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REPARATIVE GROWTH IN ADULT PERIPHERAL SENSORY NERVES

By

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ABSTRACT

This thesis involved the use of mutant and transgenic[1] mice to examine the role of various factors in reparative growth of the adult peripheral nervous system. These animals have genetic defects that could be anticipated to affect reparative growth of sensory nerves, and included mice that are transgenic for the heavy chain neurofilament (NFH)lacZ fusion construct, mice mutant either in the steel or the c-kit locus, and mice with a null mutation in the gene coding for the low-affinity neurotrophin receptor ($p75^{NGFR}$).

Both collateral sprouting and regeneration occurred in the NFHlacZ mice at a rate similar to that observed in control mice. Therefore, overexpression of the NFHlacZ construct had no effect on reparative growth of sensory nerves.

Interestingly, regeneration of all three fibre types examined was impaired in mice that have a mutation in the gene coding for Steel or c-Kit, and occured at a rate of 1/2 to 2/3 of that observed in control animals. However, the rate of collateral sprouting was normal.

Although the sensory nerves of $p75^{NGFR}$ mice underwent normal regeneration, collateral sprouting was completely absent. Interestingly, exogenous NGF induced this form of growth in these animals.

In addition, there was a significant decrease in the number of axons comprising all

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[1] An animal which has foreign DNA inserted into its genome.

three fibre types in the sensory nerves in both the Sl/Sl^d, W/W^v and $p75^{NGFR}$ mice, suggesting that the molecules involved also play a role in the development of sensory neurons.

NGF-induced hyperalgesia was not evident in the control or knockout animals upon exogenous administration of either 1 or 2.5μg/g of NGF. However, administration of 2.5μg/g of NGF resulted in a significant mechanical hyperalgesia in both the control and knockout mice. Thus, it appears that the low-affinity neurotrophin receptor does not play a role in NGF-induced hyperalgesia.

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I would like to thank Jack Diamond and everyone in his lab for all of their guidance and support.

I am dedicating this thesis to my mother, who was always there for me.

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LIST OF ABBREVIATIONS

Aβ	large, mechanosensory fibres
Aδ	small, mechano-nociceptive fibres
BSA	bovine serum albumin
C	small, heat-nociceptive fibres
^0C	degrees Celsius
DCN	dorsal cutaneous nerve
DRG	dorsal root ganglion
EtOH	ethanol
g	gram
H_2O_2	hydrogen peroxide
hr	hours
IgG	immunoglobulin
kg	kilogram
lacZ	gene coding for β-galactosidase
M	molarity
MCID	Image Analysis System
mg	milligram

min	minutes
mL	millilitre
mm^2	millimeters squared
NFH	heavy chain neurofilament
NGF	Nerve Growth Factor
o/n	overnight
PBS	phosphate-buffered saline
PO_4	phosphate buffer
$p75^{NGFR}$	low-affinity neurotrophin receptor
RT	room temperature
Sl/Sl^d	steel mutant mice
T13	sensory nerve at thoracic vertebrae 13
µg	microgram
W/W^v	c-kit mutant mice
X	number of times

INTRODUCTION

General Background

It is well-known that peripheral nerves are able to repair themselves subsequent to injury. These forms of repair include the regeneration of damaged fibres as well as the collateral sprouting of undamaged nerves into denervated regions (Diamond, 1988). After reaching their peripheral targets, the growing axons can re-establish their normal tissue contacts, resulting in a gradual recovery of function (Raivich and Kreutzberg, 1993). In contrast, fibres in the central nervous system are unable to regenerate over long distances after damage (Mocchetti and Wrathall, 1995), although they are able to undergo collateral sprouting (Lee and vanDonkelaar, 1995; Schwegler et al, 1995).

Although a number of the cellular aspects of nerve regeneration and collateral sprouting have been determined, there are many unanswered questions regarding the molecular mechanisms that lead to successful recovery of function of peripheral nerves. Many factors have been shown to increase in the cell bodies of nerves undergoing reparative growth, implying a role for these molecules in this process. In addition, there are potentially many other factors present in the environment of collaterally sprouting or regenerating fibres that may also be crucial for nerve repair.

The development of transgenic mouse technology has provided an excellent means to

study the potential functions of candidate molecules in reparative nerve growth. Transgenic mice that either overexpress or fail to express a particular factor are now readily available, and induction of reparative growth in these animals potentially allows for the role of a specific molecule to be elucidated. Furthermore, the potential contribution of a given molecule to other neuronal functions may also be readily analysed.

Reparative Growth Processes

After damage to peripheral sensory nerves, there are two forms of reparative growth that can occur: collateral sprouting and regeneration. Collateral sprouting is a growth from undamaged fibres. It can be readily evoked in adult animals by partially denervating target tissues. For example, following partial denervation of target tissue, the spared nociceptive[2] and sympathetic fibres begin to sprout into the neighbouring denervated regions, as has been demonstrated in a number of animal preparations (Diamond et al, 1987 & 1992a; Gloster and Diamond, 1992; Kinnman and Aldskogius, 1989) as well as in man (Inbal et al, 1987). This collateral sprouting can result in a functional reinnervation of the target, and usually occurs much sooner than that observed with nerve regeneration. The collateral sprouts appear to grow along the axon-denuded perineurial tubes of the dermis, and subsequently (for the C fibres) along finer perineurial tubes of the horizontal subepidermal network (Diamond et al, 1992a). Interestingly, in the adult animal, only the mechano- and heat-nociceptive fibres (Aδ

[2]Nociceptive afferents convey information about a stimulus that can cause damage to the organism.

and C fibres, respectively) undergo collateral sprouting; the low-threshold mechanosensory axons (the Aβ fibres) fail to do so (Jackson and Diamond, 1984). Collateral sprouting of nociceptive fibres is inhibited by systemic administration of antibodies to nerve growth factor (anti-NGF) (Diamond et al, 1992a). It thus appears that this growth process is dependent on the availability of endogenous NGF (described later) in the skin. In fact, if anti-NGF antibody administration is commenced after the onset of collateral sprouting, this growth process will stop almost immediately. Moreover, Diamond et al (1992a) have also shown that nociceptive axons are induced to sprout within normally innervated skin by systemic administration of NGF.

In contrast to collateral sprouting, regeneration is evoked by axotomy, and is the outgrowth of damaged fibres initiated at the ends of axons still connected to their cell bodies (Diamond and Foerster, 1992). Under optimal conditions, new axons sprout from the proximal part of the injured nerve within 24-48 hr following axotomy (Arntz et al, 1989; Gutman et al, 1942), enter the distal part, and there use the denervated Schwann tubes as guidance channels for growth to the peripheral target tissues. The regenerating axons can re-establish their normal tissue locations, leading to a gradual recovery of function (reviewed by Raivich and Kreutzberg, 1993). Unlike collateral sprouting, regeneration will occur along appropriate pathways even if a target tissue is not available. In contrast to their inability to undergo collateral sprouting, the Aβ low-threshold fibres are able to regenerate along with the Aδ and C fibres; in fact, they do so at a rate faster than that observed for the other two fibre types (Diamond et al, 1992b).

A significant finding which has helped distinguish these two growth processes is that

regeneration occurs independently of the availability of NGF. In fact, systemic anti-NGF administration either prior to or during regeneration of sensory fibres either in the target tissue (Diamond et al., 1992b) or towards the target tissue (Doubleday and Robinson, 1995) had no effect on this type of nerve growth. In addition, an *in vitro* study has shown that regeneration occurs in the absence of neurotrophins (Edbladh et al, 1994). These findings have helped establish collateral sprouting and regeneration as distinct processes.

Axotomy of cutaneous sensory nerves results in a degeneration of fibres within the target tissue, leaving a network of empty perineurial tubes. Collateral sprouting and regeneration of nerves in the skin both occur along these same empty perineurial tubes (Diamond, 1988). For this reason, the ability of anti-NGF to affect one form of reparative growth and not the other cannot simply be due to an inability for this molecule to access nerves that are undergoing collateral sprouting and not those that are undergoing regeneration. Therefore, collateral sprouting and regeneration of cutaneous nerves are distinct growth processes in that the former depends on the presence of NGF. However, regeneration of these same fibres occurs independently of the presence of this growth factor.

Cellular mechanisms of peripheral nerve regeneration

Following injury to a peripheral nerve, the fibres distal to the lesion site undergo a degenerative process known as Wallerian degeneration. This process involves the removal of cell debris, and leads to the establishment of an environment which favours the growth of regenerating axons. Axons degenerate along with myelin, leaving behind Schwann cells

surrounded by the basal lamina tube that encircled the original nerve fibre; the latter are called the Bands of Bungner (reviewed by Fawcett and Keynes, 1990). Following peripheral nerve injury, macrophages invade the site of injury and the distal nerve stump prior to the onset of Schwann cell proliferation (Perry et al, 1987). These cells are responsible for the phagocytosis of myelin debris as well as the Schwann cell proliferation that occurs following peripheral nerve injury (Baichwal et al, 1988). Furthermore, after nerve injury, macrophages secrete factors that are responsible for the upregulation of NGF mRNA in Schwann cells as well as in fibroblasts (Bandtlow et al, 1987). One of these secreted factors is interleukin-1 (IL-1); this cytokine is responsible for the increase in NGF mRNA in fibroblasts but not in Schwann cells post-nerve injury (Lindholm et al, 1987; Matsuoka et al, 1991). The factor responsible for this upregulation of NGF mRNA in Schwann cells has not yet been determined.

The Schwann cells begin to proliferate into the degenerating peripheral nerve trunk of the mouse within 19 hrs. of nerve transection; this proliferation is maximal 3 days post-axotomy (Bradley and Ashbury, 1970). The proliferating Schwann cells provide a favourable environment for regenerating axons, and in fact are a critical factor for axonal regeneration (Jeng and Coggeshall, 1987; Le Beau *et al*, 1988). When Schwann cells are prevented from proliferating into the degenerating nerve stump with the use of cytotoxic agents, the axons will either re-grow at a much reduced rate or fail to re-grow completely (Bunge, 1994; Hall, 1986). Therefore, the environment in which regeneration takes place is extremely important for successful repair of peripheral sensory nerves.

Measurement of reparative nerve growth

The techniques for measuring regeneration and collateral sprouting of nociceptive nerves in skin are well established (Diamond *et al*, 1987, 1992a,b). The method of meausuring the region of skin innervated by low-threshold mechanosensory Aβ nerve endings is determined by the electrophysiological recording of the impulses evoked by light stroking of the skin (Jackson and Diamond, 1984).

Electrophysiological techniques are not possible for measuring the area innervated by the Aδ and C fibres, since they are too small in diameter to allow resolution of their individual electrical responses. Therefore, in order to determine the area of target tissue innervated by these nerves, it is possible to stimulate them in order to evoke a reflex contraction involving the cutaneus trunci muscle (CTM) that underlies the skin; this is referred to as the CTM reflex. When the back skin of the rat is locally pinched or heated, a brisk bilateral wrinkling of the skin is produced. Following partial denervation of the skin, this response only occurs if the stimulus is applied to innervated regions of the skin, and is sustained during the period of stimulation (Nixon *et al*, 1984; Doucette and Diamond, 1987). There may be a number of nociceptive endings that are "silent" upon the initial behavioural testing and therefore, they will lie outside the area of innervation as detected by this method. Alterations in central excitability resulting from peripheral nerve damage may allow such "silent" endings to become effective in evoking the reflex response. For example, in the cat and monkey, section of peripheral nerves or dorsal roots results in changes in cutaneous receptive fields. These changes appear to result from an excitation of central neurons by

previously ineffective afferent inputs (Kirk and Denny-Brown, 1970; Dostrovsky *et al*, 1976; but see Pubols, 1984). In order to address this issue, both light and electron microscopy have been used to identify the occurence of new neuritic outgrowth in conjunction with the reappearance of the CTM reflex in initially denervated skin (see Diamond *et al*, 1987). Therefore, the expansion of a given field area after peripheral nerve damage is not due to the "unmasking" of such "silent" endings, but is a result of neuritic growth into denervated regions of target tissue.

Collateral sprouting: Precocious vs. non-precocious

The form of collateral sprouting that was evoked in the mice in the present studies is considered to be precocious collateral sprouting. This refers to the accelerated onset of this form of nerve growth that occurs when the nerve is stimulated at the time it is induced to undergo collateral sprouting. In the present experiments this stimulation would be provided as an unavoidable concomitant of the technique used to measure the initial field area after nerve isolation. Experiments in the rat have shown that although normal collateral sprouting is detectable at about 12 days post-nerve isolation, stimulation of the nerve after its isolation accelerates this process by 5-6 days (Diamond *et al*, 1992a). Since collateral sprouting of sensory fibres in the mouse was readily detectable 7 days after the initial area measurement, the time of onset of precocious collateral sprouting in the mouse was similar to that observed in the rat. Both forms of collateral sprouting proceed such that the extending nerves of the isolated field eventually begin to reach regions that are also becoming innervated by other

nerves in the target tissue, and the collateral sprouting eventually becomes undetectable. Thus, although the time of onset is different, both precocious and normal collateral sprouting have the same end result.

Use of transgenic mice to study reparative nerve growth

The differential effects of anti-NGF on collateral sprouting and regeneration referred to earlier indicates that these two processes are distinctly regulated. I have used various mutant and transgenic mice as a means to look for additional ways in which collateral sprouting and regeneration may be distinguished as different growth processes. Initially, it was necessary to establish, in normal mice, the experimental paradigms that had been previously established in the rat. A considerable amount of effort was required in order to determine the appropriate parameters for measuring the innervation territories of sensory fibres in the back skin of the mouse.

Once this was accomplished, it was then possible to use mouse models to examine the effects of various mutations on reparative nerve growth of adult cutaneous sensory fibres. The transgenic animals that were used were the NFHlacZ transgenics, the steel and c-kit mutants, and the p75 knockouts. I will describe the relevance of these various genetic manipulations to the understanding of nerve growth in the following sections.

NFHlacZ transgenics

Neurofilaments are the most abundant intermediate filaments found in sensory as well as many other types of neurons. There are three polypeptide subunits; in mature neurons these three proteins interact to form 10 nm thick neurofilaments (Geisler *et al*, 1983).

Downregulation of neurofilament levels after axotomy of DRG neurons has been well characterized (Hoffman and Cleveland, 1988; Wong and Oblinger, 1987). In contrast, major upregulation of various tubulin mRNAs has also been documented after this type of injury (Hoffman and Cleveland, 1988; Miller *et al*, 1989) as well as during collateral sprouting (Mathew and Miller, 1990). Target reinnervation is accompanied by a return to normal levels of these molecules.

In order to study the possibility that interactions between neurofilament side arms may modulate axon caliber, Eyer and Peterson have generated transgenic mice that carry a fusion protein in which the carboxyl terminus of the high molecular weight neurofilament protein (NFH) has been replaced by β-galactosidase (NFHlacZ) (Eyer and Peterson, 1994). The transgene is expressed under the control of the NFH promoter. It is present in projection neurons, as assayed by detection of β-galactosidase activity. Electron micrographs of axonal cross-sections indicate that neurofilaments are completely absent; however, microtubules appear to be present in larger than normal amounts (Eyer and Peterson, 1994). In addition, axon calibre is significantly reduced relative to normal control mice.

Since neurofilament and tubulin levels are altered during reparative nerve growth, it is expected that the above mentioned alterations in the levels of these molecules in the nerves

of NFHlacZ transgenic animals prior to damage would have an effect on the ability of their peripheral sensory fibres to undergo collateral sprouting and regeneration. Specifically, since the expression of these molecules in the undamaged peripheral nerves of these mutant mice are altered similarly to that observed after nerve damage in a normal animal, the rate of reparative growth in the mutant mice may be increased relative to that observed in a normal animal.

Steel/c-kit mutants

The proto-oncogene c-Kit encodes a transmembrane tyrosine kinase receptor that is the product of the murine dominant white-spotting (W) locus. The c-kit gene product belongs to a family that includes receptors for colony stimulating factor-1 (CSF-1) and platelet derived growth factor (PDGF) (Qiu *et al*, 1988). The ligand for c-Kit has been cloned and designated as c-Kit ligand (KL), mast cell growth factor (MGF), and stem cell factor (SCF). It has been mapped to the steel (Sl) locus (Anderson *et al*, 1990; Zsebo *et al*, 1990) and is referred to as Steel in this thesis.

Mice with mutations in either the *W* or *Sl* loci are characterized by defects in pigmentation, fertility, and haematopoiesis, implying a function for the c-Kit receptor in these processes (Russell, 1979). In agreement with this, mutant animals contain very few *c-kit* expressing cells in the affected lineages (Motro *et al*, 1991). This is consistent with a role for c-Kit in the proliferation, survival and/or migration of these cells. Furthermore, recent work has shown that mice defective in the *W* locus lack the network of interstitial cells of

Cajal associated with Auerbach's nerve plexus and intestinal pacemaker activity (Huizinga *et al*, 1995). Interestingly, *steel* and *c-kit* are expressed in other cell lineages that seem not to be affected by mutations in W or Sl loci (Nocka *et al*, 1989; Orr-Urteger *et al*, 1990; Motro *et al*, 1991), including the peripheral and central nervous systems, with one of the highest levels of expression occurring in the dorsal root ganglia (DRG) (Keshet *et al*, 1991; Motro *et al*, 1991). c-kit mRNA is first detected in the DRG at embryonic day 12.5, and persists throughout embryonic and postnatal periods. The c-kit positive DRG neurons have recently been characterized as being in the small or medium size category. Furthermore, a large percentage (44%) of these neurons also contain the neurotransmitter substance P (Hirata *et al*, 1995), implying that many of these (but not necessarily all) are nociceptive neurons (Levine *et al*, 1993).

It was recently shown that steel factor is able to induce neurite outgrowth from a sub-population of Nerve Growth Factor (NGF)-responsive murine embryonic DRG neurons *in vitro*, and that it can also act as a survival factor for these neurons (Hirata *et al*, 1993). Analogous findings have also been reported for a NGF-sensitive embryonic DRG neuronal population in the chick, where avian SCF was observed to have similar neurotrophic effects. (Carnahan *et al*, 1994). Thus, while it appears that Steel and c-Kit may have a function in the development of sensory neurons, the role of these molecules in the adult animal remains unknown. One possibility is that these factors are involved in growth processes of sensory neurons in the adult animal, analogous to those observed in embryonic neurons, although the latter findings were all derived from in vitro studies (Carnahan *et al*, 1994; Hirata *et al*, 1993).

There are several natural mutations in the genes coding for steel factor and c-kit. The Sl/Sl^d (one allele is Sl, the other is Sl^d) and W/W^v (one allele is W, the other is W^v) mutants are among the most severe mutations that allow mice to remain viable throughout adult life. Thus, one approach would be to use these two strains of mice for reparative nerve growth studies. The Sl mutation results in a deletion of most or all of the coding region for steel factor, whereas the Sl^d mutation leads to the production of a protein which lacks a transmembrane and cytoplasmic domain (Zsebo *et al*, 1990). The W mutation causes a 78 amino acid deletion that removes the membrane spanning sequences of c-kit. The W^v mutation consists of a single amino acid change in the kinase domain of c-kit, leading to a reduction in kinase activity in this receptor (Williams *et al*, 1992). Due to the severity of these mutations, it is likely that any affects of steel and c-kit on reparative nerve growth in adult mice would be detectable in the Sl/Sl^d and W/W^v mice.

$\underline{p75^{NGFR}\text{ mutants}}$

NGF (Levi-Montalcini *et al*, 1951) is the prototypic member of the neurotrophin family which includes brain-derived neurotrophic factor (BDNF; Barde *et al*, 1982), neurotrophin-3 (NT-3; Ernfors *et al*, 1990; Maisonpierre *et al*, 1990) and neurotrophin 4/5 (NT-4/5; Berkemeier *et al*, 1991). The neurotrophins are known to promote the survival of sympathetic neurons and specific populations of sensory neurons *in vitro* (Barde, 1989). Furthermore, several groups have been able to produce mutant mice in which the gene coding for one of these neurotrophins has been eliminated (Ernfors *et al*, 1993; Smeyne *et al*, 1993).

These "knockout" mice have provided insight as to the dependency of various populations of neurons on the presence of specific neurotrophic factors *in vivo* (Klein, 1994). For example, mice with a null mutation in the gene coding for NGF have an 80% reduction in their sensory neuron population, suggesting that this population of neurons requires NGF during their development (Smeyne *et al*, 1993).

Each of the neurotrophins binds to two distinct classes of membrane receptors. One class consists of a group of receptor tyrosine kinases, including trkA, trkB, trkC, and their various isoforms (reviewed by Bothwell, 1991; Chao, 1992). In addition, the neurotrophins bind to a low-affinity receptor, $p75^{NGFR}$; this receptor binds to each neurotrophin with similar affinity (Rodriguez-Tebar *et al*, 1990), but does not possess intrinsic tyrosine kinase activity (Radeke *et al*, 1987). It has been shown that trkA is essential for the high-affinity binding as well as the biological activity of NGF. This has been demonstrated in mutant pheochromocytoma cell lines (PC12) (Greene *et al*, 1977). These cell lines express significantly reduced levels of trkA, and consequently are deficient in their ability to bind NGF with high affinity and are unresponsive to this neurotrophic factor. Transfection of these cells with trkA results in an ability to respond to NGF, as evidenced by cellular hypertrophy, neurite outgrowth, and survival in serum-free medium (Loeb *et al*, 1991).

On the other hand, the relevance of the low-affinity $p75^{NGFR}$ receptor remains unclear (reviewed by Chao, 1994). It has been suggested that expression of p75 in addition to trkA is necessary in order to generate high-affinity binding sites (Hempstead *et al*, 1991), and that p75 acts to increase the association rate of NGF to the trkA receptor (Mahadeo *et al*, 1994). Others have postulated that p75 acts to increase the efficiency of NGF binding to the trkA

receptor, or possibly acts to modify the ligand specificity of trkA (Barker and Shooter 1994; Hantzopoulos *et al*, 1994; Verdi *et al*, 1994). In addition, studies have shown that $p75^{NGFR}$ has a role in apoptotic cell death; this has been demonstrated in neuronal cell lines and in cultured embryonic DRG cells (discussed later) (Rabizadeh *et al*, 1993; Barrett and Bartlett, 1994).

In addition to facilitating the interaction of NGF with trkA, $p75^{NGFR}$ may participate in intracellular signal transduction. For example, studies have shown that NGF binding to $p75^{NGFR}$ in the absence of trkA induces ceramide production in cultured T9 glioma cells (Dobrowsky *et al*, 1994). A number of protein kinases are known to associate with this low-affinity neurotrophin receptor (Omichi *et al*, 1991; Volonte *et al*, 1993a,b), and it has recently been shown that $p75^{NGFR}$ can collaborate with trkA to activate a $p75^{NGFR}$-associated protein kinase (Canossa *et al*, 1996).

Mice in which the gene coding for the $p75^{NGFR}$ receptor has been targeted by homologous recombination have been produced (Lee *et al*, 1992). These animals are both viable and fertile. Analysis of the footpad skin of these mutant mice indicated a decrease in sensory innervation relative to normal animals. This was accompanied by a loss of heat sensitivity in that region (Lee *et al*, 1992). In *in vitro* studies, embryonic sensory trigeminal neurons from mutant animals required a 3- to 4-fold higher concentration of NGF than normal neurons in order to display a similar extent of survival (Davies *et al*, 1994). In addition, in another *in vitro* study, a 2- to 3-fold higher concentration of NGF was needed to promote survival of an equivalent number of dorsal root sensory neurons from mutant mice as compared to neurons from normal animals (Lee *et al*, 1994).

In the adult animal, trkA and $p75^{NGFR}$ expression is increased in the DRG during collateral sprouting, but not during regeneration of cutaneous sensory neurons (Mearow *et al*, 1994). Expression of both receptors is significantly increased 4 days after nerve isolation, which is at least 4 days prior to the onset of normal collateral sprouting (Diamond *et al*, 1992a). The expression levels remain elevated for about 12 days after nerve isolation. The relative increase in $p75^{NGFR}$ expression is higher than that measured for trkA expression. Furthermore, $p75^{NGFR}$ expression increases during collateral sprouting of sympathetic neurons (Kuchel *et al*, 1992). This implies that collateral sprouting (which is NGF dependent), requires trkA and $p75^{NGFR}$, but that regeneration (which is NGF independent) does not.

$p75^{NGFR}$, NGF and BDNF expression are all upregulated in Schwann cells after peripheral nerve axotomy (Heumann *et al*, 1987; Meyer *et al*, 1990; Taniuchi *et al*, 1986). In fact, expression of BDNF is increased approximately 10-fold above that measured for NGF (Meyer *et al*, 1990). Thus, Schwann cells may supply BDNF to regnerating, BDNF-sensitive neurites. Althernatively, BDNF may have a local, paracrine effect on the non-neuronal cells of the injured peripheral nerve (Raivich and Kreutzberg, 1993). These effects may involve the binding of BDNF to the low-affinity neurotrophin receptor.

In conclusion, collateral sprouting is dependent of the presence of NGF (Diamond *et al*, 1992a) and may be dependent on the presence $p75^{NGFR}$ receptor. On the other hand, although regeneration of cutaneous nerves occurs independently of NGF, the finding that $p75^{NGFR}$ is upregulated in the Schwann cells of the regenerating nerve suggests that other neurotrophic factor(s) (eg. BDNF) may be involved in this reparative growth. Binding of the

relevant factor(s) to the low-affinity neurotrophin receptor as well as one of the trk receptors may be required for successful axonal regeneration.

Other neuronal functions $p75^{NGFR}$: apoptosis[3] and cell survival

In addition to the roles for $p75^{NGFR}$ discussed above, recent studies have identified a function for this receptor in the induction of apoptosis. $p75^{NGFR}$ was transfected into an immortalized neural cell line that expressed neither trkA nor $p75^{NGFR}$ (Rabizadeh *et al*, 1993). In cells cultured in medium containing serum, $p75^{NGFR}$ expression had no effect on cell death. However, when the cells were cultured in medium without serum, there was an increase in cell death in cells expression $p75^{NGFR}$ relative to those that did not. Addition of NGF to the cells resulted in an improved survival in the transfected cells relative to that observed in the control cells. Therefore, expression of $p75^{NGFR}$ may determine the dependence of certain neurons on NGF for survival.

Additional work on single cell suspensions of DRG cells have further clarified the role of $p75^{NGFR}$ in cell death (Barrett and Bartlett, 1994). Use of antisense oligonucleotides to lower the levels of $p75^{NGFR}$ expression in these sensory neurons identified a dual role for the $p75^{NGFR}$ receptor, depending on the stage of development of these neurons. Lowering $p75^{NGFR}$ expression in embryonic day 12 or day 15 neurons largely prevented the NGF-mediated survival that is normally observed. On the other hand, this treatment enhanced

[3]A process whereby a cell "decides" to undergo a program of self-destruction.

survival of embryonic day 19 and postnatal day 2 sensory neurons in the absence of NGF. Thus, $p75^{NGFR}$ is needed to mediate neuronal survival in the presence of NGF during embryogenesis. However, in the early postnatal period $p75^{NGFR}$ enhances cell death in the absence of NGF.

There is a decrease in the sensory innervation of the skin (as shown by a reduction in Substance P and CGRP protein levels) as well as a decrease in the size of DRGs in the $p75^{NGFR}$ mice relative to control animals (Lee *et al*, 1992). This can be explained by the studies described above, which has shown that the $p75^{NGFR}$ receptor is required for neuronal survival during the embryonic stages of development.

Additional work in this area has identified an exclusive role for $p75^{NGFR}$ for NGF-mediated survival effects (Cortazzo *et al*, 1996). Human neuroblastoma cells were treated with antimitotic agents, leading to loss of adherence to the tissue culture dish and apoptosis. Addition of NGF to cells expressing $p75^{NGFR}$ and not trkA resulted in protection from cell death. Furthermore, a mutant form of NGF that binds to $p75^{NGFR}$ with a 100-fold greater affinity than trkA was added to neuroblastoma cells in the presence of antimitotic agents. This form of NGF was able to completely prevent apoptosis in these cells. Moreover, a mutant form of NGF that binds to trkA with 100-fold greater affinity than to $p75^{NGFR}$ was also tested. Interestingly, this form of NGF was not able to protect these cells from apoptosis in the presence of antimitotic agents. When antibodies were used to prevent NGF binding to $p75^{NGFR}$, the cells were not protected from apoptosis induced by the presence of antimitotic agents. Therefore, this study has shown that NGF binding to the $p75^{NGFR}$ receptor is both necessary and sufficient to abrogate apoptosis in neuroblastoma cells treated

with antimitotic agents.

NGF has other functions: hyperalgesia[4]

Among the variety of functions that have been attributed to NGF is its ability to induce a profound behavioural hyperalgesia when administered exogenously to adult rats (Lewin *et al*, 1993). This property of NGF has been studied in transgenic mice that either over- or underexpress NGF in the skin; these mice express a fusion gene construct containing either a sense or antisense gene coding for NGF linked to the K14 keratin promoter (Albers *et al*, 1993). The K14 promoter is abundantly expressed in basal keratinocytes of the epidermis beginning at embryonic day 15 (Vassar *et al*, 1989). Thus, the K14-NGF fusion genes are constitutively expressed in the skin of the animal commencing at the time when the endogenous NGF gene is downregulated (Davies *et al*, 1987; Korsching and Thoenen, 1988). Animals that overexpress NGF in the skin show a profound hyperalgesia to noxious mechanical stimulation, whereas, animals that underexpress this neurotrophic factor in the skin show a reduced response to the same stimulation (Davis *et al*, 1993).

The early phase of NGF-induced heat hyperalgesia (as measured at 30min. and 3 hr. post-NGF) is thought to be a mast-cell dependent response (Lewin *et al*, 1994). These cells are known to express receptors for NGF (Horigome *et al*, 1993), and NGF can induce mast cell degranulation (Bruni *et al*, 1982). Thus, it is possible that NGF can exert its effects by

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[4]An exaggerated response to a noxious stimulus.

binding to the high-affinity trkA receptor on these cells, leading to a series of secondary events that result in the induction of a reduced threshold of response to noxious thermal stimulation (Lewin *et al*, 1994).

The role of mast cells in NGF-induced hyperalgesia was demonstrated with the use of antagonists to serotonin (5-HT), a factor known to be released from mast cells upon degranulation (Horigome *et al*, 1993), to activate and sensitize primary afferents (Rueff and Dray, 1992) and to cause hyperalgesia in rodents (Taiwo and Levine, 1992) and humans (Richardson *et al*, 1985). On the other hand, a central mechanism is thought to be involved in the late stages of NGF-induced hyperalgesia, commencing at approximately 7 hrs. post-NGF administration (Lewin *et al*, 1994). This is thought to involve the activation of NMDA receptors, leading to the sensitization of spinal circuits to peripheral input (reviewed by McMahon *et al*, 1993). In support of this data, chronic administration of the NMDA antagonist MK-801 was able to block the late-onset NGF-induced hyperalgesia (Lewin *et al*, 1994).

Mechanical hyperalgesia appears to involve different mechanisms than thermal hyperalgesia. For example, in contrast to the rapid onset of thermal hyperalgesia, NGF-induced mechanical hyperalgesia occurs approximately 7hr after exogenous NGF administration. The late onset of this response suggests that a mechanism in the central nervous system could be involved. However, this form of hyperalgesia appears to occur independently of central NMDA receptor activation, and no other mechanisms have yet been elucidated (Lewin *et al*, 1994).

Many questions remain regarding the mechanisms of NGF-induced hyperalgesia. For

example, it is not known whether NGF must bind to both the trkA and $p75^{NGFR}$ receptors in order for the events that lead to the induction of hyperalgesia to occur, or if binding to trkA is sufficient. A recent study has addressed this issue by analysing NGF receptor expression subsequent to inflammation induced by injection of complete Freund's adjuvant into the rat hindpaw (Cho *et al*, 1996). This procedure leads to a large increase in expression of NGF in the inflamed tissue, as well as an enhanced retrograde transport of NGF in the sciatic nerve (Donnerer *et al*, 1992; Woolf et al, 1994). In addition, a significant increase in mRNA levels of $p75^{NGFR}$ has been observed in the ipsilateral L5 DRG at 1 and 3 days post-injection of complete Freund's adjuvant into the rat hindpaw (Cho *et al*, 1996). This was not accompanied by a change in trkA mRNA levels. These findings suggest that p75 may be important for the events that occur subsequent to induction of hyperalgesia.

The $p75^{NGFR}$ knockout mice clearly could be a useful model to study the role of this low-affinity neurotrophin receptor in NGF-induced hyperalgesia. Therefore, the thermal and mechanical hyperalgesia that results from NGF administration was studied in $p75^{NGFR}$ mice and compared to the effects observed in control animals.

Summary of the relevance of the transgenic mice selected for study to the overall objectives

There are a variety of factors which potentially play a role in reparative growth of adult peripheral cutaneous fibres. Among the candidate molecules are the heavy chain neurofilament NFH, steel factor and its tyrosine kinase receptor c-kit, and the low-affinity neurotrophin receptor $p75^{NGFR}$. The transgenic/mutant mice described above are characterized by an alteration in a gene coding for one of these molecules. Thus, they allow

for investigation of the possible role of these various factors in reparative nerve growth. In addition, the $p75^{NGFR}$ knockout mice provide a useful model to study the role of the low-affinity neurotrophin receptor in NGF-induced hyperalgesia.

Hypotheses

1. Collateral sprouting and regeneration of cutaneous sensory fibres in adult mice will be impaired in the steel/c-kit and p75 knockout mutant mice, due to the dependency of growth on the production of the functional gene products steel factor, its tyrosine kinase receptor c-kit, and the low-affinity neurotrophin receptor $p75^{NGFR}$.

2. The rate of reparative nerve growth will be increased in the NFHlacZ mice, since the alterations in neurofilament and tubulin levels in the peripheral nerves of these mice are similar to that observed after peripheral nerve injury. This rationale will likely also apply to the growth of undamaged axons, as in the case of collateral sprouting.

3. Although the p75 receptor appears to be dispensable for a majority of NGF functions, its expression increases in conditions of experimental peripheral inflammation, and thus it is expected that the $p75^{NGFR}$ mice will exhibit abnormal hyperalgesic responses to noxious mechanical and thermal stimulation.

MATERIALS AND METHODS

1.1 Animals used for experimentation

The Sl/Sl^{d} (Zesbo *et al*, 1990), W/W^{v} (Chabot *et al*, 1988; Geissler *et al*, 1988) and p75^{NGFR} mice were obtained from Jackson Laboratories. The NFHlacZ (Eyer and Peterson, 1994) transgenics were obtained from Dr. A. Peterson (Royal Victoria Hospital, Montreal). The mice were housed in cages with microisolators, with access to food and water *ad libitum*.

Steel/c-kit and p75^{NGFR} (Lee *et al*, 1992) mice tended to develop lesions in various locations when housed beyond approximately 3-4 months. At this point they were about 5-6 months of age. The Steel/c-kit mice mainly developed lesions around the face and in back of the neck area, while the p75^{NGFR} mice tended to have this problem predominantly in their back legs and tail regions. Experiments were performed on mice that were 2-4 months of age. Only male Sl/c-kit mice were used; both male and female p75^{NGFR} and NFHlacZ mice were used. Typically, mice in the range of 20-30g were used to study reparative nerve growth. However, there were some exceptions to this, which will be discussed later.

2.1 Sensory field mapping

The area of target tissue innervated by each of the three fibres types comprising DCN

T13 were measured. The area innervated by the large low-threshold mechanosensory Aβ fibres was determined electrophysiologically. To perform these measurements for the Aδ and C fibres, a behavioural technique was necessary. These fibres are of a small calibre, therefore, individual fibres would have to be isolated from the nerve in order to perform electrophysiological measurements. The expansion of their fields into denervated territory would thus not be possible. It was necessary to remove the nerves adjacent to the desired nerve of study in order to allow the borders of the target regions to be delineated. Axotomy of these nerves was also necessary in order to evoke expansion of the nerve of interest into adjacent, denervated territory.

2.1a Collateral sprouting and regeneration nociceptive Aδ and C fibres

In order to isolate the sensory fields of selected nerves, the animals were anaesthetized with 0.04 mL/10g Equitesin (4.2g chloral hydrate, 2.1g magnesium sulphate, 15mL sodium pentobarbital, 42.8mL propylene glycol, 12.2mL 95% alcohol, 30mL 0.9% saline). A 3cm incision was made parallel to the midline, approximately 1cm to the right of the midline. The skin was lifted to expose the array of dorsal cutaneous nerves (DCNs). DCN T13 on the left side of the animal was left intact, and 4 nerves rostral and caudal to this were cut, as were 4 lateral cutaneous nerves ventral to DCN T13. After suturing the skin, the animal was allowed to recover. The animal was then re-anaesthetized either several hours later or the following morning with 20-25mg/kg sodium pentobarbital. This dose was sufficient to sedate the animal, but allowed reflex responses to remain in the animal.

Subsequently, established techniques (Doucette and Diamond, 1987) were used to test the Aδ fibre mechanonociceptive "pinch" or C fibre nociceptive "heat" fields. This involved the use of fine forceps to pinch the skin, or the application of a 60^0C heat probe to the skin, respectively. The heat probe was applied to a region of the skin for a maximum of 3-4sec. This evoked contraction of the underlying cutaneous trunci muscle (CTM reflex) which could be visualized by a distinct wrinkling of the back skin. The border between innervated and denervated areas were outlined with a marker. These were transferred to acetate transparencies which were then placed onto a light box. The size of the areas were then measured with the MCID Image Analysis System (Imaging Inc., St. Catharines, Ont.). The MCID provided the areas in mm^2. The innervation territory of the sprouting nerve was monitored over a 14-18 day period.

For nerve regeneration studies, DCN T13 was isolated as above. The animals were allowed to recover and then anaesthetized as above. Once again the "pinch" and "heat" fields were tested as described above. The animals were then given a second dose of anaesthetic. The back skin of the animal was then re-opened and DCN T13 was crushed with fine forceps for two 10sec. intervals approximately half-way between the skin and the muscle wall. The skin was re-sutured and the animals allowed to recover. The re-innervation of the regenerating nerve into the target tissue was monitored over a three week period.

Both collateral sprouting and regeneration have been determined to occur at a linear rate (Diamond *et al*, 1992a,b). There is known to be a lag phase prior to the onset of collateral sprouting, however, this growth is measurable by 7 days after its induction. Therefore, the rate of this growth was measured beginning at 7 days of collateral sprouting

and not on the day of field isolation. In order to determine the rate of collateral sprouting, the area measured at 7 days was subtracted from the area measured at 14 or 18 days, and this value was divided by the number of days, either 7 or 11.

In order to determine the rate of regeneration, the area measured at 7 days was subtracted from the area measured on the final day of the experiment, and was once again divided by the number of days between the two time points. After the nerve was crushed, it regenerated along its original pathway back to the skin; this process required about 7 days. After this occurred, it was possible to measure the expansion of the nerve field in the target tissue. The rate of both collateral sprouting and regeneration is expressed in mm^2/day, and the number of animals studied is shown in brackets after this value. Statistical significance for these experiments and all others in this thesis unless stated otherwise was considered to be $p \leq 0.05$ as determined by the Students t-test. An exception to this occurred when a comparison was made between the distributions of axon area and myelin diameter in the $p75^{NGFR}$ and control animals. In this case, the Kolmogorov-Smirnov two-sample test was used to determine whether the two independent samples were drawn from populations with the same distribution; again, the level of significance was $p \leq 0.05$.

2.1b Regeneration of the low-threshold mechanosensory Aβ fibres

The procedure leading to isolation of the field innervated by DCN T13 is described in section 2.1a. The innervation territory of DCN T13 was measured electrophysiologically. Connective tissue was removed from the nerve and the nerve was placed across bipolar

platinum electrodes connected to a differential AC preamplifier. The output of this was displayed on a Tektronix storage oscilloscope and audio amplifier. When the area of skin innervated by T13 was brushed with a fine bristle, clearly audible responses were evoked, and quickly disappeared as the stimulus crossed the innervation border. After the innervation territory of this nerve was measured, the nerve was crushed as above. The area of skin re-innervated by T13 at 12 days post-nerve crush was also measured. Only two time points are used in this case since the cutaneous nerve in the mouse is very prone to damage, and thus it would be impractical to attempt to perform numerous measurements.

3.1 NGF administration

Exogenous NGF was administered to both the $p75^{NGFR}$ knockout and control animals in order to determine whether the rate of collateral sprouting would be affected with this treatment. $p75^{NGFR}$ knockouts and controls were injected subcutaneously with either 1 or 2.5μg/g of 2.5s NGF in a volume of 0.2ml daily for a period of 10 days. Other mutant and control mice were similarly injected with 0.2ml saline daily for the same period of time.

4.1 Detection of protein

Immunocytochemistry was used to determine whether there were changes in the expression of specific molecules of interest in the DRG and/or sensory nerve during reparative nerve growth. Changes in expression of c-kit during reparative growth was

analysed in the DRG and sensory nerve during axonal regeneration and compared to the level of expression found normally as well as during collateral sprouting. In addition, the levels of trkA and IB4 lectin protein expression in the DRG were compared in $p75^{NGFR}$ and normal animals, and changes in the levels of trkA protein expression in the DRG during the collateral sprouting paradigm was compared to that observed in untreated DRGs. An antibody to S-100, a Schwann cell specific marker (Holton and Weston, 1982) was used to identify the presence of this cell type in the peripheral nerve.

4.1a Surgical procedure and tissue preparation

Initially, the animals were anaesthetized with 0.04mL/10g Equitesin. A 3cm incision was made parallel to the midline. The skin was lifted to expose the array of dorsal cutaneous nerves (DCNs). The following nerves were then crushed with forceps for two 10s intervals each: L2, L1, T12, T11, T9, T8, T6, T5 crush. T13, T10, and T7 were left intact and thus allowed to undergo collateral sprouting. The animals were allowed to recover and left for a designated number of days. In some cases the DRGs on the right side of the animal were used as controls, alternatively, the cutaneous nerves on both sides of the animal were manipulated and untreated animals were used as controls. At the specified time, the DRGs and/or relevant nerves were removed and placed directly into OCT compound. The tissue was then sectioned at a thickness of 10μm, placed on to Aptex coated slides, and stored at -70 °C.

4.1b Immunocytochemistry

Slides containing tissue sections of interest were removed from the -70^{0}C freezer and allowed to thaw at RT for 20min. Mounted sections were then encircled with a Teflon coating using a Pap pen (RBI). The tissue sections were fixed in acetone for 10 min, and then rinsed 3X in PBS over 30min. The slides were then placed into methanol containing 0.3% H_2O_2 for 30min. and rinsed 3X in PBS over 30min. This was followed by a 1hr. incubation in PBS containing 10% goat serum and 0.2% TritonX detergent. The sections were then incubated in PBS containing 2% goat serum, 0.1% BSA, and a monoclonal antibody to the Kit receptor (1:500 Santa Cruz); this incubation was carried out for 42hr. at 4^{0}C. The slides were then rinsed in PBS as above, and then incubated in PBS containing 2% goat serum, 0.1% BSA, and biotinylated goat anti-rabbit (1:200 Vector Laboratories) for 1hr. The sections were rinsed in PBS as above. As soon as the incubation in secondary antibody was complete, the ABC kit was removed from the fridge and 75μL each of solution A (avidin) and B (biotin) (ABC kit, Vector Laboratories) were added to 5mL of PBS and allowed to incubate for 30min. This procedure allowed an avidin/biotin complex to form; horseradish peroxidase was conjugated to the biotin portion of this complex. The slides were placed into the AB solution for 30min., and again rinsed in PBS as above. The reaction was then visualized by placing the slides in a solution containing 0.05% diaminobenzidine (Sigma) and 0.03% H_2O_2 (BDH) in PBS. Imidazole (Sigma) was added to this reaction at a concentration of 14mg/mL. The slides were removed from this solution as soon as the

sections turned brown and rinsed in distilled water. The sections were rinsed 3X in distilled water for 1min., then incubated in 5% copper sulphate (Sigma) for 5min. This was followed by 3 more 1min. rinses in distilled water, a 5min. rinse in tap water and a quick rinse in distilled water. The slides were then dipped consecutively in 70%, 80%, 95%, 100%, 100% EtOH for 2min. each, followed by a 2min. dip in xylene (BDH). The slides were then transferred to another container of xylene. The sections were then coverslipped with Permount (Fischer) reagent placed over the sections. Protocols using antibodies to trkA, IB4 and S-100 were similar to that used for c-kit immunostaining; changes are as follows: For S-100 (Dako) immunostaining, the dilution of primary antibody was 1:100, and normal swine serum was used instead of normal goat serum. In addition rabbit anti-swine IgG was used (at a dilution of 1:100) instead of biotinylated goat anti-rabbit secondary antibody. Finally, there was an additional step after the secondary antibody addition that required the addition of rabbit peroxidase anti-peroxidase (PAP) also at a dilution of 1:100. The sections were rinsed between these two steps as described above.

For IB4 lectin staining, the lectin was used at a concentration of 12.5μg/mL, and the secondary antibody was omitted since the lectin is already biotinylated. In addition, the PBS buffer contained 0.1mM each of calcium chloride, magnesium chloride and manganese chloride. The high salt concentration in the PBS buffer has been shown to significantly reduce background staining during avidin-biotin binding (Molliver *et al*, 1995).

4.1c Quantitative analysis

In order to determine the density of antibody staining, the MCID system was used. The sections were placed under the microscope and the image transferred to the MCID screen. The stained sections were then circled and the relative optical density value was provided by the MCID. For each slide analysed, the equipment was adjusted so that the background readings would be the same each time.

In order to determine the percentage of cells that were stained with antibody, the sections were stained with toluidine blue after the final step in the immunocytochemistry protocol. The slides were placed flat with the sections facing upward and toluidine blue (1%) was pipetted directly onto the slides. After 2-3min., the slides were dipped in distilled water to remove the stain, dehydrated in EtOH, rinsed in xylene and coverslipped as in section 4.1b. Four DRG sections were chosen per time point per animal for quantitation. The total number of cells as well as the number of antibody-stained cells were counted using a Zeiss microscope with a 40X objective. The percentage of stained cells was then averaged over the four sections.

5.1 Schwann cell proliferation

The following procedure was used to compare the proliferation of Schwann cells in control and c-kit mutant animals after sensory nerve axotomy. Surgery was performed on these animals to expose the DCNs as in section 2.1a. The DCNs on both sides of the animal were then cut midway between exit from the body wall and entry into the skin. The animals were then re-sutured and allowed to recover. After 4 days, the mice were re-anaesthetized

and the distal segments of the cut nerves removed and placed directly into OCT compound. Nerve sections were then cut immediately at a thickness of 15μm using a cryostat. The sections were then placed into 10% buffered formalin for 30s, followed by a quick rinse in distilled water and then quick rinses in EtOH in the following order: 70%, 80%, 95%, 100%, 100%, 95%, 80%, 70%. The sections were then rinsed in distilled water and placed into haematoxylin solution (Sigma) for 3min., followed by another rinse in distilled water and then 3 dips in Eosin (Sigma). The sections were rinsed in distilled water once again and then dipped into various percentages of EtOH in the following order: 70%, 80%, 95%, 100%, 100%. The slides were then dipped in xylene for 1min., Permount was placed onto them and coverslips were then placed over the sections. The number of stained nuclei in each nerve section was then counted under the 40X objective. Any profile that contained a large number of granules and thus resembled an immune cell, eg. macrophage or neutrophil was not counted.

An antibody to S-100 was also used for immunocytochemical analysis (section 5.1b) on sections of distal nerve segments; S-100 is a Schwann cell-specific marker (Holton and Weston, 1982). This procedure was used to confirm that the haematoxylin/eosin stained nuclei were in fact Schwann cell nuclei.

6.1 Analysis of axonal parameters

Since the size of the DRGs were significantly reduced in the $p75^{NGFR}$ mice as compared to normal DRGs, it was of interest to measure the axonal numbers and axonal

distributions. For example, there is a possibility that the Aβ fibres are atrophied in the nerves of the mutant mice, and are thus similar in appearance to the Aδ fibres. In order to perform these measurements, electron micrographs of sections of peripheral nerves were made and analysed.

6.1a Determination of axon numbers

DCNs were excised from the animals and placed in a fixative containing 2.5% glutaraldehyde, 2% paraformaldehyde, 1% dimethylsulfoxide, and 0.1M phosphate buffer (PO_4) (pH 7.2). The tissue was left in fixative overnight at 4^0C, then washed 3X over 15min. in PO_4, and stained with 4% osmium tetroxide, 1% potassium chromate and 0.1M PO_4 for 3-4hr at RT. The tissue was then washed in 0.1M sodium acetate 4X over 15min., and dehydrated by placing in the following solutions for 5min. each: acetone, 50% ethanol (EtOH), 70% EtOH, 95% EtOH, 100% EtOH, 100% EtOH. The tissue is then put in a solution of 1:1 acteone:Spurr's (10g ERL, 6g DER 736, 26g NSA, .4g DMAE {catalyst}) and placed of a rotator overnight at RT. On the following day the tissue was place in 3:1 Spurr's:acetone for 8hr. at RT, then 100% Spurr's o/n at RT, and then in fresh 100% Spurr's for another 8hr. Finally, the tissue was embedded in 100% Spurr's and baked at 60^0C o/n. Subsequently, 90nm thick sections were cut and placed in slot grids. Electron micrographs were then made and used to determine the number of axons in each nerve. In order to distinguish Aβ and Aδ fibres, myelin thickness was used as an indicator. Fibres with myelin thickness greater that 1μm were classified as Aβ, whereas fibres with a thickness equal to

or less than 1μm were classified as Aδ.

6.1b Determination of axonal areas and myelin thickness

The MCID image analysis system was used to measure the axonal areas and myelin thickness for random samples of axons from $p75^{NGFR}$ knockout and control mice. The myelin was excluded from the area measurements. In order to account for variability in myelin thickness, 4 measurements were performed in different regions of a given axon profile and averaged. A total of 215 axons from 6 animals were analysed for each group of animals.

7.1 Tests for presence of hyperalgesia

In order to determine whether the low-affinity neurotrophin receptor has a role in NGF-induced hyperalgesia, the effects of NGF administration on the response to noxious heat and mechanical stimulation was compared in the $p75^{NGFR}$ and normal mice.

Animals receiving daily injections of 2.5s NGF were tested for heat sensitivity by allowing the hindpaw of a lightly restrained animal to touch a 68-70^{0}C heat probe. The amount of time required for foot withdrawal was determined. If the animal did not respond within 10s, it was removed from the heat probe in order to avoid injury to the hindpaws. The procedure was repeated 6 times on each animal (or until three responses greater than 10sec. each were measured), and the feet were alternated each time.

The same animals were also tested for mechanical hyperalgesia. The mechanical threshold for eliciting a flexion reflex was determined using a modified von Frey hair that was capable of delivering various degrees of force when applied to the dorsum of the foot of the animal. This procedure was done once of each foot at each time point, and the two numbers were averaged.

RESULTS

I. REPARATIVE NERVE GROWTH

I.i Aδ and C nociceptive fibres

Ii.a NFHlacZ transgenics

The expression of neurofilament mRNA is known to decrease in the neuronal cell body subsequent to axonal injury (Hoffman and Cleveland, 1988; Wong and Oblinger, 1987). In contrast, expression of tubulin mRNA is known to significantly increase after axonal damage (Hoffman and Cleveland, 1988; Miller *et al*, 1989), as well as during collateral sprouting (Mathew and Miller, 1990). Since neurofilaments are not present and tubulin is overabundant in the axons of NFHlacZ transgenic mice, their ability to undergo reparative nerve growth was examined. Collateral sprouting and regeneration of the myelinated, nociceptive Aδ fibres was studied in mice transgenic for the NFHlacZ construct (Figs. 1a, 1b). The rate of Aδ fibre collateral sprouting was measured to be 4.6±0.7 mm^2/day (5) and 5.5±0.4 mm^2/day (11) in the transgenic and control (+/+) animals respectively. On the other hand, the rate of Aδ fibre regeneration was 7.6±0.4 mm^2/day (7) in the transgenic mice, and 8.0±0.3 mm^2/day (4) in the control mice. Both forms of nerve growth occurred in the

transgenic mice at rates that were not statistically different from that observed in control animals p=0.31 for collateral sprouting, p=0.58 for regeneration. In conclusion, both collateral sprouting and regeneration of sensory nerves is normal in NFHlacZ transgenic mice. Therefore, lack of neurofilament and elevated tubulin levels appear to be compatible with the changes that are necessary for successful reparative nerve growth.

Ii.b Steel and c-Kit mutants

Since Steel and c-Kit are expressed in the DRG (Motro *et al*, 1991), and in neuronal accessory cells of the adult mouse (Su and Federoff, 1995), there was a possibility that these molecules have a role in reparative growth of adult sensory fibres. In order to test for this possibility, both collateral sprouting and regeneration were studied in Sl/Sl^d and in W/W^v mutant mice (Figs. 2.1a,b). Examination of the ability of adult sensory fibres to undergo regeneration provided very interesting results. The rate of regeneration of the Aδ fibres was considerably reduced in both the Sl/Sl^d and W/W^v mutant animals, and was about half of that seen in +/+ animals. These rates were 4.4±0.8 mm^2/day (9) and 4.8±0.7 mm^2/day (7) for the Sl and c-kit mutants, significantly less (p=0.005, p=0.006) than in the control animals (9.8±0.9 mm^2/day (12)).

The rate of regeneration of the small, unmyelinated, heat-nociceptive C fibres was measured in the W/W^v mutant mice and compared to that observed in control animals. This rate was also significantly reduced in the W/W^v animals relative to control mice (p=0.04), but not to the same extent as that observed for Aδ fibre regeneration. The rate of

but not to the same extent as that observed for Aδ fibre regeneration. The rate of regeneration of these small, unmyelinated fibres was 2.6±0.4mm^2/day (4) in the mutant mice, and 3.8±0.5 mm^2/day (6) in the control animals. Thus, regeneration in the W/W^v mice occurred at a rate that was 68% of that observed in the control mice. Therefore, mice that have mutations in the gene coding for Steel or its tyrosine kinase receptor c-Kit have a significantly reduced ability to undergo regeneration of their sensory cutaneous fibres relative to normal control mice.

The rate of collateral sprouting of undamaged axons into adjacent denervated territory was also measured in Sl/Sl^d and W/W^v mice (Figs. 2.2a,b). The rate of Aδ fibre collateral sprouting was determined to be 5.0±0.5mm^2/day (15) in the control mice, as compared to 4.4±0.5 mm^2/day (6) and 3.9±0.5 mm^2/day± (8) in the Sl/Sl^d and W/W^v mutants, respectively. The values measured in the mutant mice were not statistically different from that observed in the control animals (p=0.9 for the Sl/Sl^d mice, p=0.08 for the W/W^v mice). In addition, C fibre collateral sprouting was analysed in the W/W^v mutant mice and compared to that observed in control animals. The rate of collateral sprouting of the heat-nociceptive C fibres was measured to be 3.1±0.2 (8) in the mutant animals, and 2.6±0.2 (8) in the control animals. These values are not statistically different from each other (p=0.3).

In conclusion, the data indicate that after nerve crush, the nociceptive Aδ and C fibres in mutant steel/c-kit animals have a significantly reduced ability to undergo regeneration relative to nociceptive fibres in wild-type animals. However, the steel and c-kit mutations do not affect the rate of collateral sprouting of sensory cutaneous fibres. Therefore, these

mutations are selectively able to influence one form of reparative nerve growth in adult mice.

Ii.c $p75^{NGFR}$ mutants

Expression levels of the $p75^{NGFR}$ low-affinity neurotrophin receptor increase in DRG neurons whose axons are undergoing collateral sprouting, an NGF-dependent process. On the other hand, there is no change in receptor expression during regeneration. The latter is known to be an NGF-independent form of reparative nerve growth (Diamond *et al*, 1992a,b; Mearow *et al*, 1993). Based on these findings, $p75^{NGFR}$ may play a role in NGF-dependent collateral sprouting. Therefore, this possibility was examined in mice that have a null mutation in the gene coding for $p75^{NGFR}$ (Lee *et al*, 1992). The rates of collateral sprouting and regeneration were measured in these mutant mice and compared to the rates observed in +/+ animals.

Examination of collateral sprouting in these knockout animals provided surprising results. There was no evidence of collateral sprouting on Aδ (6) or C (6) fibres in the $p75^{NGFR}$ knockout mice (Figs.3.1a,b). In order to determine whether the initiation of this process was merely delayed, animals were allowed to undergo the collateral sprouting paradigm for a period of 24 days. Measurement of the territories of innervation of DCN T13 at this time indicated that there was no change relative to the values obtained subsequent to field isolation. Therefore, collateral sprouting had not occurred in these animals, even after a substantial period of time. As a comparison, the rates of collateral sprouting of Aδ and C

fibres in the control mice were 3.6±0.4 mm^2/day (9) and 2.4±0.4 mm^2/day (9), respectively.

The rate of regeneration of Aδ and C fibres was also measured in the knockout mice and compared to the rates observed in +/+ animals. The rates of Aδ fibre regeneration were 7.6±0.9 mm^2/day (7) and 7.2±0.9 mm^2/day (9) for the mutant and control animals, respectively. These values were not statistically significant from each other (p=0.8). In addition, the rate of regeneration of the heat-nociceptive C fibres was measured to be 4.7±0.6 mm^2/day (7) in the $p75^{NGFR}$ mice and 4.0±0.9 mm^2/day (9) in the control animals. These values were also not statistically different from each other (p=0.6).

Both collateral sprouting and regeneration of the small, unmyelinated C fibres occurred at a slower rate relative to the small, myelinated Aδ fibres in all of the mice studied. This has been previously observed in the rat (Diamond *et al*, 1992a,b) where the rate of growth of both injured regenerating nerves and uninjured collaterally sprouting Aδ fibres always exceeded the rate of C fibre growth. Therefore, the pinch-nociceptive Aδ fibres are able to undergo reparative growth at a faster rate than that observed for the heat-nociceptive C fibre population.

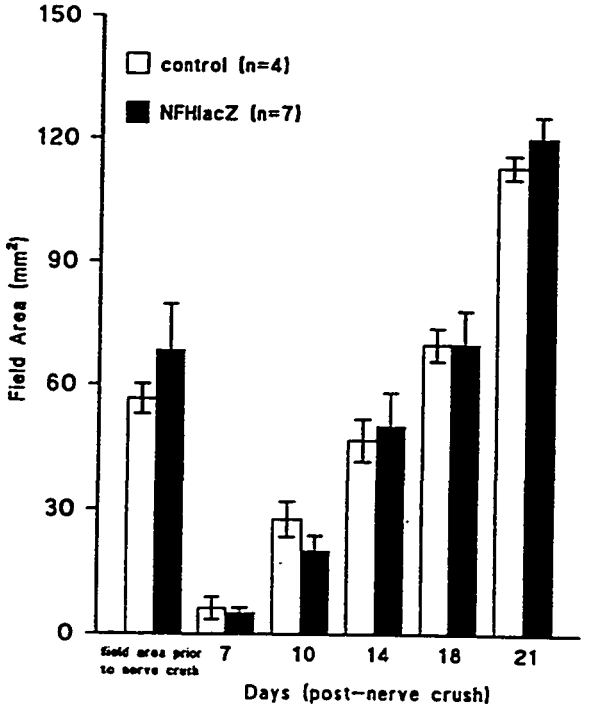
In order to determine whether the low-affinity neurotrophin receptor functions in the establishment of the innervation territories of sensory nerves, the initial areas innervated by DCN T13 were compared in +/+ and mutant mice. It initially appeared that the innervation territory of DCN T13 was smaller in the $p75^{NGFR}$ animals (34.2±3.7mm^2 for the Aδ fibres, 21.3±2.2 mm^2 for the C fibres) relative to control mice (70.2±6.5 mm^2 for the Aδ fibres, 55.4±4.7 mm^2 for the C fibres), however, the transgenic animals used in these experiments

were smaller in size than the control mice (11-14g vs. 17-22g). Thus, in order to determine whether the innervation territories were proportional to the weight of the animal, mice of comparable weights were examined (17-22g). This analysis indicated that the area innervated by the Aδ and C fibres comprising DCN T13 was similar in mutant and control mice (Fig. 3.3). The initial areas innervated by the Aδ fibres was 70.2 ±6.5 mm^2/day (9) in the control animals and 55.4±4.7 (14) in the mutant mice. The initial innervation territories of the C fibres was 36.0±3.2 (9) and 33.6±3.9 (14) in the control and knockout mice, respectively. The initial areas of target tissue innervated by DCN T13 in the mutant mice were not statistically different from the areas measured in the control animals ($p=0.09$ for the Aδ fibre; $p=0.64$ for the C fibres). In conclusion, the $p75^{NGFR}$ receptor does not appear to play a role in the establishment of innervation territories of cutaneous sensory fibres.

Fig. 1 Rates of regeneration and collateral sprouting in NFHlacZ and control mice.

Reparative nerve growth of cutaneous sensory fibres was studied in the NFHlacZ mice. These animals overexpress a fusion construct of the heavy chain neurofilament linked to the gene coding for β-galactosidase in their nervous systems. The rates of regeneration (a) and collateral sprouting (b) of Aδ fibres were measured in these animals and compared to that observed in +/+ mice. Both forms of reparative nerve growth occurred at a rate similar to that observed in control animals ($p=0.6$, $p=0.3$ for the regeneration and collateral sprouting data, respectively).

a


control (n=4)
NFHlacZ (n=7)
Field Area (mm²)
150
120
90
60
30
0
field area prior to nerve crush
7
10
14
18
21
Days (post-nerve crush)

b

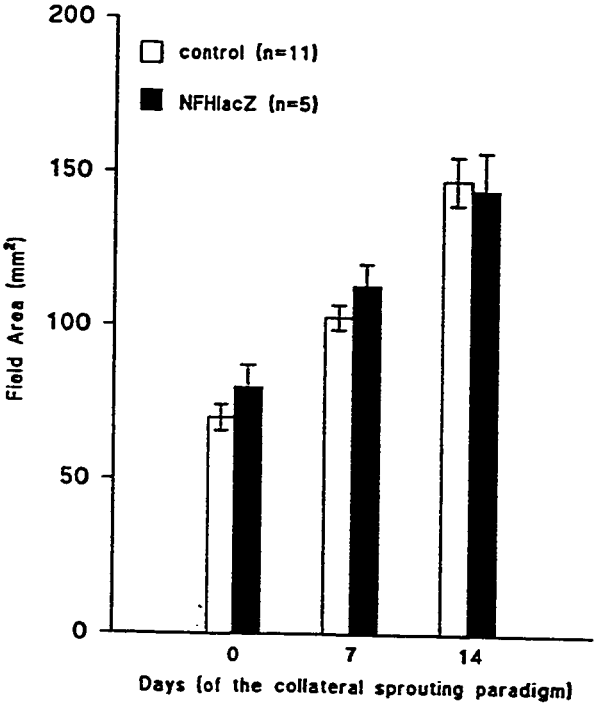
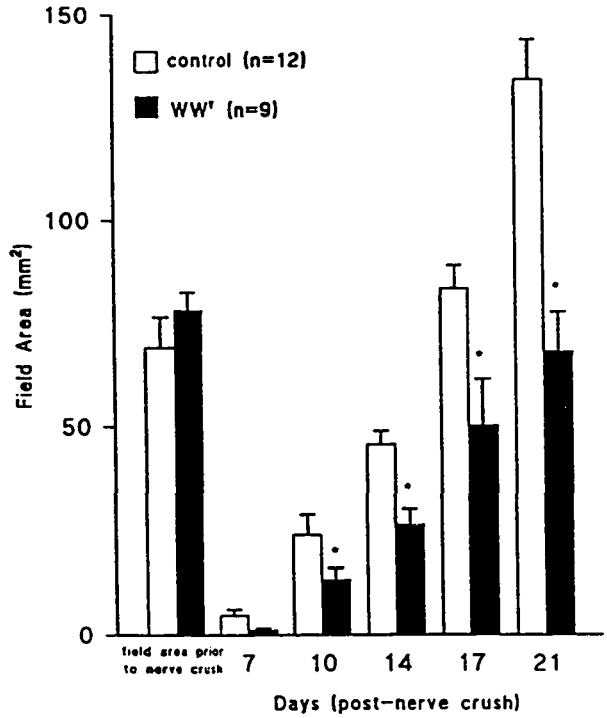
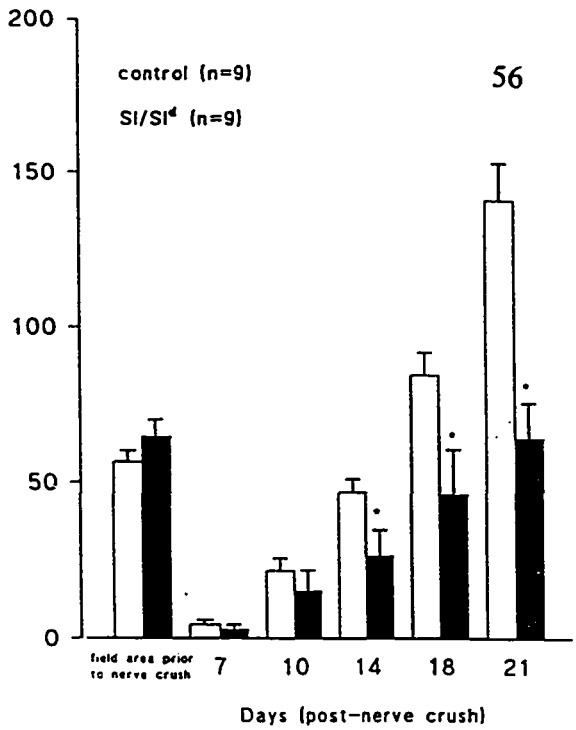

control (n=11)
NFHlacZ (n=5)
Field Area (mm²)
200
150
100
50
0
0
7
14
Days (of the collateral sprouting paradigm)

Fig 2.1 Regeneration of Aδ and C fibres in Sl/Sl^d and W/W^v mutant and control mice

The rate of regeneration of cutaneous sensory Aδ (a) was measured in Sl/Sl^d and W/W^v mutant mice and compared to the rates observed in control animals. The Sl/Sl^d mice have a mutation in the gene coding for steel factor; the W/W^v mutant mice have a defect in the gene coding for the tyrosine kinase receptor c-kit. Regeneration of the Aδ fibres was significantly reduced in both mutant mice relative to control values ($p=0.005$ for the Sl/Sl^d mice, and $p=0.06$ for the W/W^v mice). Furthermore, the rate of regeneration of the heat-nociceptive C fibres was studied in the W/W^v mutant mice (b). This rate was significantly reduced relative to that observed in control animals ($p=0.3$).

a


150
100
50
0
Field Area (mm²)
control (n=12)
WW^v (n=9)
field area prior to nerve crush
7
10
14
17
21
Days (post-nerve crush)


200
150
100
50
0
control (n=9)
Sl/Sl^d (n=9)
field area prior to nerve crush
7
10
14
18
21
Days (post-nerve crush)

b

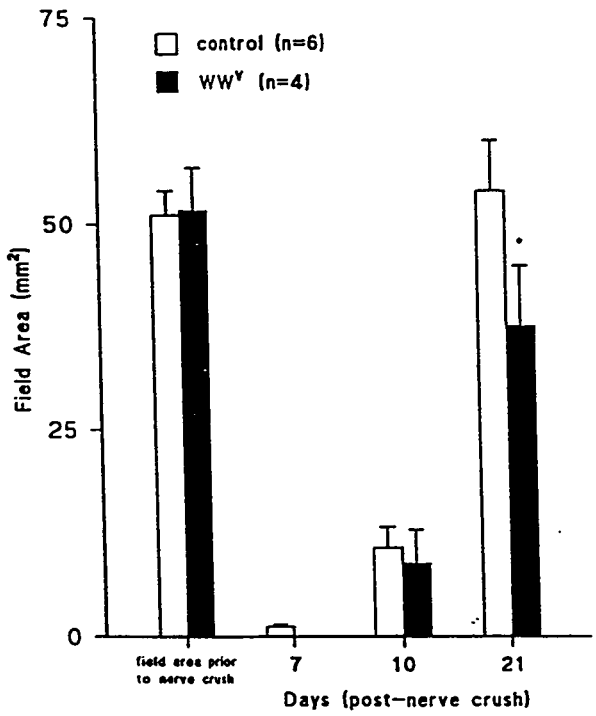
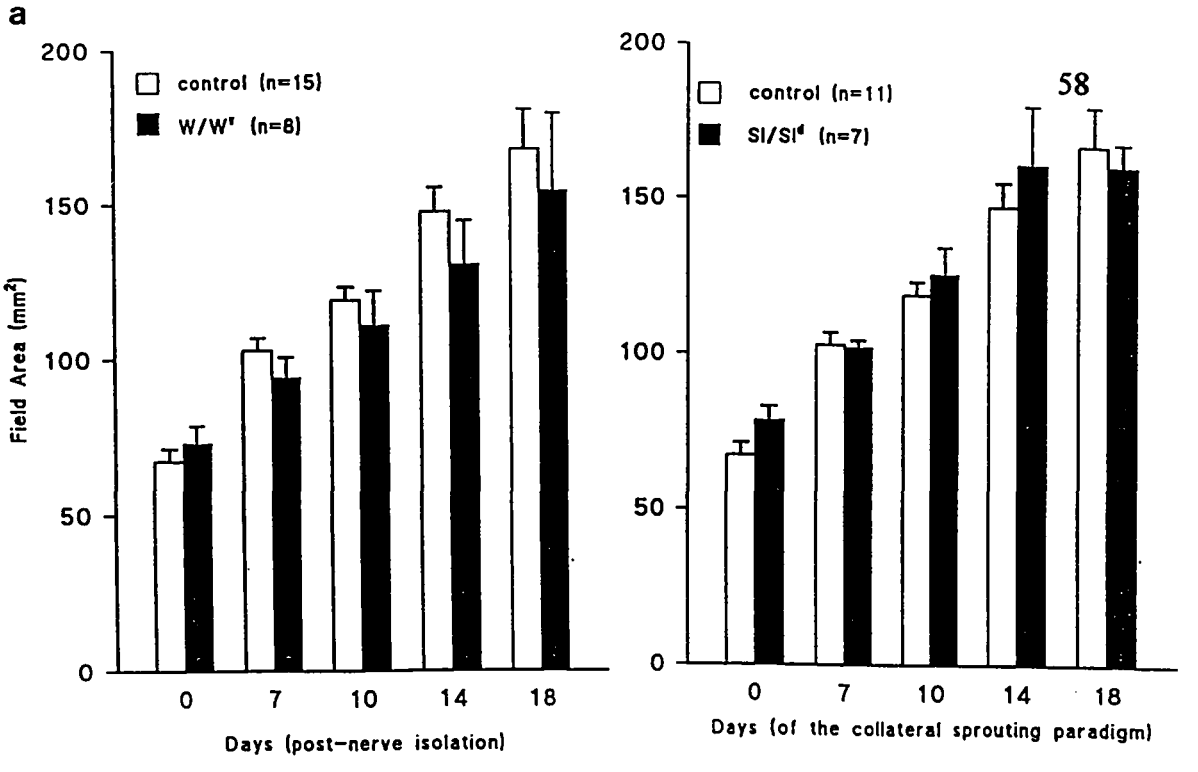

75
50
25
0
Field Area (mm²)
control (n=6)
WW^v (n=4)
field area prior to nerve crush
7
10
21
Days (post-nerve crush)

Fig. 2.2 Collateral sprouting of Aδ and C fibres in Sl/Sl^d and W/W^v mutant and control mice

The rate of collateral sprouting of cutaneous sensory Aδ fibres (a) was measured in Sl/Sl^d and W/W^v mutant mice and compared to that observed in +/+ mice. The rate of collateral sprouting was similar to that observed in control animals for both types of mutant mice ($p=0.9$ for the Sl/Sl^d mice, and $p=0.08$ for the W/W^v mice). The rate of collateral sprouting of the heat-nociceptive C fibres (b) was measured in the W/W^v mice as well as in +/+ mice. The rates of collateral sprouting of these unmyelinated fibres were not statistically different in these two types of mice ($p=0.3$).


a
200
150
100
50
0
Field Area (mm²)
control (n=15)
W/W⁵ (n=8)
0
7
10
14
18
Days (post-nerve isolation)
200
150
100
50
0
control (n=11)
Sl/Sl⁴ (n=7)
0
7
10
14
18
Days (of the collateral sprouting paradigm)

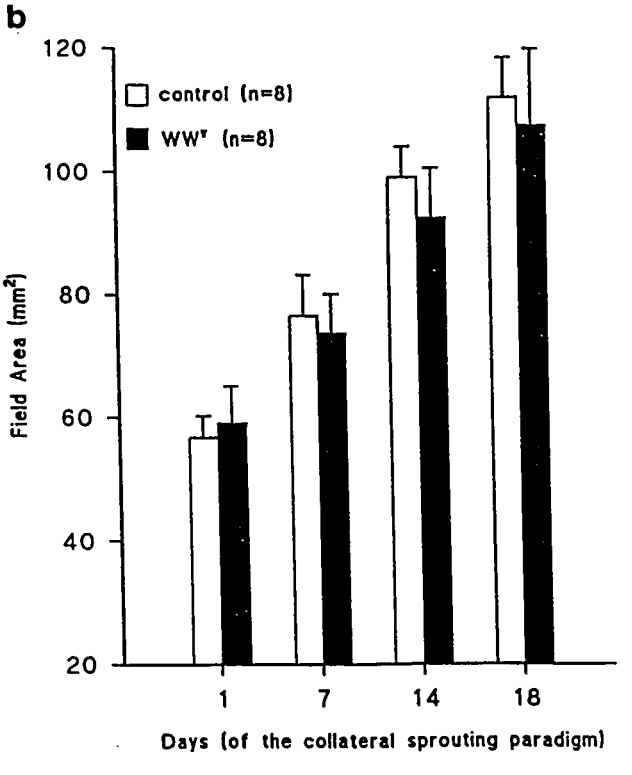
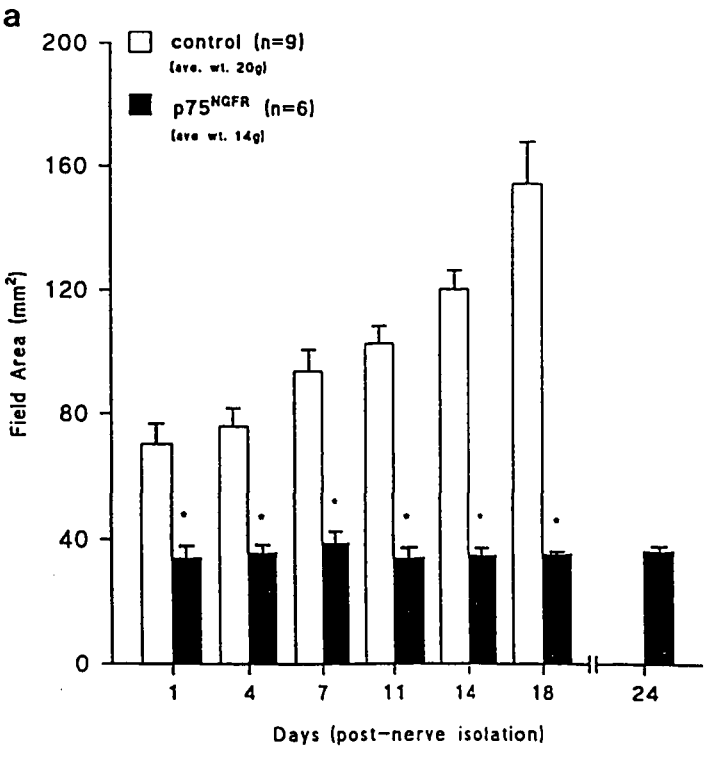

b
120
100
80
60
40
20
Field Area (mm²)
control (n=8)
WW⁵ (n=8)
1
7
14
18
Days (of the collateral sprouting paradigm)

Fig. 3.1 Collateral sprouting of cutaneous sensory fibres in $p75^{NGFR}$ and control mice.

Reparative nerve growth was analysed in mice carrying a null mutation in the gene coding for the low-affinity neurotrophin receptor. The rate of collateral sprouting of the Aδ (a) and C fibres (b) in DCN T13 was measured in these $p75^{NGFR}$ mice as well as in control animals. Collateral sprouting of the myelinated nociceptive Aδ fibres occurred at a rate of 3.6±0.4 mm^2/day in the control mice. This form of reparative growth of the unmyelinated nociceptive C fibres occurred at a rate of 2.3±0.4 mm^2/day in the control animals. However, there was no evidence of collateral sprouting of either fibre type in the $p75^{NGFR}$ mice, even as long as 24 days after target field isolation.


a
control (n=9)
(ave. wt. 20g)
p75NGFR (n=6)
(ave wt. 14g)
Field Area (mm2)
200
160
120
80
40
0
1
4
7
11
14
18
24
Days (post-nerve isolation)

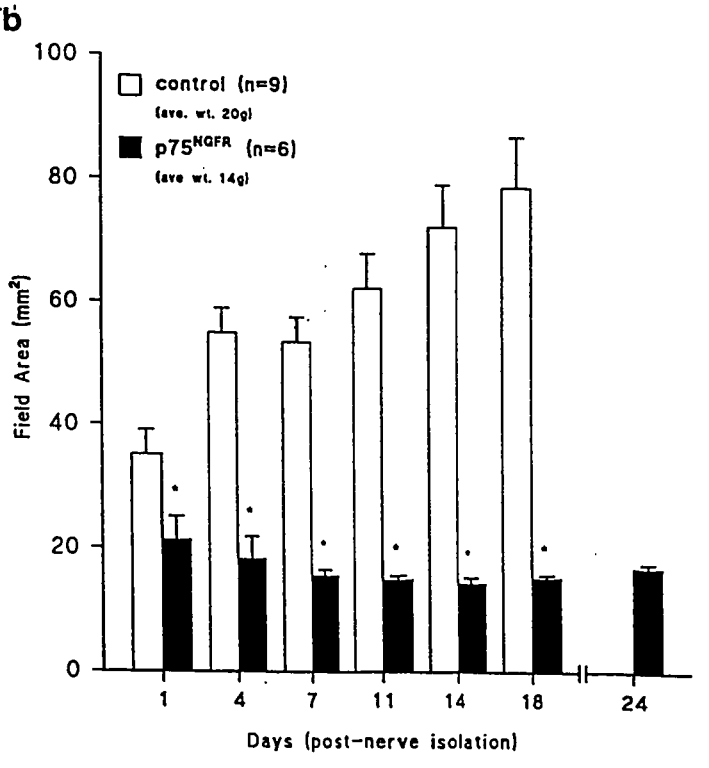
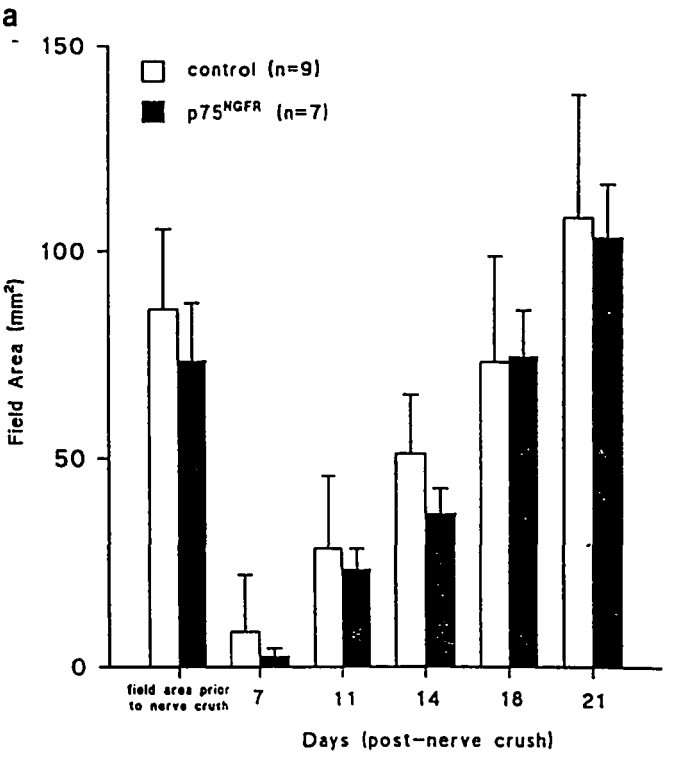

b
control (n=9)
(ave. wt. 20g)
p75NGFR (n=6)
(ave wt. 14g)
Field Area (mm2)
100
80
60
40
20
0
1
4
7
11
14
18
24
Days (post-nerve isolation)

Fig. 3.2 Regeneration of cutaneous sensory fibres in $p75^{NGFR}$ and control mice.

The rate of regeneration of Aδ (a) and C fibres (b) comprising DCN T13 was measured in $p75^{NGFR}$ mice and in control animals. This form of reparative nerve growth occurred at similar rates in both types of mice ($p=0.8$ for the Aδ fibres, and $p=0.6$ for the C fibres).


a
150
100
50
0
control (n=9)
p75NGFR (n=7)
Field Area (mm²)
field area prior to nerve crush
7
11
14
18
21
Days (post-nerve crush)

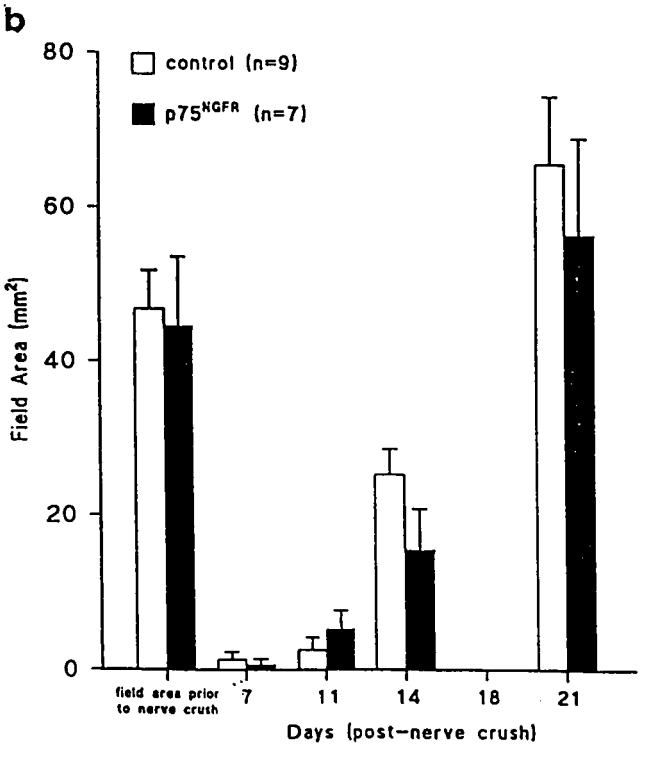
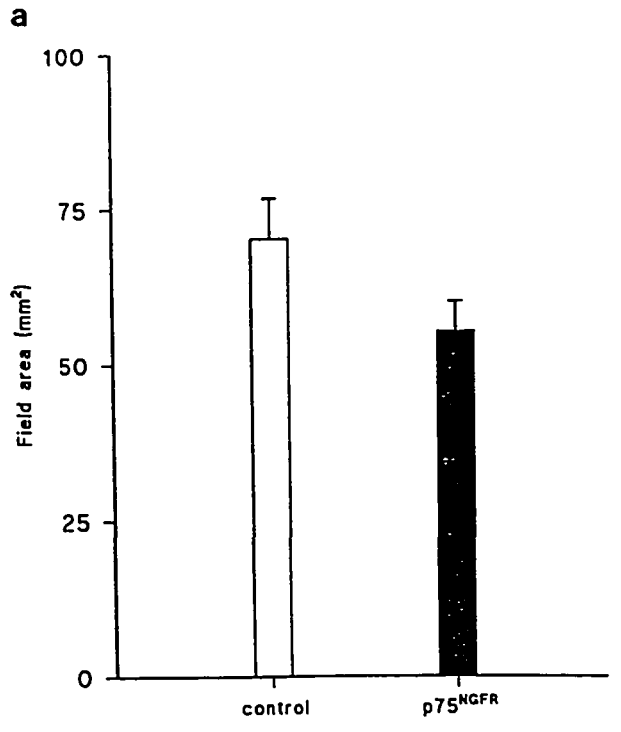
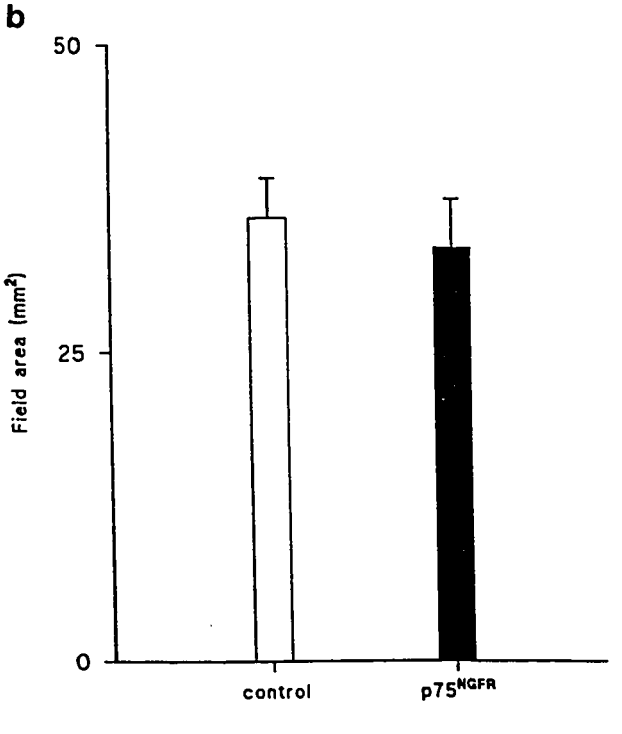

b
80
60
40
20
0
control (n=9)
p75NGFR (n=7)
Field Area (mm²)
field area prior to nerve crush
7
11
14
18
21
Days (post-nerve crush)

Fig. 3.3 Target areas innervated by Aδ and C fibres comprising DCN T13 in $p75^{NGFR}$ and control mice.

The area innervated by DCN T13 prior to reparative growth was measured in $p75^{NGFR}$ and control mice of similar weights. The area innervated by the Aδ fibres comprising T13 was similar in control and mutant mice ($p=0.09$). Similarly, the area innervated by the C fibres comprising T13 was not statistically different in the control vs. the mutant animals ($p=0.6$).


a
100
75
50
25
0
Field area (mm²)
control
p75NGFR


b
50
25
0
Field area (mm²)
control
p75NGFR

I.ii Aβ mechanosensory fibres

Regeneration of the large myelinated Aα/β fibres was measured in the c-kit mutant mice to determine whether the defect in this reparative growth of nociceptive fibres would extend to these low-threshold mechanosensory fibres. In fact, the area innervated by these fibres was significantly reduced on Day 12 post-nerve crush as compared to that measured in the control animals ($p=0.01$), (Fig. 4). Since the area of innervation was only measured at one time point during the regeneration of these fibres, it was not possible to calculate the rate of this form of reparative growth in mm^2/day. However, a comparison of the areas innervated by these fibres on Day 12 of the regeneration paradigm indicates that this growth was significantly reduced in the c-kit mutant mice; the area innervated by DCN T13 on Day 12 of the regeneration paradigm was 61± % of that measured in the control animals at the same time point.

I.iii Effects of NGF on collateral sprouting

The collateral sprouting of cutaneous sensory nociceptive fibres is known to be entirely dependent on the presence of NGF (Diamond *et al*, 1992a). There was no detectable collateral sprouting of undamaged sensory fibres in the $p75^{NGFR}$ mice, suggesting that the levels of endogenous NGF was insufficient for this reparative growth to occur in the mutant

animals. In order to determine whether administration of NGF to these animals would allow collateral sprouting to occur, mice were given daily injections of either 1or 2.5 μg/g of NGF. The ability of exogenous NGF to induce collateral sprouting in the $p75^{NGFR}$ mice was then examined. In addition, it was of interest to determine whether exogenous NGF could effect the rate of collateral sprouting in mice similar to that previously observed in rats (Diamond *et al*, 1992a). Therefore, control animals were also given daily injections of NGF and the effect on the rate of collateral sprouting was measured. The results of exogenous NGF administration on the rate of collateral sprouting of both knockout and control animals are summarized in Table 1.

The rate of collateral sprouting in control animals was increased upon daily administration of both 1 and 2.5μg/g of NGF. The rate of Aδ fibre collateral sprouting was measured to be 5.4±0.8 and 7.0±1.3 mm^2/day in animals receiving 1 and 2.5μg/g, respectively (Fig. 5a). Also, collateral sprouting of C fibres occurred at a rate of 3.2±0.9mm^2/day in animals receiving 1μg/g NGF, and at a rate of 4.4±0.8mm^2 /day in animals receiving 2.5μg/g of NGF (Fig. 5b).

Daily administration of 2.5μg/g of 2.5s NGF resulted in the onset of both Aδ and C fibre collateral sprouting in the $p75^{NGFR}$ mice (Fig. 5a,b). In fact, administration of the higher dose of NGF resulted in Aδ fibre collateral sprouting in the mutant mice at a similar rate observed in +/+ mice that did not receive NGF. Aδ fibre collateral sprouting occurred at a rate of 3.4±0.8 mm^2/day in the mutant mice receiving this dose of NGF. This value is not statistically different from such rates observed in control animals receiving 1 μg/g of

NGF daily ($p=0.06$), but is statistically different from the rates observed in control animals that did not receive NGF. The rate of collateral sprouting of C fibres in these animals was determined to be 2.3 ± 0.4 mm^2/day. This rate is similar to that observed in control mice that have not received exogenous NGF ($p=0.5$), and is statistically different from the rate observed in mutant mice that did not receive NGF ($p=0.04$).

In conclusion, exogenous administration of NGF at a dose of 2.5μg/g daily results in the onset of collateral sprouting of Aδ and C fibres in $p75^{NGFR}$ mice.

Fig. 4 Regeneration of Aβ mechanosensory fibres in W/W^v and control mice.

Since the rate of regeneration of the nociceptive sensory fibres was impaired in the Sl/Sl^d and W/W^v mice, the extent of regeneration of the large, myelinated Aβ mechanosensory fibres was also examined. The area innervated by the Aβ fibres comprising T13 prior to nerve crush and again 12 days after nerve crush was determined in the W/W^v mutant mice and compared to that measured in the +/+ animals. The extent of regeneration of the Aβ fibres 12 days after nerve crush was significantly less in the mutant mice relative to that measured in the control animals ($p=0.01$).

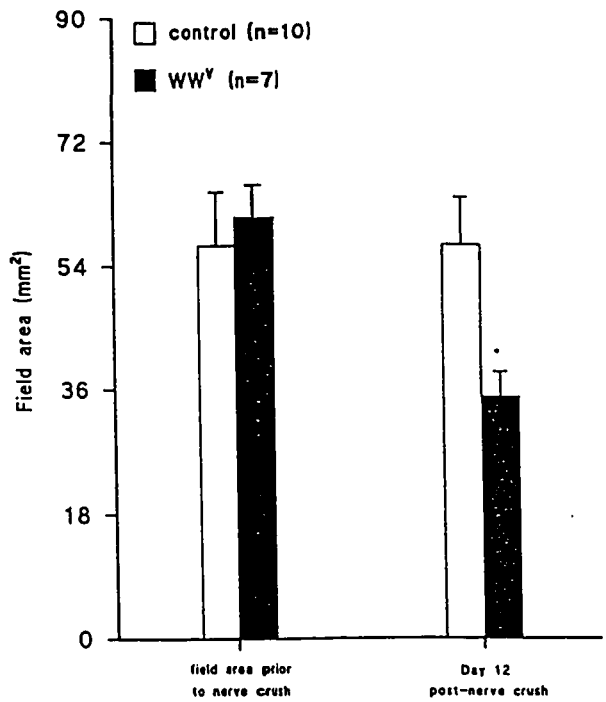
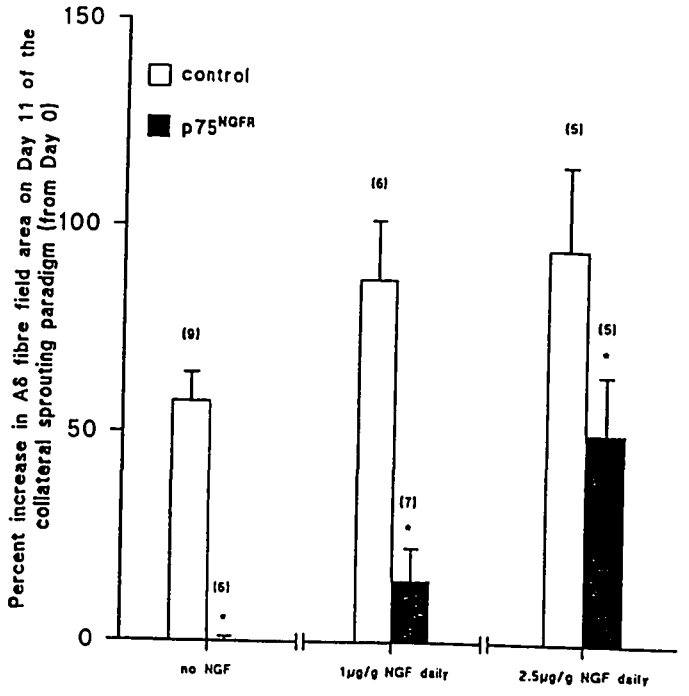

90
72
54
36
18
0
control (n=10)
WW^v (n=7)
Field area (mm^2)
*
field area prior to nerve crush
Day 12 post-nerve crush

Fig. 5 Effect of exogenous NGF on the rate of collateral sprouting in $p75^{NGFR}$ and control mice.

In order to determine whether exogenous NGF would affect the rate of collateral sprouting in control animals and allow this reparative growth to occur in $p75^{NGFR}$ mice, this neurotrophic factor was administered to control and knockout mice at a dose of either 1 or 2.5μg/g daily. The effects of these doses on the rate of collateral sprouting of Aδ (a) and C (b) fibres was then monitored over an 11 day period. The rate of collateral sprouting of both fibre types was increased with administration of either 1 or 2.5 μg/g of NGF daily to control animals. In addition, collateral sprouting of nociceptive fibres in the knockout animals was evident with daily administration of the higher dose of NGF.

a


150
100
50
0
control
p75NGFR
Percent increase in Aδ fibre field area on Day 11 of the collateral sprouting paradigm (from Day 0)
(9)
(6)
(6)
(7)
(5)
(5)
no NGF
1μg/g NGF daily
2.5μg/g NGF daily

b

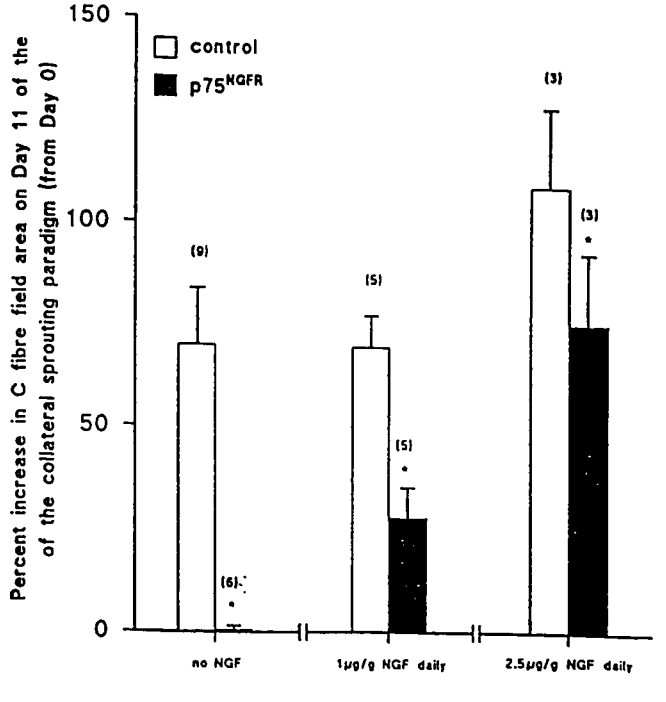

150
100
50
0
control
p75NGFR
Percent increase in C fibre field area on Day 11 of the of the collateral sprouting paradigm (from Day 0)
(9)
(6)
(5)
(5)
(3)
(3)
no NGF
1μg/g NGF daily
2.5μg/g NGF daily

Table 1. Effect of exogenous NGF on the rate of collateral sprouting of nociceptive fibres in control and $p75^{NGFR}$ mice.

Amount of exogenous NGF	Control mice		$p75^{NGFR}$ mice	
	Aδ	C	Aδ	C
0 μg/g daily	3.6±0.4	2.3±0.4	0.0±0.3	-0.5±0.3
1 μg/g daily	5.4±0.8	3.2±0.9	1.1±0.5	0.7±0.3
2.5 μg/g daily	7.0±0.3	4.4±0.8	3.4±0.8	2.3±0.4

II CELL BODY RESPONSE TO DAMAGE

II.i C-kit expression in normal mice during reparative nerve growth

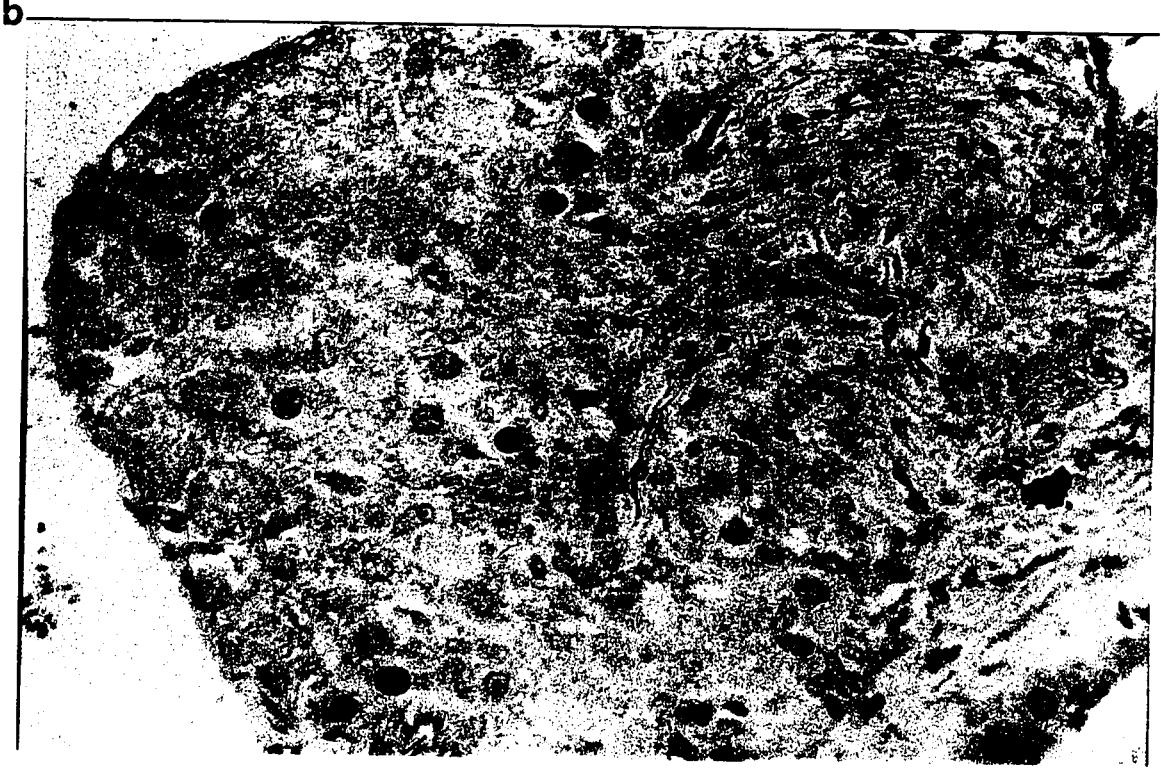
Steel factor and c-kit are both expressed in the DRG of the adult mouse (Motro *et al*, 1993). Since regeneration is impaired in animals defective in the gene coding for Steel or c-Kit, the possibility that there is an alteration in c-Kit protein levels in the DRGs of a normal animal during this reparative nerve growth was examined. There was an upregulation of c-Kit protein levels in control animals during regeneration of cutaneous sensory fibres. The upregulation was observed in the DRG as early as 1 day post-nerve crush and continued to 21 days post-nerve crush. There was an increase in both the percentage of cells stained, and the intensity of staining (Fig. 6.b,d) in the DRG.

The rate of collateral sprouting of cutaneous sensory fibres was similar in mutant and control animals. As a comparison, the levels of c-Kit protein were also measured in the DRG during collateral sprouting of a cutaneous nerve. As shown in Fig. 6c, there is no change in c-Kit protein levels during collateral sprouting in the DRG.

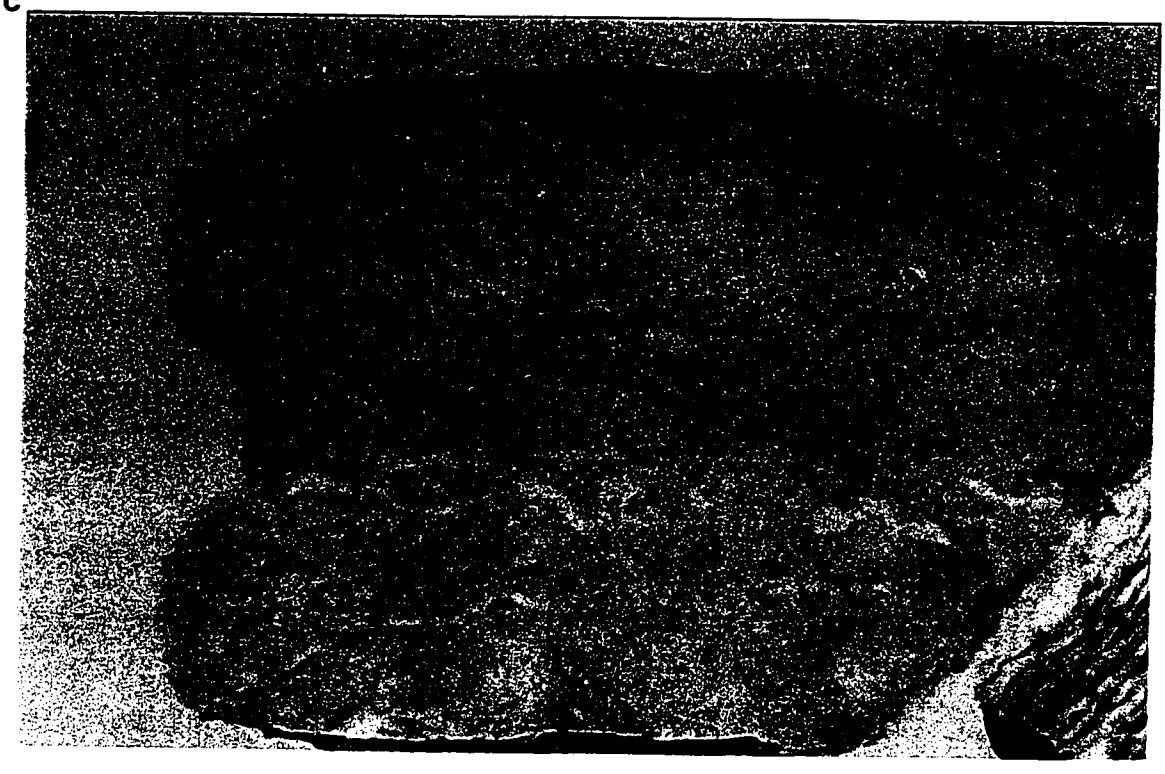
Fig. 6 C-kit protein expression in the DRG of normal mice during reparative nerve growth. Expression of c-kit protein in the DRG during reparative nerve growth was studied in normal mice. The illustrations are of DRG sections after immunostaining for c-Kit protein: (a) normal DRG (b) DRG 14 post-crush peripheral axons (c) DRG after 14 days of the collateral sprouting paradigm. The intensity of c-kit staining at various times post-nerve crush was measured as described in section 4.1c of the materials and methods (d). Magnification of photomicrographs, x290.

a

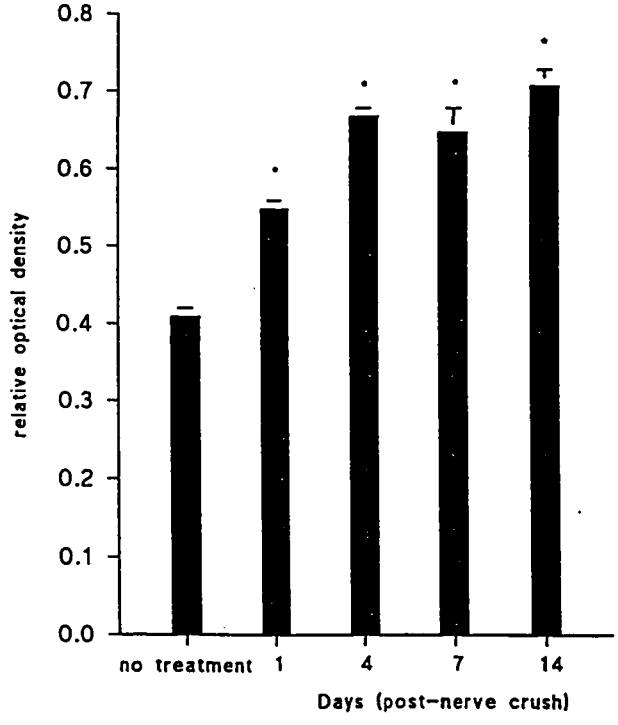
b



c



d


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
relative optical density
no treatment
1
4
7
14
Days (post-nerve crush)

II.ii trkA protein expression in the DRGs of $p75^{NGFR}$ mice

Since the DRGs of $p75^{NGFR}$ mice are significantly smaller than the DRGs of +/+ animals (Lee *et al*, 1992), it was of interest to determine whether there would be proportional decreases in specific subtypes of neurons. The DRGs of mutant mice were analysed for trkA protein expression and compared to that observed in control animals. The percentage of trkA positive cells in the control animals was 45±5%, and 42±3% in the mutant mice. These values indicate that there is a similar proportion of trkA positive cells in both groups of mice ($p>0.05$) (Fig. 7a,b).

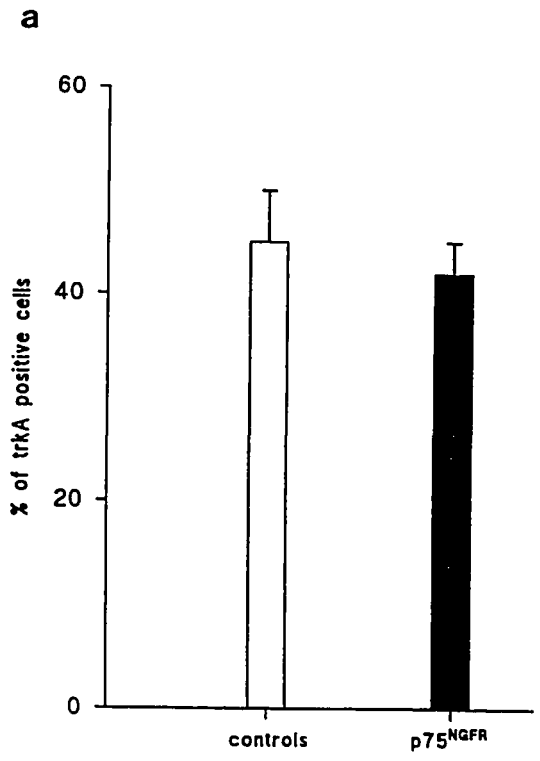
trkA protein expression increases in the DRG during collateral sprouting in rats (Mearow *et al*, 1994). In order to determine whether a similar change would occur in the control and $p75^{NGFR}$ mice, DRGs were removed from these animals on Days 0 and 12 of the collateral sprouting paradigm. Immunocytochemistry was performed on sections of DRG using a trkA antibody (L. Reichardt). The density of trkA staining on Day 12 of the collateral sprouting paradigm was increased by 66% in the control (f) DRGs relative to Day 0 (a) ($p<0.05$), and by 59% in the mutant (g) DRGs relative to Day 0 (b) ($p<0.05$). The values on Day 12 are not statistically significant from each other. Thus, there appears to be a similar increase intensity of staining in both groups of mice on Day 12 of the collateral sprouting paradigm. The intensity of trkA staining on Day 12 of the collateral sprouting paradigm appears to be similar to that observed in DRGs from mice that received 2.5μg/g of NGF daily for 11days (h,i).

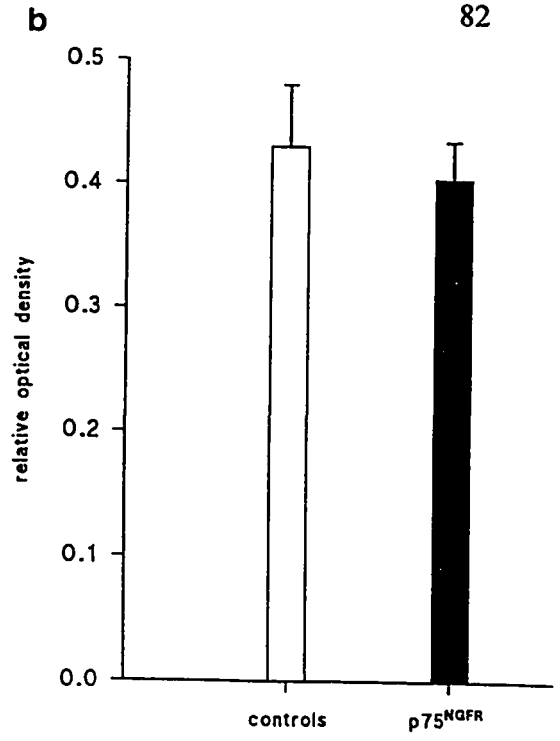
The IB4 lectin selectively binds to DRG neurons that are non-trkA positive (Averill

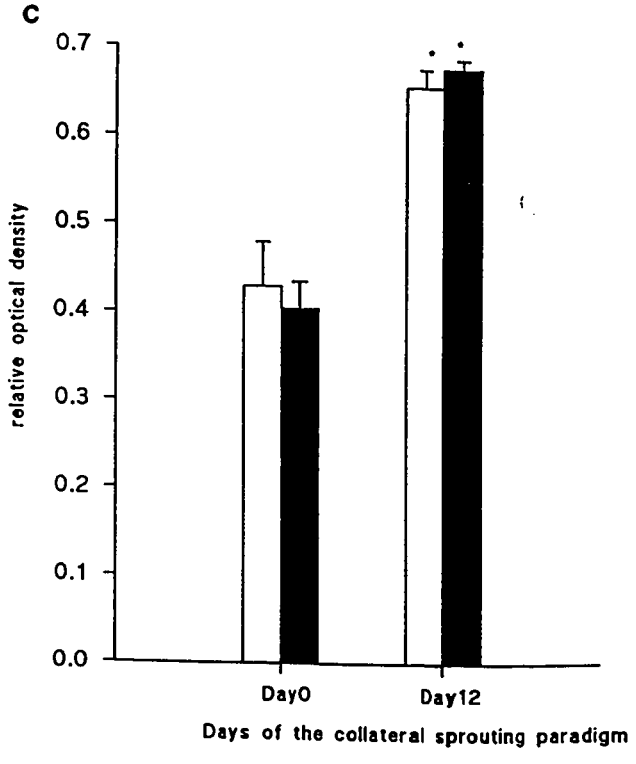
et al, 1995), and is another subtype of small DRG neurons. This compound was used to label this cell population in both the in $p75^{NGFR}$ and +/+ mice. The proportion of IB4 stained cells appears to be similar in both groups of mice (Fig. 8a). In the control DRGs, 30±3% of DRG neurons are IB4 lectin positive, and in the mutant animals, 27±4% of DRG neurons bind to this lectin. However, the density of IB4 lectin staining is significantly increased by 36% in the mutant animals relative to that measured in control mice (Fig. 8b). This suggests that DRG neurons from mutant mice express higher levels of the IB4 lectin than control cells.

Fig. 7 TrkA protein expression in the DRG of $p75^{NGFR}$ and control animals.

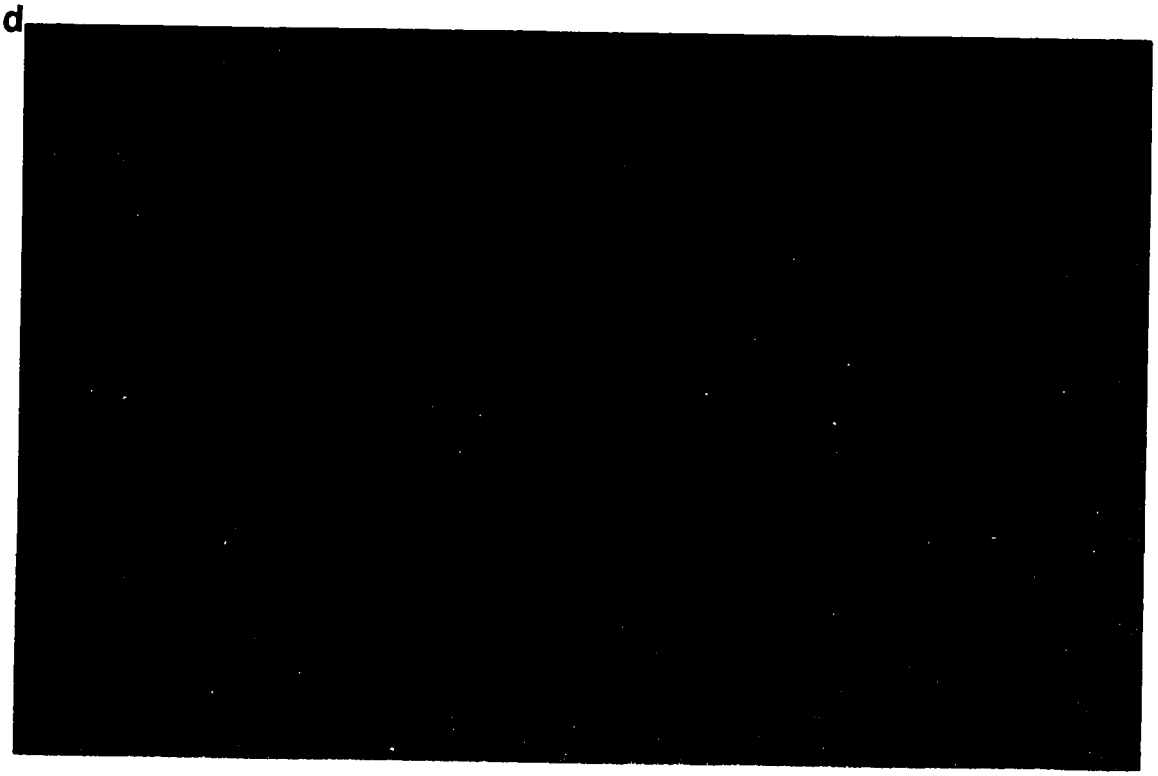
Immunocytochemistry for the high-affinity neurotrophin receptor trkA was performed in $p75^{NGFR}$ and +/+ mice. Fig. 7 shows the results of immunocytochemistry using the trkA antibody on 10μm DRG sections from both control (a) and mutant (b) mice. The proportion of trkA immunoreactive cells was determined and is shown in Fig. 7c. This value is similar in both the knockout and control animals. The density of trkA staining in these DRG sections was also measured using the MCID Image Analysis System and the results are shown in Fig. 7d. The average intensity of trkA immunoreactivity was similar in both types of mice. The intensity of trkA staining in the DRG was increased during the collateral sprouting paradigm in both control (e,f) and mutant animals (e,g) as well as after 11 days of daily injections of NGF in control (h) and mutant (i) animals. Photomicrographs; x740.


a
60
40
20
0
% of trkA positive cells
controls
p75NGFR

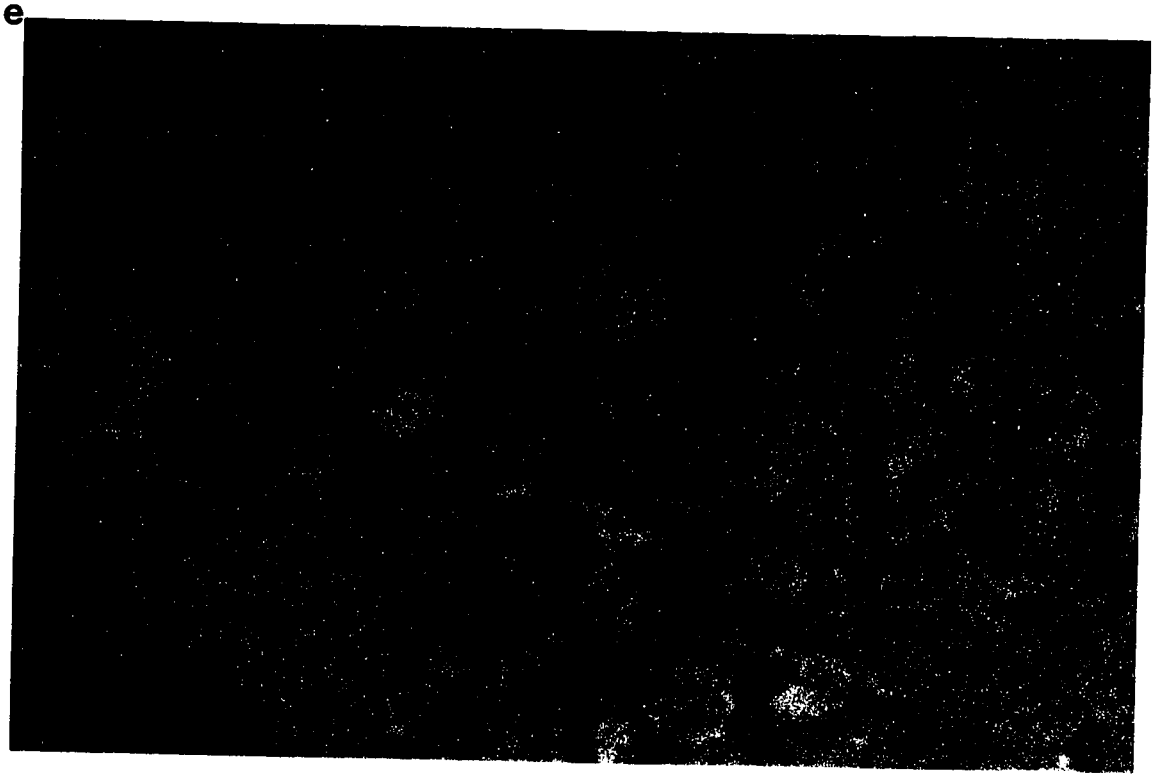

b
0.5
0.4
0.3
0.2
0.1
0.0
relative optical density
controls
p75NGFR


c
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
relative optical density
Day0
Day12
Days of the collateral sprouting paradigm

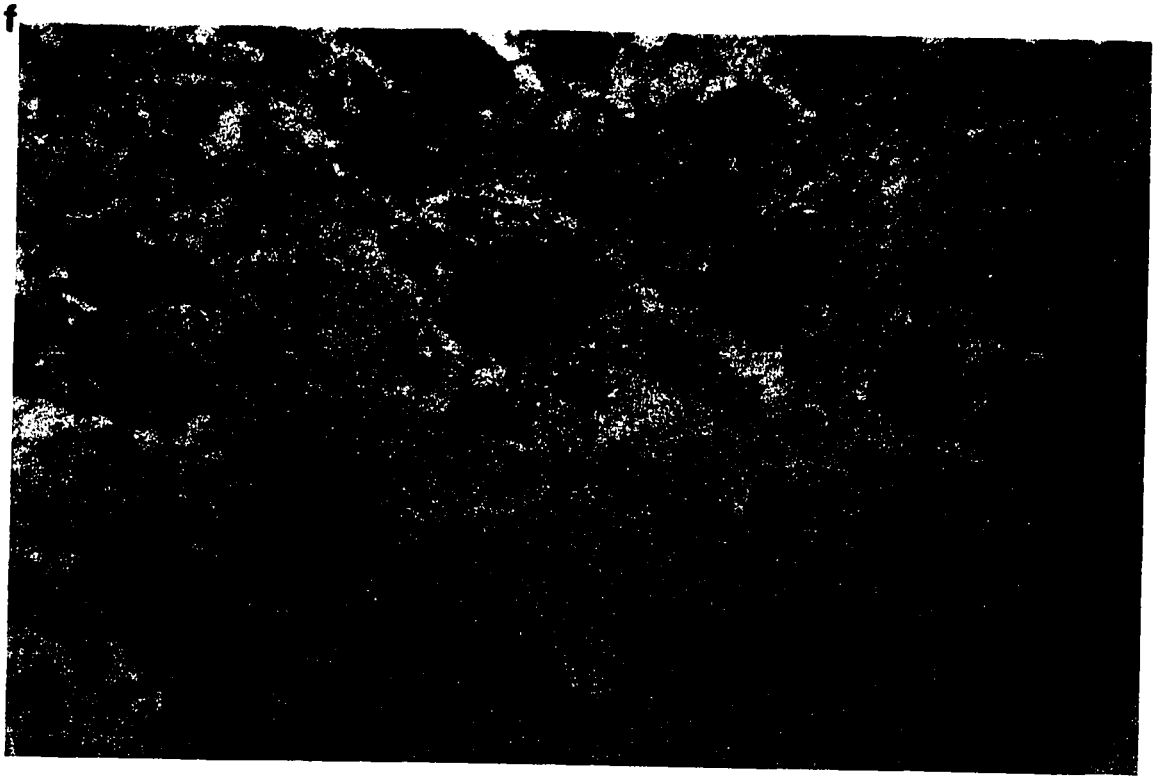
d



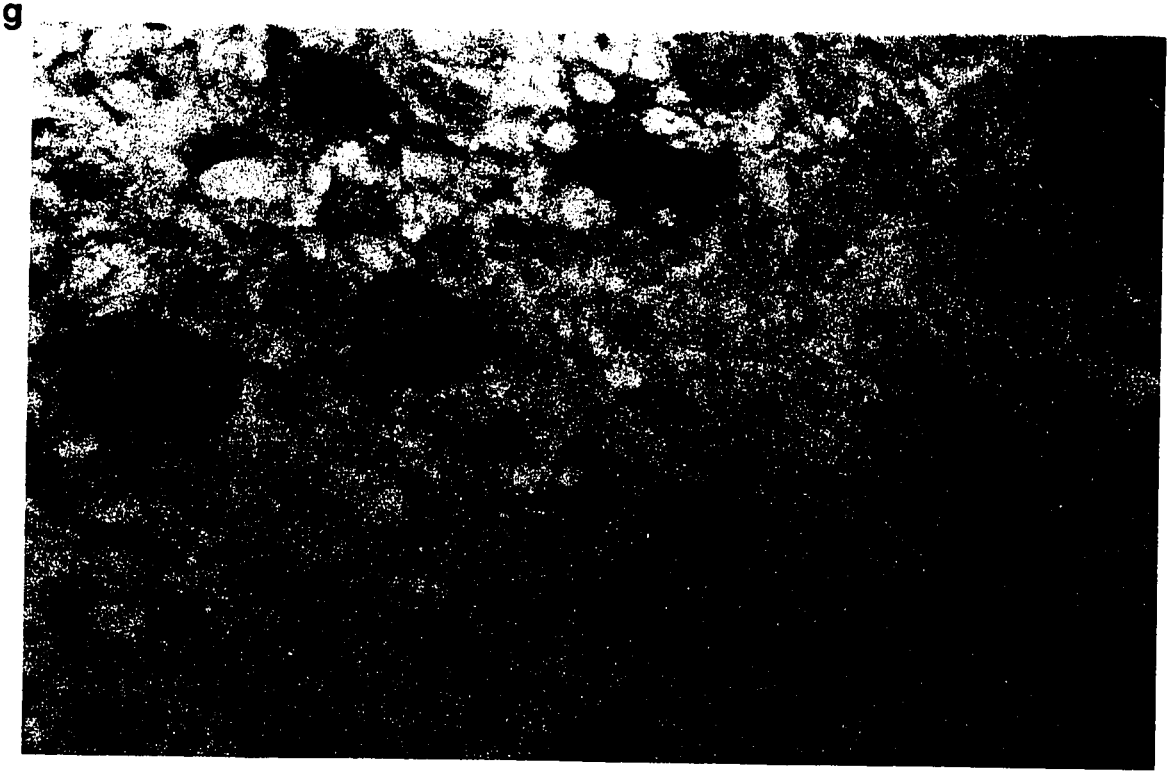
e



f



g

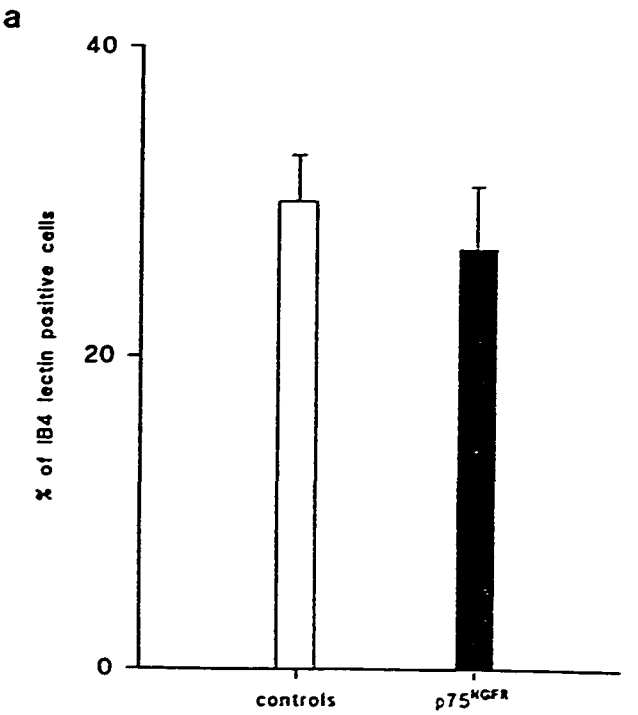


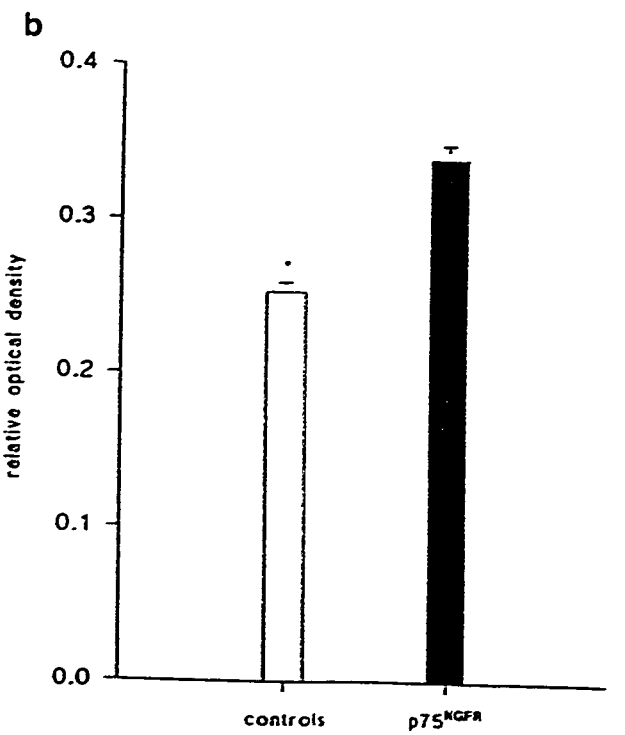
h

i

Fig. 8 IB4 lectin protein expression in the DRGs of $p75^{NGFR}$ and control mice.

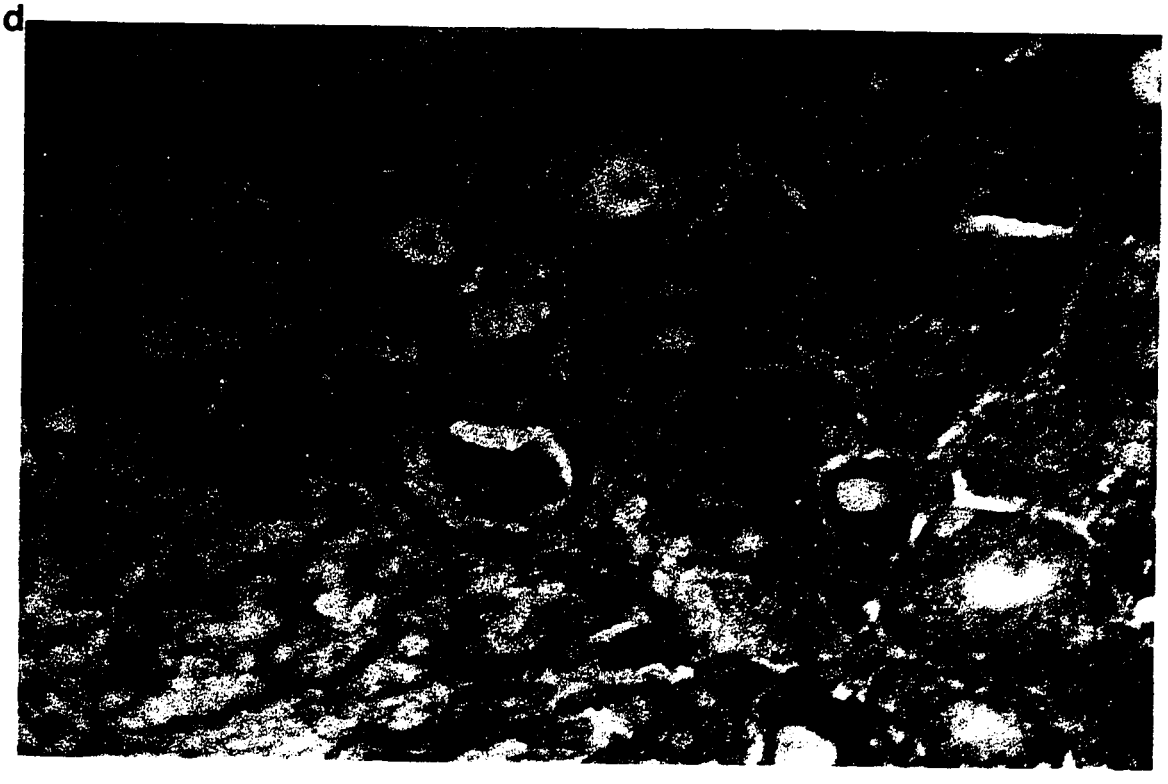
Immunocytochemistry was used to analyse the small DRG neurons that bind the IB4 lectin (Fig. 8a,b). The proportion of IB4 lectin positive cells in DRG from $p75^{NGFR}$ and control mice were determined (Fig. 8c), and the values were found to be similar. The MCID was used to measure the density of IB4 lectin staining in these DRG section (Fig. 8d). The intensity of IB4 immunoreactivity was significantly increased by 36 % in the mutant mice relative to that measured in the control animals. Magnification of photomicrographs; x740.


a
40
20
0
% of IB4 lectin positive cells
controls
p75NGFR


b
0.4
0.3
0.2
0.1
0.0
relative optical density
controls
p75NGFR

c

d



III EXAMINATION OF THE ENVIRONMENT IN WHICH REPARATIVE NERVE GROWTH OCCURS

III.i Proliferation of Schwann cells distal to nerve axotomy in Sl/c-kit mutant mice

The environment in which regeneration occurs has been shown to be an important determinant for successful nerve repair (Fawcett and Keynes,1990). Since regeneration of cutaneous sensory fibres was significantly impaired in Sl/Sl^d and W/W^v mutant mice, it was important to examine the environment in which these fibres grow subsequent to nerve crush. One factor that was specifically examined was the number of Schwann cells present distal to nerve crush. There is a significant proliferation of these cells in this region after nerve damage, with peak cell division occurring about 3 days after such an insult (Bradley and Ashbury, 1971).

In order to determine whether the impaired regeneration observed in these mutant mice was due to impaired proliferation of Schwann cells after nerve crush, cutaneous nerves from both mutant and +/+ mice were axotomized, and the distal nerve segment was removed after 4 days. A Haematoxylin/Eosin stain was then used to visualize the Schwann cells that were present on 10μm sections of nerve. Cell counts indicated that there was a similar number of these cells present in both mutant and control nerves (Fig. 9). Therefore, the impaired regeneration observed in Sl/Sl^d and W/W^v mutant mice was not due to a defect in Schwann cell proliferation in the segment distal to nerve crush.

III.ii Upregulation of c-kit expression in nerve segments after damage in Sl/c-kit mutant mice

An additional factor that was examined in nerve segments subsequent to crush injury was the expression of c-kit protein. This tyrosine kinase is expressed in sensory nerves (Hirota *et al*, 1993) as well as in non-neuronal cells (Su and Federoff, 1994). It is possible that the c-kit present in these neuronal accessory cells may be important for nerve regeneration to occur. Therefore, immunocytochemistry was used to detect c-kit protein expression in the proximal and well as distal stumps 1 day after nerve crush. C-kit was upregulated in the nerve segments both proximal (a) and distal (b) to a nerve crush site. Since the axons distal to a nerve crush would commence Wallerian degeneration very shortly after this type of injury (Fawcett and Keynes, 1991) the upregulation in the levels of this protein would have occurred in non-neuronal cells. This suggests that c-kit protein expression in the non-neuronal cells after nerve crush may be important for the efficient regeneration of sensory cutaneous fibres.

Fig. 9 Proliferation of Schwann cells after nerve axotomy in W/Wv and control mice.

The segments immediately distal to nerve axotomy were removed 4 days after this injury. A Haematoxylin/Eosin stain was used to allow visualization of the number of Schwann cells present in nerve segments from W/W^v and control mice, and the number of Schwann cells present in mutant and control nerves were counted. There was no difference in the number of Schwann cells present in the distal nerve segment 4 days after axotomy in the mutant and control animals. However, there was a significant increase in the number of Schwann cells per 10μm section 4 days post-axotomy relative to undamaged nerved in both types of mice.

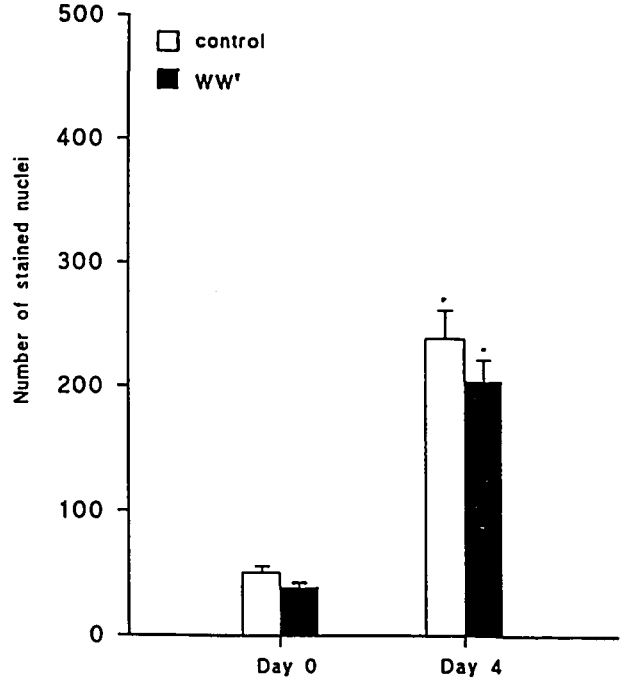
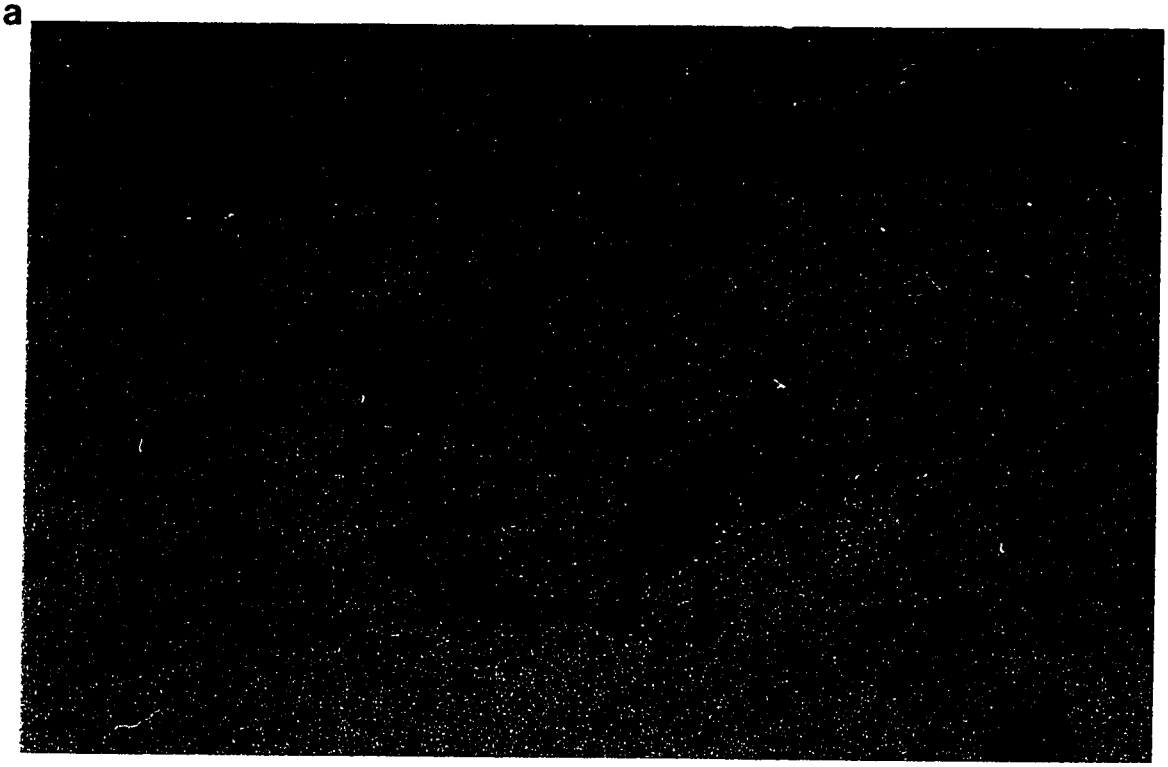

500
400
300
200
100
0
control
WW'
Number of stained nuclei
Day 0
Day 4

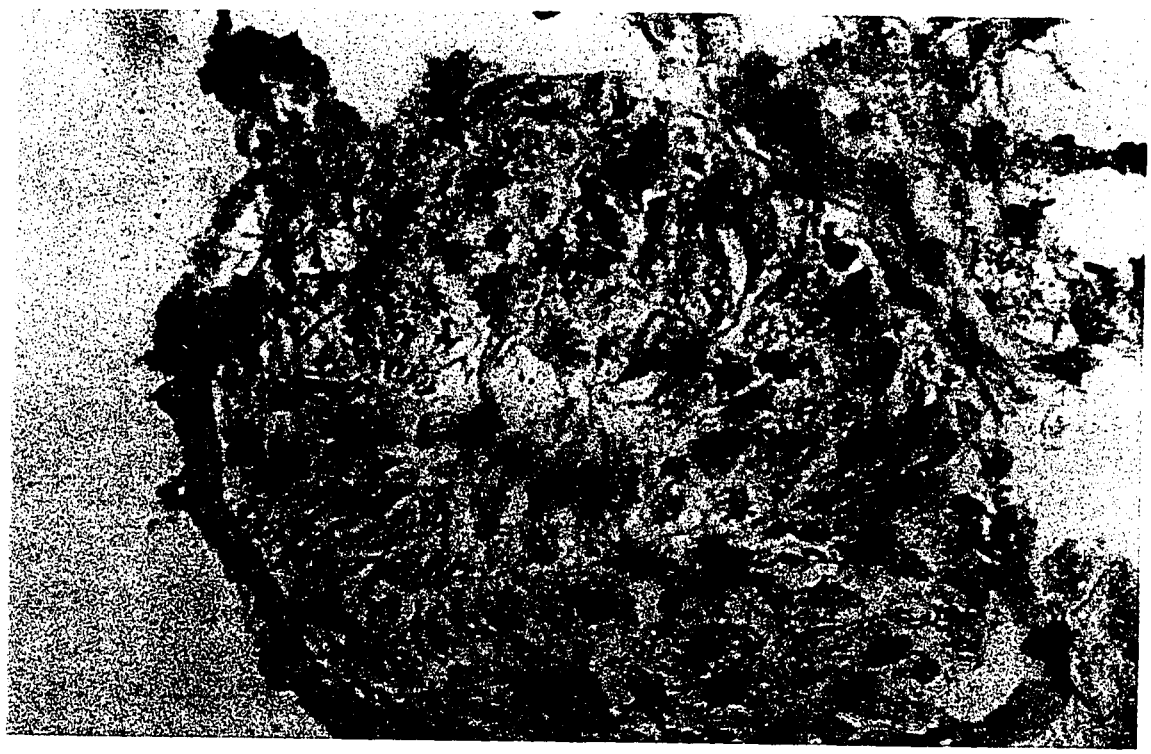
Fig. 10 Effect of nerve crush on expression of c-kit protein in the nerves of normal mice.

C-kit protein expression was studied in sensory nerves during reparative growth. The expression was analysed in the following: normal nerve (a), proximal segment 1 day after nerve crush (b), distal segment 1 day after nerve crush (c). There was an increase in the intensity of c-kit immunoreactivity in the segments proximal and distal to nerve crush. Magnification of photomicrographs; x740.

a



b



C

IV AXONAL COUNTS

i Since a comparison of reparative nerve growth was being made in mutant mice relative to normal animals, it was of interest to determine if there would be a difference in the axon numbers in these mice. The three fibre types that were analysed included the large mechanosensory Aβ, the small myelinated Aδ, and the small unmyelinated C fibres. The number of fibres in each group were counted in the Sl/Sl^d, W/W^v and $p75^{NGFR}$ mice and compared to the numbers found in the appropriate control animals. Since the rate of regeneration in the Sl/Sl^d and W/W^v mice was similar, and the mutations are of similar severity, the fibre counts from these two strains were grouped together.

There are significantly decreased numbers of all fibre types (Aβ, Aδ and C) in cutaneous sensory nerves of Sl/Sl^d and W/W^v mice as compared to control animals. The Aβ and Aδ fibre numbers were reduced to 81% and 75% ($p=0.04$) of that observed in control animals. The decrease was especially evident in the C fibre population where there was a reduction to 64% ± of control C fibre numbers ($p=0.02$) (Fig. 11).

There was a large decrease in all three types of axons in the $p75^{NGFR}$ mice relative to the numbers seen in control animals. In fact, there were 50-60% fewer axons in the nerves of mutant mice, with a similar degree of change in all three fibre types (Fig. 12). The Aα, Aδ, and C fibre populations were reduced to 49%, 50%, and 40% of control fibre numbers, respectively.

distribution of axonal type relative to that observed in control mice was also measured (Fig. 13). The parameters of axonal area (a) and myelin thickness (b) were compared in the control and mutant mice. There appeared to be a trend toward a decreased number of fibres of smaller diameter in the mutant mice, however, this is not a statistically significant finding ($p>0.05$, KS test). Furthermore, there was no difference in myelin thickness between the control and mutant animals ($p>0.05$, KS test). Therefore, there is no difference in the distribution of axonal types in knockout mice relative to that measured in control animals.

Fig. 11 Axon counts in Sl/Sl^d and W/W^v mice

The number of axons in DCN T13 belonging to each of the 3 fibre types (Aβ, Aδ, and C) comprising this sensory nerve was measured in Sl/Sl^d and W/W^v mice, and compared to numbers found in axons from control animals. Since the severity of mutation was similar in both steel and c-kit mutant mice, the numbers from the two mutant strains were grouped together. There was a significant decrease in all of the fibre types in the mutant animals relative to that measured in control animals ($p<0.05$).

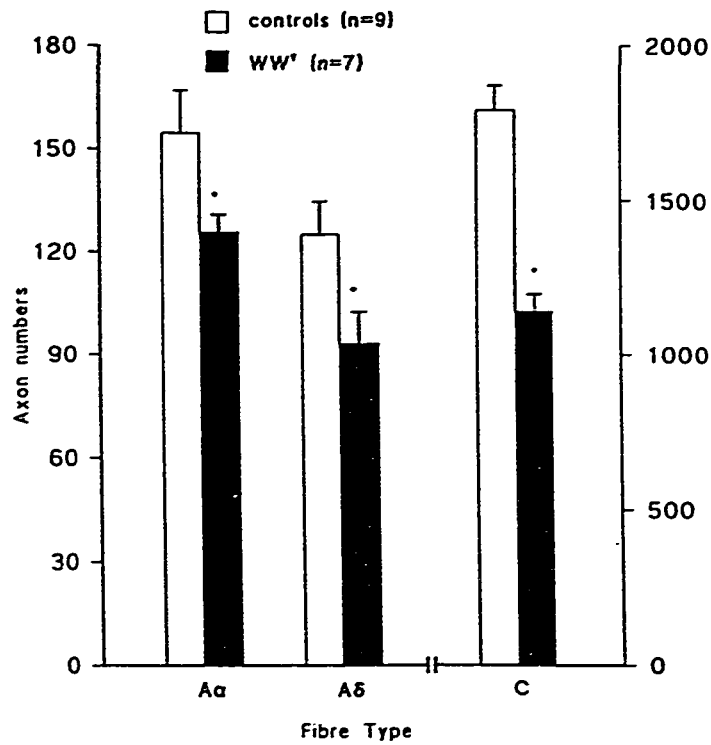

controls (n=9)
WW' (n=7)
180
150
120
90
60
30
0
2000
1500
1000
500
0
Axon numbers
Aα
Aδ
C
Fibre Type

Fig. 12 Axon counts in $p75^{NGFR}$ mice

The number of axons comprising each of the three fibre types in DCN T13 were counted in $p75^{NGFR}$ mice, and compared to the numbers found in control animals. The numbers of each of the three fibre types were significantly reduced in the knockout sensory nerves relative to that observed in control animals. The percentage decrease in fibre numbers were as follows: Aβ, 51% ($p<0.01$); Aδ, 50% ($p< 0.01$); C, 60% ($p<0.01$).

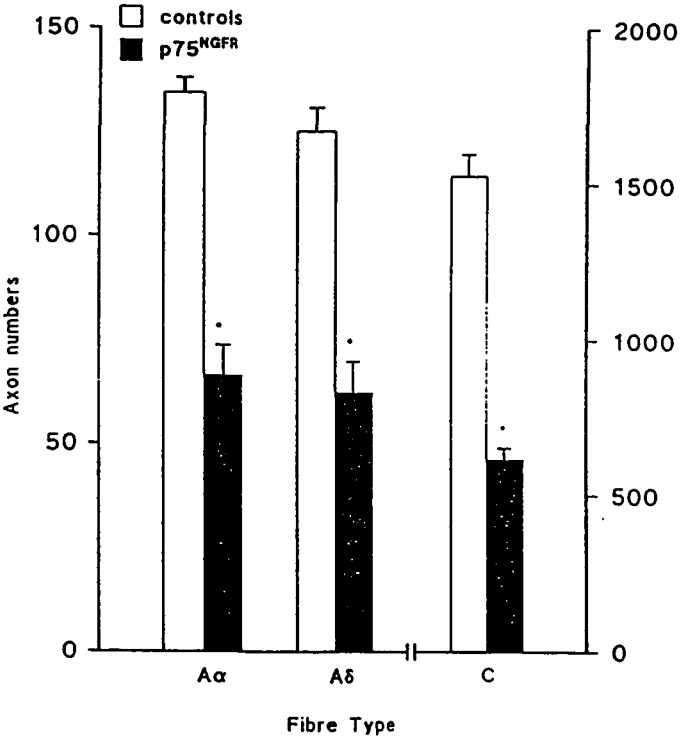
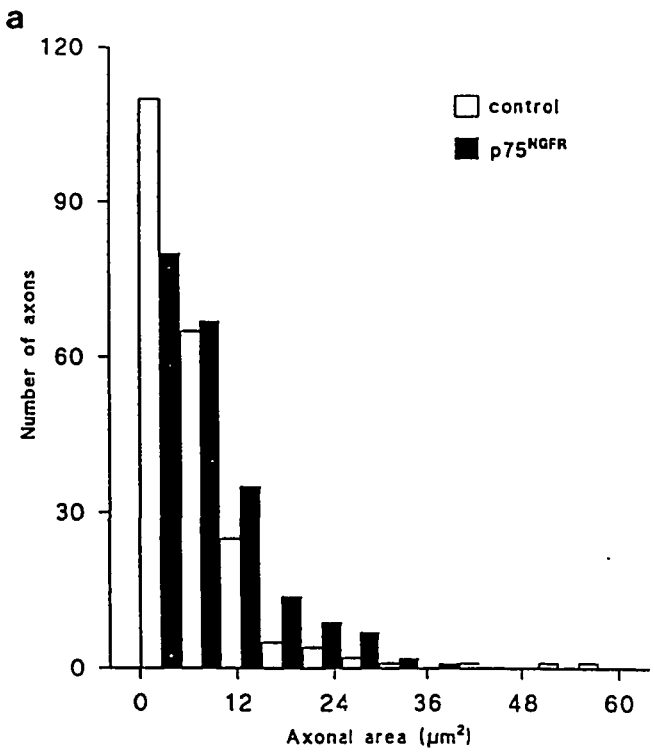
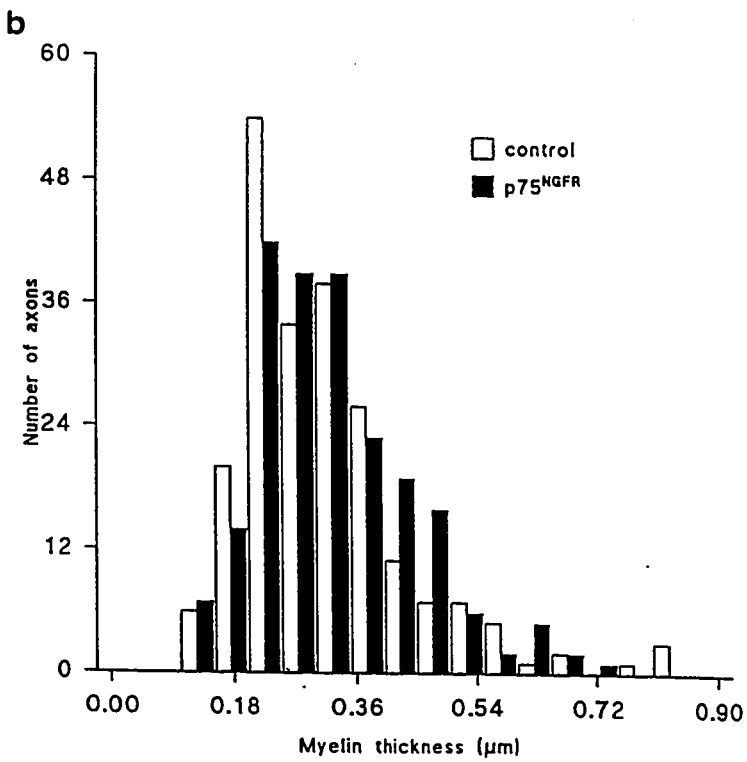

controls
p75NGFR
150
100
50
0
2000
1500
1000
500
0
Axon numbers
Aα
Aδ
C
Fibre Type

Fig. 13 Axonal distribution in p75NGFR mice

In order to determine whether there was a difference in the distribution of axon types in the $p75^{NGFR}$ and control mice, axonal areas (a) and myelin thickness (b) were measured and compared between the two groups of animals. There was no significant difference in either the axonal area, or the myelin thickness in the knockout mice relative to control animals.


a
120
90
60
30
0
Number of axons
control
p75NGFR
0
12
24
36
48
60
Axonal area (μm²)


b
60
48
36
24
12
0
Number of axons
control
p75NGFR
0.00
0.18
0.36
0.54
0.72
0.90
Myelin thickness (μm)

V INDUCTION OF HYPERALGESIA

Vi Since the $p75^{NGFR}$ mice were defective in NGF-dependent collateral sprouting, it was possible that they would also be deficient in another NGF-dependent process, the onset of hyperalgesia. Therefore, the effects of two doses of exogenous NGF on thermal and mechanical hyperalgesia was determined in these mutant mice and well as in +/+ animals.

Daily administration of 1μg/g of 2.5s NGF did not lead to either thermal or mechanical hyperalgesia in either control or mutant animals (Fig. 14a,b). Although there was a decrease in the response time to noxious heat stimulation in both groups of mice during the testing period, this effect was also observed in animals that received saline injection. This would suggest that all of the mice were able to sensitize to the stimulus over time.

However, daily administration of 2.5μg/g of 2.5s NGF did result in mechanical hyperalgesia in both +/+ and $p75^{NGFR}$ mice (Fig. 14c). The onset of hyperalgesia was evident 1.5hrs. after the second daily injection NGF in both groups of mice and continued until Day 3 of the experiment (3hrs. after the 4th injection of NGF); this was the final day of testing of the animals. The testing was not continued further since exogenous NGF administration is known to lead to the onset of thermal hyperalgesia in 30min. in rats. Furthermore, his treatment causes mechanical hyperalgesia that is detectable one day after a single injection of NGF (Lewin *et al*, 1994).

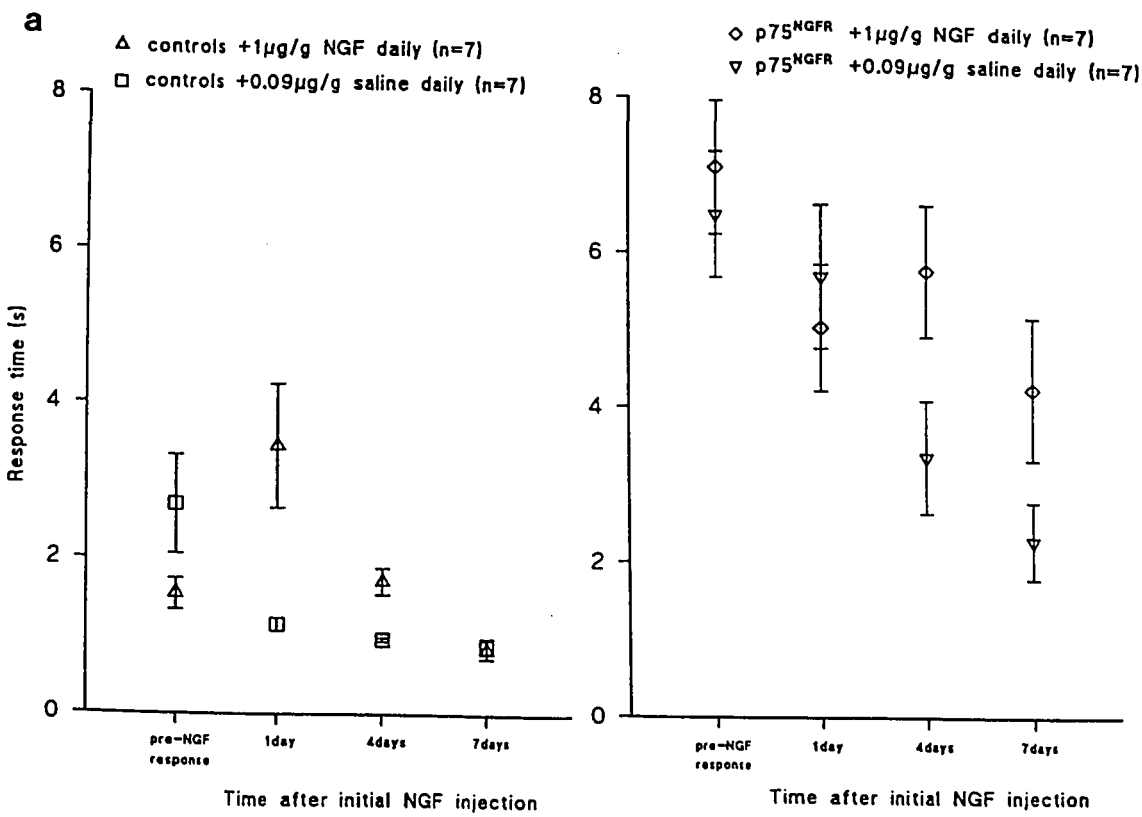
On the other hand, mice receiving this dose of NGF also responded to the noxious thermal stimulation in a similar way to animals receiving saline injections. Therefore, daily

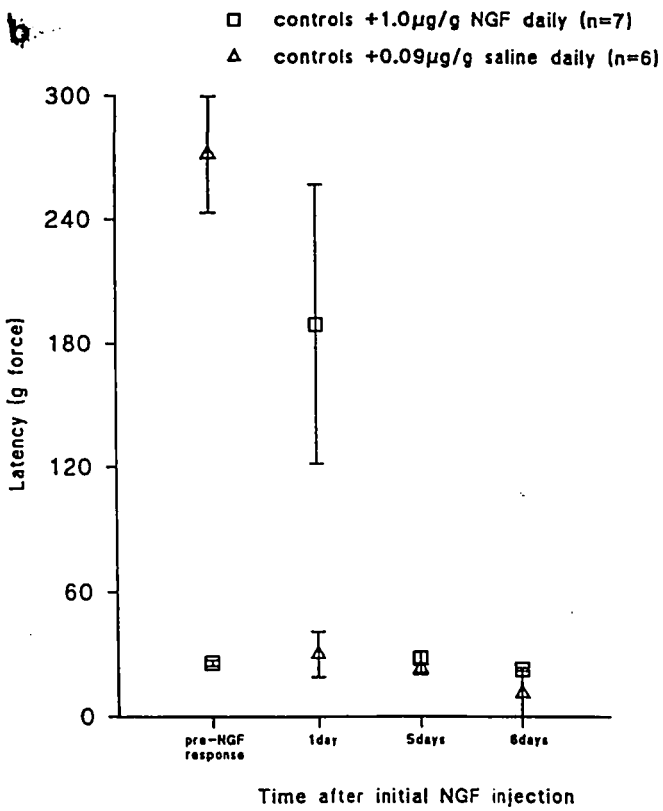
administration of 2.5μg/g of 2.5s NGF did not result in the onset of thermal hyperalgesia in either the +/+ or knockout animals (Fig. 14d).

In conclusion, daily administration of 1μg/g of 2.5s NGF did not result in the onset of either thermal or mechanical hyperalgesia in either the knockout or the control mice. Daily administration of 2.5μg/g of 2.5s NGF resulted in mechanical but not thermal hyperalgesia in both +/+ and $p75^{NGFR}$ mice. Therefore, exogenous NGF administration at a daily dose of 2.5μg/g of NGF results in mechanical hyperalgesia independently of the presence of the low-affinity neurotrophin receptor.

Fig. 14 Effect of daily administration of 1μg/g of exogenous NGF on hyperalgesia

The effect of daily administration of 1μg/g of exogenous NGF or 0.09μg/g saline on mechanical (a) and thermal (b) hyperalgesia. Mice with a null mutation in the gene coding for $p75^{NGFR}$ were compared with +/+ animals. Exogenous NGF administration did not lead to either mechanical or thermal hyperalgesia in either the knockout or control animals over the duration of the experiment.


a
△ controls +1μg/g NGF daily (n=7)
□ controls +0.09μg/g saline daily (n=7)
Response time (s)
8
6
4
2
0
pre-NGF response
1day
4days
7days
Time after initial NGF injection
◇ p75NGFR +1μg/g NGF daily (n=7)
▽ p75NGFR +0.09μg/g saline daily (n=7)
8
6
4
2
0
pre-NGF response
1day
4days
7days
Time after initial NGF injection


b
controls +1.0µg/g NGF daily (n=7)
controls +0.09µg/g saline daily (n=6)
300
240
180
120
60
0
Latency (g force)
pre-NGF response
1day
5days
8days
Time after initial NGF injection

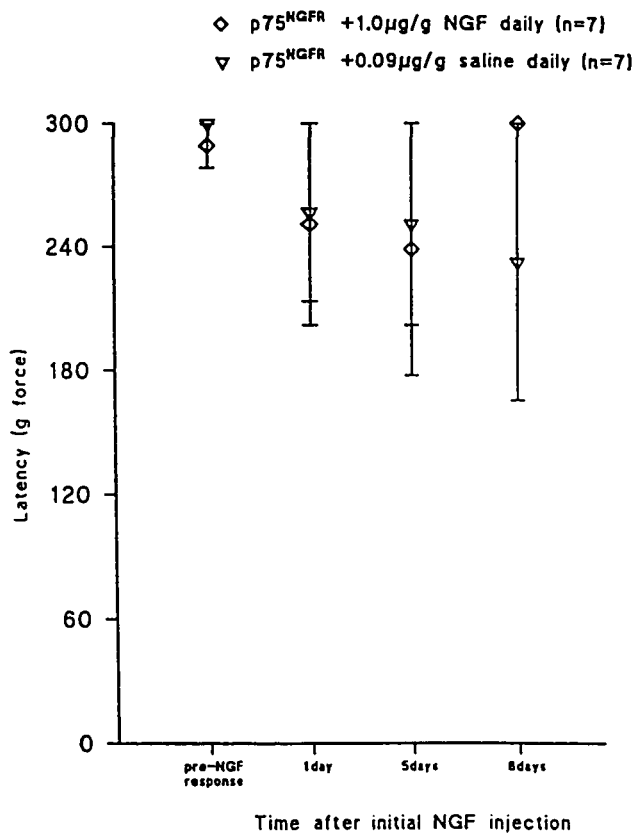
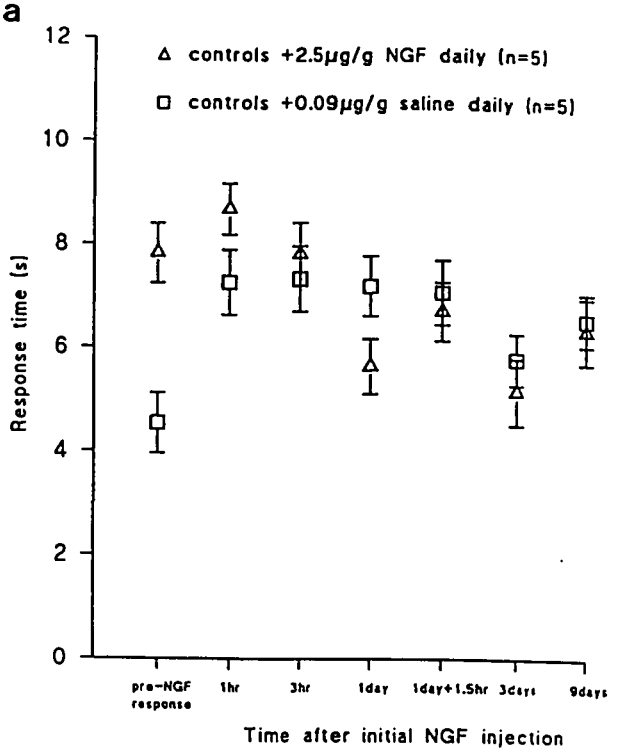
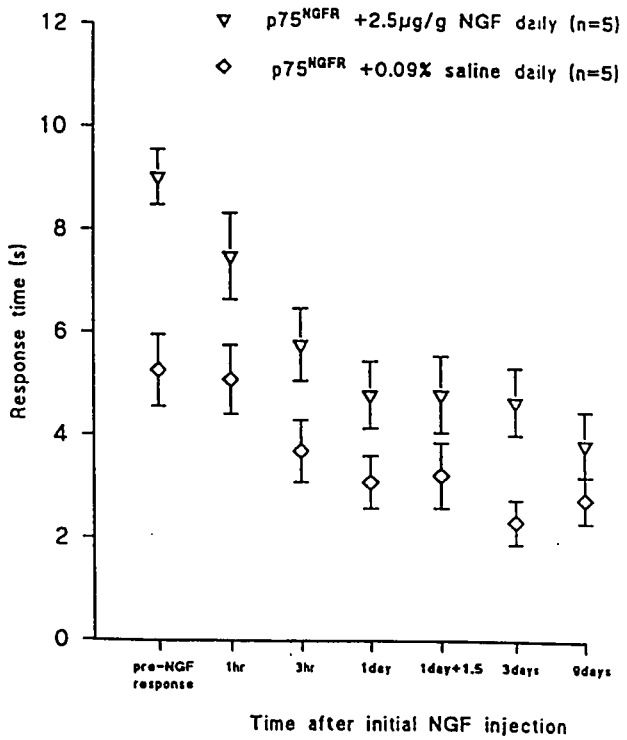

◇ p75NGFR +1.0µg/g NGF daily (n=7)
▽ p75NGFR +0.09µg/g saline daily (n=7)
300
240
180
120
60
0
Latency (g force)
pre-NGF response
1day
5days
8days
Time after initial NGF injection

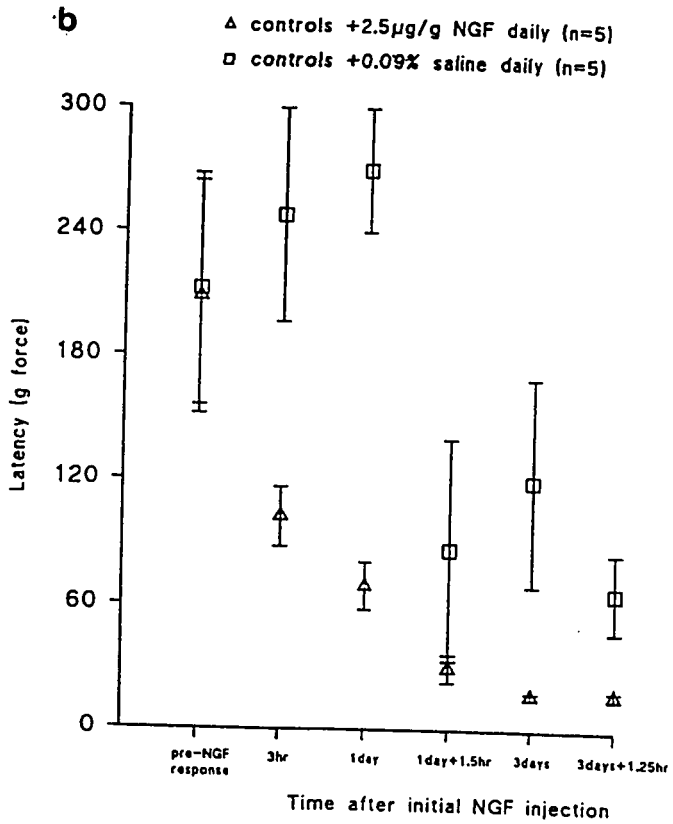
Fig. 15 Effect of daily administration of 2.5μg/g of exogenous NGF on hyperalgesia

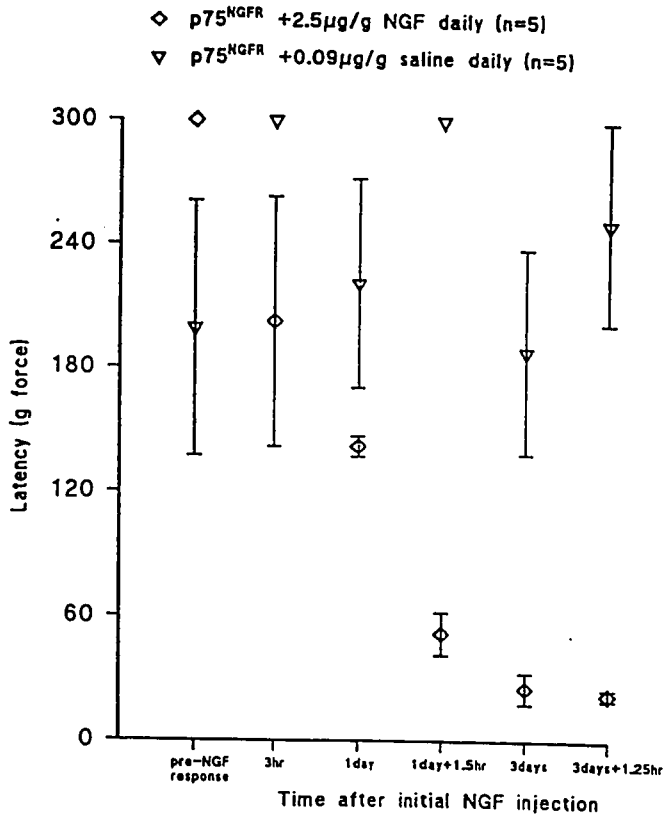
The effect of daily administration of 2.5μg/g of exogenous NGF or 0.09μg/g saline on mechanical (a) and thermal (b) hyperalgesia. Mice with a null mutation in the gene coding for $p75^{NGFR}$ were compared with +/+ animals. This dose of NGF did not result in thermal hyperalgesia over the duration of the experiment. However, mechanical hyperalgesia was evident after the second injection of exogenous NGF, and continued until after the final injection of NGF on day 4 of the experiment.

a


controls +2.5µg/g NGF daily (n=5)
controls +0.09µg/g saline daily (n=5)
Response time (s)
0
2
4
6
8
10
12
pre-NGF response
1hr
3hr
1day
1day+1.5hr
3days
9days
Time after initial NGF injection


p75NGFR +2.5µg/g NGF daily (n=5)
p75NGFR +0.09% saline daily (n=5)
Response time (s)
0
2
4
6
8
10
12
pre-NGF response
1hr
3hr
1day
1day+1.5
3days
9days
Time after initial NGF injection


b
controls +2.5μg/g NGF daily (n=5)
controls +0.09% saline daily (n=5)
300
240
180
120
60
0
Latency (g force)
pre-NGF response
3hr
1day
1day+1.5hr
3days
3days+1.25hr
Time after initial NGF injection


◇ p75NGFR +2.5μg/g NGF daily (n=5)
▽ p75NGFR +0.09μg/g saline daily (n=5)
300
240
180
120
60
0
Latency (g force)
pre-NGF response
3hr
1day
1day+1.5hr
3days
3days+1.25hr
Time after initial NGF injection

DISCUSSION

Summary of significant findings

The present findings support a role of the low-affinity neurotrophin receptor in the events leading to collateral sprouting, but not regeneration. There was no collateral sprouting in the $p75^{NGFR}$ mice, but regeneration occurred at a normal rate. In contrast, regeneration of cutaneous fibres was significantly impaired in mice mutant for either steel factor or c-kit, while collateral sprouting of these fibres was normal. In this instance then, steel and c-kit appear to play a role in the former, but not in the latter growth process. Finally, both collateral sprouting and regeneration were normal in the NFHlacZ transgenic mice, suggesting that a lack of axonal neurofilaments combined with an overexpression of microtubules has no obvious effect on either type of reparative nerve growth. In general, the results of this thesis support the distinction between the mechanisms involved in the regulation of collateral sprouting and regeneration of peripheral cutaneous sensory nerves.

Although collateral sprouting did not occur in the $p75^{NGFR}$ mice, this process was evoked by daily systemic injections of exogenous NGF. Administration of NGF to the mutant animals at a dose of 2.5μg/g resulted in the collateral sprouting of Aδ and C fibres at rate similar to that observed in control animals that did not receive NGF (The rate of C

fibre collateral sprouting was also similar to the rate observed in normal animals that received 1μg/g of exogenous NGF).

In addition, there was a significant reduction in all fibre types comprising the sensory nerves in both the $p75^{NGFR}$ mice and the Sl/Sl^{d} and W/W^{v} mice. Therefore, it appears that both $p75^{NGFR}$ and steel/c-kit play a role in the development of a population peripheral cutaneous sensory fibres.

Additional findings from this work were that mechanical hyperalgesia was induced in both $p75^{NGFR}$ and normal mice with daily administration of 2.5μg/g (but not 1μg/g) of 2.5s NGF. There was no evidence of thermal hyperalgesia with either dose of NGF. The results indicate that the low-affinity neurotrophin receptor probably does not play a significant role in NGF-induced hyperalgesia.

I MEASUREMENT OF REPARATIVE NERVE GROWTH

Accuracy of area measurements

Prior to performing any experiments using the mutant and transgenic mice, it was necessary to acquire the skills necessary to accurately measure the area of skin innervated by the cutaneous nerve selected for study (DCN T13). Previous measurements had been performed only on the rat, and these protocols had to be transferred to the mouse so that it would be possible to use mutant and transgenic animals study the effects of mutations in specific molecules on reparative nerve growth.

Analysis of these forms of growth involved monitoring the sensory modalities subserved by the three fibre groups present in the sensory nerve: Aβ, Aδ, and C fibres. As mentioned in the introduction, electrophysiological techniques were used to measure the area innervated by the Aβ fibres, whereas, a behavioural method that relied on the "CTM reflex" was used to measure the areas innervated by the Aδ and C fibres. Although measurement of the area innervated by the Aδ fibres was more difficult in the mouse than in the rat due to the small size of the fields and the difficulty in obtaining a convincing CTM response, it was much less difficult than similar measurements for Aβ and C fibres. Only two time points were obtained for the former in the c-kit mutant mice, since this nerve is very fragile in the mouse and any further measurements may have resulted in permanent damage to the entire nerve. Measurement of the initial field area and the area at a given time point after nerve crush allowed for determination of the extent of regeneration of the Aβ fibres while minimizing the chances that nerve damage would occur.

Measurement of the area innervated by C fibres was very tedious for a few reasons. A well-defined CTM response with obvious wrinkling of the skin was often difficult to obtain. This would usually mean that repeated attempts to obtain a sufficient response were necessary, possibly resulting in burn lesions to the back skin of the animal. Since it would no longer be possible to pinch or heat the skin to obtain a wrinkling response, this would preclude any chance of obtaining a measurement at the next time point. As such, it was often necessary to obtain measurements only at the beginning and end of the experiment, as was done for measurements of Aβ innervation territories. This method of measuring C fibre innervation areas provided reproducible results, and also parallelled the findings obtained by

performing Aδ fibre area measurements. Therefore, this method is useful for determining the area of target tissue innervated by these small, unmyelinated, heat-nociceptive C fibres.

In conclusion, the above-mentioned techniques were used to obtain accurate measurements of the areas innervated by the Aβ, Aδ, and C fibres that comprise DCN T13.

Initial field areas in $p75^{NGFR}$ mice

Initial measurements of collateral sprouting and regeneration on the $p75^{NGFR}$ mice were performed on animals that were of smaller size than the control mice. In fact, the average weight of the mutant mice was 14g, while that of the control mice was 20g. This was partly due to the fact that the mutant mice were obtained at 4-6 weeks of age, and are smaller in size than the control mice in the pre-adult stages, as has been observed by others (S. Landis, personal communication). The mice were used immediately upon their arrival since there was concern that lesions in the skin of their backs would develop soon after (Lee *et al*, 1992) and that this would make measurement of innervation territories difficult, if not impossible. However, some of these mice were left in their cages until they were about 2-3 months of age. Visual inspection of these mice indicated that there were no lesions present at this time, therefore, further experiments were performed on animals that were of this older age. This was necessary for a few reasons. First of all, it was of interest to determine if the area of skin innervated by DCN T13 prior to reparative growth would be similar in mutant and control mice of comparable weights. As shown in Fig. 3.3 of the Results section, there was no significant difference in the area innervated by the Aδ and C fibres of this nerve in

the control and mutant mice. Thus, although there was a large reduction in the number of sensory fibres in the p75 knockout mice, the arborization of the remaining neurons still occupied a similar territory in the skin as observed in control mice. This is not surprising, since it is thought that each neuron likely supplies the entire nerve field (Diamond *et al*, 1992c).

Significance of the density of innervation in the $p75^{NGFR}$ mice

As mentioned in the previous section, there are significantly fewer fibres occupying similar areas of target tissue in the mutant mice as compared to normal animals. In this case the density of innervation in the mutant skin may be reduced as compared to normal skin. Alternatively, there may be a greater degree of arborization of sensory fibres in the skin of the mutant mice, resulting in an innervation density similar to that in normal animals. It would be more beneficial to the animal to have fewer fibres innervating a similar area of target tissue that the other possibility, which would have been a reduced target area resulting from normal fibre arborization. This may have left areas of skin with no sensory innervation, a potentially dangerous situation for the viability of the animal.

The results of an immunocytochemical assay for neuropeptide protein levels in the skin of these mice showed a significant reduction in the amount of staining for the neuropeptides substance P (SP) and calcitonin-gene-related peptide (CGRP) in the skin of the mutant mice (Lee *et al*, 1992). This suggests that the density of target innervation of the sensory fibres that normally contain these peptides (of the Aδ and C fibre class) is reduced

in the mutant mice. Although it was possible that the levels of these neuropeptides were down-regulated in the neurons of these animals as a result of the mutation, the finding of a reduced heat-sensitivity (Lee *et al*, 1992) combined with the data regarding a reduction in axonal counts in the present study suggest that this was not the case. Therefore, there appears to be a reduction in the density of innervation of the skin by at least some fibre populations.

Lack of collateral sprouting in $p75^{NGFR}$ mice

As mentioned in the previous section, the initial field of innervation of sensory fibres is similar to that observed in control animals, and the density of innervation of at least some cutaneous sensory fibres is reduced in the skin of these animals. This may be an explanation for the lack of collateral sprouting in these mice, in that the innervation territory was occupied by significantly fewer fibres. These fibres may have extended collaterals to their maximum capabilities in order to occupy this territory during development, and were simply unable to undergo further arborization into denervated regions of skin.

There was the possibility that the lack of collateral sprouting observed in these mice could be related to their age. The younger, less mature neurons may simply not have been able to upregulate expression of various growth factors (eg. tubulins) required for this reparative growth to occur. However, the finding that regeneration was normal even in young knockout mice suggests that defects in growth mechanisms was not a likely possibility. Nevertheless, it was necessary to determine whether older animals were also

unable to undergo collateral sprouting of their peripheral sensory fibres, as was the case.

It is interesting that the sensory nerves in the $p75^{NGFR}$ mice are able to extend axons during development in order to establish normal field territories in the target tissue, but are unable to extend collaterals beyond this in the adult animal. It is likely that different mechanisms are responsible for these two processes. Of significance here are the findings that suggest that the initial outgrowth of sensory axons occurs independently of neurotrophic factor and target availability (Davies *et al*, 1987; Vogel and Davies, 1991), however, NGF may increase fibre density in the target tissue after axons have reached this point. The type of longitudinal growth toward the target tissue that occurs during axonal regeneration in the adult appears to be structurally analogous to the initial axonal outgrowth that occurs during development. Therefore, it is not surprising that both processes appear to occur independently of NGF availability (Rich *et al*, 1984; Davies *et al*, 1987; Diamond *et al*, 1987; 1992b, Vogel and Davies, 1991). In addition, most of the changes in gene expression that occur after axotomy, eg. a reduction in neurofilament mRNA levels (Hoffman and Cleveland, 1988; Wong and Oblinger, 1989) and an upregulation of Tα1 α-tubulin levels (Miller *et al*, 1989) recapitulate the program of the initial neurite outgrowth that occurs during development (Hoffman and Cleveland, 1988). Therefore, since this latter process appears to occur by an entirely different mechanism from collateral sprouting, it is not surprising that it may occur normally while the other is defective.

An insertional mutation in the gene coding for $p75^{NGFR}$ or exogenous administration of antibodies to NGF can completely halt collateral sprouting, suggesting that this growth process is completely dependent on the ability of NGF to bind to its low, and presumably

high affinity receptors (described in introduction) to induce the relevant signalling cascade. This is compatible with evidence that p75 is increased prior to and during collateral sprouting in normal rats, and that this increase is more substantial than that observed for the tyrosine kinase receptor, trkA (Mearow *et al*, 1994). This is the only example described so far in which a neuronal process is absolutely dependent on the presence of $p75^{NGFR}$ as well as trkA. As mentioned in the introduction, an exclusive role for the low-affinity receptor in protection from death of neuroblastoma cells in vitro has recently been described. This process has been shown to require NGF to bind to $p75^{NGFR}$, and occurs in the absence of the high-affinity trkA receptor (Cortazzo *et al*, 1996).

It appears collateral sprouting of sensory neurons depends entirely on the ability of endogenous NGF to bind to both its high and low-affinity receptors. On the other hand, there appear to be many factors that determine how well a nerve will regenerate after a crush injury. As will be discussed below, these include both factors present in the cell body of neurons as well as those associated with the environment in which axonal extension occurs.

The present results indicate that the p75 receptor plays a key role in NGF-dependent collateral sprouting of undamaged nociceptive axons. This suggests that p75 is required to increase the signalling efficiency of the trkA receptor, resulting in the expression of genes that are needed for collateral sprouting to occur in sensory neurons (Mearow *et al*, 1993).

Although there is the possibility that collateral sprouting cannot occur in absence of the low-affinity receptor, the effect of exogenous NGF administration on collateral sprouting indicate that this in not the case (discussed later).

Reparative growth is normal despite an absence of axonal neurofilaments: NFHlacZ transgenics

NFHlacZ transgenic mice were able to undergo reparative growth of their sensory axons similar to that seen in normal animals. Since both neurofilament and tubulin mRNA levels are decreased and increased, respectively, during regeneration, it is interesting that sensory nerves in which neurofilament is absent and tubulin is overexpressed were not affected in their ability to undergo regeneration and collateral sprouting. The alterations in the levels of these molecules in the nerves of the mutant mice may have been sufficiently similar to that observed during nerve repair, and any further increases or decreases in mRNA levels that may have occurred during reparative nerve growth were of no consequence to this process.

Alternatively, the lack of any differences in reparative nerve growth in these animals may be due to the fact that Aδ and C fibres are of a relatively small calibre and do not contain a high abundance of neurofilaments. Thus, any consequences of this mutation may only be detected in large calibre fibres that are rich in neurofilament content, for example, the low-threshold mechanosensory Aβ fibres. These fibres will regenerate in the adult animal, thus it would be possible to study the ability of these large fibres to undergo axonal regeneration in the mutant animals. However, these Aβ fibres undergo collateral sprouting during a short time period post-natally only (Jackson and Diamond, 1984), therefore, it would be impossible to measure this form of growth in the adult mutant mice. However, there is some support for the hypothesis that the large sensory fibres may be selectively

affected in the mutant mice in that it has been determined that large calibre motoneurons are able to regenerate at a faster pace in the NFHlacZ transgenics as compared to control mice (A. Peterson, personal communication). Nevertheless, there are considerable differences between sensory and motoneurons, and an experimental result in one case may not apply to a similar situation in the other.

The finding that reparative growth is normal in sensory nerves of NFHlacZ transgenic mice, together with the observation that neurofilament mRNA levels are reduced after nerve injury, suggest that this class of intermediate filaments is not required for neuronal regeneration. However, other results do not support this hypothesis. For example, anterograde transport of neurofilament in the axon is increased after nerve injury. In addition, mice that carry a deletion coding for the light chain neurofilament NF-L are defective in their ability to regenerate facial motoneurons (J.P. Julien, personal communication). In fact, only 10% of the neurons are able to regenerate, as evidenced by electron microscopic analysis of the nerve segment distal to the crush site. This data implies that neurofilaments are in fact crucial for regeneration of at least some types of peripheral nerves. This result is somewhat contrary to the above-mentioned molecular studies, which imply that neurofilaments are not required in nerve regeneration. Therefore, further studies are necessary to determine the role of these intermediate neurofilaments in reparative nerve growth.

Defective regeneration in Steel and c-kit mutant mice

Experiments using Sl/Sl^d and W/W^v mice have allowed identification of a role for steel factor and its receptor c-kit in the peripheral nervous system of adult mice, in that mutant animals show a reduced potential to regenerate sensory neurons after crush. This is an interesting example of impaired regeneration of sensory nerves as a result of a mutation of a factor that is intrinsic to the neuron itself. Another molecule that affects axonal regeneration is acidic fibroblast growth factor (aFGF) (Cordeiro *et al*, 1989; Laird *et al*, 1995), which is highly expressed in the cell bodies and axons of primary sensory neurons (Westermann *et al*, 1990). Exogenous administration of this factor has been shown to increase the rate of sensory as well as motor axonal regeneration after sciatic nerve crush (Laird *et al*, 1995).

As mentioned, it has previously been demonstrated that regeneration of nociceptive axons occurs independently of NGF (Diamond *et al*, 1992a), while the collateral sprouting of these axons is totally NGF-dependent (Diamond *et al*, 1987; Diamond *et al* 1992b). The finding that regeneration is impaired in Sl/Sl^d and W/W^v mutant mice supports that idea that Steel binds to its tyrosine kinase receptor c-Kit, resulting in the induction a signalling pathway leading to the expression of genes required for the regulation of axonal regeneration. This may be somewhat analogous to the ability of NGF to bind to its receptors and cause activation of a signalling pathway that results in the induction of regulatory genes required for collateral sprouting; these would be a separate set of genes from those required for the regulation of axonal regeneration (Campenot, 1994; Johanson *et al*, 1996). The

mechanisms of neurite extension that could then be common to both growth responses, for example, as discussed earlier there is an upregulation of tubulin in both collateral sprouting (Mathew and Miller, 1990) and regeneration (Miller *et al*, 1989). Alternatively, it is also possible that there are two parallel and independent growth systems, one for regeneration, and one for collateral sprouting.

Significance of results of NGF administration

An interesting and somewhat surprising finding in this study is that the $p75^{NGFR}$ receptor normally plays a key role in NGF-dependent collateral sprouting of undamaged nociceptive axons. In the absence of $p75^{NGFR}$, however, collateral sprouting can be partially rescued with exogenously administered NGF. This result can be considered in light of previous findings *in vitro* in the $p75^{NGFR}$ knockout mice where it has been shown that a 3- to 4-fold higher concentration of NGF is needed to promote half-maximal survival of embryonic sensory trigeminal neurons (Davies *et al*, 1993), as well as a 2- to 3-fold higher concentration of NGF in order to promote the survival of an equal number of dorsal root sensory neurons in the mutant mice relative to normal animals (Lee *et al*, 1993). In these *in vitro* studies, the data indicates that embryonic neurons from $p75^{NGFR}$ mice have a reduced sensitivity to NGF.

The finding of a lack of collateral sprouting in the sensory neurons of $p75^{NGFR}$ mice also supports a role for $p75^{NGFR}$ in increasing the availability of NGF to trkA receptors and is also consistent with the above stated studies on the survival of embryonic neurons from

$p75^{NGFR}$ mice in vitro (Davies, *et al*, 1993; Lee *et al*, 1994). Other *in vitro* studies support this hypothesis. For example, when NGF is prevented from binding to the $p75^{NGFR}$ receptor in PC12 cells, the result is a decrease in NGF binding to trkA and a reduction in NGF-mediated signal transduction (Barker and Shooter, 1994). Furthermore, similar results have been observed in MAH cells, an immortalized sympathoadrenal cell line that does not respond to NGF and expresses very low or undetectable levels of trkA and p75 (Birren and Anderson, 1990; Birren *et al*, 1992). When p75 is coexpressed in MAH cells with trkA, there is an enhancement of trkA phosphorylation as well as neuronal differentiation of MAH cells, as compared with cells expressing trkA alone (Verdi *et al*, 1994). Finally, p75 has been observed to produce an increase in discrimination in neurotrophin responsiveness (Benedetti *et al*, 1993).

Collateral sprouting can be partially rescued by exogenously administered NGF in the p75 knockout animals. The effect of exogenous NGF is distinct from that of endogenous NGF, in that collateral sprouting can only occur with exogenous NGF in the absence of the $p75^{NGFR}$ receptor. Either the amount of NGF available in the skin is not sufficient to induce collateral sprouting in the absence of p75, or this endogenous NGF is not accessing the neuron. Since this system retains the capacity to respond to exogenous NGF, this molecule may the entering neurons at locations on the axon or cell body that do not present the problems of NGF uptake that may occur when access is available solely at the terminals. In comparison to the trkA receptors at the terminals, additional receptors may be present on the axonal or cell body membranes. These may be used by exogenous NGF as a more effective way of accessing the neuron. An increase in the proportion of activated trkA receptors

would then be sufficient to trigger the appropriate intracellular signalling events leading to expression of genes relevant to the collateral sprouting process.

The results of the reparative growth experiments using the three animal models are summarized in Table 2, on the following page.

Table 2. Summary of reparative nerve growth data

Animal model	Regeneration	Collateral sprouting(C.S.)	C.S. with 1μg/g NGF	C.S. with 2.5μg/g NGF
NFHlacZ	normal	normal	-	-
Sl/Sl^d	impaired	normal	-	-
W/W^v	impaired	normal	-	-
$p75^{NGFR}$	normal	absent	some	normal

Role of $p75^{NGFR}$ in regeneration

Studies on reparative nerve growth of peripheral sensory fibres in $p75^{NGFR}$ mutant mice indicate that axonal regeneration is unaffected by the absence of the $p75^{NGFR}$ receptor. This suggests that the upregulation of p75 that is observed in Schwann cells after nerve damage (Tanuichi *et al*, 1986) may not be relevant to successful nerve regeneration. Since $p75^{NGFR}$ is the low-affinity receptor for all of the known neurotrophic factors (Chao, 1994), regeneration of all sensory fibres, including those insensitive to NGF such as the Aβ low-threshold mechanosensory ones, may involve one of the other neurotrophic factors. The results of the present study allow for the possibility that there may be compensatory mechanisms that allow a neurotrophin-independent regeneration to occur in the absence of p75. Interestingly, there is an upregulation in $p75^{NGFR}$ mRNA expression in Schwann cells associated with motoneurons post-axotomy (Heumann *et al*, 1987; Raivich and Kreutzberg, 1987) , and regeneration of these fibres occurs at a reduced rate in $p75^{NGFR}$ mice (Bisby, personal communication). Therefore, $p75^{NGFR}$ does not appear to be involved in regeneration of cutaneous sensory fibres, but may have a role in this form of reparative growth of motoneurons.

Role of $p75^{NGFR}$ in CNS collateral sprouting

The present study has demonstrated a crucial role for $p75^{NGFR}$ in collateral sprouting of peripheral sensory neurons. In addition, there is evidence that $p75^{NGFR}$ is important for

collateral sprouting of central processes.

A recent study has indicated the importance of $p75^{NGFR}$ in the collateral sprouting of CNS neurons (Lucidi-Phillipi *et al*, 1996). Normal rats were initially subjected to a unilateral lesion of the fimbria-fornix. The animals were then immediately implanted with a cannula close to the lesion site. The cannula was connected to a mini-osmotic pump, allowing for intracerebroventricular infusion of NGF or a trkA agonist (RTA IgG). The RTA IgG has been shown to stimulate trkA activation and autophosphorylation in PC12 cells and sympathetic neurons, resulting in cell survival and neurite outgrowth (Clary *et al*, 1994). Another group of rats received infusion of RTA in combination with the REX antibody. The latter is thought to cause oligomerization of $p75^{NGFR}$, inhibiting functional interactions of $p75^{NGFR}$ with other $p75^{NGFR}$ molecules or with trkA (Lucidi-Phillipi *et al*, 1996). Infusion of either NGF or RTA induced significant collateral sprouting of undamaged neurons in the dorsolateral quadrant of the septum; NGF was much more effective than the RTA antibody. Immunocytochemistry was used to demonstrate that these processes contained $p75^{NGFR}$ protein. However, infusion of RTA along with the REX antibody resulted in significantly reduced collateral sprouting of these processes, the extent of which was similar to that observed in mice infused with cerebrospinal fluid (CSF) only. These results support the hypothesis that $p75^{NGFR}$ has a significant role in NGF-induced collateral sprouting of both peripheral and central neurons.

There appears to be a difference in the role of $p75^{NGFR}$ in this form of reparative growth in peripheral and central fibres. In the central study, collateral sprouting was barely detectable in animals that were infused with either CSF or REX antibody only. In the

peripheral experiments, collateral sprouting was present in normal animals; this would approximate to the animals infused with CSF. Thus, collateral sprouting in the central system was barely detectable without some form of additional treatment. The lack of collateral sprouting in the $p75^{NGFR}$ knockout mice was approximately equivalent to the central study in which RTA was combined with REX. The role of $p75^{NGFR}$ in collateral sprouting of central fibres thus became evident only upon exogenous administration of RTA together with the REX antibody. The collateral sprouting that occurs with exogenous RTA was then almost completely prevented with co-administration of the REX antibody. However, the present study has shown that exogenous NGF can evoke collateral sprouting in the absence of the $p75^{NGFR}$ receptor. How can this be explained? One possibility is that $p75^{NGFR}$ plays a role in concentrating NGF so that it is more accessible to trkA molecules on axon terminals in the periphery. In the absence of $p75^{NGFR}$, the endogenous NGF in the skin is insufficiently concentrated. Upon administration of exogenous NGF, the concentration of this neurotrophic factor becomes sufficient to result in increased trkA binding even in the absence of $p75^{NGFR}$. An alternative explanation is that the trkA density in the axon terminals is inadequate to promote uptake of NGF to the degree necessary to induce collateral sprouting unless $p75^{NGFR}$ is present. The upregulation of trkA in the mutant animals challenged in the collateral sprouting paradigm shows, however, that at least some NGF is taken up the terminals. The exogenous NGF may be effective by virtue of its access to the much greater numbers of trkA receptors present on the axon and/or the cell body of the peripheral nerve.

In the central nervous system, exogenous NGF or RTA may not have the same access

to trkA. Therefore, there may be an upper limit to the extent that NGF can bind to trkA in the absence of functional p75, and an excess of NGF does not result in an increase in expression of genes required for nerve growth to occur. In conclusion, it appears that $p75^{NGFR}$ has a role in collateral sprouting of both peripheral and central fibres. However, there is a difference in these two systems in that exogenous NGF can induce collateral sprouting in the absence of this molecule in the peripheral nervous system. However, although trkA is activated by RTA administration in the central nervous system, suppression of $p75^{NGFR}$ activity with the use of the REX antibody prevents collateral sprouting from occurring.

The low-affinity neurotrophin receptor has recently been shown to play a role in signalling of other neurotrophic factors involved in extension of neurites. An example of this has been illustrated in cultures of basal forebrain cholinergic neurons. These neurons respond to addition of high concentrations of NT-3 with the outgrowth of neurites; addition of antibodies to $p75^{NGFR}$ is able to abrogate this effect (Sample *et al*, 1995). This would imply that NT-3 must bind to $p75^{NGFR}$ as well as its tyrosine kinase receptor TrkC in order for genes required for neurite outgrowth to be activated. Alternatively, high concentrations of NT-3 have been shown to activate trkA (Clary and Reichardt, 1994) and this may be the mechanism that leads to neurite outgrowth in this system. In conclusion, $p75^{NGFR}$ is a necessary component of reparative growth of basal forebrain cholinergic neurons evoked by NT-3.

II CELL BODY RESPONSE TO DAMAGE

C-kit increase in DRG during nerve regeneration

Examination of c-kit protein levels in the DRG and axon of normal animals after nerve crush suggests a role for this tyrosine kinase receptor and its ligand steel factor in some post-nerve crush event. The increase in the density of protein expression in individual cells as well as the increase in the number of cells that express c-kit is consistent with either an initiation or an increase in signal transduction through the c-kit receptor. Previous work has shown that c-kit dimerization and autophosphorylation leads to the association and phosphorylation of a number of SH2-containing proteins. These include PI3 kinase, phospholipase Cγ and the GTP-ase activating protein GAP (Rottapel *et al*, 1991; Reith *et al*, 1991; Herbst *et al*, 1991; Lev *et al*, 1991). Thus, it is possible that an increase in the activity of such a second messenger pathway leads to the transcription of genes that are beneficial for successful regeneration to occur. This may lead to production of one or more soluble factors that are able to influence the cell body responses of neurons that contain c-kit as well as that of neighbouring DRG neurons.

In order for neuronal c-kit to be directly involved in regeneration of sensory fibres, it would be desirable to have expression in all DRG neurons undergoing this reparative process. However, immunocytochemical analysis indicates that it is only expressed in 15-20% of DRG neurons during regeneration. There is a possibility c-kit is present in a larger population of cells but was undetectable using the rabbit polyclonal antibody to this receptor.

An in situ hybridization study to detect mRNA expression of c-kit in the DRG would allow determination of the number neurons expressing the c-kit receptor during regeneration, and this number may be much larger than the number of neurons expressing detectable c-kit protein in the DRG.

This has been illustrated in a study involving detection of basic FGF (bFGF) in the DRG after sciatic nerve transection (Ji et al, 1995). Prior to axotomy, 5.6% of all DRG neuron profiles contained bFGF mRNA, while 3.9% contained bFGF protein. Peak levels of mRNA were detected 3 days post-axotomy, where 86.6% of all DRG neuron profiles were bFGF mRNA positive. On the other hand, peak levels of bFGF protein were detected 2 days post-axotomy, where only 25.6% of all neurons contained this protein.

Many factors are likely to be involved in the regeneration of a peripheral nerve. In addition to cell body components, the environment of a regenerating nerve has also been shown to be of importance (discussed later). Although steel and its receptor c-kit appear to be one of the components required for efficient regeneration of sensory fibres, many other factors are likely to be involved, bFGF being one such example. In addition, studies on axonal regeneration in C57BL/6J mice have concluded that the delayed regeneration of the sciatic nerve in these animals likely involves multiple genetic loci (Lu *et al*, 1994). In conclusion, c-kit and its membrane-bound ligand steel factor may play a significant part in axonal regeneration, but in their absence there are ample other components involved in this process to allow successful regeneration to take place, although at a slower pace than that observed in the presence of these two molecules.

trkA and IB4 lectin in the DRG of $p75^{NGFR}$ mice

trkA positive neurons project to lamina I and the outer region of lamina II, and there is extensive colocalization between trkA and calcitonin-gene-related peptide (CGRP) (Verge *et al*, 1989). The peptide CGRP has previously been associated with nociceptive function (reviewed by Carr and Lipkowski, 1990). On the other hand, IB4 lectin positive neurons project to the inner region of lamina II, and have been reported to lack neuropeptides (Silverman and Kruger, 1990). However, recent studies suggest that there is some overlap between trkA positive and IB4 lectin positive neurons (Molliver *et al*, 1995).

The data indicates that trkA protein levels are unchanged in the DRGs of $p75^{NGFR}$ knockout mice relative to control animals except when challenged in the collateral sprouting paradigm, as mentioned above. Furthermore, although there are significantly fewer neurons in the DRGs of $p75^{NGFR}$ knockout animals, the proportion of trkA positive cells is the same as that found in control animals (40%). In addition, the amount to trkA protein present in DRG neurons appears to be similar in control and mutant mice. Thus, it appears that there is no increase in trkA expression as a compensatory reaction to the lack of a functional p75 receptor. Furthermore, the proportion of IB4 lectin positive (25%) small and medium DRG neurons is also unchanged in these mutant mice. This implies that an equal percentage of trkA responsive and trkA non-responsive small and medium DRG neurons are lost in these animals. Thus, it appears that the loss in neuron numbers is unrelated to their neurotrophin responsiveness (discussed later).

III EXAMINATION OF THE ENVIRONMENT IN WHICH REPARATIVE NERVE GROWTH OCCURS

Relevance of normal Schwann cell proliferation

It is known that nerve axotomy is accompanied by a vigorous proliferation of Schwann cells in the distal stump (Abercrombie and Johnson, 1946; Bradley and Ashbury, 1970). It is thought that this process contributes to nerve regeneration by providing a favourable environment for nerve growth. For example, Schwann cells may produce factors that can be used by the associated nerve for axonal elongation.

Analysis of Schwann cell proliferation after sensory nerve crush in Sl/Sl^d and W/W^v mice indicates that this process is not impaired in these animals. This result suggests that the environment in which sensory nerves regenerate in these mutant animals is normal. However, there are other possible defects in the environment that could contribute to an impairment in axonal regeneration, as discussed in the next section. The finding that c-kit is upregulated in the DRG of regenerating neurons in normal animals implies that defective regeneration in the Sl/Sl^d and W/W^v mice is due to an insufficient cell body response, although it certainly does not exclude the contribution of other factors.

Examples of the importance of the environment to the rate of nerve regeneration

There have been descriptions of mouse models in which impaired regeneration is due to a defect in the environment in which nerve growth must take place. A well characterized example of this is the C57Bl/Wld^{s} (Ola) mouse. The process of Wallerian degeneration is significantly delayed in these mice (Lunn *et al* 1989; Perry *et al*, 1991), and as a result, regeneration of sensory fibres is significantly impaired (Brown *et al*, 1990,1991; Bisby and Chen, 1990). However, sensory axons in these mice are able to regenerate at a similar rate to that observed in normal mice when they are exposed to the same environment as normal nerves (Brown *et al*, 1994).

Another mutant strain of mouse has recently been described (*Enr*) in which there is an impaired regeneration of peripheral nerves (Rath *et al*, 1995). Analysis of regenerating nerves in *Enr* mice indicates that the impaired regeneration is due to an inability of "early" Schwann cells to differentiate into a more mature phenotype. These examples illustrate that the environment in which regeneration takes place can be very important for the length of time required for this form of reparative nerve growth to occur.

C-kit expression in non-neuronal cells

The results of the present study indicate that c-kit protein is normally expressed in non-neuronal cells in the nerve, and levels of this protein are increased in non-neuronal cells that are located proximal and distal to a nerve crush (Fig. 6.2b,c). This has also been

observed in the sciatic nerve post-axotomy (Su and Federoff, 1995). In addition, steel factor has also been shown to be present in the non-neuronal cells (Su and Federoff, personal communication). It is possible that signalling through the c-kit receptor may lead to the production of proteins relevant to the regeneration process by a mechanism similar to that described for the possible role of c-kit in the DRG. For example, steel factor is able to upregulate expression of various cytokines and neurotrophic factors *in vitro* (Federoff, personal communication); expression of these molecules may also be upregulated *in vivo*, and may be required for the normal regeneration of sensory fibres. In any event, the role of Steel and the consequences of c-kit activation must be grossly defective in the mutant animals, and the observed impairment of axonal regeneration thus implicates these two molecules in some measure in this form of reparative growth.

IV AXONAL COUNTS

Comparison to DRG neuron counts

The number of axons present in the sensory nerves of Sl/c-kit and p75 knockout mutant mice has been quantified. This method is an alternative to counting the number of cells present in the DRG. Although the latter would be the most desirable method for quantitation, it is very tedious and time-consuming. Furthermore, there are numerous techniques for this type of measurement, and much controversy as to which protocol provides the most accurate estimate of cell numbers (Coggeshall and Lekan, 1996). In addition, only

a percentage of cells in the thoracic DRG extend collaterals into an associated dorsal cutaneous nerve; the remainder extend into ventral cutaneous and visceral nerves (Levine *et al*, 1993).

Although it is assumed that each sensory axon originates from one DRG neuron, there is evidence for branching of primary afferent fibres (Langford and Coggeshall, 1981; McCarthy *et al*, 1995; McMahon and Wall, 1987). However, the actual number of neurons involved is small, and moreover, there is no reason to assume a selective loss or preservation of DRG neurons that give rise to more than one peripheral process in the mutant mice. Therefore, since the number of axons in the mutant animals were being directly compared to those in the control mice, this method is useful for giving an accurate indication of the relative amount of fibre loss in the mutant mice.

Axon counts in steel/c-kit mutant mice

In mice carrying a mutation at the *Sl* or *W* locus, there are fewer cutaneous axons present relative to control mice. There is a small but significant reduction in Aβ and Aδ myelinated fibres. However, there is an even larger decrease (30%) in the number of C fibres present in the mutant mice relative to normal animals.

The finding of reduced numbers of sensory fibres in steel/c-kit mutant mice is the first example of a neurological deficit in these animals. It is not possible to discern whether the fibre loss is due to a failure of some neurons to develop or differentiate normally, or if they are present initially and then die at a later stage. A reduction in Aβ fibres in these animals

is especially surprising. Steel and c-kit are known to be expressed in small and medium DRG neurons consisting of a sub-population of NGF responsive neurons. As such, it does not seem likely that the large fibre population would be affected in this case. There are two possible explanations for this finding. Signalling through the Kit receptor may lead to induction of proteins needed for the development of sensory fibres. Some of these proteins may act in a paracrine manner in the DRG to affect the neurons of origin of the large mechanosensory fibre population. Alternatively, in the absence of one of these molecules, a proportion of the Aβ fibres may be unable to reach their usual sizes and may appear more like Aδ fibres. In this case, the loss of the Aδ fibres may in fact be larger than suggested by this quantitative study. However, this latter explanation is highly unlikely. As indicated previously, the area innervated by Aβ in DCN T13 was determined electrophysiologically, and required the presence of clearly audible responses upon light stroking of innervated regions of skin. The audible responses, which are the summation of numerous responses in individual axons, were of similar magnitude in both the mutant and control mice; exprerience from smaller, or damaged nerves shows that if there were fewer large myelinated fibres in the mutant animals a lower audible response would be expected.

Axon counts in $p75^{NGFR}$ mice

In mice that lack a functional p75 receptor, there is a significant reduction in all fibre types normally present in the sensory neurons. Although quantitative results of this nature have not previously been reported, Lee and colleagues have qualitatively observed a

reduction in the size of $p75^{NGFR}$ DRGs as compared to normal animals (Lee *et al*, 1992). They have also shown that the neuropeptides CGRP and Substance P are significantly reduced in the skins of the mutant animals, implying a reduced innervation by small and medium sized sensory neurons. The present results help refute the alternative explanation, that the levels of these peptides may simply be reduced in these mice.

There appears to be a trend toward a selective decrease in the proportion of small Aδ fibres in cutaneous nerves of the knockout animals as compared to numbers found in control mice, although this finding was not statistically significant. There is a possibility that some of these small fibres may have hypertrophied due to an absence of a subset of fibres of a larger axonal size. The latter include a proportion of the largest DRG neurons (associated with Aβ axons) which are thought to be unresponsive to NGF, even though a proportion do express the high affinity receptor trkA (Mearow *et al*, 1994; Wright and Snider, 1993). It seems then that the absence of a functional low-affinity neurotrophin receptor compromises the development of most or all classes of DRG neurons, regardless of their neurotrophin responsiveness.

It has been shown that p75 is expressed by approximately 60% of DRG neurons on embryonic day 15 (Dr. P.F. Bartlett, personal communication), the time in which programmed cell death is occurring (Oppenheim,1991). In addition, it has been shown that $p75^{NGFR}$ is required for survival of DRG neurons in the presence of NGF during the pre-natal stages of development (Barrett and Bartlett, 1994). Based on the results of the axonal counts in adult $p75^{NGFR}$ mice, there is a selective loss of nearly 60% of DRG neurons of all three fibre types. Thus, it seems likely that the neurons that do express this low-affinity

neurotrophin receptor during development require its expression for survival during development. In the absence of this receptors, these neurons do not survive this pre-natal period in the knockout animals. On the other hand, the approximately 40% of DRG neurons that do not express this receptor are able to survive during this period in the knockout mice. In conclusion, $p75^{NGFR}$ expression is required for the survival of significant numbers of sensory neurons of all fibre types. In its absence, neurons that normally express $p75^{NGFR}$ do not survive into adult stages.

V HYPERALGESIA

Daily administration of NGF results in a similar hyperalgesic response to noxious mechanical stimulation in both the $p75^{NGFR}$ and control mice. This implies that the low-affinity neurotrophin receptor is not required for this NGF-dependent response. Furthermore, although a dose of 1μg/g was sufficient to induce a mechanical hyperalgesia in response to exogenous NGF administration in the rat (Lewin *et al*, 1993), a dose of 2.5μg/g of NGF was needed for this response to occur in mice. This suggests that the mice are less responsive to exogenous NGF administration than the rat, and/or that the mechanism leading to NGF-induced mechanical hyperalgesia may be different between these two rodents.

Aloe and colleagues have induced a hyperalgesic response to noxious heat stimulation in mice using the higher dose of NGF (2.5μg/g) (Della Seta *et al*, 1994). In contrast to the present results, a hyperalgesic response to noxious heat stimulation was achieved at a temperature of 52^0C, and no such response was observed in the present study at a

considerably higher temperature of 68^0C. However, the protocol used in the former study differed somewhat in that the mice were placed on a hot plate instead of having a heat probe touched against the ventrum of the foot, as was done in the present study. This difference in methodology may at least in part explain the conflicting results between the two studies. Also, to account for the possibility that the lack of effect in the present study was due to the strain of mouse used (JR2448), the experiment was performed with BalbC and CD1 mice; the former is the strain used by Aloe in his study (Della Seta *et al*, 1994). However, there was no indication that NGF could induce a thermal hyperalgesia in these animals at either of the two temperatures used for testing. Therefore, the inability to induce thermal hyperalgesia with exogenous NGF in the present study is not due to strain of mouse tested, and may be explained by other factors, as will be discussed below.

Previous results have suggested that the $p75^{NGFR}$ mice are less responsive to noxious thermal stimulation than control animals when their feet were placed on a 68^0C hot plate (Lee *et al*, 1992). However, in the present study, the mutant mice were as equally responsive to this type stimulation as the control animals. This is especially surprising, since the mutant animals have significantly fewer cutaneous sensory neurons as compared to control mice. Perhaps the method of testing used in the present study elicited a response to a stimulus other than noxious heat, such as probe contact or the restraint of being placed in the testing position. This may also explain why it was not possible to detect thermal hyperalgesia in any of the mice using this method of analysis. Both the mutant and control $p75^{NGFR}$ mice that did or did not receive exogenous NGF displayed decreased latencies over time in response to noxious heat stimulation (see Figs. 14a, 15a). Therefore, it is possible that the

method of testing used allowed the mice to become conditioned to the stimulus, and that they learned to respond by withdrawing from the heat probe in order to avoid an uncomfortable sensation. Of interest to this study is the observation that Wistar rats respond to NGF administration at a dose of 1μg/g with intensive swelling and reddening of their extremities, especially their ears (M. Holmes and Y. Kril, personal observations). This would imply an inflammatory reaction, possibly involving the activation of mast cells by the exogenous NGF administration (Levi-Montalcini, 1995). This was not observed in the mice even with the higher dose of NGF. It was initially thought that this was not detectable in the mice due to the darkness of their coat colour, as both the control and mutant $p75^{NGFR}$ mice are agouti. However, the effect was also not observed in BalbC or CD1 mice; both of these strains are characterized by a white coat colour. Interestingly, addition of NGF to rat mast cells results in their rapid degranulation *in vitro*, however, this has no effect of the status of mouse mast cells after similar treatment (J. Marshall, personal communication). This may suggest that mouse mast cells do not degranulate upon exogenous administration of NGF *in vivo*. In order to address this issue further, it would be useful to test for the presence of the early phase of NGF-induced thermal hyperalgesia in the mouse; this event is known to involve mast cell activation (Lewin *et al*, 1994). Unfortunately, this early phase of NGF-induced heat hyperalgesia was not tested in these experiments, and should be addressed in the future in order to obtain a further understanding of the action of exogenous NGF in the mouse.

In summary, it seems that mice have a different response than rats to exogenous NGF administration, as evidenced by the lack of inflammatory response in the extremities as well as the finding that a higher dose of NGF was required to induce hyperalgesia. Furthermore,

the low-affinity neurotrophin receptor does not appear to play a role in this response since both $p75^{NGFR}$ and control animals responded to NGF administration with a similar mechanical hyperalgesia as well as a lack of response to noxious heat stimulation. However, there is the possibility that there were subtle differences in response in the two strains of mice that were not detected with the present testing methods.

CONCLUSIONS

The present thesis involved the use of mutant/transgenic mice to identify molecules that play a role in reparative nerve growth of cutaneous sensory fibres. Both collateral sprouting and regeneration were normal in mice that overexpress the NFHlacZ fusion construct. Since the axons of these mice contain no neurofilaments and an abundance of microtubules (Eyer and Peterson, 1994), such altered expression does not affect reparative growth of sensory fibres. On the other hand, steel factor and c-kit are needed for optimal regeneration of cutaneous nerves, as well as for the development of a proportion of all three types of fibres in these nerves, but are not required for collateral sprouting. Furthermore, $p75^{NGFR}$ is needed for collateral sprouting of these fibres but not for their regeneration. Collateral sprouting can be induced with exogenous NGF administration. Expression of this gene is also required for the development of a significant population of all three fibre types in the cutaneous nerve. Finally, thermal but not mechanical hyperalgesia occurs with exogenous NGF administration at a dose of 2.5μg/g; this effect is independent of the presence of the low-affinity neurotrophin receptor.

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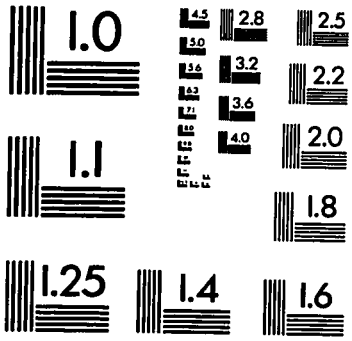

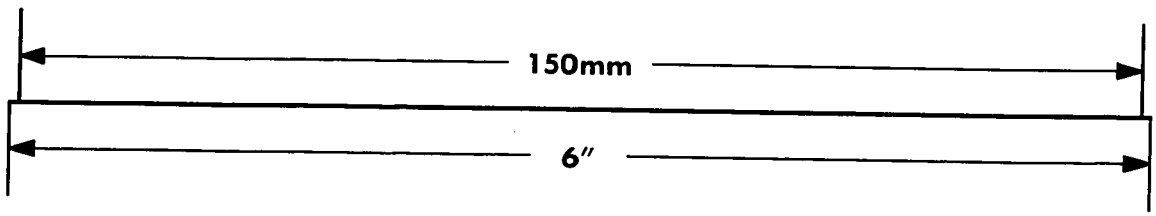

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