

Axonal regeneration and expression of
neuropeptides and neurofilaments in primary
sensory neurons *in vitro*.

by
Gürkan Öztürk

A thesis presented for the degree of Doctor of Philosophy of the
University of London

Physiology Department
Biomedical Sciences Division,
King's College London,
Strand, London WC2R 2LS

January, 1999

ABSTRACT

Following a peripheral nerve injury, a number of changes occur in the phenotype of primary sensory neurons of dorsal root ganglia (DRGs). These changes are believed to reflect a switch in metabolic activity of neurons from signal transmission to repair and regeneration. The mechanisms regulating these events are not fully known. The deprivation of neurons of certain trophic factors and synthesis or upregulation of others are possible explanations for these changes. In this thesis, explant cultures of adult mouse DRGs were used to investigate the effects of some of these factors on neuronal survival, axonal growth, and neuropeptide and neurofilament (NF) expression in primary sensory neurons and in regenerating axons using immunohistochemical techniques.

The proportion of surviving neurons was very high in cultured DRGs during 3-day incubation. Nerve growth factor (NGF) appeared to support neuronal survival and strongly stimulated growth of axons. LIF did not affect overall survival rate of these neurons and did not enhance axonal growth. However it increased the diameter of growing axons under certain conditions and also strongly upregulated galanin (GAL) expression in neurons and growing axons which was partially antagonized by NGF administration.

In adult animals, NFs play a major role in determining axonal diameter and conduction velocity, but little is known about the mechanisms by which NF expression is regulated. In this study, I investigated expressions of NF subunits in regenerating axons using immunohistochemistry and observed that NF-H was expressed before NF-M and NF-L. The neurotrophins NGF and brain-derived neurotrophic factor (BDNF) accelerated NF expression, but neurotrophin-3 (NT-3) was ineffective. However, axonal contact with the distal stump of a nerve in culture was far more effective than the neurotrophins in promoting NF expression.

Finally, the effects of diffusible factors from lesioned nerves on axonal growth were investigated and shown to be mainly due to NGF, which would be expected to promote regeneration of only those axons expressing the appropriate

NGF receptor, trkA. The effects of FGF-1 were also investigated and evidence presented that this factor may be able to stimulate regeneration of axons other than those expressing trkA.

Acknowledgements

I would like to thank my supervisor Dr. David Tonge for his excellent guidance throughout this work. I would also like to express my appreciation to Dr. Peter McNaughton and his group who have always been very helpful. Thanks to Dr. Philip Gordon-Weeks who introduced me to the confocal microscopy technique. Special thanks to Mr. Clive Daws who, many times, helped me to solve bureaucratic problems, which I experienced as an overseas student. Many thanks to all friends and the staff who made the life enjoyable at King's. Finally, I would also like to express my gratitude to my wife who has always supported me.

Abbreviations

BDNF	Brain-derived neurotrophic factor
CAM	Cell adhesion molecule
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
CM	Conditioned medium
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DRG	Dorsal root ganglion
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GAL	Galanin
GAP-43	Growth-associated protein-43
GDNF	Glial cell line-derived neurotrophic factor
HSPG	Heparan sulfate proteoglycan
IB4	Isolectin from <i>Bandeiraea simplicifolia</i>
IGF	Insulin-like growth factor
IL-1	Interleukin-1
LIF	Leukemia inhibitory factor
NeuN	Neuronal nucleus
NF	Neurofilament
NF-H	Neurofilament heavy
NF-M	Neurofilament medium (intermediate)
NF-L	Neurofilament light
NGF	Nerve growth factor
NPY	Neuropeptide tyrosine
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
NTN	Neurturin
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PNS	Peripheral nervous system
PSP	Persephin
RET	Receptor tyrosine kinase
Sc	Slow component of axonal transport
SCG	Superior cervical ganglion
SP	Substance P
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Trk	Tyrosine kinase
VIP	Vasoactive intestinal peptide

Contents

Chapter 1 : General introduction	13
1.1 Neuropeptide expressions and activity of growth factors in PNS with special reference to DRGs	15
1.1.1 Histology of DRGs	15
1.1.2 Neuropeptide expression in DRGs	15
1.1.3 Trophic support to PNS by growth factors	15
1.1.3.1 Neurotrophin family	16
1.1.3.1.1 Common properties of neurotrophin family	16
1.1.3.1.2 Nerve growth factor	19
1.1.3.1.3 Brain-derived neurotrophic factor	20
1.1.3.1.4 Neurotrophin-3	21
1.1.3.1.5 Neurotrophin-4/5	22
1.1.3.2 The cytokine family	23
1.1.3.3 Glial cell line-derived neurotrophic factor	25
1.1.3.4 Fibroblast growth factors	26
1.2 Changes in sensory neurons following peripheral nerve injury	29
1.2.1 Changes in neuropeptide expression	30
1.2.2 Changes in cytoskeleton and expression of growth-associated proteins	32
1.2.3 Explanations for the changes in gene expression following axotomy	32
1.3 Aims and rationale of this thesis	34
Chapter 2 : Material and Methods	36
2.1 Animal procedures	36
2.1.1 Removal of DRGs from mice	36
2.1.2 Peripheral nerve axotomy and crushing	36
2.2 Tissue cultures	36
2.2.1 Preparation of collagen gels	36
2.2.2 Matrigel	37
2.2.3 Setting up cultures	37
2.2.4 Fixation of preparations	38
2.2.5 Frozen sections	38
2.3 Immunofluorescence	38
2.4 Immunocytochemistry and stereography	39
2.5 Microscopy and image analysis	39
Chapter 3 : Survival of DRG neurons in explant cultures and effects of NGF and LIF	41
3.1 Introduction	41
3.1.1 The time course and extent of neuronal death following axotomy	41
3.1.2 Neuronal death and phenotype	43
3.1.3 Growth factors and sensory neuronal survival	43

3.1.4 Rationale for the study	44
3.2 Experimental procedures	46
3.2.1 Tissue cultures	46
3.2.2 Immunohistochemistry to determine neuronal numbers	46
3.2.3 Determination of live and dead cells	48
3.2.4 Immunohistochemistry to determine proportions and phenotypes of live and dead neurons	53
3.3 Results	54
3.3.1 Neuronal survival in explant DRG cultures and effects of NGF and LIF	54
3.3.2 The phenotypic properties of DRG neurons in explant cultures and neuronal death	55
3.4 Discussion	59
3.4.1 The majority of DRG neurons survived 3 days in explant cultures	59
3.4.2 There is no evidence for selective neuronal loss in explant DRG cultures	65
3.4.3 The expression of CGRP and RT97 and IB4 labelling of DRG neurons in explanted cultures	65
3.4.4 NGF increased overall neuronal survival	67
3.5 Conclusion	68
Chapter 4 : Effects of LIF and NGF on axonal growth and GAL expression in explant DRG cultures	69
4.1 Introduction	69
4.1.1 Leukemia inhibitory factor as a lesion-induced cytokine	69
4.1.2 LIF, GAL expression and NGF	71
4.1.3 Rationale for the study	72
4.2 Materials and methods	72
4.2.1 Animal procedures and tissue cultures	72
4.2.2 Immunohistochemistry, microscopy and image analysis	73
4.3 Results	74
4.3.1 Effects of LIF and NGF on axonal growth in collagen gels	74
4.3.2 GAL expression in outgrowing axons	78
4.3.3 GAL expression in sections	79
4.4 Discussion	86
4.4.1 LIF has no effect on the extension of outgrowing axons but in- creases axonal diameter	86
4.4.2 LIF up-regulates GAL expression in explanted DRG cultures	87
4.5 Conclusion	90
Chapter 5 : Expression of neurofilaments and effects of growth factors in explant DRG cultures	91
5.1 Introduction	91
5.1.1 Structure and the family of neurofilaments	91

5.1.2 Expression of neurofilaments	95
5.1.3 Axonal transport of neurofilaments	95
5.1.4 Neurofilaments and radial axonal growth.....	96
5.1.5 Neurofilament expression following axotomy	97
5.1.6 Rational for the study	98
5.2 Experimental Procedures	100
5.2.1 Tissue cultures	100
5.2.2 Immunohistochemistry and microscopy	100
5.3 Results	102
5.3.1 NF expression in outgrowing axons of normal preparations	102
5.3.2 NF expression in outgrowing axons of conditioned preparations	109
5.3.3 NF expression in DRG neurons	109
5.3.4The expression of NFs in explant DRG cultures with crushed nerves	113
5.4 Discussion	117
5.4.1 NF subunits are differentially expressed by outgrowing axons in DRG explant cultures	117
5.4.2 Growth factors increase expression of NFs in outgrowing axons	120
5.4.3 Lesioned distal nerve segment supports the expression of NFs ..	122
5.5 Conclusion	123
Chapter 6 : Effects and identification of diffusible factors released from lesioned nerves	124
6.1 Introduction	124
6.1.1 Rationale for the study	126
6.2 Experimental procedures	126
6.2.1 Explant cultures	126
6.2.2 Immunohistochemistry, microscopy and image analysis	127
6.3 Results	131
6.3.1 The effect of CM on axonal growth and the influence of trk chime- ras.....	131
6.3.2 Comparison of effects of FGF-1 and NGF on axonal growth and phenotypes of outgrowing axons	131
6.4 Discussion	132
6.4.1 CM enhances axonal outgrowth and NGF seems to be responsible for most of this effect	132
6.4.2 FGF-1 stimulates axonal outgrowth in vitro	137
6.5 Conclusion	138
Chapter 7 : General discussion.....	139
References.....	142

Tables

Chapter 1

Table 1.1 Changes in neuropeptide expression in axotomized sympathetic, sensory and motor neurons.	30
---	----

Chapter 3

Table 3.1. The concentrations and sources of factors used.	47
Table 3.2. The concentrations and sources of primary antibodies and dyes used.	47
Table 3.3. The concentrations and sources of secondary antibodies used. ..	47
Table 3.4. Numbers of neurons in freshly fixed (control) and 3 days cultured (C3) T10-T12 DRGs.	56
Table 3.5. Numbers of neurons in control and 3 days cultured preparations in the presence and absence of NGF or LIF.	56
Table 3.6. Comparison of means of neuronal numbers of 3 days cultured and freshly fixed T10-T12 DRGs.	56
Table 3.7. The percentage of dead neurons on the frozen sections of cultured thoracic DRGs.	56
Table 3.8. The comparison of the numbers of dead and total neuronal profiles stained for different markers from frozen sections of cultured DRGs.	57
Table 3.9. The comparison of the changes in phenotypic markers in cultured preparations and the freshly fixed controls.	58
Table 3.10. The survival of T10-12 DRG neurons in explant cultures exposed to alkaline environment during last 12 hours on the last day of 3 day incubation.	58

Chapter 4

Table 4.1. The concentrations and sources of factors used.	74
Table 4.2. The concentrations and sources of primary antibodies.	74
Table 4.3. The concentrations and sources of secondary antibodies used. ..	74
Table 4.4. Axonal lengths and diameters in DRG explant cultures.	76
Table 4.5. Percentages and brightness of GAL immunoreactive axons.	79
Table 4.6. Percentages and brightness of GAL immunoreactive neurons. ..	84

Chapter 5

Table 5.1. The concentrations and sources of factors used.	101
Table 5.2. The concentrations and sources of primary antibodies used. ...	101
Table 5.3. The concentrations and sources of secondary antibodies used.	101
Table 5.4. Time course of first appearance of NF subunit-immunoreactivities in outgrowing axons in explant DRG cultures.	102
Table 5.5. The effects of growth factors on the expressions of NFs in normal preparations after 3 days in culture.	102

Table 5.6. The mean numbers of axons per preparation expressing NF-M after 3 days in normal and conditioned DRG cultures.	102
Table 5.7. The total number of axons expressing NF-L in conditioned preparations.	109
Table 5.8. The mean numbers of neuronal profiles expressing NFs in optical sections.	113

Chapter 6

Table 6.1. The concentrations and sources of factors used.	128
Table 6.2. The concentrations and sources of primary antibodies.	128
Table 6.3. The concentrations and sources of secondary antibodies used.	128
Table 6.4. Lengths of the outgrowing axons in explant DRG cultures with CM.	131
Table 6.5 Lengths of the outgrowing axons in explant DRG cultures with factors.	132
Table 6.6 Percentages of outgrowing axons labelled by anti-CGRP and RT97.	132

Figures

Chapter 1

Figure 1.1 Growth factor receptor expressions define distinct populations in adult DRGs.	18
Figure 1.2 Changes in neuropeptide expression in DRG neurons following axotomy... ..	31

Chapter 2

Figure 2.1 Illustration to show the technique to explant mouse DRGs in vitro.	37
--	----

Chapter 3

Figure 3.1 The method of neuronal counting by optical sectioning.	49
Figure 3.2 NeuN antibody stained neuronal nuclei (yellow arrows) with occasional slight cytoplasmic staining (white arrows) with nucleoli visible as dark spots (red arrows)... ..	50
Figure 3.3 Determination of dead and living cells by MTT and PI methods ..	51
Figure 3.4 PI stained cytoplasm of dead cells and nuclei of all cells including microglia during processing... ..	52
Figure 3.5 The superimposition of laminin and PI staining showing a few dead neurons... ..	53
Figure 3.6 The superimposed image of all three different staining: PI, laminin and phenotypic marker, in this case CGRP.	54

Figure 3.7 The proportions of CGRP, RT97 and IB4 labelled neurons in normal DRGs and explant cultures.	59
Figure 3.8 CGRP expression in a DRG section.	60
Figure 3.9 RT97 expression in a DRG section.	61
Figure 3.10 IB4 labelling in a DRG section.	62
Figure 3.11 The estimated range of neuronal death in thoracic DRG explant cultures in 3 days.	63

Chapter 4

Figure 4.1 An illustration showing the sites of LIF induction and the changes in GAL, SP, and NPY in DRG (top) and SCG (bottom) after axotomy and source of NGF (modified from Sun et al., 1996a).	70
Figure 4.2 Axonal outgrowth from explanted DRGs into collagen gel in 48 hours.	75
Figure 4.3 Outgrowing axons from explant DRG cultures in control (A) and LIF (B) preparations.	76
Figure 4.4 Outgrowing axons from explant DRG cultures with NGF (A) or NGF+LIF (B).	77
Figure 4.5 Thickness of outgrowing axons in control (A), LIF (B), NGF (C) and NGF+LIF treated (D) preparations.	78
Figure 4.6 GAL expression in outgrowing axons in control (A) and LIF treated (B) preparations.	80
Figure 4.7 GAL expression in outgrowing axons in NGF (A) and NGF+LIF treated (B) preparations.	81
Figure 4.8 GAL immunoreactivity in outgrowing axons.	82
Figure 4.9 GAL immunoreactivity in sections of L4 DRG's.	83
Figure 4.10 GAL immunoreactivity in DRG neurons in normal (A) and axotomized (B) preparations.	84
Figure 4.11 GAL immunoreactivity in DRG neurons in control (A) and LIF treated (B) preparations after 3 days in culture.	85

Chapter 5

Figure 5.1 An illustration showing the main components of axonal cytoskeleton.	92
Figure 5.2 Structure of NF protein (A).	93
Figure 5.3 NF-H immunoreactivity in DRG explant cultures after 3 days (A and B).	103
Figure 5.4 NF-M immunoreactivity in explant DRG cultures after 3 (A) and 6 (B) days.	104
Figure 5.5 NF-L immunoreactivity in explant DRG cultures after 9 (A) and 12 (B) days.	105
Figure 5.6 High magnification images showing NF-M (A) and NF-L (B) immunoreactivities on 6th and 12th days respectively.	106
Figure 5.7 NF-M (A) and NF-L (B) labelling in the presence of NGF after 3 days in culture.	107

Figure 5.8 NF-M immunoreactivity in the presence of NT-3 (A) and BDNF (B) after 3 days in culture.	108
Figure 5.9 NF-M immunoreactivity in conditioned preparations with BDNF at low (A) and high (B) power magnifications.	110
Figure 5.10 NF-H (A) and NF-L (B) immunoreactivities in optical sections of cultured control DRGs after 3 days of incubation	111
Figure 5.11 NF-M immunoreactivity in optical sections of control (A) and BDNF-added (B) DRG preparations	112
Figure 5.12 Low (A) and high (B) magnification confocal images of a crushed DRG preparation showing NF-H immunoreactivity in regenerated axons across the gap.	114
Figure 5.13 NF-M (A) and NF-L (B) immunoreactivities in regenerated axons of crushed preparations.	115
Figure 5.14 NF-H (A), NF-M (B) and NF-L (C) immunoreactivities in totally crushed peripheral nerves of cultured DRGs after 6 days of incubation	116

Chapter 6

Figure 6.1 Axonal outgrowth in explant DRG cultures with CM (A), CM with TrkA (B) and TrkB (C) chimeras.	129
Figure 6.2 Axonal growth in explant DRG cultures with CM from which NGF was sequestered off.	130
Figure 6.3 Axonal growth in control (A), aFGF (B) and NGF (C) treated explant DRG cultures.	133
Figure 6.4 Percentages of CGRP and RT97 expressing axons growing in control preparations, aFGF, NGF and aFGF+NGF treated ones.	134

Chapter 1 :General introduction

Neurons of central and peripheral nervous systems can be classified into different groups according to their specific properties including size, shape, type of neurotransmitters they recruit, mode of action (eg. inhibitory/excitatory), location in the body, proteins they synthesize and functional aspects. For example, according to the size and shapes of their processes, most neurons can be included in one of the following categories: multipolar neurons, which have more than 2 cell processes, one being the axon and the others dendrites; bipolar neurons, with one dendrite and one axon; and pseudo-unipolar neurons, which have a single process close to the perikaryon, but which divide into 2 branches, forming a T shape, one branch extending to a peripheral ending and the other toward the central nervous system (CNS). Neurons can transmit signals to muscles and are referred to as motoneurons; they can also convey messages from sensory endings to CNS and hence are named as sensory neurons. The neurons that use acetylcholine as a neurotransmitter are known as cholinergic neurons, as some others are similarly described as nor-adrenergic, dopaminergic etc.

Despite these phenotypic differences, all neurons are basically specialized to maintain communication in and between CNS and other parts of the body. In fact, most of the time these differences serve to meet special requirements of a physiological function. For example, axon diameter determines conduction velocity (Gasser and Grundfest 1939) and may be a trigger for myelination (Arbuthnott *et al.*, 1980; Voyvadic 1989). Axon diameter, in turn, is determined by neurofilament (NF) content (Hoffman *et al.*, 1984; Wong *et al.*, 1995). Larger diameter axons are required for rapid transmission of nerve signals, for example, from motor neurons to muscle fibers.

Communication of neurons with other cells involves release of neurotransmitters. These include classical, small, fast acting neurotransmitters which act via ligand-gated receptors; as well as larger, slower acting, neuropeptides which act via G-protein linked receptors. The best known members of fast acting neurotransmitters are acetylcholine; amines:

norepinephrine, epinephrine, dopamine, serotonin and histamine; and amino acids: γ -aminobutyric acid (GABA), glycine, glutamate and aspartate. Small, fast acting neurotransmitters are synthesized in the cytosol of pre-synaptic nerve terminals and incorporated into synaptic vesicles. Other important features of these molecules are their rapid mode of action and recyclable nature.

Neuropeptides include a number of hormones which are released from parts of CNS like hypothalamus and pituitary gland. Thyrotropin-releasing hormone, growth hormone and prolactin are only a few of these. There are also members which are synthesized and act in CNS and peripheral nervous system (PNS) such as substance P (SP), calcitonin gene related peptide (CGRP), galanin (GAL), cholecystokinin (CCK), vasoactive intestinal peptide (VIP) and neuropeptide tyrosine (NPY) (Hökfelt *et al.*, 1994). Neuropeptides are generally synthesized as integral parts of large protein molecules in the neuronal cell body. They are moved to the axon terminals in the slow component of axonal transport packed into vesicles and once released they are broken down instead of being recycled. Neuropeptides are synthesized in relatively much smaller quantities compared to fast acting small transmitters.

To survive and maintain their physiological functions mainly during development and also later in adulthood, trophic support is essential for many neurons. This support is provided by a number of growth factors and may become more important under unfavorable conditions such as an injury.

The primary sensory neurons of dorsal root ganglia (DRGs) are ideal cells to study both the expression of neuropeptides and the effects of growth factors in normal animals and following an injury. This is due to their accessibility for *in vitro* and *in vivo* studies, significant involvement of growth factors in the development and functioning of DRG neurons and abundant and diverse expression patterns of neuropeptides by these cells. Throughout this thesis, I used mouse DRGs in the experiments I conducted.

Investigation of the properties and actions of neuropeptides and growth factors has been topic of intense interest in recent years but the picture is not yet complete. This thesis presents studies with emphasis on neuropeptide

expressions and actions of growth factors in DRGs.

1.1 *Neuropeptide expressions and activity of growth factors in PNS with special reference to DRGs*

1.1.1 Histology of DRGs

DRGs are located in the dorsal roots of the spinal nerves. They are pseudo-unipolar primary sensory neurons. The 2 branches of the single T-shaped process constitute one axon, and the peripheral branch has a dendritic arborization. In this instance, the dendrites are not expansion of the cell body but of an axon. The function of perikarya of pseudo-unipolar neurons is believed to be mainly metabolic. DRGs contain, side by side, small nerve cell bodies 10-20 μm in diameter and large ones up to 50 μm in diameter. In histological sections, DRG neurons are round in shape and surrounded by small satellite cells.

1.1.2 Neuropeptide expression in DRGs

Neuropeptide expression needs to be considered with reference to the substantial changes which occur following axotomy. As far as primary sensory neurons are concerned neuropeptides are often grouped as two types (see review by Hökfelt *et al.*, 1994). Type I consist of those neuropeptides that are normally expressed but down-regulated after nerve injuries e.g. CGRP, SP and SOM (Jessell *et al.*, 1979; McGregor *et al.*, 1984; Noguchi *et al.*, 1990). Type II neuropeptides on the other hand are normally expressed at very low levels but then up-regulated following nerve damage, e.g. GAL, VIP, NPY and CCK (fig. 1.2) (McGregor *et al.*, 1984; Shehab and Atkinson, 1986; Hökfelt *et al.*, 1987; Villar *et al.*, 1989; Verge *et al.*, 1992).

1.1.3 Trophic support to PNS by growth factors

Another group of proteins with diverse actions in PNS are growth factors. Members of neurotrophin family especially play important roles during development of DRG neurons. This family includes nerve growth factor (NGF), brain-derived growth factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). A number of other growth factors such as cytokines leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), glial cell line-derived

neurotrophic factor (GDNF), fibroblast growth factors (FGFs) also have significant contributions to the survival of primary sensory neurons.

1.1.3.1 Neurotrophin family

1.1.3.1.1 Common properties of neurotrophin family

All neurotrophin family members share 50-60% amino acid identity and contain six conserved cysteine residues, which in NGF form three intrachain disulfide bonds necessary for biological activities (Bradshaw, 1978). They have molecular weights of 26-27 kDa and dimer structure, formed by two identical 119 amino acid subunits except NT-4 which has 130 amino acid subunits (Levi-Montalcini and Angeletti, 1968; Leibrock *et al.*, 1989; Hohn *et al.*, 1990; Ip *et al.*, 1992).

Neurotrophin family members bind to two different types of receptors. One type includes high affinity cell surface tyrosine kinases (trk) with three different identified subtypes. These are trkA which binds NGF (Kaplan *et al.*, 1991; Klein *et al.*, 1991a), trkB which binds BDNF (Klein *et al.*, 1991b) and NT-4 (Klein *et al.* 1992) and trkC, which binds NT-3 (Lamballe *et al.*, 1991). TrkA and trkB bind also NT-3 but with much less affinity than their cognate ligands (Soppet *et al.*, 1991, Belliveau *et al.*, 1997).

The other neurotrophin receptor is low affinity receptor p75 that binds all neurotrophins with equal affinity (Chao *et al.*, 1986; Radeke *et al.*, 1987).

The two different receptors have different functional aspects. Trks are usually considered to be directly involved in the trophic activities such as axonal growth, neuronal cell survival and cell proliferation whereas p75 seems to have a modulatory effect on trk activity beside its other roles such as in programmed cell death, cellular migration and retrograde transport of neurotrophic factors (for a review see Chao and Hamstead, 1995; Bamji *et al.*, 1998).

Expression patterns of high affinity neurotrophin receptors define distinct subgroups of primary sensory neurons and also reflect their responsiveness to trophic factors during development and in adult animals. High affinity neurotrophin receptors trkA, trkB and trkC, and low affinity receptor p75

mRNAs are all expressed as early as embryonic day 12 (E12) in DRG neurons (Merlio *et al.*, 1992; Ernfors *et al.*, 1993; Mu *et al.*, 1993). The different trks are expressed by distinct populations of neurons. TrkA is expressed predominantly by small neurons with darkly staining cytoplasm. TrkB is expressed by intermediate, and trkC by large lightly staining DRG neurons (Mu *et al.*, 1993, Ernfors *et al.*, 1993; McMahon *et al.*, 1994). p75, on the other hand, is localized to the DRG neurons of different sizes (Carroll *et al.*, 1992; Schecterson and Bothwell, 1992). Until E13 which is developmentally a critical period, most DRG neurons appear to express trkC and require mainly locally synthesized NT-3 for survival (Ernfors *et al.*, 1992; Buchman and Davis, 1993, Snider and Silos-Santiago; 1996). Indeed, 55%-75% of DRG neurons are lost in NT-3 knockout mice (Ernfors *et al.*, 1994b; Fariñas *et al.*, 1994). By E13.5, in mouse, most DRG neurons down-regulate trkC and can be shown to be dependent on trkA signalling for survival (Snider and Silos-Santiago, 1996). However, the proprioceptive neurons and another subpopulation continue to express trkC into adulthood (Wright and Snider, 1995). During the period from E13 to birth, 80% of DRG neurons continue to express trkA, a finding which is consistent with the 80% loss of DRG neurons in NGF and trkA knockout mice (Silos-Santiago *et al.*, 1995). However, between birth and postnatal day 21, nearly 50% of neurons that express trkA during development down-regulate trkA (Snider and Silos-Santiago, 1996; Molliver and Snider, 1997).

In the adult animal, about 40-45% of DRG neurons express trkA (fig. 1.1) (Averill *et al.*, 1995). A small percentage of DRG neurons express high levels of trkB and do not co-express any other trks. Neurons that express trkC are the largest neurons in adult DRGs and about 15% of these also express trkA (Wright and Snider, 1995). Finally about 30% of DRG neurons which are small in size do not express any trks except a small overlap with trkA (Averill *et al.*, 1995). This population can be identified by binding lectin Isolectin B4 from *Bandeiraea simplicifolia* (IB4) and these cells have been shown to express receptor for GDNF (Molliver *et al.*, 1997; Bennet *et al.*, 1998). The low affinity receptor p75 is confined only to trk bearing neurons and co-expressed by almost all trkA, trkB neurons and about half of the trkC neurons (Wright and Snider,

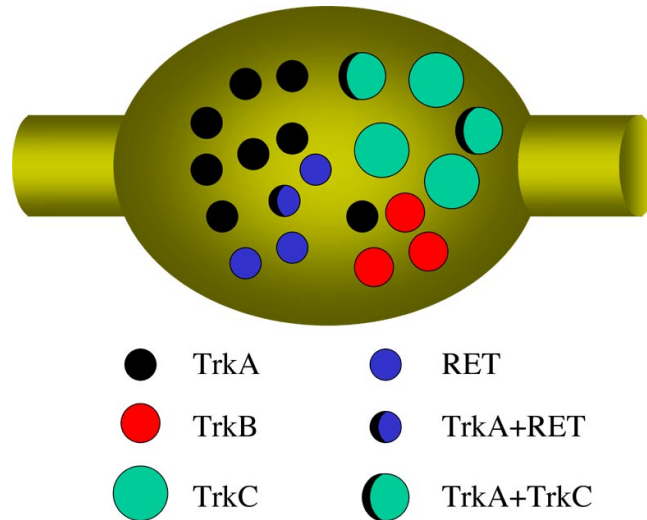


Figure 1.1 Growth factor receptor expressions define distinct populations in adult DRGs. Majority of sensory neurons express at least one of the high affinity neurotrophin receptors (trks). About 40-45% of all DRG neurons, which are mostly small and medium size, express trkA. Large and medium size neurons express trkC while some of them also express trkA. A small population of medium size neurons express trkB and they do not have co-expression of any other trks. About 30% of all DRG neurons do not have any trks and this population expresses GDNF receptor subunit RET. There is a small proportion of RET neurons which co-express trkA (see text for references)

1995).

Apart from their known trophic actions, neurotrophins may be actively involved in signal transmission in CNS and PNS. For example, NGF plays important roles in transmission of nociception, especially inflammatory pain by regulating sensitivity of nociceptive systems, regulating expression of sensory peptides such as CGRP and SP (for a review see McMahon, 1996). BDNF itself may act like a neurotransmitter and may fulfill all of the classical criteria to be considered as a neurotransmitter (for a review, see Altar and DiStefano, 1998).

Two other members of neurotrophin family have been recently isolated in fish and were termed neurotrophin-6 and neurotrophin-7 (Gotz *et al.*, 1994; Nilsson *et al.*, 1998; Lai *et al.*, 1998).

1.1.3.1.2 Nerve growth factor

NGF was the first member of neurotrophin family to be discovered (Levi-Montalcini, 1951; Cohen, 1960; Levi-Montalcini and Angeletti, 1968). It is the best known and most widely studied neurotrophic factor; partially because of its abundant availability from the salivary glands of male mice. It is produced by many peripheral tissues of the body but levels expressed are generally very low (Shelton and Reichardt, 1984).

It is widely accepted that during certain stages of embryonic development NGF plays a crucial role in the survival of sympathetic and neural crest-derived sensory neurons (Levi-Montalcini and Angeletti, 1968; Johnson *et al.*, 1980, 1983; Kessler and Black, 1980; Otten *et al.*, 1980; Lindsay, 1988a). Mice lacking genes for NGF and its high affinity receptor trkA have been found to extensively lose small DRG neurons which are responsive to pain and temperature and also some sympathetic neurons (Crowley *et al.*, 1994; Smeyne *et al.*, 1994). In parallel to these findings, NGF has been shown to rescue immature DRG and sympathetic neurons from axotomy - induced neuronal death (Hendry, 1975; Yip *et al.*, 1984).

Johnson and Yip (1985), suggested that the main trophic support for adult DRG neurons is provided by NGF together with some unknown factor(s) from the periphery and central nervous system. The most common experimental model to test this hypothesis involves the local application of NGF to the proximal stump of an transected peripheral nerve and a number of similar studies have demonstrated the effectiveness of NGF in preventing neuronal death after axotomy. In one of the earliest experiments Hendry and Campbell (1976) showed this in rat superior cervical ganglia. Rich *et al.* (1987) and Otto *et al.* (1987) reported that, after axotomy, NGF applied to the sciatic nerve in special chambers completely prevented the neuronal death which usually occurred at a rate of 16-22% and 35% respectively between 3-6 weeks in L4-L6 DRGs of untreated rats. Transient NGF treatment in the long term, on the other hand, decreased the cell loss in a similar experiment by only 55% (Rich *et al.*, 1989). Verge *et al.* (1995) demonstrated that NGF could counteract some phenotypic changes seen following axotomy. Although NGF has been shown to support DRG neurons

of even 24 month old rats (Manfridi *et al.*, 1992), there are controversial reports about the absolute requirement for NGF for survival during regeneration of axotomized sensory neurons. Gorin and Johnson (1980) and Schwartz *et al.*, (1982) demonstrated that neutralization of NGF by using antiserum did not promote neuronal loss in adult rats and guinea pigs. Similarly, chronic NGF deprivation in guinea pigs did not cause cell death in DRGs although it did cause an 18% reduction of mean neuronal size (Rich *et al.*, 1984).

In an *in vitro* study Lindsay (1988b) reported that adult sensory neurons do not require NGF for survival in culture for long periods (3-4 weeks) although it enhances axonal regeneration. This study seems to rule out the possibility of cell rescue by NGF in mature animals. However this experiment needs to be interpreted with caution. Because, some neuronal loss is likely to occur during the dissociation procedure while isolating neurons and it is difficult to exclude the possibility that these might be the ‘vulnerable’ neurons lost after axotomy.

NGF has trophic effects also in some part of CNS including striatal and septal cholinergic neurons in the brain (Gnahn *et al.*, 1983; Hefti *et al.*, 1984; Mobley *et al.*, 1985).

1.1.3.1.3 Brain-derived neurotrophic factor

BDNF was the second member of neurotrophin family to be isolated (Leibrock *et al.*, 1989; Ernfors *et al.*, 1990a). Unlike NGF, BDNF expression is not detected in the periphery, with the exceptions of lung, heart and skeletal muscles (Leibrock *et al.*, 1989; Hofer *et al.*, 1990; Maisonpierre *et al.*, 1990). However, in the CNS, expression of BDNF mRNA is quite extensive including the spinal cord (Hofer *et al.*, 1990; Maisonpierre *et al.*, 1990; Wetmore *et al.*, 1990).

Ablation of genes for BDNF or its high affinity receptor trkB led to elimination of intermediate-size DRG neurons and reduction in volume of vestibular and trigeminal ganglia and nodose-petrosal complex (Ernfors *et al.*, 1994a; Conover *et al.*, 1995). Although the animals with disrupted BDNF gene survived until postnatal day 8, trkB knockout mice had severe CNS and PNS deficiencies and died within 24 hours after birth (Klein *et al.*, 1993; Conover *et*

al., 1995). In another study, BDNF has been shown to support survival of E12.5 DRG neurons (Memberg and Hall, 1995). It has been shown that BDNF rescues axotomized motoneurons of newborn mice and rats and reduces naturally occurring retinal ganglion cell death during development (Li *et al.*, 1994; Vejsada *et al.*, 1988; Ma *et al.*, 1998).

These studies suggest that the actions of BDNF are important to developing and possibly mature sensory neurons. BDNF is also expressed by a subpopulation of adult DRG neurons which are small in size (Ernfors *et al.*, 1990b; Barakat-Walter, 1996). These are mainly trkA expressing cells and NGF dramatically increases proportion of BDNF expressing neurons in this group (Michael *et al.*, 1997). It has been demonstrated that locally produced BDNF acts in an autocrine mechanism in adult rat DRG cultures to promote survival of neurons even in the absence of other growth factors (Acheson *et al.*, 1995). An alternative mode of action on trkB cells in a paracrine manner also has been suggested based on the presence of BDNF and its high affinity receptor trkB in adult DRGs in different populations of neurons (Apfel *et al.*, 1996). This neurotrophin has been suggested to be the predominant source of trophic support for mature sensory neurons (Acheson *et al.*, 1995).

Another interesting feature of BDNF is that it is anterogradely transported to the nerve terminals from the cell body where it is produced (for a review see Altar and DiStefano, 1998). This has been shown to occur from DRG neurons to sciatic nerve or to the dorsal root (Michael *et al.*, 1997). Anterograde transport of BDNF in multiple parts of brain has also been demonstrated (Conner *et al.*, 1997; Altar *et al.*, 1997; Fawcett *et al.*, 1998)

1.1.3.1.4 Neurotrophin-3

Neurotrophin-3 has been identified and cloned through its sequence homology to NGF and BDNF (Hohn *et al.*, 1990). Disruption of expression of NT-3 or its receptor trkC caused severe sensory deficits. NT-3 knockout mice had severe movement defects of limbs, loss of substantial number of peripheral sensory and sympathetic neurons, lack of spinal proprioceptive afferents and peripheral sense organs, loss of parvalbumine and carbonic anhydrase-positive

neurons in DRG (Ernfors *et al.*, 1994b; Klein *et al.*, 1994).

The inhibition of trkC receptors by antibodies during development results in severe reductions in multiple neuronal populations (Lefcort *et al.*, 1996). NT3 has been found to be effective in promoting survival of embryonic DRG neurons (Memberg and Hall, 1995). Unlike other members of neurotrophin family its expression is not up-regulated following axotomy, on contrary, NT-3 mRNA is expressed at relatively high levels in normal animal but decreases after peripheral nerve transection and returns to normal only after about two weeks (Funakoshi *et al.*, 1993). It was reported that NT-3 promotes the survival of up to 60% of DRG neurons in culture (Maisonpierre *et al.*, 1990) and can rescue certain subpopulations of sensory neurons which normally do not respond to NGF such as the proprioceptive neurons from the trigeminal mesencephalic nucleus (Hohn *et al.*, 1990). In fact it is known that NT-3 selectively rescues sensory neurons innervating limb muscles which are probably proprioceptive (Horry-Lee *et al.*, 1993). NT-3 has also been shown to support survival of axotomized motoneurons in newborn mouse (Li *et al.*, 1994).

1.1.3.1.5 Neurotrophin-4/5

Neurotrophin-4 was first cloned from *Xenopus laevis* (Halböök *et al.*, 1991). Another mammalian neurotrophin was cloned by two groups, one of which concluded that this factor was a homologue of the frog NT-4, while the other group claimed that it was structurally too divergent to be considered a mammalian homologue of NT-4 (Berkemeier *et al.*, 1991; Ip *et al.*, 1992). Consequently, the factor was named NT-4 or NT-5, and commonly referred to as NT-4/5.

NT-4/5 shares high affinity receptor trkB with BDNF (Klein *et al.*, 1992). It differs from other members of the family in the sense that it is less important during development for survival of sensory neurons. While genetically modified mice lacking each of NGF, BDNF and NT-3 genes had severe neuronal deficits and died soon after birth, lack of the NT-4/5 gene resulted in only limited sensory neuronal loss (Conover *et al.*, 1995; Conover and Yancopoulos, 1997) which was mainly detected in nodose – petrosal and geniculate ganglia (Liu *et al.*,

1995). This deficit was greatly augmented by additional deletion of the BDNF gene (Liu *et al.*, 1995). Those and other studies have established that NT-4/5 is a survival factor for certain embryonic cranial sensory neurons (Davies *et al.*, 1993). In the adult animal, it promotes survival of different sensory neuronal populations including retinal (Cohen *et al.*, 1994; Peinado-Ramón, 1996); spiral (Zheng *et al.*, 1995) and trigeminal ganglion cells (Ibáñez *et al.*, 1993).

Although no motor or sympathetic neuronal loss has been detected in NT-4/5 knockout mice, it has been shown to support survival of facial motoneurons in neonatal animals (Koliatsos *et al.*, 1994; Li *et al.*, 1994; Liu *et al.*, 1995). It has been also demonstrated to promote survival and differentiation of developing rat striatal neurons *in vitro* (Widmer and Hefti, 1994).

1.1.3.2 The cytokine family

CNTF and LIF are two structurally related factors that belong to the cytokine family, with widespread actions throughout the body (Bazan, 1991). Other members of this family include interleukins-6 and -11, cardiotrophin-1 and oncostatin M (Patterson, 1992, Pennica *et al.*, 1995). They share a common three-dimensional configuration, a common receptor subunit, gp130 and signal transduction pathways in neuronal cells (Bazan, 1991; Ip *et al.*, 1992; for a review on this family see Murphy *et al.*, 1997)).

Human LIF is a 19.7 kDa protein containing 180 amino acid residues which was first purified and cloned in 1987 (Gearing *et al.*, 1987). LIF was so termed on the basis of its ability to differentiate the M1 murine leukemic cell line to monocytes and to inhibit their proliferation. The functional LIF receptor is composed of two transmembrane proteins, LIFR β and gp130. LIFR β binds LIF with low affinity and gp130 alone does not significantly bind LIF. However these two subunits together form a high affinity receptor complex (Ip *et al.*, 1992). Heymann *et al.* (1996), on the other hand, proposed existence of a third subunit with a molecular weight of 140-150 kDa to form a high affinity LIF receptor in human. Although LIF is normally not detectable in peripheral nervous system, its expression is induced strongly in axotomized nerve (Curtis *et al.*, 1994; for a more detailed account see chapter 4).

CNTF is a potent neural growth factor that was originally characterized as a survival factor for chick ciliary neurons *in vitro* (Varon *et al.*, 1979). The factor was first purified from chick eye and termed CNTF (Barbin *et al.*, 1984). Subsequently purified, sequenced and cloned from rat as a 22.7 kDa protein containing 199 amino acid residues (Lin *et al.*, 1989). CNTF has a specific receptor component CNTFR- α to which CNTF first has to bind in order to subsequently recruit gp130 and LIFR- β to start its signaling pathway (Davies *et al.*, 1991; Stahl and Yancopoulos, 1994). Unlike LIF, CNTF, has been shown to be constitutively present in Schwann cells of peripheral nerves in relatively large quantities (Rende *et al.*, 1992).

LIF and CNTF have trophic and survival effects on multiple cell types especially during development *in vivo* and *in vitro* (Kushima, *et al.*, 1992; Pesce *et al.*, 1993; Mayer *et al.*, 1994; Lavranos, 1995; De Miguel *et al.*, 1996). In the nervous system, their main effects are on sensory and motor neurons.

The supportive effects of CNTF and LIF on sensory neurons have been shown in several experiments. However, defects in sensory ganglia development have not been reported in any of the knockout mice for LIF, CNTF, CNTFR- α and LIFR β , indicating that neither factors are essential for the development of sensory neurons (Stewart *et al.*, 1992; Escary *et al.*, 1993; Masu *et al.*, 1993; Li *et al.*, 1995; Ware *et al.*, 1995; DeChiara *et al.*, 1995). On the other hand, Murphy *et al.* (1991) found that LIF promotes the differentiation and the survival of neural crest derived sensory neurons in culture (Murphy *et al.*, 1993a). In another study, CNTF and LIF were demonstrated to support also placode derived sensory neurons of nodose ganglion, which are normally not responsive to NGF (Thaler *et al.*, 1994). They are also effective in rescuing DRG neurons from axotomy-induced death (Lo *et al.*, 1995; Cheema *et al.*, 1994). However, LIF plays important roles in the regulation of phenotypic properties of DRG neurons *in vitro* (Mulder, 1994) and following axotomy in adult and newborn animals (see review by Zigmond *et al.*, 1996 and chapter IV for a detailed account).

The phenotypes of LIF or CNTF knockout mice did not exhibit any abnormalities in motor neurons (Stewart *et al.*, 1992; Escary *et al.*, 1993; Masu

et al., 1993). However, analysis of one group of CNTF knockout mice revealed that CNTF was required for motor neuron maintenance and survival post-natally and into adulthood (Masu *et al.*, 1993). This slowly appearing, relatively mild degeneration was substantially potentiated by simultaneous inactivation of the LIF gene (Sendtner *et al.*, 1996). These findings suggest that there is a compensation mechanism within this growth factor family where LIF possibly compensated for the loss of CNTF in CNTF knockout mice. In contrast to the ligand knockouts, the targeted deletion of receptor components LIFR β and CNTFR- α resulted in important changes in motor neuron development and death within 24 hours after birth (DeChiara *et al.*, 1995; Li *et al.*, 1995; Ware *et al.*, 1995). These findings establish that these two receptor components are essential for motor neuron development but do not show that any individual ligand is involved. The explanation of this situation is either compensation in any particular knockout animal by the remaining cytokines or the existence of additional members of the family which are not discovered yet.

1.1.3.3 Glial cell line-derived neurotrophic factor

Glial cell line-derived neurotrophic factor belongs to transforming growth factor- β (TGF- β) superfamily which specifically promotes dopamine uptake and survival of midbrain neurons. It is a disulphide-linked homodimer with a molecular weight of about 30 kDa formed by two identical 134 amino acid chains (Lin *et al.*, 1993). Other related factors identified so far are neurturin (NTN) and persephin (PSP) (Kotzbaeuer *et al.*, 1996; Milbrant *et al.*, 1998). A receptor complex, composed of GFR α -1 and the receptor tyrosine kinase (RET) binds GDNF (Treanor *et al.*, 1996). There are three more GFR α molecules (GFR α -2, GFR α -3 and GFR α -4) whose properties and ligands have not been fully identified except GFR α -2 which is known to bind NTN (for a review see Ibáñez, 1998).

Targeted deletion of GDNF gene caused renal agenesis and complete absence of enteric neurons in mice (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996). Substantial reductions in the number of motor, sensory and sympathetic neurons have been detected in these animals (Moore *et al.*,

1996).

GDNF has been shown to support DRG neurons during late embryogenesis and early postnatal period (Buj-Bello *et al.*, 1995) and these are IB4 lectin binding small sensory neurons (Molliver and Snider, 1997). GDNF expression is dramatically increased in sciatic nerve after injury in embryos and adult animals where it is normally detectable in low quantities (Trupp *et al.*, 1995; Hammarberg *et al.*, 1996). It has been shown to be retrogradely transported to L4 and L5 DRGs in neonatal rats and rescue axotomized sensory neurons in these animals (Matheson *et al.*, 1997). GDNF also promoted the survival of retinal ganglion cells following axotomy (Koeberle and Ak, 1998). Bennett *et al.* (1998) demonstrated that GDNF receptor complex, RET and GFR α -1, and NTN receptor component GFR α -2 are expressed by substantial proportions of IB4-binding DRG neurons in adult rat and this factor can prevent a series of axotomy-induced changes in these cells which are normally not responsive to NGF. Similarly, Leclerc *et al.*, (1997) showed that GDNF decreases spontaneous apoptosis occurring in neurons and satellite cells of explanted adult mouse DRGs and induces axonal growth from IB4 binding cells. These studies indicate that in adult DRGs, the trkless primary sensory neuron population is responsive to GDNF and is supported by this factor (fig. 1.1). IB4-binding neurons are known to be dependent on NGF for survival during development (Silos-Santiago, 1995). The transition from NGF to GDNF dependency in IB4-binding neurons occurs during the period when trkA is down-regulated and the expression of RET reaches peak level (Molliver *et al.*, 1997).

1.1.3.4 Fibroblast growth factors

Fibroblast growth factors are a group of polypeptide proteins which consist of at least 17 related members (for a review, see Szebenyi and Fallon; 1999). The first characterized and most extensively studied ones are acidic and basic FGFs (FGF-1, FGF-2) which were originally isolated from brain tissue and pituitary gland (Abraham *et al.*, 1986; Jaye *et al.*, 1986). Other members of the family include int-2 (FGF-3), Kaposi's FGF (FGF-4), FGF-5, FGF-6, keratinocyte growth factor (FGF-7), androgen-inducible growth factor (FGF-

8), FGF-9, FGF-10, FGF-11, FGF-12, FGF-13, FGF-14, FGF-15, FGF-16 and FGF-17. FGF's bind to heparin and other macromolecules in extracellular matrix (ECM) such as heparan sulfate proteoglycans (HSPG). This modulates their action and limits their inactivation. Due to this fact, they are also referred to as heparin binding growth factors (Gospodarowicz and Cheng, 1986; Rifkin and Moscatelli, 1989). However, FGF-1 and FGF-2 respond differently to heparin and HSPG. While the activity of FGF-1 is strongly potentiated by heparin, FGF-2 is only slightly affected by this factor (Eckenstein *et al.*, 1991). HSPG, on the other hand, is more important for the activity of FGF-2 than FGF-1 (Zhou *et al.*, 1997).

FGFs are detectable almost in all organs, solid tissues and in certain tumors (Baird *et al.*, 1986). However the main and most important localization is the nervous system, especially CNS. Both FGF-1 and FGF-2 have been localized to neurons and glial cells in the brain. FGF-1 and FGF-2 are normally confined to the intracellular milieu in the cell which is responsible for their synthesis since they lack a signal sequence in their molecular structure which is necessary for secretion of proteins (Thomas, 1987). It is widely believed that when the integrity of the cell is disrupted, FGF's are liberated to extracellular space and they exhibit their biological actions (McNeil *et al.*, 1989; Cao and Pettersson, 1993).

FGFs interact with at least two different groups of receptors. The receptors in the first group are high affinity ones and they comprise four tyrosine kinase cell surface receptors: FGFR-1, FGFR-2, FGFR-3 and FGFR-4 (see review by Johnson and Williams, 1993). They have high degree of molecular homology, the most being between FGFR-1 and FGFR-2 and the least between FGFR-1 and FGFR-4. FGFR-1 and FGFR-2 bind FGF-1 and FGF-2 equally strongly whereas FGFR-3 and FGFR-4 have higher affinity for FGF-1 (Ornitz and Leder, 1992; Vanikka *et al.*, 1992; Johnson and Williams, 1993).

The second group FGF receptors are of low affinity and structurally unconventional. These are cell surface HSPGs and their activity as low affinity FGF receptors is only hypothetical (Baird, 1994). Two of proteoglycans which

bind FGFs have been identified. These are syndecan (Kiefer *et al.*, 1990), an integral membrane proteoglycan, glypican (Brunner *et al.*, 1991), which is bound to the lipid bilayer of the cell surface and perlecan (Venstrom and Reichardt, 1993), which is found in ECM. Beside their direct binding, these molecules are also thought to deliver FGFs to their high affinity receptors (Baird 1994). It is also possible that different receptor complexes of ligand, high and low affinity receptor(s) are formed with different isoforms of these molecules to serve to regulate specificity of the cellular response.

The main biological activities of FGFs include mitogenesis, angiogenesis, chemotaxis and differentiation (Gospodarowicz *et al.*, 1987; Burgess and Maciag, 1989; Baird, 1994). Several *in vivo* and *in vitro* studies have established that they are potent survival and neurotrophic factors. Wallicke and colleagues described how these molecules are extremely effective in enhancing survival of hippocampal neurons in culture (Wallicke *et al.*, 1986, 1988; Wallicke, 1988). FGF-1 enhances regeneration from postnatal mammalian retinal ganglion cells (Lipton *et al.*, 1988). In another important study, Park and Hollenberg (1993) demonstrated that FGFs could promote retinal regeneration *in vivo*. It has been shown that FGF-2 gene expression is increased after a focal cerebral infarction and this helps neuronal survival, axonal sprouting, glial proliferation and angiogenesis (Kawamata *et al.* 1997). In the same study, they also reported that administration of exogenous FGF-2 decreased infarct size and helped functional recovery. FGF-2 also acts as a mediator in the control of cell growth and proliferation in reactive astrocytes and glioma cells (Stachowiak *et al.*, 1997).

Distribution of FGFs and their receptors in peripheral nerves is quite complex. In the intact animal the majority of DRG neurons contain both protein and mRNA of FGF-1 (Oellig *et al.*, 1995) but only a few of them express FGF-2 (Ji *et al.*, 1995). After a peripheral nerve injury, however, most of the DRG neurons start to express FGF-2 (Ji *et al.*, 1995). Intact peripheral nerve, for example sciatic, contains high amount of FGF-1 (Eckenstein *et al.*, 1991) but there are controversial reports about the presence of FGF-2. Grothe *et al.*(1997) demonstrated the expression of FGF-2 mRNA in the intact sciatic nerve.

However in an earlier study, Eckenstein *et al.* (1991) detected no FGF-2 in similar preparations. The reason of this discrepancy is not clear but may be due to different techniques they used. Following axotomy, FGF-1 is down-regulated in the distal part of sciatic nerve (Eckenstein *et al.*, 1991) while FGF-2 is strongly up-regulated in distal and proximal stumps (Grothe *et al.*, 1997).

Only two of four described high affinity receptors, FGFR-1 and FGFR-2, have been abundantly detected in the adult and embryonic DRGs (Oellig *et al.*, 1995; Grothe *et al.*, 1997) and at least for FGFR-1 the expression is not affected by nerve injury (Grothe *et al.*, 1997). In the intact sciatic nerve FGFR-1 is constitutively expressed and mRNA for FGFR-1 is strongly up-regulated following a nerve injury (Grothe *et al.*, 1997).

The source of FGFs and their receptors in peripheral nervous system is not clear. While some authors claim that Schwann cells are responsible for production of FGF-1, FGF-2 and FGFR-1 (Neuberger and De Vries, 1993; Grothe *et al.*, 1997), others demonstrated that FGF-1, FGFR-1 and FGFR-2 are solely expressed by neurons *in vitro* (Oellig *et al.*, 1995).

1.2 Changes in sensory neurons following peripheral nerve injury

Peripheral nerve injury leads to loss of some affected neurons while in surviving neurons it triggers a series of morphological, physiological and molecular changes which switches them from “transmitting mode” to a “repair and growth mode” (for a review see Fu and Gordon 1997). The morphological changes are collectively called “chromatolysis” and are characterized by breakdown of Nissl bodies, nuclear eccentricity, nucleolar enlargement and perikaryal swelling. In its fully developed state, the cell appears globular, having lost its usual angular profile, with the nucleus pressing against the cell membrane and only a narrow rim of Nissl material around the perimeter of the cell or in a cap over the nucleus. The whole process takes about 2 weeks to develop and if the axon regenerates the entire sequence of changes may be reversed over the next 4 to 6 weeks (for a review, see Lieberman, 1971).

The molecular changes during peripheral nerve regeneration include alteration in the expression of neuropeptides, new synthesis of growth-associated proteins and changes in the cytoskeletal proteins.

1.2.1 Changes in neuropeptide expression

Changes in neuropeptide expression are probably an important part of the process of switching from signal transmission to regeneration. However, motor, sympathetic and sensory neurons exhibit different modes of regulation of neuropeptide expression. While sensory neurons up-regulate NPY, GAL and VIP, and down-regulate CGRP, SP and SOM, sympathetic neurons have increased levels of SP, GAL and VIP, and decreased expression of NPY (fig. 1.2). In axotomized motor neurons, on the other hand, expressions of almost all neuropeptides are up-regulated (table 1.1) (for reviews see Hökfelt *et al.*, 1994 and Zigmond *et al.*, 1996).

In DRGs, the changes in neuropeptide expression result in attenuation of transmission, especially nociceptive transmission, by down-regulation of excitatory neuropeptides and up-regulation of others that exhibit inhibitory action. Two type I neuropeptides - CGRP and SP are both known to be excitatory modulators in nociceptive transmission (Henry, 1976; Miletic and Tan, 1988; Ryu *et al.*, 1988). It is proposed that when their expression is down-regulated after axotomy, their facilitatory role is transferred to up-regulated VIP, which is known to have trophic and survival - enhancing effects on DRG neurons *in vitro* (Shehab and Atkinson, 1986). GAL on the other hand, inhibits transmission

Table 1.1 Changes in neuropeptide expression in axotomized sympathetic, sensory and motor neurons.

Neuropeptide	Sympathetic Neurons	Sensory Neurons	Motor Neurons
NPY	↓	↑	↑
Substance P	↑	↓	↑
CGRP	=	↓	↑
Somatostatin	ND	↓	↑
CCK	ND	↓↑*	↑↓*
Galanin	↑	↑	↑
VIP	↑	↑	↑

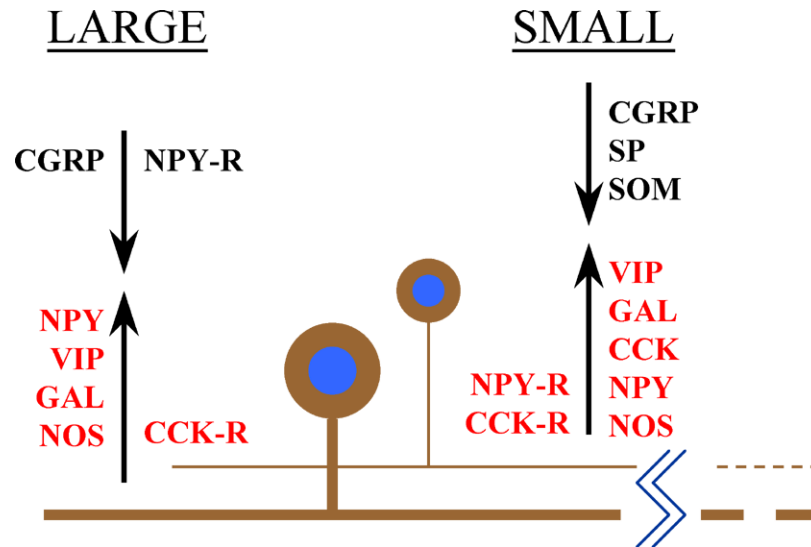


Figure 1.2 Changes in neuropeptide expression in DRG neurons following axotomy (-R designates receptor for the particular neuropeptide, NOS=nitric oxide synthetase) (Redrawn from Hökfelt *et al.*, 1994) .

in the dorsal horn and thus attenuates nociceptive signals (Yanagisawa *et al.*, 1986). However GAL has a dual effect on the spinal nociceptive flexor reflex including facilitation followed by depression in a dose dependent manner (Wiesenfeld-Hallin *et al.*, 1989). It also antagonizes the facilitation of the reflex induced by either peripheral nerve conditioning stimulation or intrathecally applied SP or CGRP (Xu *et al.*, 1990; Wiesenfeld-Hallin *et al.*, 1990). Following axotomy GAL also counteracts facilitatory effects of exogenous VIP but not exogenous SP and (Xu *et al.*, 1990). These findings may suggest that while axotomy transfers the excitatory role of CGRP and SP to VIP, up-regulated GAL antagonizes VIP instead of antagonizing SP and CGRP.

The up-regulation of certain peptides may contribute to survival of axotomized neurons as well as regeneration of axons. For example, it has been shown that VIP stimulates mitosis, promotes neurite outgrowth and enhances survival of sympathetic neuron precursors in culture and prevents cell death in sympathetic ganglion neurons after NGF withdrawal (Pincus *et al.*, 1990; Tanaka and Koike, 1994). NPY and VIP have been also demonstrated to indirectly

promote neurite outgrowth from cultured DRG neurons via spinally derived neurotrophic factor(s) (White and Mansfield, 1996).

1.2.2 Changes in cytoskeleton and expression of growth-associated proteins

Following axotomy, important changes start in the composition and dynamics of cytoskeletal elements which are mainly characterized by increased transport of tubulin and actin and the down-regulation of NF proteins (Oblinger and Lasek, 1988; Hoffman *et al.*, 1987, 1988; Hoffman and Cleveland, 1988). These changes are suggested to increase fluidity of the axoplasm and hence facilitate the elongation of growing axons (for a detailed account see chapter IV).

Axotomy induces synthesis of some proteins, whose expression is mostly confined to the period of embryonic development, and are down-regulated on target reinnervation. The examples of these are the fast transported growth-associated protein (GAP-43) and developmental isoforms of tubulin -T α and class II β (Skene and Willard, 1981; Skene *et al.*, 1986; Hoffman and Cleveland, 1988; Hoffman, 1989; Miller *et al.*, 1989). GAP-43 is a phosphoprotein that is up-regulated in growing axons and closely associated with growth cones (Meiri *et al.*, 1988). Although *in vitro* experiments indicate the involvement of this protein in axonal growth, analysis of the mice lacking the gene for GAP-43 indicated a role in axonal pathfinding rather than neurite extension (Strittmatter *et al.*, 1995).

1.2.3 Explanations for the changes in gene expression following axotomy

The mechanisms triggering the changes in gene expression following axotomy are uncertain. Loss of target-derived growth factors is the most widely accepted explanation. Another explanation is the action of various cytokines released and/or synthesized in lesioned nerve.

All neurotropic factors are retrogradely transported to DRG neurons with their receptors from the peripheral target tissue (Curtis *et al.*, 1998).

Pharmacological blockage of axoplasmic transport created some changes in neuropeptide expression similar to those seen after axotomy in sensory neurons (Keen *et al.*, 1989; Kashiba *et al.*, 1992). Therefore, changes in the gene expression in DRGs following axotomy are often attributed to the reduced supply of target-derived growth factor(s) and in particular NGF (Raivich *et al.*, 1991; Verge *et al.*, 1995). NGF is produced by keratinocytes in normal skin (Albers *et al.*, 1994) and by smooth muscle cells in the deep tissues of the body (Steers *et al.*, 1991). Following transection of a peripheral nerve, NGF mRNA is strongly up-regulated both proximal and distal to the lesion site and interleukin-1 (IL-1) secreted by macrophages invading the injury site has been shown to be involved in this induction (Heumann *et al.* 1987a, 1987b, Lindholm *et al.*, 1987). It has been demonstrated that retrograde transport of all neurotrophins including NGF was increased via receptor mediated mechanisms and/or by a generalized up-regulation of neuronal retrograde transport capacity during the first 3 days following axotomy (Curtis *et al.*, 1998). However, uptake and retrograde transport of this factor start to decline about one week after injury and becomes lower than that observed in uninjured nerve in a few days (Raivich *et al.*, 1991; Delcroix *et al.*, 1997).

Several studies have shown that the main factor that regulates neuropeptide expression in adult sensory neurons is NGF (Lindsay and Harmar, 1989; Lindsay *et al.*, 1989). Verge *et al.* (1995) demonstrated that following sciatic nerve transection, while concentrations of CGRP, SP and SOM were markedly reduced, those of VIP, NPY, GAL and CCK were elevated and that some of these changes were counteracted by delayed intrathecal infusion of NGF. However, while the decreases in CGRP and SP were reversed almost completely, the numbers of neurons expressing VIP, CCK, NPY and GAL were reduced by about 50% by NGF infusion. In another study it was shown that when different growth factors are applied to the transected sciatic nerve, NGF counteracted the down regulation of SP in DRGs (Zhang *et al.*, 1995).

It has been demonstrated that LIF mRNA is rapidly induced in the lesioned nerve and retrogradely transported to the axotomized neurons (Curtis

et al., 1994). Several *in vitro* and *in vivo* studies and analysis of LIF knockout mice have revealed that this peptide is one of the main regulators of neuropeptide expression in DRGs and superior cervical ganglia (SCGs) (see chapter IV and for a review see Zigmond *et al.*, 1996).

1.3 Aims and rationale of this thesis

The main objective of this thesis was to investigate the mechanisms and factors regulating some of the structural and molecular changes following axotomy and during nerve regeneration using a novel tissue culture technique.

The mechanism of axotomy-induced changes in DRGs has been widely studied using a few common *in vivo* and *in vitro* experimental models. However both of these types suffer some serious disadvantages. The *in vivo* models usually involve transection or ligation of a peripheral nerve, such as sciatic, application of different factors to the cut end of the nerve or local or systemic injection of these factors to the animals. During these procedures, isolation of the neurons from the sources of unknown factors or prevention of secondary and unexpected effects of interventions or substances given can not be guaranteed. For example the tissue inflammation or neuroma formation at the site of a nerve transection could produce neurotoxic cytokines such as tumor necrosis factor- α (TNF- α) which has been shown to cause apoptosis under certain conditions (Clement and Stamenkovic, 1994; Wagner and Myers, 1996) or even trigger apoptosis through an excitotoxic mechanism (Eichler *et al.*, 1994; Portera-Cailliau *et al.*, 1997). In other situations axotomized neurons can send collaterals through another nerve branch to an abnormal target, which may support survival (Rhoades *et al.*, 1987). The results of experiments such as these are therefore difficult to interpret in the context of gene expression. Another point is the fact that not all nerves receive axons from a single ganglion. Cutting sciatic nerve for instance can affect only 40% of L4-L5 DRG neurons (Devor *et al.*, 1985) and it is difficult to predict the potential contribution of remaining uninjured neurons to the phenotype and survival of axotomized ones.

The *in vitro* studies usually involve cultures of dissociated peripheral neurons. However, the procedures such as enzymatic and mechanical treatment

to isolate neurons may cause harm or changes in the cell phenotypes. In this model, neurons are subjected to a total axotomy resulting in loss of all processes. And the interactions between neurons and non-neuronal cells and the role of ECM are disrupted. Therefore dissociated neuronal cultures are usually far from being ideal models to study the axotomy-induced changes.

Throughout the experiments in this thesis I used a novel *in vitro* system in which intact mouse DRGs are explanted into collagen or matrigel gels and kept in culture medium for several days. This technique provides an experimental model that avoids some drawbacks of other *in vitro* and *in vivo* models. First of all it creates a controlled environment relatively free of interference by other factors such as immune response. It is also possible to do experiments with known amounts of added growth factors in this technique. The preservation of tissue architecture is another advantage of explant DRG cultures. Axons grow into the gels used in this model even without any growth factors; these axons can be easily identified phenotypically and changes in their growth rate can be determined. However, explant DRG cultures are not devoid of limitations. The most important one is relatively more difficult access of nutrients and O₂ to DRG neurons which may effect long-term survival of cultures.

For this study, it was important to establish that the system I used was viable for DRG cells. I therefore determined the survival of neurons in explant DRG cultures in the series of experiments described in chapter III. In chapter IV, I studied the effects of some growth factors on neuropeptide expression. I described my studies on the regulation of neurofilament expression in DRGs in chapter V. Finally in chapter VI, I presented results of my investigation on the diffusible factor(s) released from lesioned nerves.

Chapter 2 :Material and Methods

2.1 *Animal procedures*

2.1.1 Removal of DRGs from mice

Adult female mice aged 6-12 weeks (BK1 TO strain; bred at King's College) were used in the experiments. Young female animals were preferred because of easier dissection. They were anaesthetised by intraperitoneal injection of a cocktail of *Hypnorm* (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml - *Janssen*) and diazepam (5mg/ml) (4% and 20% respectively) and killed by cervical transection. According to the purpose of particular experiments, lower 4-5 thoracic or lumbar 4-5 DRGs were removed and kept in RPMI 1640 culture medium (*Sigma*) until explantation into culture or fixation. These procedures were performed as rapidly as possible under aseptic conditions. In order to identify DRGs from different spinal levels, T13 DRG, whose peripheral nerve runs caudal to the last rib was used as a reference. During removal from the mice, DRGs were usually left with a peripheral nerve attached (3-4 mm in most of the experiments, 7-8 mm in some others) whilst in some experiments the spinal roots and peripheral nerve were cut off.

2.1.2 Peripheral nerve axotomy and crushing

Animals were anaesthetised as described above. For axotomy, the skin over right thigh was incised and part of the sciatic nerve was exposed between thigh muscles and cut. For peripheral nerve crushes, after the skin over the thorax on one side was incised to expose the ribs, the intercostal nerves were compressed with forceps for about 10 seconds at a distance about 10 mm away from the DRGs. The incisions were closed with surgical staples.

2.2 *Tissue cultures*

Tissue cultures were set up either in collagen gels or matrigel - a commercially available extract of extracellular matrix (ECM), secreted by the Engelbreth-Holm-Swarm (EHS) tumour cell line.

2.2.1 Preparation of collagen gels

Collagen gels (type I - from rat tail-Sigma) were prepared using protocol of Nicosia and Ottinetti (1990) as follows:

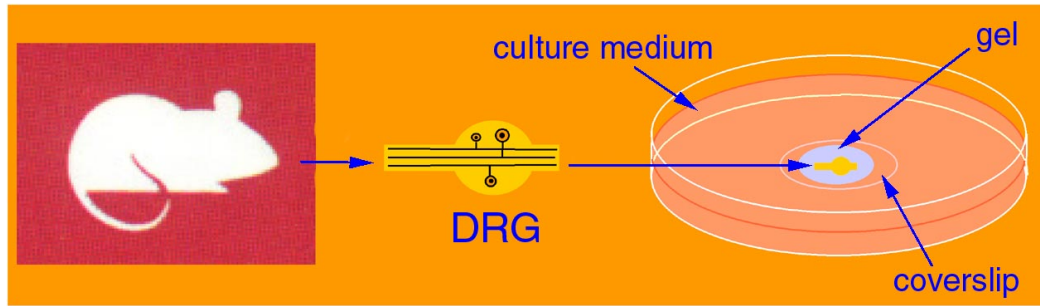


Figure 2.1 Illustration to show the technique to explant mouse DRGs *in vitro*.

50 mg collagen type I was dissolved in 2 ml of 1/1000 sterile acetic acid and left with it overnight. Next day, 9.63 ml of distilled water was added to obtain a final solution that contained 4.3 mg/ml collagen. This clear solution was used as stock and kept at 5°C. Before setting up cultures, the stock solution was mixed with DMEM (x10) and NaHCO₃ (11.7 mg/ml), in this order, at 7, 1, 2 ratios respectively to obtain working solution.

2.2.2 Matrigel

Growth factor-reduced matrigel was used in the experiments (*Becton Dickinson Labware*). The major components of matrigel matrix are laminin, collagen IV, entactin, HSPG and a number of growth factors (Kleinman *et al.*, 1982; Vukivckic *et al.*, 1992). In the growth factor-reduced form, while heavy molecular weight ECM components are conserved, the levels of a number of known growth factors are considerably reduced with the exception of ones which bind to HSPG such as TGF-β.

2.2.3 Setting up cultures

The DRGs were placed on 13 mm glass coverslips in 35 mm Petri dishes or smaller multiwells and covered with 20 - 30 µl of collagen or matrigel (fig. 2.1).

The dishes were then put into a humidified incubator for about 3 minutes (matrigel) or 6 minutes (collagen) to let the gels set. After that, preparations were covered with 2 ml (35 mm dishes) or 0.5 ml (multiwells) of RPMI

1640 medium containing 100 units of penicillin, 100 µg streptomycin and 250 ng amphotericin B per ml (*Sigma*) and subsequently maintained at 36°C in an atmosphere containing 95 % air and 5% CO₂.

In some experiments, growth factors were added to the cultures at the beginning. The details of these procedures were described in individual chapters.

2.2.4 Fixation of preparations

Preparations were fixed by immersion in 4% paraformaldehyde in phosphate buffer or 3.6 % formaldehyde for approximately 1 hr and then blocked with 3% bovine serum albumin (BSA) and 0.1% Triton X-100 in phosphate buffer saline (PBS).

2.2.5 Frozen sections

After fixation, L4 DRG preparations were embedded into a cube of liver tissue, stuck on to a piece of cork, covered with OCT, frozen by immersing into liquid nitrogen and stored at -20°C or -70°C (for longer periods of storage) until cutting. They were cut at 10 µm thickness using a cryostat (*Reichert-Jung*) and mounted on amino-propyl-triethoxy-silane (APES - from *Sigma*) – coated coverslips (22mmx22mm). APES coating was performed as follows:

Coverslips were placed on special trays and immersed in acetone. After 5 minutes they were removed and transferred into 2 % APES (in acetone) solution. After another 5 minutes, coverslips were washed with distilled water for several times and let dry at room temperature.

2.3 Immunofluorescence

In order to stain the explant cultures, primary antibodies (in 3% BSA) were left on the preparations overnight. The next day preparations were given 5 washes, each wash lasting about 1 hour, with PBS and secondary antibodies (FITC, RITC or AMCA - conjugated) were added overnight (the details of primary and secondary antibodies used were given in individual chapters). During the third day they were washed again in the same way and then mounted in Citifluor (*City University, U.K*). The procedures to stain the sections were similar

except the duration of incubation with secondary antibodies, which was 2 hours in this case and the duration of washes which was about only 10 minutes each.

2.4 Immunocytochemistry and stereography

Preparations which had already been fixed and stained using fluorescent antibodies were incubated overnight with rabbit polyclonal antibody to protein gene product 9.5 kDa (PGP 9.5- from *UltraClone*) which is a pan-neuronal marker (Wilson *et al.*, 1988). The following day, preparations were washed with 3 changes of PBS prior to an overnight incubation with biotinylated goat anti-rabbit (*Sigma*). After a further series of washes the following day, the preparations were incubated overnight with peroxidase conjugated avidin (*Sigma*). The following day, after a final series of washes with Tris-buffer (pH 7.6) preparations were incubated in Tris buffer containing 0.6 mg diaminobenzidine and 15 ml of 1% CoCl_2 per ml for 10 minutes prior to addition of 1 ml/ml of 30% H_2O_2 to visualize peroxidase activity. The stained preparations were mounted in glycerol (*Dako*) and viewed under dark -ground illumination using *Zeiss Standard* microscope with an image projection tube which was used to draw traces of the longest axons growing out of the cut ends of the peripheral nerves (usually 10-20 per preparation).

2.5 Microscopy and image analysis

The preparations labelled by fluorescent-conjugated secondary antibodies were viewed using an epifluorescence microscope (*Olympus BH2*) and images captured digitally to a computer as graphic files by an attached three chip cooled CCD camera system (*Hamamatsu 5810*). The capturing system made it possible to combine images taken using different filter blocks for FITC, RITC or AMCA.

Some preparations were scanned using *Leica TCS NT* confocal microscope to take optical sections. This microscope also supported the facility of combining different images obtained by excitation of specimens at different wavelengths.

The camera lucida drawings of the growing axons were scanned using a

flatbed scanner (*Umax Vista S12*).

When needed, images were analysed by using *Adobe Photoshop* and *NIH Image* software running on a *Power Macintosh 7500* computer.

The image analysis included marking cell profiles, measurements of diameters and lengths of outgrowing axons, intensity of fluorescent signal in axons and cells and counting number of stained and unstained neurons and axons.

Microsoft Excel software was used for statistics. The data was analysed using Student's t-test and χ^2 test where appropriate.

Chapter 3 :Survival of DRG neurons in explant cultures and effects of NGF and LIF

3.1 Introduction

It has been known for about a century that some sensory neurons axotomized by a peripheral nerve injury undergo a series of degenerative changes and some of them eventually die (Ranson, 1906; Cavanaugh, 1971; Himes and Tessler, 1989; Suzuki *et al.*, 1993; Groves *et al.*, 1997). The reaction to axotomy varies among individual neurons and the range of changes include relatively benign transformations such as simple chromatolysis and cellular atrophy as well as fatal degeneration in the form of cytoplasmic vacuolation, extreme swelling, cellular shrinkage, nuclear condensation and disintegration (Lieberman, 1971; Groves *et al.*, 1997). The surviving neurons can regenerate and restore their normal functions.

Different animal species and the members of the same species at different ages exhibit diverse responses to axotomy. The mechanisms underlying these variations and factors determining whether axotomized neurons die or survive have long been subjects of investigations but few definite conclusions have been drawn yet.

3.1.1 The time course and extent of neuronal death following axotomy

Although a consensus exists on the occurrence of neuronal death following axotomy, there is not a complete agreement about the extent and the time course of cell loss. This is maybe due to differences in quantification techniques or experimental models employed.

Groves *et al.* (1997), reported that apoptosis began about 1 week after axotomy and peaked at about 2 months. According to some studies apoptosis continues at a low level for up to 6 months after injury (Ygge and Aldskogius, 1984; Groves *et al.*, 1997). However, others found that cell death occurs only during the first 6-8 weeks, after which only little change is detectable until 6 or 12 months (Himes and Tessler, 1989; Rich *et al.*, 1989).

In most of the studies to explore the incidence of neuronal loss following axotomy the lumbar DRGs, (usually from L4 - L6), were examined after different kinds of injuries to the sciatic nerve. These experimental injury models include transection with or without capping or ligating to prevent regeneration and crushing, which permits the axons to regenerate. However, results obtained from these studies vary widely. For example Lekan *et al* (1997) reported a 30 % loss in L5 and L6 DRG neurons 6 weeks following sciatic ligation in adult rats whereas Rich *et al.* (1989) observed only an 18% and Himes and Tessler 20-25% neuronal death after sciatic nerve transection with proximal capping and ligation respectively in the same period. Some authors claimed that they detected up to 50% (Ranson, 1906) and 60% (Cavanaugh, 1951) neuronal loss post-axotomy. However, these data should be taken cautiously since the number of neurons in L4 – L5 DRGs projecting an axon into the sciatic nerve comprise only about half of the total pool as shown by Devor *et al.* (1985) electrophysiologically and histologically. Thus, it seems unlikely that the proportion of neurons lost should equal or even exceed the real number of axotomized neurons.

Thoracic DRGs and trigeminal ganglia have also been used in similar experiments and following peripheral nerve transection, significant neuronal death has been shown to occur in both (Ygge and Aldskogius, 1984; Aldskogius and Arvidsson, 1978).

It has been firmly established that the reaction of neurons in newborn animals to peripheral axotomy is more severe than in adults (Aldskogius and Risling, 1981; Bregman and Goldberger, 1982, 1983; Yip *et al.*, 1984, Schmalbruch, 1987; Himes and Tessler, 1989). Young animals lose more sensory neurons but surviving ones recover and restore normal function more rapidly than in adults; a phenomenon which is referred to as *infant lesion effect* (Bregman and Goldberger, 1982, 1983). For example, Himes and Tessler (1989) demonstrated that, while sciatic nerve transection causes only a loss of 20-25% in adult rat DRGs in 10-60 days, the neuronal death amounts to 50% by 10th day in neonates.

3.1.2 Neuronal death and phenotype

DRG neurons are usually categorized in subclasses according to their phenotypic properties based on the expression of certain neurochemical markers and also size differences (Price, 1985; Averill *et al.*, 1995). According to some authors, different subclasses of DRG neurons vary in their ability to survive axotomy (Dodd *et al.*, 1983; Lawson and Harper, 1985; Price, 1985). It has been claimed that a population of small diameter neurons, (which are assumed to have unmyelinated C fibers, as demonstrated by Ranson, 1906, 1909), are more vulnerable to axotomy (Aldskogius and Arvidsson, 1978; Rich *et al.*, 1989; White *et al.*, 1990; Groves *et al.*, 1996, 1997). These are the neurons which do not express neuropeptides and are labelled by the monoclonal antibody LA4 and IB4 lectin (Groves *et al.*, 1996). However, other reports contradict these findings. Other investigators deny the existence of selective neuronal loss (Risling *et al.*, 1983; Ygge and Aldskogius, 1984; Schmalbruch, 1987; Himes and Tessler, 1989) and some even report the loss of larger neurons after axotomy (Bondok and Sansone, 1984).

3.1.3 Growth factors and sensory neuronal survival

During development, PNS neurons depend on a number of neurotrophic factors for survival and differentiation. The receptors for these factors are expressed by different populations of sensory and motor neurons even in adult animals. Distribution of receptors for the neurotrophins and GDNF in DRGs defines distinct populations of primary sensory neurons in adult animals (Wright and Snider, 1995; Bennett *et al.*, 1998). This suggests the existence of a complementary protection and maintenance mechanism for primary sensory neurons by certain growth factors. After a peripheral nerve injury, a number of neurotrophic factors, along with some cytokines are released or their expression is up-regulated at the site of injury, in the damaged nerve or in DRGs. It seems likely that these factors may be of importance in determining whether neurons survive after axotomy (for reviews see Fu and Gordon, 1997 also see chapter I; Lindsay, 1996; Verge *et al.*, 1996).

3.1.4 Rationale for the study

Previous studies have demonstrated that axons grow in explant DRG cultures of adult animals for several days (Edström et al., 1996; Tonge et al., 1996). However, it has been also shown that apoptosis starts within 2 days in DRG neurons (Ekström, 1995; Leclere et al., 1997). Therefore, the extent of survival and neuronal loss is uncertain in these preparations. To establish a firm base for most of the experiments I include in this thesis, I needed to check the proportion of neurons surviving in the presence and absence of growth factors. I also needed to see if there was any evidence of selective cell loss in culture under the different conditions. Expressions of three different Trk receptors (A, B and C) define different subpopulation of DRG neurons and may indicate their dependency on corresponding growth factors (Wright *et al.*, 1995). Therefore, determining expression of different trk receptors in DRG neurons would be an ideal way for phenotypic identification. However, because of lack of reliable antibodies to these receptors I used alternative markers for phenotypic identification of neurons. These are CGRP, RT97 and IB4. The DRG neurons may be divided into different classes according to expression of these three markers (Price, 1985; Averill *et al.* 1995). The first population consists of those neurons which are traditionally described as “large light”, can be identified by an antibody (RT97) which recognises the phosphorylated form of heavy neurofilament 200, and is found in myelinated axons. A group of “small dark” neurons with unmyelinated axons make the second subpopulation of DRG neurons which is labelled by monoclonal antibody LA4 (Alvarez *et al.*, 1991) and IB4 lectin (Silverman and Kruger, 1990). The neurons of the third subpopulation are also mostly small and dark and express variety of neuropeptides, but mainly CGRP. However there are some medium-size neurons among them, which are also labelled by monoclonal antibody RT97. Lastly there are some small neurons which are labelled by LA4 and express CGRP (Averill *et al.* 1995). A big overlap exists between CGRP and TrkA expression - 92% of TrkA positive neurons express CGRP. Most of the RT97 expressing neurons presumably correspond to TrkB and TrkC expressing neurons (Averill *et al.*, 1995; Wright and Snider, 1995). The majority of IB4 labelled neurons

however, do not express any Trk receptor but express RET and are therefore probably responsive to GDNF (Molliver et al., 1997).

In this study I followed a 4-step approach.

Firstly, the viability of DRG neurons after 3 days in culture was determined using two different methods to assess the proportion of surviving neurons. The first one is a novel neuronal counting method. For this, I used a scanning confocal microscope to take optical sections of mouse DRGs which were stained with an antibody labelling neuronal nuclei and I counted the total number of neurons on the sections. In the second method, I used propidium iodide (PI) to assess the viability of neurons by incubating preparations with it prior to fixation.

In the second step, I tested the effects of NGF, the best-known neurotrophin, and LIF, a cytokine whose role on the cell survival in adult animal is relatively less known. A reason to choose these two factors was the possibility of existence of a reciprocity or antagonism between them as Zigmond *et al.* (1995), suggested for neuropeptide expression and Kriegstein and Unsicker (1996) for neuronal survival. A final practical point in testing NGF and LIF was to provide a reliable background information for the experiments which I included in the next chapter regarding the effects of these two factors on neuropeptide expression.

The third step involved the phenotypical identification of dead / live neurons in explant cultures under different conditions. For this, CGRP antibodies, RT97 and IB4 lectin were used to identify subgroups of DRG neurons. Together, these markers may encompass the whole population of DRG neurons.

As a last step, I determined the changes in the neuronal survival following a physical stress and the effectiveness of NGF and LIF in rescuing DRG neurons. It was important to compare the effects of these factors on neuronal survival under conditions where cell loss would be expected to be more severe.

3.2 Experimental procedures

3.2.1 Tissue cultures

Animals were anaesthetised as described in chapter II. All the explant cultures were set up in collagen gels and incubated for 3 days as described in chapter II in detail.

For the first part of the experiments, T10 - T12 DRGs from one side of 2 mice were used to set up cultures. NGF or LIF was added to one set of matching pairs from each animal (see table 3.1 for concentrations). Two sets of T10 – T12 DRGs from another mouse from the same litter were fixed immediately after removal. At the end of incubation period, cultured preparations were fixed and all the preparations from this experiment were processed in order to count neurons.

In the second part, T10 – T12 DRGs were removed from 10 mice. Some of the DRGs were immediately fixed to use as controls whilst explant cultures were set up with the rest. NGF and LIF were added to some of the preparations (table 3.1). On the second day of incubation, PI was added to the cultures and some of them transferred to a refrigerator (where the culture medium became alkaline) while the rest were returned to incubator (table 3.2). The next day all the preparations were fixed and processed to determine proportions and phenotypes of live and dead neurons.

The staining procedures with primary and secondary antibodies were performed as described in chapter 2 in detail. The concentrations of primary and secondary antibodies used are given in tables 3.2 and 3.3.

3.2.2 Immunohistochemistry to determine neuronal numbers

To determine the total number of neurons, DRGs were removed from the gel and split into 4 pieces with a razor blade under the microscope. Then they were transferred onto coverslips and stained with a mouse monoclonal antibody that specifically labels neuronal nuclei (anti-NeuN) (table 3.2) (Suhonen *et al.*, 1996). The rest of the immunocytological process was as described in chapter 2.

Table 3.1. The concentrations and sources of factors used.

Factor	Concentration	Source
NGF 2.5 S	50 ng/ml	<i>Alamone Labs/Promega</i>
LIF	30-50 ng/ml	<i>Chemicon</i>

Table 3.2. The concentrations and sources of primary antibodies and dyes used.

Antibody / dye	Concentration	Source
PI	75 μ M	<i>Sigma</i>
Guinea pig α -laminin	1/100	Zammit, PhD Thesis, 1992
Rabbit α -CGRP	1/100	<i>Affiniti</i>
Mouse monoclonal α -RT97	1/10	A gift from B. Anderton
Biotin conjugated lectin IB4	20 μ g/ml	<i>Sigma</i>
Mouse monoclonal α -NeuN	1/100	<i>Chemicon</i>

Table 3.3. The concentrations and sources of secondary antibodies used.

Antibody	Concentration	Source
AMCA-conjugated α -guinea pig IgG	1/100	<i>Jackson Immuno.</i>
FITC-conjugated goat α -mouse IgG	1/100	<i>Sigma</i>
FITC-conjugated goat α -rabbit IgG	1/100	<i>Jackson Immuno.</i>
FITC-conjugated streptavidin	1/100	<i>Sigma</i>
Alexa 488 goat α -mouse IgG	1/100	<i>Molecular Probes</i>

The mounted preparations were scanned using a *Leica* scanning confocal microscope and optical sections were obtained at a maximum of 7 μm intervals. The images were digitally stored and analysed with a *Macintosh* computer. Commercial image editing software *Adobe Photoshop* was used to create cumulative templates from series of sections by marking labelled nuclei on each one (figure 3.1). The numbers of marks on the final cumulative templates were counted using the image analysis program - *NIH Image*.

Anti-NeuN clearly labelled neuronal nuclei with slight staining of cytoplasm, which is more prominent in some neurons than others and nucleoli were clearly visible as dark spots (figure 3.2). Occasionally some distorted nuclear profiles were observed – probably an indication of an ongoing disintegration (figure 3.2).

3.2.3 Determination of live and dead cells

Before starting these experiments, I tried 3 different established labelling agents for live/dead assessment of cells and found that PI gave the best result. The other two were MTT and calcein AM.

MTT is a water soluble tetrazolium salt that is converted to insoluble purple formazan crystals in the cells with active mitochondrial machinery (Green *et al.*, 1984). It demonstrated live neurons very successfully but when I attempted to process the preparations using antibodies for phenotypic identification, I found out that formazan crystals were washed off during the histochemical procedures. This made it impossible to determine the phenotypes of living and dead cells.

Calcein AM is a fluorescent chloromethyl derivative that freely diffuses through the membranes of live cells. It is converted into a membrane-impermeant dye by esterase activity in viable cells and retained there for a few hours (Papadopoulos *et al.*, 1994). However, in my experiments calcein failed to diffuse inside DRGs and proved unsuitable for these preparations.

PI is a fluorescent stain for nucleic acids which can not cross intact cell membranes but stains the cytoplasm of dead/dying cells bright pink/red as

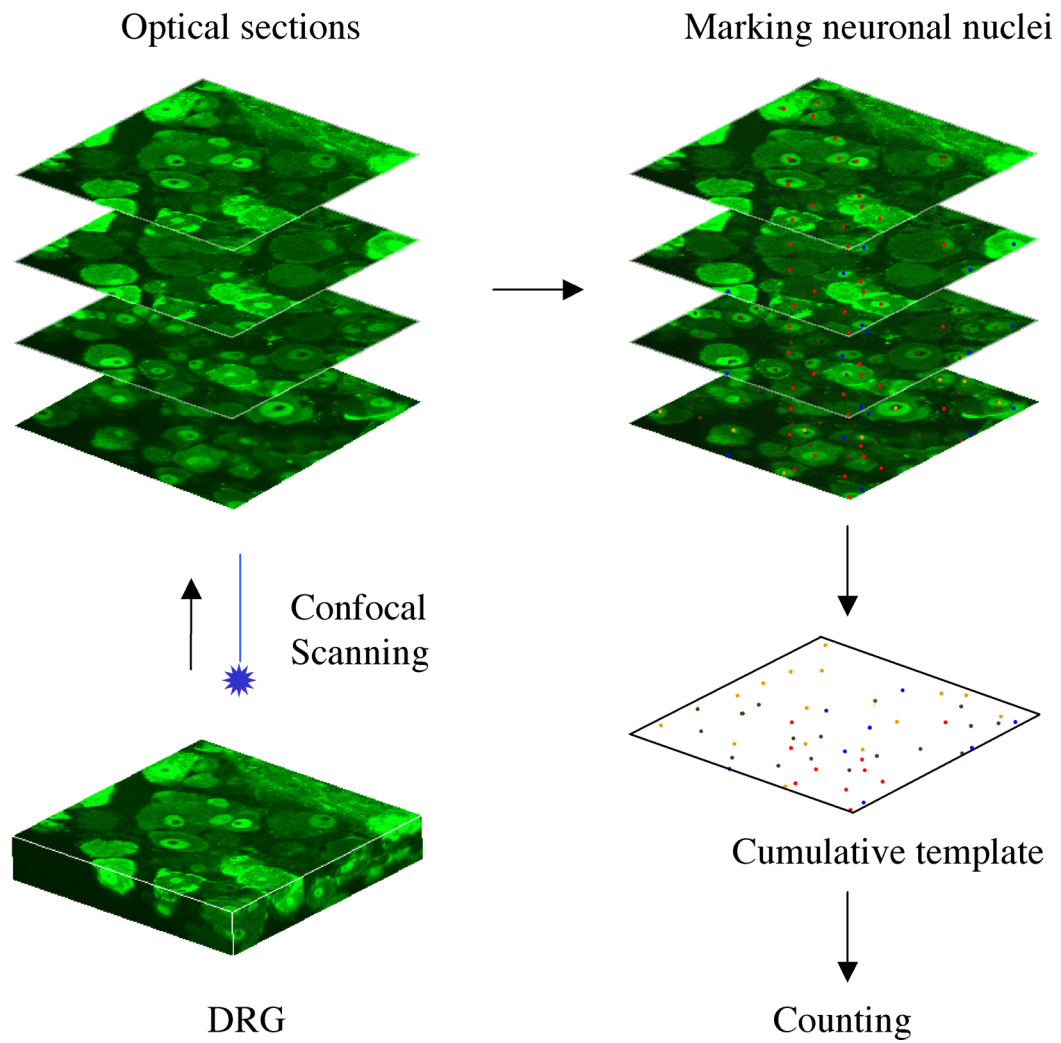


Figure 3.1 The method of neuronal counting by optical sectioning. Optical sections of DRGs are taken with a confocal microscope. Labelled neuronal nuclei are marked on each section on a template layer and a final cumulative template is obtained. The marks on this template are counted by using an image analysis program.

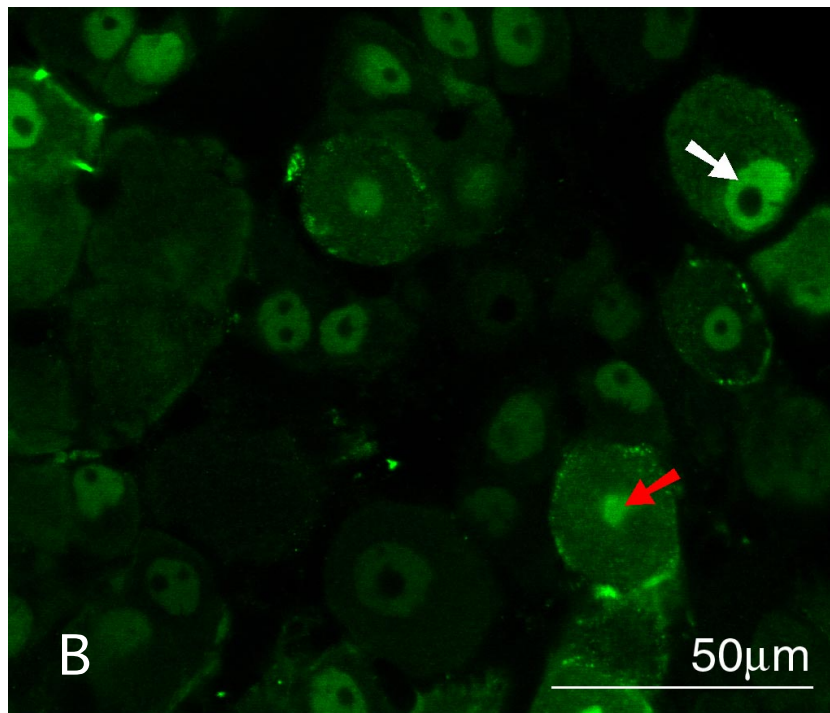
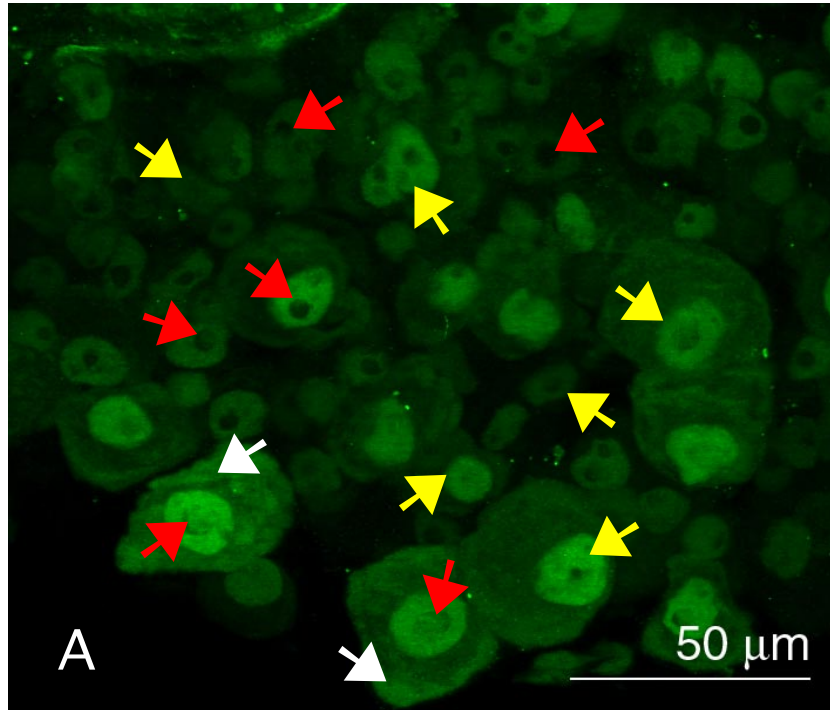


Figure 3.2 NeuN antibody stained neuronal nuclei (yellow arrows) with occasional slight cytoplasmic staining (white arrows) with nucleoli visible as dark spots (red arrows) (A). Irregular or indistinguishable nuclear boundaries were observed in some neurons which is probably a sign of nuclear disintegration in dying and/or recently died cells (red arrow); white arrow points a normal nucleus (B).

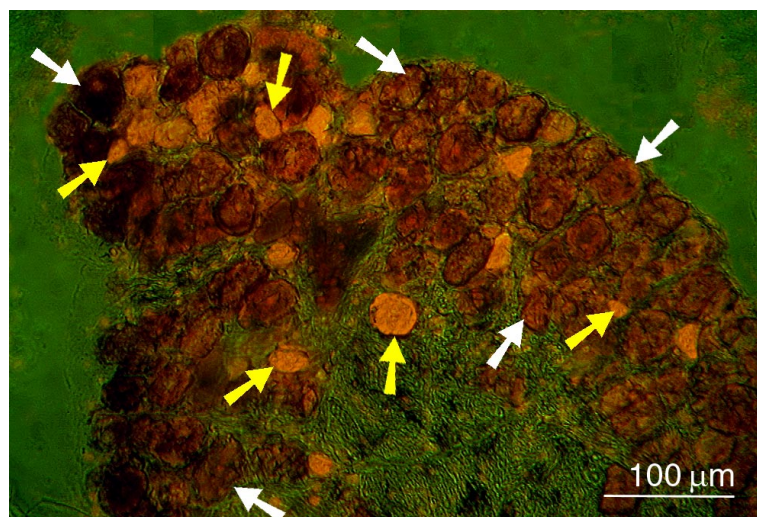


Figure 3.3 Determination of dead and living cells by MTT and PI methods. This image was taken by using a phase-contrast microscope. PI-stained dead cells appeared brightly colored due to fluorescence (yellow arrows) whilst live cells laden with formazan crystals had a dark color (white arrows).

nuclear material dispersed into cytoplasm as a result of nuclear disintegration at a very early stage (Wilde et al., 1994).

In order to determine the reliability of PI labelling and its staining pattern, I incubated some preparations with MTT and PI together overnight in a refrigerator (this was to make culture medium alkaline thus to increase death rate to make a reliable observation with sufficient number of dead cells). In these preparations, there were many cells which were homogeneously stained with PI. The rest of the cell profiles were laden with formazan crystals but lacked PI staining (fig3.3). This showed that PI staining is a reliable technique for live/dead assessment in these experiments and also that both nucleus and cytoplasm of a dead/dying cell are stained.

However, as an artefact of the technique, during the staining procedures for phenotypic markers, PI diffused over the entire sections and stained nuclei of all cells including satellite cells. Although this created extensive background labelling, dead neurons were still clearly distinguishable by their cytoplasmic staining (figure 3.4).

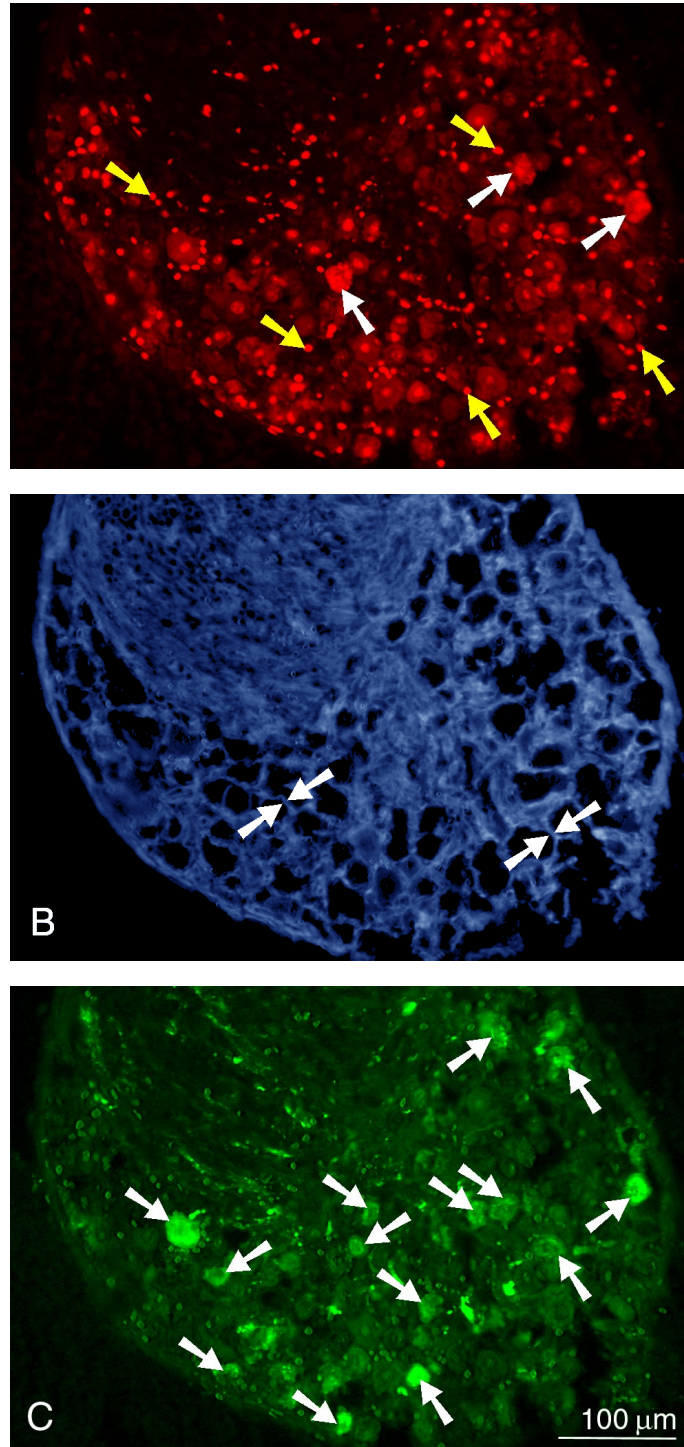


Figure 3.4 PI stained cytoplasm of dead cells and nuclei of all cells including microglia during processing (A). Dead neurons were distinguishable with cytoplasmic staining (white arrows) whereas microglia appeared as small dots (yellow arrows). Laminin staining labelled basal membrane (arrows) and made it possible to see cell boundaries (B). Phenotypic markers (in this case CGRP) were visualized with FITC (C) (arrows).

3.2.4 Immunohistochemistry to determine proportions and phenotypes of live and dead neurons

Frozen sections of preparations were cut as described in chapter 2 and stained with antibodies to CGRP, RT97 and IB4-lectin. All preparations were also counter- stained with antibodies to laminin to visualize the extracellular matrix surrounding cells.

The laminin antibody labelled basal membrane surrounding individual neurons (figure 3.4) and drawing the individual boundaries helped to identify neurons in conjunction with two basic morphological criteria, i.e. a round shape cell with a round shape nucleus. The secondary antibody to laminin was conjugated to AMCA, which is visible under UV light.

Labelling with anti-CGRP, RT97 and IB4 gave green fluorescence staining (figure 3.4). Superimposition of 3 different signals was possible with the image capturing system I used. Figure 3.5 and 3.6 shows different combinations.

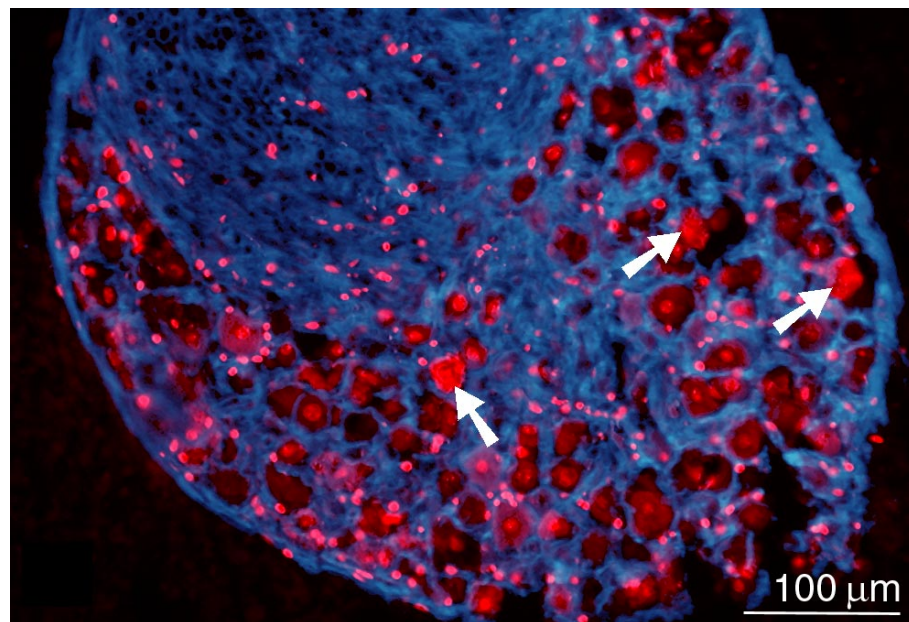


Figure 3.5 The superimposition of laminin and PI staining showing a few dead neurons (arrows). These types of superimposed images made it possible to determine the proportion of dead neurons.

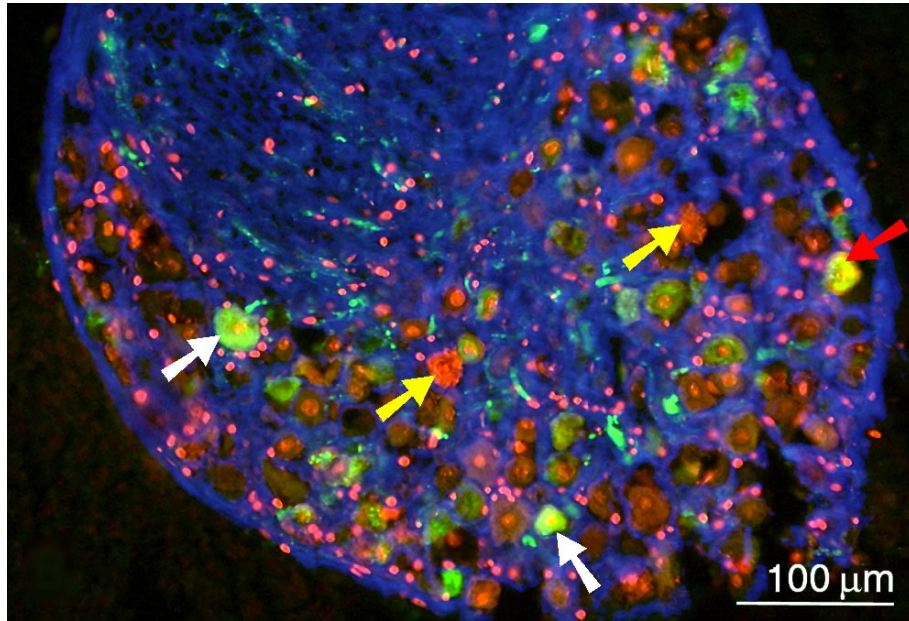


Figure 3.6 The superimposed image of all three different staining: PI, laminin and phenotypic marker, in this case CGRP. These types of combined images were helpful determining the phenotypes of dead and live neurons in conjunction with single-signal images. White arrows show some of the CGRP-positive live neurons, yellow arrows indicate CGRP-negative dead neurons and red arrow points a CGRP-positive dead neuron.

3.3 Results

3.3.1 Neuronal survival in explant DRG cultures and effects of NGF and LIF

There was only a minimal cell loss in cultured DRGs at the end of 3-day incubation. The number of identifiable neurons in optical sections after 3 days in culture was 95-98% of numbers in freshly fixed ganglia indicating neuronal loss of only 2-5% in DRGs from all three segments (table 3.4). The difference between the numbers of neurons in cultured and fresh preparations was not statistically significant as revealed by χ^2 test. However, some of the intact neurons could be dying. Therefore, to determine the proportion of viable cells, I used the PI method.

The percentage of dead cells identified by this method was 6.2% - suggesting that a proportion of the neurons counted in the optical sections were in fact dead or dying (table 3.7). However, even combining the percentages of identified dead cells with the estimated percentages of lost cells, the result

indicate that more than 90% of neurons remain viable after 3 days in culture (but see my comments regarding classification of cells as living or dying by the PI method in discussion).

To test the impact of stress on neuronal survival and to investigate the effect of NGF and LIF on a larger scale neuronal loss, some preparations were kept in an ordinary fridge overnight to make the culture medium unfavourable for the cells by letting it become more alkaline prior to fixation. As it was expected the death rate increased up to 29% in those cultures (table 3.10).

To determine the effects of growth factors on neuronal survival, preparations were incubated with NGF or LIF for 3 days and the numbers of neuronal profiles in optical sections and the percentages of dying neurons in frozen sections were determined. Since there was no significant difference in the number of neurons between optical sections of fresh and cultured preparations, even without growth factors, only the data from the frozen sections of normal cultures and those kept in alkaline medium were statistically evaluated for the effects of growth factors (tables 3.5 and 3.6). The proportion of dead neurons in the frozen sections of preparations with NGF dropped from 6.2 to 3.9% ($p < 0.01$) (table 3.7) in normal cultures and from 29 to 24% in stress-exposed preparations. LIF, on the other hand, did not change overall neuronal loss in either type of preparation. On the other hand, when the survival of different phenotypes of neurons compared, LIF appeared to significantly lower the death rate in CGRP labelled neuronal population (table 3.8). However this finding should be interpreted cautiously since the number of neurons labelled by this antibody decreased markedly in culture. It is therefore impossible to be sure of the numbers of surviving neurons which were initially CGRP-positive.

3.3.2 The phenotypic properties of DRG neurons in explant cultures and neuronal death

The changes in the phenotypic distribution of DRG neurons in explant cultures and the effects of NGF and LIF on this were compared to the control DRGs. The antibody to CGRP labelled mainly small and medium sized neurons, RT97 larger cells and IB4 small neurons (figures 3.8, 3.9 and 3.10). The normal

Table 3.4. Numbers of neurons in freshly fixed (control) and 3 days cultured (C3) T10-T12 DRGs.

	Control	C3	C3/control
T10	1832	1748	0.95
	1901	1872	0.98
T11	2115	2047	0.97
	2039	1989	0.98
T12	2642	2518	0.95
	2416	2340	0.97

The difference between control and culture preparations is evaluated with χ^2 test and found to be not significant.

Table 3.5. Numbers of neurons in control and 3 days cultured preparations in the presence and absence of NGF or LIF.

	Control*	C3*	C3+NGF		C3+LIF	
T10	1866.5	1810	1858	(1.0/1.03)	1822	(0.98/1.0)
T11	2077	2018	2060	(0.99/1.02)	1993	(0.96/0.99)
T12	2529	2429	2497	(0.99/1.03)	2441	(0.97/1.0)

Proportions of counts from NGF and LIF treated preparations to control and C3 are given in parentheses. *Mean neuronal counts (n = 2).

Table 3.6. Comparison of means of neuronal numbers of 3 days cultured and freshly fixed T10-T12 DRGs.

Treatment	n	Culture/Control ratio
None	6	0.97±0.0049
NGF	3	0.99±0.0023
LIF	3	0.97±0.0048

Table 3.7. The percentage of dead neurons on the frozen sections of cultured thoracic DRGs.

C3	C3+NGF	C3+LIF
6.2 ± 0.7	3.9 ± 0.5*	5.6 ± 0.6
n = 1203 (20)	n = 1451 (22)	n = 1387 (21)

The number of sections examined is given in parentheses. A total of 36 DRGs were removed from 6 mice and pooled. 12 DRGs were used for each type of preparations. n = total number of neurons. Student's t-test is used to compare NGF and LIF to C3 (*p < 0.01).

Table 3.8. The comparison of the numbers of dead and total neuronal profiles stained for different markers from frozen sections of cultured DRGs.

	Death rate % (marker)	Death rate % (total)
C3		
CGRP	9.1±2.9 <i>n</i> = 99 (7)	5.1±1.1 <i>n</i> = 391 (7)
RT97	8.3±4.6 <i>n</i> = 36 (6)	9.8±1.6 <i>n</i> = 357 (6)
IB4	4.3±0.04 <i>n</i> = 46 (7)	4.2±0.04 <i>n</i> = 455 (7)
C3+NGF		
CGRP	5.0±2.0 <i>n</i> = 119 (7)	2.5±0.7 <i>n</i> = 472 (7)
RT97	1.9±1.3 <i>n</i> = 106 (8)	4.6±0.9 <i>n</i> = 498 (8)
IB4	1.6±1.6 <i>n</i> = 62 (7)	4.4±0.9 <i>n</i> = 481 (7)
C3+LIF		
CGRP	2.0±1.2* <i>n</i> = 149 (9)	7.0±0.9 <i>n</i> = 762 (9)
RT97	0.0±0.0 <i>n</i> = 31 (5)	0.5±0.5 <i>n</i> = 187 (5)
IB4	1.8±1.8 <i>n</i> = 55 (7)	5.3±1.1 <i>n</i> = 438 (7)

Number of sections examined is given in parentheses. Sections were obtained from 4 DRGs for each marker. A total of 36 DRGs were removed from 6 mice, pooled. *n* = total number of neuronal profiles. Student's t-test is used to compare the death rate of marker-labelled neurons to the total death rate in that group. **p* < 0.05.

distribution of three markers and its comparison to the results from the PI experiments are given in table 3.9 and shown in figure 3.7.

The numbers of neurons labelled by all three markers decreased significantly during 3 days in culture compared to control (*p* < 0.001). When the effects of NGF and LIF on the individual markers were examined, it was found that they had no effect on the IB4-labelled neurons. However, both factors increased the number of RT97 labelled neurons significantly (*p* < 0.05) compared to control cultures and magnitudes of their effects were not statistically different

Table 3.9. The comparison of the changes in phenotypic markers in cultured preparations and the freshly fixed controls.

	Control	C3	C3+NGF	C3+LIF
CGRP	45.9±2.1** <i>n</i> = 542 (10)	25.3±2.2 <i>n</i> = 391 (7)	25.2±2.0 <i>n</i> = 472 (7)	19.6±1.4*# <i>n</i> = 762 (9)
RT97	35.7±2.2** <i>n</i> = 485 (8)	10.1±1.6 <i>n</i> = 357 (6)	21.3±1.8** <i>n</i> = 498 (8)	16.6±2.7* <i>n</i> = 187 (5)
IB4	32.9±2.6** <i>n</i> = 337 (7)	10.1±1.4 <i>n</i> = 455 (7)	12.9±1.5 <i>n</i> = 481 (7)	12.6±1.6 <i>n</i> = 438 (6)

For each phenotypic marker, sections were obtained from 4 DRGs from a pool of 12 DRGs from 2 mice in fresh control preparations and 36 DRGs removed from 6 mice in cultured preparations. The expression of each marker in C3 was compared to those in others (**p* < 0.05, ***p* < 0.001). NGF and LIF preparations are also compared (#*p* < 0.05). *n* = total number of neuronal profiles.

Table 3.10. The survival of T10-12 DRG neurons in explant cultures exposed to alkaline environment during last 12 hours on the last day of 3 day incubation.

Treatment	Dead counts	Total counts	Marker/ total ratio
None (10)	248	853	0.29±1.6
NGF(12)	285	1182	0.24±1.2*
LIF(9)	219	779	0.28±1.6

The number of sections examined is given in parentheses. For each series a total of 6 DRGs from 2 mice were used. *n* = total number of neurons. Student's t-test is used to compare NGF and LIF to C3 (**p* < 0.05).

(*p* > 0.05). On the other hand LIF caused a decrease in numbers of CGRP-labelled neurons (*p* < 0.01) while NGF did not change it.

The comparison of the susceptibility of neuronal subpopulations to death indicated that their phenotypic properties did not make any significant contribution to their fate during 3 days in culture (see table 3.8). Thus, there was no selective loss from one particular population.

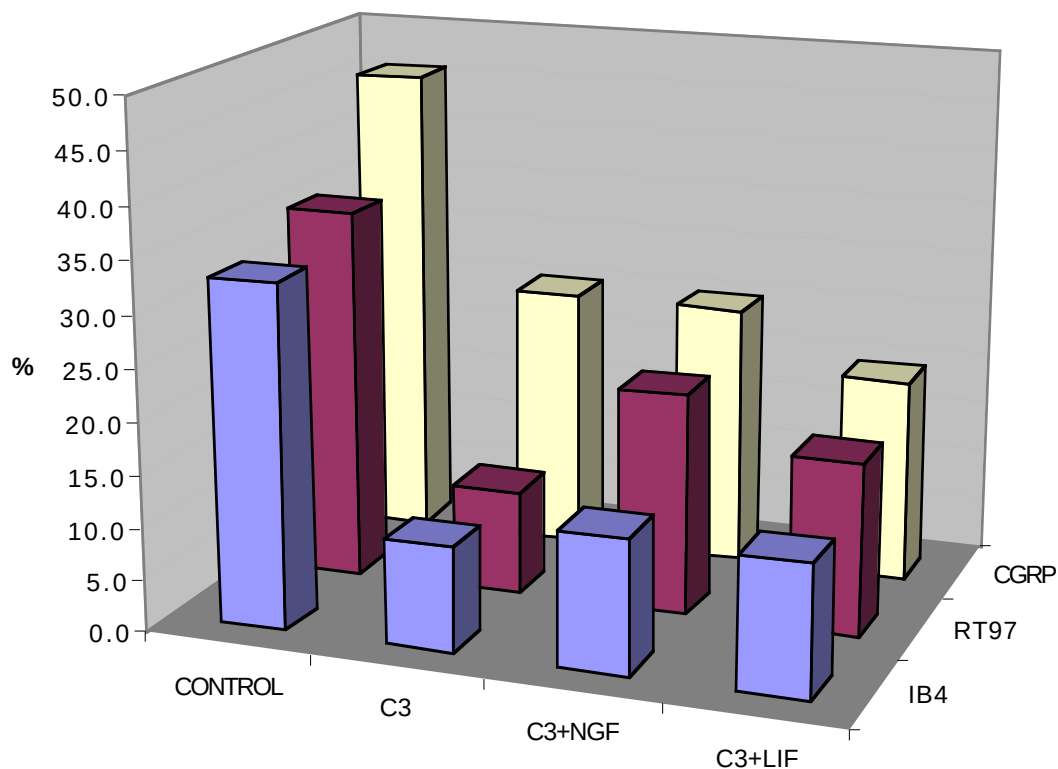


Figure 3.7 The proportions of CGRP, RT97 and IB4 labelled neurons in normal DRGs and explant cultures. The proportion of neurons labelled by all three markers decreased significantly in 3 days in culture. NGF and LIF partially prevented the reduction in number of RT97 labelled neurons while LIF accentuated the reduction of CGRP labelled neurons in culture. IB4 labelling was not affected by NGF or LIF.

3.4 Discussion

3.4.1 The majority of DRG neurons survived 3 days in explant cultures

Most studies of neuronal survival and loss in DRGs have been based on cell counts from histological sections and various mathematical correction factors to avoid double counting (Rose and Rohrlach, 1987). The small DRGs which I used and the technical possibilities of the confocal microscope allowed me to count directly the total number of neurons. Although this technique did not differentiate dead and living cells, PI staining seemed to give complementary information about neuronal survival. Since anti-NeuN slightly stains cytoplasm, it is not always possible to differentiate nuclear material leakage into the cytoplasm from a nucleus starting to disintegrate. Occasionally distorted nuclei

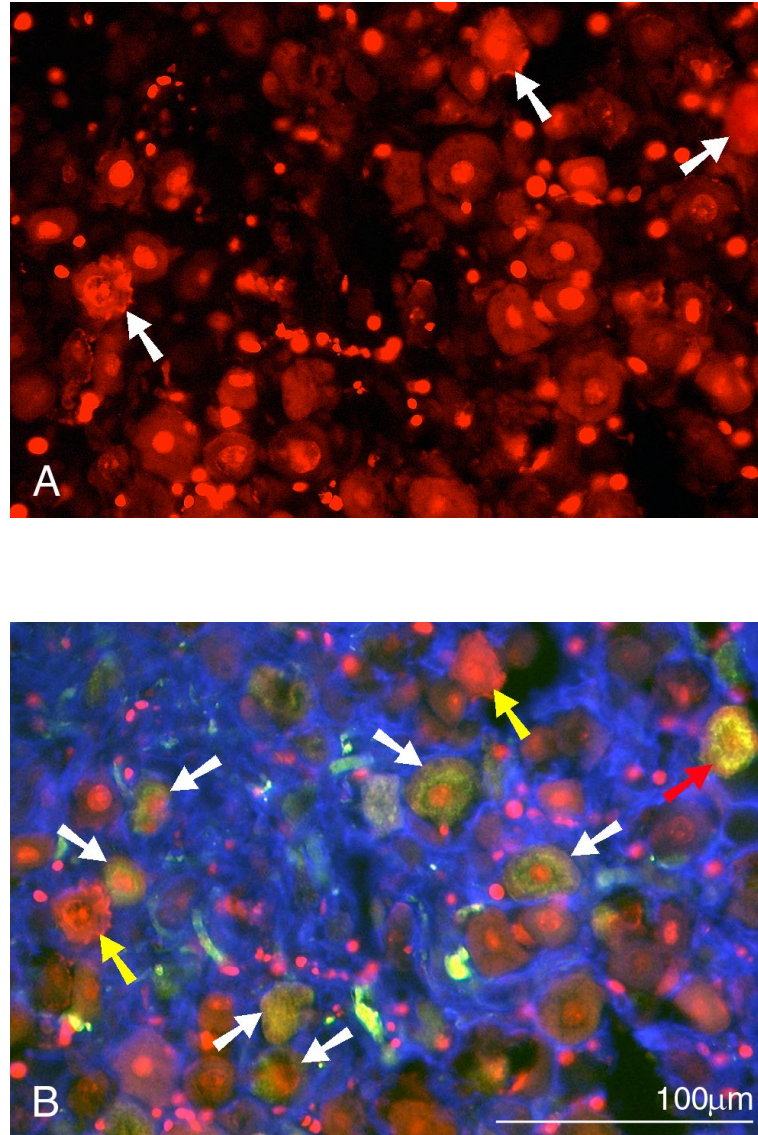


Figure 3.8 CGRP expression in a DRG section. PI staining (A) showing dead cells (arrows) and superimposed image (B) showing dead cells (yellow arrows) and CGRP labelled neurons (white arrows) one of which is dead (red arrow).

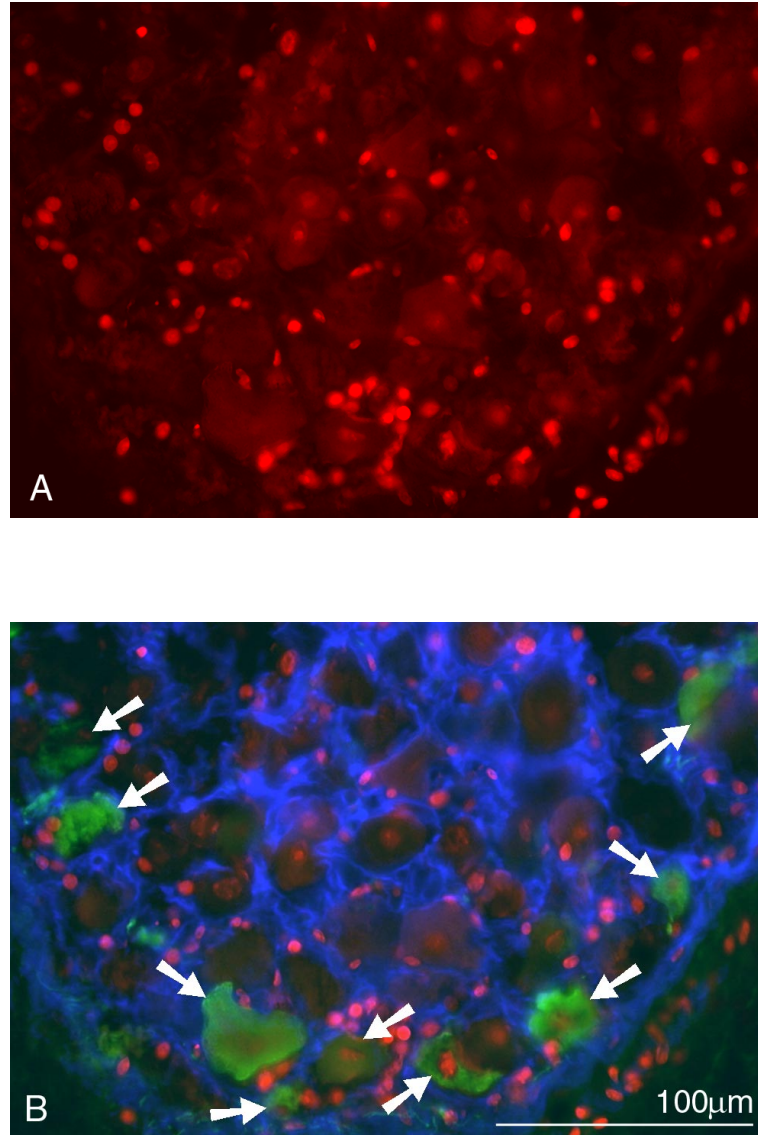


Figure 3.9 RT97 expression in a DRG section. There appear to be no dead cells in this section (A). The superimposed image (B) shows RT97 labelled neurons (arrows).

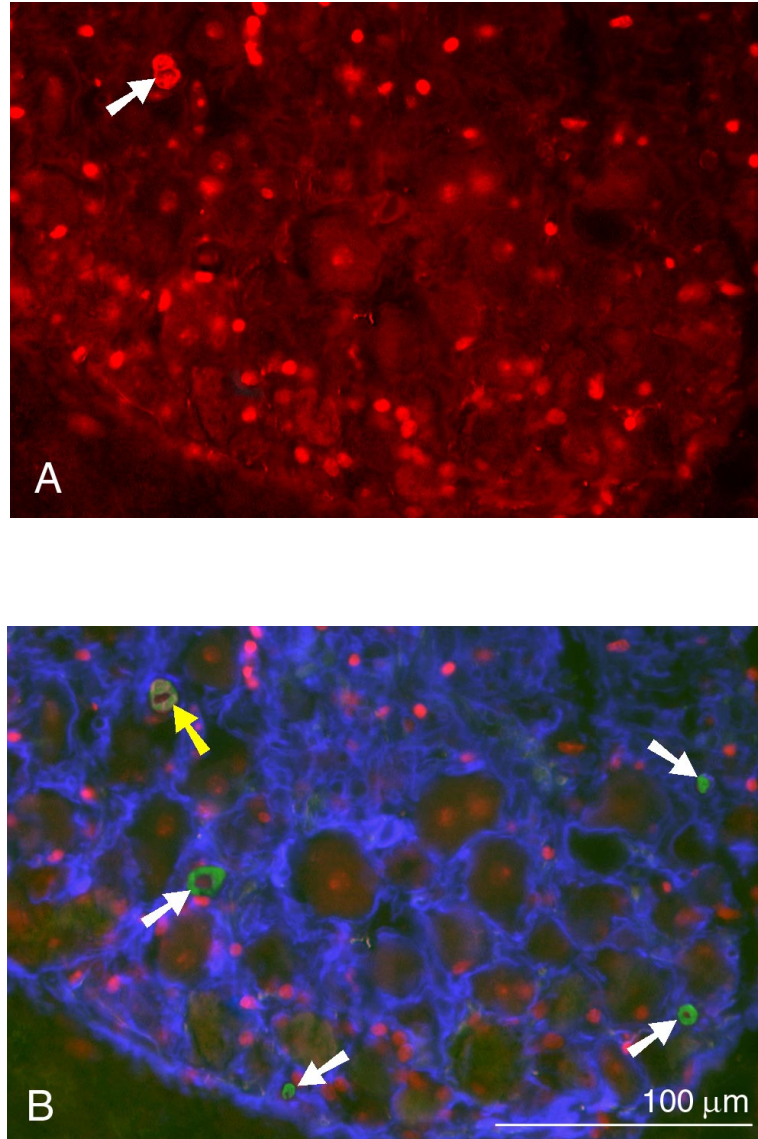


Figure 3.10 IB4 labelling in a DRG section. PI staining (A) showing a dead cell (arrow) and superimposed image (B) showing the same dead cell (yellow arrow) which is also labelled for IB4 and other IB4 labelled neurons (white arrows).

are visible in some neurons (figure 3.3), an indication of ongoing degeneration, but to differentiate them on several dozens of sections is not feasible especially when the images are taken at lower magnifications. Therefore, in these experiments, anti-NeuN proved reliable in determining only absolute numbers of neurons present on the sections. Among them may be present some dead neurons which have not disappeared completely and also some others which are in the process of deterioration. PI, on the other hand, may accurately label these latter neurons. Thus, information obtained from the preparations stained with anti-NeuN and PI provided an optimum estimation about the extent of neuronal loss in DRG cultures. The graph in figure 3.11 was prepared by merging

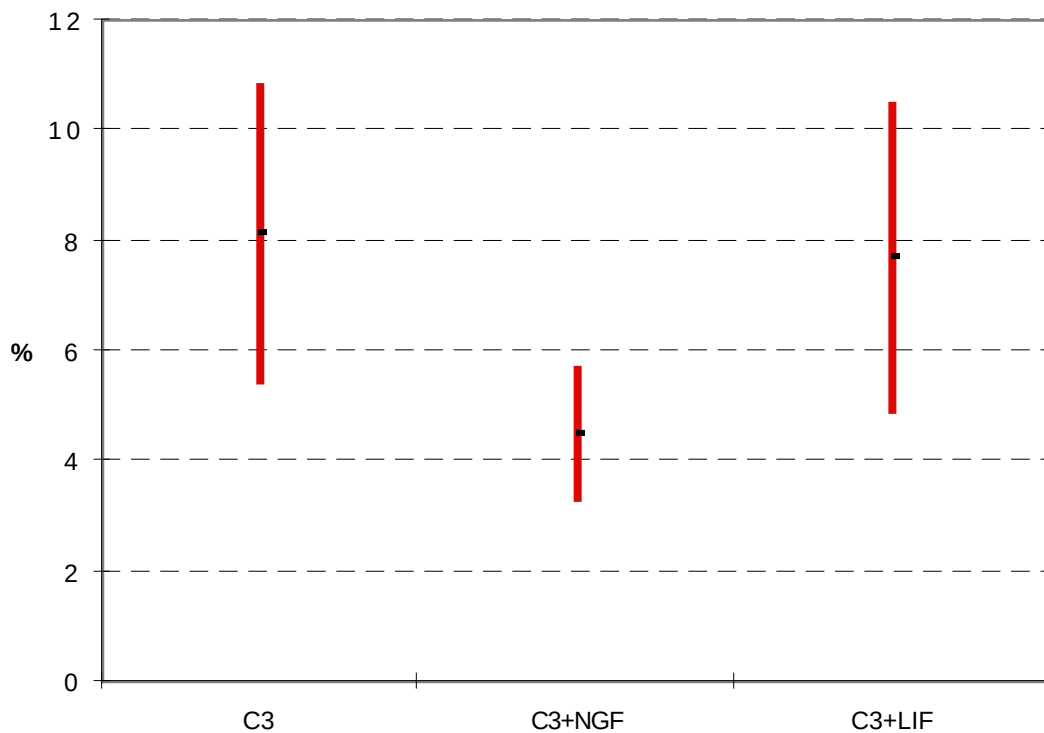


Figure 3.11 The estimated range of neuronal death in thoracic DRG explant cultures in 3 days. The graph combines both optical and frozen section data sets including S.E.M. The minimum levels of neuronal loss were calculated by subtracting SEM from the percentage of PI-stained neurons -assuming that all NeuN negative neurons might be picked up by PI. Assuming that no lost cell might be labelled by PI, the maximum points were obtained, by the summation of percentage of PI positive neurons + SEM for the percentages of PI-stained neurons + SEM of percentage of neuronal loss according to NeuN staining + percentage of neuronal loss according to NeuN staining + SEM of percentage of neuronal loss according to NeuN staining.

two series of data from anti-NeuN and PI stained preparations. A lower and upper limit of 'possible' neuronal loss was calculated incorporating standard errors. According to this figure, the possible minimum and maximum neuronal loss in explant DRG cultures at the end of 3 days is 5,5 and 10,8 percent. NGF treated preparations exhibit the lowest limits, which is 3,4 – 5,7%. LIF also very slightly lowered the both ends to 4,9 – 10,4%.

These findings suggest that the neuronal survival in explant DRG cultures is high enough to use these preparations as a reliable experimental model for studying phenomena such as axonal regeneration. The massive increase in the proportion of dying cells observed in the preparations that were kept in the refrigerator overnight demonstrated that neuronal survival is severely affected by adverse culture conditions. The high rate of survival in normal preparations compared to those in alkaline medium shows that explant DRG culture is useful *in vitro* model.

Low neuronal death rate in adult DRG cultures without any additional growth factors is consistent with Lindsay's findings (1988b). He used dissociated cell cultures and reported a maximum of 80% neuronal survival rate, which is lower than which I demonstrated in DRG explant cultures in this study.

The high rate of neuronal survival in DRG explant cultures may depend on various factors but support from non-neuronal cells is probably important. It is known that a number of growth factors are produced in explanted cut peripheral nerves and DRGs. Among them are NGF (Heumann *et al.*, 1987b), LIF (Banner and Patterson, 1994) and BDNF (Lindsay 1988a; Acheson *et al.*, 1995). These factors have been reported to support survival of mature primary sensory neurons although they are not essential (see general introduction for details).

Another explanation for the high survival rate in these preparations may be that DRGs are not exposed to enzymatic or mechanical treatments which are necessary for dissociation of neurons, but which may also kill a significant proportion of cells.

3.4.2 There is no evidence for selective neuronal loss in explant DRG cultures

The phenotypic properties of the lost neurons after axotomy *in vivo* is not clear since there are contradictory reports (see the introduction of this chapter for details). This is partly due to technical difficulties and mainly because of the disappearance of immunoreactivity to the markers shortly after axotomy (Groves *et al.*, 1996). The techniques I used in this study made it possible to determine the phenotypes of a proportion of dead/dying neurons. However, it was impossible to tell anything about the phenotypes of neurons that had disappeared. Nevertheless, considering the small extent of detected neuronal loss in optical sections, any possible phenotypic preference in this population would not greatly alter the overall outcome of the study. Another point to take into consideration is the fact that it was not possible to determine phenotype of all dead cells, as some of them had no marker, presumably as a consequence of axotomy-induced down-regulation or terminal degeneration of the cells.

I detected no selective neuronal loss in the explant DRG cultures as far as the markers CGRP, RT97 and IB4 are concerned. However, this does not mean that there is no difference by reference to other phenotypic properties. Cell size, for example, is the most widely studied criterion by other people. I did not determine the size distribution of dead and live neurons in this study, since after axotomy, a significant generalized atrophy is observed in DRG neurons and this is more evident in dying cells (Rich *et al.*, 1987; Groves *et al.*, 1997). Therefore, measurements taken from such neurons would be unreliable and misleading.

3.4.3 The expression of CGRP and RT97 and IB4 labelling of DRG neurons in explanted cultures

In this study, I showed that neurons may be labelled by anti-CGRP, RT97 and IB4 in explant DRG cultures, although the proportions of neurons which are positive for these markers decrease. This is consistent with the findings of others (Persson *et al.*, 1996) that report similar decreases in neurofilament content (Oblinger and Lasek, 1988), IB4 binding (Bennett *et al.*, 1998) and

CGRP expression after axotomy *in vivo* (Noguchi *et al.*, 1990) and CGRP expression *in vitro* (Lindsay and Harmar, 1989, Lindsay *et al.*, 1989) in DRG neurons.

NGF did not appear to affect the expression of CGRP in my study. In contrast, others have shown that NGF induces CGRP expression in dissociated sensory neuron cultures (Lindsay and Harmar, 1989, Lindsay *et al.*, 1989). One explanation for this discrepancy is that dissociated DRGs are totally axotomized and are deprived of the natural environment of satellite cells and ECM. In consequence, expression of CGRP in such neurons is generally not detected in the absence of NGF (Lindsay *et al.*, 1989). In contrast, the DRG neurons in the explant DRG cultures may continue to receive trophic support from ECM and satellite cells and therefore continue to express CGRP as they do *in vivo* during the first few days after axotomy.

Another explanation for the failure of NGF to affect CGRP expression in the explanted DRGs is that NGF and LIF may have opposing effects on expression of this peptide. LIF is known to be expressed in cultured DRGs Banner and Patterson, (1994) and Mulder (1994) showed that it decreases CGRP expression in cultured DRG neurons. Therefore, locally produced LIF might have prevented CGRP induction by added NGF.

The reduction in the proportion of neurons labelled by RT97 was partially antagonised by NGF and LIF. NGF is known to increase neurofilament expression in DRGs (Verge *et al.*, 1990) so an up-regulation in RT97 expression might be expected. However, observation of a similar effect with LIF is a new finding.

Bennett *et al.* (1998) demonstrated that intrathecal NGF administration could partially prevent the reduction in the number of IB4 binding neurons following axotomy. However, in present study, neither NGF nor LIF had such an effect in explant DRG cultures.

3.4.4 NGF increased overall neuronal survival

Consistent with previous studies that demonstrated NGF's rescue effect on DRG neurons after axotomy (see the introduction of this chapter and Johnson and Yip, 1986), this factor improved neuronal survival in explant DRG cultures. According to my findings there was no selectivity for any neuronal subpopulations labelled for three different phenotypic markers, CGRP, RT97 and IB4. However, this might not reflect the actual situation as there are some technical points to consider. The small extent of neuronal loss, together with the presence of a large proportion of unidentified neurons which presumably lost their phenotypic markers make it difficult to interpret the data. However, in parallel with the findings in this study, it has been reported by others that NGF non-selectively rescued all axotomized DRG neurons (Rich *et al.*, 1987; Otto *et al.*, 1987).

LIF did not increase the overall neuronal survival in explant DRG cultures in my experiments. Although analysis of the data suggested that it might selectively rescue CGRP labelled neurons, because of the same technical considerations mentioned above this finding must also be interpreted cautiously. However, this finding is consistent with some other reported properties of LIF. LIF is proposed to act as a target – derived neuroregulatory factor, analogous to NGF (Murphy *et al.*, 1997). In normal animal when NGF is available to the primary sensory neurons from the target tissue, CGRP level is believed to be tonically maintained by this factor. Following axotomy however, DRG neurons are deprived of NGF but they start to receive LIF in abundance, which is locally induced in injured nerve and conveyed through augmented retrograde transport (Hendry *et al.*, 1992; Curtis *et al.*, 1994). The target of LIF in DRGs are mostly those neurons which normally express CGRP as Thompson *et al.* (1997) demonstrated that when LIF was injected intraneurally it accumulated in small neurons in DRGs and 81% of them were CGRP positive, 62% TrkA-positive and 34,5% IB4-positive cells. Previously, Hendry *et al.* (1992) showed that at least 60% of sensory neurons in culture bound LIF. The selectivity of LIF binding in favour of small DRG neurons and mainly CGRP and TrkA expressing ones support my findings in this study. It could help to explain why LIF might

selectively rescue CGRP-labelled neurons and partially explain why it failed to increase the total neuronal survival in explant DRG cultures.

3.5 Conclusion

In this study I have investigated the survival and phenotype of DRG neurons in explant cultures in the absence and presence of growth factors.

- ◆ Most of the DRG neurons in explant cultures survived during 3 days in vitro with only about 5% obviously dead, although an unknown proportion of others might be dying.
- ◆ Neurons retain neurochemical markers CGRP, RT97 and IB4, although the proportions expressing them declined during 3 days in culture.
- ◆ There was no evidence of selective death of neurons of any specific phenotype although the generalized loss of markers made this difficult to determine.
- ◆ NGF seemed to promote survival of neurons but not LIF (LIF on the other hand, might have supported CGRP expressing cells).
- ◆ Expression of CGRP did not seem to be affected by NGF, but might be down-regulated by LIF.
- ◆ The proportion of neurons labelled by RT97 was supported by both NGF and LIF.

Chapter 4 :Effects of LIF and NGF on axonal growth and GAL expression in explant DRG cultures

4.1 Introduction

4.1.1 Leukemia inhibitory factor as a lesion-induced cytokine

Leukemia inhibitory factor is a pleiotropic cytokine with wide spread actions on various cell and tissue types from lipid metabolism to neuronal differentiation (for details see the general introduction) (Hilton and Gough, 1991; Hilton *et al*, 1991). It is also known as cholinergic differentiation factor as its first observed effect on nervous tissue was the induction of cholinergic differentiation in cultured noradrenergic sympathetic neurons (Fukada, 1985; Yamomori *et al*, 1989).

Although LIF mRNA is not detectable in peripheral nerves under normal conditions, the situation changes after an injury. Sympathetic and sensory neurons, however, exhibit different responses in this respect (fig. 4.1). Curtis *et al*. (1994) demonstrated that following axotomy LIF mRNA is induced rapidly in leisoned nerves. A similar increase in LIF mRNA is observed in SCGs *in vivo* after axotomy (Rao *et al.*, 1993b; Banner and Patterson, 1994). The mechanism that regulates LIF expression is not clear but IL-1 β may play a role. It has been shown that IL-1 β induces LIF production in sympathetic ganglia after axotomy (Shadiac *et al*. 1993). Macrophages infiltrating an injury site produce IL β -1 (Freidin *et al.*, 1992; Jonakait, 1993) which might be responsible for the up-regulation of LIF in the leisoned nerve. Sun *et al*. (1996), however, proposed the existence of a novel LIF inducing factor which is normally present in peripheral nerves and activated and/or released after axotomy.

In contrast to SCGs, LIF mRNA is not induced in DRGs after axotomy (Curtis *et al.*, 1994; Sun and Zigmond, 1996a). However, it was shown that it is retrogradely transported through peripheral nerves to sensory and motor neurons and that peripheral nerve injury increases the transport up to 3-fold (Curtis *et al.*, 1994). The targets of LIF in DRGs are mostly those neurons that normally express trkA and CGRP. Thompson *et al*. (1997) demonstrated that when LIF was injected into the sciatic nerve, it accumulated in small neurons in L4-L5

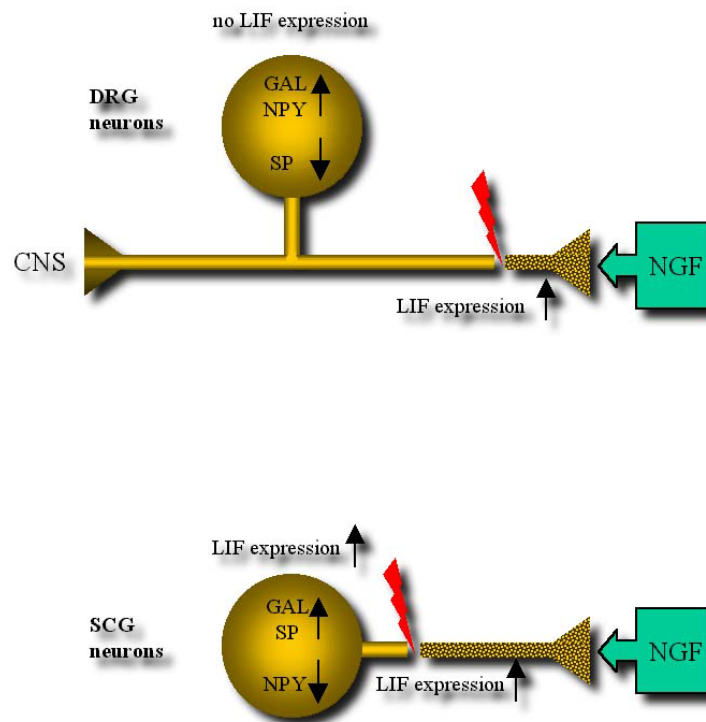


Figure 4.1 An illustration showing the sites of LIF induction and the changes in GAL, SP, and NPY in DRG (top) and SCG (bottom) after axotomy and source of NGF (modified from Sun *et al.*, 1996a).

DRGs and that 62% of these were *trkA* positive while 81% of them expressed CGRP.

LIF mRNA is induced *in vitro* in explanted SCGs (Sun *et al.*, 1994; Sun *et al.*, 1996) and also in DRG cultures (Banner and Patterson, 1994; Sun and Zigmond, 1996a). It has been demonstrated that LIF is produced by Schwann cells and fibroblasts in culture (Shadiack *et al.*, 1993; Curtis *et al.*, 1994; Sun *et al.*, 1994). However, the question why LIF mRNA is induced in explanted but not in axotomized DRGs still remains unanswered. One possible explanation is that, in axotomy, intact dorsal root axons may exert inhibition on LIF mRNA induction and this inhibition is removed in explanted DRGs since all of the axons are severed in this situation. However, Sun and Zigmond (1996a) demonstrated that dorsal rhizotomy together with peripheral axotomy produced only little LIF mRNA in rat DRGs in contrast to the large increase seen in cultured DRGs.

4.1.2 LIF, GAL expression and NGF

The role of LIF in the nerve regeneration process involves mainly the regulation of expression of certain neuropeptides (see review by Zigmond *et al.*, 1996). For example, after axotomy and in culture, VIP expression in SCGs is regulated by LIF (Sun *et al.*, 1994). The strongest relationship, however, seems to be the one between LIF and GAL expression.

GAL is a 29 amino acid peptide. It was originally isolated from porcine intestine (Tatemoto, *et al.*, 1983) and has a widespread distribution in the central nervous system (Melandar *et al.*, 1986; Skofitsch, and Jacobowitz, 1985a) as well as in primary sensory neurons (Ch'ng, *et al.*, 1985; Skofitsch, and Jacobowitz, 1985b). GAL is believed to have inhibitory actions on nociception as discussed in chapter I. However, it is expressed by developing trigeminal and DRG neurons suggesting that it may also be involved in axonal growth (Xu *et al.*, 1996a).

Recent *in vivo* and *in vitro* studies have demonstrated a strong relationship between LIF and GAL expression after peripheral axotomy. Sun and Zigmond (1996b) and Corness *et al.* (1996) demonstrated that, after axotomy, LIF deficient transgenic mice had substantially lower increases in GAL expression in DRGs compared to wild type animals. In another *in vivo* experiment, Thompson *et al.* (1998), showed that intraneurally injected LIF into a peripheral nerve causes up-regulation of GAL in corresponding DRGs.

Another factor which might affect GAL expression in DRGs is NGF. When NGF is added to adult SCG explants in culture, it blocks, by 50%, the normal increase in galanin levels (Zigmond *et al.*, 1995). This raises the possibility that NGF normally inhibits GAL expression and lack of this factor after axotomy may play a role in the induction of this neuropeptide. Support to this possibility comes from another study, in which NGF antiserum injected into unoperated rats lead to an increase in GAL mRNA and immunoreactivity in SCGs (Sun *et al.*, 1993).

4.1.3 Rationale for the study

Several recent studies have been carried out to elucidate the action of LIF during nerve regeneration and most of them have demonstrated its regulatory effects on expression of certain neuropeptides. Little is known, however, about the effect of LIF on axonal outgrowth. Mehler and Kessler (1995) demonstrated that LIF promotes neurite extension and morphological maturation in cultured embryonic neuroblasts. A recently described sympathetic sprouting from postganglionic afferents into DRGs after peripheral axotomy was also found to be induced by LIF (Thompson and Majithia, 1998).

This study was designed to investigate the effects of LIF on axonal regeneration of adult mouse DRG neuron *in vitro* and to extend the current knowledge on the contribution of LIF to GAL expression following axotomy. A possible interaction between LIF and NGF was also a major subject of investigation, as these two factors appear to have opposite effects on GAL expression. To do this, LIF, NGF and their combinations were added to the explant cultures of L4 DRGs of adult mice. A novel feature of these experiments was that it was possible to determine phenotypic changes in the cell bodies of primary sensory neurons and in outgrowing axons. The results of these *in vitro* experiments were compared to the findings from DRGs axotomized *in vivo*.

The advantage and the reliability of this *in vitro* system were explained in the previous chapter. For the first time, this *in vitro* model gave the chance to directly study the effects of LIF and NGF on GAL expression in DRGs.

4.2 Materials and methods

4.2.1 Animal procedures and tissue cultures

Animals were anaesthetised as described in chapter II. In the first part of the experiments, the lower 4-5 thoracic DRGs with short intercostal nerves attached (about 3 mm) were removed from the mice. These DRGs were then used to set up cultures which were processed later as whole-mount preparations.

In the second part, L4 DRGs were used to assess changes in the GAL expression at the cellular level using frozen sections. For this, both *in vivo* and *in vitro* experiments were done. For the *in vitro* part, L4 DRGs were removed

from the animals and the spinal roots and peripheral nerves were cut off. Most of the DRGs were used in cultures whilst some L4 DRGs were immediately fixed after removal to use as controls. In the *in vivo* part of the study, unilateral sciatic nerve transections were performed on animals and 2 days later the animals were sacrificed and L4 DRGs were removed and fixed immediately. The reason for choosing L4 DRGs in these experiments was that a substantial number of fibers from L4 run through the sciatic nerve (Devor *et al.*, 1995).

All the explant cultures were set up in collagen gels (type I, from Sigma) as described in chapter II in detail, incubated for 2 days and then fixed. LIF and NGF separately and in combination were added to some of the preparations (table 4.1 for the concentrations and the sources). 4% paraformaldehyde was used for all fixations.

4.2.2 Immunohistochemistry, microscopy and image analysis

Outgrowing axons were stained with antibodies to both GAL and the pan-axonal marker β -III tubulin. The staining procedure was performed as described in chapter II. After these preparations were scanned and quantification done, they were further processed for stereography using HRP staining (see chapter II for the details of the procedure).

The staining of frozen sections differed slightly from the explants. This included lower concentrations of primary and secondary antibodies (table 4.2) and shorter incubation time with the secondary antibodies, which is only 2 hours in this case.

The image analysis was performed as described in chapter II and included the measurement of the diameters and lengths of HRP-labelled outgrowing axons and counting numbers of labelled and unlabelled neurons and axons in the preparations stained using fluorescent secondary antibodies. In order to obtain extra information about the level of expression of GAL, intensity of fluorescent signal on the sections and explants were also analysed. Results are expressed as means $\pm$ standard error of means (S.E.M) where appropriate. The differences between means and percentages were evaluated by Student's t test and considered significant at $p < 0.05$.

Table 4.1. The concentrations and sources of factors used.

Factor	Concentration	Source
NGF 2.5 S	50 ng/ml	<i>Alamone Labs and Promega</i>
LIF	30 ng/ml	<i>Chemicon</i>
Rabbit α -NGF	10 μ l/ml	<i>Sigma</i>

Table 4.2. The concentrations and sources of primary antibodies.

Antibody	Concentration	Source
Rabbit α -GAL	1/50 (explants) 1/100 (frozen sections)	<i>Affiniti</i>
Mouse α - β -III tubulin (clone no: SDL.3D10)	1/10	<i>Sigma</i>
Rabbit α -PGP 9.5	1/200	<i>UltraClone</i>

Table 4.3. The concentrations and sources of secondary antibodies used.

Antibody	Concentration	Source
RITC -conjugated goat α -rabbit IgG	1/100	<i>Sigma</i>
FITC -conjugated goat α -mouse IgG	1/100	<i>Sigma</i>
Biotin-conjugated goat α -rabbit IgG	1/100	<i>Sigma</i>

4.3 Results

4.3.1 Effects of LIF and NGF on axonal growth in collagen gels

In control preparations after 24 hours, a few axons started to grow into the gels spontaneously and reached a mean length of 334 μ m at the end of 48 hours (table 4.4). LIF affected neither the length nor the total number of outgrowing axons significantly. NGF however induced a marked increase in the length (fig. 4.2) and total number of axons (figs. 4.3 and 4.4). There was no significant difference in the lengths and numbers of axons between NGF and NGF+LIF groups.

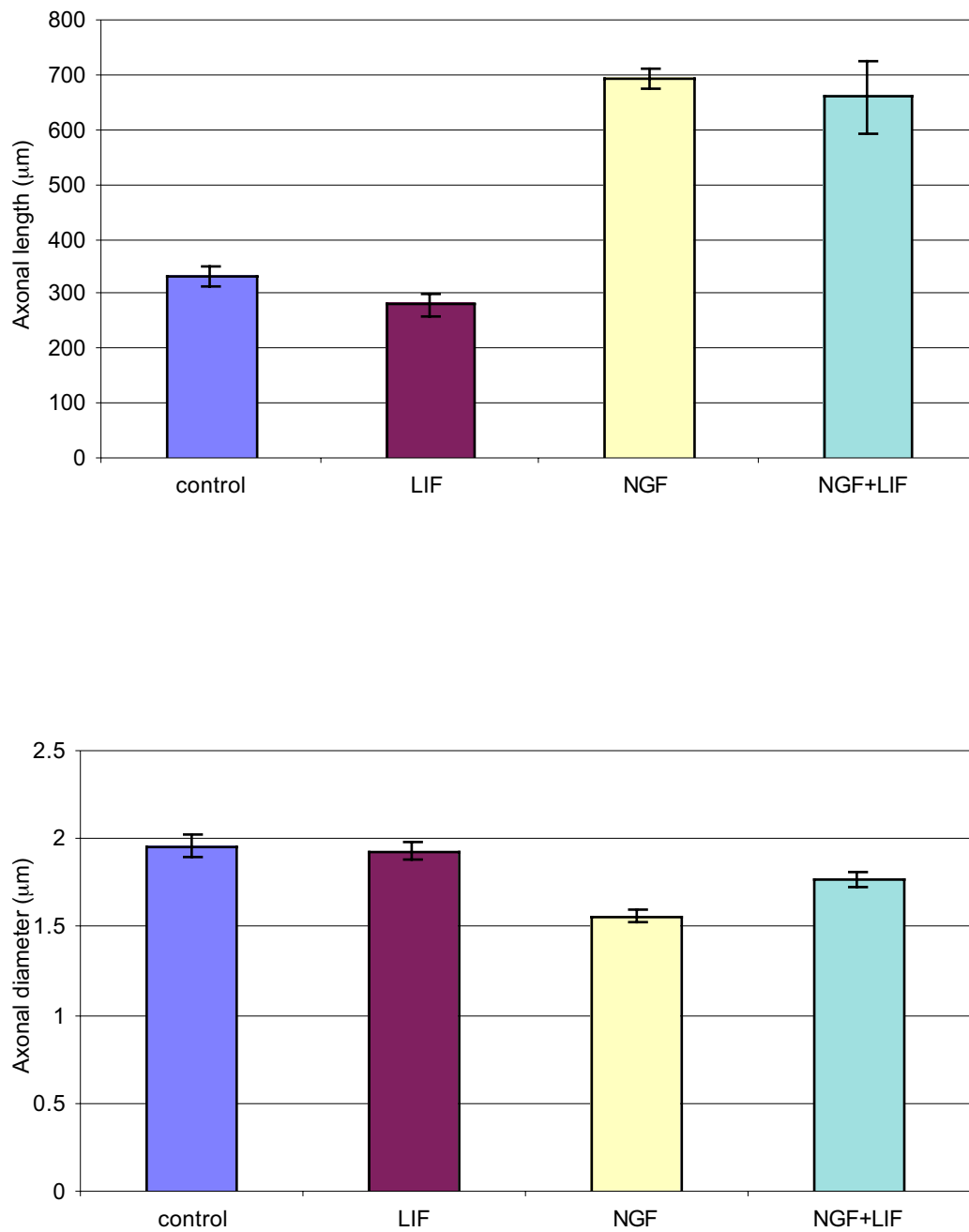


Figure 4.2 Axonal outgrowth from explanted DRGs into collagen gel in 48 hours. NGF enhanced axonal extension whereas LIF had no effect (top). NGF caused extension of smaller diameter axons (bottom). When NGF and LIF applied together, axons were thicker. LIF by itself had no significant effect on axonal diameter.

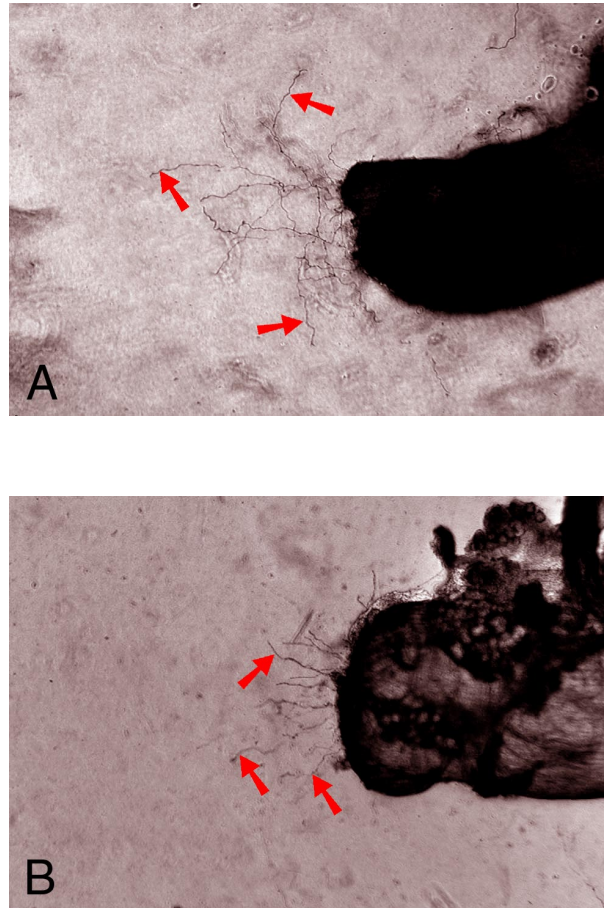


Figure 4.3 Outgrowing axons from explant DRG cultures in control (A) and LIF (B) preparations. LIF had no effect on axonal growth. Preparations were stained with PGP9.5 and labelled with HRP. Arrows indicate some of the growing axons.

Table 4.4. Axonal lengths and diameters in DRG explant cultures.

	Control	LIF	NGF	NGF+LIF
Axonal lengths	334±20 <i>n</i> = 85(6)	282±21 <i>n</i> = 85(6)	693±18 <i>n</i> = 127(6)*	660±68 <i>n</i> = 83(6)*
Axonal diameters	2±0.06 <i>n</i> = 78(6)	1.9±0.05 <i>n</i> = 96(6)	1.6±0.03* <i>n</i> = 247(6)	1.8±0.04 <i>n</i> = 224(6)

Units are in μm . The number of preparations examined is given in parenthesis. *n* = total number of axons. Student t-test is used to compare control to others in lengths and LIF to others in diameters (**p* < 0.01)

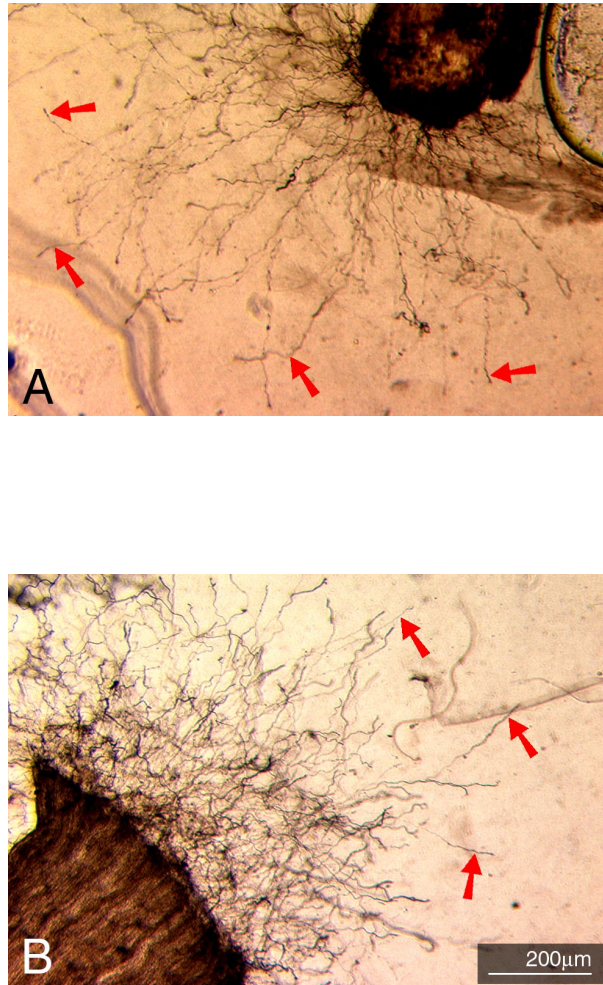


Figure 4.4 Outgrowing axons from explant DRG cultures with NGF (A) or NGF+LIF (B). NGF and NGF+LIF combination equally increased numbers and lengths of outgrowing axons. Preparations were stained with PGP9.5 and labelled with HRP. Arrows indicate some of the longest axons.

Although NGF increased the numbers and lengths of outgrowing axons, these were thinner than in controls and those with LIF (table 4.4). Interestingly, when NGF and LIF were added to the cultures together, the axons were thicker than with NGF alone ($p < 0.001$) (figs. 4.2 and 4.5).

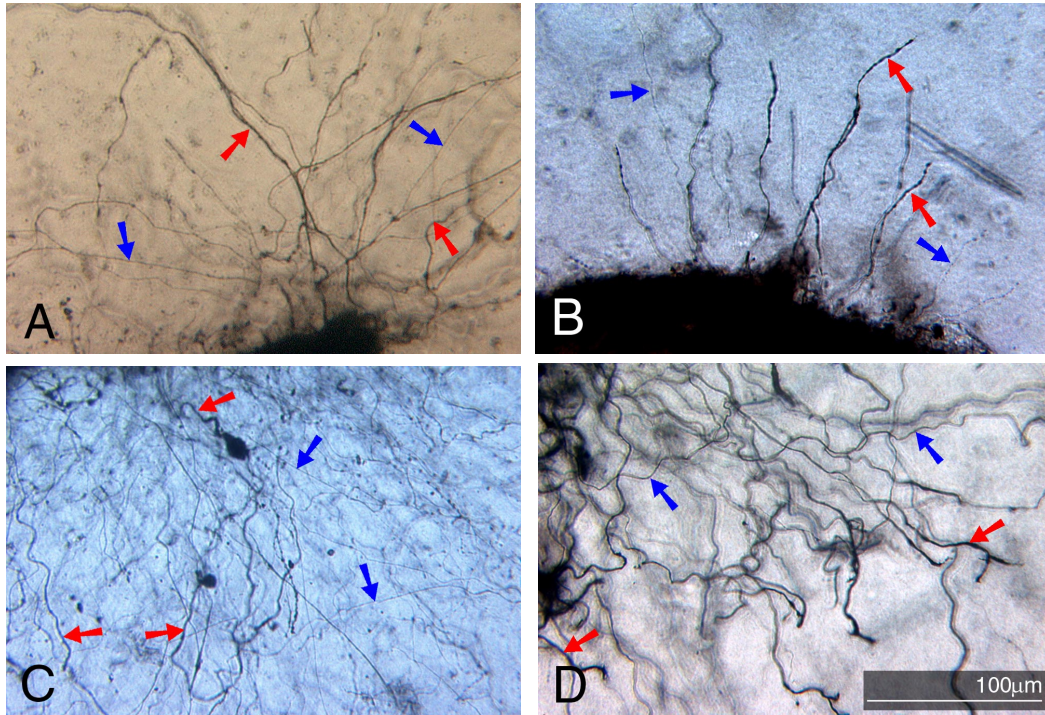


Figure 4.5 Thickness of outgrowing axons in control (A), LIF (B), NGF (C) and NGF+LIF treated (D) preparations. Note that NGF treated preparations had thinner axons than others. When LIF was added together with NGF, growing axons were thicker than NGF alone. Preparations were stained with PGP9.5 and labelled with HRP. Red and blue arrows indicate thinner and thicker axons respectively.

4.3.2 GAL expression in outgrowing axons

In control preparations, the percentage of GAL immunoreactive axons was 13% after 48 hours (table 4.5, figs. 4.6 and 4.7). This was increased to 33% by addition of LIF which reflects a real increase in the number of GAL expressing neurons since LIF did not cause an increase in the numbers of outgrowing axons (figs. 4.6 and 4.7). Similarly, when LIF and NGF were added together, this increased the proportion of GAL immunoreactive axons from 24% (NGF alone) to 36% (figs. 4.6 and 4.8). LIF + anti-NGF combination registered the least number of GAL positive axons with 10% (figs. 4.6 and 4.7).

The intensity of GAL staining in the axons was highest in the preparations treated with NGF+LIF (table 4.5, fig. 4.6). Control and LIF-treated preparations had slightly higher levels of signal than preparations cultured with NGF.

4.3.3 GAL expression in sections

In sections of normal DRGs, the proportion of GAL positive neurons was very low. The percentage of GAL positive cells in DRGs 48 hours after axotomy was significantly higher than normal DRGs (table 4.6). The proportion of GAL positive neurons in control-cultures was similar to that in axotomized DRGs but in the preparations cultured with LIF, the percentage of GAL positive neurons was significantly increased. NGF alone did not show any obvious effect on GAL expression in culture, however, the proportion of GAL positive neurons in DRGs cultured with NGF+LIF was lower than in preparations cultured with LIF alone.

Although normal preparations had a few GAL positive neurons, these cells were very brightly stained. Cut and cultured control preparations had more GAL positive neurons but intensity of staining was lower than in the few positive neurons in normal preparations. LIF caused an increase in the intensity of GAL staining compared with cut and cultured control preparations. While NGF had no effect on intensity of staining, preparations with NGF+LIF had intensities similar to those in control preparations suggesting that NGF antagonizes the stimulatory effect of LIF on GAL expression.

Table 4.5. Percentages and brightness of GAL immunoreactive axons.

	Control	LIF	NGF	NGF+LIF
Percentage	13±3* <i>n</i> = 64 (6)	33±6 <i>n</i> = 69 (6)	24±3 <i>n</i> = 196 (6)	36±4* <i>n</i> = 153 (6)
Brightness (arbitrary unit)	13±3 <i>m</i> = 7 (6)	13±2 <i>m</i> = 8 (6)	9±1* <i>m</i> = 23 (6)	27±3* <i>m</i> = 41 (6)

The number of preparations examined is given in parenthesis. *n* = total number of axons, *m* = number of GAL immunoreactive axons analysed. Student t-test is used to compare the percentages of GAL positive axons with NGF to others and the brightness of axons in cultures treated with LIF to those cultured under other conditions (**p* < 0.05, ***p* < 0.01).

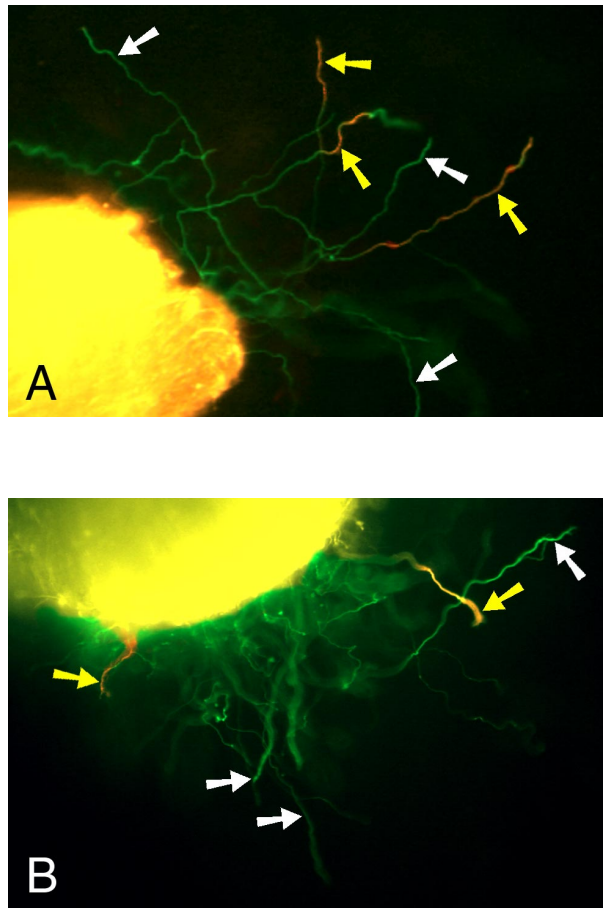


Figure 4.6 GAL expression in outgrowing axons in control (A) and LIF treated (B) preparations. Preparations were counter-stained for β -III tubulin. Axons with GAL immunoreactivity appear orange (yellow arrows) whilst the remaining axons, labelled only for β -III tubulin appear green (white arrows).

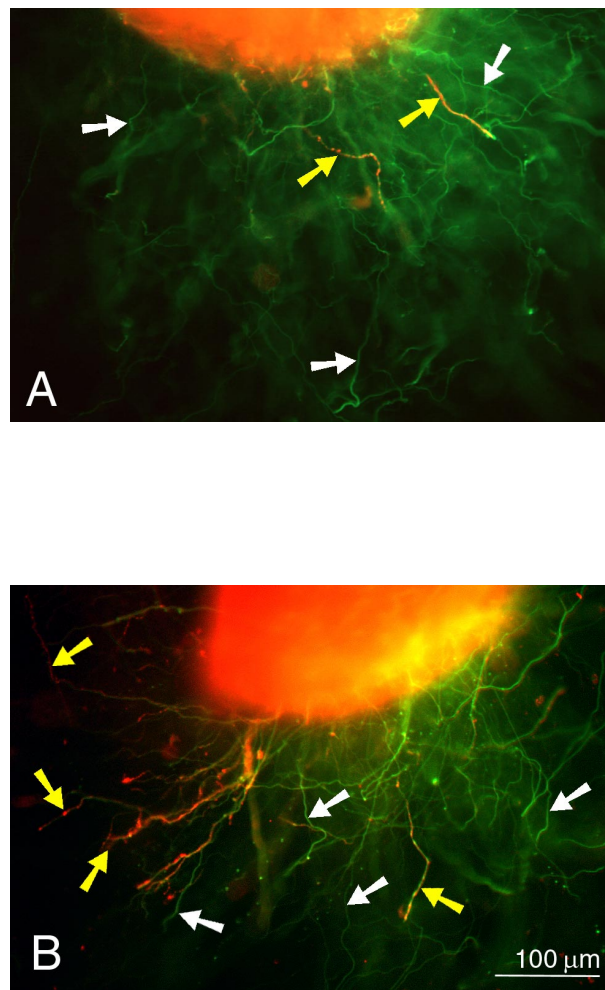


Figure 4.7 GAL expression in outgrowing axons in NGF (A) and NGF+LIF treated (B) preparations. The proportion of GAL positive axons in preparations cultured with LIF+NGF was higher than in preparations cultured with NGF alone. Preparations were counter-stained for β -III tubulin. Yellow arrows indicate some of the axons with GAL immunoreactivity and white arrows show some stained only for β -III tubulin.

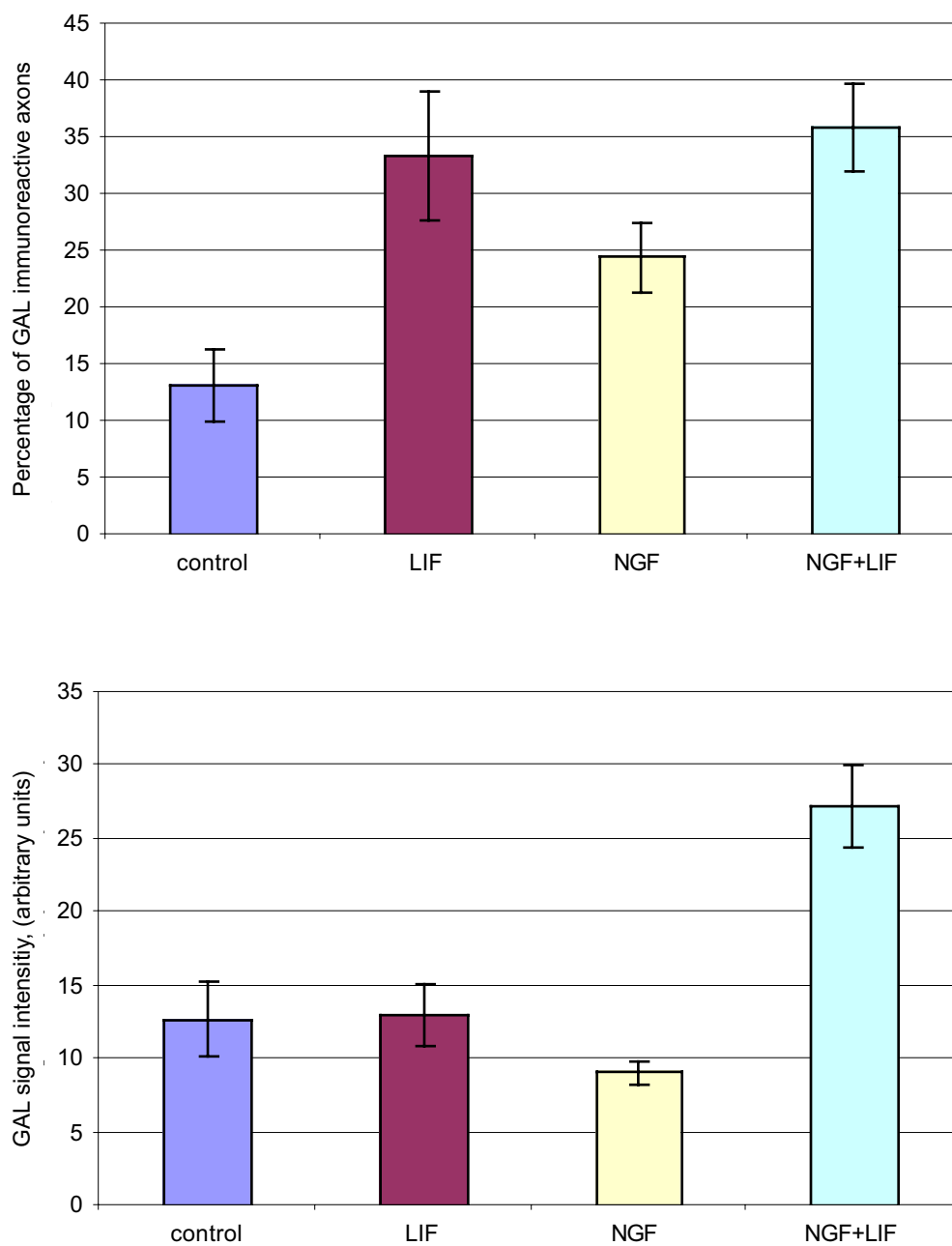


Figure 4.8 GAL immunoreactivity in outgrowing axons. The highest proportion of GAL immunoreactive axons (top) and brightest signal (bottom) were observed when NGF and LIF applied together. NGF and LIF separately increased percentage of GAL immunoreactive axons but did not significantly affect signal intensity.

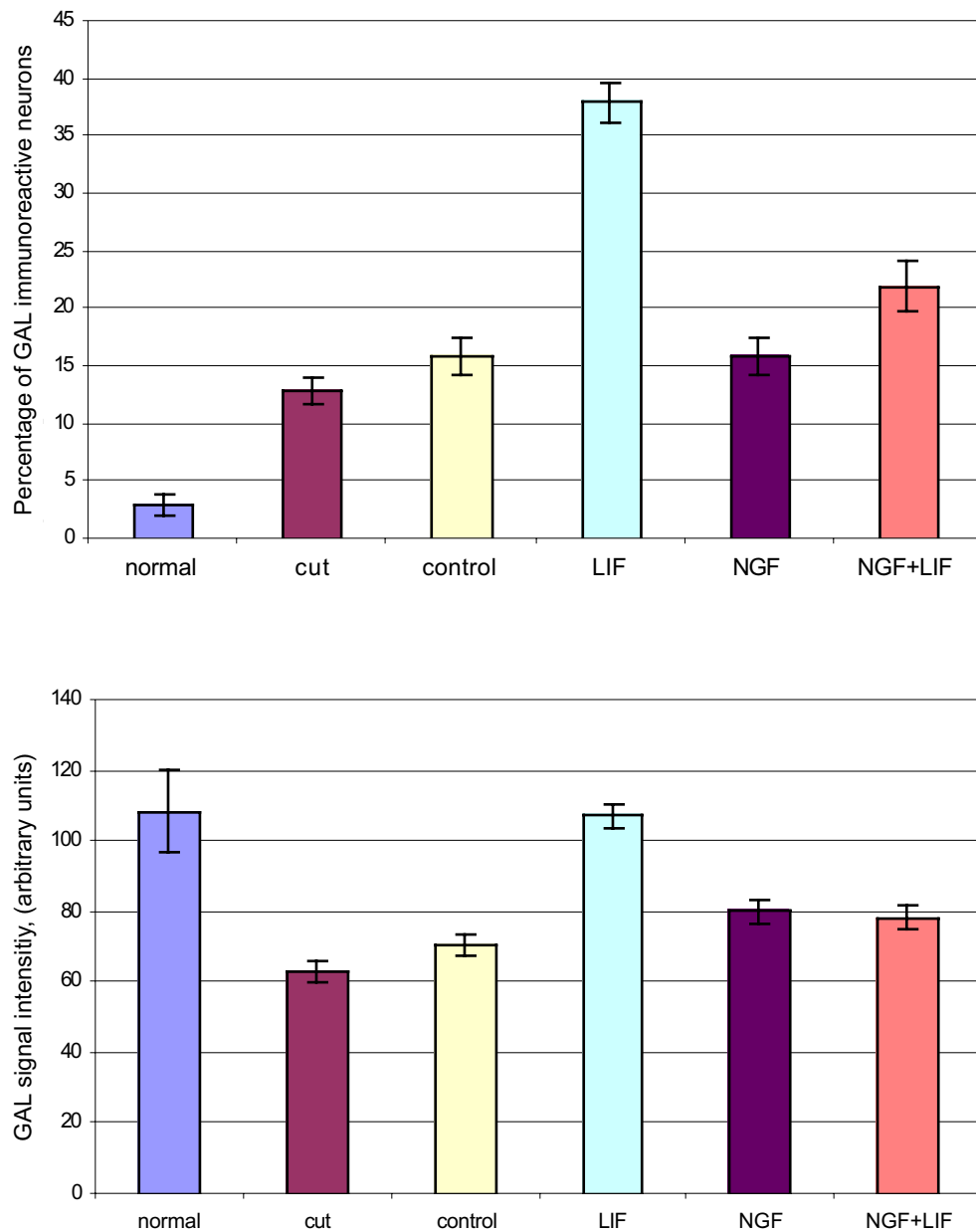


Figure 4.9 GAL immunoreactivity in sections of L4 DRG's. While normally only a few neurons expressed GAL, control preparations that had been kept in culture without additional factors also had a significantly higher proportion of GAL immunoreactive cells. LIF further increased this number. NGF on the other hand reduced this stimulation when both factors applied together (top). GAL signal intensity measurements revealed a similar situation which indicates LIF-treated preparations having the brightest signal (bottom).

Table 4.6. Percentages and brightness of GAL immunoreactive neurons.

	Normal	Control	LIF	NGF	NGF+LIF	Cut
	<i>s</i> = 14 (4)	<i>s</i> = 16 (4)	<i>s</i> = 18 (4)	<i>s</i> = 16 (4)	<i>s</i> = 17 (4)	<i>s</i> = 16 (8)
Percentage	$3 \pm 0.9^{**}$ <i>n</i> = 475	$16 \pm 1^{**}$ <i>n</i> = 541	38 ± 2 <i>n</i> = 612	$16 \pm 1^{**}$ <i>n</i> = 539	$22 \pm 2^{**}$ <i>n</i> = 597	$13 \pm 1^{**}$ <i>n</i> = 560
Brightness (arbitrary unit)	109 ± 12 <i>m</i> = 9	$71 \pm 3^{**}$ <i>m</i> = 46	107 ± 3 <i>m</i> = 180	$80 \pm 3^{*}$ <i>m</i> = 42	$79 \pm 3^{*}$ <i>m</i> = 39	$63 \pm 5^{**}$ <i>m</i> = 37

The numbers of DRGs taken are given in parenthesis. *n* = total number of counted cellular profiles, *s* = number of sections, *m* = number of analysed GAL immunoreactive neurons. Student t-test is used to compare LIF to others (**p* < 0.01, ***p* < 0.001).

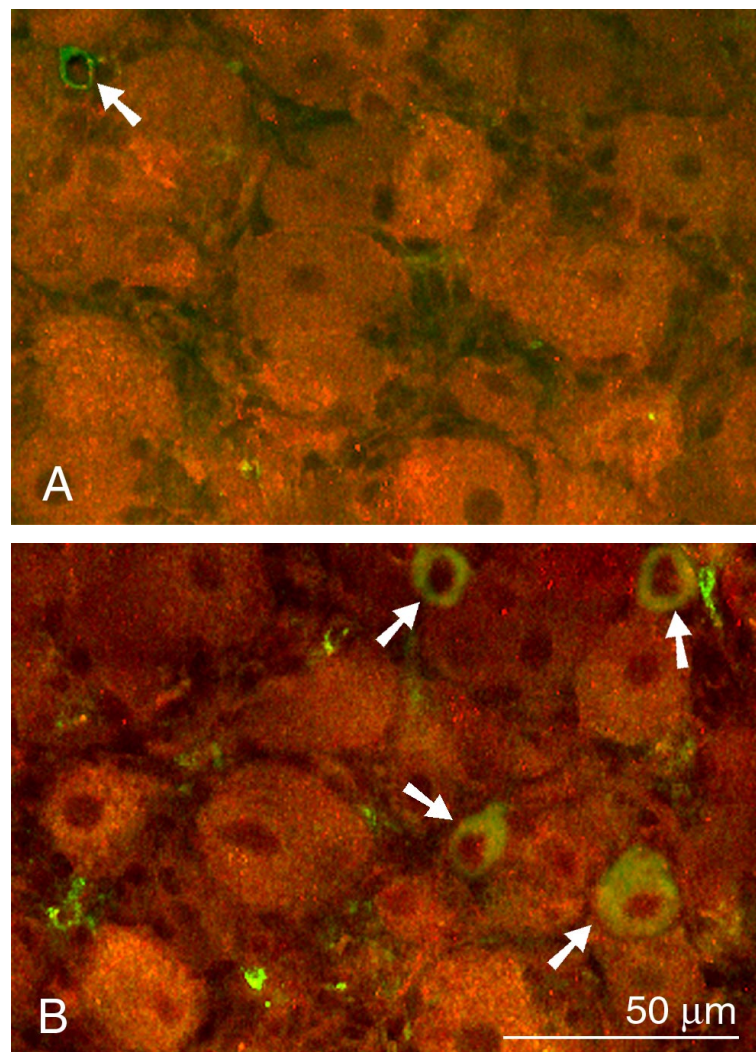


Figure 4.10 GAL immunoreactivity in DRG neurons in normal (A) and axotomized (B) preparations. The images are compositions of two different images taken using (GAL) and red (auto-fluorescence) channels. Arrows indicate GAL immunoreactive neurons.

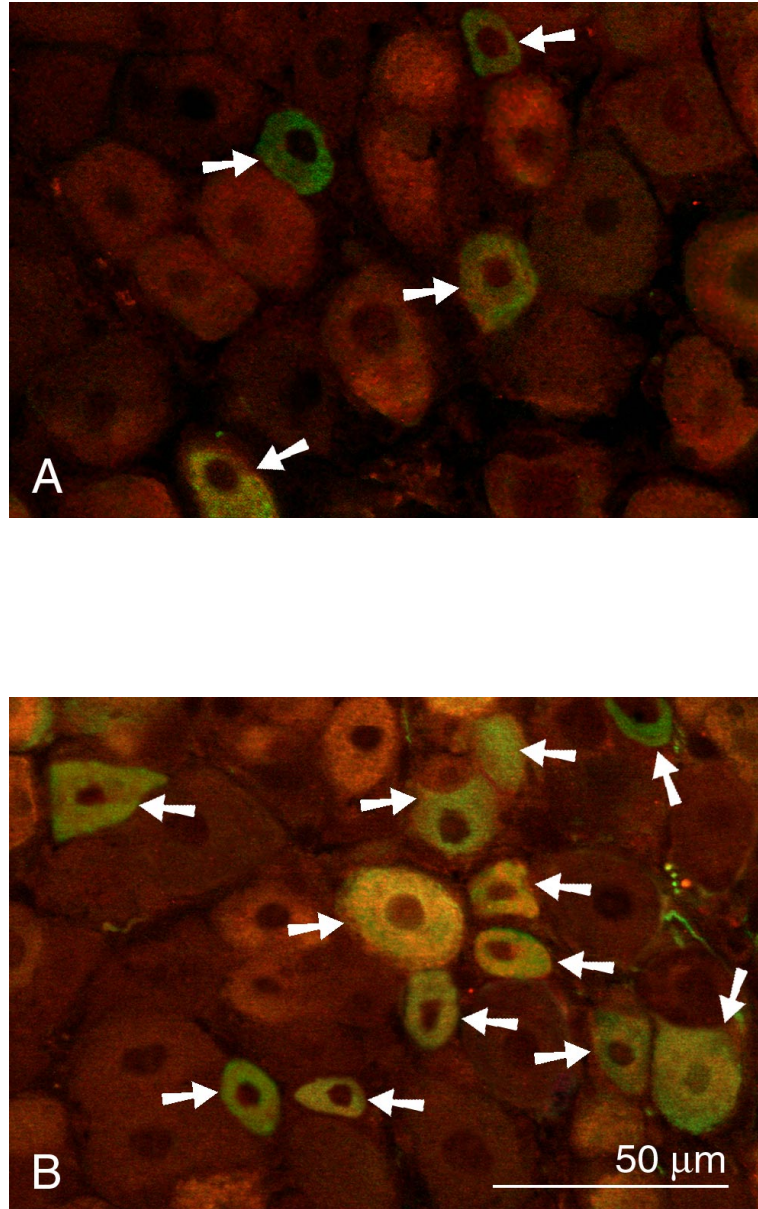


Figure 4.11 GAL immunoreactivity in DRG neurons in control (A) and LIF treated (B) preparations after 3 days in culture. The number of GAL-immunoreactive neurons markedly increased in the latter. The images are composition of two different images taken using green (GAL) and red (auto-fluorescence) channels. Arrows indicate GAL immunoreactive neurons.

4.4 Discussion

4.4.1 LIF has no effect on the extension of outgrowing axons but increases axonal diameter

Following nerve lesions, increased expression of a number of factors such as NGF, BDNF and LIF occurs and it is thought that these may be involved in regeneration (see general introduction and chapter IV for details). NGF is known to stimulate axonal growth, but less is known about LIF.

LIF is a lesion-induced, retrogradely-transported factor (Curtis *et al.*, 1994). It acts via its own receptor and also CNTF receptors and both factors support survival of different types of neurons including motoneurons (Gravel *et al.*, 1997), embryonic and neonatal sensory neurons (Thaler *et al.*, 1994) and some spinal cord neurons (Kushima *et al.*, 1992). The increased retrograde transport of LIF following peripheral nerve injury suggests that it may have a trophic role as a survival or growth factor in adult animals. However, in previous chapter my results showed that LIF did not have survival effect on adult DRG neurons in explant DRG cultures. The purpose of the experiments described in this chapter was to determine whether LIF could affect axonal regeneration and/or neuropeptide expression.

Results showed that LIF by itself had no effect on numbers, lengths or diameters of axons. In contrast, NGF caused profuse axonal growth, an effect which has been known for a long time (Horie *et al.*, 1991; Derby *et al.*, 1993). However, axons growing in the presence of NGF were thinner than in control preparations. This would be expected as the majority of NGF sensitive neurons are of small size, which have smaller unmyelinated axons. Interestingly, in preparations cultured with NGF and LIF together, axonal thickness was similar to that in control preparations, suggesting that in this situation, LIF could increase the diameter of the regenerating axons.

The mechanism by which LIF increased axonal diameter in NGF-treated preparations is uncertain. NFs are known to be a major determinant of axonal diameter. In order to test if LIF increases NF expression, I stained LIF-treated preparations using antibodies to three different NF subunits - NF-H, NF-M and

NF-L, but found no difference in labelling between these and control preparations (there were no staining for NF-M and NF-L and I observed only a few NF-H positive axons - data not shown).

This result makes it difficult to explain the observed effect of LIF on axonal diameter in this experiment. One explanation may be that at the concentration I used, LIF might not be effective on NF expression. It is also possible that, LIF might affect other cytoskeletal molecules such as tubulin.

Involvement of LIF in NF expression and radial axonal growth has been shown in some studies. For example Tham *et al.* (1997) showed that, LIF enhanced sciatic nerve regeneration in a silicone chamber model by increasing diameter of axons, number of myelinated fibers and conduction velocity. NGF is known to be a major determinant of axonal caliber in mature sensory neurons and it shows this effect by increasing NF expression (Gold *et al.*, 1991). In the next chapter, I am showing that NGF increases expression of NFs. NGF and LIF may act synergistically on radial axonal radial growth; but according to the results of the experiments presented in this chapter this does not necessarily involves regulation of NF expression. However, synergistic actions of these two factors during development have been shown. For example, Murphy *et al.* (1993a) demonstrated that in cultures of embryonic day 12 DRG, LIF promoted primary step of differentiation of NF-positive mature sensory neurons from their NF-negative precursors and NGF supported survival and further maturation of these cells. Co-localization of high affinity NGF receptor - trkA in more than half of the LIF accumulating neurons (Thomson *et al.*, 1997) also supports the idea of co-operative regulation.

4.4.2 LIF up-regulates GAL expression in explanted DRG cultures

It is well established that axotomized DRG neurons *in vivo* show increased GAL expression but the reasons for this are uncertain. This could be due to lack of target derived factors such as NGF or lesion-induced factors such as LIF. *In vivo* experiments have given results which suggest that both mechanisms may be involved since NGF infusion partially antagonizes the increased GAL expression seen after axotomy (Verge *et al.*, 1995) and

conversely, LIF injection into the sciatic nerve of normal animals causes increased GAL expression in L4 and L5 DRGs (Thompson *et al.*, 1998).

In this study, the number of GAL expressing neurons markedly increased in axotomized DRGs *in vivo* and also in cultured control preparations. The proportions of GAL positive neurons were similar in these two groups. Up-regulation of GAL in explant cultures without addition of any factors may be due to lack of NGF or spontaneous production of LIF in DRG explant cultures. Sun and Zigmond demonstrated (1996a) that LIF mRNA dramatically increases in DRGs 24 hrs after they were placed in organ cultures. In principle, the hypothesis of spontaneous LIF production could be tested by using LIF antiserum. However, I did not attempt this experiment since the access of antibodies into the explanted DRGs would be limited.

Although LIF did not affect the number and lengths of outgrowing axons, it increased the expression of GAL in explant tissue cultures. I observed increases in the proportion of neurons and outgrowing axons expressing this neuropeptide, often with a parallel increase in the intensity of the signals. This data supports some recent studies which suggested that the increased LIF transport to DRGs after axotomy up-regulates GAL expression in primary sensory neurons (Sun and Zigmond, 1996a, 1996b; Corness *et al.*, 1996; Thomson *et al.*, 1998). As a new finding I have shown here that LIF also causes a higher proportion of outgrowing axons to express GAL.

The effect of LIF on GAL expression in the absence and presence of NGF supports the idea that these two factors work reciprocally in this process. In this study, NGF counteracted most of LIF's stimulatory effect on GAL expression in DRG neurons. On the other hand, NGF failed to prevent the increase in the proportion of GAL positive axons induced by LIF. This situation may be explained by the greatly increased number of outgrowing axons which may be sensitive to LIF. Even if NGF caused a decrease in the proportion of GAL positive axons, this would not be enough to reduce it down to control levels.

If the reason for spontaneous increase in GAL expression in control cultures was the action of endogenous production of LIF then the question “why did NGF not prevent this increase?” would arise. This might be explained by the presence of LIF-sensitive DRG neurons that are *trkA*-negative. In fact, it has been demonstrated that many DRG neurons that accumulate LIF *in vivo* do not express *trkA* (Thompson *et al.*, 1997). It might also be speculated that when produced at low quantities, the effect of LIF on *trkA* positive neurons could be antagonised by locally produced NGF. Therefore, the spontaneous increase in GAL expression in control cultures may primarily be confined to those LIF-responsive neurons that do not have *trkA* and may not be reversed by NGF. Shadiack *et al.*, (1998) recently demonstrated that LIF could not induce GAL in SCG without neutralizing NGF. However, my findings have shown that this is not the case with DRGs. Even in the presence of exogenous NGF, LIF was able to significantly increase GAL expression in my experiments.

In addition to the theory that suggests an antagonism between NGF and LIF in GAL expression, an alternative explanation may be proposed based on the data I present here. According to this, NGF may increase transport of GAL to the growing axons instead of down-regulating it. Interestingly, in most of the studies on this subject, only neuronal bodies, but not growing axons, were examined to detect changes in GAL expression under different conditions. My study showed that NGF alone caused an increase in the proportion of GAL expressing axons while intensity of this signal was among the lowest. This may suggest that NGF stimulates transport of GAL from neuronal body to outgrowing axons. Conversely, when NGF and LIF were applied together, the highest signal level was obtained. This situation might be explained by the combination of an up-regulation in the expression of GAL by LIF and increased transport from the cell body by NGF.

This study also sheds some light on to the role of GAL during regeneration. It was proposed that GAL might have developmental role in DRGs (Xu *et al.*, 1996a). These investigators also suggested that it might have role in axonal growth during regeneration based on the observation that its pattern of

expression during development resembles to that of GAP-43. Holmes *et al.* (1997) demonstrated that GAL knock out mice had impaired peripheral nerve regeneration. These findings raise the question whether GAL expression is involved in axonal growth. However, my study shows that when GAL expression was increased by LIF, this did not cause positive axons to grow longer. Therefore, GAL expression does not have an effect on axonal outgrowth in explant DRG cultures.

4.5 Conclusion

In this study, I tested the effects of LIF on axonal growth and GAL expression:

- ◆ LIF does not affect axonal elongation but increases their diameters in certain circumstances.
- ◆ LIF induces GAL expression in outgrowing axons and neuronal cell bodies.
- ◆ NGF partially prevents LIF's stimulatory effect on GAL expression.

Chapter 5 :Expression of neurofilaments and effects of growth factors in explant DRG cultures

5.1 Introduction

The axonal cytoskeleton consists of three main elements (fig. 5.1). These are microtubules, neurofilaments and microfilaments (for reviews, see Lasek, 1986; Lee and Cleveland, 1996). Microtubules are hollow structures formed by circular alignment of protofilaments which are made up of α and β tubulin subunits. They can extend as long as 0.5 μ m and have a typical diameter of 22 nm. Microfilaments are composed mainly of actin with a much shorter length and smaller diameter (6 nm). They are usually concentrated around microtubules and the inner surface of plasma membrane. NFs are the most abundant axonal cytoskeletal elements and occupy most of the axonal volume. With a diameter of about 10 nm, which is between those of microtubules and microfilaments, they belong to the class of intermediate filaments. They are the main determinants of axonal caliber.

Axonal cytoskeleton can be spatially divided into compartments containing cortical and inner cytoskeletons. The cortical part mostly consists of microfilaments whereas inner cytoskeleton contains all three elements.

NFs play important roles in neurite elongation during development and also during regeneration in peripheral nervous system. Disorders in NF expression and organization have been related to a number of degenerative neuronal diseases such as *amyotrophic lateral sclerosis*, *infantile spinal muscular atrophy* and *hereditary sensory motor neuropathy*. Despite their physiological and pathological significance very little is known about the regulation of NF content. In this chapter, I studied *in vitro* expression of NFs in explant DRG cultures.

5.1.1 Structure and the family of neurofilaments

Fibrous networks in axons were first detected by 19th century anatomists and named as neurofibrils. Through the advancements in microscopy, clear visualization of these structures became possible early this century. In 1968, Schmitt 's electron microscopy studies revealed that the neurofibrils were

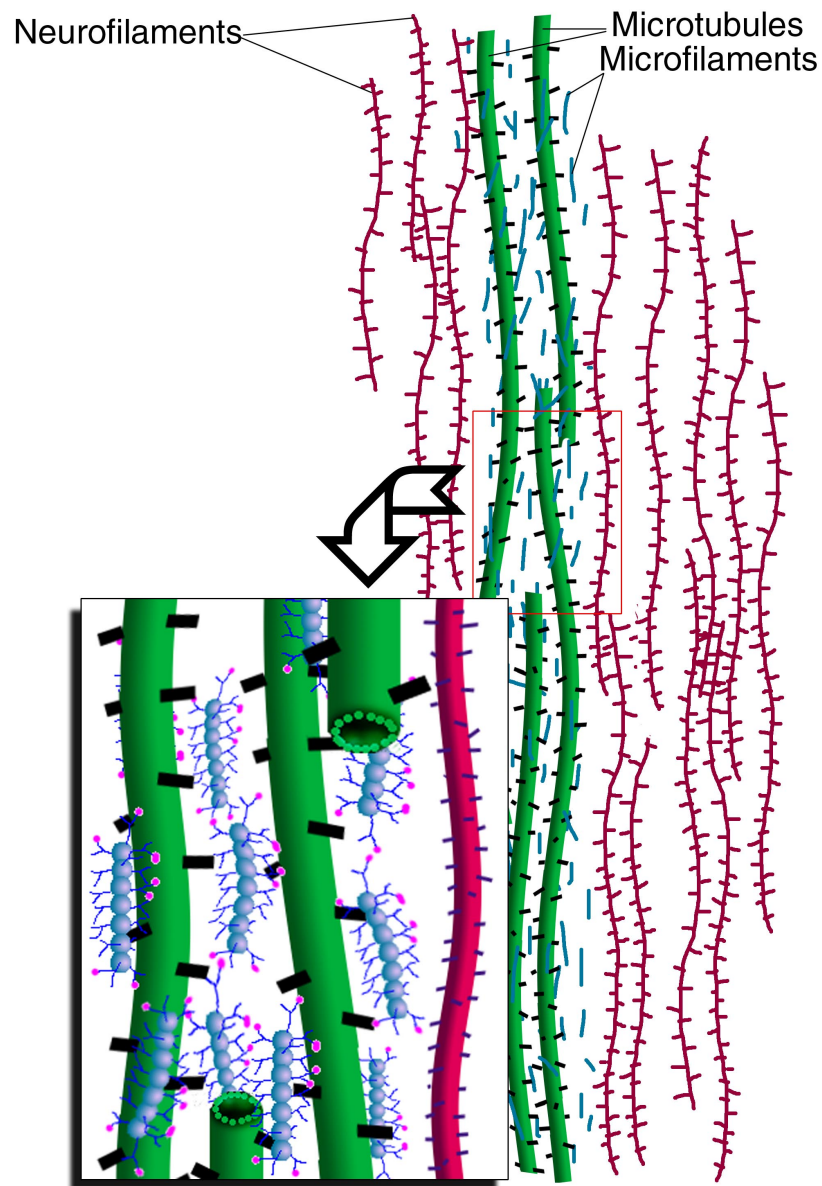


Figure 5.1 An illustration showing the main components of axonal cytoskeleton. Neurofilaments and microtubules associated with microfilaments define two separate cytoskeletal domains. The inset shows the intricate interweaving of microtubules and microfilaments. Microfilaments are also associated with globular and filamentous proteins (modified and redrawn from Lasek, 1986).

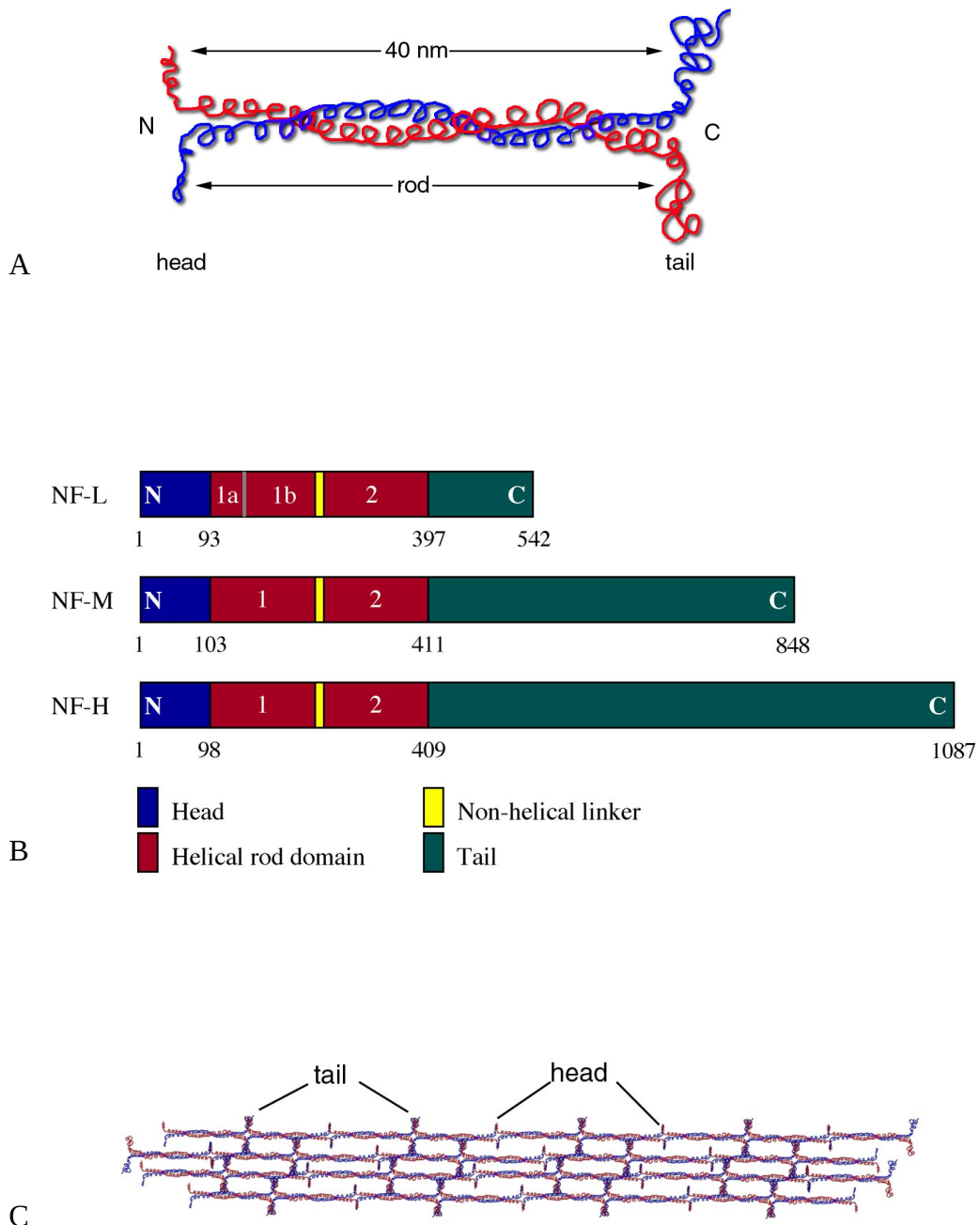


Figure 5.2 Structure of NF protein (A). Two α helices wind around to form a central rod, a globular head and tail. Mouse NFs share an approximately 310 aa long rod domain with two subdivisions (B). First division is further divided into two in NF-L. The length and constitution of the tail are the main determinants of subunit properties (The sequence data for NF-L, NF-M and NF-H is from Lewis and Cowan (1986), Levy *et al.*, (1987) and Julien *et al.*, (1988) respectively). The head domain is important for lateral association whereas tails determine the diameter of NF (C).

composed of three ~10 nm diameter subunits which were then named as neurofilaments.

NFs belong to the neuronal intermediate filament family. The identified members of this family include three NF subunits, heavy (NF-H), middle/intermediate (NF-M) and light (NF-L) molecular weight NFs, α -internexin, peripherin, and nestin. According to an intermediate filament classification based on primary amino acid sequence differences and the intron positions of the genes encoding the subunits (Steinert and Roop, 1988), the three NF subunits, nestin and α -internexin are type IV, whereas peripherin is a type III intermediate filament.

All neuronal intermediate filaments share a ~310 amino acid-long rod-like domain which is formed by two interwound α helices (fig. 5.2). The rod domain is divided into 2 coils by a non-helical short linker. Except NF-M and NF-H, first coil is further divided into 2 by an interruption. Two other domains of the neuronal intermediate filaments are the head, which is rich in threonines and serines, and the tail. Heads and tails are globular in shape and vary in length and amino acid sequence among the members of this family. The main determinant of the molecular weight of these proteins is the length of the tail. According to sequence analysis it is estimated that mouse NF-L has a molecular weight of approximately 61 kDa (Lewis and Cowan 1986), NF-M is 96 kDa (Levy *et al.*, 1987) and NF-H is 117 kDa (Julien *et al.*, 1988).

The assembly properties of NFs have been studied widely. It is accepted that NFs are obligate heteropolymers that require NF-L and substoichiometric amount of either NF-M or NF-H (Lee *et al.*, 1993). NF-H and NF-M are not capable to assemble in the absence of NF-L (Balin and Lee, 1991). NF-L, on the other hand, can form short filaments by itself (Geisler and Weber, 1981; Hisanaga and Hirokawa, 1990; Heins *et al.*, 1993). However recent studies revealed that, unlike rodents, human NF-H can self-assemble, showing an inter-species difference (Carter *et al.*, 1998). A single amino acid alteration in the peptide sequence of the rod, on the other hand, was able to make rat NF-L self-assemble and human NF-L to lose this property (Carter *et al.*, 1998). NF

assembly can tolerate up to two fold lowered NF-H and NF-M to NF-L ratio but it is impaired by an increase in this ratio (Xu *et al.*, 1996b).

Head and rod domains of subunits participate in assembly whereas tails are believed to be the major determinant of NF diameter (Heins *et al.*, 1993, Lee *et al.*, 1993) (fig. 5.2). The backbone of NF is mainly composed of NF-L. NF-M and NF-H attach to this backbone and their tails protrude to form side arms that connect with neighbouring NFs (Hirokawa *et al.*, 1984; Hisanaga and Hirokawa 1988).

5.1.2 Expression of neurofilaments

NF subunits have different expression patterns during the development. It has been suggested that they play important roles during neurite elongation, a process which requires both stability and high resilience, by maintaining characteristic shape of axons under such demanding conditions (Lin and Szaro, 1995). The different expression patterns of neuronal intermediate filaments during the development appears to be organized as to provide a partial consolidation of axonal cytoskeleton, permitting growth to continue (Schwartz *et al.*, 1990). Embryonic axons typically have smaller caliber axons which are characterized by low levels of NFs (Schwartz *et al.*, 1990) and by the absence of phosphorylated forms of NF-H and NF-M (Carden *et al.*, 1987; Szaro *et al.*, 1989). After synapse formation, NFs rapidly accumulate in the axons and this coincides with the myelination (Carden *et al.*, 1987; Schaeffer and Bruce, 1990). The expressions of peripherin and α -internexin, however, decrease in large motor and sensory neurons while they are still detectable at high levels in some small neurons that contain few NFs (Escurat *et al.*, 1990; Gorhom *et al.*, 1990; Flienger *et al.*, 1994).

5.1.3 Axonal transport of neurofilaments

Both gene transcription and translation of NF proteins take place in neuronal cell body (Lasek, 1993) where they are rapidly assembled (Black *et al.*, 1986). NFs are then transported into the axon in the 'slow component a' (Sca) of axonal transport (Hoffman and Lasek, 1975). The estimated NF transport rate is 1-1.5 mm/day in mature peripheral axons and 0.5-1 mm/day in central

fibers (Oblinger and Lasek, 1988). NFs move more rapidly in developing and regenerating axons (Hoffman and Lasek, 1980; Hoffman *et al.*, 1983; Willard and Simon 1983; McKerracher *et al.*, 1990). On the other hand, transport rates of up to 144 mm/day have been reported suggesting that NFs may be moved in rapid pulses followed by long pauses (Lasek *et al.*, 1993). It is proposed that the phosphorylation state and the level of NF-H is a major determinant of NF transport rate (Willard and Simon, 1983; Collard *et al.*, 1995; Marszalek *et al.*, 1996). It is generally believed that NFs are transported in polymer form (Morris and Lasek, 1982). There appear to be two different NF pools in axons, one transported in Sca and other stationary (Nixon andLugvinenko, 1986). There is a rapid exchange of subunits between these two pools (Okabe *et al.*, 1993; Takeda *et al.*, 1994).

5.1.4 Neurofilaments and radial axonal growth

The linear relationship between NF content and axonal caliber has long been known in normal and regenerating axons (Friede and Samorajski, 1970; Hoffman *et al.*, 1987). Through the availability of mutant animals whose genes for NFs have been genetically altered, this correlation has been firmly established.

It was demonstrated that the quail with defective NF-L gene translation survived but had reduced radial growth of axons (Yamasaki *et al.*, 1992). When transport of NF-H was prevented by expression of a NF-H- β -galactosidase fusion protein, radial growth of large myelinated axons were severely inhibited (Eyer and Peterson, 1994).

Transgenic studies have changed the earlier simple paradigm to explain the mechanism of axonal radial growth. According to that, axons would expand and contract to maintain a constant level of NF density (Friede and Samorajski, 1970; Hoffman *et al.*, 1987). However, when the expressions of individual NF subunits were increased, this caused the inhibition of radial growth (Monterio *et al.*, 1990; Wong *et al.*, 1995; Xu *et al.*, 1996b). Furthermore, while an increase in NF-L expression led to increased number of NFs, high expression of NF-M and NF-H decreased it (Xu *et al.*, 1996b). A two-fold increase in NF-M decreased

NF-H level in axons and severely inhibited radial growth (Wong *et al.*, 1995). Enhanced radial growth was observed only when NF-H or NF-M expression was increased together with NF-L expression (Xu *et al.*, 1996b).

These findings may suggest that NF-H is the main determinant of axonal radial growth. Wong *et al.* (1995) proposed that, the phosphorylated tail of NF-H modulates radial growth through long range interactions with other elements of cytoskeleton while NF-M specifies nearest neighbourhood spacing among NFs. However, recent studies with NF-H gene knock-out mice revealed some findings challenging this hypothesis (see discussion of this chapter).

5.1.5 Neurofilament expression following axotomy

After axotomy, a number of cytoskeletal changes occur and a reduction in the diameter of axotomized axons ensues. These changes have been related to the change in the NF content of axons (Hoffman *et al.*, 1984; Hoffman *et al.*, 1985). Axotomy causes a reduction in NFs in the perikarya and the axons whilst levels of tubulin increase (Oblinger and Lasek, 1988; Hoffman *et al.*, 1987, 1988). It has been shown that, decreases in both the synthesis and the transport of NFs are responsible for these changes (Hoffman and Lasek, 1980; Hoffman *et al.*, 1987; Wong and Oblinger, 1987; Oblinger and Lasek, 1988).

There are similarities between expression patterns of NFs during regeneration and development (Hoffman and Cleveland, 1988; Oblinger *et al.*, 1989). Beside decreased NF content, increased tubulin to NF ratio, higher levels of peripherin expression and faster NF transport are the main common features for both conditions (Oblinger and Lasek, 1988; Oblinger *et al.*, 1989, Hoffman and Lasek, 1980). This supports the idea that, lowered levels of NFs can help the elongation of growing axons by decreasing internal mass, thus facilitating movements of essential proteins for regeneration such as tubulin and actin (Oblinger *et al.*, 1989). The studies with conditioning lesions also revealed the inverse relationship between the content of NF and the success of regeneration. Primed axons with an earlier injury had lower NF levels but showed a faster elongation than those that were not primed (Oblinger *et al.*, 1989). In another study it was found that the reduction in NF mRNAs was proportional to the

residual volume of axons following axotomy (Jacobs *et al.*, 1997). This may indicate a regulatory mechanism within the neuron that keeps the density of NFs at a low level during regeneration. Indeed, Oblinger and Lasek, (1988) proposed an intrinsic mechanism which regulate the synthesis and/or the assembly of NFs in DRG neurons. However, the existence of an extrinsic regulation by trophic factors has stronger evidence than an intrinsic one.

When regenerating axons make synapses with their target tissue, atrophied axons regain their normal thickness (Hoffman *et al.*, 1984) and NF content returns to normal level (Greenberg and Lasek, 1988). The interruption of retrograde transport from axon tip to the DRG neurons, without transection, created similar changes in the caliber and the NF content of the axons (Gold and Dark, 1988a). These findings suggested that a target-derived, retrogradely transported factor could be responsible for the changes (Gold and Dark, 1988b). It was later demonstrated that infusion of NGF intrathecally or into the proximal stump of transected sciatic nerve partially prevented axotomy-induced NF reduction in DRG neurons (Verge *et al.*, 1990; Gold *et al.*, 1991). Therefore, deprivation of NGF after axotomy was indicated to be responsible for the changes in NF expression along with some other defined histochemical alterations in DRG neurons.

5.1.6 Rational for the study

The positive effect of NGF on NF expression is now well established. However, a number of questions remain unanswered. NGF is known to counteract injury-induced reduction of NF mRNA only in neurons with high affinity NGF receptor trkA (Verge *et al.*, 1990). These are mostly small neurons (Averill *et al.*, 1995). However, NFs are expressed mainly by larger neurons and what regulates NF synthesis in these neurons is not known. Verge *et al.*, (1990) proposed two possible explanations: The first one suggests that target-derived NGF regulates NF expression in small trkA positive neurons and an unknown analogous factor does the same thing for larger cells. The second explanation they proposed was the existence of a common factor causing reduction in NF expression, which would be counteracted by NGF in some

neurons.

DRG neurons have been classified according to the neurotrophin receptors they express (Wright *et al.*, 1995). Receptors for NT-3 and BDNF are expressed by larger size primary sensory neurons. Therefore it is conceivable that these factors may have effects on NF expression. In this chapter, I studied NF expression in explant DRG cultures and I compared the effects of NGF, NT-3 and BDNF on this. For this, I followed two different approaches.

In the first one, I determined the expression of three different NF subunits in outgrowing axons from DRGs explanted into matrigel and tested the effects of the neurotrophins. I determined the expressions of NFs in outgrowing axons as well as in DRG neurons by optical sectioning. In order to distinguish true up-regulation of NF from a selective axonal outgrowth by growth factors, I set up some cultures with DRGs that had been conditioned with an earlier peripheral nerve crush. Conditioning lesion is known to increase regeneration after a second axotomy (Lankford *et al.*, 1998). In matrigel, this strongly increases the numbers and lengths of outgrowing axons (Edström *et al.*, 1996). Furthermore, some of these axons are labelled by monoclonal antibody RT97 but not by antibodies to p75 (Tonge, unpublished observation). Since in DRGs p75 is generally co-expressed with trkA and trkB; but only by about 50 % of trkC positive neurons (Wright and Snider, 1995), it seems likely that the conditioning lesions stimulate outgrowth of axons of larger diameter DRG neurons. Thus these preparations might be expected to have most types of axons already grown without any growth factors. This in turn may make it possible to attribute any observed changes in NF expressions to the direct action of growth factors when these are added after the initial period of axon outgrowth.

In the second type of experiments, I left a relatively longer piece of nerve attached to DRG and crushed it before explantation. By determining the expression of NFs in the axons that successfully regenerated across the crush site, I studied the contribution of distal segment to NF expression.

5.2 Experimental Procedures

5.2.1 Tissue cultures

Animals were anaesthetised as described in chapter II. Three days before removing from some of the animals, peripheral nerves of the last 3 thoracic DRGs were crushed on one side.

In the first part of the experiment, explant cultures were set up in growth factor reduced matrigel as described in chapter II, incubated for 3, 6, 9 and 12 days and fixed at the end of incubations. To some of the preparations, growth factors NGF, NT-3 and BDNF were added at the beginning of incubation period. I preferred matrigel to collagen in these experiments because even without addition of any growth factors, matrigel enhances axonal outgrowth. This provides enough number of axons to observe NF immunoreactivity in all conditions. After cultures were set, BDNF, NGF and NT-3 were added to some of the preparations.

In the second part, slightly longer pieces of intercostal nerves (approximately 4 mm in length) were left attached to DRGs and were crushed by compressing with forceps. Then explant cultures were set up in matrigel as usual except that 18x18 mm square cover-slips were used instead of usual 13 mm – diameter round ones. BDNF, NGF and NT-3 were added to some of the cultures. All the preparations were incubated for 3 days and then fixed.

The concentrations and the sources of the neurotrophic factors used are given in table 5.1.

5.2.2 Immunohistochemistry and microscopy

The staining were performed as described in chapter 2, except that the preparations with crush nerves were incubated with 20 µl of primary and secondary antibodies (concentrations are given in tables 5.2 and 5.3). Preparations were counter-stained for NFs and PGP 9.5 or β -III tubulin. The later two were used as pan-axonal markers as they label almost all of the outgrowing axons.

DRGs and crushed preparations were scanned using a *Leica* scanning

Table 5.1. The concentrations and sources of factors used.

Factor	Concentration	Source
BDNF	50 ng/ml	<i>Alamone Labs</i>
NGF 2.5 S	50 ng/ml	<i>Alamone Labs and Promega</i>
NT-3	50 ng/ml	<i>Alamone Labs</i>

Table 5.2. The concentrations and sources of primary antibodies used.

Antibody	Concentration	Source
Rabbit α -NF-L	1/50	<i>Affiniti</i>
Mouse α -NF-M (clone no: NN18)	1/50	<i>Sigma</i>
Mouse α -NF-H (clone no: NE14)	1/50	<i>Sigma</i>
Mouse α - β -III tubulin (clone no: SDL3D10)	1/100	<i>Sigma</i>
Rabbit α -PGP 9.5	1/200	<i>UltraClone</i>

Table 5.3. The concentrations and sources of secondary antibodies used.

Antibody	Concentration	Source
Alexa 488 goat α -mouse IgG	1/100	<i>Molecular Probe</i>
Alexa 568 goat α -rabbit IgG	1/100	<i>Molecular Probe</i>
FITC-conjugated goat α -mouse IgG	1/100	<i>Sigma</i>
FITC-conjugated goat α -rabbit IgG	1/100	<i>Jackson ImmunoRes.</i>
RITC-conjugated goat α -mouse IgG	1/100	<i>Sigma</i>
RITC-conjugated goat α -rabbit IgG	1/100	<i>Sigma</i>

confocal microscope. Other preparations were viewed with an epifluorescence microscope and scanned using a *Hamamatsu 5810* CCD camera system as described in chapter II.

5.3 Results

5.3.1 NF expression in outgrowing axons of normal preparations

The outgrowing axons from nerve stumps were stained for NF-H, NF-M and NF-L. They were also counter-stained for PGP 9.5 (NF-H and NF-M) or β -III tubulin (NF-L). NF subunits had different time courses of expression (table 5.4). The expression of NF-H was evident on the 3rd day of incubation with numerous brightly-labelled axons (fig. 5.3). However NF-M immunoreactivity was not observed until the 6th day (fig. 5.4) and NF-L immunoreactivity until the 12th day and when they were expressed there were only a few positive axons in each preparations (table 5.4, fig. 5.5).

Table 5.4. Time course of first appearance of NF subunit-immunoreactivities in outgrowing axons in explant DRG cultures.

DAYS	3	6	9	12
	▲	▲		▲
	NF-H	NF-M		NF-L

Table 5.5. The effects of growth factors on the expressions of NFs in normal preparations after 3 days in culture.

	NF-H	NF-M	NF-L
Control	+	-	-
NGF	+	+	+
BDNF	+	+	-
NT-3	+	+	-

Table 5.6. The mean numbers of axons per preparation expressing NF-M after 3 days in normal and conditioned DRG cultures.

	Control	NGF	BDNF	NT-3
Normal	nil <i>n</i> = 8	8.3±0.4 <i>n</i> = 8	3.6±0.5* <i>n</i> = 8	3.4±0.5* <i>n</i> = 8
Conditioned	2.75±1 <i>n</i> = 4	22.5±4* <i>n</i> = 4	30±8* <i>n</i> = 4	3.8±1 <i>n</i> = 4

Student's t-test is used to compare BDNF and NT-3 to NGF in normal and control to others in conditioned preparations (**p* < 0.05). *n* = the number of preparations examined.

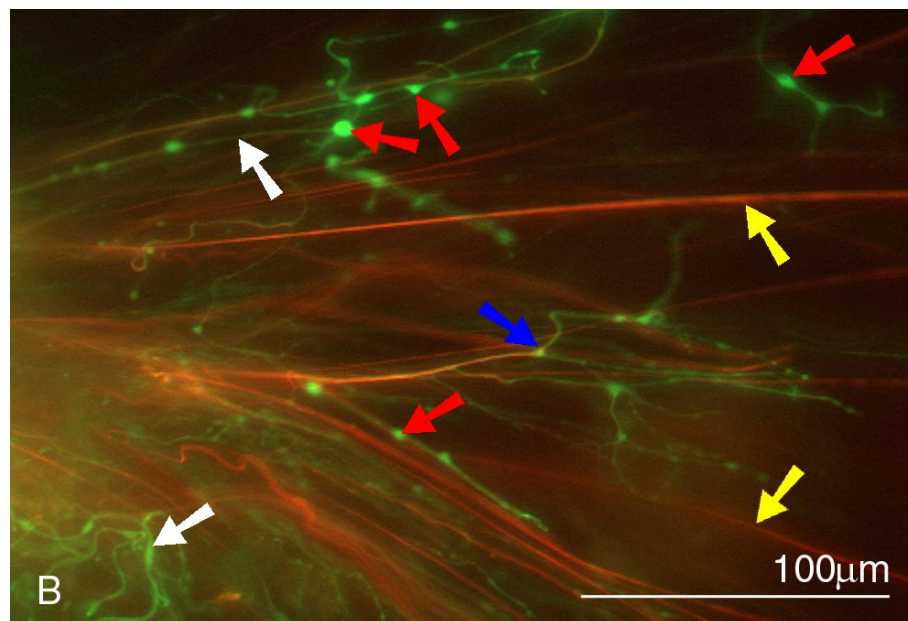
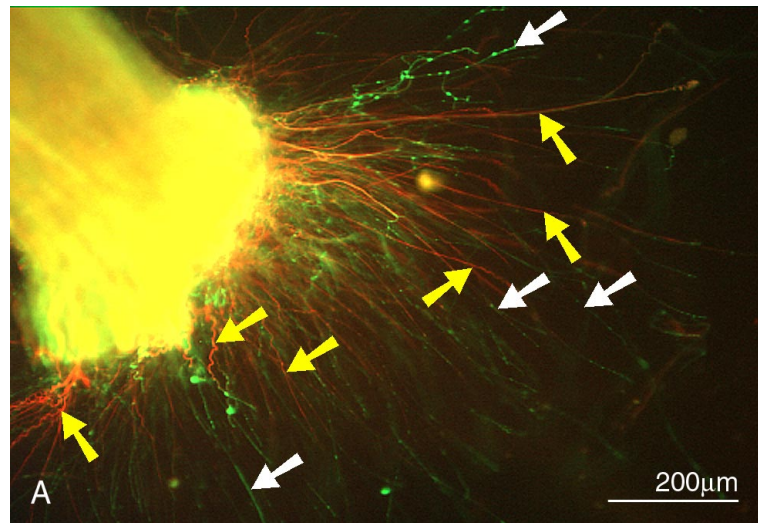


Figure 5.3 NF-H immunoreactivity in DRG explant cultures after 3 days (A and B). The preparations were double labelled for NF-H (yellow arrows) and PGP 9.5 (white arrows); secondary antibodies used were conjugated with RITC and FITC respectively. Many axons are labelled for NF-H. At higher magnification (B) some axons appear to have NF-H immunoreactivity only in proximal part which may suggest a ‘proximal to distal - deposition’ of the protein (blue arrow). Small axonal varicosities are commonly observed in outgrowing axons in DRG explant cultures (red arrows); their function, however, is unclear.

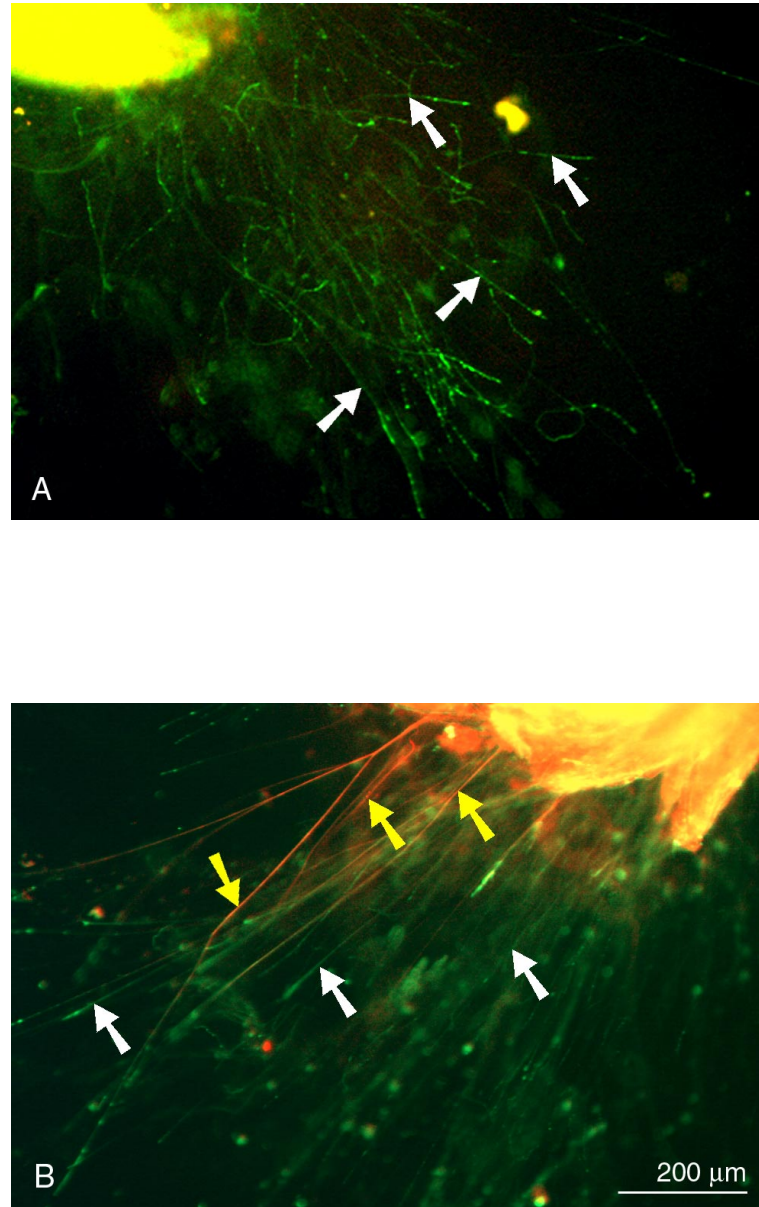


Figure 5.4 NF-M immunoreactivity in explant DRG cultures after 3 (A) and 6 (B) days. The preparations were double labelled for NF-M (yellow arrows) and PGP 9.5 (white arrows); secondary antibodies used were conjugated with RITC and FITC respectively. There was no detectable NF-M immunoreactivity in outgrowing axons after 3 days (A). On the 6th day, a few axons had NF-M labelling (B).

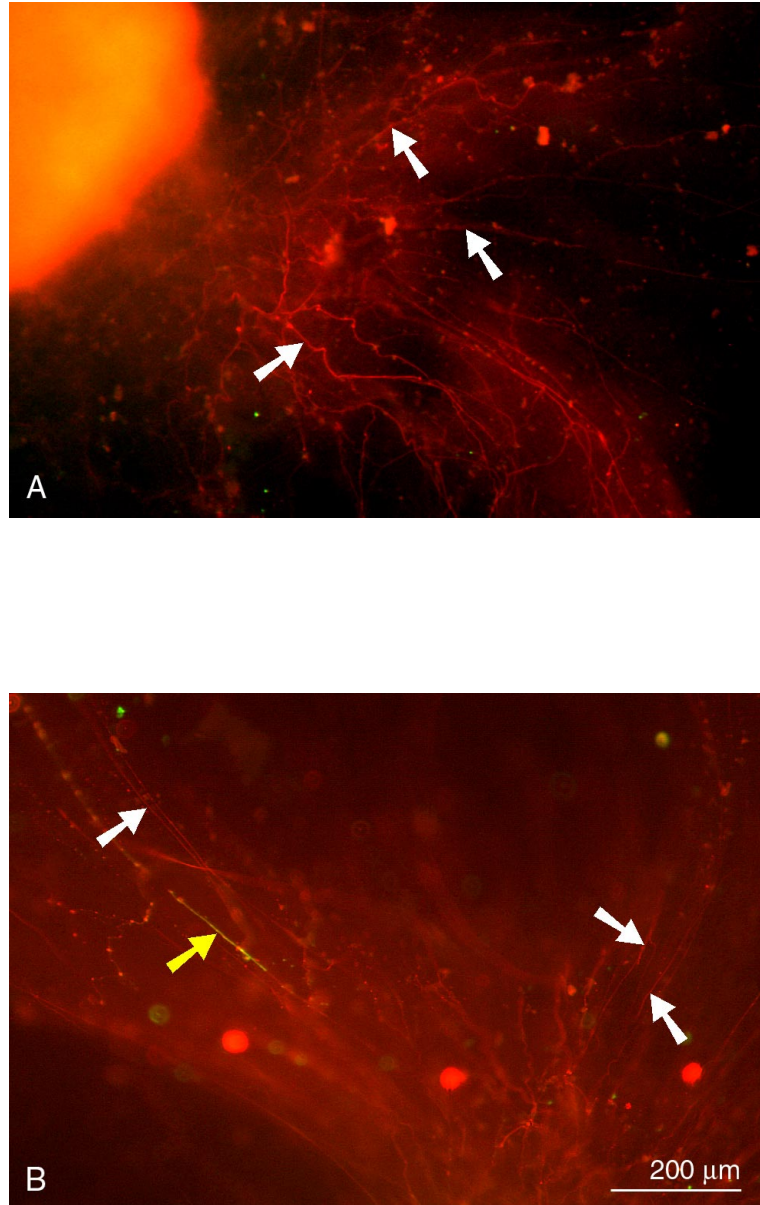


Figure 5.5 NF-L immunoreactivity in explant DRG cultures after 9 (A) and 12 (B) days. The preparations were double labelled for NF-L (yellow arrow) and β -III tubulin (white arrows); secondary antibodies used were conjugated with FITC and RITC respectively. There was no NF-L labelling until 12th day (A) by which time only a few axons had NF-L immunoreactivity (B).

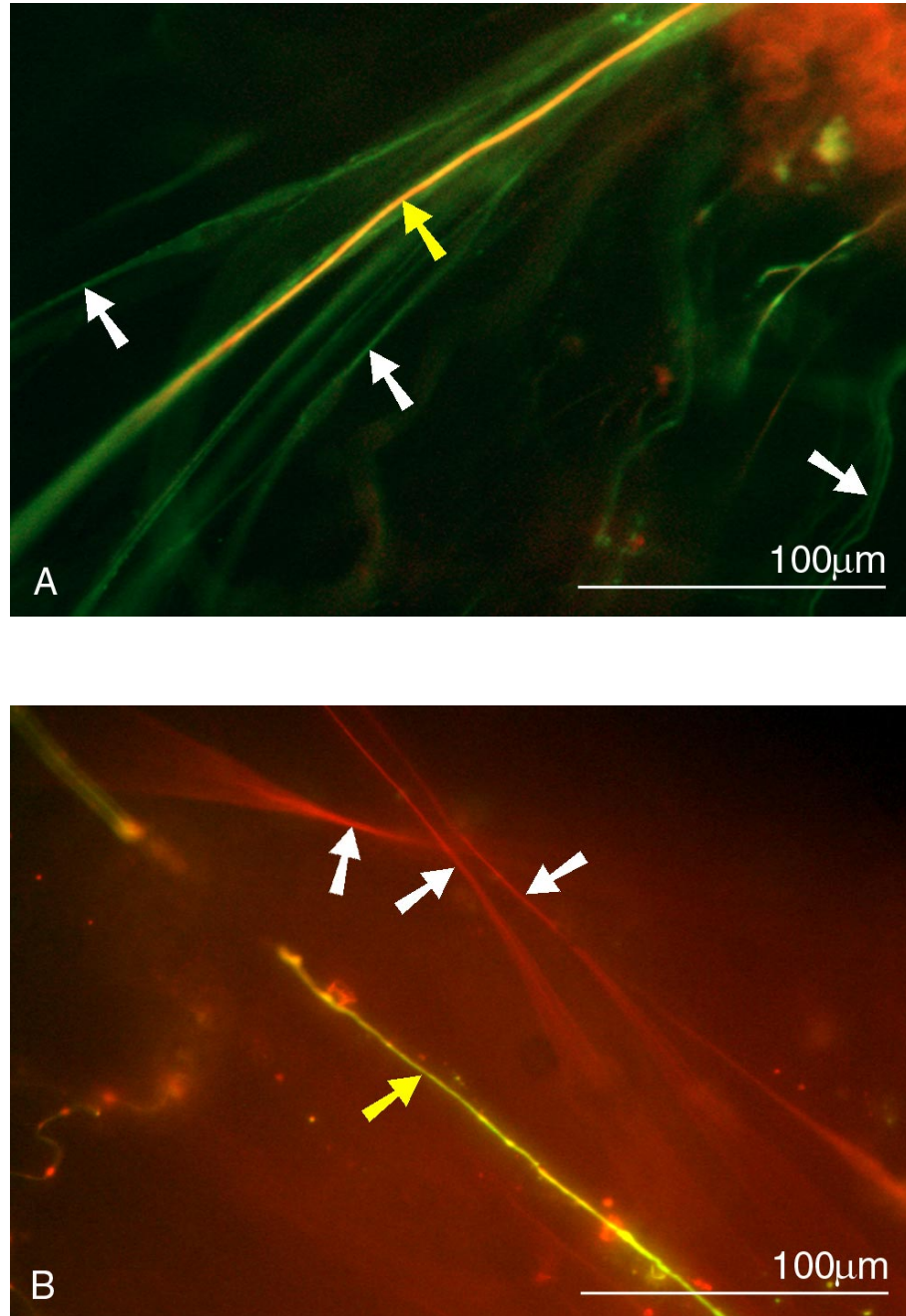


Figure 5.6 High magnification images showing NF-M (A) and NF-L (B) immunoreactivities on 6th and 12th days respectively. The preparations were double labelled for NF-M (yellow arrows) and PGP 9.5 (white arrows) (secondary antibodies used were conjugated with RITC and FITC respectively), and NF-L (yellow arrow) and β-III tubulin (white arrows) (secondary antibodies used were conjugated with FITC and RITC respectively). Similar to NF-H, NF-M appeared to be deposited from proximal to distal. However NF-L labelling appeared patchy and proximally discontinuous (see also figure 5.5b).

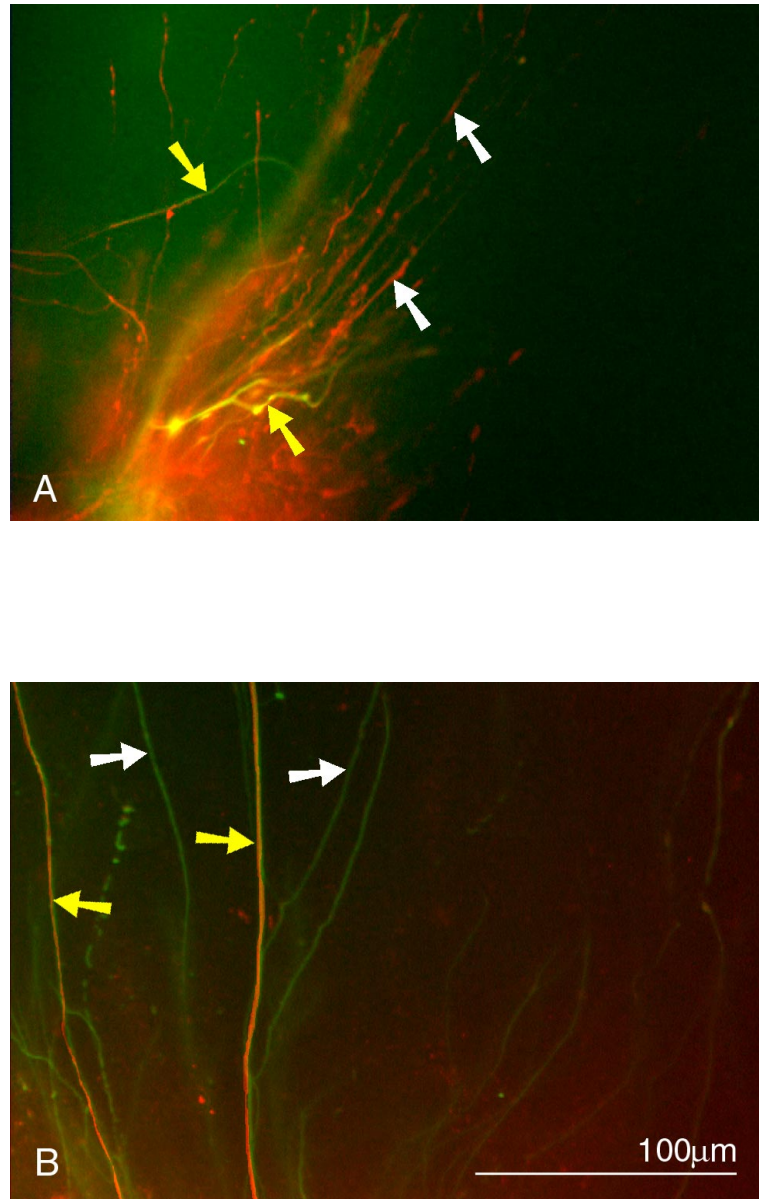


Figure 5.7 NF-M (A) and NF-L (B) labelling in the presence of NGF after 3 days in culture. The preparations were double labelled for NF-M (yellow arrows) and PGP 9.5 (white arrows) (secondary antibodies used were conjugated with FITC and RITC respectively), and NF-L (yellow arrow) and β -III tubulin (white arrows) (secondary antibodies used were conjugated with RITC and FITC respectively). While these two NFs are normally not detectable in outgrowing axons in 3 days, both were expressed when NGF was added.

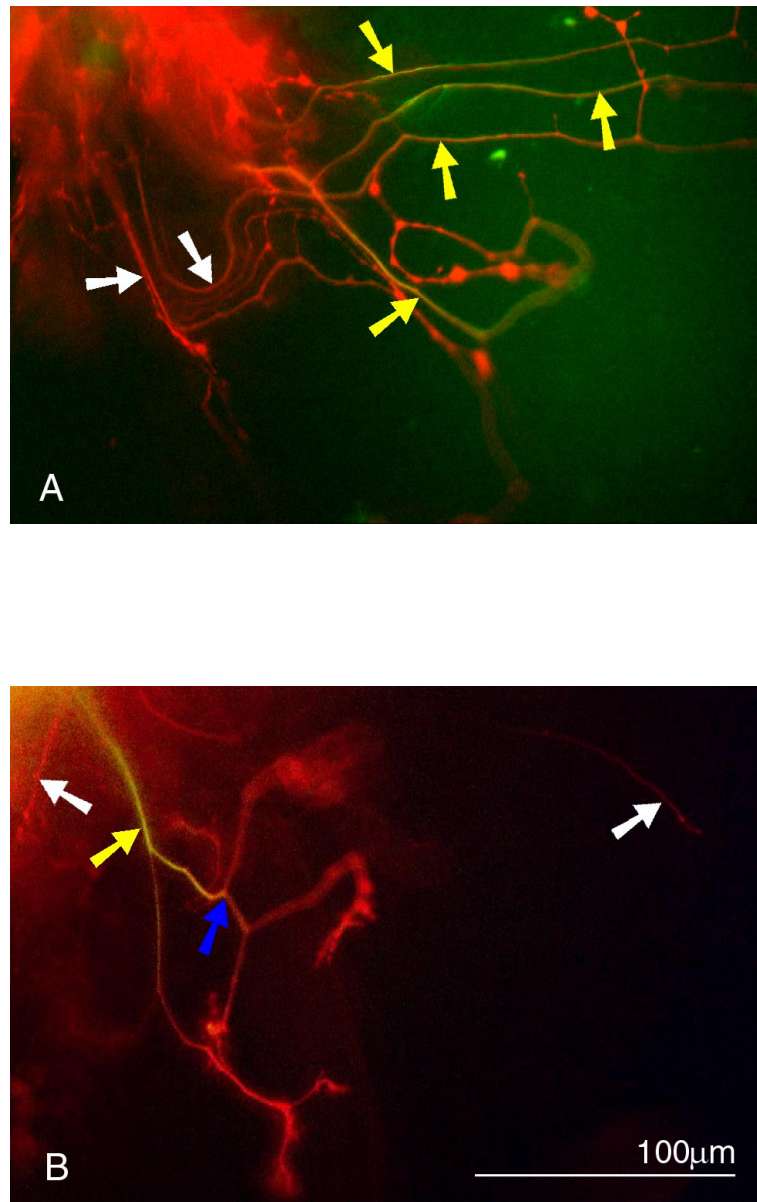


Figure 5.8 NF-M immunoreactivity in the presence of NT-3 (A) and BDNF (B) after 3 days in culture. The preparations were double labelled for NF-M (yellow arrows) and PGP 9.5 (white arrows); secondary antibodies used were conjugated with FITC and RITC respectively. NT-3 and BDNF addition caused the early appearance of immunoreactivity for NF-M, which is only detectable after 6 days without any factors. Blue arrow in B indicates the point to where NF-M immunoreactivity extends distally.

The addition of growth factors changed this pattern (table 5.5). The expression of NF-M became detectable in outgrowing axons after 3 days in the presence of NGF, NT-3 or BDNF though the latter was less effective (table 5.6, fig. 5.7 and 5.8)). Only NGF induced the expression of NF-L after a three-day incubation period (fig. 5.7). Under all conditions, whilst large numbers of neurons are NF-H-positive, the numbers positive for NF-M and NF-L (when they were expressed) were much smaller (about 2-3%).

5.3.2 NF expression in outgrowing axons of conditioned preparations

The conditioned control preparations had similar NF-H expression to the normal cultures with most axons strongly labelled. NF-M expression however was detectable after 3 days even without any growth factors (table 5.6). As in the normal cultures, NF-L expression was not observed in conditioned control preparations at the end of the same period.

NGF and BDNF markedly increased the numbers of NF-M labelled axons whereas preparations incubated with NT-3 had similar proportions of NF-M positive axons to controls in conditioned preparations (fig. 5.9). All three growth factors induced expression of NF-L in outgrowing axons after 3 days (table 5.7).

5.3.3 NF expression in DRG neurons

Optical sections of DRGs had similar proportions of NF-H and NF-L expressing neurons (table 5.8, fig.5.10). NF-M expression however, was significantly higher in BDNF and NGF treated preparations than others (fig. 5.11). I had no freshly fixed DRGs to determine the proportion of NF-positive neurons in normal animal to compare it to those in preparations cultured with

Table 5.7. The total number of axons expressing NF-L in conditioned preparations.

	Control	NGF	BDNF	NT-3
NF-L	nil	12	15	5
	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4

n = the number of preparations examined

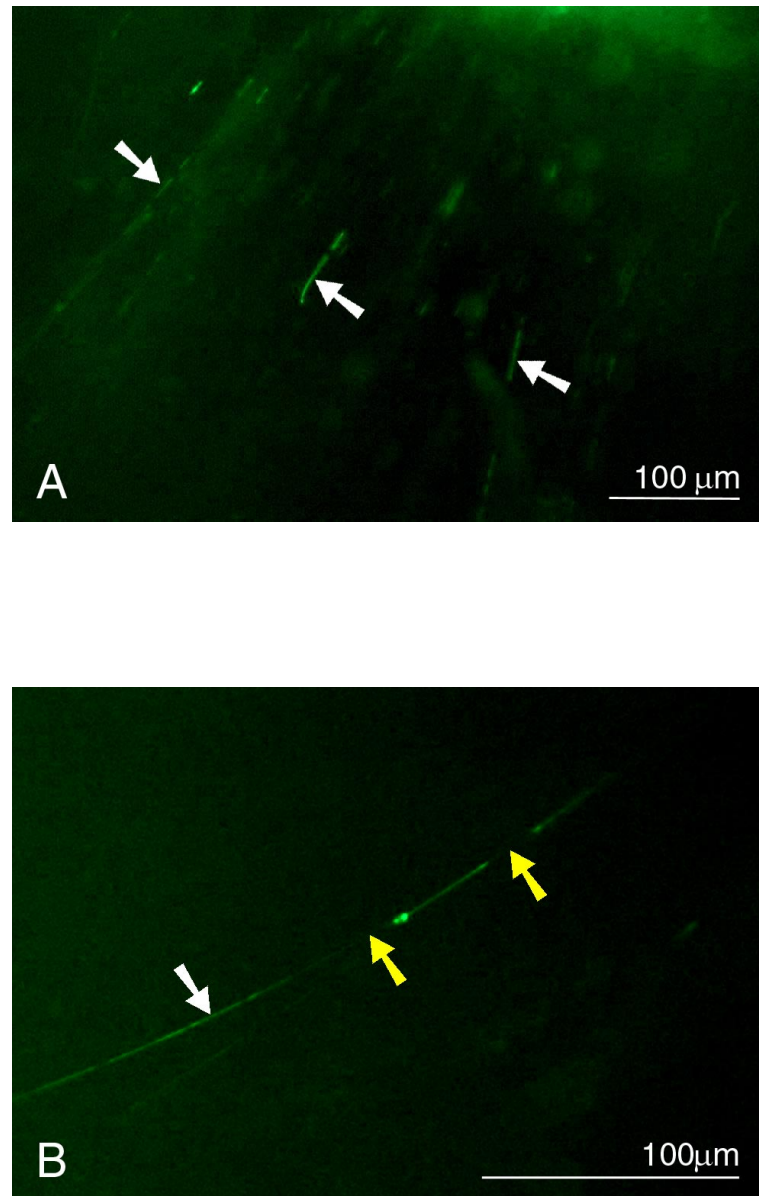


Figure 5.9 NF-M immunoreactivity in conditioned preparations with BDNF at low (A) and high (B) power magnifications; secondary antibody used was conjugated with FITC. BDNF significantly increased the number of NF-M positive axons (white arrows). The NF-M immunoreactivity is interrupted along the axons leaving frequent gaps (yellow arrows).

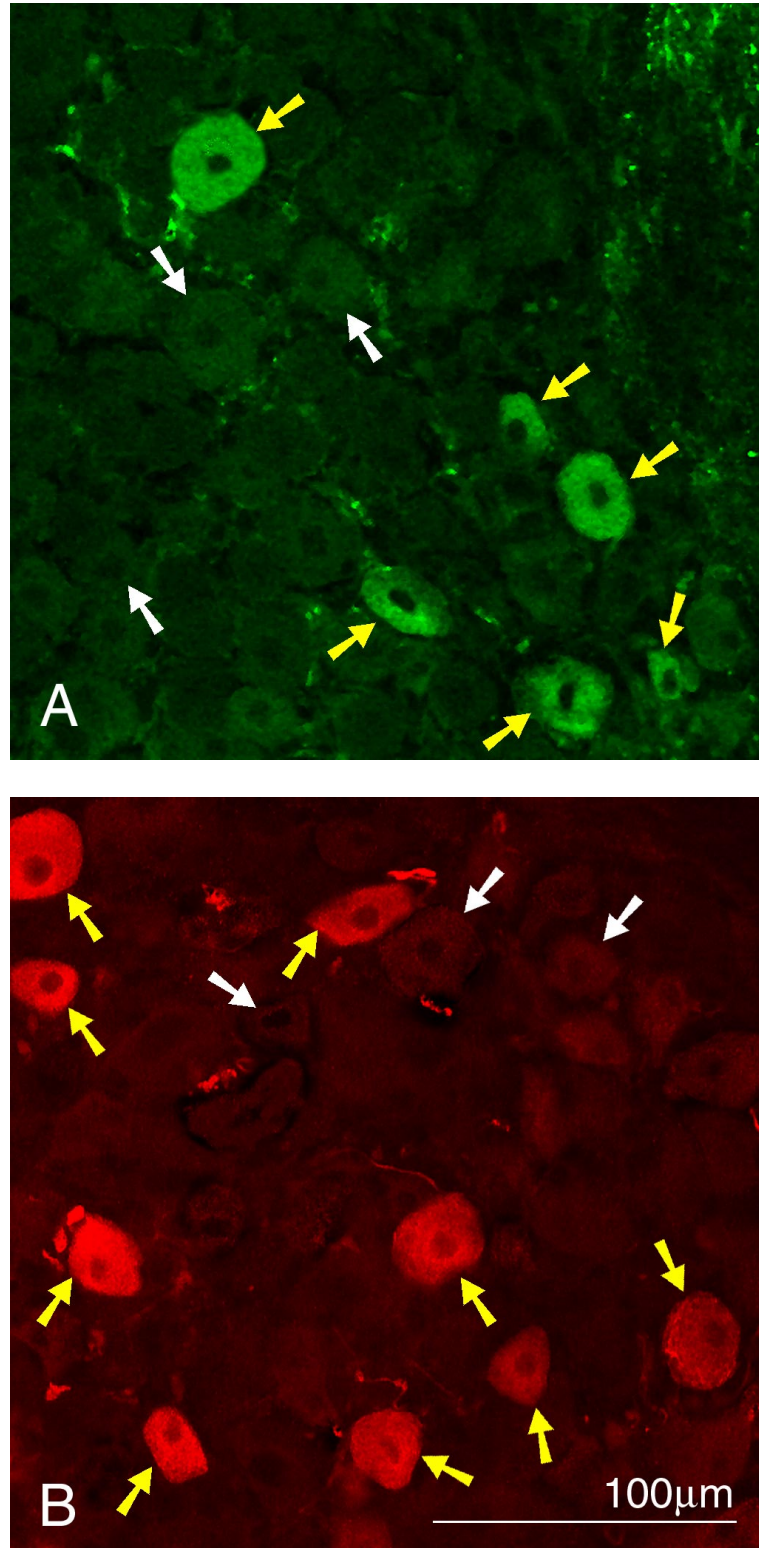


Figure 5.10 NF-H (A) and NF-L (B) immunoreactivities in optical sections of cultured control DRGs after 3 days of incubation; secondary antibodies used were conjugated with FITC and RITC respectively. Yellow arrows indicate NF expressing neurons while white arrows point some of the others.

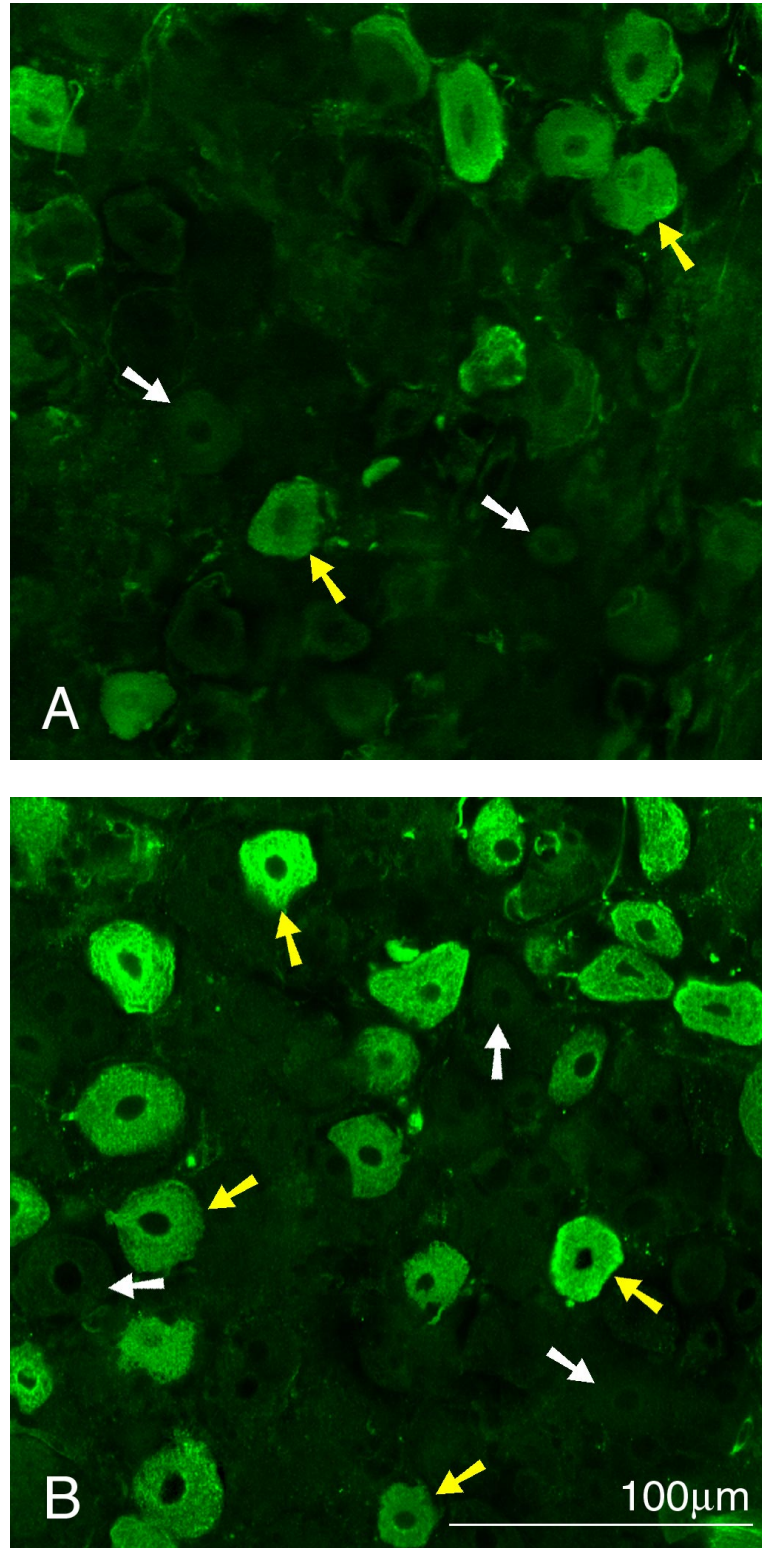


Figure 5.11 NF-M immunoreactivity in optical sections of control (A) and BDNF-added (B) DRG preparations; secondary antibody used was conjugated with FITC. There is a marked increase in the number of NF-M immunoreactive neurons in the latter. Yellow and white arrows indicate some of the positive and negative neurons respectively.

Table 5.8. The mean numbers of neuronal profiles expressing NFs in optical sections.

	Control	NGF	BDNF	NT-3
	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6
NF-H	5.8±1.6	6.5±1.2	7.5±2.5	5.7±1.4
NF-M	7.7±1.1	13±2*	19.8±4.1*	10±1
NF-L	9.7±3.4	9±2.7	7.2±1.8	5.5±1.3

Student's t-test is used to compare control to others in each group (**p* < 0.05). (*n* = the number of preparations examined).

or without growth factors. This was because at the beginning of these experiments, I planned to study only the changes of NF content of growing axons, but after obtaining my first results from these preparations I decided to determine NF contents of neurons of the same DRGs. Data from freshly fixed DRGs might have given some extra information and helped the interpretation of other data.

5.3.4 The expression of NFs in explant DRG cultures with crushed nerves

Many axons regenerated across the gap at the crush site and when stained they showed immunoreactivity for all three NFs on the 3rd day of incubation (figs. 5.12, 5.13). It was possible to trace many axons as they run through the proximal part, cross over the gap and run through the distal stump. However some axons had a pattern of interrupted NF labelling on their course across the gap which might simply be consequence of their changed plane of course or irregular deposition of NFs. The intensity of staining was much higher in the part of axons where they cross the gap. This could be due to lack of endoneural tube, in which case penetration of primary and secondary antibodies should be facilitated.

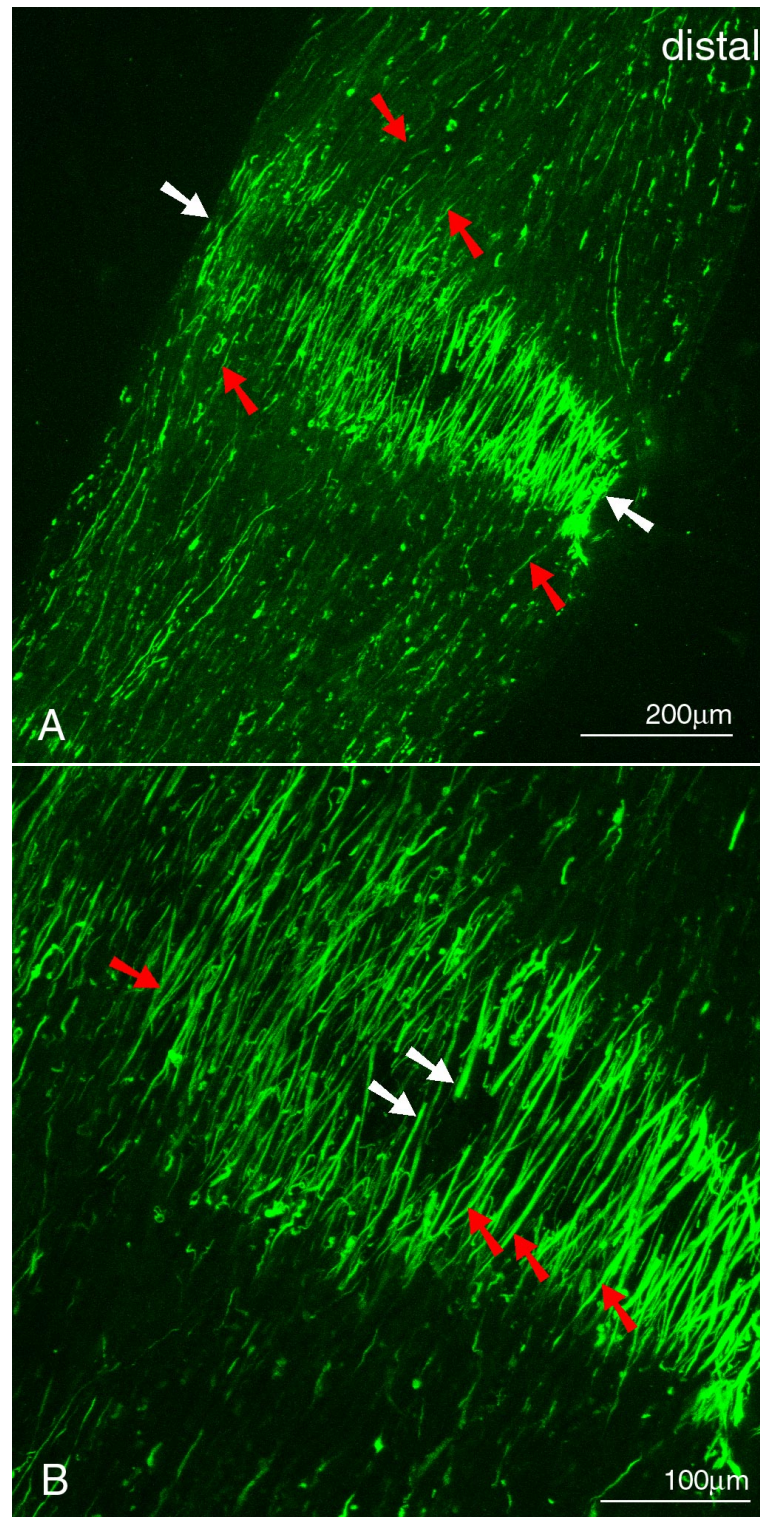


Figure 5.12 Low (A) and high (B) magnification confocal images of a crushed DRG preparation showing NF-H immunoreactivity in regenerated axons across the gap; secondary antibody used was conjugated with FITC. White arrows indicate the axis of the crush and the red ones point some of the axons running from proximal to distal part in A. In B, red arrows show some of the continuous axons crossing the gap, while white ones indicate some interrupted ones. This appearance may be due to a change in the plane of course of axon or interrupted lay-down of NF-H.

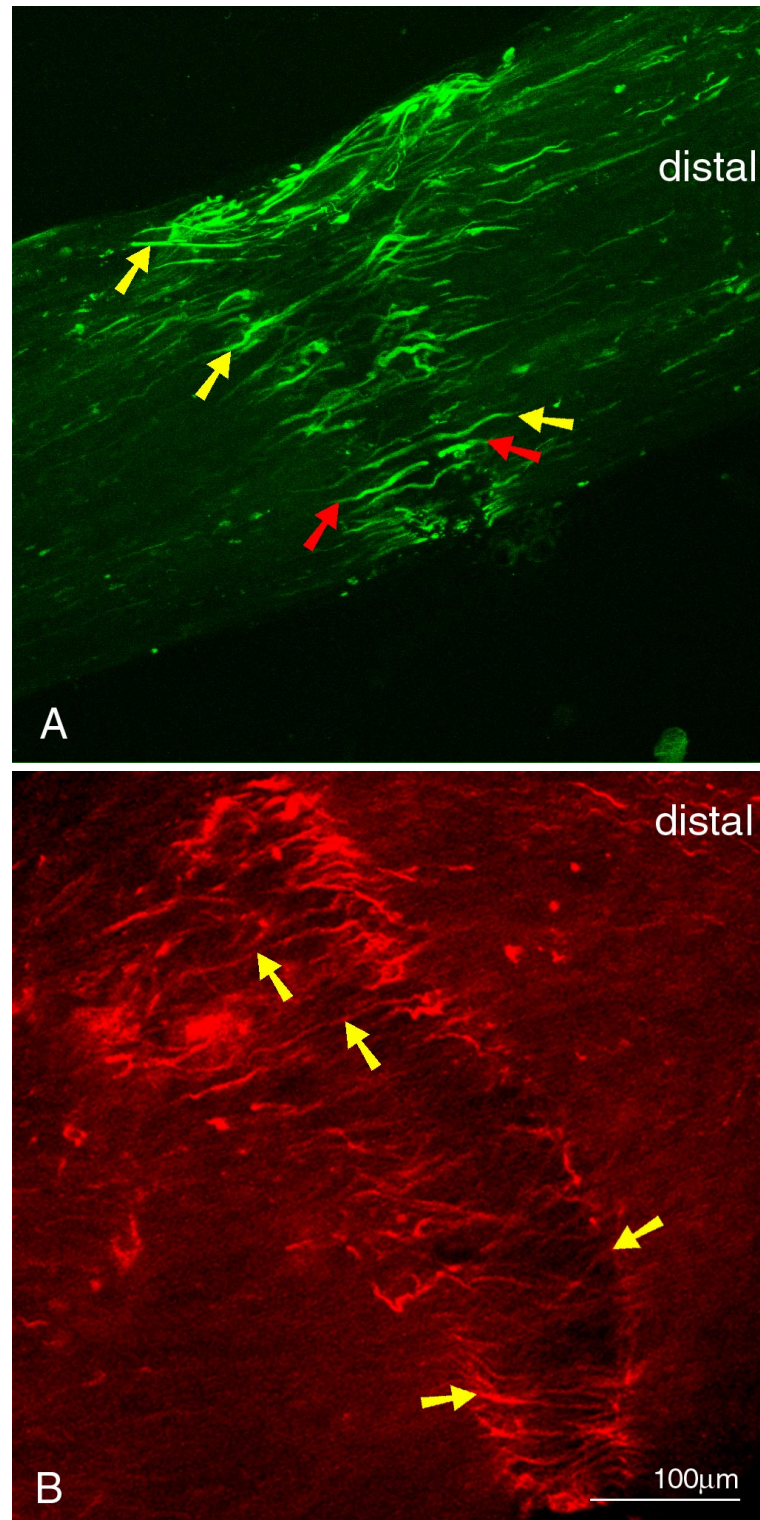


Figure 5.13 NF-M (A) and NF-L (B) immunoreactivities in regenerated axons of crushed preparations; secondary antibodies used were conjugated with FITC and RITC respectively. Yellow arrows indicate some of the continuous regenerated axons while red ones point some axons with interrupted NF immunoreactivity.

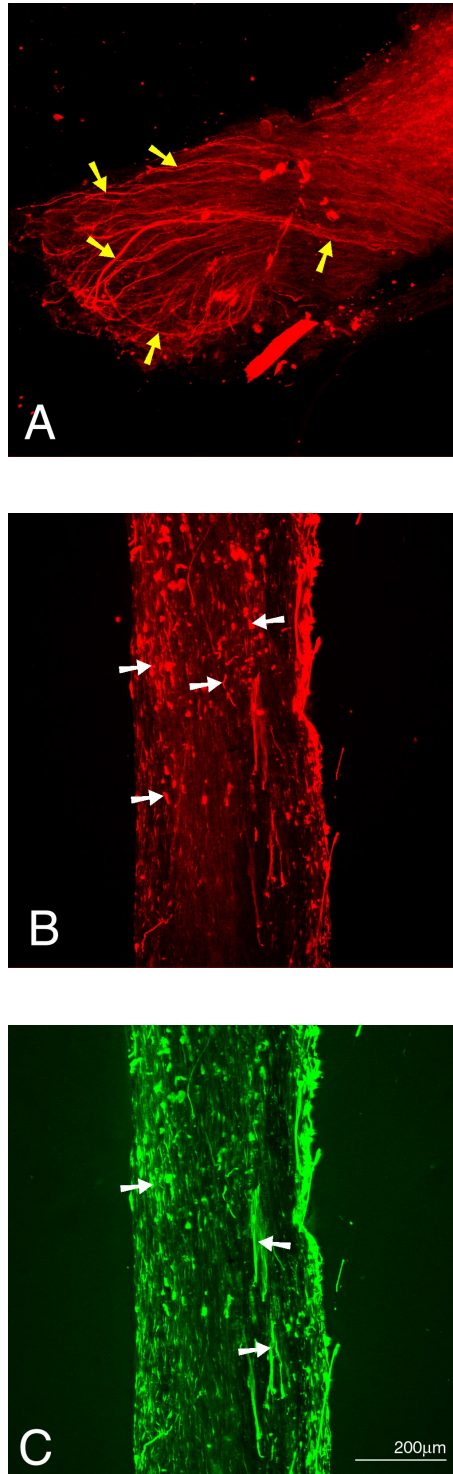


Figure 5.14 NF-H (A), NF-M (B) and NF-L (C) immunoreactivities in totally crushed peripheral nerves of cultured DRGs after 6 days of incubation; secondary antibodies used were conjugated with RITC in A and B and FITC in C. NF-H is strongly expressed by newly regenerated axons (yellow arrows in A) whereas NF-M and NF-L immunoreactivities seemed to be limited to the fragments of degenerated axons (white arrows in B and C).

5.4 Discussion

5.4.1 NF subunits are differentially expressed by outgrowing axons in DRG explant cultures

This study demonstrates that NF subunits are differentially expressed by the axons growing in matrigel in explant DRG cultures. NF-H was the first and most abundantly detected NF in the preparations. Generally, the expression pattern of NFs in regenerating axons is thought to resemble that during development (Hoffman and Cleveland, 1988; Oblinger *et al.*, 1989). It has been shown that NF-H is the last to appear among NF subunits during development (Willard and Simon, 1983). However, McKerracher *et al.*, (1990) demonstrated that retinal ganglion cell axons, growing into a piece of grafted peripheral nerve, express NF-H throughout the regeneration process although it is normally not detectable in neonates until 12th day (Willard and Simon, 1983). My finding is in parallel with those of McKerracher *et al.* and a novel observation as a first demonstration of NF expression in PNS during regeneration.

The NF turnover half-life is as short as 28 min in growing axons (Okabe *et al.*, 1993) with subunits exchanged rapidly between stationary and moving pools (Okabe *et al.*, 1993; Takeda *et al.*, 1994). The selective early expression of NF-H in outgrowing axons may be due to differential transport and/or synthesis of NF subunits. During regeneration, although the NF content is decreased in growing axons, they move more rapidly (Hoffman and Lasek, 1980). McQuarrie *et al.*, (1986) suggested that the motor for the transport of cytoskeleton is located in slow component b (Scb) and NFs may be dragged by the contents of the increased Scb during regeneration. Tetzlaff and Bisby (1989) also adopted the idea that NF movements may be determined by interaction with other cytoskeletal elements. NF-H projects side arms with its long tail and it also interacts with other cytoskeletal elements (Wong *et al.* 1995). If the mechanism suggested by McQuarrie *et al.* (1986) really exists, these properties of NF-H make it a suitable candidate to be hooked up and dragged by other fast-moving cytoskeletal elements. A selective NF transport in favour of NF-H could also help to explain why NF-H to NF-M or NF-L ratio is much greater in adult axons than in cell bodies (Hirokawa *et al.*, 1984).

Relatively higher synthesis of NF-H in the neuronal bodies may be another explanation for selective expression of NF-H in outgrowing axons. Synthesis of NF-H might be reduced less or later than that of the other NFs or be selectively regulated by an unknown mechanism. For example, Wong and Oblinger (1990) showed that mRNA levels for NF-L and NF-M were significantly reduced as early as 1 day after axotomy whereas the mRNA levels for NF-H did not change until 7 days postaxotomy. In recent studies with NF-H gene deleted mice, mRNA levels of the other two NFs were found to be not affected by NF-H gene ablation (Rao *et al.*, 1998; Zhu *et al.*, 1998; Elder *et al.*, 1998b). These findings suggest a different regulation of expression for NF-H to the other two. Some other studies have shown a correlation between the expression of NF-M and NF-L, usually in the form of an up-regulation of NF-L by NF-M (Tu *et al.*, 1995; Elder *et al.*, 1998a) indicating a mutual regulation for these two.

Analysis of optical sections from explanted DRGs might shed some light on this subject. Similar numbers of neurons expressed three different NFs in control preparations at the end of the 3-day incubation period (table 5.9). This might weaken the possibility of selective regulation of expression but not completely rule it out, because, in this study I was able to show only the numbers of neurons expressing NFs. Although the immunofluorescence technique I used also gives an idea about the amount of NFs, only the determination of exact quantities of NFs by sensitive assays like ELISA could give a conclusive result. On the other hand, the fact that NF-H content was significantly higher in growing axons and appeared to be similar to those of the others in neurons may suggest that the total amount of NF-H is greater than the other two. This may in turn suggest that more NF-H might be synthesised than the other NFs.

The significance of preferential early and abundant expression of NF-H in growing axons is not clear. There are conflicting reports about the role of NF-H in axonal growth *in vivo*. Earlier studies demonstrated a strong correlation between NF-H expression and radial axonal growth. For example, when Wong *et al.*, (1995) doubled NF-M, the level of axonal NF-H decreased to 50%

accompanied by severe reduction in axonal diameter. However, recently established transgenic NF-H gene knockout mice have proved otherwise as they did not have a significant inhibition of radial axonal growth (Rao *et al.*, 1998; Elder *et al.*, 1998b, Zhu *et al.*, 1998). On the other hand, in another study, disruption of NF-M gene led to markedly fewer axonal NFs and a suppression of radial growth (Elder *et al.*, 1998a). These findings suggest that regulation of axonal caliber is not simply determined by a single NF but probably by complex interactions between NF-H and NF-M. Future regeneration studies with NF-H gene knockout mice or production of double knockout mouse for two NFs together might shed light on this mechanism. On the other hand, NF-H may contribute to axonal growth in other ways. For example Jacobs *et al.*, (1997) demonstrated a correlation between regenerative capacity of lamprey spinal axons and the expression of NF-H. They proposed that NF-H might provide an internal propulsive force driving the growth cone forward.

Since it is known that NFs are obligate heteropolymers requiring NF-L and sub-stoichiometric amount of either NF-M or NF-H (Lee *et al.*, 1993), the presence of NF-H without NF-L in many growing axons, raises questions about its involvement in axon extension. One possibility (which was not investigated in this study) is that the growing axons might express another intermediate filament (such as peripherin or α -internexin) which might provide structural support for the growing axons. Both of these neuronal intermediate filaments are capable of self-assembly (Ching and Liem, 1993; Cui *et al.*, 1995). Expression of peripherin has been shown to be increased in axotomised PNS neurons (Troy *et al.*, 1990; Wong and Oblinger, 1990; Chadan-Milner *et al.*, 1994)

The labelling patterns of outgrowing axons for NF-H and NF-M suggest a proximal to distal deposition of these subunits in normal cultures (fig. 5.3, 5.4). NF-L immunoreactivity, however, was located more distally along the axons (fig. 5.5). Conditioned DRG preparations on the other hand, consistently exhibited more irregular and patchy staining for all 3 NFs along the axons (fig. 5.9). Axonal elongation and NF transport are known to increase after axotomy with a prior conditioning lesion while NF synthesis is severely reduced (Tetzlaff

and Bisby, 1989). The pattern of NF staining in conditioned preparations may reflect this situation. Faster moving NFs might travel further along the axons; but if their synthesis was not enough to cope with the increased demand, axons may not be paved completely and this might create the observed patchy appearance.

5.4.2 Growth factors increase expression of NFs in outgrowing axons

This study indicates that NGF increases expressions of NF-M and NF-L in explant DRG cultures. An effect of NGF on NF expression was demonstrated before (Verge *et al.*, 1990). As a novel finding, I have shown here that BDNF also increases number of axons expressing NFs. NT-3, on the other hand, did not seem to have effect on NF immunoreactivity.

The mechanism of NF induction by the growth factors might be quite complicated. Normally high affinity NGF receptor trkA is expressed by small neurons with small caliber axons. But most of these axons usually do not contain NFs (see the introduction of this chapter for details). However, there is a subgroup of trkA neurons that are also labelled by RT97, which recognizes phosphorylated form of NF-H (Averill *et al.*, 1995). Therefore, it is possible that NGF may cause increased immunoreactivity for NFs in the growing axons of these neurons.

On the other hand, it is possible to make another explanation based on the interactions between NGF and BDNF. It is known that NGF up-regulates BDNF expression in DRG neurons (Michael *et al.*, 1997). Thus, it is possible that, apart from a direct effect, NGF might also indirectly increase NF expression by inducing BDNF. BDNF in return may induce NF expression in larger trkB neurons and this might be responsible for some of the observed effects of NGF on NFs. If this is the case, then BDNF might appear to be the main growth factor regulating NF expression. However, in order to test this hypothesis, the time course and the level of BDNF expression and also the threshold level of BDNF to increase NF expression in DRG cultures should be known. On the other hand, there is some evidence in this study in favour of this hypothesis.

First of all, according to optical sections of cultured DRGs, only BDNF increased the number of NF-M positive neurons significantly. Secondly, in the NGF-treated normal preparations the mean number of NF-M positive axons per preparation was 8.3. A conditioning lesion however markedly increased this number to 22.5. NGF is the most potent known neurotrophic factor and induces axonal growth from trkA positive neurons. The most likely explanation for this 'extra effect' of NGF in conditioned preparations is that the lesions lead to the outgrowth of greater number of axons which include types which respond to the neurotrophins with changes in NF expression, but which do not grow out into the gels without the conditioning lesion. However, one might also speculate that the further increase in NF-M expression in conditioned preparations with NGF might not be due to a direct effect of this factor but the effect of endogenous BDNF that was induced by NGF.

TrkC receptors are expressed by a subpopulation of mostly larger DRG neurons which have high NF content and unlike the other neurotrophins, NT-3 is constitutively expressed in normal peripheral nerves and therefore might be expected to influence axonal phenotype (Funakoshi *et al.*, 1993). Indeed it has been shown that NT-3 decreases up-regulation of NPY in axotomized DRG neurons (Sterne *et al.*, 1998).

However, NT-3 had no significant effect on NF content under the conditions of my experiments. There may be different explanations for this observation. Firstly, the concentration of NT-3 I used might not be suitable to cause a detectable change in NF contents of growing axons. A second possibility is that, the time course of any potential effect of this factor on regeneration might be different from those of other neurotrophins. For example, Stern *et al.*, (1997) demonstrated that, NT-3 promoted axonal growth when it was locally applied to a transected sciatic nerve and this effect was maximum at day 15, with significantly greater number of myelinated axons by 8 months after repair. Finally it is also possible that, NT-3, indeed, has no direct effect on NF content of growing axons directly.

The observed NF-M expression with NT-3 in normal cultures could be

due to its stimulatory effect on the growth of axons from some NF-M expressing trkC neurons. This is evident from the conditioned preparations where NT-3 - added and control cultures had no difference in NF-M expression.

5.4.3 Lesioned distal nerve segment supports the expression of NFs

Detection of immunoreactivities for all three NFs after 3 days in crushed cultures suggest that distal nerve segment supports NF expression even in the absence of additional growth factors. It is known that degenerating distal part of a lesioned nerve provides a favourable medium for the regenerating axons. Bedi *et al.* (1992), showed that adult DRG neurons extend neurites on frozen sections of formerly degenerated nerves, but not on the sections of normal nerves. The non-neuronal cells of distal segment secrete a number of proteins that support axonal growth. For example after loss of contact with the axons, Schwann cells start to express cell adhesion molecules with neurotrophic actions such as NCAM and L1 (for review, see Martini, 1994) and the neurotrophins NGF, BDNF and NT-4 (Meyer *et al.*, 1992; Funaoshi *et al.*, 1993).

I designed another experiment to elucidate whether the ECM components or secreted factors from the non-neuronal cells in distal part of lesioned peripheral nerves might be responsible for induction of NF expression in crushed preparations. In this experiment, I crushed the distal half of the nerve repeatedly along its length under high mechanical pressure before setting up cultures. This was to kill all the cells in this part, which might potentially affect NF expression by secreting factors.

NF-H was strongly expressed by newly growing axons from proximal stump through the crushed section of nerve (fig 5.14). In contrast, there were only small fragments of NF-M and NF-L-positive material; presumably degenerated bits of axons.

These findings may suggest that the main factors that induce NF-M and NF-L expression are not ECM components but the factor(s) expressed by the non-neuronal cells in degenerating peripheral nerve.

Consistent with my other findings in the first part of this study, NF-H exhibited a pattern of expression that is independent of other two NFs and did not require support from non-neuronal cells in the preparations I described above. However, with the existing data, it is not possible to speculate about the effect of ECM on NF-H expression.

More studies will need to be done to elucidate the details of the links between NGF, BDNF and expression of NFs. It also needs further experiments to identify the factor(s) participating in the regulation of the expression of NF-H.

5.5 Conclusion

In this experiment I studied NF expression in explant DRG cultures:

- ◆ NF-H expression was detected earlier than NF-M and NF-L in growing axons.
- ◆ Conditioning lesions led to earlier expression of NF-M but not NF-L in growing axons.
- ◆ NGF and BDNF caused increased labelling for NF-M in growing axons while BDNF also increased the number of NF-M labelled DRG neurons.
- ◆ Regenerated axons across a crush site in DRG cultures had all three NFs at the end of 3-day incubation. I obtained some evidence that live non-neuronal cells are required for NF expression in these preparations.

Chapter 6 :Effects and identification of diffusible factors released from lesioned nerves

6.1 Introduction

After a peripheral nerve has been cut, regenerating axons tend to grow towards the distal stump (Cajal, 1928; Kuffler, 1989; Politis, 1982). One explanation for this is that, diffusible growth factors are released from the distal stump, attracting the axons.

Indeed, several different factors are released at the site of a peripheral nerve injury including neurotrophins, cytokines and other growth factors such as FGFs and GDNF (see discussion). Schwann cells, fibroblasts, axotomized neurons, macrophages and platelets are the main sources of secreted factors. For example, after loss of axonal contact, Schwann cells start to express neurotrophins NGF and BDNF (Meyer *et al.*, 1992), LIF (Banner and Patterson, 1994) and FGFs (Neuberger and De Vries, 1993; Grothe *et al.*, 1997). Fibroblasts in degenerating peripheral nerves are also known to secrete NGF (Bandtlow *et al.*, 1987; Heumann *et al.*, 1987a). Platelets which accumulate at the site of injury release a number of factors including platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), epidermal growth factor (EGF) and insulin-like growth factors (IGFs) (Oka and Orth, 1983; Assoian and Sporn, 1986; Ross *et al.*, 1986; Karey *et al.*, 1989). Macrophages invading lesion sites also produce PDGF, TGF- β and IGF-1 and in addition, FGF-2, TGF- α , and IL-1 (for review see Rappolle and Werb, 1992).

These proteins may be involved in regulation of different parts of the regeneration process. An obvious effect of these factors is the stimulation of neurite outgrowth from the proximal stump of cut nerves (Longo *et al.*, 1984; Richardson and Ebendal, 1982; Tedeschi and Liuzzi, 1992; Windebank and Poduslo, 1986). Diffusible factors may also be involved in the proliferation and migration of Schwann cells and fibroblasts which occurs in lesioned nerves (Perry *et al.*, 1987; Tonge *et al.*, 1996). Some factors also support the survival of axotomized neurons (see general introduction and chapter III for a detailed accounts). Guidance of regenerating axons towards their correct targets may

also depend on the actions of these factors (Thomas, 1989; Aaronson *et al.*, 1995).

Several studies have shown that the activity of the factors released from lesioned nerves which stimulate neurite outgrowth from embryonic sensory and sympathetic ganglia may be largely neutralized by antibodies to NGF (Richardson & Ebendal, 1982; Windebank & Poduslo, 1986; Tonge *et al.*, 1996). In view of the fact that expression of trkA is restricted to sympathetic and mainly small diameter sensory axons, this raises the question as to what might stimulate the growth of the motor and sensory axons which do not express trkA.

One possible factor might be FGF, which is synthesized by macrophages which accumulate at the lesion sites in injured nerves and in cellular bridge which forms between proximal and distal nerve stumps (Baird *et al.*, 1985; Grothe *et al.*, 1997; Fu and Gordon, 1997). Expression of FGF receptors by large diameter neurons in DRGs, which might be expected to express trks B or C, might also make it a candidate for this role (Oellig *et al.*, 1995).

Several studies have shown that FGF-1 and FGF-2 enhances peripheral nerve regeneration *in vivo* (Danielsen *et al.*, 1988; Aebischer *et al.*, 1989; Corderio *et al.*, 1989; Vergara *et al.*, 1993; Laquerriere *et al.*, 1994). They also stimulate neurite outgrowth from spinal cord neurons (Peulve *et al.*, 1994a; Iwasaki *et al.*, 1995) and PC12 cells (Togari *et al.*, 1983, 1985; Rydel and Greene, 1987; Schubert *et al.*, 1987; Lin *et al.*, 1996). FGF-1 enhances regeneration from adult rat DRG neurons in culture (Mohiuddin *et al.*, 1996).

Apart from direct trophic action on neurite extension, FGFs can also indirectly contribute to peripheral nerve regeneration by different mechanisms.

First of all, they may facilitate regeneration by stimulating the division of Schwann cells (Davis and Stroobant, 1990; Peulve *et al.*, 1994b). Interaction of FGFs with ECM components may also help nerve regeneration. It has been demonstrated that FGF-2 increases collagen fiber degradation, speeds up ECM turnover and regulates tissue remodelling in vascular smooth muscle cells and human gliomas by up-regulating the interstitial collagenase expression and

differentially regulating expression of matrix metalloproteinases and their inhibitors (Kennedy *et al.*, 1997; Miyagi *et al.*, 1998; Pickering *et al.*, 1997). There is another interaction between FGFs and cell adhesion molecules (CAMs - NCAM, N-Cadherin, LI). It has been shown that CAMs support neurite outgrowth from PC12 cells and developing neurons via FGF receptors (Walsh *et al.*, 1997).

Finally, FGFs can induce production and secretion of NGF by astrocytes, fibroblasts and cultured hippocampal neurons (Ono *et al.*, 1991; Yoshida and Gage, 1992; Ferhat *et al.*, 1997). These findings have always created arguments about whether FGFs have a direct tropic and survival effects or whether they induce NGF production which in turn produces these effects and indirectly show these actions. However there is strong evidence against the latter case suggesting the existence of direct neurotrophic effect for FGFs on cultured hippocampal neurons and in peripheral nerve regeneration independently of glial and Schwann cells (Wallicke and Baird, 1988; Mohuiddin *et al.*, 1996).

6.1.1 Rationale for the study

This study was designed as two parts. In the first part, an answer to the question ‘whether the main secreted factor from lesioned nerve with stimulatory effect on axonal growth is NGF or there are other factor(s) which are involved?’ was sought. For this, I prepared conditioned media (CM) from lesioned nerves in order to obtain secreted proteins from lesioned nerves to use in cultures. The contribution of NGF and BDNF/NT-4 (if there is any) to the total stimulatory effect of CM was determined. For this, trkA-IgG and trkB-IgG fusion proteins (trkA and trkB chimeras) were added to some preparations to sequester NGF and BDNF/NT-4 the changes in the axonal growth were determined. In the second part of the study, I investigated the effect of FGF-1 on axonal growth and determined the phenotypic properties of outgrowing axons.

6.2 Experimental procedures

6.2.1 Explant cultures

Explant cultures of thoracic DRGs were set up in collagen gels as described in chapter II. All cultures kept in incubation for 3 days. In the first

part, CM was used instead of regular RPMI medium. To prepare CM sciatic nerve of some animals were axotomized as described in chapter II. The animals were killed 4 days later and the distal stump of sciatic nerve (~7-8 mm) and a piece of the unoperated sciatic nerve of same size from contralateral side were removed and kept in RPMI medium at 37°C for 4 days. Then, CM was collected, pooled and added directly to the explant DRG cultures.

NGF, FGF-1 and trk chimeras were used at the concentrations given in table 6.1. Heparin was added to FGF-1 treated preparations to potentiate FGF's action. In the first part of the experiments, trkA and trkB chimeras were added to some of the CM preparations. In order to determine if a known concentration of NGF could cause a similar stimulation of axonal growth, NGF was sequestered from the CM before setting up cultures and 5 ng/ml NGF was added to these preparations. To sequester NGF, trkA chimeras were added to the CM, incubated for 2-3 hours, then protein A (a suspension of *staph. aureus* membranes) was added to bind trkA chimeras that had bound NGF molecules. The medium was spun in a centrifuge, the supernatant was collected and used to set up some cultures.

6.2.2 Immunohistochemistry, microscopy and image analysis

The concentrations and sources of primary and secondary antibodies used in this study were given in table 6.2 and 6.3.

The ideal way to determine axonal phenotype would be to use antibodies to trkA. However, results with this antibody (a gift from Dr D. O Clary) were not clear because of faint staining and high background. Therefore, I used antibodies to CGRP, which is usually co-expressed with trkA, and RT97, which is a marker for large diameter axons (presumably trkB and trkC) although these antibodies do not define the trk populations exactly (Averill *et al.*, 1995). Antibodies to pan-axonal markers β -III tubulin and PGP9.5 were used to counter-stain these preparations. To determine lengths of the growing axons, after viewing with fluorescence microscope, preparations labelled with PGP9.5 were further processed for HRP staining as described in chapter II. The sources and concentrations of antibodies used in this study were given in table 6.2.

Table 6.1. The concentrations and sources of factors used.

Factor	Concentration	Source
NGF 2.5 S	50 and 5 ng/ml	<i>Promega</i>
FGF-1	10 ng/ml	<i>Promega</i>
Heparin	20 µl/ml	<i>Sigma</i>
TrkA-IgG	10 µg/ml	<i>A gift from Genentech</i>
TrkB-IgG	10 µg/ml	<i>A gift from Genentech</i>
Protein A	1/20	<i>Sigma</i>

Table 6.2. The concentrations and sources of primary antibodies.

Antibody	Concentration	Source
Rabbit α -CGRP	1/100	<i>Affiniti</i>
Mouse monoclonal α -RT97	1/10	A gift from B. Anderton
Mouse α - β -III tubulin (clone no: SDL.3D10)	1/10	<i>Sigma</i>
Rabbit α -PGP 9.5	1/200	<i>UltraClone</i>

Table 6.3. The concentrations and sources of secondary antibodies used.

Antibody	Concentration	Source
RITC -conjugated goat α -rabbit IgG	1/100	<i>Sigma</i>
FITC -conjugated goat α -mouse IgG	1/100	<i>Sigma</i>
Biotin-conjugated goat α -rabbit IgG	1/100	<i>Sigma</i>

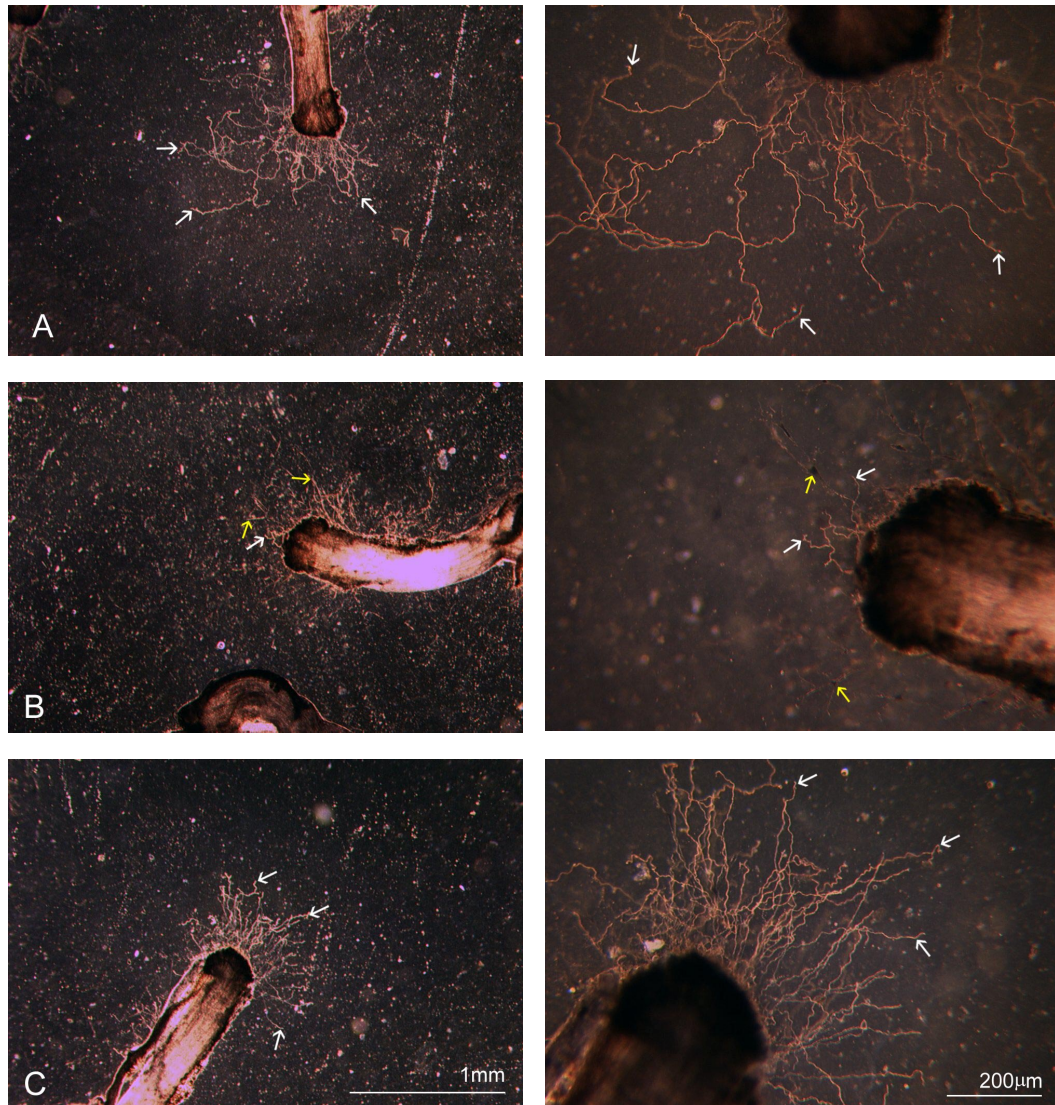


Figure 6.1 Axonal outgrowth in explant DRG cultures with CM (A), CM with TrkA (B) and TrkB (C) chimeras. Axonal growth was profuse with CM. TrkA chimeras blocked this almost completely. TrkB chimeras also caused a decrease in axonal growth but was not as effectively as TrkA chimeras. Preparations were labelled by antibodies to PGP9.5 and processed for HRP staining. Lower and higher magnification dark-ground images of the same preparation were shown in the same row. White arrows indicate some of the longest axons and yellow arrows point some migrating Schwann cells, which are abundant in the preparations treated with TrkA chimeras (B).

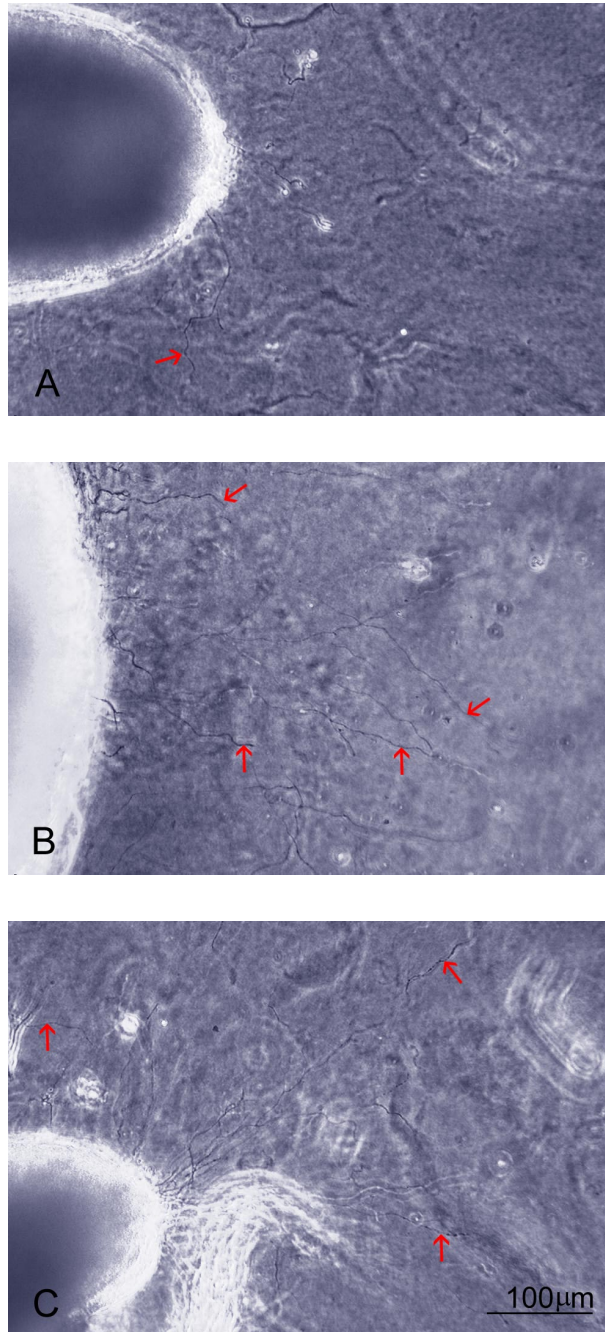


Figure 6.2 Axonal growth in explant DRG cultures with CM from which NGF was sequestered off (A), Addition of 5 ng/ml NGF to this preparations caused an axonal growth similar to that observed with normal medium with the same amount of additional NGF (B and C respectively). Arrows indicate some of the long axons. Fixed preparations were imaged with a phase-contrast microscope without staining.

6.3 Results

6.3.1 The effect of CM on axonal growth and the influence of trk chimeras

CM increased the numbers and the lengths of outgrowing axons (fig. 6.1, table 6.4). Addition of trkA chimeras significantly inhibited this stimulation. trkB chimeras also partially inhibited the enhanced axonal outgrowth by CM but not as strongly as trkA ones (fig. 6.1, table 6.4). These results confirmed the conclusions of other studies using antibodies that NGF is the most effective diffusible factor released from lesioned nerves. (Richardson and Ebendal, 1982; Windebank and Poduslo, 1986; Tonge *et al.*, 1996). In addition to NGF, lesioned nerves might release other factors which might enhance effects of NGF but have no effect on axonal growth by themselves. Therefore, I removed NGF from CM with the trkA chimeras and then added a known amount of NGF (5 ng/ml) but could not find any evidence that its effect was enhanced by unknown factors (fig. 6.2).

Table 6.4. Lengths of the outgrowing axons in explant DRG cultures with CM.

CM	TrkA-IgG fusion protein	TrkB-IgG fusion protein
600±23	164±18*	452±19*
n = 69(6)	n = 27(4)	n = 46(6)

Number of preparations examined is given in parentheses, n = number of axons measured. Student t-test is used to compare CM to others, *p < 0.001.

6.3.2 Comparison of effects of FGF-1 and NGF on axonal growth and phenotypes of outgrowing axons

Preparations cultured with FGF-1 showed significantly increased axonal lengths compared to control preparations (fig. 6.3, table 6.5). The majority of outgrowing axons (more than 75%) induced by FGF were labelled by antibody to CGRP (fig. 6.4, table 6.6). RT97 labelled approximately one third of all outgrowing axons in similar preparations.

Then I compared these results to the effects of NGF which should act only via trkA. NGF yielded a more profuse outgrowth than FGF-1 both in numbers and lengths of axons (fig. 6.3, table 6.5). Almost all axons in the

Table 6.5 Lengths of the outgrowing axons in explant DRG cultures with factors.

Control	FGF-1	NGF
338±29	539±25*	888±62*
n = 40 (6)	n = 120 (10)	n = 100 (8)

The number of preparations examined is given in parentheses, n = number of axons measured. Student t-test is used to compare control to others,*p < 0.001.

Table 6.6 Percentages of outgrowing axons labelled by anti-CGRP and RT97.

	Control	FGF-1	NGF	FGF-1+NGF
CGRP	43,6±5,11** n = 94 (8)	75,3±3,5 n = 150 (8)	90,2±1,3** n = 488 (8)	90,3±1,6** n = 330 (8)
RT97	8,8±2,3** n = 148 (8)	37,6±3,2 n = 229 (8)	49,5±2,4* n = 418 (8)	71,5±3,6** n = 158 (8)

The number of preparations examined is given in parentheses, n = number of axons measured. Student t-test is used to compare aFGF to others,*p < 0.01, **p < 0,001.

preparations with NGF had CGRP immunoreactivity and about 50% were labeled by RT97 (fig 6.4, table 6,6).

In order to have an idea about the extent of overlap between axonal populations induced by these two factors, I added both NGF and FGF-1 to some cultures. The percentages of CGRP labelled axons were not different than NGF alone in these preparations. However they had a substantial increase in the number of RT97 labelled axons compared to FGF or NGF alone.

6.4 Discussion

6.4.1 CM enhances axonal outgrowth and NGF seems to be responsible for most of this effect

Peripheral nerve injury triggers a series of reactive and restorative changes in the distal and proximal nerve stumps, DRGs and even in the spinal cord. Regulation of synthesis of a variety of growth factors at the site of injury is an important part of these changes. Expression of NGF, BDNF, NT-4, GDNF

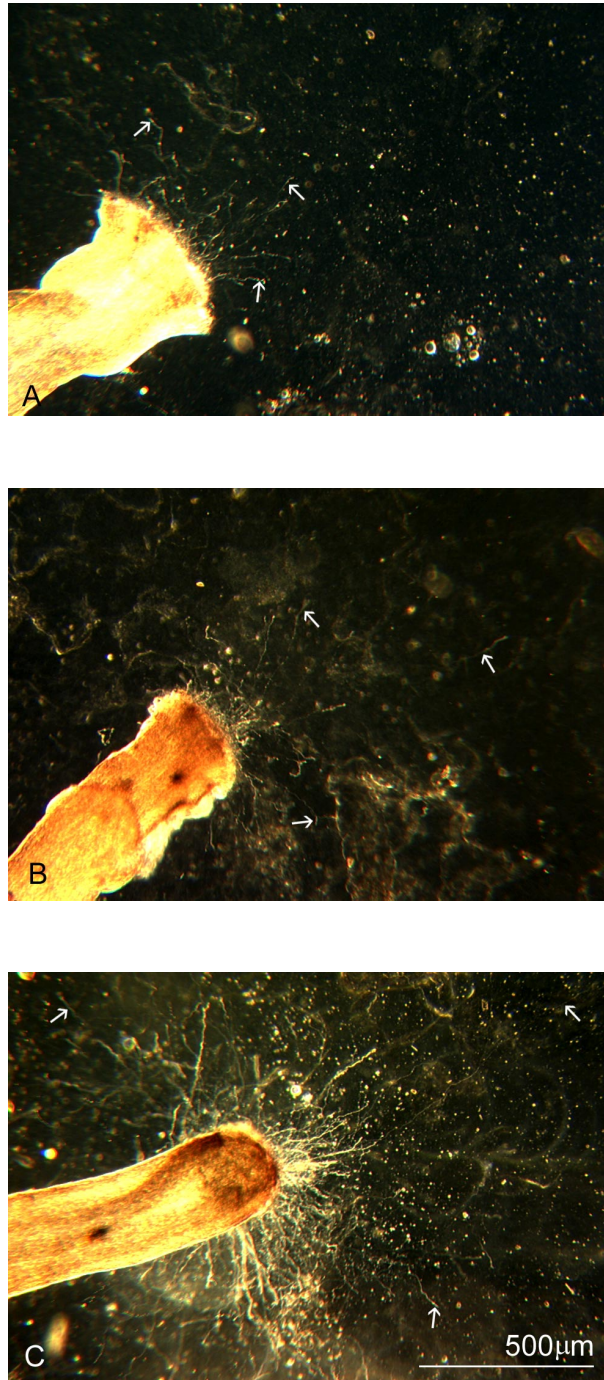


Figure 6.3 Axonal growth in control (A), aFGF (B) and NGF (C) treated explant DRG cultures. aFGF enhanced axonal outgrowth but not as strongly as NGF. Preparations were labelled by antibodies to PGP9.5 and processed for HRP staining. Arrows indicate some of the long axons.

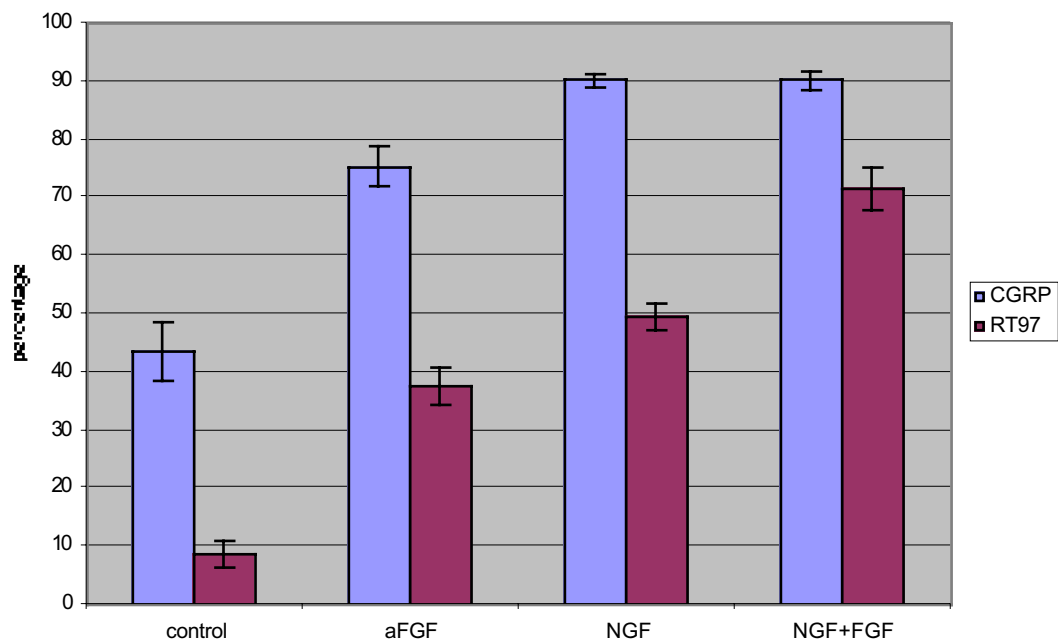


Figure 6.4 Percentages of CGRP and RT97 expressing axons growing in control preparations, aFGF, NGF and aFGF+NGF treated ones.

and FGF-2 increases in lesioned nerve while that of NT-3 decreases (Funakoshi, *et al.*, 1993; Meyer *et al.*, 1992, Hammarberg *et al.*, 1996; Grothe *et al.*, 1997). Whether these factors support initiation of axonal regeneration in adult animal is not certain. NGF, BDNF, NT-3, GDNF and FGF-1 have been demonstrated to stimulate axonal outgrowth from DRG neurons *in vitro* (Lindsay, 1988b; Mohiuddin *et al.*, 1995; Edström *et al.*, 1996; Leclerc *et al.*, 1997)). FGF-1 and FGF-2 are also known to enhance peripheral nerve regeneration *in vivo* (Corderio *et al.*, 1989; Danielsen *et al.*, 1988; Vergara *et al.*, 1993). The neurotrophins support survival of developing neurons which express high affinity trkA, trkB and trkC receptors respectively (see review by Barbacid, 1995). In adult animal, receptors for NGF, BDNF/NT-4 and NT-3 are expressed by distinct groups of DRG neurons with some overlap (Wright and Snider; 1995). If the neurotrophins have the same effects on axonal growth *in vivo* as *in vitro*, this may suggest that, after nerve injury, while NGF and BDNF support small and medium diameter axons expressing trkA and trkB, larger trkC expressing axons which are responsive to NT-3 will not be supported by this factor as its synthesis

decreases (Funakoshi, *et al.*, 1993). It is not fully known either which factors are required to initiate growth of small diameter axons which do not express any trk receptors although GDNF might be a candidate (Leclere *et al.*, 1997).

Several previous studies have shown that lesioned nerves release factors which can stimulate neurite outgrowth from sympathetic ganglia and DRGs and that this activity can be neutralized by antibodies to NGF. However, polyclonal antibodies raised against 2.5S NGF can cross-react with other neurotrophins (Acheson *et al.*, 1991; Murphy *et al.*, 1993b) and might therefore neutralize their activity as well as that of NGF.

Trk chimeras are produced by combining extracellular domain of trk molecules to the Fc domain of human IgG. The molecules obtained are soluble and retain the binding specificity of the native receptor and are capable of blocking the biological activity of their cognate neurotrophins (Shelton *et al.*, 1995).

The neutralization of activity of CM with trkA chimeras indicates that the major axonal growth-stimulating factor released by peripheral nerves is NGF. The apparent lack of effect of the other growth factors in CM could mean that their abilities to stimulate growth of adult mouse sensory axons *in vitro* might differ from NGF - or that levels of synthesis might be lower in my assay system. It has been demonstrated before that NT-3 and GDNF stimulate axonal growth in matrigel (Edström *et al.*, 1996; Leclere *et al.*, 1997). This probably means that the levels of the other factors are too low to have detectable effects in the assay system I used. However, the actual levels of various factors in CM are not known. The minimum concentration of NGF that causes detectable increases in axonal length is about 0.5 ng/ml (Edström *et al.*, 1996). In my study, CM caused mean axonal lengths nearly to double (338 μ m in control, 600 μ m in CM, tables 6.4 and 6.5). This might suggest that NGF is present in CM at high concentrations. However, according to unpublished observations by Tonge *et al.*, it seems to be lower than 1 ng/ml. The capacity of such low doses of NGF in CM to induce substantial axonal growth in CM is quite interesting. This might suggest that neuronally-derived NGF might have a higher

potency than that derived from salivary gland. Another possibility is that lesioned nerves release factors that might potentiate effects of NGF, although perhaps having little effect by themselves. To investigate this possibility, NGF from CM was sequestered by trkA chimeras and then a known concentration of NGF was added to the preparations. The result of this experiment did not provide evidence for any “synergistic” factor and suggests that the activity is therefore due to NGF alone- but whether the form released from lesioned nerves is more effective than that extracted from mouse salivary glands in stimulating axonal growth remains to be determined.

Incubation of preparations with TrkB chimeras also led to inhibition of axonal growth to some extent but not as strongly as the trkA chimeras did. This finding seems paradoxical since trkA chimeras were able to block axonal growth almost completely. This situation might not be necessarily due to the inhibition of action of BDNF and/or NT4 which recognize trkB but might be explained by cross-reaction of trkB chimeras with NGF. In fact, it has been shown that trkB chimeras may also bind NGF especially at high concentrations (Shelton *et al.*, 1995). BDNF was found to stimulate onset of neurite outgrowth from dissociated neurons of adult rat (Lindsay, 1988b) although contrary findings were reported by Malgrange *et al.* (1994). The reasons for the relative ineffectiveness of BDNF and NT-4 in CM are uncertain. Both factors interact with trkB, which is related to trkA, the NGF receptor and might therefore be expected to produce similar effects on axonal growth. Indeed, in amphibia Tonge *et al.* (unpublished observation) found that both factors strongly stimulated axonal growth of DRG neurons. However, when added to explant cultures of mouse DRGs, exogenous BDNF failed to induce axonal growth (Edström *et al.*, 1996). These findings may reflect differences in the activities of endogenous and exogenous BDNF or might simply be consequences of inter-species differences. However, the experimental model I used in this study needs alternative explanations since I did not use exogenous BDNF or NT-4. One possible explanation to this situation may be low levels of BDNF and NT-4 in the *in vitro* model I used; but this needs to be investigated.

6.4.2 FGF-1 stimulates axonal outgrowth *in vitro*

Although levels of growth factors other than NGF in CM were apparently not sufficient to stimulate axonal growth, it is possible that production of factors *in vivo* might be high enough locally to influence axonal growth. FGF is expressed by macrophages and these accumulate in the bridge of tissue formed by migrating cells which link proximal and distal stumps of lesioned nerves (for reviews see Martini, 1994; Fu and Gordon, 1997). Since FGF-1 and FGF-2 promote nerve regeneration *in vitro* (see introduction) it might be expected that they should stimulate axonal growth and that this might include populations of axons which do not express trkA.

In this study, I have demonstrated that FGF-1 induces axonal outgrowth from explanted adult mouse peripheral nerves with attached DRGs. Although FGF-1 stimulated both the number (data not shown) and the lengths of outgrowing axons, the latter effect was more prominent. However, the stimulation was not as strong as with NGF. NGF greatly enhanced axonal outgrowth and most axons were stained by α -CGRP, which may be expected from the known co-expression of this neuropeptide with trkA (Averill *et al.*, 1995). Interestingly, a smaller proportion of axons was labelled by RT97, which may be explained by the fact that about 18% of DRG neurons that express trkA are labelled by RT97 (Averill *et al.*, 1995).

Axons growing in the presence of FGF-1 were also labelled by anti-CGRP and RT97 but the proportions of axons labelled by each of these antibodies were different than those obtained using NGF. The proportion of CGRP-positive axons was lower and that of RT97-positive axons was higher with FGF-1 than NGF. One interpretation of these results is that, FGF-1 stimulates outgrowth of a population of axons which are RT97-positive but CGRP-negative and it is likely that these will include axons which are trkB and / or trkC-positive. Direct confirmation of this point may require further experiments using antibodies specific for trkB and trkC.

Consistent with the hypothesis that NGF and FGF stimulate outgrowth of separate (but possibly overlapping) populations of axons, in cultures incubated

with both factors, the proportion of RT97-positive axons was higher.

6.5 Conclusion

- ◆ In this chapter, I studied the effect of CM and FGF-1 on the axonal growth from explant DRG cultures:
- ◆ CM stimulated axonal growth.
- ◆ The main stimulatory factor in CM appeared to be NGF.
- ◆ FGF-1 also promoted growth of axons which could be labelled by anti-CGRP and RT97.
- ◆ The proportion of RT97-positive axons was higher in FGF-1 treated cultures than those incubated with NGF.
- ◆ Local release of FGF from lesioned nerve might be one mechanism by which, trkA-negative axons are stimulated to grow.

Chapter 7 :General discussion

Nerve injuries are followed by a series of changes in expression of a number of proteins including neuropeptides and neurofilaments. The reasons for these changes are uncertain, but could involve lack of target-derived trophic factors or exposure to 'lesion' factors.

In this thesis, I used a novel *in vitro* system to investigate the effects of various growth factors on expression of neuropeptides, NFs and axonal growth.

In order to establish that this *in vitro* model was suitable for the survival of DRG neurons, first, I determined the extent of cell loss in culture. This was very important for the interpretation of the results of experiments. In the third chapter, I demonstrated that, DRG neurons are viable, with a very high survival rate, for at least 3 days in culture. Furthermore, in chapter V, I showed that axonal regeneration continues for at least 12 days *in vitro* even without changing the medium, providing additional evidence for reliability of explant DRG cultures and raising the possibility that these preparations could be used in future for the study of effects of other growth factors and drugs on neuronal viability.

Neuropeptide plasticity is an important mechanism for axotomized neurons to switch from "transmitting mode" to "regeneration and growth". The trigger for changes in expression of neuropeptides in lesioned nerves are uncertain, although the decline in expression of CGRP and SP is widely believed to be due to lack of target-derived NGF. However other factors, target-derived or lesion-induced, might also be involved in regulation of neuropeptide expression. In this study, I showed that LIF strongly up-regulates GAL. I also demonstrated that, NGF has a negative effect on the expression of this neuropeptide as I showed that the stimulatory effect of LIF on GAL expression was partially antagonized by NGF. As a novel technical opportunity, cultured DRG preparations made it possible to detect changes in neuropeptide expression not only in the DRG neurons but also in the axons growing into gels. In principle, these preparations could be used to investigate the role of other cytokines and growth factors on neuropeptide expression. It is also possible to

investigate the relation of a particular neuropeptide to the regeneration propensity of the axons that express it. For example, in this study, I had no evidence suggesting that GAL-expressing axons may grow longer.

Reduction in NF expression is another characteristic structural change observed following axotomy. The significance of this reduction in the main component of axonal cytoskeleton is believed to increase axoplasmic fluidity, facilitating transport of other molecules essential for axonal elongation such as tubulin and actin. However, the mechanism regulating these changes is not clear although lack of target-derived NGF is indicated to be responsible for some of them. In PNS neuropathies, a series of defects have been demonstrated such as neuronal loss, reduction in expression of NFs, decrease in conduction velocity and impaired capacity of regeneration of axons. There is currently much interest in finding therapies for these conditions. NGF has been shown to promote expression of NFs, but may be expected to affect only trkA-expressing neurons, although it may also affect trkB-expressing ones indirectly via BDNF expression. BDNF and NT-3 might be expected to have similar effects to NGF on NF expression in responsive neurons. In chapter V, I studied the effects of the neurotrophins on NF expression in regenerating axons. NGF and BDNF accelerated expression of NFs, but NT-3 was ineffective. However, when axons were able to regenerate into a distal nerve stump, they promptly expressed all 3 NFs. The distal stump was therefore more effective than any of the neurotrophins in promoting NF expression and this effect was dependent on viable cells in distal nerve stump. However the nature and the identity of the factors involved remains to be determined.

Several diffusible factors are released from lesioned nerves and they participate in various parts of regeneration process. Conditioned medium, which is prepared using previously injured nerves has a strong stimulatory effect on axonal growth in DRG cultures. In this study, I found that this effect of CM appears to be due mainly to NGF. However, NGF is expected to promote regeneration of only those axons with the appropriate NGF receptor, trkA. What stimulates regeneration of the other axons that do not express trkA remains to

be investigated. However, in the last chapter, I showed that FGF-1 might promote growth of some *trkB* and *trkC* axons as well as some *trkA* ones.

This study has demonstrated that, the *in vitro* system I used is a suitable model to study effects of various factors on peripheral nerve regeneration. Potentially, this model could be improved further to study the longer-term consequences of nerve injury. With small modifications and technical improvements, this model could also be used for dynamic studies and also for time-lapse analysis of axonal regeneration.

References

- Aaronson, O. S., Golding, J. P., and Tonge, D. A. (1995) Oriented growth of regenerating axons in axolotl forelimbs is consistent with guidance by diffusible factors from distal nerve stumps. *Neuroscience* **66**, 201-213.
- Abraham, J. A., Mergia, A., Whang, J. L., Tumolo, A., Friedman, J., Hjerrild, K. A., Gospodarowicz, D., and Fiddes, J. C. (1986) Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* **233**, 545-548.
- Acheson, A., Barker, P.A., Alderson, R.F., Miller, F.D. and Murphy, R.A. (1991) Detection of brain-derived neurotrophic factor-like activity in fibroblasts and Schwann cell: inhibition by antibodies to NGF. *Neuron* **7**, 265-275.
- Acheson, A., Conover, J. C., Fandl, J. P., DeChiara, T M., Russell, M., Thadani, A., Squinto, S. P., Yancopoulos, G. D., Lindsay, R. M. (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature* **374**, 450-453.
- Aebischer, P., Salessiotis, A. N., and Winn, S. R. (1989) Basic fibroblast growth factor released from synthetic guidance channels facilitates peripheral nerve regeneration across long nerve gaps. *J. Neurosci. Res.* **23**, 282-289.
- Albers, K. M., Wright, D. E., and Davis, B. M. (1994) Overexpression of nerve growth factor in epidermis of transgenic mice causes hypertrophy of the peripheral nervous system. *J. Neurosci.* **14**, 1422-1432.
- Aldskogius, H., and Arvidsson, J. (1978) Nerve cell degeneration and death in the trigeminal ganglion of the adult rat following peripheral nerve transection. *J. Neurocytol.* **7**, 229-250.
- Aldskogius, H., and Risling, M. (1981) Effect of sciatic neurectomy on neuronal number and size distribution in the L7 ganglion of kittens. *Exp. Neurol.* **74**, 597-604.
- Altar, C. A., and DiStefano, P. S. (1998) Neurotrophin trafficking by anterograde transport. *Trends Neurosci.* **21**, 433-437.
- Altar, C. A., Cai, N., Bliven, T., Juhasz, M., Conner, M., Acheson, A. L., Lindsay, R. M., and Wiegand, S. J. (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature* **389**, 856-860.
- Alvarez, F. J., Morris, H. R., and Priestley, J. V. (1991) Sub-population of smaller diameter trigeminal primary afferent neurons defined by expression of calcitonin gene-related peptide and the cell surface oligosaccharide recognized by monoclonal antibody LA4. *J. Neurocytol.* **20**, 716-731.
- Apfel, S. C., Wright, D. E., Wiideman, A. M., Dormia, C., Snider, W. D.,

and Kessler, J. A. (1996) Nerve growth factor regulates the expression of brain-derived neurotrophic factor mRNA in the peripheral nervous system. *Mol. Cell. Neurosci.* **7**, 134-142.

Arbuthnott, E. R., Boyd, I. A., and Kalu, K. U. (1980) Ultrastructural dimensions of myelinated peripheral nerve fibres in the cat and their relation to conduction velocity. *J. Physiol.* **308**, 125-157.

Assoian, R. K. ,and Sporn, M. B. (1986) Type beta transforming growth factor in human platelets: release during platelet degranulation and active on smooth muscle cells. *J. Cell Biol.* **102**, 1217-1223.

Averill, S., McMahon, S. B., Clary, D. O., Reichardt, L. F., and Priestley, J. V. (1995) Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur. J. Neurosci.* **7**, 1484-1494.

Baird, A. (1994) Fibroblast growth factors: activities and significance of non-neurotrophin neurotrophic factors *Cur. Op. Neur.* **4**, 78-86.

Baird, A., Esch, F., Mormede, P., Ueno., N., Ling, N., Böhlen, P., Ying, S. - Y., Wehrenberg, W. B., and Guillemin, R. (1986) Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues. *Recent Prog. Horm.Res.* **42**, 143-205.

Baird, A., Mormede, P., and Böhlen, P. (1985) Immunoreactive fibroblast growth factor in cells of peritoneal exudate suggests its identity with macrophage-derived growth factor. *Biochem. Biophys. Res. Com.* **126**, 358-364.

Balin, B. J., and Lee, V. M. (1991) Individual neurofilament subunits reassembled in vitro exhibit unique biochemical, morphological and immunological properties. *Brain Res.* **556**, 196-208.

Bamji, S. X., Majdan, M., Pozniak, C. D., Belliveau, D. J., Aloyz, R., Kohn, J., Causing, C. G., and Miller, F. D. (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. *J. Cell Biol.* **140**, 911-923.

Bandtlow, C. E., Heumann, R., Schwab, M. E., and Thoenen, H. (1987) Cellular localization of nerve growth factor synthesis by in situ hybridization. *EMBO J.* **6**, 891-899.

Banner, L.R., and Patterson, P. H. (1994) Major changes in the expression of the mRNAs for cholinergic differentiation factor/leukemia inhibitory factor and its receptor after injury to adult peripheral nerves and ganglia. *Proc. Natl. Acad. Sci. USA* **91**, 7109-7113.

Barakat-Walter, I. (1996) Brain derived neurotrophic factor like immunore-

activity is localized mainly in small sensory neurons of rat dorsal root ganglia. *J. Neurosci. Methods* **68**, 281-288.

Barbacid, M. (1995) Neurotrophic factors and their receptors. *Curr. Opin. Cell Biol.* **7**, 148-155.

Barbin, G., Manthorpe, M., and Varon, S. (1984) Purification of the chick eye ciliary neurotrophic factor. *J. Neurochem.* **43**, 1468-1478.

Barrett, G. L., and Bartlett, P. F. (1994) The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proc. Natl. Acad. Sci. USA* **91**, 6501-6505.

Bazan, J. F. (1991) Neuropoietic cytokines in the hematopoietic fold. *Neuron* **7**, 197-208.

Belliveau, D. J., Krivko, I., Kohn, J., Lachance, C., Pozniak, C., Rusakov, D., Kaplan, D., and Miller, F. D. (1997) NGF and neurotrophin-3 both activate TrkA on sympathetic neurons but differentially regulate survival and neuritogenesis. *J. Cell Biol.* **136**, 375-388.

Bennett, D. L., Michael, G. J., Ramachandran, N., Munson, J. B., Averill, S., Yan, Q., McMahon, S. B., and Priestley, J. V. (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J. Neurosci.* **18**, 3059-3072.

Berkemeier, L. R., Winslow, J. W., Kaplan, D. R., Nikolics, K., Goeddel, D. V., and Rosenthal, A. (1991) Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron* **7**, 857-866.

Black, M. M., Keyser, P., and Sobel, E. (1986) Interval between the synthesis and assembly of cytoskeletal proteins in cultured neurons. *J. Neurosci.* **6**, 1004-1012.

Bondok, A. A., and Sansone, F. M. (1984) Retrograde and transganglionic degeneration of sensory neurons after peripheral nerve lesion at birth. *Exp. Neurol.* **86**, 322-330.

Bradshaw, R. A. (1978) Nerve growth factor. *Annu. Rev. Biochem.* **47**, 191-216.

Bredesen, D. E., and Rabizadeh, S. (1997) p75^{NTR} and apoptosis: trk-dependent and trk-independent effects. *Trends Neurosci.* **20**, 287-290.

Bregman, B. S., and Goldberger, M. E. (1982) Anatomical plasticity and sparing of function after spinal cord damage in neonatal cats. *Science* **217**, 553-555.

Bregman, B. S., and Goldberger, M. E. (1983) Infant lesion effect: III. Anatomical correlates of sparing and recovery of function after spinal cord damage in

newborn and adult cats. *Dev. Brain Res.* **9**, 137-154.

Brunner, G., Gabrilove, J., Rifkin, D. B., and Wilson, E. L. (1991) Phospholipase C release of basic fibroblast growth factor from human bone marrow cultures as a biologically active complex with a phosphatidylinositol anchored heparan sulfate proteoglycan. *J. Cell Biol.* **114**, 1275-1283.

Buchman, V. L., and Davies, A. M. (1993) Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic neurons. *Development* **118**, 989-1001.

Buj-Bello, A., Buchman, V. L., Horton, A., Rosenthal, A., and Davies, A. M. (1995) GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* **15**, 821-828.

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

Cao, Y., and Pettersson, R. F. (1993) Release and subcellular localization of acidic fibroblast growth factor expressed to high levels in HeLa cells. *Growth Factors* **8**, 277-290.

Carden, M. J., Trojanowski, J. Q., Schlaepfer, W. W., and Lee, V. M. (1987) Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. *J. Neurosci.* **7**, 3489-3504.

Carroll, S. L., Silos-Santiago, I., Frese, S. E., Ruit, K. G., Milbrandt, J., and Snider, W. D. (1992) Dorsal root ganglion neurons expressing trk are selectively sensitive to NGF deprivation in utero. *Neuron* **9**, 779-788.

Carter, J., Gragerov, A., Konvicka, K., Elder, G., Weinstein, H., and Lazzarini, R. A. (1998) Neurofilament (NF) assembly; divergent characteristics of human and rodent NF-L subunits. *J. Biol. Chem.* **273**, 5101-5108.

Cavanaugh, M. V. (1971) Quantitative effects of the peripheral innervation area on nerve and spinal ganglion cells. *J. Comp. Neurol.* **94**, 181-219.

Cavanaugh, M. W. (1951) Quantitative effects of the peripheral innervation area on nerves and spinal ganglion cells. *J. Comp. Neurol.* **94**, 181-218.

Ch'ng, J. L. C., Christofides, N. D., Anand, P., Gibson, S. J., Allen, Y. S., Su, H. C., Tatemoto, K., Morrison, J. F. B., Polak, J. M., and Bloom, S. R. (1985) Distribution of galanin immunoreactivity in the central nervous system and the responses of galanin containing neuronal pathways to injury. *Neuroscience* **16**, 343-354.

Chadan-Milner, S., Moya, K. L., Portier, M. M., and Filliatreau, G. (1994) Identification of a peripherin dimer: Changes during axonal development and re-

generation of the rat sciatic nerve. *J. Neurochem.* **62**, 1894-1905.

Chao, M. V., and Hempstead, B. L. (1995) p75 and Trk: a two - receptor system. *Trends Neurosci.* **18**, 321-326.

Chao, M. V., Bothwell, M. A., Ross, A. H., Koprowski, H., Lanahan, A. A., Buck, C. R., and Sehgal, A. (1986) Gene transfer and molecular cloning of the human NGF receptor. *Science* **232**, 518-521.

Cheema, S. S., Richards, L., Murphy, M., and Bartlet, P. F. (1994) Leukemia inhibitory factor prevents the death of axotomised sensory neurons in the dorsal root ganglia of the neonatal rat. *J. Neurosci. Res.* **37**, 213-218.

Ching, G. Y., and Liem, R. K. (1993) Assembly of type IV neuronal intermediate filaments in nonneuronal cells in the absence of preexisting cytoplasmic intermediate filaments. *J. Cell Biol.* **122**, 1323-1335.

Clement, M. V., and Stamenkovic, I. (1994) Fas and tumor necrosis factor receptor-mediated cell death: similarities and distinctions. *J. Exp. Med.* **180**, 557-67.

Cohen, A., Bray, G. M., and Aguayo, A. J. (1994) Neurotrophin-4/5 (NT-4/5) increases adult rat retinal ganglion cell survival and neurite outgrowth in vitro. *J. Neurobiol.* **8**, 953-959.

Cohen, S. (1960) Purification of a nerve-growth promoting protein from the mouse salivary gland and its neurocytotoxic antiserum. *Proc. Natl. Acad. Sci. USA* **46**, 302-311.

Collard, J. F., Cote, F and Julien, J. P. (1995) Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. *Nature* **375**, 61-64.

Conner, J. M., Lauterborn, J. C., Yan, Q., Gall, C. M., and Varon, S. (1997) Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. *J. Neurosci.* **17**, 2295-2313.

Conover, J. C., and Yancopoulos, G. D. (1997) Neurotrophin regulation of the developing nervous system: analyses of knockout mice. *Rev. Neurosci.* **8**, 13-27.

Conover, J. C., Erickson, J. T., Katz, D. M., Blanchi, L. M., Poueymirou, W. T., McClain, J., Pan, L., Helgren, M., Ip, N. Y., Boland, P., Friedman, B., Wiegand, S., Vejsada, R., Kato, A. C., DeChiara, T. M., and Yancopoulos, G. D. (1995) Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. *Nature* **375**, 235-238.

Corderio, P. G., Seckel, B. R., Lipton, S. A., D'Amore, P. A., Wagner, J., and

Madison, R. (1989) Acidic fibroblast growth factor enhances peripheral nerve regeneration in vivo. *Plast. Reconstr.Surg.* **83**, 1013-1019.

Corness, J., Shi, T. J., Xu, Z. Q., Brulet, P., and Hökfelt, T. (1996) Influence of leukemia inhibitory factor on galanin/GMAP and neuropeptide Y expression in mouse primary sensory neurons after axotomy. *Exp. Brain Res.* **112**, 79-88.

Crowley, C., Spencer, S. D., Nishimura, M. C., Chen, K. S., Pitts-Meek, S., Armanini, M. P., Ling, L. H., McMahon, S. B., Shelton, D. L., Levinson, A. D., and Philips, H. (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* **76**, 10001-10011.

Cui, C., Stambrook, P. J., and Parysek, L. M. (1995) Peripherin assembles into homopolymers in SW13 cells. *J. Cell Sci.* **108**, 3279-3284.

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

Curtis, R., Tonra, J. R., Stark, J. L., Adryan, K. M., Park, J. S., Cliffer, K. D., Lindsay, R. M., and DiStefano, P. S. (1998) Neuronal injury increases retrograde axonal transport of the neurotrophins to spinal sensory neurons and motor neurons via multiple receptor mechanisms. *Mol. Cell. Neurosci.* **12**, 105-118.

Danielsen, N., Pettman, B., Vahlsing, H. L., Manthorpe, M., and Varon, S. (1988) Fibroblast growth factor effects on peripheral nerve regeneration in a silicone chamber model. *J. Neurosci. Res.* **20**, 320-330.

Davies, A. M., Horton, A., Burton, L. E., Schmelzer, C., Vandlen, R., and Rosenthal, A. (1993) Neurotrophin-4/5 is a mammalian-specific survival factor for distinct populations of sensory neurons. *J. Neurosci.* **13**, 4961-4967.

Davies, S., Aldrich, T. H., Valenzuela, D. M., Wong, V. V., Furth, M. E., Squinto, S. P., and Yancopoulos, G. D. (1991) The receptor for ciliary neurotrophic factor. *Science* **253**, 59-63.

Davis, J. B., and Stroobant, P. (1990) Platelet - derived growth factors and fibroblast growth factors are mitogens for rat schwann cells. *J. Cell Biol.* **110**, 1353-1360.

De Miguel, M. P., De Boer-Brouwer, M., Paniagua, R., van den Hurk, R., De Rooij, D. G., and Van Dissel-Emiliani, F. M. (1996) Leukemia inhibitory factor and ciliary neurotrophic factor promote the survival of Sertoli cells and gonocytes in coculture system. *Endocrinology* **137**, 1885-1993.

DeChiara, T. M., Vejsada, R., Poueymirou, W. T., Acheson, A., Suri, C., Conover, J. C., Friedman, B., McClain, J., Pan, L., Stahl, N., et al (1995) Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound motor neuron deficits at birth. *Cell* **83**, 313-322.

Delcroix, J. D., Tomlinson, D. R., and Fernyhough, P. (1997) Diabetes and axotomy-induced deficits in retrograde axonal transport of nerve growth factor correlate with decreased levels of p75^{LNTR} protein in lumbar dorsal root ganglia. *Brain Res. Mol. Brain Res.* **51**, 82-90.

Derby, A., Engleman, V. W., Frierdich, G. E., Neises, G., Rapp, S. P., and Roufa, G. (1993) Nerve growth factor facilitates regeneration across nerve gaps: morphological and behavioral studies in rat sciatic nerve. *Exp. Neurol.* **119**, 176-191.

Devor, M., Govrin-Lippmann, R., Frank, I., and Raber, P. (1985) Proliferation of primary sensory neurons in adult rat dorsal root ganglion and the kinetics of retrograde cell loss after sciatic nerve section. *Somatosens. Res.* **3**, 139-167.

Dodd, J., Jahr, C. E., Hamilton, P. N., Heath, M. J. D., Matthew, W. D., and Jessell, T. M. (1983) Cytochemical and physiological properties of sensory and dorsal horn neurons that transmit cutaneous sensation. *Cold Spring Harbor Symp. Quant. Biol.* **48**, 685-695.

Eckenstein, F. P., Shipley, G. D., and Nishi, R. (1991) Acidic and basic fibroblast growth factors in the nervous system: distribution and differential alteration of levels after injury of central versus peripheral nerve. *J. Neurosci.* **11**, 412-419.

Edström, A., Ekström, P. A. R., and Tonge, D. (1996) Axonal outgrowth and neuronal apoptosis in cultured adult mouse dorsal root ganglion preparations: Effects of neurotrophins, of inhibition of neurotrophin actions and of prior axotomy. *Neuroscience* **75**, 1165-1174.

Eichler, M. E., Dubinsky, J. M., Tong, J., and Rich, K. M. (1994) The ability of diphenylpiperazines to prevent neuronal death in dorsal root ganglion neurons in vitro after nerve growth factor deprivation and in vivo after axotomy. *J. Neurochem.* **62**, 2148-2157.

Ekström, P. A. (1995) Neurons and glial cells of the mouse sciatic nerve undergo apoptosis after injury in vivo and in vitro. *NeuroRep.* **6**, 1029-1032.

Elde, R., Chao, Y., Cintra, A., Brelje, T. C., Pelto-Huikko, M., Juntilla, T., Fuxe, K., Pettersson, R. F., and Hökfelt, T. (1991) Prominent expression of acidic fibroblast growth factor in motor and sensory neurons. *Neuron* **7**, 349-364.

Elder, G. A., Friedrich, Jr. V., Bosco, P., Kang, C., Gourov, A., Tu, P-H., Zhang, B., Lee, V. M. -Y., and Lazzarini, R. A. (1998a) Absence of the mid-sized neurofilament subunit decreases axonal calibers, levels of light neurofilament (NF-L) and neurofilament content. *J. Cell Biol.* **141**, 727-739.

Elder, G. A., Friedrich, Jr. V., Kang, C., Bosco, P., Gourov, A., Tu, P-H., Zhang, B., Lee, V. M. -Y., and Lazzarini, R. A. 1998b Requirement of heavy neurofilament subunit in the development of axons with large calibers. *J. Cell Biol.* **143**, 195-205.

Ernfors, P., and Persson, H. (1991) Developmentally regulated expression of BDNF/NT-3 mRNA in rat spinal cord motor neurons and expression of BDNF mRNA in embryonic dorsal root ganglion. *Eur. J. Neurosci.* **3**, 953-961.

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

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

Ernfors, P., Lee, K. F., and Jaenisch, R (1994a) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* **368** 147-150.

Ernfors, P., Lee, K. F., Kucera, J., and Jaenisch, R. (1994b) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* **77**, 503-512.

Ernfors, P., Merlio, J. -P., and Persson, H. (1992) Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. *Eur. J. Neurosci.* **4**, 1140-1158.

Ernfors, P., Rosario, C. M., Merlio, J. P., Grant, G., Aldskogius, H., and Persson, H. (1993) Expression of mRNAs for neurotrophin receptors in the dorsal root ganglion and spinal cord during development and following peripheral or central axotomy. *Brain Res. Mol. Brain Res.* **17**, 217-226.

Ernfors, P., Wetmore, C., Olson, L., and Persson, H. (1990b) Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* **4**, 511-526.

Escary, J. -L., Perreau, J., Dumenil, D., Ezine, S. and Brulet, P. (1993) Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. *Nature* **363**, 361-364.

Escurat, M., Djabali, K., Gumpel, M., Gros, F., and Portier, M. M. (1990) Differential expression of two neuronal intermediate filament proteins, peripherin and the low-molecular-mass neurofilament protein (NF-L), during the development of the rat. *J. Neurosci.* **10**, 764-784.

Eyer, J., and Peterson, A. (1994) Neurofilament deficient axons and perikaryal aggregates in viable transgenic mice expressing a neurofilament-beta-galactosidase fusion protein. *Neuron* **12**, 389-405.

Fariñas, I., Jones, K. R., Backus, C., Wang, X. Y., and Reichardt, L. F. (1994) Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. *Nature* **369**, 658-661.

Fawcett, J. P., Bamji, S. X., Causing, C. G., Aloyz, R., Ase, A. R., Reader, T. A., McLean, J. H., and Miller, F. D. (1998) Functional evidence that BDNF is an anterograde neuronal trophic factor in the CNS. *J. Neurosci.* **18**, 2808-2821.

Ferhat, L., Represa, A., Zouaoui-Aggoun, D., Ferhat, W., Ben-Ari, Y., Khrestchatisky, M. (1997) FGF-2 induces nerve growth factor expression in cultured rat hippocampal neurons. *Eur. J. Neurosci.* **9**, 1282-1289.

Flienger, K. H., Kaplan, M. P., Wood, T. L., Pintar, J. E., and Liem, R. K. (1994) Expression of the gene for the neuronal intermediate filament protein alpha-internexin coincides with the onset of neuronal differentiation in the developing rat nervous system. *J. Comp. Neurol.* **342**, 161-173.

Freidin, M., Bennett, M. V. L., and Kessler, J.A. (1992) Cultured sympathetic neurons synthesize and release the cytokine interleukin 1 β . *Proc. Natl. Acad. Sci. USA* **89**, 10440-10443.

Friede, R. L., and Samorajski, T. (1970) Axon caliber related to neurofilaments and microtubules in sciatic nerve fibers of rats and mice. *Anat. Rec.* **167**, 379-387.

Fu, S. Y., and Gordon, T. (1997) The cellular and molecular basis of peripheral nerve regeneration. *Mol. Neurobiol.* **14**, 67-116.

Fukada, K. (1985) Purification and partial characterization of a cholinergic differentiation factor. *Proc. Natl. Acad. Sci. USA* **82**, 8795-8799.

Funakoshi, H., Frisen, J., Barbany, G., Timmusk, T., Zachrisson, O., Verge, V. M. K., and Persson, H. (1993) Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *J. Cell Biol.* **123**, 455-465.

Gasser, H. S., and Grundfest, H. (1939) Axon diameters in relation to the spike dimensions and the conduction velocity in mammalian A fibers. *Am. J. Physiol.* **127**, 393-404.

Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kilso, A., and Metcalf, D. (1987) Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). *EMBO J.* **6**, 3995-4002.

Geisler, N. and Weber, K. (1981) Self-assembly in vitro of the 68,000 molecular weight component of the mammalian neurofilament triplet proteins into intermediate sized filaments. *J. Mol. Biol.* **151**, 565-571.

Gnahn, H., Hefti, F., Heumann, R., Schwab, M. E., and Thoenen, H. (1983) NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: evidence for a physiological role of NGF in the brain? *Brain Res.* **285**, 45-52.

Gold, B. G., and Dark, C. (1988a) Somatofugal axonal atrophy produced by colchicine application to the sciatic nerve. *Ann. Neurol.* **24**, 481.

Gold, B. G., and Dark, C. (1988b) Evidence that the loss of retrogradely transported muscle-derived factor initiates the axon reaction. *Soc. Neurosci. Abstr.* **14**, 255.

Gold, B. G., Moblye, W. C., and Matheson, S. F. (1991) Regulation of axonal caliber, neurofilament content and nuclear localization in mature sensory neurons by nerve growth factor. *J. Neurosci.* **11**, 943-955.

Gorham, J. D., Baker, H., Kegler, D., Ziff, E. B. (1990) The expression of the neuronal intermediate filament protein peripherin in the rat embryo. *Dev. Brain Res.* **57**, 235-248.

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

Gospodarowicz, D. and Cheng, J. (1986) Heparin protects basic and acidic FGF from inactivation. *J. Cell. Physiol.* **128**, 475-484.

Gospodarowicz, D. Neufeld, G., and Schweigerer, L. (1987) Fibroblast growth factor: structural and biological properties *J. Cell. Physiol. (Suppl.)* **5**, 15-26.

Gotz, R., Koster, R., Winkler, C., Raulf, F., Lottspeich, F., Scharl, M., and Thoenen H. (1994) Neurotrophin-6 is a new member of the nerve growth factor family. *Nature* **372**, 266-269.

Gravel, C., Götz, R., Lorrain, A., and Sendtner, M. (1997) Adenoviral gene transfer of ciliary neurotrophic factor and brain-derived neurotrophic factor leads to long-term survival of axotomized motor neurons. *Nat. Med.* **3**, 765-770.

Green, L. M., Reade, J. L., and Ware, C. F. (1984) Rapid colorimetric assay

for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J. Immunol. Methods* **70**, 257-268.

Greenberg, S., and Lasek, R. J. (1988) Neurofilament protein synthesis in DRG neurons decreases more after peripheral axotomy than after central axotomy. *J. Neurosci.* **8**, 1739-1746.

Grothe, C., Meisinger, C., Hertenstein, A., Kurz, H., Wewetzer, K. (1997) Expression of fibroblast growth factor-2 and fibroblast growth factor receptor 1 messenger RNAs in spinal ganglia and sciatic nerve: regulation after peripheral nerve lesion. *Neurosci.* **76**, 123-135.

Groves, M. J., Christopherson, T., Giometto, B., and Scaravilli, F. (1997) Axotomy induced apoptosis in adult rat primary sensory neurons. *J. Neurocytol.* **26**, 615-624.

Groves, M. J., Ng, Y.-W., Ciardi, A.b and Scaravilli, F. (1996) Sciatic nerve injury in the adult rat: comparison of effect on oligosaccharide, CGRP and GAP43 immunoreactivity in primary afferents following two types of trauma. *J. Neurocytol.* **26**, 219-231.

Halböök, F., Ibáñez, C. F., and Persson, H. (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in *Xenopus* ovary. *Neuron* **6**, 845-858.

Hammarberg, H., Piehl, F., Cullheim, S., Fjell, J., Hokfelt, T., and Fried, K. (1996) GDNF mRNA in Schwann cells and DRG satellite cells after chronic sciatic nerve injury. *NeuroRep.* **7**, 857-860.

Hefti, F., Dravid, A., and Hartikka, J. (1984) Chronic intraventricular injections of nerve growth factor elevate hippocampal choline acetyl-transferase activity in adult rats with partial septo-hippocampal lesions. *Brain Res.* **293**, 305-311.

Heins, S., Wong, P. C., Muller, S., Goldie, K., Cleveland, D. W., and Aebi, U. (1993) The rod domain of NF-L determines neurofilament architecture, whereas the end domains specify filament assembly and network formation. *J. Cell Biol.* **123**, 1517-1533.

Hendry, I. A. (1975) The response of adrenergic neurons to axotomy and nerve growth factor. *Brain Res.* **94**, 87-97.

Hendry, I. A., and Campbell, J. (1976) Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. *J. Neurocytol.* **5**, 531-560.

Hendry, I. A., Murphy, M., Hilton, D. J., Nicola, N. A., and Bartlett, P. F. (1992) Binding and retrograde transport of leukemia inhibitory factor by the sen-

sory nervous system. *J. Neurosci.* **12**, 3427-3434.

Henry, J. L. (1976) Effects of substance P on functionally identified units in cat spinal cord. *Brain Res.* **114**, 439-451.

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

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

Heymann, D., Godard, A., Raher, S., Bentouimou, N., Blanchard, F., Cherel, M., Hallet, M-M., Jaques, Y. (1996) Leukaemia inhibitory factor (LIF) and oncostatin M (OSM) high affinity binding require additional receptor subunits besides GP130 and GP190. *Cytokine* **8**, 197-205.

Hilton, D. J., and Gough, N. M. (1991) Leukemia inhibitory factor: a biological perspective. *J. Cell Biochem.* **46**, 21-26.

Hilton, D. J., Nicola, N. A., and Metcalf, D. (1991) Distribution and comparison of receptors for leukemia inhibitory factor on murine hemopoietic and hepatic cells. *J. Cell. Physiol.* **146**, 207-215.

Himes, B.T., and Tessler, A. (1989) Death of some dorsal root ganglion neurons and plasticity of others following sciatic nerve section in adult and neonatal rats. *J. Comp. Neurol.* **284**, 215-230.

Hirokawa, N., Clicksman, M. A., and Willard, M. B. (1984) Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton. *J. Cell. Biol.* **98**, 1523-1536.

Hisanaga, S., and Hirokawa, N. (1988) Structure of the peripheral domains of neurofilaments revealed by low angle rotary shadowing. *J. Mol. Biol.* **202**, 297-305.

Hisanaga, S., and Hirokawa, N. (1990) Molecular architecture of the neurofilament. II. Reassembly process of neurofilament L protein in vitro. *J. Mol. Biol.* **211**, 871-882.

Hofer, M., Pagliusi, S. R., Hohn, A., Leibrock, J., and Barde, Y. -A. (1990) Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO J.* **9**, 2459-2464.

Hoffman, P. N. (1989) Expression of GAP-43, a rapidly transported growth-

associated protein, and class II beta tubulin, a slowly transported cytoskeletal protein, are coordinated in regenerating neurons. *J. Neurosci.* **9**, 893-897.

Hoffman, P. N., and Cleveland, D. W. (1988) Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: induction of a specific beta-tubulin isotype. *Proc. Natl. Acad. Sci. USA* **85**, 4530-4533.

Hoffman, P. N., and Lasek, R. J. (1980) Axonal transport of the cytoskeleton in regenerating motor neurons: constancy and change. *Brain Res.* **202**, 317-333.

Hoffman, P. N., and Lasek, R. J. (1975) The slow component of axonal transport: identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J. Cell. Biol.* **66**, 351-366.

Hoffman, P. N., Cleveland, D. W., Griffen, J. W., Landes, P. W., Cowan, N. J., and Price, D. L. (1987) Neurofilament gene expression : a major determinant of axon caliber. *Proc. Natl. Acad. Sci. USA* **84**, 3472-3476.

Hoffman, P. N., Griffin, J. W., and Price, D. L. (1984) Control of axonal caliber by neurofilament transport. *J. Cell Biol.* **99**, 705-714.

Hoffman, P. N., Lasek, R. J., Griffin, J. W., and Price, D. L. (1983) Slowing of the axonal transport of neurofilament proteins during development. *J. Neurosci.* **3**, 1694-1700.

Hoffman, P. N., Thompson, G.W., Griffin, J. W., and Price, D. L. (1985) Changes in neurofilament transport coincide temporally with alterations in the caliber of axons in regenerating motor fibers. *J. Cell Biol.* **101**, 1332-1340.

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

Hököfelt, T. Hallin, Z. W., Villar, M., and Melander, T. (1987) Increase of galanin like immunoreactivity in rat dorsal root ganglion cells after peripheral axotomy. *Neurosci. Lett.* **83**, 217-220.

Hököfelt, T., Zhang, X., and Wiesenfeld-Hallin, Z (1994) Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends Neurosci.* **17**, 22-30.

Holmes, F. E., McMahon, S. B., Murphy, D., and Wynick, D. (1997) Targeted disruption of galanin reduces nerve regeneration and neuropathic pain. *Soc. Neurosci. Abstr.* **23**, 1954.

Horie, H., and Akahori, Y. (1994) Three-dimensional cell aggregation en-

hances growth-promoting activity of NGF in adult DRG. *Neurorep.* **6**, 37-40.

Hory-Lee, F., Russell, M., Lindsay, R. M., and Frank, E. (1993) Neurotrophin-3 supports the survival of developing muscle sensory neurons in culture. *Proc. Natl. Acad. Sci. USA* **90**, 2613-2617.

Ibáñez, C. F., Ernfors, P., Timmusk, T., Ip, N. Y., Arenas, E., Yancopoulos, G. D., and Persson, H. (1993) Neurotrophin-4 is a target-derived neurotrophic factor for neurons of the trigeminal ganglion. *Development* **117**, 1343-1353.

Ip, N. Y., Ibáñez, C. F., Nye, S. H., McClain, J., Jones, P. F., Gies, D. R., Belluscio, L., LeBeau, M. M., Espinosa, R., Squinto, S. P., Persason, H., and Yancopoulos, G. (1992) Mammalian neurotrophin 4: structure, chromosomal location, tissue distribution, and receptor specificity. *Proc. Natl. Acad. Sci. USA* **89**, 3060-3064.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N., and Yancopoulos, G. D. (1992) CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* **69**, 1121-1132.

Iwasaki, Y., Shiojima, T., Ikeda, K., Tagaya, N., Kobayashi, T., and Kinoshita, M. (1995) Acidic and basic fibroblast growth factors enhance neurite outgrowth in cultured rat spinal cord neurons. *Neurol. Res.* **17**, 70-72.

Jacobs, A. J., Swain, G. P., Snedeker, J. A., Pijak, D. S., Gladstone, L. J., and Selzer, M. E. (1997) Recovery of neurofilament expression selectively in regenerating reticulospinal neurons. *J. Neurosci.* **17**, 5206-5220.

Jaye, M., Howk, R., Burgess, W., Ricca, G. A., Chiu, I-M., Ravera, M. W., O'Brien, S. J., Modi, W. S., Maciag, T., and Drohan, W. N. (1986) Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosome localization. *Science* **233**, 541-545.

Jessell, T. M., Tsunoo, A., Kanazawa, I., and Otsuka, M. (1979) Substance P: depletion in the dorsal horn of rat spinal cord after section of the peripheral processes of primary sensory neurons. *Brain Res.* **168**, 247-259.

Ji, R-R., Zhang, Q., Zhang, X., Piehl, F., Reilly, T., Pettersson, R. F., Hökfelt, T. (1995) Prominent expression of bFGF in dorsal root ganglia after axotomy. *Eur. J. Neurosci.* **7**, 2458-2468.

Johnson, E. M., Taniuchi, M., Clark, H. B., Springer, J. E., Koh, S., Tayrien, M. W., and Loy, R. (1987) Demonstration of the retrograde transport of nerve growth factor (NGF) receptor in the peripheral and central nervous system. *J. Neurosci.* **7**, 923-929.

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

Johnson, Jr., and Yip, H. K. (1985) Central nervous system and peripheral nerve growth factor provide trophic support critical to mature sensory neuronal survival. *Nature* **314**, 751-752.

Johnson, Jr., and Yip, H. K. (1986) The role of NGF in sensory neurons in vivo. *Trends Neurosci.* **9**, 33-37.

Johnson, Jr., E. M., Gorin, P. D., Brandeis, L. D., and Pearson, J. (1980) Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. *Science* **210**, 916-918.

Johnson, Jr., Osborne, P. A., Rydel, R. E., Schmidt, R. E., and Pearson, J. (1983) Characterization of the effects of autoimmune nerve growth factor deprivation in the developing guinea pig. *Neurosci.* **1983**, 631-642.

Jonakait, G. M. (1993) Neuronal-immune interactions in sympathetic ganglia. *Trends Neurosci.* **16**, 419-423.

Julien, J. P., Cote, F., Beaudet, L., Sidky, M., Flavell, D., Grosveld F., and Mushynski, W. (1988) Sequence and structure of the mouse gene coding for the largest neurofilament subunit. *Gene* **68**, 307-314.

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

Karey, K. P., Marquardt, H., and Sirbasku, D. A. (1989) Human platelet-derived mitogens. I. Identification of insulinlike growth factors I and II by purification and N alpha amino acid sequence analysis. *Blood* **74**, 1084-1092.

Kashiba, H., Senba, E., Kawai, Y., Ueda, Y., and Tohyama, M. (1992) Axonal blockade induces expression of vasoactive intestinal polypeptide and galanin in rat dorsal root ganglion neurons. *Brain Res.* **577**, 19-28.

Kato, A. C., and Lindsay, R. M. (1994) Overlapping and additive effects of neurotrophins and CNTF on cultured human spinal cord neurons. *Exp. Neurol.* **130**, 196-201.

Kawamata, T., Speliotes, E. K., Finklestein, S. P. (1997) The role of polypeptide growth factors in recovery from stroke. *Adv. Neurol.* **73**, 377-382.

Keen, P., Nielsch, U., and Watts, P. A. (1989) An investigation into the mechanism of induction of vasoactive intestinal peptide gene expression in rat sensory neurons following nerve injury. *J. Physiol.* **410**, 62.

Kennedy, S. H., Rouda, S., Qin, H., Aho, S., Selber, J., Tan, E. M. (1997)

Basic FGF regulates interstitial collagenase gene expression in human smooth muscle cells. *J. Cell Biochem.* **65**, 32-41.

Kessler, J. A., and Black, I. B. (1980) Nerve growth factor stimulates the development of substance P in sensory ganglia. *Proc. Natl. Acad. Sci. USA* **77**, 649-652.

Kiefer M. C., Stephans, J. C., Crawford, K., Okino, K., Barr, P. J. (1990) Ligand affinity cloning and structure of a cell surface heparan sulfate proteoglycan that binds basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* **87**, 6985-6989.

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

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

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

Klein, R., Silos-Santiago, I., Sneyne, R. J., Lira, S. A., Brambilla, R., Bryant, S., Mang, L., Snider, W. D., and Barbacid, M. (1994) Disruption of the neurotrophin-3 receptor gene TrkC eliminates Ia muscle afferents and results in abnormal movement. *Nature* **368**, 249-251.

Klein, R., Smeyne, R. J., Wurst, W., Long, L. K., Auerbach, B. A., Joyner, A. L., and Barbacid, M. (1993) Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. *Cell* **75**, 113-122.

Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K., and Martin, G. R. (1982) Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry* **21**, 6188.

Koeberle, P. D., and Bal, A. K. (1998) Effects of GDNF on retinal ganglion cell survival following axotomy. *Vision Res.* **38**, 1505-1515.

Koliatsos, V. E., Cayouette, M. H., Berkemeier, L. R., Clatterbuck, R. E., Price, D. L., and Rosenthal, A. (1994) Neurotrophin 4/5 is a trophic factor for mammalian facial motor neurons. *Proc. Natl. Acad. Sci. USA* **91**, 3304-3308.

Korsching, S., and Theonen, H. (1983) Quantitative demonstration of retrograde transport of endogenous nerve growth factor. *Neurosci. Lett.* **39**, 1-4.

Kotzbauer, P.T., Lampe, P.A., Heuckeroth, R. O., Golden, J. P., Creedon, D.

J., Johnson, E. M. Jr., and Milbrandt, J. (1996) Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature* **384**, 467-470.

Kriegstein, K., and Unsicker, K. (1996) Distinct modulatory actions of TGF-beta and LIF on neurotrophin-mediated survival of developing sensory neurons. *Neurochem. Res.* **21**, 843-850.

Kushima, Y., and Hatanaka, H. (1992) Interleukin-6 and leukemia inhibitory factor promote the survival of acetylcholinesterase-positive neurons in culture from embryonic rat spinal cord. *Neurosci. Lett.* **143**, 110-114.

Lai, K. O., Fu, W. Y., Ip, F. C., and Ip, N. Y. (1998) Cloning and expression of a novel neurotrophin, NT-7, from carp. *Mol. Cell. Neurosci.* **11**, 64-76.

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

Lankford, K. L., Waxman, S. G., and Kocsis, J. D. (1998) Mechanism of enhancement of neurite regeneration in vitro following a conditioning sciatic nerve lesion. *J. Comp. Neurol.* **391**, 11-29.

Laquerriere A., Peulve, P., Jin, O., Tiollier, J., Tardy, M., Vaudry, H., Hemet, J., and Tadie, M. (1994) Effect of basic fibroblast growth factor and alpha-melanocytic stimulating hormone on nerve regeneration through a collagen channel. *Microsurg.* **15**, 203-210.

Lasek, R. J. (1986) Polymer sliding in axons. *J. Cell Sci. Suppl.* **5**, 161-179.

Lasek, R. J., Paggi, P., and Katz, M. J. (1993) The maximum rate of neurofilament transport in axons: a view of molecular transport mechanisms continuously engaged. *Brain Res.* **616**, 58-64.

Lavranos, T. C., Rathjen, P. D., Seemark, R. F. (1995) Trophic effects of myeloid leukaemia inhibitory factor (LIF) on mouse embryos. *J. Reprod. Fertil.* **105**, 331-338.

Lawson, S. N., and Harper, A. A. (1985) Cell types in rat dorsal root ganglia: morphological, immunocytochemical, and electrophysiological analyses. In Willis, W. D., and Rowe, L. (eds.): *Somatosensory Pathways*. New York: Alan R. Liss, Inc. pp. 97-103.

Leclerc, P., Ekström, P., Edström, A., Priestley, J., Averill, S., and Tonge, D. A. (1997) Effects of glial cell line-derived neurotrophic factor on axonal growth and apoptosis in adult mammalian sensory neurons in vitro. *Neurosci.* **82**, 545-558.

Lee, M. K., and Cleveland, D. W. (1996) Neuronal intermediate filaments. *Annu. Rev. Neurosci.* **19**, 187-217.

Lee, M. K., Xu, Z., Wong, P.C and Cleveland, D. W. (1993) Neurofilaments are obligate heteropolymers in vivo. *J. Cell. Biol.* **122**, 1337-1350.

Lefcort, F., Clary, D. O., Rusoff, A. C., and Reichardt, L. F. (1996) Inhibition of the NT-3 receptor TrkC, early in chick embryogenesis, results in severe reductions in multiple neuronal subpopulations in the dorsal root ganglia. *J. Neurosci.* **16**, 3704-3713.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengeler B., Masiakowski, P., Thoenen, H., and Barde, Y. A. (1989) Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**, 149-152.

Lekan, H. A., Chung, K., Yoon, Y. W., Chung, J.M., and Coggeshall, R. E. (1997) Loss of dorsal root ganglion cells concomitant with dorsal root axon sprouting following segmental nerve lesions. *Neurosci.* **81**, 527-534.

Levi-Montalcini, R. (1951) Selective growth-stimulating effects of mouse sarcomas on the sensory and sympathetic nervous system of chick embryos. *J. Exp. Zool.* **116**, 321-362.

Levi-Montalcini, R. and Angeletti, P. V. (1968) Nerve growth factor. *Physiological Review* **48**, 534-569.

Levy, E., Liem, R. K., D'Eustachio, P., and Cowan, N. J. (1987) Structure and evolutionary origin of the gene encoding mouse NF-M, the middle - molecular - mass neurofilament protein. *Eur. J. Biochem.* **166**, 71-77.

Lewis, S. A., Cowan, N. J. (1986) Anomalous placement of introns in a member of the intermediate filament multigene family: an evolutionary conundrum. *Mol. Cell. Biol.* **6**, 1529-1534.

Li, L., Oppenheim, R. W., Lei, M., and Houenou, L. J. (1994) Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. *J. Neurobiol.* **25**, 759-766.

Li, M., Sentner, M., and Smith, A. (1995) Essential function of LIF receptor in motor neurons. *Nature* **378**, 724-727.

Lieberman, A. R. (1971) The axon reaction. A review of the principal features of perikaryal responses to axon injury. *Int. Rev. Neurobiol.* **14**, 49-124.

Lin, H-Y., Xu, J., Ornitz, D. M., Halegoua, S., and Hayman, M. J. (1996) The fibroblast growth factor receptor-1 is necessary for the induction of neurite outgrowth in PC12 cells by aFGF. *J. Neurosci.* **16**, 4579-4587.

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S., and Collins, F. (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130-1132.

Lin, L. F., Mismar, D., Lile, J. D., Armes, L. G., Butler, E. B., Vannice, J. L., and Collins, F. (1989) Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). *Science* **246**, 1023-1025.

Lin, W., and Szaro, B. G. (1995) Neurofilaments help maintain normal morphologies and support elongation of neurites in xenopus laevis cultured embryonic spinal cord neurons. *J. Neurosci.* **15**, 8331-8344.

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

Lindsay, R. M. (1996) Role of neurotrophins and trk receptors in the development and maintenance of sensory neurons: an overview. *Phil. Trans. R. Soc. Lond.* **351**, 365-373.

Lindsay, R. M. (1988a) The role of neurotrophic factors in development, maintenance and regeneration of sensory neurons. In *The Making of the Nervous System*. J. Parnavelas, C. D. Stern, and R. V. Stirling, eds., pp. 148-165, Oxford U.P. 148-165.

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

Lindsay, R. M., and Harmar A.J. (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons *Nature* **337**, 362-364.

Lindsay, R. M., Lockett C., Sternberg, J., and Winter, J. (1989) Neuropeptide expression in cultures of adult sensory neurons: modulation of substance P and calcitonin gene related peptide levels by nerve growth factor. *Neurosci.* **8**, 53-65. .

Lipton, S. A., Wagner, J. A., Madison, R. D., and D'amore, P. A. (1988) Acidic fibroblast growth factor enhances regeneration of processes by postnatal mammalian retinal ganglion cells in culture. *Proc. Natl. Acad. Sci. USA* **85**, 2388-2392.

Liu, X., Ernfors, P., Wu, H., and Jaenisch, R. (1995) Sensory but not motor neuron deficits in mice lacking NT4 and BDNF. *Nature* **375**, 238-241.

Lo, A. C., Li, L., Oppenheim, R. W., Prevet, D., and Houenou, L. J. (1995) Ciliary neurotrophic factor promotes the survival of spinal sensory neurons following axotomy but not during the period of programmed cell death. *Exp. Neurol.* **134**, 49-55.

Longo, F. M., Hayman, E. G., Davis, G. E., Ruoslahti, E., Engvall, E., Manthorpe, M., and Varon, S. (1984) Neurite-promoting factors and extracellular

matrix components accumulating in vivo within nerve regeneration chambers. *Brain Res.* **309**, 105-117.

Ma, Y. T., Hsieh, T., Forbes, M. E., Johnson, J. E., and Frost, D. O. (1988) BDNF injected into the superior colliculus reduces developmental retinal ganglion cell death. *J. Neurosci.* **18**, 2097-2107.

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

Malgrange, B., Delree, P., Rigo, J. M., Baron, H., and Moonen, G. (1994) Image analysis of neuritic regeneration by adult rat dorsal root ganglion neurons in culture: quantitation of the neurotoxicity of anticancer agents and of its prevention by nerve growth factor or basic fibroblast growth factor but not by brain-derived ne J. *Neurosci. Methods* **53**, 111-122.

Manfridi, A., Forloni, G. L., Arrigoni-Martelli, E., and Mancina, M. (1992) Culture of dorsal root ganglion neurons from aged rats: effects of acetyl-L-carnitine and NGF. *Int. J. Dev. Neurosci.* **10**, 321-329.

Marszalek, J. R., Williamson, T. L., Lee, M. K., Xu, Z., Hoffman, P. N., Becher, M. W., Crawford, T. O., and Cleveland, D. W. (1996) Neurofilament subunit NF-H modulates axonal diameter by selectively slowing neurofilament transport. *J. Cell Biol.* **135**, 711-724.

Martini, R. (1994) Expression and functional roles of neural cell surface molecules and extracellular matrix components during development and regeneration of peripheral nerves. *J. Neurocytol.* **23**, 1-28.

Masu, Y., Wolf, E., Holtman, B., Sendtner, M., Brem, G. and Thoenen, H. (1993) Disruption of the CNTF gene results in motor neuron degeneration. *Nature* **365**, 27-32.

Matheson, C. R., Carnahan, J., Urich, J. L., Bocangel, D., Zhang, T. J., and Yan, Q. (1997) Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor for sensory neurons: comparison with the effects of the neurotrophins. *J. Neurobiol.* **32**, 22-32.

Mayer, M., Bhakoo, K., and Noble, M. (1994) Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes in vitro. *Development* **120**, 143-153.

McGregor, G.P., Gibson, S.J., Sabate, I. m., Blank, M.A., Chiristofides, N.D., Wall, P.D, Polak, J.M., and Bloom, S.R. (1984) Effect of peripheral nerve section and nerve crush on spinal cord neuropeptides in the rat; increased VIP and PHI in

the dorsal horn. *Neurosci.* **13**, 207-216.

McKerracher, L., Vidal, S. M., and Aguayo, A. J. (1990) Slow transport rates of cytoskeletal proteins change during regeneration of axotomized retinal neurons in adult rats. *J. Neurosci.* **10**, 641-648.

McMahon, S. B. (1996) NGF as a mediator of inflammatory pain. *Phil. Trans. R. Soc. Lond.* **351**, 431-440.

McMahon, S. B., Armanini, M. P., Ling, L. H., and Philips, H. S. (1994) Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. *Neuron* **12**, 1161-1171.

McNeil, P. L., Muthukrishnan, L., Warder, E., and D'Amore, P. A. (1989) Growth factors are released by mechanically wounded endothelial cells. *J. Cell Biol.* **109**, 811-822.

McQuarrie, I. G., Brad, S. T., and Lasek, R. J. (1986) Diversity in the axonal transport of structural proteins: major differences between optic and spinal axons in rat. *J. Neurosci.* **6**, 1593-1605.

Mehler, M. F., and Kessler, J. A. (1995) Cytokines and neuronal differentiation. *Critical Reviews in Neurobiology.* **9**, 419-426.

Meiri, K. F., Willard, M., and Johnson, M. I. (1988) Distribution and phosphorylation of the growth-associated protein GAP-43 in regenerating sympathetic neurons in culture. *J. Neurosci.* **8**, 2571-2581.

Melander, T., Hökfelt, T. and Rökaeus, Å. (1986) Distribution of galanin-like immunoreactivity in the rat central nervous system. *J. Comp. Neurol.* **248**, 475-517.

Memberg, S. P., and Hall, A. K. (1995) Proliferation, differentiation, and survival of rat sensory neuron precursors in vitro require specific trophic factors. *Mol. Cell. Neurosci.* **6**, 323-335.

Merlio, J.P., Ernfors, P., Jaber, M., and Persson, H. (1992) Molecular cloning of rat trkC and distribution of cells expressing messenger RNAs for members of the trk family in the rat central nervous system. *Neurosci.* **51**, 513-532.

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

Michael, G. J., Averill, S., Nitkunan, A., Rattray, M., Bennett, D. L., Yan, Q., and Priestley, J. V. (1997) Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells

and in their central terminations within the spinal cord. *J. Neurosci.* **17**, 8476-8490.

Milbrandt, J., de Sauvage, F. J., Fahrner, T. J., Baloh, R. H., Leitner, M. L., Tansey, M. G., Lampe, P. A., Heuckeroth, R. O., Kotzbauer, P. T., Simburger, K. S., Golden, J. P., Davies, J. A., Vejsada, R., Kato, A. C., Hynes, M., Sherman, D., Nishimura, (1998) Persephin, a novel neurotrophic factor related to GDNF and neurturin. *Neuron* **20**, 245-253.

Miletic, V., and Tan, H. (1988) Iontophoretic application of calcitonin gene-related peptide produces a slow and prolonged excitation of neurons in the cat lumbar dorsal horn. *Brain Res.* **446**, 169-172.

Miller FD, Tetzlaff W, Bisby MA, Fawcett JW, Milner RJ (1989) Rapid induction of the major embryonic alpha-tubulin mRNA, T alpha 1, during nerve regeneration in adult rats. *J. Neurosci.* **9**, 1452-1463.

Miyagi N., Kato, S., Terasaki, M., Shigemori, M., Morimatsu, M. (1998) Fibroblast growth factor-2 and -9 regulate proliferation and production of matrix metalloproteinases in human gliomas. *Int. J. Oncol.* **12**, 1085-1090.

Mobley, W. C., Rutkowski, J. L., Tennekoon, G. I., Buchanan, K., and Jonhston, M. V. (1985) Choline acetyltransferase activity in striatum of neonatal rats increased by nerve growth factor. *Science* **229**, 284-287.

Mohiuddin, L., Fernandez, K., Tomlinson, D. R., and Fernyhough, P. (1995) Nerve growth factor and neurotrophin-3 enhance neurite outgrowth and up-regulate the levels of messenger RNA for growth-associated protein GAP-43 and T α 1 α -tubulin in cultured adult rat sensory neurons. *Neurosci. Lett.* **185**, 20-23.

Mohiuddin, L., Fernyhough, P., Tomlinson, D. R. (1996) Acidic fibroblast growth factor enhances neurite outgrowth and stimulates expression of GAP-43 and T α 1 α -tubulin in cultured neurons from adult rat dorsal root ganglia. *Neurosci. Lett.* **215**, 111-114.

Molliver, D. C., and Snider, W. D. (1997) Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. *J. Comp. Neurol.* **381**, 428-438.

Molliver, D. C., Radeke, M. J., Feinstein, S. C., and Snider, W. D. (1995) Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. *J. Comp. Neurol.* **361**, 404-416.

Molliver, D. C., Wright, D. E., Leitner, M. L., Parsadanian, A. S., Doster, K., Wen, D., Yan, Q., and Snider, W. D. (1997) IB4-binding nociceptors switch from NGF to GDNF dependence in early postnatal life. *Neuron* **19**, 849-861.

Monteiro, M. J., Hoffman, P. N., Gearhart, J. D., and Cleveland, D. W. (1990) Expression of NF-L in both neuronal and nonneuronal cells of transgenic mice: increased neurofilament density in axons without affecting caliber. *J. Cell Biol.* **111**, 1543-1557.

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Philips, H. S., Reichardt, L. F., Ryan, A. M., Carver-Moore, K., and Rosenthal, A. (1996) Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76-79.

Morris, J. R., and Lasek, R. J. (1982) Stable polymers of the axonal cytoskeleton: the axoplasmic ghosts. *J. Cell Biol.* **92**, 192-198.

Mu, X., Silos - Santiago, I., Carroll, S., and Snider, W. (1993) Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. *J. Neurosci.* **13**, 4029-4041.

Mulderry, P. K. (1994) Neuropeptide expression by newborn and adult sensory neurons in culture effects of nerve growth factor and other neurotrophic factors. *Neurosci.* **59**, 673-688.

Murphy, M., Dutton, R., Koblar, S., Cheema, S., and Bartlett, P. (1997) Cytokines which signal through the LIF receptor and their actions in the nervous system. *Prog. Neurobiol.* **52**, 355-378.

Murphy, M., Reid, K., Brown, M.A., and Bartlett, P. F. (1993a) Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons. *Development* **117**, 1173-1182.

Murphy, M., Reid, K., Hilton, D. J., and Bartlett, P. F. (1991) Generation of sensory neurons is stimulated by leukemia inhibitory factor. *Proc. Natl. Acad. Sci. USA* **88**, 3498-3501.

Murphy, R. A., Acheson, A., Hodges, R., Haskins, J., Richards, C., Reklow, E., Chlumecky, V., Barker, P. A., Alderson, R. F., and Lindsay, R. M. (1993b) Immunological relationships of NGF, BDNF and NT-3: recognition and functional inhibition by antibodies to NGF. *J. Neurosci.* **13**, 2853-2862.

Neuberger, T. J., and De Vries, G. H. (1993) Distribution of fibroblast growth factor in cultured dorsal root ganglion neurons and Schwann cells: localization during maturation in vitro. *J. Neurocytol.* **22**, 436-448.

Nicosia, R. F., and Otinetti, A. (1990) Growth of microvessels in serum-free matrix cultures of rat aorta. *Lab. Invest.* **63**, 115-122.

Nilsson, A. S., Fainzilber, M., Falck, P., and Ibáñez, CF. (1998) Neurotrophin-7: a novel member of the neurotrophin family from the zebrafish. *FEBS Lett.* **424**, 285-290.

Nixon, R. A., and Logvinenko, K. B. (1986) Multiple fates of newly synthesized neurofilament proteins: evidence for a stationary neurofilament network distributed nonuniformly along axons of retinal ganglion cell neurons. *J. Cell Biol.* **102**, 647-659.

Noguchi, K., Senba, E., Morita, Y., Sato, M., and Tohyama, M. (1990) α -CGRP and β -CGRP mRNA's are differentially regulated in the rat spinal cord and dorsal root ganglion *Mol. Brain Res.* **7**, 299-304..

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

Oblinger, M. M., Szumlas, R. A., Wong, J., and Liuzzi, F. J. (1989) Changes in cytoskeletal gene expression affect the composition of regenerating axonal sprouts elaborated by dorsal root ganglion neurons in vivo. *J. Neurosci.* **9**, 2645-2653.

Oellig, C., Pirvola, U., Taylor, L., Elde, R., Hökfelt, T., and Pettersson, R. F. (1995) Acidic FGF and FGF receptors are specifically expressed in neurons of developing and adult rat dorsal root ganglia. *Eur. J. Neurosci.* **7**, 863-874.

Oka, Y., and Orth, N. (1983) Human plasma epidermal growth factor/beta-urogastrone is associated with blood platelets. *J. Clin. Invest.* **72**, 249-255.

Okabe, S., Miyasaka, H., and Hirokawa, N. (1993) Dynamics of the neuronal intermediate filaments. *J. Cell Biol.* **121**, 375-386.

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

Ornitz, D., and Leder, P. (1992) Ligand specificity and heparin dependence of fibroblast growth factor receptors 1 and 2. *J. Biol. Chem.* **267**, 16305-16311.

Otten, U., Goedert, M., Moyer, N., and Lembeck, F. (1980) Requirement of nerve growth factor for development of substance P containing sensory neurons. *Nature* **287**, 158-159.

Otto, D., Unsicker, K., and Grothe, C. (1987) Pharmacological effects of nerve growth factor and fibroblast growth factor applied to the transected sciatic nerve on neuron death in adult rat dorsal root ganglia. *Neurosci. Lett.* **83**, 156-160.

Papadopoulos, N. G., Dedoussis, G. V., Spanakos, G., Gritzapis, A. D., Baxevas, C. N., and Papamichail, M. (1994) An improved fluorescence assay for the determination of lymphocyte-mediated cytotoxicity using flow cytometry. *J. Immunol. Methods* **177**, 101-111.

Park, C. M., and Hollenberg, M. J. (1993) Growth factor induced retinal

regeneration in vivo. *Int. Rev. Cytol.* **146**, 49-74.

Patterson, P. H. (1992) The emerging neurotrophic cytokine family: first CDF/LIF, CNTF, and IL-6: next ONC, MGF, GCSF? *Curr. Opin. Neurobiol.* **2**, 94-97.

Peinado-Ramón, P., Salvador, M., Villegas-Pérez, M. P., and Vidal-Sanz, M. (1996) Effects of axotomy and intraocular administration of NT-4, NT-3, and brain-derived neurotrophic factor on the survival of adult rat retinal ganglion cells. A quantitative in vivo study. *Invest. Ophthalmol. Vis. Sci.* **37**, 489-500.

Pennica, D., King, K. L., Shaw, K. J., Luis, E., Rullamas, J., Luoh, S. M., Darbonne, W. C., Knutzon, D. S., Yen, R., Chien, K. R., et al. (1995) Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. *Proc. Natl. Acad. Sci. USA* **92**, 1142-1146.

Perry, V. H., Brown, M. C., and Gordon, S. (1987) The macrophage response to central and peripheral nerve injury. A possible role of macrophages in regeneration. *J. Exp. Med.* **165**, 1218-1223.

Persson, J. K., Lindh, B., Elde, R., Robertson, B., Rivero-Melián, C., Eriksson, N. P., Hökfelt, T., and Aldskogius, H. (1996) The expression of different cytochemical markers in normal and axotomised dorsal root ganglion cells projecting to the nucleus gracilis in the adult rat. *Exp. Brain Res.* **105**, 331-344.

Pesce, M., Farrace, M. G., Piacentini, M., Dolci, S., and De Felici, M. (1993) Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). *Development* **118**, 1089-1094.

Peulve, P., Laquerriere, A., Hemet, J., and Tadie, M. (1994a) Comparative effect of alpha-MSH and bFGF on neurite extension of fetal rat spinal cord neurons in culture. *Brain Res.* **654**, 319-323.

Peulve, P., Laquerriere, A., Paresy, M., Hemet, J., and Tadie, M. (1994b) Establishment of adult rat schwann cell cultures: effect of b-FGF, α -MSH, NGF, PDGF and TGF- β on cell cycle. *Exp. Cell Res.* **214**, 543-550.

Pichel, J.G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J., Sariola, H., and Westphal, H. (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73-76.

Pickering, J. G., Ford, C. M., Tang, B., and Chow, L. H. (1997) Coordinated effects of fibroblast growth factor-2 on expression of fibrillar collagens, matrix metalloproteinases, and tissue inhibitors of matrix metalloproteinases by human

vascular smooth muscle cells. Evidence for repressed collagen production and act
Arterioscler. Thromb. Vasc. Biol. **17**, 475-482.

Pincus, D. W., DiCicco-Bloom, E. M., and Black, I. B. (1990) Vasoactive intestinal peptide regulates mitosis, differentiation and survival of cultured sympathetic neuroblasts. *Nature* **343**, 564-567.

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

Portera-Cailliau, C., Price, D. L., and Martin, L. J. (1997) Excitotoxic neuronal death in the immature brain is an apoptosis-necrosis morphological continuum. *J. Comp. Neurol.* **378**, 70-87.

Price, J. (1985) An immunohistochemical and quantitative examination of dorsal root ganglion neuronal subpopulations. *J. Neurosci.* **5**, 2051-2059.

Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A., and Shooter, E. M. (1987) Gene transfer and molecular cloning of the rat NGF receptor. *Nature* **325**, 593-597.

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

Ranson, S. W. (1909) Alterations in the spinal ganglion cells following neurotomy. *J. Comp. Neurol.* **19**, 125-153.

Ranson, S.W. (1906) Retrograde degeneration in the spinal nerve. *J. Comp. Neurol.* **16**, 265-293.

Rao, M. V., Houseweart, K. M., Williamson, T. L., Crawford, T. O., Folmer, J., and Cleveland, D. W. (1998) Neurofilament-dependent radial growth of motor axons and axonal organization of neurofilaments does not require the neurofilament heavy subunit (NF-H) or its phosphorylation. *J. Cell Biol.* **143**, 171-181.

Rao, M.S., Sun, Y., Vaidyanathan, U., Landis, S. C., and Zigmond, R. E. (1993) Regulation of substance P is similar to that of vasoactive intestinal peptide after axotomy or explantation of the rat superior cervical ganglion. *J. Neurobiol.* **24**, 571-580.

Rappollee, D. A., and Werb, Z. (1992) Macrophage-derived growth factors. *Curr. Top. Microbiol. Immunol.* **181**, 87-140.

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

Rhoades, R. W., Chiaia, N. L., Mooney, R. D., Klein, B. G., Renehan, W. E., and Jacquin, M. F. (1987) Reorganization of the peripheral projections of the trigeminal ganglion following neonatal transection of the infraorbital nerve. *Somatosens. Res.* **5**, 35-62.

Rich, K. M., Disch, S. R., and Eichler, M. E. (1989) The influence of regeneration and nerve growth factor on the neuronal cell body reaction to injury. *J. Neurocytol.* **18**, 569-76.

Rich, K. M., Luszczynski, J. R., Osborne, P. A and Johnson, E. M. Jr. (1987) Nerve growth factor protects adult sensory neurons from cell death and atrophy caused by nerve injury. *J. Neurocytol.* **16**, 261-268.

Rich, K. M., Yip, H. K., Osborne, P. A., Schmidt, R. E., and Johnson, E. M., Jr. (1984) Role of nerve growth factor in the adult dorsal root ganglia neuron and its response to injury. *J. Comp. Neurol.* **230**, 110-118.

Richardson, P. M., and Ebendal, T. (1982) Nerve growth activities in rat peripheral nerve. *Brain Res.* **246**, 57-64.

Rifkin, D. B., and Moscatelli, D. (1989) Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell Biol.* **109**, 1-6.

Risling, M., Aldskogius, H., Hildebrand, C., and Remahl, S. (1983) Effects of sciatic nerve section on L7 spinal roots and dorsal root ganglia in adult cats. *Exp. Neurol.* **82**, 568-580.

Rose, R. D., and Rohrlich, D. (1987) Counting sectioned cells via mathematical reconstruction. *J. Comp. Neurol.* **263**, 365-386.

Ross, R., Raines, E. W and Bowen-Pope, D. F. (1986) The biology of platelet-derived growth factor. *Cell* **46**, 155-169.

Rydel, R., and Greene, L. A. (1987) Acidic and basic fibroblast growth factors promote stable neurite outgrowth and neuronal differentiation in cultures of PC12 cells. *J. Neurosci.* **7**, 3639-3653.

Ryu, P. D., Gerber, G., Murase, K., and Randic, M. (1988) Actions of calcitonin gene related peptide on rat spinal dorsal horn neurons. *Brain Res.* **441**, 357-361.

Sanchez, M. P., Silos-Santiago, I., Frisen, J., He, B., Lira, S. A., and Barbacid, M. (1996) Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **383**, 70-73.

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

Schlaepfer, W. W., and Bruce, J. (1990) Simultaneous upregulation of neurofilament proteins during the postnatal development of the rat nervous system. *J. Neurosci. Res.* **25**, 39-49.

Schmalbruch, H. (1987) Loss of sensory neurons after sciatic nerve section in the rat. *Anat. Rec.* **219**, 323-329.

Schmitt, F. O. (1968) Fibrous proteins-neuronal organelles. *Proc. Natl. Acad. Sci. USA* **60**, 1092-1101.

Schubert, D., Ling, N., and Baird, A. (1987) Multiple influences of a heparin-binding growth factor on neuronal development. *J. Cell Biol.* **104**, 635-643.

Schwartz, J. P., Pearson, J., and Johnson, E. M., Jr. (1982) Effect of exposure to anti-NGF on sensory neurons of adult rats and guinea pigs. *Brain Res.* **244**, 378-381.

Schwartz, M. L., Shneidman, P. S., Bruce, J., and Schlaepfer, W. W. (1990) Axonal dependency of the postnatal upregulation in neurofilament expression. *J. Neurosci. Res.* **27**, 193-201.

Sendtner, M., Gotz, R., Holtmann, B., Escary, J. L., Masu, Y., Carroll, P., Wolf, E., Brem, G., Brulet, P., and Thoenen, H. (1996) Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF. *Curr. Biol.* **6**, 686-694.

Shadiack, A. M., Vaccariello, S. A., Sun, Y., and Zigmond, R. E. (1998) Nerve growth factor inhibits sympathetic neurons' response to an injury cytokine. *Proc. Natl. Acad. Sci. USA* **95**, 7727-7730.

Shadiack, A.M., Hart, P. P., Carlson, C. D., and Jonakait, G. M. (1993) Interleukin-1 induces substance P in sympathetic ganglia through the induction of leukemia inhibitory factor (LIF). *J. Neurosci.* **13**, 2601-2609.

Shehab, S.A.S., and Atkinson, M. E. (1986) Vasoactive intestinal polypeptide increases in spinal cord after peripheral axotomy of the sciatic nerve originate from primary afferent neurons. *Brain Res.* **372**, 37-44.

Shelton, D. L., and Reichardt, L. F. (1984) Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation of effector organs. *Proc. Natl. Acad. Sci. USA* **81**, 7951-7955.

Shelton, D. L., Sutherland, J., Gripp, J., Camerato, T., Armanini, M. P., Philips, H. S., Carroll, K., Spencer, S. D., and Levinson, A. D. (1995) Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. *J. Neurosci.* **15**, 477-491.

Silos - Santiago, I., Molliver, D. C., Ozaki, S., Smeyne, R. J., Fagan, A. M.,

Barbacid, M., Snider, W. D. (1995) Non -TrkA expressing small DRG neurons are lost in TrkA deficient mice. *J. Neurosci.* **15**, 5929-5942.

Silverman, J. D., and Kruger, L. (1990) Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. *J. Neurocytol.* **19**, 789-801.

Skene, J. .H., Jacobson, R. D., Snipes, G. J., McGuire, C. B., Norden, J. J., and Freeman, J. A. (1986) A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes. *Science* **233**, 783-786.

Skene, J. H., and Willard, M. (1981) Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous systems. *J. Cell Biol.* **89**, 96-103.

Skofitsch, G., and Jacobowitz, D. M. (1985a) Immunohistochemical mapping of galanin-like neurons in the rat central nervous system. *Peptides* **6**, 509-546.

Skofitsch, G., and Jacobowitz, D. M. (1985b) Galanin-like immunoreactivity in capsaicin sensitive sensory neurons and ganglia. *Brain Res. Bull.* **15**, 191-195.

Smeyne, R. J., Klein, R., Schnapp, A., Long, L. K., Bryant, S., Lewin, A., Lira, S. A., and Barbacid, M. (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. *Nature* **368**, 246-249.

Snider, W. D., and Silos-Santiago, I. (1996) Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development. *Phil. Trans. R. Soc. Lond.* **351**, 395-403.

Soppet, D., Escandon, E., Maragos, J., Middlemas, D. S., Reid, S. W., Blair, J., Burton, L. E., Stanton, B. R., Kaplan, D. R., Hunter, T., Nikolics, K., and Parada, L. F. (1991) The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. *Cell* **65**, 895-903.

Stachowiak, M. K., Moffett, J., Maher, P., Tucholski, J., Stachowiak, E. K. (1997) Growth factor regulation of cell growth and proliferation in the nervous system. A new intracrine nuclear mechanism. *Mol. Neurobiol.* **15**, 257-283.

Stahl, N. and Yancopoulos, G. D. (1994) The tripartite CNTF receptor complex: activation and signaling involves components shared with other cytokines. *J. Neurobiol.* **25**, 1454-1466.

Steers, W. D., Kolbeck, S., Creedon, D., and Tuttle, J. B. (1991) Nerve growth factor in the urinary bladder of the adult regulates neuronal form and function. *J. Clin. Invest.* **88**, 1709-1715.

Steinert, P. M., and Roop, D. R. (1988) Molecular and cell biology of inter-

mediate filaments. *Annu. Rev. Biochem.* **57**, 593-625.

Sterne, G. D., Brown, R. A., Green, C. J., and Terenghi, G. (1997) Neurotrophin-3 delivered locally via fibronectin mats enhances peripheral nerve regeneration. *Eur. J. Neurosci.* **9**, 1388-1396.

Sterne, G. D., Brown, R. A., Green, C. J., and Terenghi, G. (1998) NT-3 modulates NPY expression in primary sensory neurons following peripheral nerve injury. *J. Anat.* **193**, 273-281.

Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F., and Abbondanzo, S. J. (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359**, 76-79.

Strittmatter, S.M., Fankhauser, C., Huang, P. L., Mashimo, H., and Fishman, M. C. (1995) Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. *Cell* **80**, 445-452.

Suhonen, J. O., Peterson, D. A., Ray, J., and Gage, F. H. (1996) Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature* **383**, 624-627.

Sun, Y., and Zigmond, R. E. (1996a) Leukaemia inhibitory factor induced in the sciatic nerve after axotomy is involved in the induction of galanin in sensory neurons. *Eur. J. Neurosci.* **8**, 2213-2220.

Sun, Y., and Zigmond, R. E. (1996b) Involvement of leukemia inhibitory factor in the increases in galanin and vasoactive intestinal peptide mRNA and the decreases in neuropeptide Y and tyrosine hydroxylase mRNA in sympathetic neurons after axotomy. *J. Neurochem.* **67**, 1751-1760.

Sun, Y., Landis, S. C., and Zigmond, R. E. (1996) Signals triggering the induction of leukemia inhibitory factor in sympathetic superior cervical ganglia and their nerve trunks after axonal injury. *Mol. Cel. Neurosci.* **7**, 152-163.

Sun, Y., Rao, M. S., Zigmond, R. E., and Landis, S. C. (1994) Regulation of vasoactive intestinal peptide expression in sympathetic neurons in culture and after axotomy: the role of cholinergic differentiation factor/leukemia inhibitory factor. *J. Neurobiol.* **25**, 415-430.

Sun, Y., Shadiack, A. M., Landis, S. C., and Zigmond, R. E. (1993) Differential effects of cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) and NGF on peptide expression in adult rat superior cervical ganglion (SCG). *Soc. Neurosci. Abstr.* **19**, 664.

Suzuki, H., Oyagani, K., Takahashi, H., Kono, M., Yokoyama, M., and Ikuta, F. (1993) A quantitative pathological investigation of the cervical cord, roots and

ganglia after long-term amputation of the unilateral upper arm. *Acta Neuropathol.* **85**, 666-673.

Szaro, B. G., Lee, V. M. -Y, and Gainer, H. (1989) Spatial and temporal expression of phosphorylated and non-phosphorylated forms of neurofilament proteins in the developing nervous system of *Xenopus laevis*. *Dev. Brain Res.* **48**, 87-103.

Szebenyi, G., and Fallon, J. F. (1999) Fibroblast growth factors as multi-functional signaling factors. *Int. Rev. Cytol.* **185**, 45-106.

Takeda, S., Okabe, S., Funakoshi, T., and Hirokawa, N. (1994) Differential dynamics of neurofilament-H protein and neurofilament-L protein in neurons. *J. Cell Biol.* **127**, 173-185.

Tanaka, S., and Koike, T. (1994) Vasoactive intestinal peptide suppresses neuronal cell death induced by nerve growth factor deprivation in rat sympathetic ganglion cells in vitro. *Neuropeptides* **26**, 103-111.

Tatemoto, K., Rökaeus, Å., Jörnvall, H., McDonald, T. J., and Mutt, V. (1983) Galanin - a novel biologically active peptide from porcine intestine. *FEBS Lett.* **164**, 124-128.

Tedeschi, B., and Liuzzi, F. J. (1992) Axotomized frog sciatic nerve releases diffusible neurite-promoting factors. *Dev. Brain Res.* **69**, 97-107.

Tetzlaff, W., and Bisby, M. A. 1989 Neurofilament elongation into regenerating facial nerve axons. *Neurosci.* **29**, 659-666.

Thaler, C. D., Suhr, L., Ip, N., and Katz, D. M. (1994) Leukemia inhibitory factor and neurotrophins support overlapping populations of rat nodose sensory neurons in culture. *Dev. Biol.* **161**, 338-344.

Tham, S., Dowsing, B., Finkelstein, D., Donato, R., Cheema, S. S., Bartlett, P. F., and Morrison, W. A. (1997) Leukemia inhibitory factor enhances the regeneration of transected rat sciatic nerve and the function of reinnervated muscle. *J. Neurosci. Res.* **47**, 208-215.

Thomas, K. A. (1987) Fibroblast growth factors. *FASEB J.* **1**, 434-440.

Thomas, P. K. (1989) Focal nerve injury: guidance factors during regeneration. *Muscle Nerve* **12**, 796-802.

Thompson, S. W. N., and Majithia, A. A. (1998) Leukemia inhibitory factor induces sympathetic sprouting in intact dorsal root ganglia in the adult rat in vivo. *J. Physiol.* **506**, 809-816.

Thompson, S. W. N., Priestley, J. V., and Southall, A. (1998) GP130 cytokines, leukemia inhibitory factor and interleukin-6, induce neuropeptide expression in in-

tact adult rat sensory neurons in vivo: time-course, specificity and comparison with sciatic nerve axotomy. *Neurosci.* **84**, 1247-1255.

Thompson, S. W., Vernallis, A. B., Heath, J. K., and Priestley J. V. (BYG), 9 (6) (1997) Leukemia inhibitory factor is retrogradely transported by a distinct population of adult rat sensory neurons: co-localization with trkA and other neurochemical markers. *Eur. J. Neurosci.* **9**, 1244-1251.

Togari, A., Baker, D., Dickens, G., Guroff, G. (1983) The neurite promoting effect of fibroblast growth factor on pc12 cells. *Biochem. Biophys. Res. Com.* **114**, 1189-1193.

Tonge, D. A., Aarson, O. S., Golding, J.P., and Jagers, D. (1996) Cellular migration and axonal outgrowth from adult mammalian peripheral nerves in vivo. *J. Neurobiol.* **29**, 151-164.

Treanor, J. J., Goodman, L., de Sauvage, F., Stone, D. M., Poulsen, K. T., Beck, C. D., Gray, C., Armanini, M. P., Pollock, R. A., Hefti, F., Phillips, H. S., Goddard, A., Moore, M. W., Buj-Bello, A., Davies, A. M., Asai, N., Takahashi, M., Vandle, R., Henderson, C. E., and Rosenthal, A. (1996) Characterization of a multi-component receptor for GDNF. *Nature* **382**, 80-83.

Troy, C. M., Muma, N. A., Greene, L. A., Price, D. L., and Shelanski, M. L. (1990) Regulation of peripherin and neurofilament expression in regenerating rat motor neurons. *Brain Res.* **529**, 232-238.

Trupp M., Rydén M., Jörnvall H., Funakoshi H., Timmusk T. Arenas E. and Ibanéz C.F. (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. *J. Cell Biol.* **130**, 137-148.

Tu, P. -H., Elder, G., Lazzarini, R. A., Nelson, D., Trojanowski, J. Q., and Lee, V. M. -Y. (1995) Overexpression of human NFM subunit in transgenic mice modifies the level of endogenous NFL and the phosphorylation state of NFH subunits. *J. Cell Biol.* **129**, 1629-1640.

Vanikka, S., Partanen, J., Bellosta, P., Couloir, F., Basilico, C., Jaye, M. and Alitalo, K. (1992) Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction. *EMBO J.* **11**, 4273-4280.

Varon, S. Manthorpe, M., and Adler, R. (1979) Cholinergic neurotrophic factors: I. Survival, neurite outgrowth and choline acetyltransferase activity in monolayer cultures from chick embryo ciliary ganglia. *Brain Res.* **173**, 29-45.

Vejsada, R., Tseng, J. L., Lindsay, R. M., Acheson, A., Aebischer, P., and Kato, A. C. (1988) Synergistic but transient rescue effects of BDNF and GDNF on

axotomized neonatal motoneurons. *Neurosci.* **84**, 129-139.

Venstrom, K. A., and Reichardt L. F. (1993) Extracellular matrix 2: role of extracellular matrix molecules and their receptors in the nervous system. *FASEB J.* **7**, 996-1003.

Vergara, J., Medina, L., Maulen, J., Inestrosa, C., and Alvarez, J. (1993) Nerve regeneration is improved by insulin-like growth factor I (IGF-I) and basic fibroblast growth factor (bFGF). *Restor. Neuro. Neurosci.* **5**, 181-189.

Verge, V. M. K., Richardson, P.M., Wiesenfeld-Hallin, Z., Hökfelt, T.J. (1995) Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons *Neurosci.* **15**, 2081-2096.

Verge, V. M. K., Tetzlaff, W., Bizby, M. A., and Richardson, P. M. (1990) Influence of nerve growth factor on neurofilament gene expression in mature primary sensory neurons. *J. Neurosci.* **10**, 2018-2025.

Verge, V. M., Gratto, K. A., Karchewski, L. A., and Richardson, P. M. (1996) Neurotrophins and nerve injury in the adult. *Phil. Trans. R. Soc. Lond.* **351**, 423-430.

Verge, V.M.K., Wiesenfeld Hallin, Z., and Hökfelt, T. (1992) Cholecystokinin in mammalian primary sensory neurons and spinal cord: in situ hybridization studies on rat and monkey spinal ganglia. *Eur. J. Neurosci.* **5**, 240-250.

Villar, M. J., Cortes, R., Theodorsson, E., Wiesenfeld-Hallin, Z., Schalling, M., Fahrenkrug, J., Emson, P.C., and Hökfelt, T. (1989) Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin. *Neurosci.* **33**, 587-604.

Voyvodic, J. T. (1989) Target size regulates caliber and myelination of sympathetic axons. *Nature* **342**, 430-432.

Vukicevic, S., Kleinman, H. K., Luyten, F. P., Roberts, A. B., Roche, N. S., and Reddi, A. H. (1992) Identification of multiple active growth factors in basement membrane matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. *Exp. Cell Res.* **202**, 1-8.

Wagner, R., and Myers, R. R. (1996) Schwann cells produce tumor necrosis factor alpha: expression in injured and non-injured nerves. *Neurosci.* **73**, 625-629.

Wallicke, P. A. (1988) Basic and acidic fibroblast growth factors have trophic effects on neurons from multiple CNS regions. *J. Neurosci.* **8**, 2618-2627.

Wallicke, P. A., and Baird, A. (1988) Neurotrophic effects of basic and acidic fibroblast growth factors are not mediated through glial cells. *Dev. Brain Res.* **40**, 71-79.

Wallicke, P. A., Cowan, W. M., Ueno, N., Baird, A., and Guillemin, R. (1986) Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc. Natl. Acad. Sci. USA* **83**, 3012-3016.

Walsh, F. S., Meiri, K., and Doherty, P. (1997) Cell signalling and CAM-mediated neurite outgrowth. *Soc. Gen. Physiol. Ser.* **52**, 221-226.

Ware, C. B., Horowitz, M. C., Renshaw, B. R., Hunt, J. S., Liggitt, D., Koblar, S. A., Gliniak, B. C., McKenna, H. J., Papayannopoulou, T. and Thoma, B. et al (1995) Targeted disruption of the low-affinity leukaemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development* **121**, 1283-1299.

Wetmore, C., Ernfors, P., Persso, H., and Olson, L. (1990) Localization of brain-derived neurotrophic factor mRNA to neurons in the brain by in situ hybridization. *Exp. Neurol.* **109**, 141-152.

White, D. M., and Mansfield, K. (1996) Vasoactive intestinal polypeptide and neuropeptide Y act indirectly to increase neurite outgrowth of dissociated dorsal root ganglion cells. *Neuroscience* **73**, 881-887.

White, F. A., Bennett-Clarke, C. A., Mac Donald, G. J., Enfiejian, H. L., Chiaia, N. L., and Rhoades, R. W. (1990) Neonatal infraorbital nerve transection in the rat: comparison of effects on substance P immunoreactive primary afferents and those recognised by the lectin *Bandiera simplicifolia*-I. *J. Comp. Neurol.* **300**, 249-262.

Widmer, H. R., and Hefti, F. (1994) Neurotrophin-4/5 promotes survival and differentiation of rat striatal neurons developing in culture. *Eur. J. Neurosci.* **6**, 1669-1679.

Wiesenfeld-Hallin, Z., Villar, M.J., and Hökfelt, T. (1989) The effects of intrathecal galanin and C-fiber stimulation on the flexor reflex in the rat. *Brain Res.* **486**, 205-213.

Wiesenfeld-Hallin, Z., Xu, X.-J., Villar, M.J., and Hökfelt, T. (1990) Intrathecal galanin potentiates the spinal analgesic effect of morphine: electrophysiological and behavioral studies. *Neurosci. Lett.* **109**, 217-221.

Wilde, G. J., Sundstrom, L. E., and Iannotti, F. (1994) Propidium iodide in vivo: an early marker of neuronal damage in rat hippocampus. *Neurosci. Lett.* **180**, 223-226.

Willard, M., and Simon, C. (1983) Modulations of neurofilament axonal transport during the development of rabbit retinal ganglion cells. *Cell* **35**, 551-559.

Wilson, P. O. G., Barber, P. C., Hamid, Q. A., Power, B. F., Dhillon, A. P.,

Rhode, J., Day, I. N. M., Thompson, R. J., and Polak, J. M. (1988) Immunolocalization of protein gene product 9.5 using rabbit polyclonal and mouse monoclonal antibodies. *Br. J. Exp. Path.* **69**, 91-104.

Windebank, A. J., and Poduslo, J. F. (1986) Neuronal growth factors produced by adult peripheral nerve after injury. *Brain Res.* **385**, 197-200.

Wong, J., and Oblinger, M. M. (1987) Changes in neurofilament gene expression occur after axotomy of dorsal root ganglion neurons: an insitu hybridization study. *Metab. Brain. Dis.* **2**, 291-303.

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

Wong, P. C., Marszalek, J., Crawford, T. O., Xu, Z., Hsieh, S-T., Griffin, J. W., and Cleveland, D. W. (1995) Increased neurofilament subunit NF-M expression reduces axonal NF-H, inhibits radial growth, and results in neurofilamentous accumulation in motor neurons. *J. Cell. Biol.* **130**, 1413-1422.

Wright, D. E., and Snider, W. D. (1995) Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia. *J. Comp. Neurol.* **351**, 329-338.

Xu, X-J, Wiesenfeld-Hallin, Z., Villar, M.J., Fahrenkrug, J., and Hökfelt, T. (1990) On the role of galanin, substance P and other neuropeptides in primary sensory neurons of the rat: studies on spinal reflex excitability and peripheral axotomy. *Eur. J. Neurosci.* **2**, 733-743.

Xu, Z-Q., Shi, T-J., and Hökfelt, T. (1996a) Expression of galanin and galanin receptor in several sensory systems and bone anlage of rat embryos. *Proc. Natl. Acad. Sci. USA* **93**, 14901-14905.

Xu, Z., Marszalek, J. R., Lee, M. K., Wong, P. C., Folmer, J., Crawford, T. O., Hsieh, S-T., Griffin, J. W., Cleveland, D. W. (1996b) Subunit composition of neurofilaments specifies axonal diameter. *J. Cell Biol.* **133**, 1061-1069.

Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M. J., and Patterson, P. H. (1989) The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science* **246**, 1412-1416.

Yamasaki, H., Bennett, G. S., Itakura, C., and Mizutani, M. (1992) Defective expression of neurofilament protein subunits in hereditary hypotrophic axonopathy of quail. *Lab. Invest.* **66**, 734-743.

Yanagisawa, M., Yagi, N., Otsuka, M., Yanaihara, C., and Yanaihara, N. (1986) Inhibitory effects of galanin on the isolated spinal cord of the newborn rat.

Neurosci. Lett. **70**, 278-282.

Ygge, J., and Aldskogius, H. (1984) Intercostal nerve transection and its effect on the dorsal root ganglion. A quantitative study on ganglion cell number and sizes. *Exp. Brain Res.* **55**, 402-408.

Yip, H. K., Rich, K. M., Lampe, P. A., and Johnson, E. M. Jr. (1984) The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal root ganglia. *J. Neurosci.* **4**, 2986-2992.

Yoon, S. O., Casaccia-Bonnel, P., Carter, B., and Chao, M. V. (1998) Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. *J. Neurosci.* **18**, 3273-3281.

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

Zhang, Q., Ji, R-R, Lindsay, R., and Hökfelt, T. (1995) Effect of growth factors on substance P mRNA expression in axotomized dorsal root ganglia. *NeuroRep.* **6**, 1309-1312.

Zheng, J. L., Stewart, R. R., and Gao, W. Q. (1995) Neurotrophin-4/5 enhances survival of cultured spiral ganglion neurons and protects them from cisplatin neurotoxicity. *J. Neurosci.* **15**, 5079-5087.

Zhou, F.Y., Owens, R.T., Hermonen, J., Jalkanen, M., and Höök, M. (1997) Is the sensitivity of cells for FGF-1 and FGF-2 regulated by cell surface heparan sulfate proteoglycans? *Eur. J. Cell Biol.* **73**, 166-174.

Zhu, Q., Lindenbaum, M., Levavasseur, F., Jacomy, H., and Julien, J. -P. (1998) Disruption of the NF-H gene increases axonal microtubule content and velocity of neurofilament transport: relief of axonopathy resulting from the toxin beta,beta'-iminodipropionitrile. *J. Cell Biol.* **143**, 183-193.

Zigmond, R. E., Hyatt-Sachs, H., Mohny, R. P., Schreiber, R. C., Shadiack, A. M., Sun, Y., and Vaccariello, S. A. (1996) Changes in neuropeptide phenotype after axotomy of adult peripheral neurons and the role of leukemia inhibitory factor. *Perspec. Dev. Neurobiol.* **4**, 75-90.

Zigmond, R. E., Shadiack, A. M., and Sun, Y. (1995) Antiserum to nerve growth factor (NGF) alters neuropeptide expression in the superior cervical ganglion (SCG) and dorsal root ganglion (DRG) in vivo. *Soc. Neurosci. Abstr.* **21**, 1025.