the study (a "mixed" vs a "pure" nerve) and/or the type of nerve lesion used (crush or section). Although it might be predicted that if NGF positively regulates NGF receptor mRNA levels, a lack of NGF should result in a down-regulation of the mRNA; the results of this study suggest that this is not the case, at least over the relatively short term. Analogous observations have been made by Lindsay et al. (1990) who demonstrated that while added NGF resulted in increased expression of $p75^{NGFR}$ in DRG neurons in vitro, expression of $p75^{NGFR}$ was not significantly altered compared to control when NGF was absent. Indeed, $p75^{NGFR}$ levels in DRG neurons were observed to remain at baseline values following a prolonged anti-NGF treatment to adult rats (Zhou and Rush, 1996), thus lending further support to the present findings, and to the notion that a short-term disruption of NGF does not lead to a decrease in $p75^{NGFR}$ mRNA.

The finding that $p75^{NGR}$ and *trk*A mRNA levels not only recovered but were increased above baseline values upon target reinnervation (Krekoski et al., 1996) suggests the possibility that NGF may be involved in the regulation of at least some aspect of the regenerative program, especially during the latter stages of axonal regeneration, i.e, when the regenerating neurites begin to establish terminal arbors within their target tissues. However, this possibility does not seem likely based on two observations. Firstly, mRNA levels for both NGF receptors reach baseline levels even when target reinnervation is prevented (Krekoski et al., 1996). Secondly, target reinnervation by regenerating axons proceeds quite normally in the absence of endogenous NGF (Diamond et al., 1992b).

Thus, the present study supports the hypothesis that NGF is not an obligatory requirement for successful nerve regeneration; however, it does not completely eliminate the possibility that NGF may still play some role during axonal regeneration, for example in relation to the expression of neuronal phenotype upon target reinnervation (see earlier comments; reviewed by Verge et al., 1996). Furthermore, should there be extraordinary redundancy in the mechanisms regulating the regenerative capacity of sensory neurons, the possibility exists that the elimination of one of the supporting trophic molecules, including NGF, might not detectably impair it. Notably, several insulin-like growth factors produced in the degenerating nerve stump have been demonstrated to play an important role in the success of regenerating sensory axons (reviewed by Ishii, 1994).

6.4. Role of GAP-43 In Neurite Growth in the Adult Rodent

It is well described that increases in GAP-43 protein and mRNA levels occur during both development and regeneration of rat DRG neurons (Skene, 1989; Fitzgerald et al., 1991; Chong et al., 1992). GAP-43 protein expression during regeneration appears to peak at a time when axons are actively elongating and falls once targets are innervated (Bisby, 1988). Based on these and similar studies, it was hypothesized that the increase resulted from a loss of an inhibitory influence following axotomy of a peripheral nerve (Skene, 1989). However, since the results of this study demonstrate an increase in GAP-43 mRNA expression in both regenerating and sprouting DRG neurons, it is apparent that this view cannot apply to the increased expression observed in undamaged DRG neurons undergoing collateral sprouting.

In uninjured DRG neurons in the adult rat, expression of GAP-43 mRNA has been correlated with the presence of NGF high-affinity binding sites; most of the GAP-43 positive neurons express NGF receptors and almost all of the neurons with NGF receptors express high levels of basal GAP-43 mRNA (Verge et al., 1990). These authors suggest that the high levels of GAP-43 may be maintained by endogenous NGF, an interpretation that supports the speculation that a relatively high basal concentration of GAP-43 in some neurons may relate to their ability to undergo collateral sprouting. Clearly, the present findings suggest that the collateral sprouting of NGF-responsive sensory neurons is associated with an increase in GAP-43 mRNA levels. The possible role of NGF in the regulation of collateral sprouting may be related to its capacity to upregulate GAP-43 expression in the uninjured neurons and to post-transcriptional alterations in GAP-43 in neuronal growth cones (Meiri and Burdick, 1991). The finding that NGF exposure of neuroblastoma cells transfected with trkA cDNA leads to a novel increase in GAP-43 expression and the formation of neurites (Matsushima and Bogonmann, 1993) suggests the existence of a relationship between NGF-evoked intracellular signalling mechanisms and the expression of GAP-43. However, as presented in this study, GAP-43 mRNA expression is also increased in regenerating DRG neurons, even in some neurons which did not possess NGFRs; this result is similar to those found by Verge and collegues (1990). Although the trigger responsible for evoking the upregulation following axonal injury remains to be established, the fact that GAP-43 mRNA expression is increased in two very different situations (axonal damage with loss of peripherally supplied NGF and collateral sprouting evoked by an increased supply of NGF) argues that there is

certainly not a single mechanism responsible for its regulation. This notion is supported by the present finding in which the level of GAP-43 mRNA remains at a baseline value in DRG neurons whose axons are prevented from sprouting during an anti-NGF treatment but are increased in regenerating DRG neurons during the same anti-NGF treatment paradigm (K.M. Mearow, personal communication).

6.5. Role of NGF in the Postnatal Period on Nerve Growth Responses in the Adult Rat

The most profound finding in this thesis was that anti-NGF, when administered during a critical postnatal period, severely compromised the ability of nociceptive afferent neurons to undergo NGF-dependent collateral sprouting in the skin of *adult* rats. Moreover, this anti-NGF-induced impairment was also demonstrated to be highly dependent upon the developmental stage at which NGF-responsive neurons were deprived of NGF; treatment to juvenile rats led to a less severe impairment relative to the neonatal treatment, whilst the same treatment to adult rats was without effect on denervation-evoked collateral sprouting of nociceptive axons. The age-dependency of the effect on the collateral sprouting capacity of mature Aδ- and C-nociceptive fibres is entirely consistent with the notion that the role of NGF changes during development depending on maturational stage of the neuron (see Introduction). In direct contrast to the effects of neonatal anti-NGF treatment on sprouting, the same treatment did not affect the regenerative capacity of the nociceptive neuron; the functional reinnervation of the mechano- and thermo-nociceptive innervation fields by regenerating Aδ- and C-axons proceeded quite normally in adult rats that were administered anti-NGF as neonates.

The defect in sprouting is unlikely to be due to NGF insufficiency since NGF mRNA levels increased normally in denervated skin. Moreover, as a result of the substantial loss of neurons in the sprouting-impaired animals, NGF levels in their skin would be increased relative to the levels in the skin of control littermates (cf. Woolfe et al., 1996). It is also unlikely that the remaining neurons were NGF-sensitive, or that their sprouting had reached some sort of a "sprouting ceiling", since systemically administered NGF evoked normal collateral sprouting. Although possible impairments in axoplasmic transport mechanisms were not examined in this thesis, it is doubtful that the transport systems were impaired; sensory thresholds were normal, the caliber of sensory axons were normal, regeneration was normal, and exogenous NGF-evoked collateral sprouting was normal, findings that are inconsistant with a permanent reduction of axonal transport (Grafstein and Forman, 1980; Leon and McComas, 1984; Riaz and Thomlinson, 1996). Having eliminated all of these possibilities as being likely candidates to explain the defective sprouting, I am left to conclude that the defect in spontaneous collateral sprouting resides in the nociceptive terminals, as mentioned earlier. Furthermore, I hypothesize that the defect involves a reduced capacity of the nerve terminals to take up a sufficient amount of endogenous NGF that is necessary to either evoke and/or maintain normal sprouting. However, some degree of NGF uptake would be required to account for the apparently healthy state of the neurons, and to explain the minor sprouting that was observed to occur in a small number of adult animals that were neonatally treated with anti-NGF. Although the usual sprouting-related increase in $p75^{NGFR}$ expression in DRG (Mearow et al., 1994) was absent, *trk*A mRNA levels in the same DRG

increased normally (Mearow et al., 1994). Taken together, these findings suggest that smaller increases in NGF uptake are required to induce this reaction, than are needed to evoke sprouting or to induce increased $p75^{NGFR}$ expression (cf. Mearow and Kril, 1995). Although the mechanism responsible for the proposed reduction in NGF uptake is not known, it is conceivable that the ratio of $p75^{NGFR}$:TrkA in the nociceptive terminals (see above), and/or the absolute levels of one or the other of these receptors (at the terminal) have values that do not promote adequate uptake of NGF. Support for this theory is derived from the present finding that the basal level of *trk*A gene expression was normal whilst that of $p75^{NGFR}$ was increased in sprouting-compromised DRG neurons. Nevertheless, the predicted consequence of such an alteration would be a less than sufficient uptake of endogenous NGF necessary to evoke TrkA signalling required for normal sprouting and $p75^{NGFR}$ gene expression, but one that is sufficient for evoking an increase in *trk*A mRNA levels. That exogenous NGF rescued the impaired sprouting, and evoked an increase in the expression of both NGF receptors, can be explained by the exogenous NGF activating TrkA receptors present along the entire axon shaft and the cell body of the primary afferent (but see Campenot, 1994).

6.6. Role of NGF in DRG Neuron Survival

The axon profile in a selected cutaneous sensory nerve (mDCN T13) was used as an alternative method to counting the number of neurons present within the DRG of treated and control rats. Although actual neuronal counts would be the method of choice for quantitation, it is extremely tedious and time-consuming, and as yet there is much controversy as to which protocol provides the most accurate count of cell numbers (Coggeshall, 1996).

Although it is assumed that each DRG neuron gives rise to only one peripheral projection, there is evidence of branching; however, the actual number of neurons estimated to be involved is relatively small. Nevertheless, since the number of axons in mDCN T13 from treated rats was compared to those in control rats, the method used in this study (axon profile) is an effective method for obtaining an accurate indication of the relative amount of neuron loss following neonatal anti-NGF treatment.

Examination of the mDCN T13 revealed a loss in the total number of cutaneous afferents in adult rats which were treated with anti-NGF between P0 to P14, a finding that is entirely consistent with previous reports (Jenq et al., 1984; Hulsebosch et al., 1987; Ritter et al., 1991). The reduction in Aβ-fibres, the majority of which most likely arise from large DRG neurons (Lawson, 1992) and probably innervate hair follicles (Perl, 1992), is consistent with the finding that some large DRG neurons express TrkA protein (Averill et al., 1995; Molliver et al., 1995). However, since the anti-NGF antiserum used in the present study also blocks NT-3 function, in conjunction with the finding that some 15% of large diameter cutaneous afferents express trkC mRNA (McMahon et al., 1994), it is possible that a portion of the observed Aβ-fibre loss resulted from reduced NT-3 levels during the neonatal treatment paradigm. This result is the first example showing the survival of a proportion of Aβ fibres in normal rodents continues to depend on a trophic source in the postnatal period, consistant with previous observations in NT-3 deficit mutant mice (Airaksinen et al., 1996). In addition, the present finding that NGF deprivation between P13-27 led to a reduction in the number of unmyelinated fibres in the adult rat is also the first example that the survival of

small-diameter DRG neurons remains dependent on NGF well into postnatal life. This finding is in contrast to those reported by Lewin and colleagues (1992) who saw no neuronal loss when anti-NGF was administered after P2. Although this discrepancy is not easily explained, it is conceivable that differences between the two anti-NGF treatment regimes (duration of treatment, amount of anti-NGF injected) might be contributing factors to the observed differences. Nonetheless, the present findings demonstrate for the first time that at least a portion of the small-sized DRG neurons exhibit a prolonged dependency on NGF for their survival.

6.7. Conclusions

In summary, four conclusions are reached from the results of this thesis. First, NGF synthesis in the skin of adult rats is under the control of the innervating cutaneous axons. Second, the denervation-evoked increase in endogenous NGF influences $p75^{NGFR}$ and *trk*A gene expression in DRG neurons whose axons are undergoing collateral sprouting but has no affect on their expression during axonal regeneration. Third, during early life, NGF plays a critical role in the establishment of NGF-dependent growth mechanisms. Finally, small-sized DRG neurons exhibit a prolonged dependency on NGF for their survival during the latter stages of postnatal development.

ADDENDUM

During the oral defense of this thesis it was brought to the attention of the thesis author that the choice of post-hoc statistical analyses utilized in Chapters Two and Three were not the most appropriate. The author agrees with this criticism; however, since these chapters consist of published material, they are under copyright protection by the respective journals, and as such, the author was not authorized to alter their formats at the time of thesis submission.

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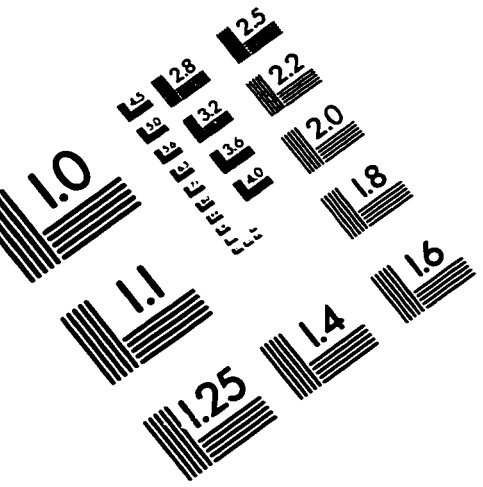
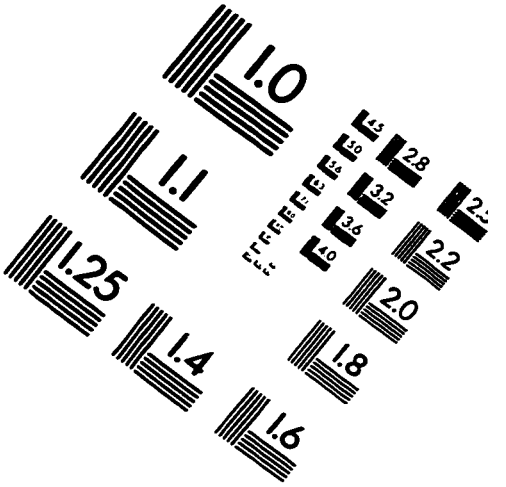
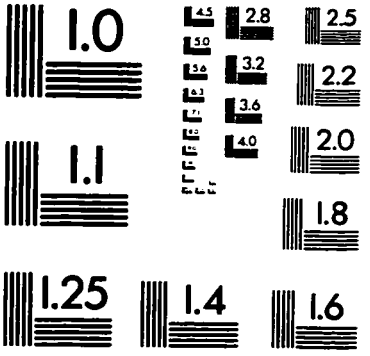
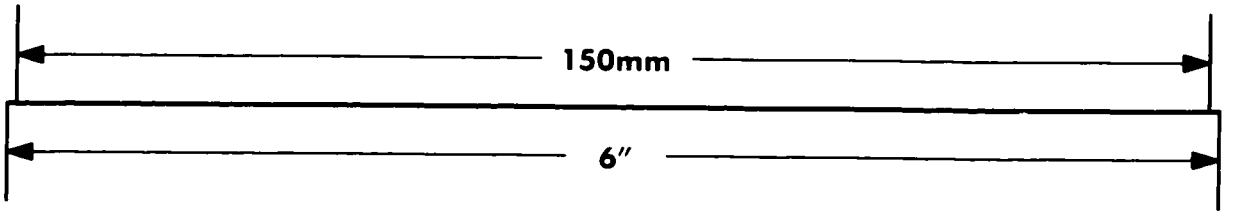
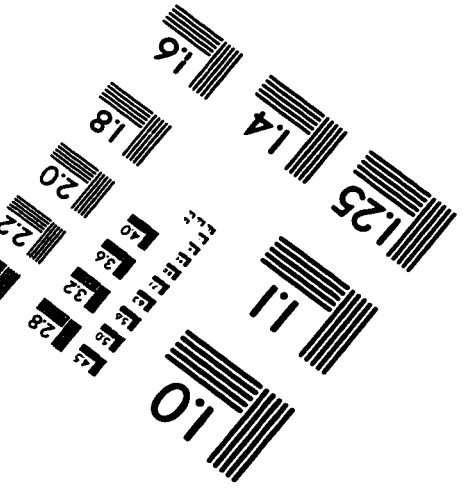
IMAGE EVALUATION TEST TARGET (QA-3)

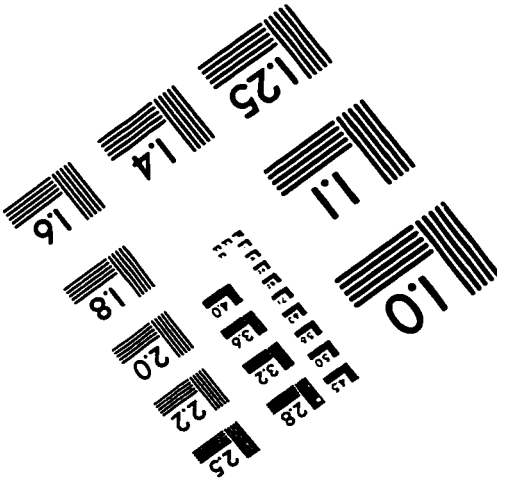












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