THE ROLE OF NERVE GROWTH FACTOR IN THE REINNERVATION OF DENERVATED SKIN BY SYMPATHETIC NERVES

By

ANDREW GLOSTER, B.Sc.

A Thesis
Submitted to the school of Graduate Studies
in partial Fulfiment of the Requirements
for the degree
Doctorate of Philosophy

McMaster University

TROPHIC REGULATION OF THE REINNERVATION OF DENERVATED SKIN

DOCTOR OF PHILOSOPHY (1993) McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: The role of Nerve Growth Factor in the Re-innervation of Denervated Skin By Sympathetic Nerves

Author: Andrew Gloster, B.Sc. (Manitoba)

Supervisor: Professor Jack Diamond

Number of Pages: 193

Abstract

This thesis examines the nerve growth factor (NGF) dependency of collateral sprouting (the growth of undamaged axons) and axonal regeneration (the growth of damaged axons) by sympathetic nerves in the adult rat. Sympathetic pilomotor fields were revealed by the electrical stimulation of selected dorsal cutaneous nerves. After the removal of neighbouring nerves, sympathetic fibers underwent collateral sprouting, causing the pilomotor fields to expand into the surrounding deprived territory; this sprouting was completely blocked by polyclonal anti-NGF serum. Crushing a nerve eliminated its associated pilomotor field and evoked regeneration of sympathetic fibers. Anti-NGF treatment impaired neither the subsequent restoration of the pilomotor field, nor its continued expansion into surrounding skin regions. A single 6-OHDA treatment, which destroys sympathetic terminals but leaves the axons damage-free, immediately eliminated or drastically reduced the size of pilomotor fields. The pilomotor field reestablished over the following 20 days, and if the surrounding nerves had been surgically eliminated, they continued to expand into the surrounding denervated territory. Anti-NGF administration did not prevent the initial phase of

pilomotor field recovery, but expansion ceased when the fields were about 60% of normal size. After multiple 6-OHDA injections however, the expansion was almost double this value before the anti-NGF treatment was able to halt it. It is concluded that, although the cellular mechanisms underlying neurite outgrowth are probably common to collateral sprouting and axonal regeneration, only the former is regulated by NGF; the latter is driven by a NGF-independent "regeneration response" whose size is determined by the degree of axonal damage, and probably the degree of the associated cell body reaction.

Acknowledgements

I wish to thank Steve Langford for all his encouragement, and for introducing me to Dr. John Rowe. I will always owe a debt of gratitude to Dr. John Rowe for his backing and assistance in turning around my academic progress. I thank Dr. Jack Diamond for providing me with a superb scientific education, and for his never ending enthusiasm and passion for science. I also wish to the people associated with the Diamond lab, especially Mike Holmes, Michael Cameron, Jim Fawcett, Yvonne Kril, Elizabeth LaRose, and Christine Bourgeau, for their assistance and friendship. Thanks is also due to Dr Colin Nurse and Dr. Michael Coughlin for their comments and advice on this thesis. Thanks also to Carrie Causing for her support throughout the past few hectic months. Finally, I wish to thank my parents for their continual encouragement over the years. I wish to acknowledge that this work was funded by the Medical Research Council of Canada.

Statement indicating which experiments in this thesis were performed in person by Andrew Gloster.

All the experiments described in this thesis were performed by Andrew Gloster with the following exceptions 1) the NGF antisera was produced by Jolanta Stanisz 2) the cutting and examination of the electron microscopic tissue sections was performed by Evi Pertens and Jack Diamond 3) Some of the daily antisera injections were performed by Michael Holmes, Christine Bourgeau, Yvonne Kril, and Michael Cameron 4) Surgical assistance was provided by Mike Holmes and Christine Bourgeau

Table of Contents

First Paper

Sympathetic nerves in adult rats regenerate normally and restore pilomotor function during an anti-NGF treatment that prevents their collateral sprouting

Second Paper

NGF-dependent and NGF-independent recovery of functional pilomotor activity after chemical sympathectomy with 6-hydroxydopamine.

List of Illustrations

Paper 1

Sympathetic nerves in adult rats regenerate normally and restore pilomotor function during an anti-NGF treatment that prevents their collateral sprouting

Paper 2

NGF-dependent and NGF-independent recovery of functional pilomotor activity after chemical sympathectomy with 6-hydroxydopamine.

Introduction - Review of Literature

Neuronal Outgrowth

The primary focus of this thesis is directed towards discovering some of the fundamental differences in the adult rat between the regulation of collateral sprouting, which is a growth of undamaged nerve fibres, and axonal regeneration, a regrowth of damaged nerve fibres. In particular the experiments explored the dependency upon nerve growth factor (NGF) of the neuronal outgrowth of the postganglionic neurons of the paravertebral sympathetic trunk *in vivo*. Neuronal outgrowth occurs in a number of biological situations including the initial embryological outgrowth of axons, the growth and regrowth of undamaged and damaged nerves in the adult, and *in vitro*, the neurite extension that occurs from both dissociated neurons and neuronal explants.

Regulation of neuronal outgrowth can be mediated by molecules associated with the extracellular matrix, by cell-cell signals, and by diffusible trophic factors (Dodd and Jessell, 1988). For example, the extracellularly matrix bound proteins, laminin and fibronectin promote neurite outgrowth (Rogers et al., 1983) through their binding to

integrin receptors on growth cones (Reichardt and Tomaselli, 1991). In contrast, some cell-cell interactions inhibit nerve growth. A glycoprotein on the surface of posterior-half sclerotomal cells of the somite causes collapse of sensory growth cones, resulting in nerve outgrowth through the permissive anterior-half sclerotome, and thus plays an important role in regulating the initial outgrowth of the segmental peripheral nerves (Davies et al., 1990). Similarly, there is evidence for inhibitory molecule(s) on oligodendrocytes which inhibit the extension of regenerating neurites in the adult mammalian central nervous system (CNS) (Bandtlow et al., 1990). All of the examples discussed above involve interactions between molecules and individual growth cones, which suggests that the growth cone may integrate positive and negative growth signals found in its environment. Ultimately, all neurite growth depends upon the cell body for the provision of the raw materials required for neurite extension, and therefore, regulation is also possible at the level of the cell body, where the regulation determines whether a growth cone is competent to respond to environmental signals. An interesting example of neurite outgrowth that involves regulation at the level of both the cell body and the growth cone is the NGF stimulated outgrowth of neurites from the pheochromocytoma cell line PC12. In this situation, an initial brief exposure to NGF,

a diffusible trophic factor, first "primes" the cells, a step which involves changes in gene transcription, and initiates neurite extension. A second exposure to NGF, a few days later, promotes, in a transcriptionally independent manner, neurite extension, at the level of the growth cone (Greene, 1984). While there is no evidence for cell body regulation during the initial outgrowth of axons during development, there is evidence for regulation of neurite outgrowth by the neuronal cell body during regeneration and collateral sprouting, which is discussed below.

Some of the evidence favouring a role for NGF in supporting neuronal outgrowth is problematical. As will be discussed below, because NGF is required for neuronal survival in some circumstances, it can not be concluded whether the process of neurite outgrowth in these cases is specifically dependent upon NGF, unless neuronal survival and neurite growth are manipulated separately (e.g. Campenot, 1982, see below). In some experiments, evaluations of innervation density were based upon the measurement of levels of various neuronal enzymes or neurotransmitters which are found in neuronal fibres. These indirect evaluations may not accurately reflect the actual innervation density, since NGF directly regulates the levels of these same enzymes and neurotransmitters, independently of NGF's effects upon fibre density. Therefore, a higher

level of an enzyme or neurotransmitter may indicate either a greater number of endings or a greater concentration of enzymes in each ending. Most of the information on the role of NGF in controlling neurite outgrowth comes from experiments involving either *in vitro* neuronal explants or the initial neurite outgrowth to a target tissue during development. In the text that follows I will provide a brief review of the literature which particularly relates to the question of whether NGF is or is not an obligatory requirement for axonal growth and/or arborization.

NERVE GROWTH FACTOR

i) The neurotrophins

NGF, the first neurotrophic molecule discovered (Levi-Montalcini and Booker, 1960a,b), is the prototype of the neurotrophin family, which includes brain-derived neurotrophic factor (BDNF) (Barde et al. 1982; Lindsay et al., 1985; Hofer and Barde, 1988), neurotrophin-3 (NT-3) (Maisonpierre et al., 1990; Ernfors et al., 1990; Rosenthal et al., 1990; Hohn et al., 1990), and neurotrophin-4 (NT-4) (Hallböök et al., 1991; Berkemeier et al., 1991). These neurotrophins support the survival of various neuronal subpopulations during development (Lindsay et al., 1985; Hallböök et al., 1991). In this thesis there will be only a limited discussion of the non-NGF neurotrophins, because of

the limited information concerning their role on sympathetic neurons to date. However, it is important to keep in mind that the NGF antisera used in many of the investigations referenced in this thesis, and in the present experiments, may have also recognized other neurotrophins besides NGF.

ii) Sites of NGF production

It has been suggested that the amount of NGF synthesized in various tissues regulates the density of sympathetic and sensory innervation in the adult, since relatively high NGF protein and mRNA expression levels are found in adult tissues which are richly innervated by sympathetic or sensory fibres. Specifically, there are high levels of NGF and NGF mRNA in adult iris, whisker pad, submandibular gland, heart atria, and skin, all tissues with a dense sympathetic and/or sensory innervation (Korsching and Thoenen, 1983; Heumann et al., 1984; Bandtlow et al., 1987). NGF has been shown to be produced by many different cell types, such as epithelial, smooth muscle and Schwann cells, fibroblasts, and keratinocytes (Bandtlow et al., 1987; Thoenen et al., 1988; Creedon and Tuttle, 1991; Marco et al., 1991). Recently, both BDNF and NT-3 have been shown to be expressed embryonically in mesenchymal cells of the mouse dermis (Schecterson et al., 1992).

While NGF acted as a chemoattractant for chick

sensory axons *in vitro* (Gundersen and Barrett, 1979), the NGF concentration required for this effect was vastly higher than endogenous NGF levels. Results from a number of experiments make it seem unlikely that NGF acts as a chemoattractant *in vivo*. Experiments using transgenic mice, in which NGF is overexpressed in specific tissues, have shown that elevated NGF levels alone are not sufficient to induce sympathetic fibre ingrowth. Ectopic NGF production in the anterior pituitary, which is not normally innervated by sympathetic fibres, does not induce ingrowth of nearby sympathetic fibres (Borrelli et al., 1992). Similarly, increased expression of NGF by the pancreatic β cells leads to hyperinnervation of the β cells by only the subpopulation of sympathetic fibres which normally innervate the β cells, and not by inappropriate sympathetic fibres which supply neighbouring cells (Edwards et al., 1989). Thus, elevated NGF levels in a target tissue increase the density of local sympathetic innervation, but not the ingrowth of neighbouring sympathetic innervation. The possible role of NGF in a dynamic turnover of endings by regulating collateral sprouting is discussed below.

The normally high levels of NGF production in the testis, epididymis, and male mouse submandibular gland are not correlated with the expected high density of sympathetic or sensory innervation, indicating that NGF probably has

non-neuronal functions (Angeletti et al., 1968; Ayer-Le Lievre, 1988). For example, in the testis NGF is known to regulate the progression through the meiosis phase of spermatogenesis (Parvinen et al., 1992).

iii) Nerve growth factor receptor and the trk family

Two types of receptors for neurotrophins have been described; the nerve growth factor receptor (p75 or NGFR) and the trk family of proteins. The NGF receptor p75 is a transmembrane protein which binds all members of the neurotrophin family examined to date (NGF, BDNF, NT-3, NT-4) with equal affinity (Rodriguez-Tébar et al., 1990; Squinto et al., 1991; Hallböök et al., 1991). trk (also called trkA) is the prototype of the trk protein family, which also includes trkB and trkC; it is also a transmembrane receptor, containing a cytoplasmic tyrosine kinase domain (Martin-Zanca et al., 1986). trk binds NGF, NT-3, and NT-4 (Cordon-Cardo et al., 1991; Berkemeier et al., 1992), trkB binds BDNF, NT-3, and NT-4 (Klein et al, 1991a,1992; Squinto et al., 1991), and trkC binds only NT-3 (Lamballe et al., 1991). Thus, there is not a unique receptor for each neurotrophin, and in some of the studies referenced below which utilized exogenous NGF, the endogenous molecule that normally evokes the observed responses *in vivo* may be a neurotrophin other than NGF. The point is similar to that

made earlier regarding the use of polyclonal antibodies raised against NGF, which might interfere with the biological functions of other members of the neurotrophin family. The binding of NGF by trk (Klein et al., 1991b, Meakin et al., 1992), and NT-3 by trkC (Lamballe et al., 1991) has been demonstrated to be of the high affinity type. Before the discovery of either p75 or trk, NGF was known to bind to high and low affinity NGF receptors, also referred to as the slow and fast receptors respectively, definitions that were based upon rates of NGF dissociation (Sutter et al., 1979). The high affinity (or slow) receptor is coded for, at least in part, by the trk gene, while the low affinity (or fast) receptor is coded for by the p75 gene (Johnson et al., 1986; Radeke et al., 1987; Meakin et al., 1992). It is controversial whether p75 is required for the formation of the high affinity receptor complex, with evidence for (Hempstead et al. 1991) and evidence against (Klein et al., 1991b; Radeke and Reinstein, 1991; Ibanez et al., 1992; Meakin et al., 1992) this possibility (reviewed by Barker and Murphy, 1992). Embryonic sympathetic neurons express trk (Schecterson et al., 1992), trkB (Klein et al., 1990), and p75 (Yan and Johnson, 1988), but not trkC. Adult SCG neurons express trkB (Klein et al, 1990), but it is not known whether they also express trk or trkC. The expression of trkB, the receptor for BDNF, in embryonic sympathetic

neurons is curious because BDNF does not support their survival *in vitro* (Barde et al., 1982), while NT-3 (Maisonpierre et al., 1990; Ernfors et al., 1990) and NT-4 (Hallböök et al., 1991) only weakly promote neurite extension from SCG explants. Further evidence of the role of p75 and trk in mediating NGF's effects are discussed below.

iv) Mechanism of NGF actions

Although the binding of NGF to trk and possibly p75 is the first step in transducing the NGF signal, the exact mechanism by which NGF mediates its actions is uncertain. It has been hypothesized that the high affinity NGF receptor mediates all of the biological actions of NGF independently of p75, (Sonnenfeld and Ishii, 1985). NGF binding activates trk's tyrosine kinase, which results in not only autophosphorylation (Kaplan et al., 1991a) but also the phosphorylation of a number of other proteins, including phospholipase C-γ1 (Vetter et al., 1991; Ohmichi et al., 1991), and probably some of the extracellular signal-regulated kinases (Boulton et al., 1991) as well as increasing the levels of ras-guanosine nucleotide exchange factor, guanosine triphosphatase activating protein and the ratio of Ras-GTP/Ras-GDP (Li et al., 1992). All of these proteins are components of second messenger systems and by

their modification, NGF binding is hypothesized to lead to a cascade of events which results in the modulation of gene expression (Maher 1988; Klein et al. 1991b; Kaplan et al, 1991b; Meakin et al., 1992). It is clear that both NGF and p75 are endocytosed and then retrogradely transported, possibly as a NGF-p75 complex (Johnson et al., 1987), to the cell body (Hendry et al. 1974; Stokel et al., 1975; Schwab, 1977; Johnson et al., 1978; Palmatier et al. 1984; Johnson et al., 1987; Raivach et al. 1991), where NGF is localized to the inner nuclear membrane (Yanker and Shooter, 1979). Direct NGF infusion into the cytoplasm of PC12 cells is without biological activity indicating that the binding of NGF with the membrane NGF receptors is an essential step for biological function (Heumann et al., 1981). The binding of NGF to p75 is at least partially responsible for mediating the biological responses, since p75 plays a role in regulating sympathetic and sensory neuronal survival (see below). Transfection of PC12 cells with chimeric receptors which combine the extracellular domain of the epidermal growth factor (EGF) receptor with the intracellular domain of p75, alters the PC12 cells so that they extend neurites in response to EGF as well as NGF (Yan et al., 1991). This result is highly suggestive that p75 can promote neurite outgrowth independently of trk. Thus, p75 most likely plays an important role in mediating NGF response, although the

details of this role are still unclear.

NGF has two types of effect. Firstly, a rapid response is generated locally in the region of the NGF binding sites (e.g. specific protein phosphorylation) that is independent of new transcription or protein synthesis, and secondly, a slower effect that occurs far from the terminal binding site (e.g. induction of enzymes at the cell body that requires new transcriptional and protein synthesis changes (reviewed by Greene, 1984)). An example of a local fast effect can be seen in cultured neonatal superior cervical ganglia (SCG) neurons. In this situation, NGF must be delivered directly to the nerve terminals in one compartment in order to stimulate neurite outgrowth from these terminals; application of NGF to the cell body, to axons, or to nerve terminals in a second culture compartment does not induce terminal neurite sprouting in the first compartment (Campenot, 1982). Rapid changes in membrane permeability and neurite outgrowth occur within 5 minutes of NGF addition, and like changes in cell adhesiveness, are not dependent upon new RNA or protein synthesis (Horii and Varon, 1975, 1977; Schubert and Whitlock, 1977; Seeley and Greene, 1983); indeed, after axotomy NGF can initiate neurite outgrowth by the distal axonal segments (Seeley and Greene, 1983). Thus, NGF can regulate local neurite outgrowth independently of the cell body, even though

ultimately all the substrates required for neurite outgrowth are produced in the cell body. The "slow" NGF mediated events, including the initial transformation of PC12 cells, and the prevention of natural cell death, depend upon the retrograde transportation of NGF to the cell body (or simply the uptake of NGF in the case of PC12 cells), and are also transcriptionally dependent (reviewed by Greene, 1984; Heumann et al., 1981). The regulation of gene expression by NGF is thought to occur via immediate early genes, such as c-fos (Gizang-Ginsberg and Ziff, 1990). Neuronal death occurs if cultured embryonic day 21 (E21) rat sympathetic neurons are deprived of NGF, if E10 chick tregiminal neurons are deprived of NGF or BDNF, or if E8 chick ciliary neurons are deprived of ciliary neurotrophic factor. The finding that both transcriptional and protein synthesis inhibitors prevent this cell death led to the hypothesis that during the period of natural cell death, neurotrophic factors suppress the synthesis of suicide proteins (Martin et al., 1988; Scott and Davies, 1990). Whether NGF acts locally on neurites and/or on the cell body during regeneration and collateral sprouting is discussed below.

v) Role of NGF during development

a) Sympathetic neurons

The SCG is the most studied component of the

peripheral sympathetic nervous system, and has been used as a model for the entire system. For this reason almost all the studies on sympathetic neurons referred to in this thesis involved SCG neurons. The particular sympathetic neurons studied in this thesis are those contained within the paravertebral sympathetic trunk which innervate pilomotor muscle in the rat dorsal skin. However, neurons that innervate rat pilomotor muscle in other areas are also located within the SCG (Lichtman et al., 1979), and therefore, the referenced studies on SCG neurons are particularly relevant to this thesis.

During development many populations of neurons, including those of the sympathetic ganglia, go through a period of natural cell death which is regulated by neurotrophins. The survival of mouse embryonic SCG cells are NGF independent at E14, but do become NGF dependent by E16 (Coughlin and Collins, 1985). Injections of exogenous NGF into neonatal mice decreased the amount of natural cell death in the SCG (Levi-Montalcini and Booker, 1960b; Hendry and Campbell, 1976), while injections of antiserum to NGF (anti-NGF) into neonatal mice (Levi-Montalcini and Booker, 1960a) or autoimmunization of pregnant rats and guinea pigs (Johnson et al., 1980) resulted in a 90-97% loss of the normal number of SCG neurons in the developing foetuses. Thus, NGF regulates the process of natural cell death for

sympathetic neurons, with the post-ganglionic sympathetic nerves competing for a limited amount of NGF produced in the target tissues. The greater the amount of target tissue, the greater the amount of NGF produced, and the greater the fraction of post-ganglionic neurons which obtain enough NGF to survive. In this way the size of the neuronal pool is matched to the size of the target tissue (Purves, 1988).

The survival of postnatal SCG neurons is not supported by BDNF (Lindsay et al., '85; Hofer and Barde, '88), and neurite extension from SCG explants is only weakly supported by NT-3 (Maisonpierre et al., '90; Ernfors et al., '90) and NT-4 (Hallböök et al., '91; Berkemeirer et al., '91). It is not completely clear whether the NGF receptor p75 is necessary for sympathetic neuron survival, since daily injections of monoclonal antibodies to p75 into rats from postnatal day 1 (P1) to P5, results in a 50% decrease in SCG cell numbers, while two consecutive injections of the p75 antibodies, once into the E16 fetuses and then once into the newborn rat pups, results in an 85% decrease in SCG cell numbers (as well as a loss of 50% of sensory DRG neurons) (Johnson et al., 1989). However, the mechanism by which these p75 antibodies mediate this decrease in sympathetic neuronal survival during development is not clear because these same antibodies do not affect either the NGF dependent survival of, or neurite extension from, newborn SCG cell

cultures (Johnson et al., 1989). In addition, while mutation of the p75 gene in transgenic mice, by the insertion of extraneous DNA into the third exon (of p75), substantially decreased the density of cutaneous sensory fibres (not determined quantitatively), the mutation did not affect the development of sympathetic innervation in either the iris or sweat glands (Lee et al., 1992). Thus, while all of the above evidence indicates that the survival of sensory neurons requires p75 during development, most of the evidence suggests that p75 is not necessary for sympathetic neuronal survival, although the results from the *in vivo* p75 antibody experiments are inconsistent with this latter proposition. Similar experiments investigating the development of sympathetic neurons in animals receiving antibodies to trk, or in transgenic mice with trk gene deletions, have not yet been reported.

In addition to supporting neonatal sympathetic cell survival, NGF regulates neonatal sympathetic neuron synapse formation, enzyme levels, neurite extension, and neuronal morphology. *In vitro*, NGF is necessary for neurite outgrowth from chick embryonic SCG neurons (Levi-Montalcini et al., 1954), and this outgrowth forms the basis of a functional assay for NGF using dissociated mouse neonatal SCG neurons (cf. Coughlin and Collins, 1985). However, since NGF is required for sympathetic neuronal survival it

is not possible to conclude whether the neurite outgrowth itself is NGF dependent. As mentioned previously, this outgrowth could be NGF independent, with NGF being simply required for neuron survival. This problem does not apply to the *in vitro* experiments of Campenot (1982) who demonstrated that NGF delivered directly to the nerve terminals in one compartment, stimulated neurite outgrowth only in that compartment. This NGF stimulated nerve growth was independent of NGF's effects on neuronal survival, since the neuronal survival could be supported by the addition of NGF to a second compartment containing the neuronal somata (Campenot, 1982). In addition to NGF's stimulation of neonatal sympathetic neurite formation *in vitro*, in the neonate NGF regulates the number of primary dendrites on SCG neurons *in vivo*. Neonatal NGF injections increased the number of primary dendrites which remained on the adult sympathetic neurons, while neonatal anti-NGF injections decreased the number (Ruit and Snider, 1991). The ability of NGF to upregulate many parameters of SCG neuronal morphology continues in the adult animal (discussed below).

NGF also modulates enzymatic changes of sympathetic neurons during development. The normal postnatal increase in the activities of dopamine ß-hydroxylase and tyrosine hydroxylase, rate limiting enzymes involved in norepinephrine synthesis, are enhanced upon addition of

exogenous NGF; furthermore, the increased activities are more than that expected due to the increased SCG neuronal survival and hypertrophy (Thoenen et al., 1971). Similarly, the uptake of norepinphrine appears to be upregulated by NGF, since neonatal injections of anti-NGF decrease norepinephrine uptake in the SCG (Angeletti et al., 1971). Again, as is the case with NGF regulation of neuronal morphology, NGF continues to be involved in the regulation of transmitter related enzymes in the adult (see below).

The morphological and enzymatic changes in the post-ganglionic neurons of the SCG, due to NGF and anti-NGF administration, are accompanied by changes in cell number, morphology, and enzyme levels of the pre-ganglionic neurons contained in the thoracolumbar column of Terni of the spinal cord, which innervate the SCG. Neonatal anti-NGF treatment led to a decrease in the number of synapses on postganglionic neurons, as well as a decrease in their intracellularly recorded synaptic responses (Nja and Purves, 1978). In contrast, neonatal NGF treatment increased the number of pre-ganglionic fibres and synapses, and also the activity of choline acetyl transferase in the SCG (Aguayo et al., 1976; Schafer et al., 1983; Black et al., 1972). Neonatal injections of NGF also reduces the developmental process of natural cell death in pre-ganglionic neurons, as well as causing the surviving neurons to hypertrophy

(Oppenheim et al., 1982). Oppenheim et al. (1982) hypothesized that the post-ganglionic neurons produced a retrogradely transported factor which the pre-ganglionic neurons competed for, and which inhibited the natural cell death of the pre-ganglionic neurons. Since NGF injections reduced the amount of post-ganglionic sympathetic neuronal natural cell death, the treatment would have increased the amount of the factor produced by the post-ganglionic neurons, and hence decreased the amount of pre-ganglionic neuronal death. By this mechanism, the number of pre-ganglionic neurons is hypothesized to be matched to the size of the target tissue (the post-ganglionic neurons), in an analogous manner to the way NGF, produced by sympathetically innervated tissue, is hypothesized to control the number of post-ganglionic neurons (Black et al., 1972; Schafer et al., 1983).

Thus, embryologically and neonatally, NGF functions to regulate the sympathetic innervation of target tissue. In the adult, NGF regulates sympathetic innervation of target tissues through similar mechanisms as those operating during development (see below).

b) Non-sympathetic neurons

Other peripheral and central neuronal populations besides sympathetic neurons are dependent upon NGF during

development (reviewed in Greene and Shooter, 1980; Whittemore and Seiger, 1987; Dreyfus, 1989). NGF is required for the survival of, and neurite extension from, a subpopulation of pre- and postnatal dorsal root ganglion cells (the "small dark" cells) (Johnson et al., 1980; Aloe et al., 1980; Hamburger et al., 1981; Goedert et al., 1984; Yip et al., 1984; Straznicky and Rush, 1985; Keller et al., 1990). This subpopulation of cells express trk; however the cells whose survival is NGF independent are trkB and trkC positive (Carroll et al., 1992). Similar experiments to examine if all sympathetic neurons which express trk are destroyed by immunosympathetomy have yet to be reported. The survival of, but not neurite extension from, CNS cholinergic striatal interneurons and the cholinergic neurons which project from the basal ganglia to the cerebral cortex and hippocampus may be dependent upon NGF during development (reviewed in Dreyfus, 1989). Embryonic motoneurons produce p75 (Yan and Johnson, 1988; Eckenstein, 1988), and bind and retrogradely transport NGF (Raivich et al., 1987; Wayne and Heaton, 1990a); however *in vitro* they do not require NGF for survival, although NGF does stimulate neurite outgrowth from embryonic motoneurons in culture (Wayne and Heaton, 1990b). While embryonic motoneurons are sensitive to NGF in culture, a physiological role for endogenous NGF has yet to be demonstrated. The electrical

properties of sensory neurons can also be influenced by NGF; exogenous NGF for example decreases the duration of the Ca^{2+} component of action potentials in cultured fetal mouse DRG neurons (Chalazonitis, et al., 1987). NGF also regulates developmental changes in neurotransmitter-related enzyme levels in non-sympathetic neurons. Exogenous NGF injections enhanced the normal postnatal increase in substance P and somatostatin levels in DRG neurons (Kessler and Black, 1981), and choline acetyltransferase and acetylcholine esterase activities in the basal forebrain-medial septum, hippocampus, and neocortex (Honegger and Lenoir, 1982; Gnahn et al., 1983; Mobley et al., 1986; Martinez et al., 1987). Interestingly, anti-NGF injections did not prevent the normal neonatal increase in substance P in the DRG or choline acetyltransferase in the CNS (Kessler and Black, 1980; Gnahn et al., 1983; Martinez et al., 1987), even though anti-NGF is known to down regulate these molecules in the adult (see below).

vi) NGF and initial axonal outgrowth

Based upon comparisons between the timings of the initiation of NGF and NGF receptor production, and of the time of arrival of sympathetic and sensory axons at their target tissues in the embryonic mouse and chick, NGF has been concluded not to be necessary for the initial outgrowth

of sympathetic and sensory axons. NGF production does not begin in the mouse heart ventricle and mandibular gland until the time (E12-13) of arrival of the sympathetic fibres (Korsching and Thoenen, 1988), which is before the time (E14) that the SCG neurons become dependent on NGF (Coughlin and Collins, 1985). Similarly, in the early embryonic mouse maxillary pad NGF production begins at the time of arrival of the sensory trigeminal axons (E11); but the NGF concentration then decreases at the time of onset of natural cell death (E13) in the trigeminal ganglia (Davies et al., 1987). This decrease in NGF concentration in the maxillary pad is thought to be due to the retrograde transport of NGF by the sensory axons from the target to the trigeminal ganglia (Davies et al., 1987). A similar increase in NGF synthesis in chick leg skin occurs coincident with the arrival of sensory fibres, but the increase in NGF concentration is not dependent upon this innervation; the increase in NGF synthesis occurs even when the normal innervation of the skin is surgically prevented (Rohrer et al, 1988). It has also been shown that trigeminal neurons express p75 mRNA at low levels prior to the arrival of their axons at the maxillary pad, at which point p75 expression in these neurons is increased fivefold (Wyatt et al., 1990). The survival of trigeminal neurons does not become NGF dependent until after their respective axons reach their

target tissues. The same is true for the vestibular, geniculate, petrosal, and nodose sensory neurons although the specific neurotrophin in these cases is BDNF. Interestingly, when these neurons are dissociated, and allowed to mature in culture, they become dependent upon neurotrophins at the age when their axons would have been reaching their target tissues *in vivo*. This indicates that the survival of these sensory neurons are initially NGF and BDNF independent, that is, until their axons have completed their initial axonal outgrowth, and that the target tissue is not necessary for the conversion to trophic dependency (Davies et al., 1987; Vogel and Davies, 1991). Other *in vitro* evidence, which also supports the concept of the NGF independence of initial axon outgrowth has come from experiments using cocultures of trigeminal ganglia with their appropriate target (maxillary pad) and an inappropriate target (distal forelimb bud) tissue, when the ganglia and target tissues were taken prior to the trigeminal axons reaching the maxillary pad. In these experiments the addition of anti-NGF to the culture prevented neither the initial outgrowth of axons nor the characteristic nature of the outgrowth (Lumsden and Davies, 1983). Thus, it is unlikely that neurotrophins are required for either the initial axonal outgrowth, or the survival of sympathetic or sensory neurons before the axons reach their

target tissues. It is difficult to understand how, *in vivo*, NGF could direct neurite growth to particular targets within a tissue since so many different cell types produce NGF. For example, in the skin, sympathetic fibres make contact with blood vessels, and pilomotor muscle, but not other cells in the dermis even though NGF production is not localized to specific cell types in embryonic skin, but rather is distributed throughout the presumptive dermis and epidermis (Davies et al., 1987). These findings, combined with the results from the transgenic mice which ectopically expressed NGF, as mentioned above, rule out NGF acting as a chemoattractant molecule *in vivo* during initial axonal outgrowth, even though NGF is a chemoattractive molecule at pharmacological doses *in vitro* (Gundersen and Barrett, 1979). Thus the initial outgrowth of sympathetic and sensory axons to their target tissue appears to be NGF independent, although NGF may increase the sympathetic and sensory fibre density in the target tissue after this target contact (see below).

vii) NGF and adult neurons

a) sympathetic neurons

Adult sympathetic neurons, in comparison to embryonic and neonatal ones, have a diminished dependency on NGF, although NGF is still involved in the regulation of

their neuronal morphology, transmitter levels, and long-term survival. *In vitro*, as neonatal sympathetic cells mature, the proportion of neurons that appear to be dependent on NGF for their survival decreases; 10 days after dissociated neonatal sympathetic cells were placed in culture 0-10% of the cells survived 10 days of NGF deprivation, while after 50 days in culture, 20-50% survived the NGF deprivation (Lazarus et al., 1976; Chun and Patterson, 1977a). Neonatal sympathetic neurons appeared to be even less NGF-dependent in the experiments of Campenot (1982); after 4 days in culture with added NGF, a population of dissociated neonatal neurons survived over 40 days of NGF deprivation (Campenot, 1981). However, this NGF dependency does not completely disappear in the adult. In adult mice, rats, and rabbits, elimination of endogenous NGF by either daily anti-NGF administration (for 3 weeks), or by autoimmunization against NGF, leads to neuronal cell atrophy in the SCG (Levi-Montalcini and Booker, 1960a; Angeletti et al., 1971; Gorin and Johnson, 1980; Johnson et al., 1982); the atrophy had reversed by three months after cessation of the anti-NGF treatment, and there was no obvious neuronal loss (Angeletti et al., 1971). However, in rats and rabbits autoimmunized against NGF, the SCG neuronal numbers had fallen by 25-85% 5-10 months after treatment had been initiated (Gorin and Johnson, 1980; Johnson et al., 1982; Ruit et al., 1990).

NGF appears to regulate the morphology of adult sympathetic neurons. In the SCGs of mice injected with exogenous NGF, their neuronal cell bodies and dendritic arbours were larger (Ruit et al., 1990). Conversely, anti-NGF treatment decreased the size of SCG neurons, their dendritic arbour, and the number of synapses on them. As well, after 4-5 days of anti-NGF injections, there was a decrease in the post-synaptic potentials recorded intracellularly from post-ganglionic SCG cells in response to electrical stimulation of the pre-ganglionic nerve (Nja and Purves, 1978). Exogenous NGF itself, however, affected neither the number of synapses nor synaptic function (Nja and Purves, 1978; Ruit et al., 1990). As stated previously, during early embryonic and postnatal development, NGF regulates the production of rate limiting enzymes in the synthesis of the sympathetic neurotransmitters norepinephrine and acetylcholine. In the adult, levels of tyrosine hydroxylase and dopamine ß-hydroxylase activities, norepinephrine, norepinephrine reuptake, and acetylcholine are upregulated by NGF (Angeletti et al., 1971; Bjerre et al., 1975; Chun and Patterson 1977b; Gorin and Johnson., 1980; Rich et al., 1984; Raynaud et al., 1988). We can conclude from these results that adult sympathetic neurons utilize NGF for their survival, and to maintain their enzyme activities, and morphological properties. As will be

discussed below, these NGF effects are very relevant in the assessment of NGF's role in regulating the collateral sprouting and regeneration of sympathetic neurons.

b) NGF and other adult neurons

While the survival of DRG sensory neurons in adult animals is NGF independent (Lindsay et al., 1988), if deprived of NGF, sensory neurons exhibit a slight atrophy (Rich et al., 1984), as well as a decrease in both axon calibre and neurofilament content (Gold et al., 1991). NGF also regulates neurotransmitter levels in these neurons. The levels of substance P and CGRP, (and interestingly, p75) in DRG neurons (Otten et al., 1980; Schwartz et al., 1982; Lindsay et al., 1989) and of choline acetyl transferase in basal ganglia neurons (Gnahn et el., 1983), are all increased by exogenous NGF. These results show that NGF's role in the adult nervous system is not confined to sympathetic neurons but rather, many of the properties that are highly regulated by NGF in sympathetic neurons, can be modulated to a greater and lesser extent in other neurons.

viii) Non-neuronal effects of NGF

Certain non-neuronal cells are responsive to NGF. For example NGF is a growth and differentiation factor for human hemopoietic colonies (Matsuda et al., 1988), a growth

factor for cultured chromaffin cells (Lillien and Claude, 1985), accelerates wound healing in skin (Li et al., 1980), and induces mast cell degranulation (Mazurek et al., 1986). In addition, NGF is made in the testis, epididymis, and submandibular gland, where it is very likely regulating non-neuronal functions in these tissues, such as regulating spermatogenesis (Angeletti et al., 1968; Ayer-Le Lievre, 1988). Possible non-neuronal roles for NGF in non-neuronal cells are important when considering the possible roles of NGF during regeneration and are discussed below.

REGENERATION

i) Introduction

Axonal regeneration refers to the regrowth of axons from the proximal stump after a nerve cut or crush. Axonal regeneration occurs extensively in the mammalian peripheral nervous system, including sensory (Ramón y Cajal, 1928), motor (Ramón y Cajal, 1928), parasympathetic (Landmesser and Pilar, 1970), pre-ganglionic sympathetic (Langley, 1885;1887), and (of more relevance to the present studies) the post-ganglionic sympathetic fibres, including those innervating, the pineal gland (Owman, 1964), the sweat glands (Navarro and Kennedy, 1989), the heart (Goodall, 1951), spleen (Kirpekar et al., 1970), vas deferens (Kirpekar et al., 1970), and blood vessels (Kilvington and

Osborne, 1907). In the CNS of adult mammals, axonal regeneration does not normally occur spontaneously (but see Foerster, 1982), but the regenerative potential exists, as proved by the provision of an appropriate environment (e.g. an implanted peripheral degenerated nerve) along which regrowth will occur (So and Aguayo, 1985).

Axotomy of peripheral nerves is followed by several events that include the degeneration of the distal stump, a neuronal cell body reaction, and an outgrowth of axons from the proximal stump. There are also transneuronal changes that occur after post-ganglionic sympathetic axotomy. These changes include a decrease in the length and complexity of the undamaged dendrites of the neuronal cell bodies (Yawo, 1987), as well as a decrease in the number of pre-ganglionic synapses (Nja and Purves, 1978). A potential role for NGF in peripheral nerve regeneration has been proposed by some (e.g. Bjerre et al., 1975; Johnson et al., 1988) and discounted by others (eg. Rich et al., 1984; Diamond et al., 1987; 1992b; Raivich et al., 1991). The results presented in this thesis strongly support the view that axonal regeneration is NGF-independent.

As stated above, axotomy results in the degeneration of the distal stump, a process called Wallerian degeneration (reviewed by Allt, 1976, Fawcett and Keynes, 1990). During Wallerian degeneration Schwann cells in the

distal nerve stump proliferate, the blood-nerve barrier breaks down, and macrophages invade the nerve, clearing the degenerated debris.

From the proximal stump of the damaged nerve, many neurites extend and retract with a number of the neurites continuing to grow into the degenerating distal stump (Shawe, 1955, Allt, 1976). Each axon in the peripheral stump can give rise to more than one axon in the distal stump, and even months later more axons may be present 3 cm distal to the lesion site than in the proximal segment (Shawe, 1955).

The type of neuronal injury that is sustained is a very important factor when determining the success of regeneration. In the intact nerve, axons are contained within endoneurial tubes formed from Schwann cell basal lamina (Sunderland, 1978). Subsequent to a crush injury the continuity of the endoneurial tubes is often maintained (Haftek and Thomas, 1968), and consequently, fibres will readily regenerate down their original pathways (Allt, 1976). However, neuronal transection results in a discontinuity of the endoneurial tube, and axonal growth becomes unselective; both myelinated and unmyelinated sensory and motor axons can grow into endoneurial tubes that originally contained different fibre types (Langley and Anderson, 1904; Kilvington, 1941; Simpson and Young, 1945).

Since the endoneurial tubes still lead to their original targets, the result is often a regeneration down inappropriate endoneurial tubes, producing a non-functional regeneration (Sunderland, 1990). However, regenerating fibre growth is not completely unselective in this respect. More regenerating motor axons regenerate down a motor nerve stump than down a sensory nerve stump when the proximal stump, a sensory stump, and a motor stump are placed in the three ends of a "Y" junction silicone chamber (Brushart and Seiler, 1987). Nevertheless, the large amount of non-functional regeneration generally observed after nerve cuts argues against specific coded pathways within the nerve trunk for different fibre types. Even when only cutaneous nerve modalities are involved, there is a striking difference in the quality of the functionally appropriate regeneration that is achieved between nerves that were cut, and those that were crushed (Horch, 1979).

The last observation probably reflects differences in the selection and/or accessing of endoneurial tubes, rather than differences in target site end-organ selection within the target tissue itself. Fibres regenerating after nerve cut can reveal functional selectivity once they are within their target tissue. The post-ganglionic SCG neurons innervated by pre-ganglionic fibres from different spinal levels innervate characteristic end-organs. After axotomy,

regenerating pre-ganglionic fibres selectively reinnervate the post-ganglionic SCG neurons appropriate for the their spinal cord level origin (Langley, 1887; Guth and Bernstein, 1961). Similarly, in the chick the two types of presynaptic parasympathetic fibres after oculomotor nerve axotomy functionally and selectively appear to reinnervate their original targets in the ciliary ganglion, the post-ganglionic choroid and ciliary post-ganglionic neurons, while the motor fibres of the oculomotor nerve do not make any inappropriate synapses within the ganglion (Landmesser and Pilar, 1970). However, these functional studies did not exclude the presence of non-functional regenerated fibres which had regenerated to inappropriate targets and could be based on an unusually extensive arborization of a relatively small number of axons which successfully reached the vicinity of their appropriate target regions (Landmesser and Pilar, 1970).

ii) Cell body reaction and changes in molecular synthesis during regeneration

The soma of injured neurons undergoes a morphological change termed the cell body reaction whereby the appearance of the cytoplasm is altered, primarily due to the dissolution of Nissl substance that is associated with changes in transcription and translation (reviewed in

Lieberman, 1974). The cell body reaction, is a critically important feature in the interpretation of the results of this thesis, especially in light of the "near vs. far crush" phenomenon, which is discussed below. An increase in total protein synthesis (Oblinbger and Lasek, 1988), as well as alterations in specific gene expression and protein synthesis, have been described for regenerating neurons. In sympathetic neurons there is a down-regulation in the levels of mRNA coding for tyrosine hydroxylase, as well as a concomitant decrease in its activity (Raynaud et al., 1988, Koo et al., 1988). Axotomy leads to a decrease in p75 mRNA in sensory DRG neurons (Verge et al., 1992), and to an increase in p75 mRNA in motor neurons (Wood et al., 1990). After axotomy the levels of mRNA coding for certain structural proteins such as neurofilament proteins NF68 and NF140, are also reduced (Hoffman and Cleveland, 1988; Wong and Oblinger, 1990). In contrast, levels of mRNA coding for other structural proteins such as Tα1 α-tubulin (Miller et al., 1989), class II β-tubulin (Hoffman and Cleveland, 1988; Hoffman, 1989), and peripherin (a type IV intermediate filament; Wong and Oblinger, 1990), as well as the growth associated protein GAP-43/B50 (Skene et al., 1989), are upregulated in sensory and motor neurons during their regeneration. Levels of mRNA coding for still other structural proteins type I, IV β-tubulin, and T26 α-tubulin

remain unchanged during regeneration (Hoffman and Cleveland, 1988; Miller et al., 1989). During regeneration there is a decrease in the ratio of neurofilament to tubulins that are transported axonally, which has been hypothesized to facilitate axonal growth (Oblinger and Lasek, 1988). This change is primarily due to the changes in neurofilament synthesis (Oblinger and Lasek, 1988). It is not known what the signal is which induces these changes in gene expression (described above) after axotomy, however most of the changes in gene expression during regeneration, described above, recapitulate the developmental program of initial axonal outgrowth (Hoffman and Cleveland, 1988), which, as discussed earlier, is NGF-independent, at least for sympathetic and sensory nerves. This parallel between the developmental programs of gene expression for initial axonal outgrowth and axonal regeneration constitutes circumstantial evidence favouring NGF-independence of axonal regeneration.

The extent of the cell body reaction depends upon the distance of the axonal crush site from the cell body (reviewed by Lieberman, 1974). Crushes made close to the cell body induce a more severe, and shorter latency of onset of the cell body reaction (chromatolysis), and results in more neuronal cell death in both the peripheral nervous system (PNS) and CNS, than do crushes made further from the cell body. These observations are referred to as the "near

vs. far crush" phenomenon. In addition, the closer CNS axons are cut to their cell bodies, the more likely they are able to regenerate down an implanted peripheral pathway (Aguayo et al., 1991). The most distal form of axonal injury is that induced in sympathetic axons by 6-OHDA, whereby only the terminals and paraterminal regions of the axon tips are destroyed (Tranzer and Thoenen, 1968). In accordance with the extremely distal location of the lesion, a less vigorous cell body reaction would be expected compared to the one associated with a cut or crush lesion of the axon (which necessarily would be closer to the cell body). Consistent with this expectation, cutting (Bianchine et al., 1964; Matthews and Raisman, 1971) or crushing (Purves and Nja, 1978) post-ganglionic sympathetic nerves initiates a vigorous cell body reaction, while 6-OHDA lesions do not cause any ultrastructural changes in sympathetic cell bodies (Tranzer et al., 1969; Angeletti and Levi-Montalcini, 1970; Bjerre et al., 1974). Multiple lesions of an axon have also been shown to increase the cell body reaction when compared to single lesions (Howe and Bodian, 1941; Romanes, 1951; Oliver et al., 1969). These differences between the intensity of the cell body reaction induced by nerve crush and by 6-OHDA, and by single and multiple lesions, are proposed as key factors in explaining the results of the present studies.

iii) Changes in the gene expression in the distal stump associated with Wallerian degeneration and with axonal regeneration

During Wallerian degeneration and axonal regeneration changes in Schwann cell gene expression recapitulate many of the changes in Schwann cell gene expression that accompany the initial outgrowth of axons to their target tissues (Hoffman, 1989). Prior to the stage of myelination, human embryonic (E91-98) Schwann cells in femoral nerves express elevated levels of p75 (Scarpini et al., 1988), and embryonic Schwann cells in rat sciatic nerve express elevated levels of NGF (Bandtlow et al., 1987; Heumann et al., 1987b). There is a decreased expression of NGF by Schwann cells beginning at the time of birth, and also a decrease in expression of p75 beginning one week after birth; expression of NGF and p75 reaches adult levels three weeks after birth (Heumann et al., 1987b). This decrease in NGF and p75 expression is reversed after axotomy. Schwann cells in the degenerating distal stump express elevated levels of NGF, BDNF, and p75, (Taniuchi et al., 1986, 1988; Heumann et al., 1987a, 1987b; Meyer et al., 1992), and low levels of myelin proteins P_0, P_1, P_2, and myelin associated glycoprotein (MAG) (Politis et al., 1982) compared to Schwann cells in the normal adult nerve. Infiltration of macrophages into the degenerating distal

stump, and their subsequent release of IL-1, is responsible for sustaining the observed increases in Schwann cell NGF mRNA (Heumann et al., 1987b; Lindholm et al., 1987). After successful axonal regeneration, Schwann cell expression of NGF and p75 in the distal stump had dropped by two thirds by 20 days after a nerve crush (Heumann et al., 1987b). In contrast, production of Po, P1, P2, and MAG in the distal stump increased significantly 3 weeks after regeneration had begun into the distal stump (Politis et al., 1982). Based on the above findings the changes in Schwann cell p75, MBP, Po, and GMF-beta expression have been hypothesized to be regulated by axonal contact (Politis et al., 1982; Taniuchi et al., 1986; Heumann et al., 1987b; Borsch et al., 1989). Therefore, gene expression in the degenerating distal stump (after axotomy) is similar to the gene expression in the developing nerve trunk (at the time of the initial outgrowth of axons), and the changes in gene expression which occur after successful axonal regeneration are similar to those which occur after the initial outgrowth of axons.

iv) Regeneration and NGF

Based on the augmented levels of NGF and p75 expression in Schwann cells, it has been hypothesized that the NGF synthesized by Schwann cells binds to Schwann cell p75. The bound NGF is proposed to then act as a local

source of NGF for the regenerating sympathetic and sensory axons, which are dependent upon this molecule for their continued growth and as a chemotactic guide (Taniuchi et al., 1988). This hypothesis requires the direct transfer of NGF from the low-affinity NGF receptors on Schwann cells to the high-affinity NGF receptors on regenerating axons (Taniuchi et al., 1988). However, a direct transfer of NGF from high to low affinity receptors has yet to be demonstrated.

Experiments relevant to whether the resumption of Schwann cell NGF and p75 production is required for successful regeneration comes from studies using the mutant mice strain Ola, where Wallerian degeneration in the distal stump after axotomy is severely retarded, and where there is only a slight increase of Schwann cell NGF and p75 (Brown et al., 1991). In these mutant mice nerve regeneration, although not prevented, of both motor and sensory unmyelinated fibres is slowed (Lunn et al., 1989; Bisby and Chen, 1990; Brown et al., 1991; Brown et al., 1992). The very slow degeneration has been attributed to the demonstrated inability of macrophages to infiltrate the distal stump, which would be reasonably expected to result in very little increase in expression of p75 and NGF in the distal stump (Brown et al., 1991). However, the injection of exogenous NGF into the degenerating nerves produced only

a very slight increase in the rate of sensory fibre regeneration (Brown et al., 1991). Thus, it seems that low NGF levels can not be held responsible for the slow regeneration rate. More important, Schwann cells have been shown not to be necessary for successful regeneration after a nerve cut or crush, although the Schwann cell basal lamina is important. Limited regeneration occurred down nerves which were frozen to eliminate Schwann cells; the Schwann cell basal lamina was however, intact. Regeneration failed to occur at all down nerves which were scalded to destroy both Schwann cells and the integrity of the basal lamina (Gulati, 1988; Sketelj et al., 1989; Ide and Kato, 1990). However, it must be pointed out that this seemingly Schwann cell independent regeneration was limited to distances of less than 2 cm (Gulati, 1988). Thus, the above evidence does not support the hypothesis that NGF bound to the Schwann cell p75 receptor is necessary for regeneration of peripheral nerves.

Evidence that NGF is not involved in regeneration has come from studies of NGF binding and retrograde transport of NGF after axotomy. After axotomy the amount of high affinity NGF binding is decreased in a subset of DRG cell bodies which normally exhibit such binding (Verge et al, 1989). Recently, the levels of the mRNAs coding for p75 and for trk were reported to decrease after axotomy (Verge

et al., 1992). The decrease in expression of p75 and trk, and the decrease in high affinity NGF binding suggests a diminished role for NGF during sensory nerve regeneration, at least until after reconnection of axons to the target tissue reestablishes the NGF supply. After sciatic nerve crush the amount of NGF and NGF binding activity that is transported back to the cell body falls to 40% of the normal value (Heumann et al., 1987a; Raivich et al., 1991), while the level of NGF binding saturation remains at 7% (Raivich et al., 1991). The combination of a decrease in retrograde transport of NGF, NGF receptor expression, and high affinity binding, while not disproving the hypothesis that NGF taken up by regenerating sensory axons is necessary for regeneration, makes it seem unlikely.

In contrast to the axotomy induced decrease in high affinity NGF binding and p75 mRNA in sensory neurons, there is an increase in p75 mRNA in axotomised rat motor neurons, but its functional significance is not clear since motoneurons are not dependent upon NGF either during development or in the adult (Ernfors et al., 1989; Wood et al., 1990; Saika et al 1991).

Experiments on the neurite extension from adult sympathetic and sensory neurons *in vitro*, indicate that NGF is not necessary for their regeneration. Regeneration of SCG neurites occurs to a limited extent in dissociated

neonatal neurones, which had been kept in culture for one month, in the presence of anti-NGF (Campenot, 1981). Also anti-NGF did not prevent the innervation of whole irises by adult SCG fibres when the irises and SCGs are placed in direct contact in culture (Silberstein et al., 1971). The growth of neurites from dissociated adult sensory (DRG) neurons in culture was not prevented by anti-NGF (Lindsay, 1988). However, in all of the above cases exogenous NGF stimulated the extent of regeneration (Silberstein et al., 1971; Campenot, 1981; Lindsay, 1988). Thus, the regeneration of sensory and sympathetic neurons for which NGF has been hypothesised to be necessary (Taniuchi et al., 1986, 1988) does not require NGF for their regeneration *in vitro*, although NGF does appear to enhance this regeneration.

In vivo, when exogenous NGF was added to silicone chambers, there was an increase in the number of myelinated fibres which regenerated across the chamber after 4 weeks (Rich et al., 1989); after 10 weeks however, there was no difference in the number of myelinated fibres between chambers with or without exogenous NGF (Hollowell et al., 1990). Exogenous NGF also increased the calibre of motor axons regenerating across silicone chambers (Chen et al., 1989), as well as the calibre of sensory axons proximal to the lesion site (Gold et al., 1991). This NGF induced

increase in axon calibre would likely result in a greater amount of myelination, since myelination does not proceed until an axon calibre has increased to a threshold diameter (Voyvodic, 1989). Thus, the findings of Rich et al., (1989) and Hollowell et al., (1990) may result from a more rapid myelination of axons, brought on by an NGF-induced faster increase in axon calibre. In order to exclude this possibility, measurements and counts of both myelinated and unmyelinated axons need to be performed in the chamber experiments of Rich et al., (1989). Regeneration of sciatic nerve sensory axons after nerve crush proceeded normally in guinea pigs deprived of endogenous NGF through NGF autoimmunization; there was no alteration in the number of fibres regenerating through a tube connecting proximal and distal stumps, or an increase the amount of cell death after axotomy (Rich et al., 1984). Therefore, NGF does not appear to be necessary for sensory nerve regeneration across a silicone chamber *in vivo*, although it may enhance the myelination of the regenerating axons. Other studies on the *in vivo* regeneration of sensory nerves demonstrated that NGF was not necessary for sensory regeneration. Daily injections of anti-NGF did not prevent the regeneration and recovery of function by three classes of cutaneous sensory fibres, (those subserving thermo-nociception, mechano-nociception, and light touch) in the rat, even though the

anti-NGF treatment regime was many-fold greater than that which totally prevented the collateral sprouting of sensory fibres (Diamond et al., 1987; 1992a; 1992b, and see below).

The strongest evidence in favour of NGF being necessary for regeneration *in vivo* comes from experiments on the regeneration of post-ganglionic sympathetic axons after 6-OHDA treatment. Bjerre and colleagues concluded that NGF was involved in sympathetic nerve regeneration since exogenous NGF increased the number of sympathetic nerve fibres which regenerated after 6-OHDA treatment (Bjerre et al., 1973), while a single dose of anti-NGF decreased the number (Bjerre et al., 1974). The restoration of sympathetic innervation in a number of tissues was evaluated morphologically, by determining the number of sympathetic fibres, and the extent of their arborization, as revealed by staining for norepinephrine content by the Falck-Hillarp method. A complicating feature of these experiments is that NGF regulates the level of the rate limiting enzyme (Levitt et al., 1965) that is required for norepinephrine synthesis (Gorin and Johnson, 1980; Raynaud, et al., 1988). An alternate interpretation of their data is that the apparent effect on fibre regeneration was attributable to the effects of NGF in upregulating, and anti-NGF in down-regulating, the norepinephrine content of fibres, thereby affecting their histological visibility. It is possible, therefore, that

fibre regeneration per se was not affected. This problem is particularly relevant to the present studies, which also utilised a histological stain for sympathetic fibres which depends upon endogenous norepinephrine.

Even if NGF is not required for regeneration, NGF ameliorates some of the effects of axotomy. NGF reverses the decrease in SCG synapses and synaptic input after post-ganglionic cell axotomy (Nja and Purves, 1978). NGF also decreases the amount of axotomy induced atrophy and cell death of DRG neurons (Rich et al., 1987), as well as reversing the associated decrease of immunohistochemical staining within the spinal cord for a marker (fluoride-resistant acid phosphatase) of a subset of DRG terminals (Csillik et al., 1985). The sensory neurons with high affinity NGF binding are the most likely to have atrophied one month after axotomy (Verge et al., 1989), and delivery of exogenous NGF to the proximal nerve stump restores neuronal volume, high affinity NGF binding, trk and p75 mRNA levels in DRG neurons after axotomy (Rich et al., 1987; Verge et al., 1989, 1992). Axotomy is associated with the loss of target derived trophic factor(s), and the effects of axotomy which are reversed by NGF (or prevented in the case of cell death) are also produced by NGF deprivation without axotomy. Therefore, the reversal of these effects of axotomy by NGF does not demonstrate that NGF is necessary

for sensory or sympathetic nerve regeneration, but rather only that NGF can reverse the effects of NGF deprivation which accompany regeneration.

Thus, while it is reasonably clear that NGF is not necessary for sensory nerve regeneration, it is not clear whether NGF is necessary for post-ganglionic sympathetic nerve regeneration. This thesis addressed the question of whether NGF is necessary for post-ganglionic sympathetic fibre regeneration.

COLLATERAL SPROUTING

i) Introduction

Collateral sprouting refers to the branching growth emanating from undamaged nerve axons, which *in vivo* almost invariably occurs at or within the target tissue of the nerve concerned. In adult organisms collateral sprouting is commonly evoked experimentally by partially denervating a target tissue, which induces neurite outgrowth from the remaining undamaged fibres into the deprived regions of the tissue. Evidence will be presented which suggests that collateral sprouting may be an on-going process in normal non-denervated tissues, playing a role in a dynamic turnover of endings. Collateral sprouting of sensory endings was first observed in the frog tadpole tail fin by Speidel in 1935, and later was demonstrated for sensory fibres in the

rabbit ear by Weddel et al., (1941). It has also been demonstrated in the sympathetic system (Murray and Thompson, 1957; Guth and Berstein, 1961; Olson and Malmfors, 1970), the parasympathetic system (Roper, 1976), and for motor peripheral axons (Edds, 1953), and also in the CNS (see Tsukahara, 1981). The regulation of collateral sprouting by trophic factors and how such regulation may control innervation density, is one of the major themes of this thesis.

ii) Collateral Sprouting in the PNS and CNS

a) autonomic neurons

Both pre- and post-ganglionic sympathetic fibres, as well as parasympathetic fibres, undergo collateral sprouting. Collateral sprouting of post-ganglionic sympathetic fibres has been demonstrated in a number of experimental models. In the iris, collateral sprouting of these fibres is induced by increasing the amount of target tissue, established by the insertion of supernumerary irises into the anterior chamber of the eye (Olson and Malmfors, 1970). After partial denervation of the target tissues, sprouting of the surviving post-ganglionic sympathetic fibres has been described for the sweat (Kennedy and Sakuta, 1984) and pineal glands (Lingappa and Zigmond, 1987; Dornay et al., 1985). Postganglionic SCG sympathetic fibres also

sprout in dentate gyrus and CA3 region of the hippocampus following partial deafferentiation of the hippocampus by fimbria/fornix lesions (Crutcher et al., 1979, 1981; Springer and Loy, 1985). These sprouted fibres may have originated either from the sparse sympathetic innervation of the hippocampus or from the sympathetic fibres innervating cerebral blood vessels (Crutcher et al., 1979).

Evidence for the functional sprouting of pre-ganglionic sympathetic fibres within the partially denervated superior cervical ganglia has also been demonstrated (Murray and Thompson, 1957; Guth and Berstein, 1961). As described previously, following axotomy of post-ganglionic neurons there is a retraction of pre-ganglionic fibres in the SCG. After regeneration the pre-ganglionic fibres sprout collaterals, restoring the number of pre-ganglionic fibres in the SCG to normal levels (Purves and Nja, 1978). Whether pre-ganglionic fibre sprouting accompanies post-ganglionic sprouting is not known. Therefore, in the experiments performed for this thesis studying post-ganglionic axonal regeneration and collateral sprouting, pre-ganglionic fibre retraction and sprouting may be occurring.

b) sensory neurons

The collateral sprouting of sensory neurons has been extensively studied in various animals, revealing differences in the regulation of such sprouting between different species and between different modalities, as well as differences in the territorial constraints of such sprouting (reviewed by Diamond et al., 1992). After adjacent denervation, sprouting of the fibres mediating light-touch in adult salamander skin is limited to specific territorial domains (Diamond et al., 1982), as is the sprouting of the analogous fibres that can occur in the hairy skin of rats during a brief period of post-natal life (Jackson and Diamond, 1984; Mills et al., 1989). These same fibres, however, regenerate after a crush in both adult rats and adult salamanders, and their regeneration does not exhibit domain constraints (Scott et al., 1981; Jackson and Diamond, 1984).

The ability of sensory nerves to undergo collateral sprouting is related to their modality. After adjacent denervations in the adult rat, the large myelinated fibres that mediate light touch (Horsch 1981; Jackson and Diamond, 1981; 1984) and taste (Kinnman and Aldskogius, 1988) fail to sprout, although the dermal and lingual nociceptive fibres (Aδ and C) sprout readily (Weddel, 1941; Nixon et al., 1984; Doucette and Diamond, 1987; Kinnman and Aldskogius, 1988).

Although the taste, low threshold touch, and nociceptive fibres differ in their sprouting ability, all can regenerate with apparently equal ease (Zalena, 1964; Diamond et al., 1987). From these observations, then, it would seem that the axonal regeneration and collateral sprouting of sensory fibres are differentially regulated.

c) motoneurons

The sprouting of motor axons, first demonstrated by Edds (1953), has recently been reviewed (Brown et al., 1981). The motoneuron sprouts originate from both internodes and unmyelinated nerve terminals (Brown, 1981). The possibility that sprouting can occur from both terminals and internodes is relevant to the interpretation of experiments performed in this thesis. Angaut-Petit et al., (1982, 1987) have suggested that the collateral sprouting and regeneration of motor fibres is restricted to the continuous perineurial pathways, and cannot continue into neighbouring non-continuous perineurial pathways.

d) CNS neurons

Collateral sprouting in the central nervous system has been observed in the spinal cord and brain (reviewed by Tsukahara, 1981; Cotman et al., 1981). For example, the spinal roots of spinal nerves sprout in the dorsal horn of

the spinal cord after adjacent rhizotomy (McNeil et al., 1990), while elimination of the entorhinal cortex input induces the undamaged septo-hippocampal and commissural-associational inputs to sprout in the outer molecular layer of the dentate gyrus (Scheff et al., 1980).

iii) Regulation of Collateral sprouting

a) Trophic molecules.

Sensory nerves in the salamander are induced to sprout not only by the surgical removal of neighbouring nerves, as described above, but also by the application of colchicine, to neighbouring nerves (Aguilar et al., 1973); colchicine is a blocker of axonal transport (Dahlström, 1968). Similarly, application of colchicine to one of the spinal nerves supplying the plantaris muscle induced the collateral sprouting of the other spinal nerve innervating plantaris (Guth et al., 1980), while application of colchicine to the fimbria caused the collateral sprouting of commissural fibres in the hippocampus (Goldowitz and Cotman, 1980). Aguilar et al., (1973) hypothesized that axoplasmic transport is normally involved either with delivery of a substance which prevents collateral sprouting, or the removal of a substance which inhibits collateral sprouting, and that colchicine treatment blocks these processes.

NGF has now been demonstrated to be a molecule which controls collateral sprouting in a couple of experimental situations. NGF, as described above, is a trophic molecule retrogradely transported in sensory and sympathetic neurons. Local injections of anti-NGF, prevents the collateral sprouting of sympathetic neurons into the hippocampus after elimination of its septal input by fimbria fornix lesions (Springer and Loy, 1985). However, the elevation of NGF levels in the hippocampus, by infusion of exogenous NGF into the ventricles, while raising NGF levels in the hippocampus to triple that found after fimbria fornix lesion, failed to induce the ingrowth of sympathetic fibres into the hippocampus, suggesting that NGF may be necessary but not sufficient for, sympathetic sprouting in the hippocampus (Saffran et al., 1989). The experiments using transgenic mice ectopically expressing NGF, as described previously, also support this contention. Daily subcutaneous injections of anti-NGF reversibly inhibited the collateral sprouting of intact cutaneous heat- and mechano-nociceptive sensory nerves into adjacent denervated skin, while direct intradermal injections of exogenous NGF enhanced the rate of this sprouting, and evoked sprouting in normal innervated skin (Diamond et al., 1987; 1992a). Since the anti-NGF in the latter experiments recognizes NT-3 and to a lesser extent BDNF the actual neurotrophin necessary

for collateral sprouting of cutaneous nociceptive fibres is not certain (Diamond et al., 1992a).

Spared motor axons sprout when muscle is partially denervated, but intact motor fibres will also sprout when neuromuscular transmission is prevented by by blocking acetylcholine release using botulinum toxin injections (Duchen and Strich, 1968), or by blocking nerve impulse using tetrodotoxin (Brown and Ironton, 1977). These last observations led to the hypothesis that a neurotrophic molecule is released by inactive muscle which is responsible for the collateral spouting of motor axons, and that the production of this molecule is down-regulated with increased muscle activity. Thus, the production of trophic molecules by a target tissue may be a common mechanism by which collateral sprouting is controlled. The regulation of collateral sprouting by these trophic molecules could be acting at the level of the growth cone and/or at the level of the cell body (Brown et al., 1981).

b) Impulses

The partial denervation of a target tissue may not be sufficient to induce collateral sprouting from the remaining axons. The sprouting of post-ganglionic sympathetic fibres in the pineal gland is dependent upon the integrity of pre-ganglionic input in the SCG, and fails to

occur after the pre-ganglionic denervation of the ganglion (Dornay et al., 1985). Surprisingly, however, the analogous sprouting of post-ganglionic SCG fibres in the hippocampus (Crutcher et al., 1979), or to a supernumary iris implanted in the anterior chamber of the eye, is not prevented by SCG decentralization (Olson and Malmfors, 1970). One possible relevant factor that has not been examined in these experimental situations was the extent to which SCG decentralization reduced or abolished impulse activity in the post-ganglionic fibres. It is not known whether pre-ganglionic inputs regulate the collateral sprouting of post-ganglionic sympathetic neurons in the experimental system used in this thesis. As will be discussed later, such activity might influence collateral sprouting, although there is no direct evidence for this in the autonomic system.

Following partial denervations, cutaneous heat- and mechano-nociceptive fibres sprout in the rat dorsal skin after a latency of 10-12 days. Impulse activity, evoked either by pinching the skin or by electrical stimulation of the nerve, at the time of isolation and up to two days before isolation, reduced this latency by 5-6 days, resulting in the "precocious sprouting" of these fibres (Nixon et al., 1984, Doucette and Diamond, 1987, Diamond et al., 1992a). Since the impulses must proceed to the cell

body for this effect (Nixon et al., 1984, Doucette and Diamond, 1987), this is an example of control of collateral sprouting at the cell body. This influence of impulse activity on collateral sprouting could constitute a mechanism by which nerve activity may mediate the morphology of neuronal projections. The possibility that impulses play a role in regulating the collateral sprouting of post-ganglionic sympathetic neurons is considered further in the Discussion.

The regulation of collateral sprouting by trophic factors and impulses are interrelated. The induction of precocious sprouting by impulses in cutaneous nociceptive nerves is prevented in rats which are treated with an anti-NGF "umbrella" at the time of nerve stimulation (Diamond et al., 1992a). The sprouting then proceeds with the normal or non-precocious time course. Thus, the induction of precocious sprouting, as well as the normal process of collateral sprouting of the cutaneous sensory nerves is NGF dependent (Diamond et al., 1992a).

iv) Molecular correlates of collateral sprouting

Almost all SCG neurons (Richardson et al., 1986) and a subpopulation of DRG neurons (Verge et al., 1989; 1990) possess high affinity NGF binding. These same neurons express GAP-43, considered to be a marker of neuronal

plasticity (Skene, 1989). Since SCG neurons and a subpopulation of DRG neurons can undergo collateral sprouting, there may be a connection between an undamaged neuron's expression of high-affinity NGF binding and its ability to undergo collateral sprouting, consistent with a role for NGF in mediating collateral sprouting of sensory and sympathetic neurons (Verge et al., 1990). High affinity NGF binding is also noted in various CNS neuronal populations, in particular, the cholinergic neurons of the basal forebrain (Richardson et al., 1986), whose sprouting is also NGF dependent (Van der Zee et al., 1992). The significance of high affinity NGF binding will be discussed below.

Whatever the external factors are which induce collateral sprouting, ultimately they promote the appropriate gene expression required to provide the materials needed to support neurite outgrowth. Little work has been done on this topic compared to the regeneration studies described above. One correlate of collateral sprouting, that has been demonstrated in sprouting SCG neurons, is the increased expression of Tα1 α-tubulin, a form of tubulin found in growing neurons (Mathew and Miller, 1990). Another correlate is the upregulation of the p75 NGF receptor, demonstrated for the sympathetic neurons sprouting in the pineal (Kuchel et al., 1992). Since NGF also

increases expression of Tα1 α-tubulin (Mathew and Miller, 1990) and p75 (Ma et al., 1992) in sympathetic neurons, the increase in their expression during collateral sprouting may be due to the increase in NGF available for retrograde transport in the partially denervated tissue (Mathew and Miller, 1990). As mentioned earlier Tα1 α-tubulin and p75 are upregulated during regeneration. Since after axotomy the retrograde transport of NGF falls, the signal for the increased expression of these proteins during regeneration can not be NGF levels. Because both regeneration and collateral sprouting involve neurite extension, it can be anticipated that many of the changes in expression of genes coding for structural proteins occur during the two forms of nerve growth, although the mechanism by which these changes are made may be different between collateral sprouting and regeneration.

vi) Functional plasticity - dynamic turnover

While collateral sprouting is revealed experimentally by partial denervations, it may also occur in the normal intact animal as part of a dynamic turnover of endings. Barker and Ip (1966) hypothesized that the density of innervation is maintained by the opposing processes of collateral sprouting and fibre retraction. They proposed a turnover of endings based upon their observations in cat and

rat hindlimb muscles of fusimotor and extrafusal motor fibres and endplates, which in some instances had the characteristics of growing fibres and newly formed endplates, and in other instances had the characteristics of degenerating endplates and fibres. The former they hypothesised, were growing towards the muscle to innervate it. Similarly, a dynamic turnover of endings in the CNS was proposed by Sotelo and Palay (1971) based upon their observations of what they interpreted as occasional spontaneously degenerating nerve terminals in the brains of normal animals. There is substantial evidence for the continual turnover of synapses in many PNS and CNS systems based on observations of regressing and newly formed endings in normal tissue (reviewed by Cotman et al. 1981). A dramatic example of this turnover is the almost complete degeneration of sympathetic innervation of the uterus which occurs during pregnancy, followed by the regrowth of these fibres after delivery (Sporrong et al., 1978). Direct observations of nerve terminals by fluorescent dye techniques, has revealed an ongoing process of remodelling of postganglionic SCG neuronal dendrites (Purves et al., 1986), of pre-ganglionic synapses on parasympathetic neurons (Purves et al., 1987), of sensory fibres in the mouse cornea (Harris and Purves, 1989), and of motor terminals at frog neuromuscular junctions (Herrera et al.,, 1990, 1991);

interestingly, little or no remodelling was observed at neuromuscular junctions in mice (Lichtman et al., 1987). Since the proposed dynamic turnover of endings, like experimentally induced collateral sprouting, probably involves the growth of undamaged fibres, it is possible that the mechanisms mediating them may be identical (see Diamond et al., 1988). If so, neurotrophins and impulse activity could be involved in the regulation of a normal dynamic turnover of endings, as in collateral sprouting.

vii) Density of innervation and NGF

NGF has been implicated in regulating the density of sympathetic and sensory innervation. Levels of NGF (Korsching and Thoenen, 1983; Shelton and Reichardt, 1984) and its message (Heumann et al., 1984) are positively correlated with the density of sympathetic innervation. Other experimental results also suggest a role for NGF in the regulation of innervation density. Exogenously applied NGF increases the number of sympathetic fibres in many tissues including iris, salivary gland, and heart (Bjerre et al., 1975). From these observations, Bjerre et al., (1975) concluded that NGF controlled the innervation density by regulating the collateral sprouting of sympathetic axons. As discussed earlier, experiments in which either NGF is

over expressed in specific cells, or NGF is ectopically expressed, have shown that increased NGF expression increases the density of sympathetic innervation only if the tissue is normally sympathetically innervated. Therefore, NGF is unlikely to be involved in the initial sympathetic ingrowth into a target tissue but only in regulations of the neuronal growth within it (Borrelli, et al., 1992). Neurotrophins also appear to be involved in regulating the density of dendritic branches emanating from neuronal somata. The dendritic arbors of adult mice postganglionic sympathetic neurons are increased in length and complexity by elevated levels of NGF and decreased by lower levels of NGF (Ruit et al., 1990).

viii) Competition for NGF by different neuronal populations

Different neuronal subpopulations can compete for and respond to the same neurotrophins. In 1985, Korsching and Thoenen showed that competition occurs between sensory and sympathetic fibres for available NGF; after sympathetic nerve terminals have been destroyed (by systemic 6-OHDA injections), the amount of NGF retrogradely transported to the dorsal root ganglia is increased within two days, presumably because the NGF levels in the target tissues have risen due to the elimination of NGF uptake by the sympathetic fibres (Korsching and Thoenen, 1985). The NGF-

dependent sprouting of sympathetic fibres into the hippocampus, described earlier, is induced by the elimination of its cholinergic septal input, suggesting that the septal pathway neurons and the sympathetic neurons are competing for NGF or a related molecule (Springer and Loy, 1985). In support of this, the sprouting of the septohippocampal fibres in the outer molecular layer of the dentate gyrus is inhibited by anti-NGF infusions into the ventricles (Van der Zee et al., 1992). Indirect evidence for competitive interactions comes from studies of transmitter levels; elimination of the sympathetic, sensory, or parasympathetic fibres from the iris increases the levels of markers for the remaining two fibre types (tyrosine hydroxylase for sympathetic fibres, substance P for sensory fibres, and choline acetyltransferase for parasympathetic fibres) (Kessler 1983, 1985). Administration of exogenous NGF also increased the concentration of the markers for each of the three fibre types, leading Kessler (1985) to conclude that the three neuronal populations were competing for NGF, and that NGF thereby increased the number of fibres or the amount of marker per fibre, or both. It follows that the production of multiple neurotrophins in a target tissue should permit the differential regulation of the innervation densities of different neuronal subpopulations, these subpopulations each responding to the separate

neurotrophins.

Sympathetic nerves in adult rats regenerate normally and restore pilomotor function during an anti-NGF treatment that prevents their collateral sprouting

Andrew Gloster and Jack Diamond

Published in the Journal of Comparative Neurology 236:263-274

Abstract

We have used nerve growth factor (NGF) anti-sera administration to study the NGF dependency of the re-innervation of denervated skin by sympathetic nerves in the adult rat. Sympathetic pilomotor fields were revealed by electrical stimulation of selected dorsal cutaneous nerves; the affected skin rapidly assumed a "gooseflesh" appearance, sharply demarcated from surrounding unstimulated skin. Evoked 2-5 days after section of neighboring nerves, the "isolated" pilomotor field of the spared nerve was found to be coextensive with an area of amine-fluorescent fibers that were associated with pilomotor muscles and blood vessels. After its isolation, a pilomotor field begins to expand into the surrounding deprived territory, reaching a maximum size at approximately 40 days. Fluorescence studies confirmed that new sympathetic fiber growth had occurred into the expanded regions of such fields. Daily injections of polyclonal anti-NGF serum completely prevented these pilomotor field expansions. Following termination of the

anti-NGFtreatment, expansion proceeds normally. Finally, if the onset of anti-NGF treatment was delayed until pilomotor field expansion had already commenced, further expansion was halted. Regeneration of sympathetic fibers was evoked by crushing a selected nerve. Recovery of pilomotor function in the totally denervated skin was first detected at about 20 days post-crush, and the field progressively enlarged over the next 40 days. Although the imposed NGF deprivation is known to cause a demonstrable shrinkage, and presumably atrophy (Diamond et al., '92a) of sympathetic ganglia, the anti-NGF treatment appeared to impair neither the restoration of a pilomotor field after nerve crush, nor its continued expansion into skin regions well beyond that originally supplied by the nerve, i.e. into territory whose invasion by collateral sprouts would have been totally prevented by the treatment. During such NGF deprivation fluorescent regenerating fibers were visualised in the nerve trunk. We conclude that even though the regenerating and collaterally sprouting sympathetic fibers probably utilise the same degenerating dermal pathways to reach and functionally re-innervate the same denervated targets, only the collateral sprouting of the uninjured axons is dependent upon endogenous NGF. These findings extend the results described earlier for nociceptive fibers, and suggest that these contrasting dependencies upon growth factors by

sprouting and regeneration might apply throughout the adult nervous system.

Introduction

During development the differentiation and survival of sympathetic post-ganglionic neurons depend critically upon the availability of endogenous nerve growth factor (NGF) (Levi-Montalcini and Booker, '60; Klingman and Klingman, '67). This NGF dependency persists into adult life, albeit at a reduced level (Angeletti et al., '71; Gorin and Johnson, '80; Ruit et al., '90). It seemed unlikely, therefore, that either of the two potential growth behaviors of adult sympathetic neurons, the collateral sprouting of undamaged axons (Olson and Malmfors, '70), or the regeneration of damaged ones (Tuckett, 1896; Kilvington and Osborne, '07), would occur independently of NGF. Nevertheless, an examination of this possibility seemed an especially challenging test of the proposal concerning these two growth responses that emerged from our study in adult sensory neurons (Diamond et al., '92 a,b). Like sympathetic neurons, the sensory neurons of the dorsal root ganglion (DRG) also derive from the neural crest, and most, if not all, of the nociceptive population are also NGF dependent during development (Johnson et al., '80; Aloe et al., '81; Goedert et al., '84). Soon after birth, however, this absolute dependency upon NGF disappears (Goedert et al., '84; Yip et al., '84). Unexpectedly, while the collateral

sprouting of cutaneous nociceptive nerves in the adult rat was entirely blocked by a maintained anti-NGF regime, their regeneration was totally unaffected; yet the two types of nerve growth utilized the same pathways within denervated skin (Diamond et al., '92a,b). These and related findings led to the proposal that axonal regeneration is sustained by relatively non-selective mechanisms, while collateral sprouting is dependent upon specific growth factors such as NGF. We now report the further testing of this concept on adult sympathetic neurons.

A maintained NGF deprivation in adult animals seemed particularly likely to compromise <u>any</u> new growth of sympathetic axons, since it causes an appreciable shrinkage of sympathetic ganglia (Diamond et al., '92a; cf. Angeletti et al., '71) and loss of sympathetic neurons (Gorin and Johnson, '80; Snider et al., '88; Ruit et al., '90, but see Angeletti et al., '71). Moreover the ability to visualize sympathetic axons by the preferred approach, namely the glyoxylic acid-induced fluorescence technique (cf. de la Torre, '80) would likely be compromised by anti-NGF treatment which eventually leads to a marked reduction in transmitter levels in sympathetic neurons (Gorin and Johnson, '80). Despite these concerns, the results show that during a maintained anti-NGF administration which appeared to totally prevent their collateral sprouting, and

coincidentally to bring about the anticipated compromising of sympathetic neurons, sympathetic axons not only regenerated but restored autonomic functions. This distinction between the NGF dependency of the two types of nerve growth in the sympathetic system greatly strengthens our proposal relating to axonal regeneration and collateral sprouting (Diamond et al., '92a,b), suggesting that it might be applicable to the adult nervous system generally.

Materials and Methods

Preparation of NGF antiserum

The preparation and purification of 2.5S NGF from male mouse salivary glands followed the procedures of Mobley et al. ('76) and Darling and Shooter ('84), and are further detailed in the report of Diamond et al. ('92a). Neurotrophic biological activity was measured in the dissociated cell assay (Greene, '77) with neonatal mouse superior cervical ganglion neurons (Coughlin and Collins, '85); half-maximal response was at 1 ng/ml. The preparation of 7S NGF followed the procedure of Varon et al. ('67), as modified by Stach et al. ('77). Half-maximal activity in the dissociated cell assay was also at 1 ng/ml.

Sheep (ewes) were used for the preparation of NGF antiserum (anti-NGF). The animals received initial intradermal injections (at 20-30 sites) of 0.5 mg of 2.5S NGF in phosphate buffered saline mixed with 2 volumes of Freund's complete adjuvant. Thereafter, monthly boosts of these amounts of 2.5S NGF were made in Freund's incomplete adjuvant. Blood was collected from the jugular veins, beginning 10 days after the second boost, allowed to clot at room temperature, and the serum was heat inactivated at 56 C for 30 min, cooled, and filter sterilized.

To determine serum titers of anti-NGF, serial

dilutions of the antiserum in culture medium were combined with equal volumes of medium containing 20 ng/ml 7S NGF (final concentration 10 ng/ml 7S NGF), incubated at room temperature for 1 hr and assayed by the dissociated cell method, as above. A 10,000-fold dilution of antiserum completely blocked the activity of 10 ng/ml 7S NGF.

Specificity of the antibody was determined by the Ouchterlony double diffusion analysis and by Western blot, as described in Diamond et al., ('92a). The antibody recognized 2.5S and 7S NGF, but not mouse serum proteins, renin, or epidermal growth factor. We now know that our antibody recognizes both neurotrophin 3 (NT-3), and to a lesser extent, BDNF (R.A. Murphy personal communication).

Administration of immune and non-immune serum

Rats were injected subcutaneously, in the nape of the neck. The standard daily dosage of 2.5 µl/g body weight was based on the findings from our previous sensory nerve studies, and is some 30-40% higher than the minimum dosage required to prevent the collateral sprouting of cutaneous nociceptive fibers (Diamond et al., '92a).

Mapping of sympathetic pilomotor fields

The objective here was to define the area of skin containing the pilomotor muscles innervated by the

sympathetic fiber population contained within a selected dorsal cutaneous nerve (DCN). The hair along the dorsal midline was first clipped and depilated with a commercial depilating agent (Neet). To reveal the "pilomotor field" of the DCN, the pilomotor muscles were activated by electrical stimulation of the sympathetic fiber population of the nerve via a pair of platinum wire electrodes, by stimulus parameters previously found to be supramaximal for activation of the similarly sized sensory C fibers (approximately 8V at 20Hz). Approximately 20 sec of stimulation was found to result in a distinct area of altered skin, displaying a clear demarcation between affected and unaffected regions (Fig. 1). The contraction of the pilomotor muscle caused an elevation of the touch domes and a characteristic "fine-grained" ruffling of the skin; the latter was quite different from the coarse skin corrugations involved in the cutaneous trunci muscle (CTM) reflex response to nociceptive stimulation described in previous reports (Nixon et al., '84; Doucette and Diamond, '87). About 10 min later, when the skin texture had returned to normal, the nerve was re-stimulated to confirm the accuracy of the original mapping. Nerve stimulation was performed only when the skin was completely smooth; on occasion some ruffling was present, attributable to circulating catecholamines, which can also activate

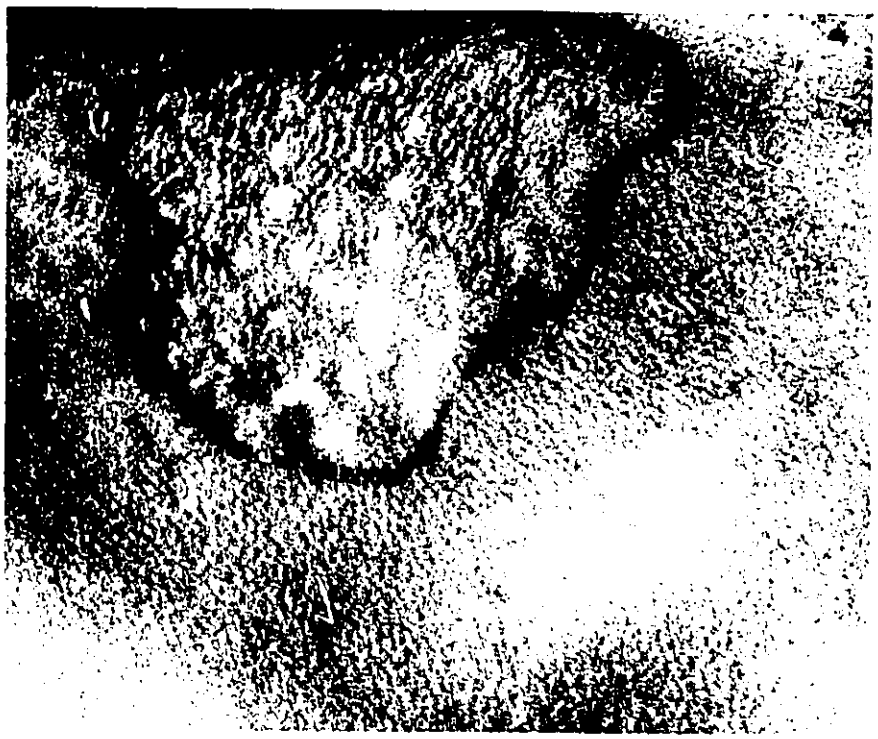

Figure 1. The pilomotor field. This animal was prepared, as explained in the text, for pilomotor field measurement and the photographs were taken 1-3 minutes after nerve stimulation. A distinct area of the skin shows the characteristic "goose flesh" appearance, with elevated touch domes and a finer dimpling between them. A line has been drawn along the functional border. While the clearness of the demarcation is evident, it is very dependent on the angle of lighting. To reveal the whole border the light needed to be moved from the position which gave this viewing. When that was done, all the border was easily seen. This map was then traced onto acetate for measurement and to provide a permanent record. Scale bar is 500μm.

pilomotor muscles (Koizumi and Brooks, '80). Other distinguishing features of this sympathetic pilomotor response (see Results) were that (i) it developed over a period of 5 sec after the onset of stimulation; (ii) the appearance was maintained for some 1-2 min after stimulation ended; (iii) the response could be obtained by exciting the distal portion of the DCN after it was sectioned, i.e. it was a directly evoked efferent response, and not a reflexly evoked one; and finally, (iv) the response could be prevented by the α-adrenergic blocker phentolamine. The area of pilomotor response was defined as the functional sympathetic field for the stimulated nerve. (The area of pilomotor innervation was found to be essentially co-extensive with the area of sympathetic innervation of the cutaneous vasculature for a given DCN, see Results later).

The isolated sympathetic field "paradigm"

The objective here was to produce a field of sympathetic innervation within a surround of totally denervated skin, thus "isolating" the sympathetic field. The operative procedures were similar to those described for the analogous isolation of sensory fields in Doucette and Diamond ('87), and Diamond et al., ('92a). Briefly, the DCNs were exposed in female Wistar rats (150 - 300 g) anesthetized by intraperitoneal injection of 45 mg/kg sodium

pentobarbital. Body temperature was maintained by a heating pad. The permanent elimination of selected DCNs was achieved by cutting them near their site of emergence from the body wall and by excising the entire distal portion of the nerve (some 2-3 cm, almost to the skin). The thoracic DCNs 9, 10, 11 and 12, the lumbar DCNs 1, 2 and 3, and the lateral cutaneous nerves T10-13 and L1-3, were removed on the left side, leaving only DCN-T13 intact; the lateral of the 2 branches of this nerve was then cut a few mm from the body wall, ligated, and the central stump buried deep under the latissimus dorsi muscle. This procedure isolated the cutaneous field of the medial branch of DCN-T13 (mDCN-T13) on the left side, within a relatively vast surround of denervated skin. The skin was sutured with a continuous silk thread.

When a field had been isolated and defined, a few spots were tattooed into the skin, some 7-8 mm outside its border, by puncturing the skin with a 27 gauge hypodermic needle dipped in India ink. These spots provided reference points to be used in the comparison of field mappings done at later times. To make permanent records, a sheet of transparent acetate was placed over the skin of the rat, and the positions of the field borders and the reference tattoos were transferred to it with waterproof colored markers. Areas of the fields were determined with an image analyzer

(Bioquant).

Light touch field mapping

The low threshold mechanosensory ("touch") field, which was used as a "reference" nerve field in some of the experiments, was delineated electrophysiologically (Jackson and Diamond, '84). The dissected-free and cleaned nerve trunk was laid across bipolar platinum wire electrodes connected to a differential AC preamplifier, the signal from which was displayed on a Tektronix storage amplifier and audio amplifier. The brushing of innervated skin with a fine bristle evoked clearly audible responses, which disappeared abruptly as the stimulus crossed the touch field border.

Collateral sprouting of sympathetic fibers

The selected nerve, mDCN-T13, was stimulated to reveal its pilomotor field after this was isolated by denervation of the surrounding skin as described above. The mapping procedure was repeated at intervals of approximately 10-20 days, for a total span usually of 40-120 days. Changes in field areas resulted from the collateral sprouting of sympathetic fibers (see below), and were always evaluated with respect to the original field size. The proportional enlargement of the nerve field associated with

the normal growth of the animal, but unrelated to its isolation, was analysed with the area enclosed by reference tattoos as an internal standard. This provided a correction factor, which turned out to be needed only in animals examined at times greater than 40 days after field isolation. The occurrence of scarring and self-mutilation limited the number of times a pilomotor field could be examined in any one particular animal; in most instances one to three mappings were possible.

Regeneration of sympathetic fibers

Axonal regeneration was initiated by crushing with a pair of fine watchmaker's forceps (3 times for 1 min), a selected DCN, whose field, in some instances, had been previously isolated as described earlier (cf. Diamond et al., '92). To assess the completeness of the crushes in the present study, the most obvious approach would have been to stimulate the nerve proximal to the crush site and examine appropriate skin area for the presence or absence of the pilomotor response. However, because the necessary chemical depilation could cause scarring, particularly in denervated skin, it was not used immediately after initial denervations. Instead, pilomotor function was first examined when evidence of functional regeneration was first sought. Since the sympathetic fibers are similar in size to

the unmyelinated sensory fibres, the completeness of nerve crushes was determined 3-4 days afterwards by testing for the presence of surviving sensory fibers distal to the crush. This testing was accomplished in one of two ways. The first was a straightforward test for the persistence of a surviving mechano- and heat-nociceptive innervation within the deprived region of skin by pinching it or by applying a heated probe, respectively, and noting whether a CTM reflex response was evoked as described in earlier reports (Nixon et al., '84; Doucette and Diamond, '87). The second approach was used particularly in studies in which the nerve field had not been previously isolated at the time of nerve crush; it utilized a brief episode of electrical stimulation of the nerve trunk distal to the crush site, again to see if the sensory-evoked CTM response reflex occurred (Doucette and Diamond, '87). In some of these regeneration studies the skin was extensively denervated, and the incidence of scarring was much higher than that in the study of collateral sprouting, which involves the denervation of smaller skin areas. When the scarring was judged as more than slight the experiment was discontinued.

Examination of sympathetic fiber fluorescence

The presence of sympathetic fibers in the skin was revealed by a modification of the glyoxylic acid technique

for monoamine fluorescence (de la Torre, '80). This approach took advantage of the monoamine uptake system of sympathetic fibers taking up not only their normal transmitter, norepinephrine, but also the false transmitter, α-methyl norepinephrine (α-CH_3-NE) (Malmfors, '65); this monoamine also fluoresces, and enhances the fiber fluorescence seen with the glyoxylic acid technique. Animals were injected intraperitoneally with 1 ml of α-CH_3-NE solution (1mg/ml, Sigma). Skin samples (10 x 2 mm) were taken 30-90 min later, frozen in a -10 C freezer, and stored at -70° C. Cryostat sections (30 μm thick) were placed on a microscope slide and covered with a 2% glyoxylic acid solution (in 0.2 M sucrose, 0.25 M phosphate buffer, pH 7.4) for 5 min. The slides were then air dried under a stream of cool air, and put in a 100° C oven for 2-4 min. The specimens were then covered with light paraffin oil and coverslipped. Slides were examined with a Zeiss universal microscope equipped with an epifluorescence condenser (RS III; filter set: BP 390-420 excitation, FT 425 beam splitter, LP 450 barrier). Segments of the cutaneous nerve trunk were also examined distal to the point of crush; these were simply immersed in the glyoxylic acid solutions for 5 min, placed on a slide, and then treated as for the skin sections described above.

GAP-43/B50 Immunocytochemistry.

The dorsal T9-L4 and lateral T9-L3 cutaneous nerves were surgically removed, completely denervating a large region of the rat dorsum. Skin samples were taken from this region 7 days later, and placed in a 4% paraformaldehyde, 4% picric acid solution overnight. After being transferred to 10% and then 20% sucrose solutions over a 2 day period, the skin blocks were frozen and stored at -20° C. Thirty μm sections were cut on a cryostat from the frozen skin blocks, and placed on slides, on which they air-dried over night. Slides were incubated with GAP-43 (B50) monoclonal antibodies (1:1000 dilution) [(a generous gift of Dr. D.J. Schreyer (Schreyer and Skene, '91)] for 5 hrs, rinsed 3 times for 10 min in 0.1 M phosphate buffered saline (PBS) and then incubated with fluorescein labelled anti-mouse antibodies (1:100) (Sigma) for 1 hr, rinsed 3 times for 10 min in 0.1 M PBS, once for 5 min in 4mM carbonate buffer, and then finally coverslipped with a 0.1% paraphenylene diamine in glycerol mounting media. Slides were viewed as described for the glyoxylic acid technique, but with a BP 390-420 excitation, FT 425 beam splitter, and LP 450 barrier, filter set.

Results

The pilomotor fields

Field areas

In approximately 150 animals whose weights ranged from 149-288 g (mean 205 g; ± 31 SD) the physiological pilomotor field of mDCN-T13 varied from 110-853 mm^2 (mean 354 mm^2; ± 97 SD). The fields were roughly elliptical in shape with the long axis running medio-laterally, but their borders were somewhat irregular. The stimulation period routinely used to evoke the pilomotor fields was 30-60 sec, but they were usually clearly defined after 5-10 sec of stimulation. Following cessation of stimulation the fields remained well characterized for approximately one min and then began to fade, disappearing completely over the following 3-10 min. The field border was sharply delineated, remaining essentially unchanged in location and sharpness of quality over repeated stimulation episodes (at intervals of more than 10 min). In only 2 out of more than 150 instances was there any difficulty in defining the border between the affected region and the surrounding unaffected area. In 3 of the fields studied, a small but distinct pilomotor response was noticed outside the main field area, possibly involving only one or two pilomotor muscles. Such outlying responses were noted, but since they occurred infrequently, were of extremely small size, and

their location was some 4 or 5 mm outside the main defined field, they were excluded from the measurement of the field area. In 15 animals electrophysiological mapping was also done to define the mDCN-T13 touch field, subserved by Aα axons (Jackson and Diamond, '84); these were similar in size to those found in the previous studies. The average sympathetic field area was twice (204%) that of the touch fields, making them distinctly larger than both the heat- and mechano-nociceptive fields of the same nerves, which have been previously described (Nixon et al., '84; Doucette and Diamond; '87; Diamond et al., '92a). However, the pilomotor fields varied considerably more in size among different animals than did the corresponding sensory fields, and the weak relationship between the pilomotor field and touch field area for the same DCN branch disallowed the use of the "touch ratio" approach (cf. Diamond et al., '92a) to evaluate pilomotor field expansions in individual animals.

Morphological correlates

The glyoxylic acid technique clearly revealed fluorescing axons running along the pilomotor smooth muscle bands that extended obliquely from the hair follicles to the dermal-epidermal border (Fig. 2). These axons were taken to be the sympathetic pilomotor fibers. Fluorescent axons were also readily identified in association with blood vessel

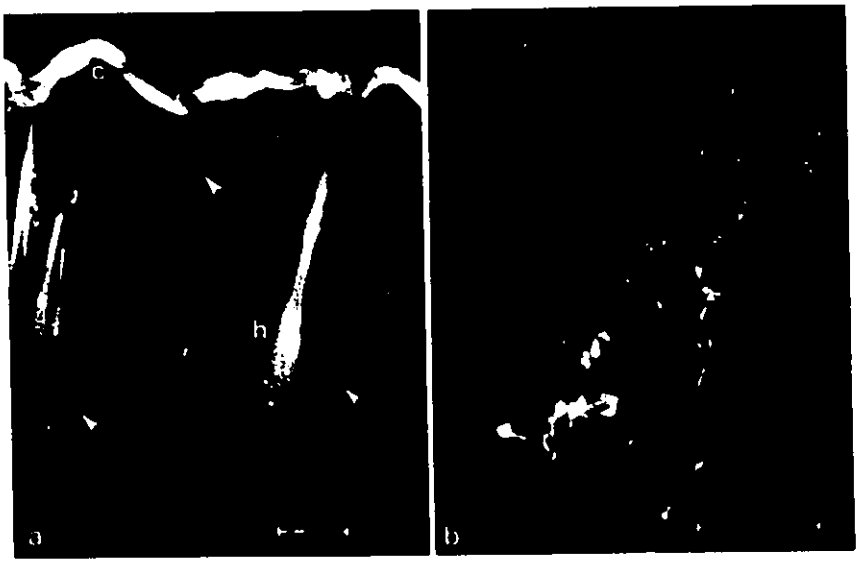

Figure 2. Sympathetic innervation of pilomotor muscle. a) The typical glyoxylic acid fluorescent fibers (arrows) are visible coursing over the pilomotor muscle. b) Fibers at higher magnification. These fibers are the same as those by the lower left arrow in "a". Scale bar is 200 μm for a, 50 μm for b. c=cornified epithelium h=hair follicle

walls and coursing in bundles within the dermis. In 3 animals a correlation between the morphological and functional pilomotor field of the same nerve (mDCN-T13) was sought. Two to five days after the cutaneous field was isolated by surrounding denervation, the pilomotor field border was delineated physiologically, and marked for a few millimeters of its length by a groove cut into the epidermis. Successive transverse sections of the frozen skin were then cut in a plane orthogonal to this marked field border, including some millimeters of the field on either side (cf. Nixon et al., '84). Pilomotor axons displaying amine fluorescence were clearly visible within the field, as were fluorescent axons on the dermal blood vessels. Pilomotor fibers were never found more than 0.5 mm beyond the functional field border (see Fig. 4). The same was true for fluorescent fibers on blood vessels in four out of the five animals included in this study. In the other animal a few fluorescent fibers were seen on blood vessels at a distance of 4.0 mm beyond the border. Both the number and length of the fibers on individual pilomotor muscle were observed to progressively decrease as the field border was approached from the field center.

Expansion of pilomotor fields following isolation

Periodic nerve stimulations were done to follow the

anticipated expansion of an isolated field into the surrounding denervated skin; as indicated below, such expansions must result from the collateral sprouting of intact fibers from the spared (stimulated) nerve, into the neighboring territory (cf. Diamond et al., '92a). Since each field was mapped initially (and repeatedly), the experiment incorporated a potential "conditioning" stimulation paradigm like that which evokes precocious sprouting of nociceptive nerves (Nixon et al., '84; Doucette and Diamond, '87). The approach was adhered to, nevertheless, since: (i) because of the large variability in pilomotor field sizes the alternative approach of comparing field sizes in groups of animals examined once only (at a selected interval after the fields were isolated) would have required the use of a very large number of animals; (ii) in the absence of a proportional relationship between the pilomotor area and that of the touch field of the same cutaneous nerve (see above), there was no simple way of identifying whether a later expansion of the pilomotor field had occurred <u>in a single animal</u> (cf. Diamond et al., '92a); (iii) the tonic background discharge of efferent impulses occurring normally in sympathetic nerves (Koizumi and Brooks, '80), made the experimental control of conditioning stimulation problematical (see Discussion).

An important potential source of error was that the

afferent sensory volleys evoked in the nerve by the testing stimulation might indirectly induce pilomotor activity in skin, due to the reflex activation of the adrenals with subsequent release of catecholamines (Koizumi and Brooks, '80); this could be especially problematical if the pilomotor muscles were denervated and perhaps had become supersensitive (Trendelenburg, '66). This possibility was examined by comparing the pilomotor fields revealed by stimulation of intact nerves with those evoked some minutes later by stimulation of the distal segments of the same nerves after they had been sectioned. No differences were found between the two fields in each of the 20 animals examined, which included normal fields, and fields re-established either by collateral sprouting or regeneration.

The results of the field expansion studies are shown in Figure 3. An enlargement was just detectable by approximately 10 days after a field was isolated, and over the group of animals studied the expansions appeared to have reached a ceiling by 40 days post-isolation, by which time the fields had increased to about 200-215% of their initial size. In a few animals followed up for over 100 days, no significant further expansion seemed to have occurred when the correction factor for the animal's growth was utilized (see Methods).

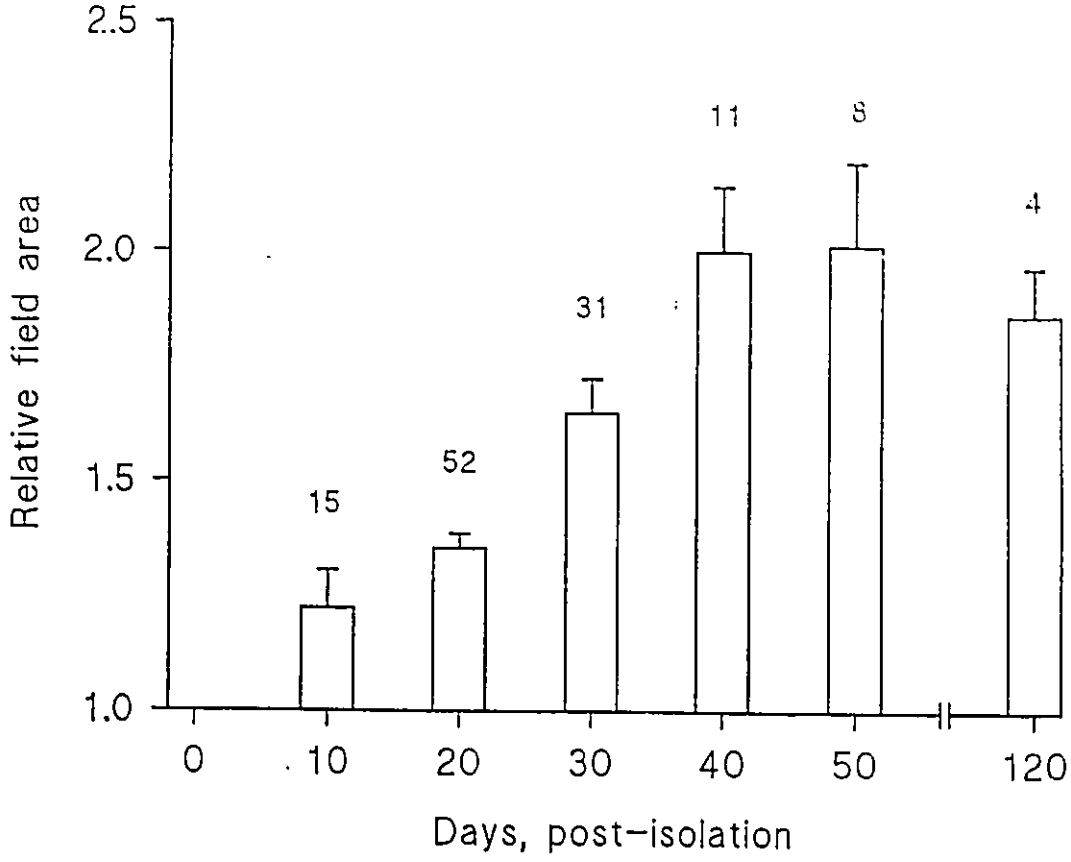

Figure 3. Expansion of the mDCN-T13 field into surrounding denervated skin following their isolation. The fields were mapped at the indicated times after their isolation, and are expressed on the ordinate relative to the original field size. In this and subsequent figures the "n" value above each histogram is the number of animals in the group and the error bars are standard errors of the mean.

Visualizing the sprouted axons.

In 3 animals a detailed study was made to examine the correlation between the morphological (shown by fluorescence) and functional field borders in the expanded fields. The findings were consistent with those for original fields, showing an approximate correspondence between the two borders, although the fiber density near the border was lower for expanded fields than for original fields (Fig. 4). Essentially similar findings came from measurements of fluorescent fibers on blood vessels (data not shown). Fluorescent fibers were observed on pilomotor muscles and blood vessels both within the original area of this field and within the expanded region, and an occasional fiber was seen a few millimeters beyond the new border. Of course some of these latter fibers may have sprouted in from the innervated skin surrounding the original area of the isolated field; an analogous result was described for sensory fibers (Nixon et al., 1984). Our conclusion is that the pilomotor field expansions were indeed attributable to the collateral sprouting of intact sympathetic fibers in the spared nerve.

In some animals collateral sprouting was significantly delayed

Interesting information came when the individual

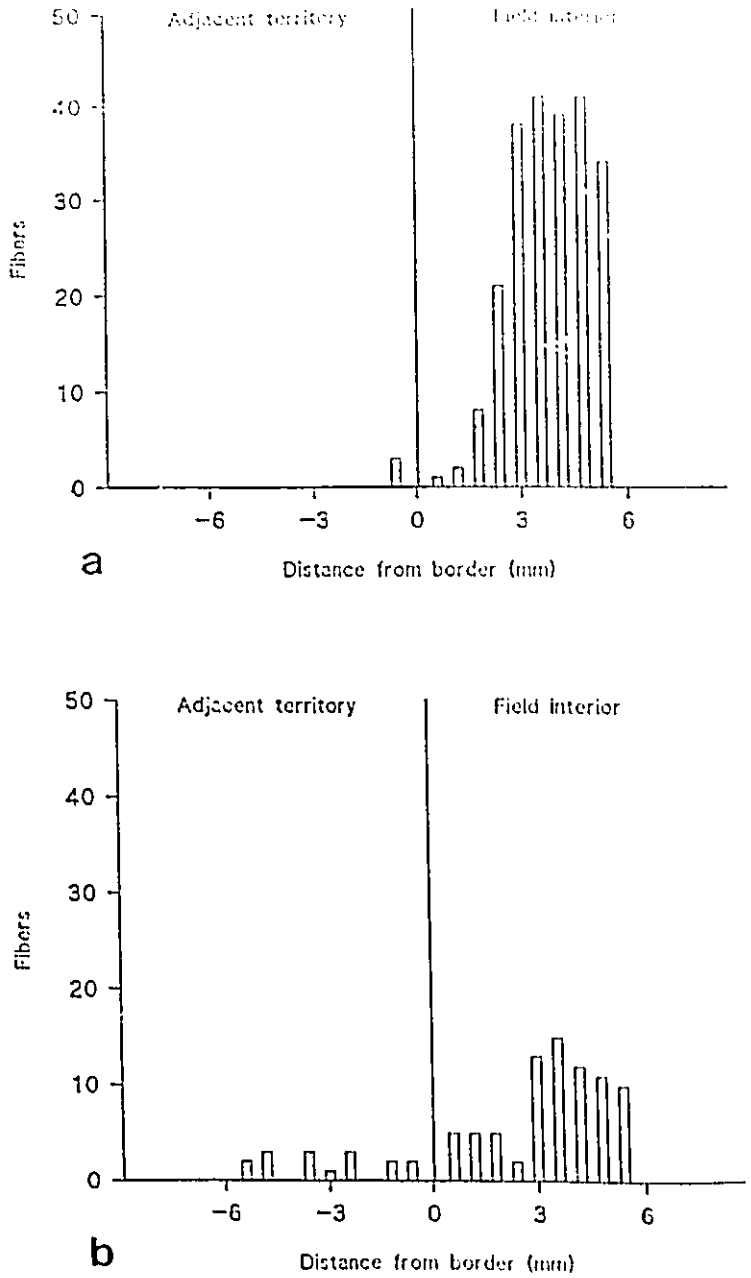

Figure 4. Correlation between functional and morphological borders of normal and sprouted fields. The histograms show the number of fibers observed, in skin sections from 3 animals, at various distances from the functional pilomotor border, for fields isolated 3-6 a) or 40-97 b) days previously. The border is indicated by the vertical line at the 0 point of the abscissa.

findings from all the animals in this particular series (cf. Fig. 3) were presented in the form of a scattergram (Fig. 5). A proportion of animals showed little or no field expansion by 20 or 30 days. However, expansions had occurred in all animals that were examined at 40 days or later, including animals which at 20 and 30 days had no field expansions. There were no obvious differences in age, or in the initial sizes of fields, between the animals whose pilomotor fields had failed to expand by 20-30 day, and those in which expansion occurred. The possible significance of these observations is discussed later.

Recovery of pilomotor function by regenerating sympathetic nerves.

DCNs were crushed, as described in the Methods, to evoke axonal regeneration. The time course of the subsequent recovery of pilomotor fields in the initially totally deprived skin is shown in Figure 6. The first signs of pilomotor function appeared at about 20 days after nerve crush. Although regeneration rates of the sympathetic axons were not studied quantitatively, a minimum value can be obtained by using the initial recovery time, and by taking the approximate length of the regeneration pathway as extending from the nerve crush to the entrance zone of the nerve into the skin. This minimum rate of sympathetic fiber

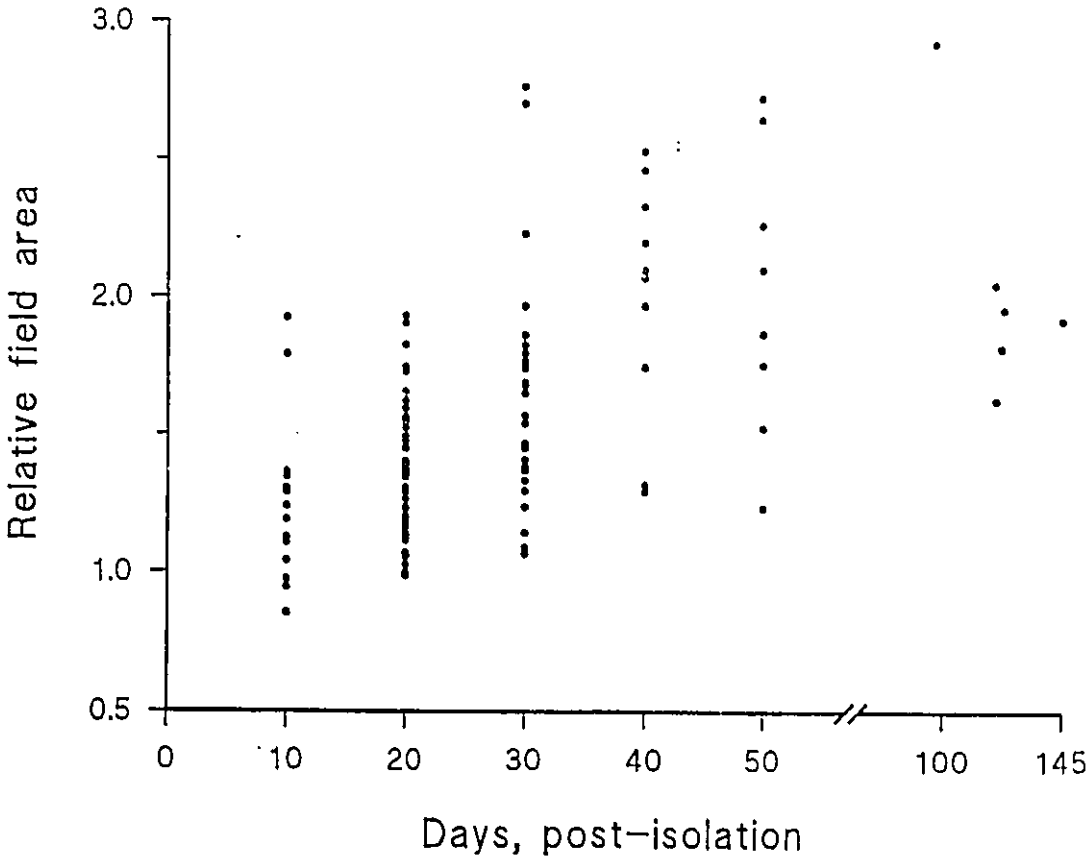

Figure 5. Scattergram showing the extent of field expansion for individual animals, after adjacent denervation. The fields were mapped at the indicated times after their isolation, and are expressed on the ordinate relative to the original field size.

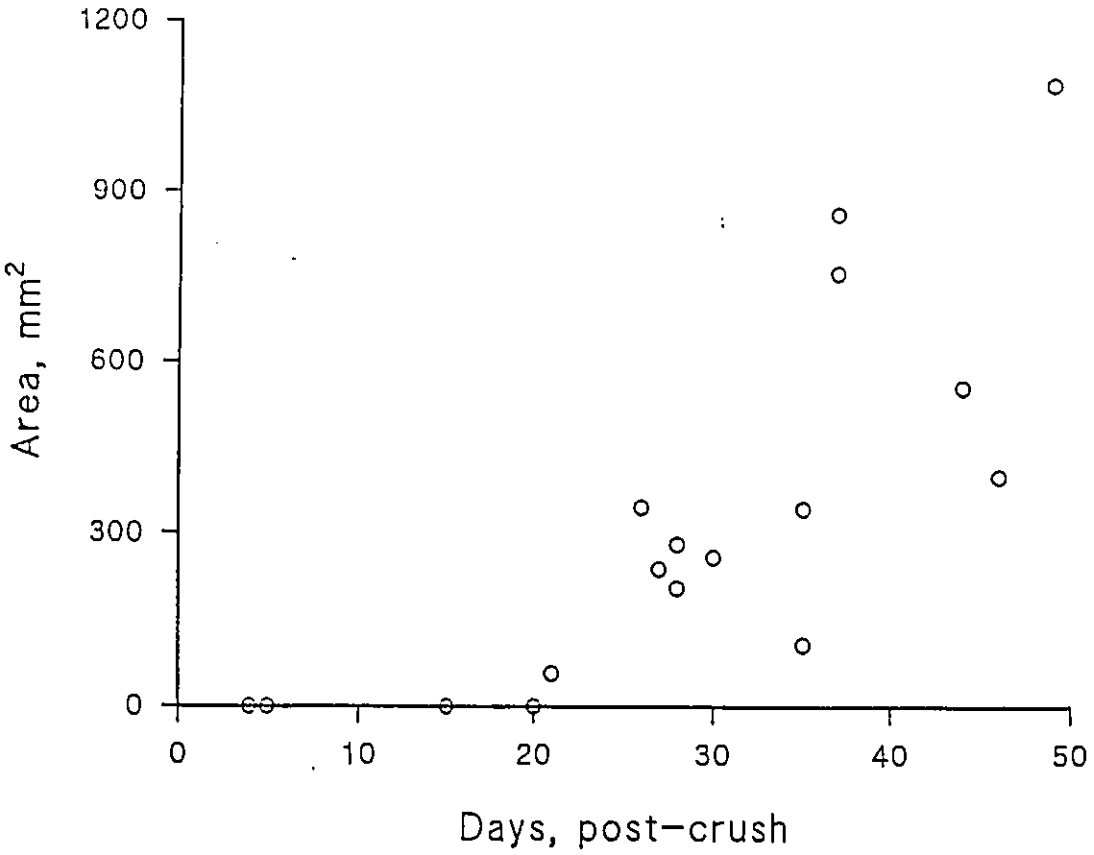

Figure 6. Restoration of pilomotor field after nerve crush. Plotted here are the absolute sizes of the functional pilomotor fields that were measured in animals at the indicated times after nerve crush was made.

regeneration works out to be approximately 2 mm/day. In the animals studied beyond 35 days the enlarging pilomotor field extended well beyond the average original field size (Fig. 6), i.e. into territory into which collateral sprouting would normally occur. The regenerating sympathetic fibers were able to be visualized within the restored field by the glyoxylic acid technique. although the intensity of their fluorescence was lower than normal. These studies did not examine the correlation between the functional and morphological borders since the much lower density of regenerating fibers compared to normal fields made quantification impractical. At approximately 30 days after nerve crush fluorescent fibers were also visualized in the nerve trunk distal to the crush site, by applying the glyoxylic acid technique some 30-60 min after tying a ligature around the nerve to promote a build up of transported axoplasmic materials to increase the fluorescence levels (see below).

The effects of anti-NGF treatment

(i) On collateral sprouting

Anti-NGF treatment totally prevented the usual expansion of the isolated pilomotor field (Fig. 7). This finding, however, does not exclude the possibility that a non-functional sprouting occurred, i.e. one that failed to

establish pilomotor function. The hypothesised sprouting of physiologically invisible nerve growth was excluded in an analogous study of sensory nerves by morphological techniques (Diamond et al., '92a). However in these anti-NGF treated animals the intensity of the fluorescence was always weak, and the number of visible fluorescent fibers was reduced even within the area of the original field. A failure to observe fluorescent axons outside this field, therefore, could not be taken as evidence of an absence of sympathetic fibers, particularly since newly grown axons would likely be thinner than normal. In some experiments immunohistochemical staining was done for the growth associated protein GAP43 (B-50) (Schreyer and Skene, '91; Benowitz and Routtenberg, '87) in an attempt to detect newly grown, but possibly non-functional, fibers. This approach had to be abandoned, however, because immunostaining could be demonstrated in skin which had been totally denervated 7 days earlier (Fig 8); all axons in such skin can be assumed to be degenerating or absent (Nixon et al., '84; Doucette and Diamond, '87). A post-denervation increase in B-50 (GAP43) immunoreactivity has been described in degenerating neuromuscular junctions (Verhaagen et al., '88) and comparable evidence that Schwann cells in peripheral denervated pathways express B-50 (GAP43) has been recently published (Curtis, et al., '92). An EM search was

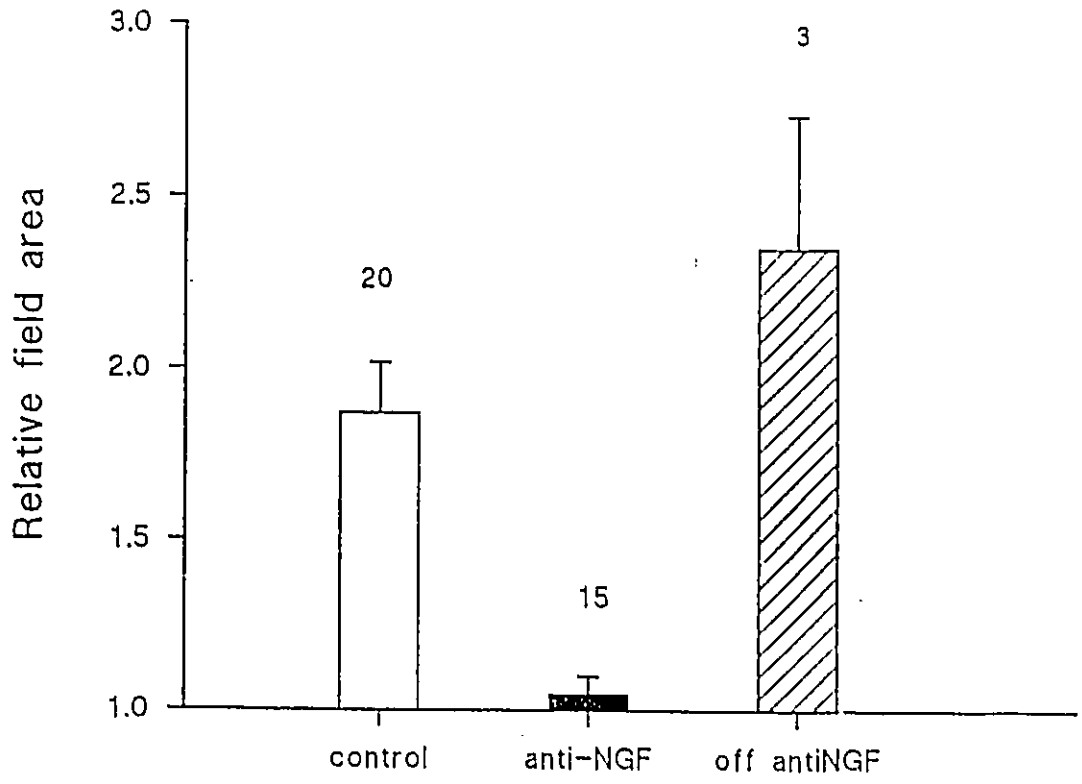

Figure 7. Anti-NGF treatment reversibly blocks collateral sprouting. The pilomotor fields were measured in two groups of animals at 35-40 days after isolation. The open histogram shows the result from the control group, which received daily injections of non-immune serum throughout (comparison with Fig. 3 shows that these fields had already expanded close to the maximum extent); the solid histogram shows the result of the group receiving anti-NGF serum. The treatment was discontinued in three animals of the latter group, and their fields were re-measured 20 days later (hatched histogram).

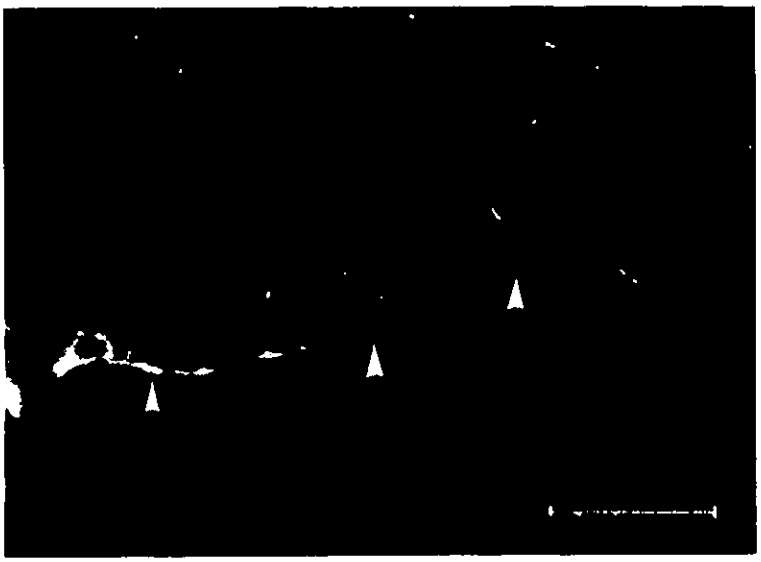

Figure 8. GAP-43 (B50) Immunostaining in denervated skin. GAP-43 (B-50) immunoreactive staining (arrows) in skin which had been completely denervated 7 days earlier. The pattern of the staining is consistent with the distribution of blood vessels.

undertaken for sympathetic fibers in the skin region outside the non-expanded pilomotor field in anti-NGF treated animals, and none was observed. However, this approach was also considered to be inadequate when it was found that in untreated animals, whose physiological pilomotor fields had expanded, only a small proportion of the Schwann tubes associated with pilomotor muscle in the region of expansion had become reinnervated (in normal skin all such tubes were found to contain axons). It was clearly impracticable to make an exhaustive EM search to establish unequivocally the absence of sympathetic fibers from the physiologically unresponsive skin outside the pilomotor field.

One reason for believing that physiologically silent fibers were absent came from the findings on newly grown regenerating axons in anti-NGF treated animals; these axons were unambiguously shown to be present by functional tests (see below). However, a more direct finding that substantiated the absence of an expanded sympathetic innervation during anti-NGF treatment came from experiments in which the initiation of anti-NGF treatment was delayed until a significant expansion of isolated pilomotor fields had occurred. The newly acquired pilomotor areas remained fully functional when anti-NGF treatment was instituted. In one such study the isolated fields of 4 animals were allowed to expand for 10 days, by which time they had increased by

an average of 30% (SEM ± 4), before daily anti-NGF treatment was begun. When the fields were re-mapped 20 days later, they were found to be only slightly changed (some 42% larger than their original sizes; SEM ± 10); in non-injected animals pilomotor fields increased on average by 65% (SEM ± 7) over the same time period. This result is consistent with the anti-NGF treatment rapidly bringing the collateral sprouting to a halt. Comparable findings came from 4 animals whose fields had expanded by 82% (SEM ± 22) after 40 days, when sprouting would have virtually ceased. Anti-NGF treatment was then started, and continued for a further 40 days; again, when the fields were re-mapped, their functional areas had hardly changed (89%; SEM ± 43 larger than their original size).

The effects of anti-NGF treatment were fully reversible. As seen in Figure 7, 20 days after discontinuation of a treatment that had totally blocked expansion of the pilomotor field for 35-40 days, the field had doubled in size. This expansion is similar to the largest field expansion seen in normal (untreated) animals at 20d (see Fig. 5), clearly indicating that the anti-NGF treatment did not impair the subsequent collateral sprouting of the sympathetic fibers.

These findings, considered in light of the analogous ones described for sensory nerves (Diamond et al.,

'92a), lead us to conclude that the absence of field expansion during anti-NGF was indeed due to the prevention of collateral sprouting of sympathetic axons, despite our inability to prove this by morphological approaches.

(ii) On Regeneration

Anti-NGF injections were started on the day of nerve crush and maintained on a daily basis thereafter. On selected days the usual nerve stimulation was done to test for a recovery of pilomotor function by regenerating sympathetic axons. The results were unexpected; physiological pilomotor function returned to the initially deprived skin in the anti-NGF treated animals just as with the non-injected animals (Fig. 9). The recovery included an eventual extension of pilomotor function into skin well beyond the area originally supplied by the regenerating nerve, i.e. into skin whose invasion by collateral sprouts from neighboring sympathetic axons would have been totally prevented by an identical anti-NGF administration. For example, after 30 days of regeneration, 10 out of 15 fields were at least one standard deviation larger than normal field size (570 mm^2 vs the normal value of 354 mm^2), and 7 of these had increased by at least two standard deviations (to an average of 623 mm^2). There were no indications that the anti-NGF treatment had delayed the onset of recovery of

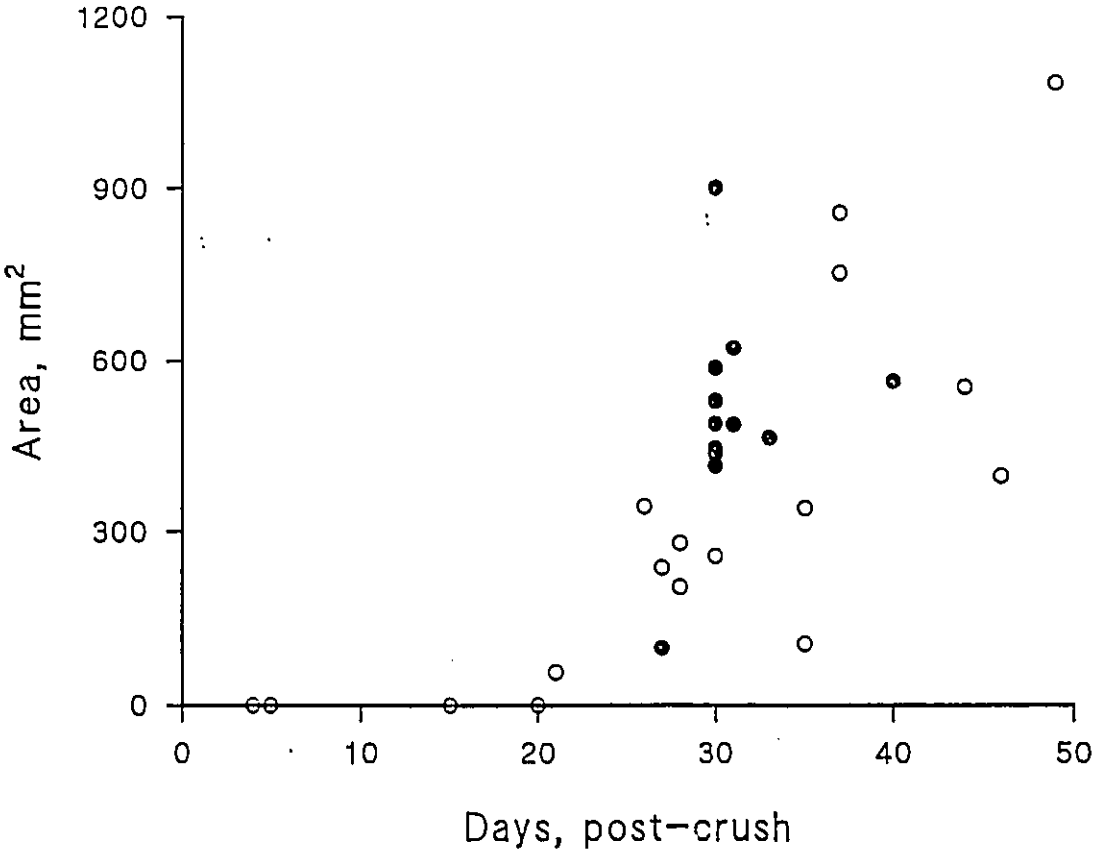

Figure 9. Anti-NGF treatment fails to affect regeneration. This figure repeats the data of figure 6, but now the plot includes the data obtained from animals receiving daily anti-NGF treatment (solid circles) following the nerve crush. There was no statistically significant difference between the two groups in the field sizes measured 30-40d after nerve crush.

pilomotor responses, or the rate at which the pilomotor fields enlarged; nor did the extent of the expansions achieved seem to be affected (Fig 9). In these conditions of anti-NGF treatment the functionally detectable regenerating axons were able to be visualized by the fluorescent technique within the cutaneous nerve trunk distal to the crush site (Fig. 10).

The physiological character of the pilomotor function restored by either collateral sprouting or axonal regeneration was indistinguishable from that of normal fields. However, a preliminary, purely qualitative, EM examination revealed that many of the apparent fiber bundles associated with the pilomotor muscle consisted, in fact, of axon-free Schwann tubes. Thus the terminal density of the reinnervation achieved by either collateral sprouting or axonal regeneration must have been considerably less than in normal skin. The pharmacological character of the newly grown sympathetic endings appeared to be normal. Intravenous injections of phentolamine effectively blocked pilomotor responses in doses of 10mg/kg, and reduced their intensity in doses of 5mg/kg, both in the skin of normal animals, and in skin whose pilomotor function was restored by axons regenerating during a maintained anti-NGF treatment.

Figure 10. Regenerated sympathetic fibers in an anti-NGF treated rat. The entire nerve segment shown here was distal to the crush site. A ligature was tied around the nerve (at the top of the picture). The nerve was excised 60 minutes later, treated with glyoxylic acid to reveal adrenergic fibers and mounted in a squashed preparation. Scale bar is 500 μm.

Discussion

Reinnervation of denervated skin by sympathetic nerves

Sympathetic nerves were able to restore pilomotor function to denervated skin of the rat dorsum both by collateral sprouting (from undamaged axons) and by axonal regeneration (following nerve crush). In some animals there appeared to have been a delay in the onset of collateral sprouting following isolation of the sympathetic field. We are interested in the possibility that these variations in sprouting latency may relate to variations in the ongoing tonic discharge in the sympathetic neurons involved in the sprouting response (Koizumik and Brooks, '80). In sensory neurons the sprouting latency is dramatically affected by impulse activity (Nixon et al., '84; Doucette and Diamond, '87; Diamond et al., '92a).

The role of NGF in sympathetic reinnervation

Collateral sprouting

The inability of isolated sympathetic fields to expand during anti-NGF administration would seem to demonstrate that the collateral sprouting of sympathetic fibers is dependent on endogenous NGF. However, removal of endogenous NGF leads to a decreased synthesis of tyrosine hydroxylase (Gorin and Johnson, '80; see also Raynaud et

al., '88), one of the rate limiting enzymes in norepinephrine synthesis (Levitt et al., '65). Thus, collateral sprouting might have occurred, but because of reduced neurotransmitter levels in the newly grown collaterals these fibers were unable to activate the pilomotor muscle in the expanded region of the field. An indirect, but powerful, argument against such "silent sprouting" came from the experiments in which the initiation of anti-NGF treatment was delayed until fields had expanded for either 10 or 40 days. Not only were their functionally delineated areas fully maintained, but the newly sprouted fibers retained the ability to drive the pilomotor muscles even when the anti-NGF treatment was continued for 40 days. A relevant finding, which is discussed below, is that sympathetic axons which had <u>regenerated</u> during anti-NGF treatment could be demonstrated as being unambiguously present by their ability to drive pilomotor muscles. Therefore both functional and morphological evidence is consistent with a prevention of collateral sprouting by the anti-NGF regime.

When these findings are considered in light of those from the analogous studies of sensory nerve sprouting (Diamond et al., '92a), the conclusion seems inescapable that the collateral sprouting of sympathetic nerves is indeed NGF dependent.

Regeneration

The surprising finding in the present study was that sympathetic axons regenerating after crush seemed to be entirely unaffected by a maintained anti-NGF treatment, achieving the same functional restoration of pilomotor fields as nerves regenerating in the absence of such treatment. Regeneration appeared not even to be hindered, much less prevented, by the identical treatment which totally prevented collateral sprouting. The conclusion of Bjerre et al. ('74), based on morphological evidence, that anti-NGF inhibits the regeneration of sympathetic fibers after 6-hydroxydopamine (6-OHDA) treatment in mice, thus needs to be re-examined. Since their morphological examinations depended upon norepinephrine staining, and NGF upregulates tyrosine hydroxylase levels (Gorin and Johnson, '88; Raynaud et al., '88), one of the rate limiting steps in norepinephrine synthesis, the anti-NGF treatment might have made newly grown fibers invisible by lowering norepinephrine levels. In studies soon to be reported, we provide evidence that sympathetic fiber regeneration after 6-OHDA also occurs independently of endogenous NGF (Gloster and Diamond, in preparation).

Were the regenerating sympathetic axons accessible to the anti-NGF antibodies?

To gain access to the regenerating fibers the injected antibodies must enter the endoneurial compartment, a process which would normally be hindered by the blood-nerve barrier (Olsson, '66; de la Motte and Allt, '76; Sparrow and Kiernan, '81, Seitz et al., '89). However, not only is the blood-nerve barrier to macromolecules known to break down both at the site of nerve crush and in the degenerating distal nerve trunk (Olsson, '66; de la Motte and Allt, '76; Seitz et al., '89), but an advancing zone of increased permeability to macromolecules accompanies the growing axons as they regenerate down the distal stump (de la Motte and Allt, '76; Sparrow and Kiernan, '81). Accordingly, the findings of Campenot ('82) are very relevant here; they indicate that in order to evoke the elongation of sympathetic axons, at least in vitro, NGF must be present in the vicinity of the growing nerve endings. There is an additional reason to reject the "inaccessibility" explanation. The present (quantitative) findings showed that regenerating sympathetic fibers eventually extended into the same denervated skin regions that collateral sprouts would normally invade to innervate the same pilomotor targets. The pathways utilised within the dermis by growing sympathetic axons to reach their

autonomic targets would almost certainly be the same for both regenerating and collaterally sprouting fibers. (Regenerating sensory nerves, for example, use the same dermal pathways as collaterally sprouting ones; Diamond et al., '87). Since anti-NGF treatment prevented sympathetic nerve sprouting, these pathways are certainly accessible to antibodies. It follows then that sympathetic axons regenerating within the dermis would have been equally accessible to the injected anti-NGF. We conclude that there is a genuine difference between collateral sprouting and regeneration of sympathetic fibers with respect to their ability to occur during anti-NGF treatment. A similar differential effect of anti-NGF administration was demonstrated for regenerating and sprouting sensory nerves (Diamond et al,. '92a,b).

Significance of the NGF independence of sympathetic axonal regeneration

There is considerable circumstantial data to support the frequent assumption that NGF is involved in axonal regeneration. Expression of NGF (Heumann et al., '87) and the low affinity NGF receptor (NGFR) (Taniuchi et al., '88) by Schwann cells are both upregulated after nerve crush and then down regulated following successful nerve regeneration. However, during regeneration high affinity

binding of NGF is decreased both on the cell body (Verge et al., '89) and in the regenerating axons (Raivich et al., '91). In addition, axonal regeneration can proceed down peripheral nerve segments which had been frozen to eliminate Schwann cells (Sketelj et al., '89; Ide and Kato., '90). The view we are promoting is that the trophic support for regeneration is relatively non-selective among different classes of axons, endogenous NGF being perhaps just one of many potentially growth-promoting molecules produced in degenerating nerve pathways. Our evidence for sensory axons (Diamond et al., '92b), and now for sympathetic ones, is that at best NGF is relatively unimportant to their regeneration.

There are better candidates for molecules able to regulate nerve regeneration. Insulin-like growth factor (IGF-1), as well as NGF, accumulates in the distal stump of a transected nerve and is able to stimulate regeneration of sensory nerves, and antibodies to IGF-1 have been shown to partially inhibit regeneration (Kanje et al., '89; Sjöberg and Kanje, '89). Basic fibroblast growth factor and human growth hormone have also been reported to enhance axonal regeneration (Danielsen et al., '88; Kanje et al., '88). In experiments in which exogenous NGF apparently promoted regeneration (e.g. Rich et al., '89; but see Hollowell et al., '90) it is possible that NGF was effective by virtue of

a primary action on Schwann cells, enhancing their ability perhaps to myelinate growing fibers (cf. Rich et al., '89), or to produce non-NGF growth factors that promote axonal elongation.

Recent findings indicate that our polyclonal antibody against NGF recognizes, besides NGF, BDNF, and NT-3 (R. A. Murphy, personal communication). This raises the possibility that any one, or a combination of NGF, BDNF, and NT-3, might be necessary for the collateral sprouting, though not the regeneration, of sympathetic axons. However, embryonic sympathetic neurons are unresponsive to BDNF (Lindsay et al., '85; Hofer and Barde, '88), and are only weakly responsive to NT-3, compared to NGF (Maisonpierre et al., '90; Ernfors et al., '90), and also to the latest additions to this family of neurotrophic agents, NT-4 (Halböok et al., '91) and NT-5 (Berkemeirer et al., '91). Thus NGF remains the most likely candidate for regulating the collateral sprouting of sympathetic neurons.

Implications of the findings.

The present results extend similar findings for cutaneous nociceptive nerves (Diamond et al., '87; '92a,b). In this instance an efferent nerve modality, the sympathetic axons, was studied and their collateral sprouting was found to be NGF dependent, while their axonal regeneration was

not. The difference in trophic requirement for these two forms of nerve growth may thus apply more generally within the adult nervous system. Both the levels of NGF message (Shelton and Reichardt, '84) and NGF protein (Korsching and Thoenen, '83) in target tissues are correlated with the density of their sympathetic innervation. However, the amount of NGF available to the cell body i · very different during collateral sprouting and axonal regeneration. Collateral sprouting occurs under conditions when the endogenous NGF availability within the target tissue may be confidently assumed to be higher than normal (cf. Korsching and Thoenen, '85; Diamond et al., '92a). During regeneration the cell body is cut off from its target tissue, and the amount of NGF available from the distal nerve trunk is less than half the normal supply (Heumann et al., '87, Raivich et al., '91). Both the level of NGF and high affinity NGF binding activity in the proximal stump falls to 40% of normal after nerve crush (Raivich et al., '91). The level of high affinity NGF binding is similarly down-regulated in DRG neurons whose axons were cut (Verge et al., '89). We have recently shown that NGFR mRNA expression is increased in DRG neurons whose axons are undergoing collateral sprouting, but reduced, at least for the first 6-7 days, when they are regenerating (Mearow et al., '91; see also Kuchel et al., '91). It is perhaps not surprising that

regeneration is NGF independent, given that it occurs at a time of decreased NGF availability. However, NGF is certainly required for maintenance of the normal phenotype of sympathetic neuron, and even for their long-term sympathetic survival (Angeletti et al., '71; Gorin and Johnson, '80; Snider et al., '88; Ruit et al., '90; Diamond et al., '92a). These facts make the successful functional regeneration of sympathetic fibers in the NGF-deprived situation particularly striking. Paradoxically, it seems that such regeneration is driven by mechanisms that override the adverse effects of the concomitant trophic deficit, even though this deficit could ultimately lead to atrophy and ultimately the death of the neuron.

We are interested in the possibility that the mechanisms which operate during the kind of experimentally-evoked collateral sprouting we study may be involved in a continual process of axonal sprouting and regression, resulting in a dynamic turnover of endings (Diamond et al., '88; see also Bjerre et al., '75). We suggest that NGF has a role in regulating the normal density of innervation in adult skin, and that this represents the biological significance of our findings concerning collateral sprouting. Recently we have found that NGF mRNA expression is increased in denervated skin (Mearow, Kril, Ross and Diamond, unpublished observations), indicating that the

nerve itself may also contribute to the regulation of such dynamic processes. Axonal regeneration presents a contrasting picture to collateral sprouting in these regards. It is a reparative response to nerve damage, and its primary requirement is an appropriate pathway to the correct target region. The availability of non-selective growth-promoting processes (cf. Dodd and Jessel, '88) in such pathways (possibly involving substrate-bound molecules) would seem of obvious benefit to the organism, provided that within the target tissue itself there are more selective mechanisms (including diffusible growth factors) which can operate to facilitate a functionally useful connectivity of the arriving axons.

NGF-dependent and NGF-independent recovery of functional pilomotor activity after chemical sympathectomy with 6-hydroxydopamine.

Andrew Gloster and Jack Diamond

To be submitted to Journal of Neuroscience

Abstract

Collateral sprouting refers to the arborising growth of undamaged axons which occurs usually within their target tissues; axonal regeneration, in contrast, is a growth response evoked by axonal damage, and it begins at the injury site. We have previously demonstrated that endogenous NGF is an absolute requirement for the collateral sprouting of cutaneous sensory and sympathetic nerves, and that the regeneration of the same nerves is essentially an NGF-independent growth. We have now examined the recovery of sympathetic nerve function in adult animals in which the sympathetic terminals were destroyed by 6-OHDA, leaving the axons damage-free. The principal question addressed was: to what extent would the reestablishment of the sympathetic terminal field involve regeneration, and to what extent collateral sprouting? Sympathetic pilomotor activity was evoked in the depilated back skin by electrical stimulation

of selected dorsal cutaneous nerves in adult rats. Pilomotor (smooth muscle) contractions elevated touch domes and produced a fine-grained ruffling of the skin within a clearly defined discrete area; this area represented the functional pilomotor field of the stimulated nerve. A single 6-OHDA treatment appeared to abolish pilomotor fields, although a few terminals might have survived. By 20d the average normal pilomotor field area was reestablished, and if the surrounding nerves had been surgically eliminated at the time of 6-OHDA treatment, this area continued to expand into the surrounding denervated territory, tripling the normal size by about 70d. Daily anti-NGF administration was used to "challenge" this functional recovery. The initial phase of pilomotor recovery occurred essentially normally, though at a somewhat reduced rate, but expansion of the pilomotor fields ceased when they reached about 60% of their normal size. However, the expansion was almost double this value if the animals initially received not one, but 4 treatments of 6-OHDA (over a 12d period); by 20d after the last 6-OHDA injection the fields had reached their normal field sizes, but no further expansion occurred even when the surrounding skin had been denervated. Upon cessation of anti-NGF treatment, pilomotor fields resumed their normal expansion into the surrounding denervated territory.

In the light of our earlier findings from crushed sympathetic nerves, which could regenerate fields of triple normal sizes during daily anti-NGF treatment, we interpret the early, NGF-independent, phase of the recovery after 6-OHDA lesion as corresponding largely to regeneration of the chemically lesioned sympathetic terminals. We take the second, NGF-dependent, phase of expansion to be due to the collateral sprouting of sympathetic axons. A number of observations, however, indicate that when 6-OHDA treatment was combined with the surgical elimination of the surrounding (and overlapping) nerve fields, regeneration and sprouting are probably initiated simultaneously, rather than being sequential events. We suggest that NGF-independent axonal regeneration is associated with the "cell body reaction", a response that is related to the severity of axonal injury. Since this reaction is known to be minimal after a 6-OHDA lesion, the ensuing regenerative response would be small, ceasing when the cell body reaction disappeared. The increased extent of NGF-independent recovery following multiple 6-OHDA lesions, and the essentially unlimited NGF-independent recovery that follows nerve crush, are both consistent with the hypothesis that the duration of the regenerative response is determined largely by the extent of the cell body reaction. Following the cessation of the regenerative response, the recovery

would become NGF-dependent, since it would be subserved entirely by collateral sprouting, which appears to occur independently of a cell body reaction, and to be determined entirely by the existing levels of target-tissue derived neurotrophic agents. We conclude that although the cellular mechanisms sustaining neuritic outgrowth are likely to be common to both axonal regeneration and collateral sprouting, their regulation must differ in the two growth states.

Introduction

During development nerve growth factor (NGF) is required for the survival of sympathetic (Levi-Montalcini and Booker, 1960; Klingman and Klingman, 1967) and certain populations of sensory neurons (Johnson et al., 1980; Aloe et al., 1981; Goedert et al., 1984). The morphological phenotype of sympathetic neurons is also regulated by NGF, not only in the neonate (Ruit and Snider, 1991) but also in the adult (Bjerre et al., 1975; Snider et al., 1988; Ruit et al., 1990). Although NGF probably continues to be necessary in the adult for the long-term survival of sympathetic neurons (Gorin and Johnson, 1980; Johnson et al., 1982), their vulnerability to NGF deprivation is reduced (cf. Angelleti et al., 1971). Because of this ability to survive anti-NGF administration, at least in the short term, such treatment constitutes a practicable approach to the study of the possible role of NGF in adult axonal growth. Using this strategy, we recently showed that while NGF is an absolute requirement for the collateral sprouting of undamaged sympathetic axons, after the same axons are crushed, they regenerate and restore sympathetic function, independently of NGF (Gloster and Diamond, 1992); these results are similar to that reported earlier for sensory neurons by Diamond et al., (1992a,b). Our finding that sympathetic nerve regeneration occurs independently of NGF appears to be

at odds with the earlier study of Bjerre et al. (1974), who reported that after 6-OHDA treatment anti-NGF treatment inhibited the regeneration of sympathetic terminals. We have now reexamined the NGF dependency of the recovery of sympathetic nerve function after 6-OHDA treatment. The results may reconcile the seemingly contradictory findings, while confirming our earlier conclusion that sympathetic nerve regeneration is NGF-independent. If the proposed interpretation of the findings is correct, the distinction between collateral sprouting and axonal regeneration becomes more explainable.

Materials and Methods

Measurement of sympathetic pilomotor field area.

The procedure for pilomotor field mapping was described in detail by Gloster and Diamond (1992). Briefly, the dorsal back skin of female Wistar rats (150-220 g), anaesthetized with sodium pentobarbitol (45mg/kg), was shaved and depilated using a commercial product (Neet). The selected dorsal cutaneous nerve (DCN), or a defined DCN branch, was then exposed as described below and placed over a pair of platinum stimulating electrodes. Sympathetic axons were excited by 500 μsec 8V square wave pulses, parameters already established in this laboratory as supramaximal to activate the similarly sized sensory C fibers in the same DCNs (Doucette and Diamond, 1987). Stimulation at 20Hz for 3-10 seconds resulted in contractions of pilomotor muscle, leading to elevation of touch domes and a fine-grained ruffling of the skin; the region affected was clearly visible as a discrete area (see Fig 1, in Gloster and Diamond, 1992), which defined the functional pilomotor field of the stimulated nerve.

"Isolation" of a pilomotor field.

The denervations required to produce a sympathetic field isolated within a surround of totally denervated skin

were essentially the same as those described in Gloster and Diamond (1992) with the exception that, in the present experiments, the denervations did not need to be so extensive, a strategy which minimized denervation-related scarring of the skin. The fur of the anesthetized animals was shaved over the thoracolumbar region of the back, a 5 cm long incision was made about 1 cm to the right of the midline, and the skin reflected to reveal the segmental DCNs. Approximately one cm lengths of DCNs T10-12, DCN L1-3, and lateral cutaneous nerves T12-L1 on the left side, were dissected free of connective tissue from their point of exit from the body wall to their point of entrance into the skin; the exposed nerves were then cut and removed, allowing the central stump to retract behind the body wall musculature. The *lateral* of the two branches of T13 was cut about 1cm from the body wall, and the proximal stump was then ligated and buried in the body wall. These procedures successfully prevented the regeneration of the cut nerves to the skin during the duration of these experiments.
Following completion of these denervations, only the sensory and sympathetic fields of the medial branch of the DCN-T13 (mDCN-T13) remained on the left side of the dorsal region of interest, surrounded by totally denervated skin. Of relevance to the fluorescent study, which utilized skin from the <u>opposite</u> side of the midline as a "control" (see below),

the effect of the skin incisions used in the denervation surgery, plus the denervations themselves, was to eliminate a small proportion of the sympathetic innervation from that side. Generally the mDCN-T13 fields were surgically isolated and first mapped 2d after 6-OHDA treatment; however in one group of animals the fields were isolated and mapped the day prior to the 6-OHDA treatment (see below).

6-OHDA destruction of sympathetic terminals.

To achieve this objective, a single 6-OHDA injection (150 mg/kg, in 0.1% ascorbic acid, 0.1 M phosphate buffer) was given intraperitoneally (I.P.). This treatment leads to an almost complete loss of adrenergic post-ganglionic sympathetic terminals (Tranzer and Thoenen, 1968; Tranzer et al., 1969; Champlain, 1970). In some experiments, animals received a total of 4 injections of 6-OHDA, evenly spaced over a 12d period. When 6-OHDA treatment was combined with the surrounding denervations described above, the end result was a sympathetic field that was both chemically lesioned, and isolated within a surround. However, because of the extensive overlapping of adjacent sympathetic fields (Gloster and Diamond, 1992), the area of the isolated field was itself also partially denervated, i.e. a subpopulation of its normal complement of sympathetic axons was eliminated.

The evaluation of functional restoration of the sympathetic innervation.

The anticipated recovery of pilomotor function within the extensive region of denervated skin created by the combined surgical and chemical lesions described above would involve a reestablishment of sympathetic terminals within the original field area, and the outgrowth of sympathetic collaterals into the surrounding area. The first mapping of the pilomotor field was done either immediately after its isolation (i.e. just prior to the chemical lesion) to measure the initial size of the pilomotor field, or it was done 2 days after the 6-OHDA injection, an approach originally intended to test for any pilomotor function that survived the treatment. As will be described later, however, it seems likely that some regeneration of the initially lesioned terminals might well have occurred by 2d; thus the apparent residual field at that time probably overestimates the extent to which terminals survived the 6-OHDA treatment, and indeed none of them might have done so. For certain of the studies done on animals first examined at 2d, an estimate was required of their initial field sizes; since these had not been examined prior to treatment, we used instead average values of the fields measured in a group of normal unoperated and untreated control animals (see Results). Usually an

interval of at least 10 days was left between successive pilomotor field mappings to reduce the extent of the scarring that occasionally followed the depilation procedure; such scarring presented problems in only about 10% of the animals.

Glyoxylic acid staining of sympathetic fibers.

The glyoxylic method of monoamine staining (Furness and Costa, 1975, as modified by de la Torre, 1980) was used to fluorescently label sympathetic nerve fibers. Because of the likelihood that the anti-NGF treatment would lower norepinephrine levels (Gorin and Johnson, 1980; Raynaud et al., 1988), the animals were injected I.P. with 1 ml of a 1mg/ml solution of α-methyl-norepinephrine (Sigma) solution, which is taken up by the sympathetic nerve moncamine reuptake system (Malmfors, 1965) and increases the intensity of fiber fluorescence. When glyoxylic acid staining of skin was done soon after the pilomotor fields had been mapped, by electrical stimulation of the DCNs, unusually variable results were obtained, a troublesome finding which we have not yet explained. To eliminate this concern, the skin samples used to provide fluorescent evidence of the presence or absence of sympathetic fibers following 6-OHDA treatments were almost always taken from the rat dorsum contralateral to the side used for pilomotor field mapping. (As mentioned

earlier, by a few days after denervation surgery, the population of sympathetic fibers would have been somewhat less than normal even on that side, because of the fibers cut by the skin incisions, and the elimination of overlapping nerves). A razor blade was used to cut skin samples (4 x 4 mm), which were then pinned flat before being frozen and stored at -20° C. Sections (30 μm thick) were cut on a cryostat and placed on 1.5% alum coated slides. The sections were then covered with a 2% glyoxylic acid solution (in 0.2 M sucrose, 0.25 M phosphate buffer, pH 7.4) for 1 min, and air dried under a stream of cool air for 15 min. Dried slides were placed in a 100° C oven for 2-4 min. and immediately coverslipped under light paraffin oil. Slides were viewed with a Zeiss universal microscope equipped with an epifluorescence condenser (RS III; filter set; BP 436/17, FT460 beam splitter, LP 478 barrier filter).

Antibody production.

The production of polyclonal antibodies to NGF was identical to that described in detail in Diamond et al., (1992a). In brief, NGF from mouse salivary glands was purified and assayed with the dissociated cell technique (Greene, 1977) using neonatal mouse superior cervical ganglia (SCG) (Coughlin and Collins, 1985). Female sheep were immunized and boosted monthly with the purified NGF in

Freund's complete adjuvant. The collected immune serum was heat inactivated and filter sterilized. A 10,000 fold dilution of the serum blocked the neurite promoting activity of 10ng/ml 7S NGF in the dissociated cell assay. Specificity of the antibody was determined using Ouchterlony double diffusion analysis and by Western blotting. We now know (R.A. Murphy, personal communication) that our polyclonal antibody also recognizes neurotrophin-3 (NT-3; Maisonpierre et al., 1990) to the same extent as NGF, and to a lesser degree, brain derived neurotrophic factor (BDNF; Hofer and Barde, 1988).

Antibody treatment.

Animals received either no serum treatment, or daily sub-cutaneous injections of either anti-NGF or control serum, for periods ranging from 30-40 d. To exclude the possibility that differences between the anti-NGF and control treated groups might be due to an accentuation by the anti-NGF treatment of the 6-OHDA mediated damage, in one group of rats the anti-NGF treatment was started two days after 6-OHDA treatment (see Results).

Results

Normal pilomotor fields.

We define the discrete area of skin displaying elevation of touch domes and fine ruffling of the skin after DCN stimulation as the pilomotor field for that DCN (Gloster and Diamond, 1992). In the group of animals mapped prior to 6-OHDA administration, the pilomotor fields for the mDCN-T13 (309 ± 8 mm^2; n=21) were similar to those described previously (Gloster and Diamond, 1992). When skin samples were examined a few days after surgical isolation of a pilomotor field, numerous fluorescent sympathetic fibers were seen to be spiralling around blood vessels and coursing along the pilomotor muscles within the area of the field, but not outside it (cf. Gloster and Diamond, 1992).

Acute effects of 6-OHDA.

Except for specific animals mentioned later, the standard procedure of 6-OHDA administration was to inject the drug I.P. two days before carrying out the denervations to isolate the selected pilomotor field. Within 5-30 minutes of the injection the animals displayed both an increased heart rate and elevated fur; these effects are due to the sympathomimetic actions of 6-OHDA (Kostrezewa and Jacobowitz, 1974). One of these animals was tested 3 hours after the injection, and no pilomotor field could be

elicited by nerve stimulation. By five hours all the rats exhibited ptosis, indicating a loss of sympathetic innervation of the levator palpebrae superioris. This ptosis typically lasted about 2 days, and disappeared over the following day. In one animal which received multiple injections of 6-OHDA treatment, one every three days (see below), ptosis was noted after each injection, essentially disappearing before the next injection 3d later. We conclude from these observations that a single injection of 6-OHDA effectively eliminated at least the vast majority of sympathetic terminals, and that by 2d a number of these had recovered to the extent that some sympathetic function was restored.

Recovery after combined 6-OHDA lesion and surgical isolation of the field.

(i) 2d post-injection.

Nine animals that received either no serum injections or injections of non-immune serum were examined two days after 6-OHDA (injection) treatment. Nerve stimulation failed to evoke any pilomotor function in three of these, but in the other six animals pilomotor fields were revealed; their areas were approximately 30% (109 $\pm$ 8 mm^2)of the original field size. The intensity of the evoked pilomotor muscle contractions was clearly weaker than normal

in these 5 animals; the fine ruffling of the skin was greatly diminished in intensity, and usually became apparent only after stimulation times of 20-40 seconds, instead of the normal 3-10 seconds. Despite this requirement, the pilomotor responses appeared more or less uniformly across the (markedly sub-normal) field area, and this area did not enlarge with continued stimulation. The indications that the sympathetic innervation density in the skin was considerably reduced is supported by the morphological studies, which revealed no fluorescent fibers in association with either pilomotor muscle or blood vessels. Occasional fibers were seen, however, coursing over the cutaneus trunci muscle, which is a skeletal muscle sheet lying just below the dermis, and a few fluorescent fibers were also observed running deep within the dermis itself, usually close to the base of a hair follicle. The likelihood that a substantial denervation supersensitivity was involved in these residual pilomotor responses is discussed later. As pointed out above, these responses observed at 2d post-injection included at least a component which probably was due to a recovery of terminal function following the destructive effects of 6-OHDA (this recovery could have accounted for a substantial fraction of the fields observed at 2 days).

(ii) 10-70d post-injection.

Since the pilomotor fields were both surgically and chemically denervated, any observed recovery within the original field of the stimulated nerve could represent both the regrowth of the former terminals, and the sprouting of new collaterals to compensate for the loss of the "overlapping" population normally subscribed by the adjacent nerves. Ten days after 6-OHDA treatment the pilomotor fields (average = 236 ± 48 mm^2; n=3; Fig 1) were approaching, but had not yet totally recovered, their normal sizes (approximately 309 mm^2); however, the duration of stimulation required to evoke the pilomotor response had returned to normal, as had the intensity of the fine ruffling of the skin which marked the response. By 20d post-injection the fields (504 ± 52 mm^2, n=5) were significantly larger than normal ($p < 0.00002$), expanding therefore into skin previously not innervated by the spared nerve (mDCN-T13). By 30d the pilomotor fields were double the normal size (average 628 ± 31 mm^2; n=12), and this expansion continued for up to 70d (956 ± 130 mm^2; n=4), the longest time examined. The most rapid increase in functional field area, 24 mm^2/d, occurred between 2 and 20d; the rate of expansion then decreased to about 9 mm^2/d between 20 and 70d.

We also examined a group of animals that had been

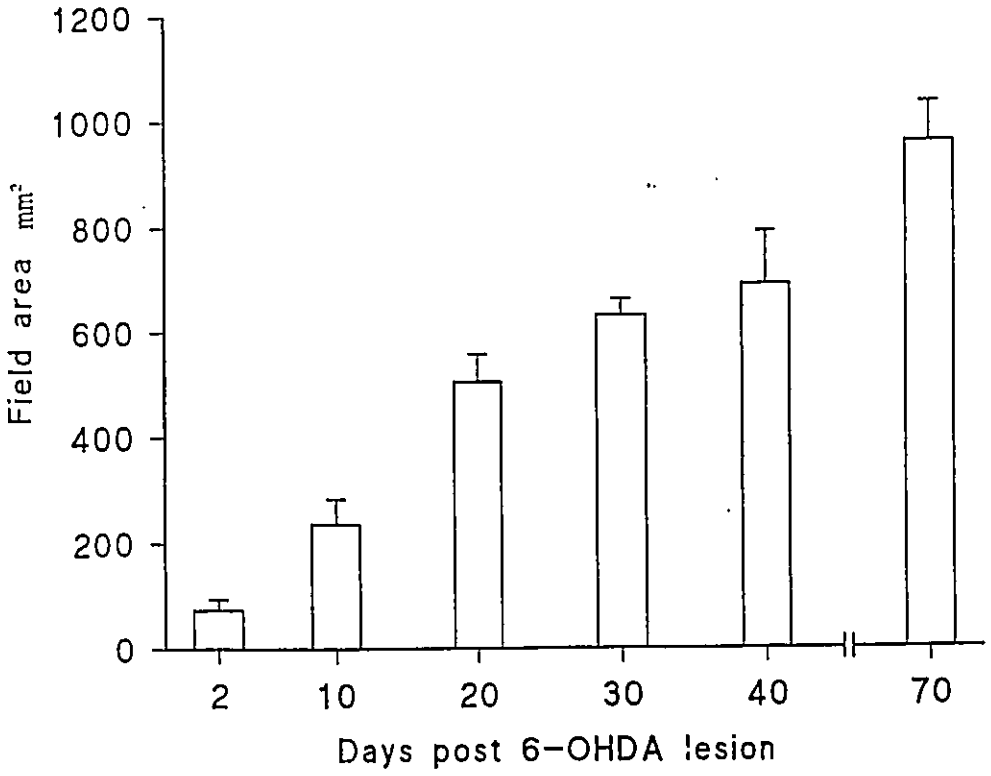

Figure 1.
Expansion of the mDCN-T13 pilomotor field after combined 6-OHDA lesion and adjacent surgical denervation. The fields were mapped at the indicated times after the 6-OHDA lesion. The error bars are standard errors of the mean. The normal field size is approximately 309 mm^2.

treated with 6-OHDA, but whose pilomotor fields had not been isolated by adjacent nerve lesions. In these animals, then, the restoration of functional terminals could be achieved without the need for an additional compensatory collateral sprouting like that described above. In keeping with this view the pilomotor fields that recovered by 20d after 6-OHDA treatment without denervations were significantly smaller (374 ± 18 mm^2; n=3) than the recovered fields in the combined chemically and surgically lesioned animals ($p < 0.05$), and were not significantly different from normal field sizes. Thus, it appears that in the absence of adjacent denervation, field recovery at 20d after 6-OHDA injection fails to extend beyond the normal field size. The significance of this difference between the two groups of animals is discussed below.

Effects of anti-NGF treatment on recovery after 6-OHDA lesion.

Daily injections of anti-NGF serum inhibited both the extent and the rate of restoration of pilomotor fields ($p < 0.0002$; Fig 2). Since overall there were no differences in the results from the animals in which the anti-NGF treatment was begun at the time of 6-OHDA lesion,

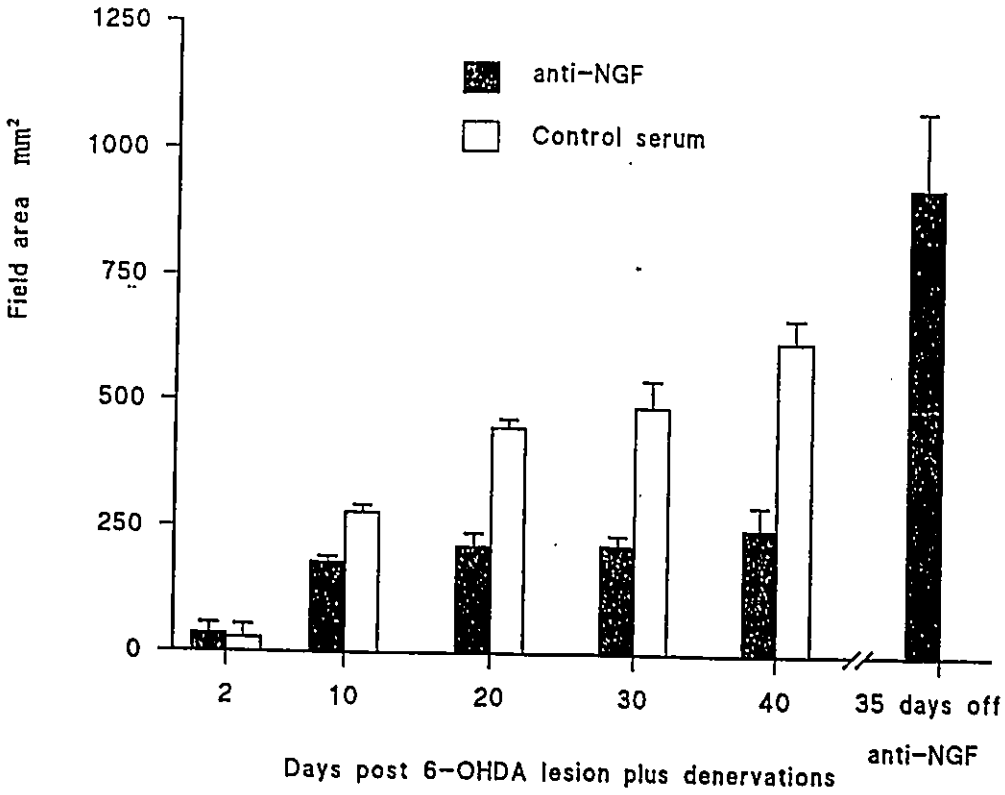

Figure 2.
Anti-NGF treatment blocks field recovery after the combined 6-OHDA lesion and surgical denervations. The open bars show the results from animals treated daily with nonimmune serum, the solid bars relate to animals treated with anti-NGF serum.

and those in which it began 2d after, the data for both groups have been pooled. The measured fields at 2d were the same for all groups, (anti-NGF, control, and non-injected). By 10d the fields had expanded in all the groups; however, in the anti-NGF group the increases, were significantly less than in the controls (180 $\pm$ 32 mm^2, n=9, as compared to 279 $\pm$ 14 mm^2, n=4, $p < 0.0002$), and over the following 30d of anti-NGF treatment no further significant increase in size was observed. In the control serum injected, or non-injected animals, expansion continued up to the longest time point studied (40d) when their fields were 2-3 times larger than the fields in the anti-NGF group (Fig 2). The rate of the (curtailed) recovery in the anti-NGF group between 2 and 10d (16 $\pm$ 3 mm^2/day) was also significantly less ($p < 0.03$) than that in control serum injected rats (32 $\pm$ 5 mm^2/day). (Unexpectedly, in animals receiving control serum injections, the fields were smaller at 30d ($p < 0.05$), but not significantly different at 10, 20, or 40d from those in the uninjected animals). Fluorescent studies confirmed that a regrowth of the chemically lesioned sympathetic fibers had occurred in both the anti-NGF and control treated animals (Fig 3). As explained in the Methods section and above, the skin samples were taken from the <u>contralateral</u> side of the midline. Since no surgical denervations were done on that side, the recovery is assumed to comprise only regrowth of

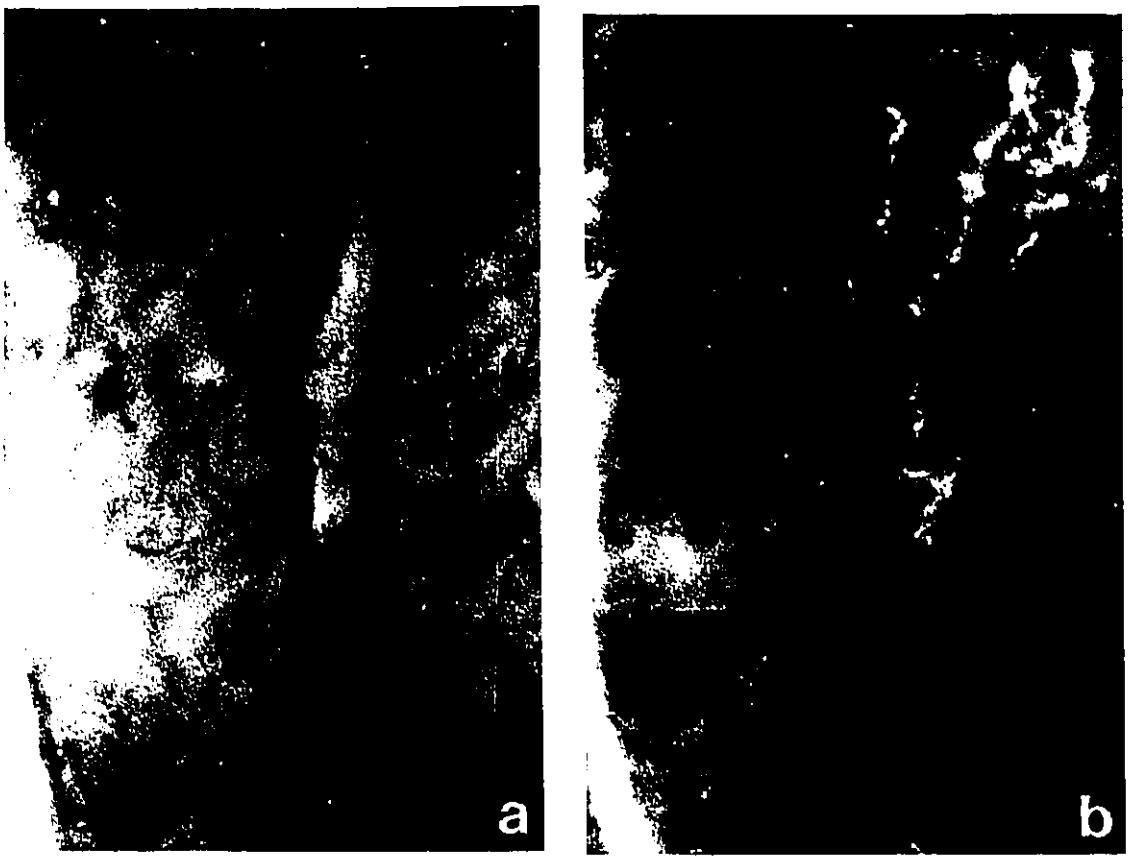

Figure 3
Fluorescent demonstrations of the sympathetic innervation of pilomotor muscle: a skin taken from an animal 2d after 6-OHDA injection; note the absence of fluorescent fibers on the pilomotor muscle (arrows). b, skin from an animal which had received anti-NGF treatment throughout the 40d period following a 6-OHDA injection.

lesioned terminals, and not collateral sprouting to nearby vacated target sites. These findings of abundant fibers both on pilomotor muscle and blood vessels were in marked contrast to the fluorescent results from the skin samples viewed at 2d post 6-OHDA injection (Fig 3).

The inhibitory effect of anti-NGF administration on the expansion of the isolated pilomotor field was reversible. Thirty-five days after animals were removed from 30 days of such treatment (the recovery had ceased totally at 10 days), the pilomotor fields had enlarged ($p < 0.0001$) to almost triple the normal average size (930 $\pm$ 151 mm^2, n=3). Moreover an anti-NGF treatment begun after 10 days was able to halt a normal pilomotor field recovery that was already well under way. In this study 3 animals were first reexamined at 30d after combined field isolation plus 6-OHDA injection; their pilomotor fields had approximately doubled in size by this time (606 $\pm$ 66 mm^2); had this expansion been allowed to continue, they would have reached values of almost 700 mm^2 over the next 10 days (see fig 1). At the 30 day time point, however, daily anti-NGF treatment was started and 10d later the measured pilomotor field areas were 504 $\pm$ 64 mm^2; although this result might point to a slight <u>reversal</u> of the expansion (cf. the analogous findings for sensory sprouting, Diamond et al., (1992a)) the apparent reduction was not significant.

Effects of anti-NGF treatment on recovery after repeated 6-OHDA lesions.

For reasons explained in the Discussion, we also examined animals which received multiple 6-OHDA lesions (4 injections of 6-OHDA over 12 days). During maintained anti-NGF administration, these animals recovered to a significantly greater extent than those receiving only a single injection (Fig 4). Indeed, measured at 20d after the last 6-OHDA injection, the fields of anti-NGF treated animals (307 mm^2; n=2) and controls (309 mm^2; n=2) were of (almost) equal size. As described earlier, in anti-NGF treated animals which had received only a single 6-OHDA injection, the fields had effectively ceased their expansion at 10d, when they were only 213 mm^2 in size. At 20d the fields in the anti-NGF treated multiply lesioned group were 50% larger than those after the single 6-OHDA treatment, a significant difference ($p < 0.02$). By 30d the field sizes in the controls were significantly increased (386 11 mm^2, n=3 $p < 0.001$) compared to the now static anti-NGF treated animals (308 $\pm$ 28 mm^2; n=4). No significant difference was found in the field recovery between the control groups (i.e. animals that were not given anti-NGF treatment) receiving multiple and single 6-OHDA injections.

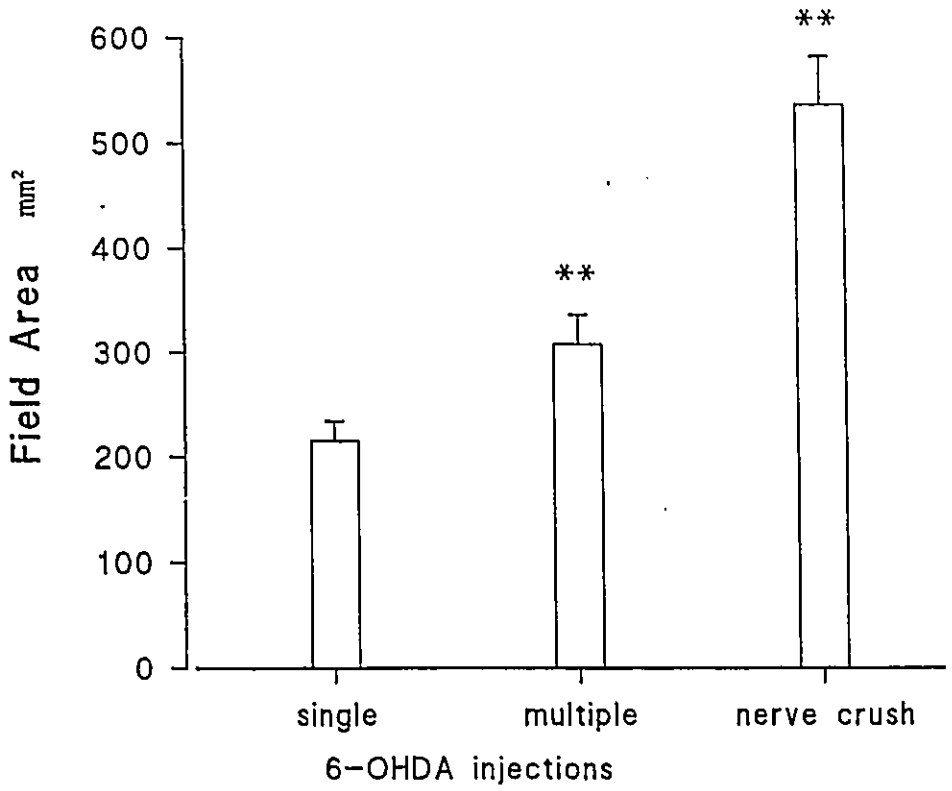

Figure 4
Comparison between the mDCN-T13 field sizes recovered by 30d in animals treated with daily injections of anti-NGF which had received, in addition to adjacent surgical denervation, either a single 6-OHDA injection (left hand bar), multiple 6-OHDA injections (middle bar, see text), or a crush of the mDCN-T13 nerve (right histogram).

Discussion.

Effects of 6-OHDA on sympathetic axons.

Systemic 6-OHDA treatment, in doses similar to, or less than, those used in this study, bring about an almost complete destruction of the terminal and preterminal regions of sympathetic axons (Tranzer and Thoenen, 1968; Malmfors and Sachs, 1968; Champlain, 1970; Bjerre et al., 1974). In the present experiments, the extent of this destruction was indicated by the invariable absence of fluorescent fibers on pilomotor muscle and blood vessels 2 days after 6-OHDA lesion. Nevertheless, in 6 out of 9 cases examined at this time point there was a sparse responsive area, about 30% the normal field size of clearly attenuated pilomotor function. The few fluorescent fibers observed in these instances running close to the bases of hair follicles were presumably making enough functional contact with a proportion of the pilomotor muscles to account for the (reduced) pilomotor function. The ability of such a sparse innervation to drive pilomotor function may have been facilitated by the very large supersensitivity that is known to develop after 6-OHDA lesions (Haeusler et al., 1969). Denervation-induced supersensitivity after surgical denervation of smooth muscle is largely complete by 2d (Langer et al., 1967). The presence of a surviving but reduced pilomotor field need not, however, imply that the fibers subserving the response

were spared by the drug. A functional nerve regrowth could well have occurred during the two days following 6-OHDA treatment. We observed ptosis after each injection of the drug, even during the repeated series of injections, but this ptosis invariably disappeared by 2-3 days. The simplest explanation of these findings is that of a regrowth of lesioned terminals, such that the fastest-growing ones succeeded in reestablishing functional contact with their target smooth muscle cells by 2 days.

Recovery of pilomotor function after lesion.

In the back skin of the rat there is a large overlap between adjacent sympathetic nerve fields (Gloster and Diamond, 1992). Because of this, when adjacent surgical denervations were combined with 6-OHDA treatment, a proportion of the endings which originally occupied the now-isolated field (those deriving from the adjacent nerves) were irreversibly eliminated. In these circumstances a two-part process of sympathetic recovery can be anticipated. One is the restoration of the spared nerve's chemically-lesioned terminals, a process confined to the area of the original field. The second is the collateral sprouting of fibers into surgically denervated skin sites; these sites would occur within the skin of the original field, as well as the surrounding skin. The regrowth of the damaged

terminals and the initiation of true collateral sprouting need not be sequential events, but could proceed in parallel. After combined 6-OHDA treatment and adjacent denervations, the original field areas were reestablished in a little more than 10 days, and by 20 days the fields were almost double their original sizes. However, in the absence of associated adjacent denervations, the fields at 20 days post 6-OHDA injection were indistinguishable in size from normal fields, and no invasion of the adjacent, "foreign", territory had occurred. This result was anticipated; the concurrent recovery of sympathetic terminals in the surrounding skin would remove any inducement for that region to become reinnervated by collateral sprouting from axons supplying the field of study.

The elongating regrowth (regeneration) of the chemically lesioned sympathetic terminal presumably begins at the most distal undamaged point of the axon. Since the 6-OHDA treatment eliminated all stainable sympathetic fibers on pilomotor muscle, the regrowth is presumed to originate from sympathetic axons at a level somewhat deeper in the dermis than that where the pilomotor muscles are inserted on the hair follicle (which is just below the sebaceous gland). The region of the sympathetic axon where collateral sprouting (to nearby denervated target sites) is initiated is more problematical. It is unlikely, however, to be the

terminal itself, or even the immediate preterminal region. When surgically denervated skin is in the process of becoming reinnervated by collateral sprouts emanating from neighboring sympathetic axons, the fluorescent fibers are always seen to arrive at the pilomotor muscles from deeper levels (Gloster and Diamond, unpublished observations); as well, collaterals have never been seen to arise from the fluorescent terminals on the normally innervated pilomotor muscles in the neighboring skin. Thus collateral sprouting of spared sympathetic axons, like the axonal regrowth after 6-OHDA lesions, is presumed to originate below the level where the pilomotor muscles are inserted on the hair follicle. As pointed out earlier, following combined 6-OHDA treatment and adjacent denervations, collateral sprouting and terminal regrowth (regeneration) could be occurring at the same time; the added possibility has to be entertained, moreover, that the two differentially regulated neuritic outgrowths could originate at nearby regions of the same axon, a seemingly paradoxical concept which is clarified below.

Why is the entire original field area not restored synchronously?

Presumably all the sympathetic terminals regrowing within the original field area would reach their former

pilomotor muscles more or less synchronously. It might be expected therefore that pilomotor function would be restored simultaneously across the entire field (as it was, though in an attenuated form, across the reduced field at 2d post-6-OHDA injection in some animals). Instead, after combined 6-OHDA treatment and surgical isolation of the field, a small pilomotor field was always established first, which then expanded to the normal field size and beyond. However, this pattern of recovery is understandable on the basis of the innervation density pattern across a single nerve field. The field center has a higher innervation density than the outer regions, because the latter are normally overlapped by the adjacent nerves (Gloster and Diamond, 1992). If a minimum number of axonal varicosities (the presumed neurotransmitter release sites; Vehara et al., 1976) has to be present alongside the pilomotor muscle for it to be activated, then the center of the original field would be the first region to become functionally reconstituted. In the outer regions of the field the relatively fewer reforming terminals would need to achieve progressively longer lengths, and thus release points, along the muscles, for the pilomotor function to become reestablished there. This requirement for extra growth in the outer regions could account for the progressive nature of the field redevelopment.

Effects of anti-NGF treatment on the recovery of pilomotor function after 6-OHDA lesions.

Daily injections of anti-NGF slowed the recovery of the pilomotor field during the first 20d, at which point, at about 2/3 of the original field area, a plateau was reached, and further recovery ceased. The fluorescent findings indicate clearly that the observed recovery was associated with the regrowth of sympathetic fibers. We believe it is unlikely that a higher dose of anti-NGF would have halted recovery earlier than 20d; the dosage used was that which completely blocks the collateral sprouting both of sympathetic fibers (Gloster and Diamond, 1992) and of sensory fibers (Diamond et al., 1992a), and in the latter study this level of treatment was shown to be in excess of the minimum needed to produce the effect. Rather, the reconstitution of sympathetic terminals seemed to involve a NGF-independent growth response. A factor to be considered here is the possibility that the anti-NGF treatment simply caused a progressive impairment of neurotransmission, eventually resulting in a loss of pilomotor function that would not, therefore, be attributable to a failure of neurite outgrowth. NGF can upregulate norepinephrine levels in sympathetic neurons via its effect on tyrosine hydroxylase levels (Raynaud et al., 1988), the enzyme for

the rate limiting step in norepinephrine synthesis (Levitt et al., 1965). Conceivably, the anti-NGF treatment might have had the converse effect. However, this explanation of the observed effects seems unlikely. The same daily anti-NGF treatment failed to diminish functional pilomotor fields in rats in which post-6-OHDA reinnervation had already proceeded for 30d before the anti-NGF administration was begun. Thus the treatment did not lower norepinephrine content to levels that interfered with activation of pilomotor muscle by newly grown sympathetic fibers. An important finding of relevance to our results is that of Bjerre et al. (1974); these authors reported an inhibition of sympathetic axon regeneration following 6-OHDA by a single dose of anti-NGF serum. The differences in protocol between those studies and the present investigations make a comparison between the two sets of results difficult. A significant feature of the Bjerre et al. study was its reliance on the presence or absence of fluorescently-revealed fibers to reveal the presence or absence of new growth. Given this fact, the two sets of findings seem reconcilable, and explainable on the basis of the considerations to be described below.

Recovery after 6-OHDA is due to combined axonal regeneration and collateral sprouting.

The present findings reveal two components of pilomotor field recovery after combined 6-OHDA and adjacent denervations, a limited anti-NGF resistant phase, and a more extensive phase entirely blocked by anti-NGF treatment. The anti-NGF independent recovery is reminiscent of the massive recovery of pilomotor function achieved by sympathetic axons regenerating after a crush lesion, which was totally unaffected by the same anti-NGF treatment (Gloster and Diamond, 1992), as was the analogous restoration of cutaneous sensory fields by regenerating sensory afferent nerves (Diamond et al., 1987, 1992a,b). The anti-NGF sensitive phase of recovery is clearly analogous to the NGF-independent collateral sprouting of undamaged sympathetic fibers (Gloster and Diamond, 1992). These considerations provide a clear interpretation of the recovery of pilomotor sympathetic function after a combined surgical field isolation and 6-OHDA lesion: there is an initial NGF-independent regenerative phase, involving the regrowth of lesioned terminals, closely followed by a NGF-dependent sprouting phase, involving collateral outgrowth into the adjacent denervated skin. As pointed out earlier, within the area of the field both types of growth, regeneration and collateral sprouting, would occur after a 6-OHDA lesion

combined with adjacent denervations, and these two growths could well be initiated concurrently. However, only the collateral sprouting contribution to the reestablishment of the original field area would be prevented by the anti-NGF treatment. This selective block also explains the observation that in the presence of anti-NGF the initial rate of increase of the recovering pilomotor field was slower than in the absence of anti-NGF, presumably reflecting the absence of a contribution from collateral sprouting in the former situation. In addition, if collateral sprouting was required to restore the original field area after 6-OHDA lesions even in the absence of adjacent denervations, anti-NGF treatment should hinder this recovery. While the effect of anti-NGF treatment on field recovery after 6-OHDA treatment alone was not studied, the effect of anti-NGF treatment on recovery after combined 6-OHDA treatment and surgical denervation was. The recovery after the combined 6-OHDA treatment and surgical denervations would be expected to be equal to or greater than after 6-OHDA treatment alone, due to the elimination of possible competition from neighboring nerves in the former case. The fields recovered by 20d after combined 6-OHDA lesion and surgical denervation in anti-NGF treated animals were smaller than the fields recovered after 6-OHDA treatment alone (surgical denervations) in non-serum

injected animals. Therefore, collateral sprouting is likely required for the restoration of the original field area after the 6-OHDA treatment alone.

A likely explanation of why the proposed regenerative growth of sympathetic fibers after a 6-OHDA lesion differs from that after a nerve crush is suggested by the near vs. far crush phenomenon.

Near vs. Far phenomena.

Axonal axotomy initiates a cell body reaction (CBR, or axon reaction) which encompasses a series of changes in the neuronal cell body. These include disintegration of granular endoplasmic reticulum (chromatolysis) and displacement of the nucleus from a central to a more peripheral location (Lieberman, 1974), as well as changes in molecular synthesis, e.g. increased production of Tα1 α-tubulin (Miller et al., 1989). It seems that at least some of the features of the CBR are associated with the regenerative response of the axon (Lieberman, 1974). One of the variables which affects the CBR is the distance of the axonal cut or crush from the cell body, often referred to as the near vs. far crush phenomenon. Nerve lesions closer to the cell body induce a more severe CBR than do more distal lesions (reviewed by Lieberman, 1974). Significantly, close lesions evoke a more vigorous regenerative response than do

distal lesions, both in the peripheral nervous system (Lieberman, 1974) and in the central nervous system (CNS) (Aguayo et al., 1991). Moreover, when compared to a single crush, two consecutive axonal cuts or crushes increase the extent of a number of events, including chromatolysis (Howe and Bodian, 1941; Romanes, 1951), the rate of regeneration (McQuarrie and Grafstein, 1973; McQuarrie et al., 1977), and the transport of cytoskeletal proteins in rat sciatic nerve (McQuarrie and Jacob, 1991). Thus, there is a good correlation between the intensity of the CBR and the magnitude of the regenerative response. The lesion produced by 6-OHDA represent an axonal injury located at the most extreme distance from the cell body (Tranzer and Thoenen, 1968; Tranzer et al., 1969; Champlain, 1970). In the SCG a 6-OHDA lesion does not appear to cause the ultrastructural alterations that characterize the CBR (chromatolysis or nuclear eccentricity) (Tranzer et al., 1969; Angeletti and Levi-Montalcini, 1970; Bjerre et al., 1974). In contrast, cutting (Bianchine et al., 1964; Matthew and Raisman, 1972) or crushing (Purves and Nja, 1978) of the post-ganglionic nerve initiates a vigorous CBR in the SCG.

From what has been said above, a paucity of detectable CBR should correlate with an absence, or extremely limited, regenerative response. We suggest that the minimal CBR which occurs after 6-OHDA is capable only of

sustaining a minimal degree of (NGF-independent) regeneration of sympathetic axons. This, regeneration however, involving perhaps only some hundreds of micrometers of terminal, is able to restore most of the original field before the regenerative drive disappears, and the neurons revert to their normal (undamaged) status. At this stage continued neuritic outgrowth becomes possible only in the presence of adjacent denervated skin, which evokes collateral sprouting, an event regulated (in this instance) by endogenous NGF. In contrast to the situation with 6-OHDA, the intense CBR induced by nerve crush would be associated with a powerful regenerative response, and as is observed, this is capable of sustaining a NGF-independent growth of axons and terminals which continues well beyond the original field area (Gloster and Diamond, 1992), eventually waning as the CBR disappears and the neurons presumably revert to their normal undamaged status.

The multiple 6-OHDA treatment experiments were done to test this proposed relationship between regeneration and the CBR since, as already mentioned, a repeated lesion is known to increase the severity of the CBR (Howe and Bodian, 1941; Romanes, 1951; Oliver et al., 1969). On this basis, repeated 6-OHDA injections might be expected to induce a larger NGF-independent recovery than after a single injection. The results were entirely consistent with this

proposal, in anti-NGF treated animals the recovery observed after repeated 6-OHDA lesions was significantly greater than that after a single 6-OHDA lesion, remaining NGF-independent for a significantly longer time.

We suggest that the two growth modes able to establish a neuritic arborization within a target tissue, namely the collateral sprouting of undamaged axons, and the regeneration of damaged ones, differ mainly in the nature of the signals that evoke and regulate them. Collateral sprouting appears to be initiated by and dependent on growth factors derived from the target tissue, and is relatively independent of a CBR. Regenerative growth, in contrast, is driven by a central program which is intimately associated with a CBR, and is induced by a signal generated in response to axonal damage, possibly involving the elimination of a molecule normally delivered retrogradely along the axon (cf. Cragg, 1970). The concept of a role for the CBR, in this instance in relation to regeneration within the CNS, is supported by the findings of Aguayo et al. (1991) on the regeneration of retinal axons along an implanted peripheral pathway.

The access question

Our interpretation of the findings, and our conclusions, assume an axonal regenerative growth that

occurs independently of NGF. If NGF were indeed to be responsible for regeneration, its source would need to be inaccessible to the injected anti-NGF molecules, and furthermore this NGF source could not be responsible for the collateral sprouting of the same axons, since this is totally blocked by anti-NGF treatment (Gloster and Diamond, 1992). A key finding relevant to this issue came from our earlier sensory studies, which provided the first evidence for NGF-independent regeneration of axons whose collateral sprouting was NGF-dependent; this was that the regenerating and sprouting neurites grew along the same (denervated) perineurial pathways (Diamond et al., 1992a,b). Furthermore the most plausible hindrance to axonal access, the blood-nerve barrier, breaks down during Wallerian degeneration (Olsson, 1966; de la Motte and Allt, 1976; Seitz et al., 1989), and permeability actually increases in the vicinity of newly regenerating axons (de la Motte and Allt, 1976; Sparrow and Kiernan, 1981). In the present studies the 6-OHDA lesions involve only the nerve terminals, and in this region the perineurial sheath is open ended (Burkel, 1967), so that the blood-nerve barrier is normally absent (Low, 1976). Finally, it seems unreasonable to suppose that sympathetic fibers regenerating after a single 6-OHDA lesion are shielded from antibodies by the blood-nerve barrier more effectively than are fibers regenerating after repeated 6-

OHDA treatment; yet this proposal would be needed to account for the differential ability of anti-NGF to prevent the field recovery after the two treatment protocols.

Conclusions

The total evidence now available indicates overwhelming that sympathetic and sensory nerve regeneration is not dependent on NGF. We are now proposing that the extent of the "regeneration drive" is tightly linked to the CBR to axonal injury, and that it occurs independently of NGF-like molecules generated within the target tissue; in contrast, the collateral sprouting of the same axons when uninjured is dependent entirely on the provision of local growth factor. As noted previously (Diamond et al., 1982), there are numerous observations which point to other important differences between regeneration and collateral sprouting. Our present findings strengthen these. Ultimately, however, the two growth states can lead to a virtually identical reinnervation of deprived target tissue. The key issue are the location, and the nature, of the signal(s) which evokes one or the other form of growth. These considerations may well apply to the adult mammalian CNS, in which collateral sprouting is a widespread phenomenon, while spontaneous axonal regeneration is absent, or occurs only under special conditions (cf. Foester, 1982).

It is interesting that in most, and possibly all, the situations in which regenerating nerves are able to "compete" for target tissue with collaterally sprouting ones, it is the regenerating ones which are ultimately successful regeneration (see Diamond et al., 1982). the growth drive associated with the CBR thus appears to be associated with the still unknown properties of the growing axon tip which determine the ability to establish itself at the target site, and to compete successfully with the analogous collaterally sprouting neurites.

Unifying Discussion.

This thesis has examined the regulation of neurite extension from adult postganglionic sympathetic neurons *in vivo* in three principle situations; following crush injury of the axons, chemical lesion of the terminals by 6-OHDA, and adjacent denervations. Differences were found between the trophic support required for neurite outgrowth in these three conditions. The outgrowth can be categorized as collateral sprouting, which is usually defined as occurring from undamaged axons and regeneration which is a regrowth of damaged ones. The primary conclusion of the thesis is that NGF, or a closely related molecule (see below), is necessary for the collateral sprouting, but not for the regeneration of postganglionic sympathetic neurons. The results however, further suggest that regenerative outgrowth is not only NGF-independent, but firstly, that its extent is determined by the degree of axonal damage suffered by the parent neuron, and secondly that in appropriate circumstances the regenerative phase of outgrowth can proceed into a collateral sprouting mode, the major difference between the two being the regulatory mechanisms involved.

Antibody specificity.

In all these experiments the anti-NGF serum used was a sheep polyclonal antibody which recognizes, in addition to NGF, NT-3, and to a lesser extent, BDNF (R. Murphy personal communication). Therefore, all references to NGF dependent or independent growth should more accurately refer to "NGF or related neurotrophins" dependent or independent growth. However, it is not yet known if the polyclonal anti-NGF used in these studies is able to block the biological actions of BDNF and NT-3. Since BDNF does not support the survival of embryonic sympathetic neurons, BDNF is unlikely to be the neurotrophin involved in these studies. Embryonic sympathetic neurons have been reported to extend neurites in response to NT-3 and NT-4, but to a lesser degree than that elicited in response to NGF (Maisonpierre et al., 1990; Ernfors et al., 1990; Hallböök et al., 1991). The determination of whether adult sympathetic neurons also respond to NT-3 and NT-4, and the development of more specific antibodies to these neurotrophins, will be required before the uncertainty is clarified as to the exact molecule responsible for the collateral sprouting of sympathetic axons.

Antibody access at the site of axonal growth.

An important consideration in the studies is

whether the failure of anti-NGF to prevent outgrowth could be attributed to a failure of antibody access to the critical regions where an NGF dependent outgrowth is initiated. Daily injections of anti-NGF serum were used to eliminate endogenous NGF. Ever since Campenot (1982) found that NGF must be supplied directly to nerve terminals (and not the somata) of embryonic sympathetic neurons to support neurite extension from these terminals, it has been accepted that the site of neurite outgrowth is the important region for a growth promoting agent to have its effects. Thus, the observed results could be explained on the basis of a "global" growth-promoting action of NGF on sympathetic neurons, if the distal regions of the axons undergoing collateral sprouting were accessible to the injected antibodies, while the regions of nerve crush from which regeneration began, were inaccessible. This explanation of the differential effects of anti-NGF in blocking axonal outgrowth is unlikely however; after nerve crush there is a rapid disappearance or reduction in the blood nerve barrier to large macromolecules at the site of crush, and a comparable breakdown in the blood nerve barrier during the process of Wallerian degeneration within the distal nerve stump along which regeneration usually proceeds (Olsson, 1966; de la Motte and Allt, 1976; Seitz et al., 1989). Furthermore, the permeability to macromolecules appears to

be yet further enhanced as the regenerating axons grow down the degenerating stump, an effect postulated to result from the action of molecules released by the growing axons (de la Motte and Allt, 1976; Sparrow and Kiernan, 1981). Three features of the present studies are of special interest. The first was the ability of anti-NGF treatment to prevent collateral sprouting, but not regeneration after nerve crush, within the denervated skin surrounding the original pilomotor fields. In that skin, both the collateral sprouting and the regenerating sympathetic axons are very likely to be contained within the same degenerating pathways created by the elimination of neighbouring nerves, and therefore antibody access would be similar for both of the nerve-growth states. This likelihood cannot be stated with complete certainty for sympathetic axons however, since the exact pathway followed by the growing sympathetic axons within the skin is not known. However, it is known that the analogous collateral sprouting and regeneration of cutaneous nociceptive nerves, which exhibit the same differential requirement for NGF as sympathetic nerves, do utilize the same dermal pathways for their outgrowth into the surrounding skin (Diamond et al., 1992a).

The second consideration applies to the 6-OHDA lesion studies. Here it can be confidently assumed that the growing sympathetic neurites growing from the chemically-

traumatized region of the terminal would have been accessible to the antibodies. The blood-nerve barrier ends near the terminal region of both myelinated and unmyelinated fibres (Burkel, 1967; Low, 1976), enabling released neurotransmitter to diffuse readily to the target tissues. This terminal region is where the neurite outgrowth after 6-OHDA lesions originates (Tranzer and Thoenen, 1968; Malmfors and Sachs, 1968; de Champlain, 1970; Bjerre et al., 1974). It can be presumed, therefore, that such outgrowth is accessible to the systemically administered anti-NGF antibodies. Despite this, in animals receiving daily anti-NGF injections, sympathetic function was restored to 60% of the original field area, indicating the development of a regenerative response whose NGF independence could not be attributable to a diffusion hinderance created by a blood-nerve barrier.

Finally, the recovery after multiple 6-OHDA lesions was greater than that after single 6-OHDA lesions. If antibody accessibility is determining the extent of the apparent NGF-independent neurite growth, then a difference would need to be postulated in the antibody access to the growth initiating region after single and multiple 6-OHDA injections. Given the known locus of action of 6-OHDA this seems an unlikely proposition. From all these considerations, the most reasonable conclusion is that the

demonstrated differences in NGF-dependency of neurite outgrowth are not due to the differential access of the injected antibodies.

Regulation of collateral sprouting.

The collateral sprouting of the postganglionic sympathetic fibres innervating pilomotor muscle has some distinctive features that deserve comment.

1) Timecourse.

The collateral sprouting, induced by adjacent denervations, had begun by 10 days, and it reached a plateau value by 40-50 days; this timecourse of sprouting is similar to that of the postganglionic sympathetic neurons which innervate the rat iris (Olson and Malmfors, 1970) and the sweat glands (Kennedy and Sakutat, 1984; Kennedy et al., 1988), of the SCG fibres that follows implantation of a supernumerary iris in the anterior chamber of the eye (Olson and Malmfors, 1970), and for sympathetic fibres re-innervating sweat glands in the partially denervated rat foot pad (Kennedy and Sakutat, 1984; Kennedy et al., 1988). In contrast, the sprouting of sympathetic nerves within the partially denervated pineal gland was reported as essentially complete by 10 days (Dornay et al., 1985, see also Lingappa and Zigmond, 1987). The difference between the sprouting within the pineal and the various instances of

sprouting just described, might be attributable to the fact that in the case of the pineal gland, only the sprouting of fibres to denervated tissue within the original field of innervation was examined, whereas in the other cases the fibre sprouting studied was to tissue outside of the original field of innervation. The duration of neurite growth in the latter cases would obviously require more time than that required to extend neurites only within the field. It remains to be determined whether sprouting of sympathetic fibres within the original field has a shorter timecourse.

2) Variability of the timecourse of sprouting.

While the duration of the pilomotor fibre sprouting in the present experiments was very similar to the duration of the SCG fibre sprouting in the supernumary iris studies just mentioned, the timecourse of pilomotor fibre sprouting was more variable. Some animals had pilomotor fields which had enlarged close to the maximum extent by 10 days, while others had pilomotor fields which had not enlarged at all by 30 days. All pilomotor fields, however, were observed to have expanded in animals examined at 50 days after isolation. In contrast, the timecourse of sprouting both of sympathetic fibres to implanted irises (Olson and Malmfors, 1970) and to sweat glands in the partially denervated foot (Kennedy et al., 1988) exhibited little variability, in this

respect resembling the sprouting of dermal nociceptive fibres, which run within the same cutaneous nerves as the sympathetic fibres studied in the present investigation (Nixon et al., 1984; Doucette and Diamond, 1987). The variability of the pilomotor fibre sprouting is not due to a variability intrinsic to the mapping technique used to test for the functional presence of sympathetic fibres, since the same test was used to follow the regeneration of the same fibres after combined nerve crush and 6-OHDA lesions, where the timecourses of field expansion were relatively constant. One possible basis for the variability in postganglionic fibre sprouting that was not controlled, however, was the impulse activity in the sympathetic fibres. In the case of cutaneous nociceptive axons, the generation in them of impulse activity around the time of field isolation can markedly reduce the latency of the collateral sprouting of the spared axons (Nixon et al., 1984; Doucette and Diamond, 1987). Thus, one possibility is that in the present studies, impulse activity was a factor, despite the common occurrence of a barrage of impulses during the initial field mapping at the time of field isolation, differences in "spontaneous" preganglionic input activity during the sprouting process were a potential source of sprouting variability. In the cat there is a lack of ongoing impulse activity in either the axons of postganglionic pilomotor

neurons or in the axons of the preganglionic neurons which specifically innervate these neurons (Jänig and Szulczyk, 1981). SCG decentralization reduced the amount of sympathetic sprouting to a supernumary iris, measured at 2 weeks after the iris implant (Olson and Malmfors, 1970), and that occurring within the dentate and CA3 region of the hippocampus after lesion of the fimbria (Crutcher et al., 1979); in the pineal gland no sprouting at all occurred after SCG decentralization (Dornay et al., 1985).

3) Correlation between pilomotor and vasculature fields.

While the functional fields of the sympathetic fibres innervating blood vessels were not studied, the morphological borders of both pilomotor and blood vascular sympathetic fields were found to be coextensive. This was true for both the original fields and the fields which had expanded by collateral sprouting, suggesting that the regulation of the area of skin innervated by these fibres may be the same even though their targets (pilomotor muscle versus blood vessel) differ. The alternative possibility, that the fibres innervating pilomotor muscle and blood vessels are branches of the same axons, can probably be discounted; in the cat and guinea pig in which this point was specifically studied, the fibres innervating these two targets were demonstrated to be two distinct fibre

populations (Jänig and Szulczyk, 1981; Gibbins, 1992).

4) Inhibition of neurite outgrowth by daily anti-NGF treatment.

Continued daily injections of anti-NGF serum completely prevented the collateral sprouting of pilomotor sympathetic fibres for up to 40 days after the adjacent denervations. This effect of anti-NGF was reversible. After cessation of the treatment, collateral sprouting proceeded normally into the surrounding skin. This result is consistent with the previously reported ability of a local injection of anti-NGF to prevent the sprouting of sympathetic fibres into the hippocampus following fimbrial lesion (Springer and Loy, 1985), and suggests that the collateral sprouting of all sympathetic fibres may be NGF-dependent.

The possibility that the anti-NGF treatment did not prevent the collateral sprouting of pilomotor fibres, but merely made the newly grown fibres functionally invisible by reducing norepinephrine levels below that required for neurotransmission, is unlikely for two reasons. Firstly, when recently sprouted fibres were "challenged" with daily anti-NGF treatment, the sizes of the pilomotor fields were not reduced, even over a period of 40 days. As well, anti-NGF treatment halted, but did not reverse, the expansion of fields that were still enlarging when treatment was

instituted. Thus newly sprouted fibres were always able to drive pilomotor function even after the establishment of a maintained anti-NGF treatment regime. Secondly, newly grown sympathetic fibres regenerating after nerve crush lesions were capable of restoring pilomotor function in animals receiving the same daily anti-NGF treatment which completely prevented collateral sprouting. Therefore, sympathetic fibres can indeed grow and restore apparently normal pilomotor function in animals receiving anti-NGF treatment. From these findings, the conclusionn seems inescapable that the anti-NGF treatment prevented collateral sprouting, and did not simply give the appearance of this by lowering levels of the neurotransmitter in newly grown fibres below the values necessary for driving pilomotor function.

Site of NGF's regulation of collateral sprouting.

As discussed in the introduction, NGF can act locally at neurite terminals, independently of new RNA or protein synthesis, or distally after retrograde transport of the NGF signal from the terminals to the cell body, modifying transcription and protein synthesis. Therefore, NGF may be required for directly promoting growth at the terminals and/or for inducing the production in the cell body of the raw materials required to support sustained axonal growth.

Regulation of axonal regeneration.

The results from this thesis clearly show that adult rat cutaneous sympathetic fibres regenerate independently of endogenous NGF. Daily anti-NGF treatment did not prevent or even inhibit the regeneration of sympathetic fibres after nerve crush, but did markedly curtail the recovery after 6-OHDA lesions. The latter result in part confirms Bjerre et al., (1974) findings that the anti-NGF treatment inhibits the recovery of sympathetic fibres after 6-OHDA treatment. While it may be argued that a higher dose of anti-NGF sera might have prevented the axonal regeneration after nerve crush, this is unlikely; the same daily anti-NGF dose completely prevented the collateral sprouting of both sympathetic and sensory fibres, and in the latter case a six times higher dose did not prevent their regeneration after nerve crush (Diamond et al., 1987, 1992a,b). This unexpected NGF-independence of regrowth after crush, but limited NGF-independent growth after 6-OHDA treatment, can be understood by the following arguement.

Intensity of the regenerative response.

A number of studies have suggested that the intensity of the cell body reaction, which consists of chromatolysis and changes in RNA and protein synthesis, is related to the extent of the regenerative response

(Lieberman, 1974; Bernstein, 1975). Multiple lesions, which increase the intensity of the cell body reaction, also increase the rate of regeneration (McQuarrie, 1973; McQuarrie et al., 1977). Much experimental work has shown that axonal lesions closer to the cell body induce a greater chromatolytic reaction than lesions farther from the cell body (reviewed by Lieberman, 1974). While both cutting and crushing of SCG axons generates a pronounced cell body reaction (Bianchine et al., 1964; Nja and Purves, 1978), destruction of sympathetic terminals using systemic 6-OHDA administration (in the adult) does not result in any ultrastructural changes of the cell body (Tranzer et al., 1969; Angeletti and Levi-Montalcini, 1970; Bjerre et al., 1974). A consideration of the findings described in this thesis, which utilised both 6-OHDA lesions and nerve crush, suggest that the extent of the NGF independent phase of sympathetic nerve regeneration is related to the intensity of the cell body reaction. The closer to the cell body the nerve lesion is, the greater the cell body reaction, and the longer the period of time before the neurons revert to normal from the NGF independent regenerative mode. In their normal state, the neurons are capable of changing to a NGF dependent collateral sprouting mode. The 6-OHDA injection, by inducing a lesion only at the distal end of the axon, induces only a modest NGF-independent neurite extension,

compared to that initiated by a crush injury which lesions the axon much closer to the cell body (and which evokes chromatolysis (Nja and Purves, 1978). Multiple lesions induce a greater cell body reaction (Howe and Bodian, 1941; Romanes, 1951; reviewed in Lieberman, 1974), a faster regeneration rate (McQuarrie, 1973; McQuarrie et al., 1977), and a more successful regeneration (Gutman, 1942) than do single lesions. Therefore multiple 6-OHDA injections should induce a greater cell body reaction than single 6-OHDA reactions, and would be predicted therefore to evoke a greater degree of regeneration. The latter was observed. There was a longer period of NGF-independent nerve growth after multiple 6-OHDA injections than after single injections. The results overall thus also indicate that the regenerative program induced by nerve damage is not only NGF independent, but that it is not an all or nothing phenomenon.

Anti-NGF induced neuronal death.

It is remarkable, since chronic NGF deprivation is known to cause cell death of sympathetic neurons (Gorin and Johnson, 1980; Johnson et al., 1982; Ruit et al., 1990), that the sympathetic neurons in this study were regenerating and restoring function while many of them may well have been dying. However, the conditions in which there is an

increased risk of cell death may actually favour rather than hinder regeneration. As mentioned above, cutting retinal axons close to their cell bodies, as opposed to further away, increases the success of regeneration along an implanted peripheral pathway, yet also increases the proportion of retinal neurons which die (Aguayo et al., 1991). The mechanism by which the regenerative drive and neuronal death are related is unknown, but it seems likely that greater axonal damage while increasing the regenerative drive, may be also be so extensive as to cause cell death.

Role of other trophic molecules in supporting regeneration.

While the results show that the axonal regeneration of postganglionic sympathetic neurons after nerve crush is not dependent upon NGF, they do not rule out a role for NGF during axonal regeneration. There may be redundancy in the system: a combination of trophic molecules, including NGF, might so "excessively" (i.e. with a large safety factor) support regeneration, that the elimination of any one trophic molecule does not detectably impair it. However, there are two molecules which may be involved in regeneration which are not members of the NGF neurotrophin family. These are insulin-like growth factor (IGF) I and II; both of these are produced in the degenerating nerve, and antibodies which recognize both IGF-I and IGF-II have

been shown to inhibit sensory nerve regeneration in the sciatic nerve (Kanje et al., 1989; Sjöberg and Kanje, 1989; Ishii et al., 1992).

NGF and regeneration: Implications.

The regeneration of sensory cutaneous fibres after nerve crush, like that of the sympathetic axons, is similarly not inhibited by daily anti-NGF treatment (Diamond et al., 1987, 1992b). Thus, two neuronal populations, one afferent and one efferent, which both require NGF for their survival during the embryological period of natural cell death, and for at least a component of their maintenance as adult neurons, do not require NGF for their regeneration. It follows, then, that a neuronal responsiveness to a trophic factor either during development or later in the adult does not imply that the axonal regeneration of the same neurons is dependent upon the same trophic factor. This NGF independence of regeneration parallels the NGF independency of the initial outgrowth of sympathetic and sensory nerves to their target tissue (see Introduction), raising the possibility that both the axonal regeneration and the initial outgrowth of axons to their target tissues may be similarly regulated.

Literature Cited

Aguayo AJ, Peyronnard JM, Terry LC, Romaine JS, Bray GM (1976) Neonatal neuronal loss in rat superior cervical ganglia: Retrograde effects on developing preganglionic axons and Schwann cells. J Neurocytol 5:137-155.

Aguayo AJ, Rasminsky M, Bray GM, Carbonetto S, McKerracher L, Villegas-Perez MP, Vidal-Sanz M, Carter DA (1991) Degenerative and regenerative responses of injured neurons in the central nervous system of adult mammals. Phil Trans R Soc (Lond) 331:337-343.

Aguilar CE, Bisby MA, Cooper E, Diamond J (1973) Evidence that axoplasmic transport of trophic factors is involved in the regulation of peripheral nerve fields in salamanders. J Physiol (London) 234:449-464.

Allt G (1976) Pathology of the peripheral nerve. In: The peripheral nerve (Landon DN, ed) pp 666-739. London: Chapman and Hall.

Aloe L, Cozzari C, Calissano P, Levi-Montalcini R (1980) Somatic and behaviourial postnatal effects of fetal injection of nerve growth factor antibodies in the rat. Nature 291:413-415.

Angaut-Petit D, Faille L (1987) Inability of regenerating mouse motor axons to innervate a denervated target. Neurosci Lett 75:163-168.

Angaut-Petit D, Mallart A, Faille L (1982) Role of denervated sheaths and endplates in muscle reinnervation by collateral sprouting in the mouse. Biol Cell 46:277-290.

Angeletti PU, Levi-Montalcici R (1970) Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine. Proc Natl Acad Sci USA 65:114-121.

Angeletti PU, Levi-Montalcini R, Caramia F (1968) The nerve growth factor: Chemical properties and metabolic effects. Adv Enzymol 31:51-75.

Angeletti PU, Levi-Montalcini R, Carminia F (1971) Analysis of the effects of the antiserum to the nerve growth factor in adult mice. Brain Res 27:343-355.

Ayer-Le Lievre C, Olson L, Ebendal T, Halbröök F, Persson H (1988) Nerve growth factor mRNA and protein in the testis and epididymis of mouse and rat. Proc Natl Acad Sci USA 85:2628-2632.

Bandtlow CE, Heumann R, Schwab, ME, Thoenen, H (1987) Cellular localization of nerve growth factor synthesis by in situ-hybridization. EMBO J 6:891-899.

Bandtlow C, Zachleder T, Schwab ME (1990) Oligodendrocytes arrest neurite growth by contact inhibition. J Neurosci 10:3837-3848.

Barde YA, Lindsay RM, Monard D, Thoenen H (1982) Purification of a new neurotrophin factor from mammalian brain. EMBO J 1:549-553.

Barker D, Ip MC (1966) Sprouting and degeneration of mammalian motor axons in normal and de-afferentated skeletal muscle. Proc Roy Soc B 163:538-554.

Barker PA, Murphy RA (1992) The nerve growth factor receptor: A multicomponent system that mediates the actions of the neurotrophin family of proteins. Mol Cell Biochem 110:1-15.

Berkemeier LR, Wilslow JW, Kaplan DR, Nikolics K, Goedel DV, Rosenthal A (1992) Neurotrophin-5: A novel neurotrophic factor that activates trk and trkB. Neuron 7:857-866.

Bianchine JR, Levy L, Farah AE (1964) Chromatolysis in the superior cervical ganglion of the cat. Exp Mol Path 3:128-140.

Bisby MA, Chen S (1990) Delayed Wallerian degeneration in sciatic nerves of C57BL/Ola mice is associated with impaired regeneration of sensory axons. Brain Res 530:117-120.

Bjerre B, Björklund A, Edwards DC (1974) Axonal regeneration of peripheral adrenergic neurons: Effects of antiserum to nerve growth factor in mouse. Cell Tiss Res 148:441-476.

Bjerre B, Björklund A, Mobley W, Rosengren E (1975) Short- and long-term effects of nerve growth factor on the sympathetic nervous system in the adult mouse. Brain Res 94:263-277.

Bjerre B, Björklund A, Stenevi U (1973) Stimulation of growth of new axonal sprouts from lesioned monoamine neurones in adult rat brain by nerve growth factor. Brain Res 60:161-176.

Black IB, Hendry IA, Iversen LL (1972) The role of post-synaptic neurones in the biochemical maturation of presynaptic cholinergic terminals in a mouse sympathetic ganglion. J Physiol (Lond) 221:149-159.

Borrelli E, Sawchenko PE, Evans RM (1992) Pituitary hyperplasia induced by ectopic expression of nerve growth factor. Proc Natl Acad Sci USA 89:2764-2768.

Bosch EP, Zhong W, Lim R (1989) Axonal signals regulate expression of glia maturation factor-beta in Schwann cells: An immunohistochemical study of injured sciatic nerves and cultured Schwann cells. J Neurosci 9:3690-3698.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD (1991) ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65:663-675.

Brown MC, Holland RL, Hopkins WG (1981) Motor nerve sprouting. Ann Rev Neurosci 4:17-42.

Brown MC, Ironton R (1977) Suppression of motor nerve terminal sprouting in partially denervated mouse muscles. J Physiol (Lond) 272:70-71P.

Brown MC, Lunn ER, Perry VH (1992) Consequence of slow Wallerian degeneration for regenerating motor and sensory axons. J Neurobiol 23:521-536.

Brown MC, Perry VH, Lunn ER, Gordon S, Heumann R (1991) Macrophage dependence of peripheral sensory nerve regeneration: Possible involvement of nerve growth factor. Neuron 6:359-370.

Brushart TM, Seiler WA (1987) Selective reinnervation of distal motor stumps by peripheral motor axons. Exp Neurol 97:289-300.

Burkel WE (1967) The histological fine structure of perineurium. Anat Rec 158:177-190.

Burstein DE, Greene LA (1978) Evidence for RNA synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. Proc Natl Acad Sci USA 75:6059-63.

Campenot RB (1981) Regeneration of neurites in long-term cultures of sympathetic neurons deprived of nerve growth factor. Proc Natl Acad Sci USA 214:579-581.

Campenot RB (1982) Development of sympathetic neurons in compartmentalized cultures 11. Local control of neurite survival by nerve growth factor. Dev Biol 93:13-21.

Carroll SL, Silos-Santiago I, Frese SE, Ruit KG, Milbrant J, Snider WD (1992) Dorsal root ganglion neurons expressing trk are selectively sensitive to NGF deprivation in utero. Neuron 9:779-788.

Chalazonitis A, Peterson ER, Crain SM (1987) Nerve growth factor regulates the action potential duration of mature sensory neurons. Proc Natl Acad Sci USA 84:289-293.

Chen Y-S, Wang-Bennett LT, Coker N (1989) Facial nerve regeneration in the silicone chamber: The influence of nerve growth factor. Exp Neurol 103:52-60.

Chun LLY, Patterson PH (1977a) Role of nerve growth factor in the development of rat sympathetic neurons in vitro II Developmental studies. J Cell Biol 75:705-711.

Chun LLY, Patterson PH (1977b) Role of nerve growth factor in the development of rat sympathetic neurons in vitro III Effects on acetylcholine production. Developmental studies. J Cell Biol 75:712-718.

Cordon-Cardo C, Tapley P, Jing S, Nanduri V, O'Rourke E, Lamballe F, Kovary F, Klein R, Jones KV, Reichardt LF (1991) The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. Cell 66:173-183.

Cotman CW, Nieto-Sampedro M, Harris EW (1981) Synapse replacement in the nervous system of adult vertebrates. Physiol Rev 61:684-784.

Coughlin MD, Collins MB (1985) Nerve growth factor-independent development of embryonic mouse sympathetic neurons in dissociated cell culture. Dev Biol 110:392-401.

Creedon D, Tuttle JB (1991) Nerve growth factor synthesis in vascular smooth muscle. Hyper 18:730.

Crutcher KA, Brothers L, Davis JN (1979) Sprouting of sympathetic nerves in the absence of afferent input. Exper Neurol 66:778-783.

Crutcher KA, Brothers L, Davis JN (1981) Sympathetic noradrenergic sprouting in response to central cholinergic denervation: a histochemical study of neuronal sprouting in the rat hippocampal formation. Brain Res 210:115-128.

Csillik B, Schwab ME, Thoenen H (1985) Transganglionic regulation of central terminals of dorsal root ganglion cells by nerve growth factor (NGF). Brain Res 331:11-15.

Curtis R, Stewart HJS, Hall SM, Wilkin GP, Mirsky R, Jessen KR (1992) GAP-43 is expressed by non-myelinatin-forming Schwann cells of the peripheral nervous system. J Cell Biol 116:1455-1464.

Dahlström A (1968) Effect of colchicine on transport of amine storage granules in sympathetic nerves of rat. Eur J Pharmacol 5:111-112.

Davies AM, Bandtlow C, Jeumann R, Korsching S, Rohrer H, Thoenen H (1987) Time and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. Nature 326:353-358.

Davies JA, Cook MW, Stern CD, Keynes RJ (1990) Isolation from chick somites of a glycoprotein fraction that causes collapse of dorsal root ganglion growth cones. Neuron 2:11-20.

de Champlain J (1970) Degeneration and regrowth of adrenergic nerve fibers in the rat peripheral tissues after 6-hydroxydopamine. Can J Physiol Pharmacol 49:345-355.

de la Motte DJ, Allt G (1976) Crush injury to peripheral nerve: An electron microscope study employing horseradish peroxidase. Acta Neuropathol (Berl) 36:9-19.

de la Torre JC (1980) An improved approach to histofluorescence using the SPG method for tissue monoamines. J Neurosci Meth 3:1-5.

Diamond J (1982) Modelling and competition in the nervous system: Clues from the sensory innervation of skin. Curr Top Dev Biol 17:147-205.

Diamond J, Coughlin M, Holmes M (1992a) Endogenous NGF and nerve impulses regulate the collateral sprouting of sensory axons in the skin of the adult rat. J Neurosci 12:1454-1466.

Diamond, J, Coughlin M, MacIntyre L, Holmes M, Visheau B (1987) Evidence that endogenous β nerve growth factor is responsible for the collateral sprouting, but not the regeneration, of nociceptive axons in adult rats. Proc Natl Acad Sci USA 84:6596-6600.

Diamond J, Holmes M, Foerster A, Coughlin M (1992b) Sensory nerves in adult rats regenerate and restore sensory function to the skin independently of endogenous NGF. J Neurosci 12:1467-1476.

Diamond J, Holmes M, Visheau B (1988) NGF-regulated plasticity in the adult nervous system. Soc Neurosci Abstr 14:245.6.

Dodd J, Jessell TM (1988) Axon guidance and the patterning of neuronal projections in vertebrates. Science 242:692-699.

Dornay M, Gilad VH, Gilad GM (1985) Compensatory changes in contralateral sympathetic neurons of the superior ganglion and in their terminals in the pineal gland following unilateral ganglionectomy. J Neurosci 5:1522-1526.

Doucette R, Diamond J (1987) The normal and precocious sprouting of heat nociceptors in the skin of adult rats. J Comp Neurol 261:592-603.

Dreyfus C (1989) Effects of nerve growth factor on cholinergic brain neurons. Trends Pharmacol Sci 10:145-149.

Duchen LW, Strich SJ (1968) The effects of botulinum toxin on the pattern of innervation of skeletal muscle in the mouse. Q J Exp Physiol 53:84-89.

Eckenstein F (1988) Transient expression of NGF-receptor-like immunoreactivity in postnatal rat brain and spinal cord. Brain Res 446:149-154.

Edds MV (1953) Collateral nerve regeneration. Quart Rev Biol 28:260-276.

Edwards RH, Rutter WJ, Hanahan D (1989) Directed expression of NGF to pancreatic β cells in transgenic mice leads to selective hyperinnervation of the islets. Cell 58:161-170.

Ernfors P, Henschen A, Olson L, Persson H (1989) Expression of nerve growth factor receptor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motoneurons. Neuron 2:1605-1613.

Ernfors P, Ibanez CF, Ebendal T, Olson L, Persson H (1990) Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: Developmental and topographical expression in the brain. Proc Natl Acad Sci USA 87:5454-5458.

Fawcett JW, Keynes RJ (1990) Peripheral nerve regeneration. Annu Rev Neurosci 13:43-60.

Finn PJ, Ferguson IA, Renton FJ, Rush RA (1986) Nerve growth factor immunohistochemistry and biological activity in the rat iris. J Neurocytol 15:169-176.

Foerster AP (1982) Spontaneous regeneration of cut axons in adult rat brain. J Comp Neurol 210:335-356.

Furness JB, Costa M (1975) The use of glyoxylic acid for the fluorescence histochemical demonstration of peripheral stores of noradrenaline and 5-hydrotrytamine in whole mounts. Histochemistry 41:335-352.

Gibbins IL (1992) Vasoconstrictor, vasodilator and pilomotor pathways in sympathetic ganglia of guinea pigs. Neuroscience 47:657-672.

Gizang-Ginsberg E, Ziff EB (1990) Nerve growth factor regulates tyrosine hydroxylase gene transcription through a nucleoprotein complex that contains c-Fos. Genes Dev 4:477-491.

Gnahn H, Hefti F, Heumann R, Schwab ME, Thoenen H (1983) NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: Evidence for a physiological role of NGF in the brain. Dev Brain Res 9:45-52.

Goedert M, Otten U, Hunt SP, Bond A, Chapman D, Schlumpf M, Lichtensteiger W (1984) Biochemical and anatomical effects of antibodies against nerve growth factor on developing rat sensory ganglia. Proc Natl Acad Sci USA 81:1580-1584.

Gold BG, Mobley WC, Matheson SF (1991) Regulation of axonal caliber, neurofilament content, and nuclear localization in mature sensory neurons by nerve growth factor. J Neurosci 11:943-955.

Goldowitz D, Cotman CW (1980) Axonal transport and axon sprouting in the adult rat dentate gyrus: An autoradiographic study. Neuroscience 5:2163-2174.

Goodall M (1951) Studies of adrenalin and noradrenaline in mammalian heart and suprarenals. Acta Physiol Scand 24:suppl 85, 1-31.

Gorin PD, Johnson EM (1980) Effects of long-term growth factor deprivation on the nervous system of the adult rat; an experimental autoimmune approach. Brain Res 198:27-42.

Grafstein B (1975) The nerve cell body response to axotomy. Exp Neurol 48:32-51.

Greene LA (1977) A quantitative bioassay for nerve growth factor (NGF) activity employing a clonal phaeochromocytoma cell line. Brain Res 133:350-353.

Greene LA (1984) The importance of both early and delayed responses in the biological actions of nerve growth factor. Trends Neurosci 7:91-94.

Greene LA, Shooter EM (1980) The nerve growth factor: Biochemistry, synthesis and mechanism of action. Annu Rev Neurosci 3:353-402.

Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci 7:2424-2428.

Gulati AK (1988) Evaluation of acellular and cellular nerve grafts in repair of rat peripheral nerve. J Neurosurg 68:117-123.

Gundersen RW, Barrett JN (1979) Neuronal chemotaxis; chick dorsal root axons turn towards high concentrations of nerve growth factor. Science 206:1079-1080

Guth L, Bernstein JJ (1961) Selectivity in the re-establishment of synapses in the superior cervical sympathetic ganglion of the cat. Exp Neurol 4:59-69.

Guth L, Smith S, Donati EJ, Albuquerque EX (1980) Induction of intramuscular collateral sprouting by neurally applied colchicine. Exp Neurol 67:513-523.

Haeusler G, Haefely W, Thoenen H (1969) Chemical sympathectomy of the cat with 6-hydroxydopamine. J Pharmacol Exp Ther 170:50-61.

Haftek J, Thomas PK (1968) Electron microscope observations on the effect of localized crush injuries on the connective tissues of peripheral nerve. J Anat 103:233-243.

Hallböök F, Ibanez CF, Persson H (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 6:845-858.

Hamburger V, Brunso-Bechtold JK, Yip JW (1981) Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. J Neurosci 1:60-71.

Harris L, Purves D (1989) Rapid remodelling of sensory endings in the cornea of living mice. J Neurosci 9:2210-2214.

Hempstead B, Martin-Zanca D, Kaplan D, Parada L, Chao M (1991) High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 350:678-683.

Hendry IA, Campbell J (1976) Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. J Neurocytol 5:351-360.

Hendry IA, Stockel K, Thoenen H, Iversen LL (1974) The retrograde axonal transport of nerve growth factor. Brain Res 68:103-121.

Herrera AA, Banner LR, Nagaya N (1990) Repeated *in vivo* observation of frog neuromuscular junctions: Remodelling involves concurrent growth and retraction. J Neurocytol 19:85-99.

Herrera AA, Banner LR, Werle MJ, Regnier M, Nagaya-Stevens N (1991) Post-metamorphic development of neuromuscular junctions and muscle fibers in the frog cutaneous pectoris. J Neurobiol 22:15-28.

Heumann R, Korsching S, Bandtlow C, Thoenen H (1987a) Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection. J Cell Biol 104:1623-1631.

Heumann R, Korsching S, Scott J, Thoenen H (1984) Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. EMBO 3:3183-3189.

Heumann R, Lindholm D, Bandtlow C, Meyer M, Radeke MJ, Misko TP, Shooter E, Thoenen H (1987b) Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration and regeneration: Role of macrophages. Proc Natl Acad Sci USA 84:8735-8739.

Heumann R, Schwab M, Thoenen H (1981) A second messenger required for nerve growth factor biological activity? Nature 292:838-840.

Hofer MM, Barde YA (1988) Brain-derived neurotrophic factor prevents neuronal death *in vivo*. Nature 331:261-262.

Hoffman PN (1989) Expression of GAP-43, a rapidly transported growth-associated protein, and class II beta tubulin, a slowly transported cytoskeletal protein, are coordinated in regenerating neurons. J Neurosci 9:893-897.

Hoffman PN, Cleveland DW (1988) Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: Induction of a specific β-tubulin isotype. Proc Natl Acad Sci USA 85:4530-4533.

Hohn A, Leibrock J, Bailey K, Barde Y-A (1990) Identification of a novel member of the nerve growth factor/brain derived neurotrophic factor family. Nature 344:339-341

Hollowell JP, Villadiego A, Rich KM (1990) Sciatic nerve regeneration across gaps within silicone chambers: Long-term effects of NGF and consideration of axonal sprouting. Exp Neurol 110:45-51.

Honegger P, Lenoir D (1982) Nerve growth factor (NGF) stimulation of cholinergic telencephalic neurons in aggregating cell cultures. Dev Brain Res 3:229-238.

Howe HA, Bodian D (1941) Refractoriness of nerve cells to poliomyelitis virus after interruption to their axons. Johns Hopkins Hosp Bull 69:92-103.

Horch K (1979) Guidance of regrowing sensory axons after cutaneous nerve lesions in the cat. J Neurophysiol. 42:1437-1449.

Horch K. (1981) Absence of functional collateral sprouting of mechanoreceptor axons into denervated areas of mammalian skin. Exp Neurol 74:313-317.

Horii Z-I, Varon S (1975) Nerve growth factor-induced rapid activation of RNA labelling in dorsal root ganglionic dissociates from the chick embryo. J Neurosci Res 1:361-375.

Horii Z-I, Varon S (1977) Nerve growth factor action on membrane permeation to exogenous substrates in dorsal root ganglionic dissociates from the chick embryo. Brain Res 124:121-133.

Ibanez CF, Ebendal T, Barbany G, Murray-Rust J, Blundell TL, Persson H (1992) Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the trk gene product. Cell 69:329-341.

Ide C, Kato S (1990) Peripheral nerve regeneration. Neurosci Res Suppl 13:S1157-164.

Jackson P, Diamond J (1984) Temporal and spatial constraints on the collateral sprouting of low-threshold mechanosensory nerves in the skin of rats. J Comp Neurol 226:336-345.

Jänig W, Szulczyk P (1981) The organization of lumbar preganglionic neurons. J Auto Nerv Syst 3:177-191.

Johnson EM, Andres RY, Bradshaw (1978) Characterization of the retrograde transport of nerve growth factor (NGF) using high specific activity [125I] NGF. Brain Res 150:319-331.

Johnson EM, Gorin PD, Brandeis LD, Pearson J (1980) Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. Science 210:916-918.

Johnson EM, Gorin PD, Osborne PA, Rydel RE, Pearson J (1982) Effects of autoimmune NGF deprivation in the adult rabbit and offspring. Brain Res 240:131-140

Johnson D, Lanahan A, Buck CR, Seghal A, Morgan C, Mercer E, Bothwell M, Chao M (1986) Expression and structure of the human NGF receptor. Cell 47:545-554.

Johnson EM, Osborne PA, Taniuchi M (1989) Destruction of sympathetic and sensory neurons in the developing rat by a monoclonal antibody against the nerve growth factor (NGF) receptor. Brain Res 478:166-170.

Johnson EM, Taniuchi M, Clark HB, Springer JE, Koh S, Tayrien MW, Loy R (1987) Demonstration of the retrograde transport of nerve growth factor receptor in the peripheral and central nervous system. J Neurosci 7:923-929.

Johnson EM, Taniuchi M, DiStefano PS (1988) Expression and possible function of nerve growth factor receptors on Schwann cells. Trends Neurosci 11:299-304.

Johnston MV, Rutkowskin JL, Wainer BH, Long JB, Mobley WC (1987) NGF effects on developing forebrain cholinergic neurons are regionally specific. Neurochem Res 12:985-994.

Jonsson G, Sachs C (1970) Effects of 6-hydroxydopamine on the uptake and storage of noradrenaline in sympathetic adrenergic neurons. Eur J Pharmacol 9:141-155

Kanje M, Skottner A, Lundborg G (1988) Effects of growth hormone treatment on the regeneration of rat sciatic nerve. Brain Res 475:254-258.

Kanje M, Skottner A, Sjöberg J, Lundborg G (1989) Insulin-like growth factor (IGF-1) stimulates regeneration of the rat sciatic nerve. Brain Res 486:396-398.

Kaplan D, Hempstead B, Martin-Zanca D, Chao M, Parada L (1991b) The trk proto-oncogene product: A signal transducing receptor for nerve growth factor. Science 252:554-558.

Kaplan DR, Martin-Zanca D, Parad LF (1991a) Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature 350:158-160

Keller A, Williams R, Vahvviolos J, Auffray C, Rush RA (1990) Immunohistochemical localization of nerve growth factor in a subpopulation of chick spinal ganglion neuron. J Neurosci Res 27:136-143.

Kennedy WR, Sakuta M (1984) Collateral reinnervation of sweat glands. Annals Neurol. 15:73-78.

Kennedy WR, Navarro X, Kamei H (1988) Reinnervation of sweat glands in the mouse: Axonal regeneration versus collateral sprouting. Muscle Nerve 11:603-609.

Kessler JA (1985) Parasympathetic, sympathetic, and sensory interactions in the iris: Nerve growth factor regulates cholinergic cilary innervation *in vivo*. J Neurosci 5:2719-2725.

Kessler JA, Bell WO, Black IB (1983) Interactions between the sympathetic and sensory innervation of the iris. J Neurosci 3:1301-1307.

Kessler JA, Black IB (1980) Nerve growth factor stimulates the development of substance P in sensory ganglia. Proc Natl Acad Sci USA 77:649-652.

Kessler JA, Black IB (1981) Similarities in development of substance P and somatostatin in peripheral sensory neurons: Effects of capsaicin and nerve growth factor. Proc Natl Acad Sci USA 78:4644-4647.

Kilvington B, Osborne WA (1907) The regeneration of post-ganglionic vasoconstrictor nerves Part II. J Physiol (London) 35:460-485.

Kinnman E, Aldskogius H (1988) Collateral reinnervation of taste buds after chronic sensory denervation: A morphological study. J Comp Neurol 270:569-574.

Kirpekar SM, Wakade AR, Prat JC (1970) Regeneration of sympathetic nerves to the vas deferens and spleen of the cat. J Pharmacol Exp Ther 175:197-205.

Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991b) The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189-197.

Klein R, Lamballe F, Bryant S, Barbacid M (1992) The trkB tyrosine protein kinase is a receptor for neurotrophin-4. Neuron 8:947-956.

Klein R, Martin-Zanca D, Barbacid M, Parada LF (1990) Expression of tyrosine kinase receptor gene trkB is confined to the murine embryonic and adult nervous system. Development 109:845-850.

Klein R, Nanduri V, Jing S, Lamballe F, Tapley P, Bryant S, Cordon-Cardo C, Jones K, Reichardt L, Barbacid M (1991a) The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66:395-403.

Klingman GI, Klingman JD (1967) Catecholamines in peripheral tissues of mice and cell counts of sympathetic ganglia after the prenatal and postnatal administration of the nerve growth factor antiserum. Int J Neuropharmacol 6:501-508.

Koo EH, Hoffman PN, Price DL (1988) Levels of neurotransmitter and cytoskeletal protein mRNAs during nerve regeneration in sympathetic ganglia. Brain Res 449:361-363.

Korsching S, Thoenen H (1983) Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: Correlation with density of sympathetic innervation. Proc Natl Acad Sci USA 80:3513-3516.

Korsching S, Thoenen H (1985) Nerve growth factor supply for sensory neurons: Site of origin and competition with the sympathetic nervous system. Neurosci Lett 54:201-205.

Korsching S, Thoenen H (1988) Developmental changes of nerve growth factor levels in sympathetic ganglia and their target organs. Dev Biol 126:40-46.

Kostrezewa RM, Jacobowitz DM (1974) Pharmacological actions of 6-hydroxydopamine. Pharmacol Rev 26:199-288.

Kuchel GA, Hellendall R, Blum M (1992) Transynaptic regulation of low-affinity p75 nerve growth factor receptor mRNA precedes and accompanies lesion-induced collateral neuronal sprouting. Exp Neurol 118:73-84.

Lamballe F, Klein R, Barbacid M (1991) trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66:967-979.

Landmesser L, Pilar G (1970) Selective reinnervation of two cell populations in the adult pigeon cilary ganglion. J Physiol (Lond) 211:203-216.

Langer SZ, Draskócky PP, Trendelenburg U (1967) Time course of the development of supersensitivity to various amines in the nictitating membrane of the pithed cat after denervation or decentralization. J Pharm Exp Ther 157:255-273.

Langley JN (1885) Note on regeneration of pre-ganglionic fibres of the sympathetic. J Physiol (Lond) 18:280-284.

Langley JN (1887) On the regeneration of pre-ganglionic and post-ganglionic visceral nerve fibres. J Physiol (Lond) 22:215-230.

Langley JN, Anderson HK (1904) On the union of the fifth cervical nerve with the superior cervical ganglion. J Physiol (Lond) 30:439-442.

Lazarus KJ, Bradshaw RA, West NR, Bunge RP (1976) Adaptive survival of rat sympathetic neurons cultured without supporting cells or exogenous nerve growth factor. Brain Res 113:159-164.

Lee K-F, Li EN, Huber J, Landis SC, Sharpe AH, Chao MV, Jaenisch R (1992) Targeted mutations of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell 69:737-749.

Levi-Montalcini R, Booker B (1960a) Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein. Proc Natl Acad Sci USA 46:384-391.

Levi-Montalcini R, Booker B (1960b) Excessive growth of the sympathetic ganglia evoked by a protein isolated from mouse salivary glands. Proc Natl Acad Sci USA 46:373-384.

Levi-Montalcini R, Meyer H, Hamburger V (1954) *In vitro* experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. Cancer Res 14:49-57.

Levitt M, Spector S, Sjoerdsma A, Undenfriend S (1965) Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. J Pharmacol Exp Ther 148:1-8.

Li BQ, Kaplan D, Kung H, Kamata T (1992) Nerve growth factor stimulation of the ras-guanine nucleotide exchange factor and GAP activities. Science 256:1456-1459.

Li AKC, Koroly MJ, Schattenkerk ME, Malt RA, Young M (1980) Nerve growth factor: Acceleration of the rate of wound healing in mice. Proc Natl Acad Sci USA 77:4379-4381.

Lichtman JW, Magrassi L, Purves D (1987) Visualization of neuromuscular junctions over periods of several months in living mice. J Neurosci 7:1215-1222.

Lieberman AR (1974) Some factors affecting retrograde neuronal responses to axonal lesions. In: Essays on the nervous system. (Bellairs R, Gray EG, eds) pp 71-104. Oxford: Clarendon Press.

Lillien LE, Claude P (1985) Nerve growth factor is a mitogen for cultured chromaffin cells. Nature 317:632-634.

Lindholm D, Heumann R, Meyer M, Thoenen H (1987) Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of the rat sciatic nerve. Nature 330:658-659.

Lindsay RM (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. J Neurosci 8:2394-2405

Lindsay RM, Harmar AJ (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 337:362-365.

Lindsay RM, Shooter EM, Radeke MJ, Misko TP, Dechant G, Thoenen H, Lindholm D (1990) Nerve growth factor regulates expression of the nerve growth factor receptor gene in adult sensory neurons. Eur J Neurosci 2:389-396.

Lindsay RM, Thoenen H, Barde Y-A (1985) Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. Dev Biol 112:319-328.

Lingappa JR, Zigmond RE (1987) A histochemical study of the adrenergic innervation of the rat pineal gland: Evidence for overlap of the innnervation from the innervation from the two superior cervical ganglia and for sprouting following unilateral denervation. Neurosci 3:893-902.

Litchman JW, Purves D, Yip JW (1979) On the purpose of selective innervation of guinea-pig superior cervical ganglion cells. J Physiol (Lond) 292:69-84.

Liu C-N (1955) Time pattern in retrograde degeneration after trauma of central nervous system of mammals. In: Regeneration in the central nervous system (Windle WF, ed) pp 84-93. Springfield: Thomas.

Low FN (1976) The perineurium and connective tissue of peripheral nerve In: The peripheral nerve (Landon DN, ed) pp 159-187. London: Chapman and Hall.

Lunn ER, Perry VH, Brown MC, Rosen H, Gordon S (1989) Absence of Wallerian degeneration does not hinder regeneration in peripheral nerve. Eur J Neurosci 1:27-33.

Lumsden AGS, Davies AM (1983) Earliest sensory nerve fibres are guided to peripheral targets by attractants other than nerve growth factor. Nature 306:786-788.

Lunn ER, Perry VH, Brown NC, Rosen H, Gordon S (1989) Absence of Wallerian degeneration does not hinder regeneration in peripheral nerve. Eur J Neurosci 1:27-33.

Ma Y, Campenot RB, Miller FD (1992) Concentration-dependent regulation of neuronal gene expression by nerve growth factor. J Cell Biol 117:135-141.

Maher P (1988) Nerve growth factor induces protein-tyrosine phosphorylation. Proc Natl Acad Sci USA 85:6788-6791.

Maisonpierre P, Belluscio L, Squinto S, Ip N, Furth M, Lindsay R, Yancopoulos G (1990) Neurotrophin-3: A neurotrophic factor related to NGF and BDNF. Science 247:1446-1451.

Malmfors T (1965) Studies on adrenergic nerves. The use of rat and mouse iris for direct observations on their physiology and pharmacology of cellular and subcellular levels. Acta Physiol Scand 248 (Suppl):1-93.

Malmfors T, Sachs C (1968) Degeneration of adrenergic nerves produced by 6-hydroxydopamine. Eur J Pharm 3:89-92.

Marco E, Marchisio PC, Bondanza S, Franzi AT, Cancedda R, De Luca M (1991) Growth-regulated synthesis and secretion of biologically active nerve growth factor by human keratinocytes. J Biol Chem 266:21718-21722.

Martin DP, Schmidt RE, Distefano PS, Lowry OH, Carter JG, Johnson EM (1988) Inhibitors of protein synthesis prevent neuronal death caused by nerve growth factor deprivation. J Cell Biol 106:829-844.

Martin-Zanca D, Mitra G, Long LK, Barbacid M (1986) Molecular characterization of the human trk oncogene. Cold Spring Harbor Symp Quant Biol 51:938-992.

Martin-Zanca D, Barbacid M, Parada LF (1990) Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. Genes Dev 4:683-694.

Martinez HJ, Dreyfus CF, Jonakait GM, Black IB (1987) Nerve growth factor selectively increases cholinergic markers but not neuropeptides in rat basal forebrain in culture. Brain Res 412:295-301.

Matsuda H, Coughlin MD, Bienenstock J, Denburg JA (1988) Nerve growth factor promotes human hemopoietic colony growth and differentiation. Proc Natl Acad Sci USA 85:6508-6512.

Mathew TC, Miller FD (1990) Increased expression of Tα1 α-tubulin mRNA during collateral and NGF-induced sprouting of sympathetic sprouting. Dev Biol 141:84-92.

Matthew MR, Raisman G (1972) A light and electron microscopic study of the cellular response to axonal injury in the superior cervical ganglion of the rat. Proc R Soc Lond B 181:43-79.

Mazurek N, Westamp G, Erne P, Otten U (1986) Nerve growth factor induces mast cell degranulation without changing intracellular calcium levels. FEBS Lett 198 No 2:315-320.

McNeill DL, Carlton SM, Coggeshall RE, Hulsebosch CE (1990) Denervation-induced synaptogenesis of calcitonin gene-related peptide containing primary afferent terminals. J Comp Neurol 296:263-268.

McQuarrie IG, Grafstein B (1973) Axon outgrowth enhanced by a previous injury. Arch Neurol 29:53-55.

McQuarrie IG, Grafstein B, Gershon MD (1977) Axonal regeneration in the rat sciatic nerve: Effect of a conditioning lesion and dbcAMP. Brain Res 132:444-453.

McQuarrie IG, Jacob JM (1991) Conditioning nerve crush accelerates cytoskeletal protein transport in sprouts that form after a subsequent crush. J Comp Neurol 305:139-147.

Meakin SO, Suter U, Drinkwater CC, Welcher AA, Shooter EM (1992) The rat trk protoncogene product exhibits properties characteristic of the slow nerve growth factor receptor. Proc Natl Acad Sci USA 89:2374-2378.

Mearow K, Ross G, Gloster A, Holmes M, Diamond J. (1991) Changes in NGF Receptor and Gap-43/B-50 mRNA associated with collateral sprouting of cutaneous nerves. Soc Neurosci Abstr 17:526.2

Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: Different mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol 119:47-54.

Miller FD, Tezlaff WT, Bisby MA, Fawcett JW, Milner RJ (1989) Rapid induction of the major embryonic α-tubulin mRNA, Tα1, during nerve regeneration in adult rats. J Neurosci 9:1452-1463.

Mobley WC, Rutkowski JL, Tennekoon GI, Gemski J, Buchanoan K, Johnston MV (1986) Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. Mol Brain Res 1:53-62.

Mobley WC, Schenker A, Shooter EM (1976) Characterization and isolation of proteolytically modified nerve growth factor. Biochemistry 15:5543-5551.

Murray JG, Thompson JW (1957) The occurrence and function of collateral sprouting in the sympathetic nervous system of the rat. J Physiol (Lond) 135:133-162.

Navarro X, Kennedy W (1989) Sweat gland reinnervation by sudomotor regeneration after different types of lesions and graft repairs. Exp Neurol 104:229-234.

Nja A, Purves D (1978) The effects of nerve growth factor and its antiserum on synapses in the superior cervical ganglia of the guinea-pig. J Physiol (Lond) 277:53-75.

Oblinger MM, Lasek RJ (1988) Axotomy-induced alterations in the synthesis and transport of neurofilaments and microtubules in dorsal root ganglion cells. J Neurosci 8:1747-1758.

Ohmichi M, Decker S, Pang L, Sattiel A (1991) Nerve growth factor binds to the 140 kd trk prot-oncogene product and stimulates its association with the src homology domain of phospholipase c-γ1. Biochem Biophys Res Comm 179:217-223.

Oliver JE, Bradley WE, Fletcher TF (1969) Identification of preganglionic parasympathetic neurons in the sacral spinal cord of the cat. J Comp Neurol 137:321-328.

Olson L, Malmfors T (1970) Growth characteristics of adrenergic nerves of adrenergic nerves in the adult rat. Fluorescence histochemical and ^{3}H-noradrenaline uptake studies using tissue transplantations to the anterior chamber of the eye. Acta Physiol Scand Suppl 348:1-111.

Olsson Y (1966) Studies on vascular permeability in peripheral nerves I. Distribution of circulating fluorescent serum albumin in normal, crushed and sectioned rat sciatic nerves. Acta Neuropathol (Berl) 7:1-15.

Oppenheim RW, Maderdrut JL, Wells DJ (1982) Cell death of motoneurons in the chick embryo spinal cord. VI Reduction of naturally occurring cell death in the thoracolumbar column of Terni by nerve growth factor. J Comp Neurol 210:174-189.

Otten U, Goedert N, Mayer N, Lembeck F (1980) Requirements of nerve growth factor for development of substance P-containing sensory neurones. Nature 301:158-159.

Owman CH (1964) Sympathetic nerves probably storing two types of monoamines in the rat pineal gland. Int J Neuropharmacol 3:105-112.

Palmatier M, Hartman BK, Johnson EM (1984) Demonstration of retrogradely transported endogenous nerve growth factor in axons of sympathetic neurons. J Neurosci 4:751-756.

Parvinen M, Pelto-Huikko M, Soder O, Schultz R, Kaipia A, Mali P, Toppari J, Hakovirta H, Lonnerberg P, Ritzen EM, Ebendal T, Olson L, Hokfelt T, Persson H (1992) Expression of β-nerve growth factor and its receptor in rat seminiferous epithelium: Specific function at the onset of meiosis. J Cell Biol 117:629-641.

Politis MJ, Sternberger N, Ederle K, Spencer PS (1982) Studies on the control of myelinogenesis iv. Neuronal induction of Schwann cell myelin-specific protein synthesis during nerve fiber regeneration. J Neurosci 2:1252-1266.

Purves D (1988) Body and brain: A trophic theory of neural connections. Cambridge: Harvard Univ Press.

Purves D, Hadley RD, Voyvodic JT (1986) Dynamic changes in the dendritic geometry of individual neurons visualized over periods of up to three months in the superior cervical ganglia of living mice. J Neurosci 6:1051-1060.

Purves D, Nja A (1978) Trophic maintenance of synaptic connections in autonomic ganglia. In: Neuronal plasticity (Cotman C, ed) pp 27-47. New York: Raven Press.

Purves D, Voyvodic JT, Magrassi L, Yawo H (1987) Nerve terminal remodelling visualized in living mice by repeated examination of the same neuron. Science 238:1122-1126.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593-597.

Radeke MJ, Feinstein SC (1991) Analytical purification of the slow, high affinity NGF receptor: Identification of a novel polypeptide. Neuron 7:141-150.

Raivich G, Hellweg R, Kreutzberg GW (1991) NGF receptor-mediated reduction in axonal NGF uptake in retrograde transport following sciatic nerve injury and during regeneration. Neuron 7:151-164.

Raivich G, Zimmerman A, Sutter A (1987) Nerve growth factor (NGF) expression in chicken cranial development. J Comp Neurol 256:229-245.

Ramón y Cajal S (1928) Degeneration and regeneration of the nervous system. (May RM, trans). New York: Oxford University Press.

Raynaud B, Faucon-Biguet N, Vidal S, Mallet J (1988) Regulation of neurotransmitter metabolic enzymes and tyrosine hydroxylase mRNA level by nerve growth factor in cultured sympathetic neurones. Development 102:361-368.

Reichardt LF, Tomaselli KJ (1991) Extracellular matrix molecules and their receptors: Functions in neural development. Ann Rev Neurosci 14:531-570

Rich KM, Alexander TD, Pryor JC, Hollowell JP (1989) Nerve growth factor enhances regeneration through silicone chambers. Exp Neurol 105:162-170.

Rich KM, Luszczynxki JR, Osborne PA, Johnson EM (1987) Nerve growth factor protects adult sensory neurons from cell death and atrophy caused by nerve injury. J Neurocytol 16:261-268.

Rich KM, Yip HK, Osborne PA, Schmidt RE, Johnson EM (1984) Role of nerve growth factor in the adult dorsal root ganglia neuron and its response to injury. J Comp Neurol 230:110-118.

Richardson PM, Verge VMK, Riopelle RJ (1986) Distribution of neuronal receptors for nerve growth factor in the rat. J Neurosci 6:2312-2321.

Rodriguez-Tebar A, Dechant G, Barde Y-A (1990) Binding of brain derived neurotrophic factor to the nerve growth factor receptor. Neuron 4:487-492.

Rogers SL, Letourneau PC, Palm SL, McCarthy J, Furcht LT (1983) Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev Biol 98:212-220

Rohrer H, Heumann R, Thoenen H (1988) The synthesis of nerve growth factor (NGF) in developing skin is independent of innervation. Dev Biol 128:240-244.

Romanes GJ (1951) The motor columns of the lumbosacral spinal cord of the cat. J Comp Neurol 94:313-364.

Roper S (1976) Sprouting and regeneration of synaptic terminals in the frog cardiac ganglion. Nature 261:148-149.

Rosenthal A, Goeddel DV, Nguyen T, Lesi M, Shih A, Laramee GR, NIkolics K, Winslow JW (1990) Primary structure and biological activity of a novel human neurotrophic factor. Neuron 4:767-773.

Ruit KG, Osborne PA, Schmidt RE, Johnson EM, Snider WD (1990) Nerve growth factor regulates sympathetic ganglion cell morphology and survival in the adult mouse. J Neurosci 10:2412-2419.

Ruit K, Snider WD (1991) Administration or deprivation of nerve growth factor during development permanently alters neuronal geometry. J Comp Neurol 314:106-113.

Saffran BN, Woo JE, Mobley WC, Crutcher KA (1989) Intraventricular infusion in the mature rat brain enhances sympathetic innervation of cerebrovascular targets but fails to elicit sympathetic ingrowth. Brain Res 492:245-254.

Saika T, Senba E, Noguchi K, Sato M, Yoshida S, Kubo T, Matsunaga T, Tohyama M (1991) Effects of nerve crush and transection on mRNA levels for nerve growth factor receptor in the rat facial motoneurons. Mol Brain Res 9:157-160.

Scarpini E, Ross AH, Rosen JL, Brown MJ, Rostami A, Koprowski H, Lisak RP (1988) Expression of nerve growth factor receptor during human peripheral nerve development. Dev Biol 125:301-310.

Schafer T, Schwab ME, Thoenen H (1983) Increased formation of pre-ganglionic synapses and axons due to a retrograde trans-synaptic action of nerve growth factor in the rat sympathetic nervous system. J Neurosci 3:1501-1510.

Schecterson LC, Bothwell M (1992) Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron 9:449-463.

Scheff SW, Benardo LS, Cotman CW (1980) Decline in reactive fiber growth in the dentate gyrus of aged rats compared to young adult rats following entorhinal cortex removal. Brain Res 199:21-38.

Schreyer DJ, Skene JH (1991) Fate of GAP-43 in ascending spinal axons of DRG neurons after peripheral nerve injury: Delayed accumulation and correlation with regenerative potential. J Neurosci 11:3738-3751.

Schubert D, Whitlock C (1977) Alteration of cellular adhesion by nerve growth factor. Proc Natl Acad Sci USA 74:4055-4058.

Schwab ME (1977) Ultrastructural localization of a nerve growth factor-horseradish peroxidase (NGF-HRP) coupling product after retrograde axonal transport in adrenergic neurons. Brain Res 130:190-196.

Schwartz JP, Pearson J, Johnson EM (1982) Effects of exposure to anti-NGF on sensory neurons of adult rats and guinea pigs. Brain Res 244:378-381.

Scott SA, Cooper E, Diamond J (1981) Merkel cells as targets of the mechanosensory nerves in salamander skin. Proc R Soc Lond B 211:455-470.

Scott SA, Davies AM (1990) Inhibition of protein synthesis prevents cell death in sensory and parasympathetic neurons deprived of neurotrophic factor in vitro. J Neurobiol 21:630-638.

Seeley PJ, Greene LA (1983) Short-term local actions of nerve growth factor at the growth cone. Proc Natl Acad Sci USA 80:2789-2793.

Seitz R, Reiners K, Himmelmann F, Heininger K, Hartung H, Toyka K (1989) The blood-nerve barrier in Wallerian degeneration: A sequential long-term study. Muscle Nerve 12:627-635.

Shawe GDH (1955) On the number of branches formed by regenerating nerve-fibres. Br J Surg 474-488.

Shelton D, Reichardt LF (1984) Expression of the ß-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. Proc Natl Acad Sci USA 81:7951-7955.

Silberstein SD, Johnson DG, Jacobowitz DM, Kopin IJ (1971) Sympathetic reinnervation of the rat iris in organ culture. Proc Natl Acad Sci USA 68:1121-1124.

Simpson SA, Young JZ (1945) Regeneration of fibre diameter after cross-unions of visceral and somatic nerves. J Anat 79:48-65.

Sjöberg J, Kanje M (1989) Insulin-like growth factor (IGF-1) as a stimulator of regeneration in the freeze-injured rat sciatic nerve. Brain Res 485:102-108.

Skene JHP (1989) Axonal growth-associated proteins. Annu Rev Neurosci 12:127-156.

Sketelj J, Bresjanac M, Popovic M (1989) Rapid growth of regenerating axons across the segments of sciatic nerve devoid of Schwann cells. J Neurosci Res 24:153-162.

Snider WD (1988) Nerve growth factor enhances dendritic arborization of sympathetic ganglion cells in developing mammals. J Neurosci 8:2628-2534.

So KF, Aguayo AJ (1985) Lengthy regrowth of cut axons from ganglion cells after peripheral nerve transplantation into the retina of adult rats. Brain Res 328:349-354.

Sonnenfeld K, Ishii D (1985) Fast and slow nerve growth factor binding sites in human neuroblastoma and rat pheochromocytoma cell lines: Relationship of sites to each other and to neurite formation. J Neurosci 5:1717-1728.

Sotelo C, Sanford LP (1971) Altered axons and axon terminals in the lateral vestibular nucleus of the rat. Lab Invest 25:653-671.

Sparrow JR, Kiernan JA (1981) Endoneurial vascular permeability in degenerating and regenerating peripheral nerves. Acta Neuropathol (Berl) 53:181-188.

Speidel CC (1935) Studies of living nerves III Phenomena of nerve irritation and recovery, degeneration and repair. J Comp Neurol 61:1-82.

Sporrong B, Alm P, Owman C, Sjöberg N-O, Thorbert G (1978) Ultrastructural evidence for adrenergic nerve degeneration in the guinea pig uterus during pregnancy. Cell Tiss 195:189-193.

Springer JE, Loy R (1985) Intrahippocampal injections of nerve growth factor inhibit sympathohippocampal sprouting. Brain Res Bull 15:629-634.

Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM, DiStefano PS, Yancopoulos GD (1991) trkB encodes functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell 65:885-893.

Stach RW, Wagner BJ, Stach BM (1977) A more rapid method for the isolation of the 7S nerve growth factor complex. Anal Biochem 83:26-32.

Stokel K, Schwab M, Thoenen H. (1975) Comparison between the retrograde axonal transport of nerve growth factor and tetanus toxin in motor, sensory and adrenergic neurons. Brain Res 99:1-16.

Straznicky C, Rush RA (1985) The effect of nerve growth factor on developing primary sensory neurons of the trigeminal nerve in chick embryos. Anat Embryol 171:91-95.

Sunderland S (1990) The anatomy and physiology of nerve repair. Muscle Nerve 13:771-784.

Sunderland S (1978) *Nerves and Nerve Injuries* (2nd ed). New York: Churchill-Livingston.

Sutter A, Riopelle R, Harris-Warrick R, Shooter E (1979) Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. J Biol Chem 254:5972-5982.

Taniuchi M, Clark HB, Johnson EM (1986) Induction of nerve growth factor receptor in Schwann cells after axotomy. Proc Natl Acad Sci USA 83:4094-4098.

Taniuchi M, Clark HB, Schweitzer JB, Johnson EM (1988) Expression of nerve growth factor receptors by Schwann cells of axotomized peripheral nerves: Ultrastructural location, suppression by axonal contact, and binding properties. J Neurosci 8:664-681.

Theonen H, Angeletti PU, Levi-Montalcini R, Kettler R (1971) Selective induction by nerve growth factor of tyrosine hydroxylase and dopamine ß-hydroxylase in the rat superior cervical ganglia. Proc Natl Acad Sci USA 7:1598-1602.

Thoenen H, Bandtlow C, Heumann R, Lindholm D, Meyer M, Roher H (1988) Nerve growth factor: Cellular localization and regulation of synthesis. Cell Mol Neurobiol 8:35-40.

Towbin H, Staehlin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some application. Proc Natl Acad Sci USA 76:4350-4354.

Tranzer JP, Thoenen H (1968) An electron microscopic study of selective, acute degeneration of sympathetic nerve terminals after administration of 6-hydroxydopamine. Experimentia 24:155-156.

Tranzer JP, Thoenen H, Snipes RL, Richards JG (1969) Recent developments on the ultrastructural aspect of adrenergic nerve endings in various experimental conditions. Progr Brain Res 31:33-46.

Trendelenburg U (1966) Mechanisms of supersensitivity and subsensitivity to sympathomimetic amines. Pharmacol Rev 18:629-640.

Tsukahara N (1981) Synaptic plasticity in the mammalian central nervous system. Annu Rev Neurosci 4:351-379.

Tuckett ILI (1896) On the structure and degeneration of non-medullated nerve fibres. J Physiol (Lond) 19:267-307.

Van der Zee CEEM, Fawcett J, Diamond J (1992) Antibody to nerve growth factor inhibits collateral sprouting of septohippocampal fibers following entorhinal cortex lesion in adult rats. J Comp Neurol 326:91-100.

Varon S, Nomura J, Shooter EM (1967) Isolation of mouse nerve growth factor in high molecular weight form. Biochemistry 6:2202-2209.

Verge VMK, Merlio JP, Grondin J, Ernfors P, Persson H, Riopelle RJ, Hökfelt T, Richardson PM (1992) Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor in primary sensory neurons: Responses to injury and infusion of NGF. J Neurosci 12:4011-4022.

Verge VMK, Riopelle RJ, Richardson PM (1989) Nerve growth factor receptors on normal and injured sensory neurons. J Neurosci 9:914-922.

Verge VMK, Tetzlaff W, Richardson PM, Bisby MA (1990) Correlation between GAP43 and nerve growth factor receptors in rat sensory neurons. J Neurosci 10:926-934.

Verhaagen J, Oestreicher AB, Edwards PM, Veldman H, Jennekens FGI, Gispen WH (1988) Light- and electron-microscopical study of phosphoprotein B-50 following denervation and reinnervation of the rat soleus muscle. J Neurosci 8:1759-1766.

Vetter M, Martin-Zanca D, Parada L, Bishop J, Kaplan D (1991) Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-γ1 by a kinase activity associated with the product of the trk proto-oncogene. Proc Natl Acad Sci USA 88:5650-5654.

Vogel KS, Davies AM (1991) The duration of neurotrophic factor independence in early sensory neurons is matched to the time course of target field innervation. Neuron 7:819-830

Voyvodic JT (1989) Target size regulates calibre and myelination of sympathetic axons. Nature 342:430-433.

Wayne DB, Heaton MB (1990a) The ontogeny of specific retrograde transport of nerve growth factor by motoneurons of the brainstem and spinal cord. Dev Biol 138:484-498.

Wayne DB, Heaton MB (1990b) The response of cultured trigeminal and spinal cord motoneurons to nerve growth factor. Dev Biol 138:473-483.

Weddel GL, Guttmann L, Gutmann E (1941) The local extension of nerve fibres into denervated areas of skin. J Neurol Neurosurg Psychiatr 4:206-255.

Whittemore SR, Seiger A (1987) The expression, localization and functional significance of β-nerve growth factor in the central nervous system. Brain Res Rev 12:439-464.

Wood SJ, Pritchard J, Sofroniew MV (1990) Re-expression of nerve growth factor receptor after axonal injury recapitulates a developmental event in motor neurons: Differential regulation when regeneration is allowed or prevented. Eur J Neurosci 2:650-657.

Wong J, Oblinger MM (1990) Differential regulation of peripherin and neurofilament gene expression in regenerating rat DRG neurons. J Neurosci Res 27:332-341.

Wyatt S, Shooter EM, Davies AM (1990) Expression of the NGF receptor gene in sensory neurons and their cutaneous targets prior to and during innervation. Neuron 2:421-427.

Yan Q, Johnson EM (1988) An immunohistochemical study of the nerve growth factor receptor in developing rats. J Neurosci 8:3481-3498.

Yan H, Schlessinger J, Chao MV (1991) Chimeric NGF-EGF receptors define domains responsible for neuronal differentiation. Science 252:561-563.

Yanker BA, Shooter EM (1979) Nerve growth factor in the nucleus: Interaction with receptors on the nuclear membrane. Proc Natl Acad Sci USA 76:1269-1273.

Yawo (1987) Changes in the dendritic geometry of mouse superior cervical ganglion cells following postganglionic axotomy. J Neurosci 7:3703-3711.

Yip HK, Rich KM, Lampe PA, Johnson EM (1984) The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal ganglia. J Neurosci 4:2986-2992.

Zelena J (1964) Development, degeneration and regeneration of receptor organs. Progr Brain Res 13:175-213.