PROPERTIES OF INTACT AND INJURED SENSORY NEURONS WITH NERVE GROWTH FACTOR RECEPTORS

by

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ABSTRACT

The role of nerve growth factor (NGF), a neurotrophic molecule, and its high-affinity receptor in intact and injured adult rat lumbar sensory neurons was examined at a cellular level using quantitative receptor radioautography to localize the NGF high-affinity receptor-positive subpopulation, in conjunction with histochemistry on adjacent sections. The 40-50% of sensory neurons displaying NGF receptors were characterized. Virtually all neurons containing substance P or CGRP were NGF receptor-positive, but not those with somatostatin or thiamine monophosphatase activity. The ability of a neuron to bind NGF with high-affinity correlates positively with growth-associated protein (GAP43) expression but not with neurofilament (NFM) expression. Following injury, sensory neurons atrophy, lose NGF receptors, decrease NFM expression, while GAP43 expression is elevated in all neurons irrespective of their ability to bind NGF. Infusion of NGF for 1 week, at the time of injury or 3 weeks following injury counteracts NGF receptor loss, cell atrophy, and decreased NFM expression, but only in those neurons bearing NGF receptors. GAP43 expression remained high in all neurons despite infusion. NGF's function in normal sensory neurons appears to be modulatory, permitting regulation of intrinsic properties. Injury disrupts this permissive state, which can be restored with exogenous NGF.

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RESUME

Le rôle du facteur de croissance NGF, une molécule aux effets neurotrophiques, chez les neurones sensitifs lombaires intacts et endommagés fut étudié au niveau cellulaire chez le rat en localisant les récepteurs à haute affinité pour ce facteur par la méthode de radioautographie quantitative et par histochimie des sections adjacentes. Ces récepteurs se retrouvent chez 40 à 50% des neurones observés. Les neurones qui contiennent la substance P ou le CGRP expriment tous le récepteur à haute affinité pour le facteur NGF contrairement aux neurones somatostatinergiques ou aux neurones contenant l'enzyme, thiamine monophosphatase chez lesquels aucune trace de ce récepteur ne peut être visualisée. Cette capacité de lier avec haute affinité le facteur NGF chez les neurones est associée avec l'expression de la protéine de croissance GAP43 sans toutefois requérir l'expression du neurofilament NFM. La lésion des neurones sensitifs est suivie d'une atrophie, d'une perte des récepteurs à haute affinité pour le NGF et d'une diminution de l'expression du neurofilament NFM alors que l'expression de la protéine GAP43 s'élève chez tous les neurones peu importe qu'ils lient le facteur NGF ou non. Les conséquances, à l'exception de l'élèvation de la protéine GAP43, sont prévenue par l'infusion du facteur NGF pendant une semaine débutant au moment de la lésion ou trois semaines après mais

seulement pour les neurones liant le facteur NGF. Il est a noter que l'expression accrue de la protéine GAP43 suite a une lésion n'est pas dépendante de la présence de recepteurs au facteur NGF mais qu'elle est observée pour la totalite des neurones. Le facteur NGF joue un important rôle dans la régulation des propriétés intrinsèques des neurones sensitifs. Suite à une lésion, l'addition de facteur NGF exogene permet de perpétuer cette homéostasie des neurones sensitifs.

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CLAIM FOR ORIGINAL WORK

My gratitude is extended to my supervisor, Dr. Peter Richardson and collaborators, Drs. Richard Riopelle, Wolfram Tetzlaff, Mark Bisby and Robert Benoit for valued assistance in manuscript preparation and specific contributions as follows:

- Dr. Peter Richardson surgically implanted the osmotic pumps used to administer NGF, assisted in lesioning operations, and created of many of the computer programs used in data analysis;
- Dr. Wolfram Tetzlaff prepared the GAP43 and NFM cDNA probes used in Chapters 5 & 6, and instructed and assisted the Candidate in conducting the in situ hybridizations using these probes. Dr. Tetzlaff also prepared the Northern blot identifying GAP43 mRNA in normal and injured sensory neurons which led to Figure 1 in Chapter 5;
- Dr. Robert Benoit made the somatostatin-28 antibody (S-309) and peptide used in Chapters 2 & 5, and instructed the Candidate on the working dilutions.

Except where otherwise stated the results presented in this thesis represent an original contribution to knowledge concerning the cellular function of the trophic molecule, nerve growth factor (NGF) in normal and injured sensory neurons. The findings are presented below.

Technical advancements:

- a quantitative NGF receptor radioautographic technique was developed that yields morphometric data, and enables determination of binding site densities and analysis of binding properties for individual neurons;
- immersion-fixation histochemical techniques for 5 μm frozen sections were developed by modifying existing protocols that allow detection of the enzyme thiamine monophosphatase and localization of peptides using specific antisera.

Scientific findings :

Intact sensory neurons - approximately 40-50 % of neurons in the lumbar dorsal root ganglion of adult rats display high-affinity receptors for nerve growth factor, and are thus presumably responsive to NGF;

- Scatchard analysis of the concentration dependence of binding for heavily labelled neurons indicates saturable high-affinity binding with a dissociation-equilibrium constant of 15-50 pM and additional binding of lower affinity. For neurons with only background levels of labelling, no high-affinity binding component is detected by Scatchard analysis, reinforcing the apparent lack of NGF receptors on these neurons;
- characterization of the population of NGF-responsive neurons revealed that growth associated protein (GAP43) mRNA, substance P, and calcitonin gene-related peptide (CGRP), are strongly expressed only in neurons capable of responding to NGF. The

positive correlation between NGF-responsiveness and GAP43 expression is also observed for another population of NGF-sensitive neurons, the sympathetic neurons in the superior cervical ganglion. The presence of high-affinity NGF receptors on sensory neurons containing high levels of GAP43, substance P and CGRP provides the anatomical substrate necessary to support the hypothesis of a direct role for endogenous NGF in regulation of these molecules in vivo, as has already been shown by others in vitro. The fact that only 50% of NGF-responsive neurons contain substance P implies that NGF may be necessary, but is not sufficient for expression of this peptide. Neurons containing somatostatin or thiamine monophosphatase invariably lack high-affinity NGF receptors and have low basal levels of GAP43 mRNA. The display of high-affinity NGF receptors is not correlated with the expression of the medium neurofilament subunit (NFM), however, NFM mRNA levels correlate positively with neuronal volume.

Injured sensory neurons - in response to sciatic nerve transection, sensory neurons atrophy, lose receptors for NGF, and have decreased levels of NFM mRNA. The axotomy-induced loss of receptors is attributable to a loss of high-affinity NGF binding, while low-affinity NGF and nonspecific binding are apparently unchanged. One month after the sciatic nerve is cut the mean number of NGF high-affinity sites falls to less than 20% of normal values because of reduced receptor density and cell volume. The loss of perikaryal NGF receptors following

injury does not merely reflect translocation of receptors into peripheral or central axonal processes as receptors on both these processes are also reduced. The posttraumatic reduction in NFM mRNA expression is very apparent for large neurons with or without NGF receptors which normally synthesize NFM abundantly and is proceeded by a reduction in neuronal volume more evident at 3 weeks than 1 week after injury. Neurons with high-affinity receptors are more liable to atrophy after injury than those lacking such receptors. The correlation between GAP43 expression and display of NGF high-affinity receptor is lost following axotomy. Almost all neurons have high concentrations of GAP43 mRNA, suggesting the synthesis of GAP43 is regulated by a signal other than NGF soon after injury.

- **Infusion of NGF** for 1 week either at the time of injury or three weeks after, counteracts NGF receptor loss, cell atrophy, and decreased NFM expression but only in neurons with high-affinity NGF receptors. One week after nerve transection GAP43 expression remained high in all neurons despite infusion. These findings demonstrate:

i. The actions of infused NGF appear specific, effecting the changes studied only in those neurons displaying receptors for NGF;

ii. NGF can regulate synthesis of its own receptor in vivo;

ii. NGF can partially restore neuronal cell volume after injury, but only in neurons with NGF receptors;

iii. NGF is neither necessary nor sufficient for high levels of

neurofilament synthesis because some normal neurons with high concentrations of NFM mRNA do not bear high-affinity NGF receptors, and others with NGF receptors have low concentrations of NFM mRNA even following infusion of NGF. However, NGF is relevant to NFM synthesis because it counteracts postaxotomy down-regulation of NFM mRNA in a subset of the NGF-responsive neurons. Thus, it is postulated that NGF permits NGF-sensitive neurons to respond differentially to a second factor stimulating neurofilament synthesis;

iv. Some signal other than NGF or lack of NGF is responsible for the early posttraumatic induction of GAP43;

v. The ability of exogenous NGF to counteract some of the axotomy-induced changes described in this thesis, suggests the reduced retrograde supply of NGF to the cell body that occurs following injury could be responsible for these changes.

To my parents

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CHAPTER 1 - INTRODUCTION

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1.0 INTRODUCTION

When a peripheral sensory neuron is injured molecular and morphological changes within the proximal and distal portions of the neuron ensue (Grafstein and McQuarrie, 1978; Aldskogius et al., 1985). Some of the changes in the proximal stump and perikaryon are thought to be initiated by the reduction in the normal retrograde transport of trophic molecules derived from cells in the endoneurium or target tissues (Lieberman 1974; Aldskogius et al., 1985). One such molecule is nerve growth factor (NGF). NGF is unquestionably the best characterized neurotrophic factor and has been shown to play a critical role in the development, maintenance and survival of peripheral sympathetic and sensory neurons and more recently the central cholinergic neurons of the basal forebrain and striatum (reviewed in Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Greene and Shooter 1980; Whittemore and Seiger, 1987; Barde 1989). That reduced retrograde transport of NGF influences the axotomy response of sensory neurons is best demonstrated by the ability of exogenous NGF to counteract many of the degenerative changes that occur following injury, most notably the loss of substance P, neuronal atrophy or death (Johnson, E.M., et al., 1986; Fitzgerald et al., 1985; Miyata et al., 1986; Otto et al., 1987; Rich et al., 1987). The injured neurons if allowed to regenerate, would once again contact a

large source of endogenous NGF, as cells within the distal endoneurial segment greatly increase their normal synthesis of NGF in response to axotomy (Heumann et al., 1987; Richardson and Ebendal 1982). However, the molecular mechanisms underlying the role of NGF in intact and injured sensory neurons remain for the most part enigmatic. Elucidation of this role at a cellular level can be approached by first identifying and characterizing the population of neurons capable of responding to NGF - those with specific high-affinity NGF receptors. Analysing how this population of sensory neurons change in their responsiveness to NGF, and other neural markers following injury, might give insights into whether these changes contribute to what would be regarded as a general degenerative or regenerative axotomy response. Finally, this approach would allow one to determine the ability of NGF to counteract some of these axotomy-induced changes and whether it is a direct effect on NGF-responsive neurons or a less specific intraganglionic effect. Because NGF acts on only a subpopulation of neurons, defining the molecular mechanisms underlying NGF actions in normal and injured sensory neurons would serve as a paradigm for other neurotrophic molecules not yet available for such detailed analysis.

This thesis will attempt to elucidate the role of NGF in sensory neurons at a cellular level by:

(i) reviewing what is known about the NGF molecule and receptor(s), its mechanism of action, and role in sensory neurons (Chapter 1);

(ii) presenting work that addresses the molecular role of NGF at a cellular level in intact and injured mature sensory neurons. In order to do so a quantitative receptor radioautographic technique was developed that allows identification of the subpopulation of sensory neurons displaying high-affinity NGF binding sites, and thus capable of responding to NGF. This technique was used alone or in conjunction with adjacent section histochemical or in situ hybridization techniques to characterize the NGF-responsive population, study the binding properties, and define changes in this population following injury with respect to morphology, NGF binding properties, or mRNA for two markers of neural injury. Finally, this approach was used to study the regulatory mechanisms by which exogenous NGF might counteract these injury-induced changes. (Chapters 2,3,4,5,6);

(iii) summarizing the findings of the studies presented in objective (ii), and discussing their relevance (Chapter 7).

2.0 NGF - THE MOLECULE

2.1 EARLY HISTORY

The concept of neurotrophism was put forth by Cajal almost a century ago (Cajal, 1928). However, it was not until the early fifties that proof for a specific neurotrophic factor emerged. The discovery of NGF was a direct extension of work which showed

mouse sarcoma 180 tumor transplants in the developing chick embryo were hyperinnervated by fibers derived from adjacent sensory ganglia (Bueker 1948). Levi-Montalcini and Hamburger noted that this effect was even greater for the sympathetic ganglia and was due to a diffusible growth factor secreted by the tumor which they called "nerve growth factor" (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini, 1951). Development of a bioassay that could detect small quantities of NGF allowed duplication of the initial results and was instrumental in isolating and purifying the factor (Levi-Montalcini et al., 1954). In an attempt to determine whether NGF was contained in the nucleic acid or protein of the mouse sarcoma 180 tumor extract, snake venom was employed for its phosphodiesterase activity with the intent of enzymatically inactivating the nucleic acids in the active fraction. This resulted in the serendipitous observation that snake venom was a more potent source of NGF than the tumor itself (Cohen and Levi-Montalcini, 1956). An extrapolation of this last finding led to the discovery of the richest source of NGF - the male mouse submandibular gland, the mammalian analogue of the snake venom gland (Cohen 1960). The subsequent production and administration to neonatal mice of antibodies to mouse NGF resulted in the specific destruction of the sympathetic ganglia and demonstrated for the first time the critical role that this trophic molecule plays in the normal development of the nervous system (Cohen 1960). The inexplicably high amount of NGF found

in the male mouse submandibular gland has provided the quantities of starting material necessary for its purification, molecular characterization, and insights into its biosynthesis.

2.2 NGF BIOSYNTHESIS

NGF that is synthesized and released by the mature male mouse submandibular gland can be isolated as a complex of three dissimilar polypeptide chains designated α, β, and Γ with a stoichiometry of $\alpha_2\beta\Gamma_2$ (Varon et al., 1967a,b; Burton et al., 1978). The stoichiometric structure has been recently disputed by Young et al., (1988), who employing multiple physicochemical approaches determined the subunit formula to be $\alpha_2\beta\Gamma$. The intact complex has a molecular weight of ≈130,000, a pI of 5.15 and is referred to as 7s NGF due to its sedimentation coefficient. One or two zinc ions are associated with the intact complex and are believed to increase its stability (Pattison and Dunn, 1975; Bothwell and Shooter, 1978; Young et al.,1988). The complex is stable at neutral pH, but dissociates into separate subunits outside the pH 5-8 range (Server and Shooter, 1977).

All of the biological activity attributed to NGF is found within the β subunit, which is a stable noncovalently linked, basic (pI=9.3) homodimer weighing 26,500. Each chain of the dimer is 118 aa in length with six cysteines and three disulphide bridges (Greene et al., 1971; Hogue Angeletti et al.,

1971; Hogue Angeletti and Bradshaw, 1971). Biologically active β-NGF has been isolated with proteolytic N- and C-terminal modifications (2S NGF) (Mobley et al., 1976), in monomer (Frazier et al., 1973) or dimer (Stach and Shooter, 1974) form, or in a glycosylated state (Murphy et al., 1989). However, it must be dissociated from the 7S complex in order to be active (Silverman and Bradshaw, 1982). Sequencing of the entire mouse NGF gene revealed a gene 45 kilobases long with several small 5' exons. Four different mRNA species were identified that result from alternative splicing and initiation from two independent promoter elements (Selby et al., 1987). This and earlier work (Server and Shooter, 1977) confirmed that the mature form of the protein, β-NGF, is located at the C-terminal and synthesized from a larger precursor protein, the size of which may differ as a result of alternative RNA splicing or independent promoter initiation. The two major transcripts predict precursor proteins of 27kDa and 34kDa differing in the location of the signal peptide sequence which may be responsible for different cellular localization (Edwards et al.,1986). Both precursors give rise to apparently the same protein as infection of a number of mammalian cell types with a virus vector containing either the long or short NGF mRNA transcript resulted in the production of precursors that were then processed to mature proteins identical to NGF in weight and in vitro bioactivity (Edwards et al., 1988).

The α and Γ subunits of the 7S NGF complex, both members of

the kallikrein family of specific serine proteases, are localized in the same cell type as β-NGF (Mowry et al., 1984; Murphy et al., 1986) and believed to be involved in the processing of the larger NGF precursor to its mature form (Mason et al., 1983). The Γ subunit is an arginyl-esteropeptidase that is capable of cleaving pro-NGF at the C-terminal arginine residue (position -2), the amino acid that is critical for 7S complex stability (Stach et al., 1976; Server and Shooter, 1977; Berger and Shooter, 1978; Jongstra-Bilen et al.,1989). Cleavage of pro-NGF not only processes the protein to its mature form, but also causes a 10-20 fold increase in its bioactivity as measured by in vitro bioassay (Edwards et al., 1988), and might be a basis for regulation of activity in vivo. The α subunit, whose function is unknown, is an inactive serine esteropeptidase possibly due to crucial amino acid substitutions (Silverman and Bradshaw, 1982; Isackson et al., 1985). It associates with the NH_3-terminal of the β subunit and is speculated to be involved in 7S complex stability or protection from degradation. The mandatory requirement for these specific proteases in the biosynthesis of NGF outside the submandibular gland is questionable, as there are many cell types that synthesize NGF in the apparent absence of either the α or Γ subunits (Pantazis, 1983; Murphy et al., 1986). Both trypsin and the EGF binding protein can effectively process the NGF precursor protein to its mature form (Berger and Shooter, 1977; Edwards et al., 1988). This raises the possibility that similar proteases could perform

this function in the other cell types.

The large amounts of NGF found in a variety of exocrine sources has facilitated its purification not only from male mouse submandibular glands, but also from snake venoms (Hogue Angeletti et al., 1976; Angeletti et al., 1967, Perez-Polo et al., 1978), bovine seminal plasma (Harper et al.,1982), and rabbit, bull and guinea pig prostate gland (Harper and Thoenen, 1980; Chapman et al., 1981) Mouse DNA probes have been used to isolate the NGF gene for human (Ullrich et al., 1983), bovine (Meier et al., 1986), chicken (Ebendal et al., 1986; Meier et al., 1986; Wion et al., 1986), and rat (Whittemore et al., 1988). The NGF coding regions in the genes are highly conserved across the species, including all six cysteines (Ebendal et al., 1986). Probes derived from portions of the gene sequences have allowed insights into the regional, and sometimes cellular location of the endogenous sites of NGF synthesis, those believed to be physiologically important for specific neuronal interactions (Shelton and Reichardt, 1984; Korsching et al., 1985; Whittemore et al., 1986; Bandtlow et al., 1987; Ayer-LeLievre et al., 1988). However, the minute levels of synthesis are largely responsible for the unsuccessful isolation, poor characterization and cellular localization of NGF from these sources, and it still remains to be shown that this NGF is identical to the β-NGF derived from exocrine sources.

Transcriptional regulation of submandibular gland NGF has been addressed using cloned promoter and 5' flanking regions

from mouse and rat genes which are highly conserved in the promoter regions (Zheng and Heinrich, 1988). Transgenic male mice were constructed using a NGF-human growth hormone fusion gene and the developmental induction of NGF gene expression in the submandibular gland at puberty was studied (Alexander et al.,1989). The results of the study indicated that cell-specific and developmental expression of the NGF gene are regulated by cis elements within 5 kb upstream of the promoter. This approach should prove useful in the study of NGF gene regulation in the regions representing the endogenous sources of NGF once the sensitivity of the molecular techniques is capable of detecting the low levels in these regions.

3.0 NGF RECEPTOR(S)

3.1 ACTIONS OF NGF

NGF has a diverse range of actions on its target neurons in vivo, including regulation of survival of peripheral sympathetic and neural crest-derived neurons during specific periods of their development, and promotion of nerve fiber outgrowth (for reviews see Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980). In addition to these trophic effects, NGF plays a role in the differentiation and maintenance of a number of biochemical properties, such as regulation of transmitter enzymes and peptides of specific peripheral and central

cholinergic neurons (Thoenen and Barde, 1980; Gnahn et al., 1983; Mobley et al., 1985; Vantini et al., 1989; Mobley et al., 1989; Hagg et al., 1989). In vitro work has focused on many NGF-responsive cell types, in attempts to unravel mechanisms underlying the areas of NGF-induced cell differentiation, neurite extension and signal transduction. The rat adrenal pheochromocytoma (PC12) cell line has proved most useful as a model because PC12 cells do not require NGF for survival and therefore allow separation of NGF effects on cellular and differentiation events from cell viability events (Greene and Tischler, 1976). NGF-induced differentiation of PC12 cells into a neuronal phenotype have been broadly categorized as rapid and membrane-associated not requiring transcription, or slow and nuclear-associated requiring transcriptional change (reviewed in Greene & Shooter 1980; Fujita et al., 1989). In the former category are transient morphological responses, changes in second messenger levels, ion fluxes, transport, and protein phosphorylations including nuclear proteins. The latter category of transcription-dependant alterations include some very rapidly transcribed protooncogenes and more slowly transcribed enzymes, transcription factors, cytoskeletal,and surface proteins (Fujita et al., 1989). The key to understanding how NGF acts however, is to understand the receptor(s) involved, as the first step in the transduction of NGF-mediated actions on any cell type is the interaction of NGF with specific cell membrane-bound receptors.

3.2 ONE VERSUS TWO RECEPTORS

Initial studies to determine the binding characteristics of NGF receptors on sensory and sympathetic neuronal membranes gave conflicting results. They showed both heterogenous binding with negative cooperativity accounting for the two observed affinities with dissociation equilibrium constants (Kds) of 10^{-10}M and 10^{-7}M (Frazier et al., 1974a,b), and binding to receptors with a single affinity of approximately 10^{-10}M (Banerjee et al.,1973; Herrup and Shooter, 1973). Subsequent studies however, clearly demonstrated the existence of two distinct, saturable classes of binding sites on many cell types, differing in kinetic and molecular properties. Both peripheral sensory and sympathetic and central cholinergic NGF-responsive neurons, as well as rat PC12 pheochromocytoma and human LAN-1 neuroblastoma cells bind NGF with two affinities (Sutter et al.,1979; Riopelle et al., 1980; Olender and Stach, 1980; Mobley et al., 1989; Bernd and Greene, 1984; Landreth and Shooter, 1980; Schecter and Bothwell, 1981; Marchetti and Perez-Polo, 1987). The two classes of receptors differ in their affinity for NGF and numbers (approximately 10 times as many low- compared to high-affinity receptors) . There is a high-affinity (type I) receptor with a Kd of approximately 10^{-11}M and a low-affinity receptor (type II), with a Kd of 10^{-9}M . Both receptors have similar association rate constants but are distinguished by dissociation rate constants that differ by approximately two orders of

magnitude. Experimentally, the low-affinity NGF receptor is identified by its trypsin sensitivity and rapid dissociation of prebound ^{125}I-NGF with excess unlabelled NGF. The high-affinity receptor is trypsin insensitive, insoluble in low concentrations of Triton-X 100, dissociates prebound ^{125}I-NGF very slowly and its Kd corresponds well with the concentration of NGF required to induce a half-maximal neurite outgrowth response in sensory neurons (Greene, 1977; Ishii, 1978; Sutter et al., 1979; Landreth and Shooter, 1980; Schecter and Bothwell, 1981; Vale and Shooter, 1982; Buxser et al., 1983; Godfrey and Shooter, 1986). The two receptors are also referred to as "fast" and "slow" on the basis of the dissociation rate of NGF from them. Table I summarizes the various properties of the two NGF receptor classes.

TABLE I
PROPERTIES OF THE TWO NGF RECEPTORS

		low-affinity (fast)	high-affinity (slow)
Kd (^{125}I-NGF binding)		$\approx 10^{-9}$M	$\approx 10^{-11}$M
Dissociation rate ($t_{1/2}$)		Fast ≈10 sec	Slow ≈30 mins
Trypsin sensitivity		Labile	Stable
Triton X-100 solubility		Soluble	Insoluble
Mol. Wt.	Complex	≈100,000	≈158,000
	Receptor	≈80,000	≈140,000

Much of the work done on characterization of the NGF receptor(s) has centered on resolving whether the heterogeneity of binding derives from a single or different protein moieties. If only one receptor protein were involved then negative cooperativity or ligand-induced conversion from low- to high-affinity could explain the heterogeneity. In agreement with others (Sutter et al., 1979; Riopelle et al., 1980; Olender et al., 1981), Godfrey and Shooter (1986) demonstrated that negatively cooperative interactions among a single class of NGF receptors could not account for the observed heterogeneity. If they were, then increased occupancy of receptors should result in a decreased affinity for NGF, and an accelerated dissociation rate under dilution plus chase conditions. In addition, the increase in the dissociation rate observed should be close to the differences in the Kd's for the two sites. When chick embryo sympathetic neurons were incubated to steady state with $5x10^{-12}M$ ^{125}I-NGF, then diluted 15-fold into buffer with or without a large excess of unlabelled NGF (1500-fold), the dissociation rate was increased, but only two-fold and not the expected 100-fold if there were negative cooperativity. Furthermore, this slight increase in dissociation was also observed when the excess of unlabelled NGF was less than the concentration of ^{125}I-NGF used for preloading the cells, thus occurring regardless of increased or decreased receptor occupancy. This argues in favor of two apparently different binding sites for NGF.

It has been suggested that the binding sites are

interconvertible, with the high-affinity site being formed as a result of NGF binding to its low-affinity site. Landreth and Shooter (1980) first put forth this hypothesis when a lag period in the appearance of high-affinity binding was observed following the incubation of naive PC12 cells with 14 pM ^{125}I-NGF. The involvement of a conversion process was further supported by the continuing formation of high-affinity sites following the removal of ^{125}I-NGF from the incubation medium. However, several important explanations for these observations were not considered. It is possible that dissociation of ^{125}I-NGF from the low-affinity sites and rebinding to high-affinity sites accounted for the increase, because low concentrations of ^{125}I-NGF preferentially bind to the high-affinity sites (Sutter et al., 1979). In addition, Schecter and Bothwell (1981), who failed to observe the lag in appearance of NGF bound to high-affinity sites, sought to determine if high-affinity binding sites existed in the absence of NGF by briefly digesting naive PC12 cells with trypsin before exposure to ^{125}I-NGF. This treatment resulted in the complete loss of low-affinity binding normally observed with no apparent loss in high-affinity binding sites, and suggests that both receptor subtypes are present on naive PC_{12} cells in the absence of NGF. Similar work on cultured human neuroblastoma cell support these conclusions (Sonnenfeld and Ishii, 1985). Solubilized membrane preparations were also shown to bind NGF with high-affinity in the absence of low-affinity sites (Marchetti and Perez-Polo, 1987). Finally,

perhaps the strongest argument against negative cooperativity or ligand-induced conversion of low- to high-affinity binding sites is the existence of cells that bind NGF with only low-(Buxser et al., 1983) or high-affinity (Sonnenfeld and Ishii, 1985). These studies demonstrate a ligand-induced conversion from low- to high-affinity states is not necessary for the high-affinity binding site to exist, but do not preclude the possibility that the two classes are related.

An alternate hypothesis to explain the observed binding heterogeneity would be a common binding protein, which differentially associates with submembranous elements such as an effector protein that could account for the differing trypsin sensitivity and induce the conformational change resulting in a higher affinity for NGF. Multiple approaches have been used in an attempt to resolve this issue including studies of lectin-binding, chemical cross-linking, and molecular characterization of the low-affinity binding site.

3.3 BIOCHEMICAL AND MOLECULAR CHARACTERIZATION

Wheat germ agglutin (WGA) was the only lectin found to effect NGF binding. It is a multivalent lectin that crosslinks cell surface proteins and is hypothesized to crosslink these proteins to others normally unrestrained in the lipid bilayer thereby altering the solubility properties and conformation of the proteins (Vale et al., 1985). Exposure of PC12 cells to WGA

either before or after addition of ^{125}I-NGF conferred on the rapidly dissociating receptors two properties associated with high-affinity binding, namely, slow dissociation and Triton-X 100-insolubility (Vale and Shooter 1982). The effect of WGA was not specific for NGF receptors, as >90% of cell surface glycoprotein receptors for WGA become associated with Triton-X 100-insoluble material at concentrations slightly less than those employed for the NGF receptor effects (Vale et al., 1985). However, these results correlate the affinity of binding with a cytoskeletal-associated state and support the theory of NGF receptor transmembrane interactions in high-affinity binding.

Chemical cross-linking studies where ^{125}I-NGF binds to its receptor and is covalently linked in place by addition of a bifunctional cross-linking agent, have been used to determine the molecular weights of the receptor species. Two different cross-linking agents have been routinely employed - ethyldimethylisopropylaminocarbodiimide (EDAC) and hydroxysuccinimidyl-p-azidobenzoate (HSAB). The major difference between EDAC and HSAB is that EDAC is impermeable to biological membranes while HSAB is able to penetrate the membrane, and thus the more likely candidate for detection of receptor-associated effector proteins. Once cross-linked, the labelled receptor-ligand complexes can then be analysed by gel electrophoresis and/or processed for radioautography to determine the molecular weights of the complexes (Bothwell 1989). A major common binding protein of 87-100 kDa was revealed in all cell types examined

(Massague et al.,1981; Grob et al., 1985; Buxser et al., 1983, Puma et al., 1983; Hosang and Shooter, 1985; Kouchalakos and Bradshaw, 1986; Taniuchi et al., 1986). A minor band of 190-220 kDa is believed to represent multimers of the 87-100 kDa NGF receptor, as disulfide reducing agents such as 2-mercaptoethanol were effective in reducing the higher molecular weight species to a 90 kDa protein and supports the existence of NGF receptor disulphide linked dimers (Buxser et al., 1985; Kouchalakos and Bradshaw, 1986; Stach and Perez-Polo, 1987; Bothwell 1989). A second major band of 143-158 kDa was found only on cells capable of both binding and responding to NGF (Massague et al., 1981; Hosang and Shooter, 1985). The 158 kDa receptor species preferentially labelled at low concentrations of NGF, was chase-stable at 0°C and trypsin-resistant, thus likening it to the high-affinity form of the receptor. The 100kDa species was fast-dissociating and identified as the low-affinity binding site (Hosang and Shooter, 1985). It is unlikely that the 87-100 kDa species is a proteolytic fragment of the 143-158 kDa species, because neither the addition of several protease inhibitors nor prolonged incubation at 37°C altered the ratio of the two species (Hosang and Shooter, 1985).

The production of monoclonal antibodies against the low-affinity NGF receptor on human melanoma (ME20.4) (Ross et al., 1984), and rat PC12 cells (192 IgG) (Chandler et al., 1984) have enhanced the specificity and sensitivity of isolating crosslinked NGF-R complexes by allowing for immunoprecipitation.

Lack of crossreactivity however has limited these probes to specific genus lines. In both rat and human cells a major NGF receptor species of 70-80 kDa was observed, representing a phosphorylated glycoprotein (Ross et al., 1984; Taniuchi et al., 1986; Marano et al., 1987). A common binding protein of ≈ 90 kDa for both receptor subtypes was found (Green and Greene, 1986) when EDAC was used as a crosslinking reagent, followed by immunoprecipitation, on cell types displaying either high-affinity, low-affinity or both classes of receptor. These results reinforce the hypothesis that the high-affinity binding site consists of a common binding protein complexed with a 50-60 kDa cytoskeletal effector protein which is only detectable when a membrane-permeant crosslinking reagent such as HSAB is used to cross-link both components of the receptor (Hosang and Shooter, 1985).

3.4 STRUCTURE OF THE LOW-AFFINITY RECEPTOR

The monoclonal antibodies have also been used to isolate both rat and human NGF low-affinity receptor cDNA clones (Johnson,D., et al., 1986; Radeke et al., 1987) with the hope that sequence information might give insights into the relationship between the two receptor classes and the possible

signal transduction mechanism of the high-affinity receptor. Cloning and sequencing of the rat and human NGF low-affinity receptor genes indicated a very strong homology between species (> 90%) with the only major difference being the number of putative N-linked glycosylation sites. Amino acid sequence information revealed that the cloned NGF receptor belonged to a new class of receptor molecule bearing no homology to any other known receptor protein. Recently, two molecules showing strong sequence and structural homology to the extracellular domain of the NGF low-affinity receptors have been isolated and sequenced. They include a putative cytokine-inducible B cell and primary carcinoma growth factor receptor (CDw40) (Braesch-Anderson et al., 1989; Stamenkovic et al., 1989) and the 55kd tumor necrosis factor receptor (Loetscher et al., 1990; Schall et al., 1990). It is also anticipated that the receptor for brain derived neurotrophic factor (BDNF) whose sequence and structure are strikingly similar to NGF (Leibrock et al., 1989), will bear common stuctural features to the NGF receptor (Leibrock et al., 1989) as well as a common pathway for initial signal transduction (Borasio et al.,1989). In fact, recent evidence supports the existence of a common low-affinity receptor for NGF and BDNF with selective binding and biological response to the individual trophic factor being effected via the respective high-affinity receptors (Rodriguez-Tébar et al., 1990). It will be interesting to see if the newly cloned member of the NGF/BDNF neurotrophic factor family (Hohn et al., 1990; Maisonpierre et

al., 1990) also possesses this common feature in binding properties.

The NGF low-affinity receptor gene is a single copy gene (Chao et al., 1986) whose cDNA encodes a message of 3.8 kb (Johnson,D., et al., 1986; Radeke et al., 1987; Misko et al., 1988). The core protein is 386 aa after signal peptide cleavage and has a predicted molecular mass of 42 kDa, considerably smaller than that observed in crosslinking studies. Additional post-translational modifications such as N- and O-linked glycosylation account for most of this discrepancy. The receptor has a single membrane spanning domain of 22 aa with a long 222 aa extracellular domain and a 151 aa long cytoplasmic domain. The extracellular domain contains four cysteine-rich repeats which are conserved among human, rat and chicken species (Large et al., 1989) and the conservation of these negative charges is speculated to be involved in the binding of the highly positively charged NGF molecule (Hogue Angeletti and Bradshaw, 1971). The intracellular domain lacks the consensus sequence for an ATP binding protein and is too short to encode tyrosine kinase activity. However, it does contain a conserved PEST sequence (Large et al., 1989) which has been correlated with turnover rates of less than 5 hours (Rechsteiner et al., 1987). If there is rapid turnover then it could necessitate and account for the large intracellular pools of NGF binding sites observed (Ross et al., 1984; Raivich et al., 1985; Richardson et al., 1986; Marano et al., 1987).

Successful transfection of fibroblasts and melanoma cell lines with cDNA clones encoding the NGF low-affinity binding site yields cells expressing only the low-affinity form of the receptor (Chao et al., 1986; Johnson,D et al., 1986; Radeke et al., 1987). It is speculated that because these cells do not normally display high-affinity binding sites, they lack cellular components necessary to generate a high-affinity receptor, or, perhaps the cDNA isolated encodes only the low-affinity receptor and is not involved in high-affinity receptor formation. To resolve this NR18 cells, a mutant PC12 cell line that lack functional receptors and do not respond to NGF (Bothwell et al., 1980), were transfected with a full length human receptor cDNA (Hempstead et al., 1989). The transfectants displayed heterogenous binding of ^{125}I-NGF with Kds of $6x10^{-11}$M and $2x10^{-8}$M similar to NGF binding on normal PC12 cells (Sonnenenfeld and Ishii, 1985). The binding sites were categorized as functional because exposure of the cells to physiological concentrations of NGF specifically induced the protooncogene c-fos, recognized as an early response of normal PC12 cells to NGF (Curran et al., 1985 Kruijer et al., 1985) However, none of the morphological or differentiative responses of PC12 cells to NGF exposure were observed and are perhaps a limitation of this mutant cell line. The results of Hempstead et al., (1989) support the biochemical and molecular findings discussed in the previous paragraphs that both classes of receptor are related and probably share a common binding protein. Perhaps the ability to express functional high-

affinity receptors is limited to those cell types capable of performing the post-translational modifications and/or expressing the additional protein(s) necessary to form the high-affinity binding site. The exact nature of these modifications required to generate the high-affinity species of receptor remain speculative. Further, with no definitive structural information available on the NGF high-affinity receptor one should not preclude the possibility that the receptor may consist of two ligand-binding chains such as the high-affinity interleukin-2 receptor (Wang and Smith, 1987) with the yet unidentified additional binding subunit containing the signal transduction component.

Finally, the recent generation of transgenic mice carrying multiple copies of the human low-affinity NGF receptor gene, has shown that constructs containing significant amounts of flanking sequences are capable of directing high level expression in a tissue-specific manner (Patil et al., 1990). The resulting protein binds NGF, is recognized by the monoclonal antibody specific to the human receptor, and appears to be regulated in an appropriate manner following injury of the sciatic nerve. The ability of the flanking sequences to direct the receptor to appropriate regions should provide an interesting tool for the targeting of heterologous gene expression in the NGF-responsive subpopulations of neurons such as the cholinergic basal forebrain neurons involved in many neurodegenerative disorders.

4.0 MECHANISM OF ACTION OF NGF

4.1 DESCRIPTION

The mechanisms by which NGF elicits its numerous physiological actions are still not clearly delineated, even though much is known about specific aspects of the NGF response (reviewed in Greene and Shooter, 1980; Yanker and Shooter, 1982; Bradshaw et al., 1985; Fujita et al., 1989). The sequence of events by which NGF acts on responsive neurons involve synthesis, uptake via specific receptors located on the axons and terminals, and retrograde transport back to the cell soma. NGF is synthesized within peripheral nerve and even more abundantly in regions densely innervated by NGF responsive neurons in both the peripheral and central nervous systems (Shelton and Reichardt, 1984; Korsching et al., 1985; Shelton and Reichardt, 1986b; Whittemore et al., 1986; Large et al., 1986; Bandtlow et al., 1987; Heumann et al., 1987b; Ayer-LeLievre et al., 1988). NGF released from its synthesizing cells, interacts with specific receptors and is subsequently internalized (Olender and Stach, 1980; Levi et al., 1980; Layer and Shooter, 1983; Hosang and Shooter, 1987). Ligand-induced endocytosis of NGF and most if not all the physiological responses to NGF are mediated via the high-affinity receptors (Olender and Stach, 1980; Bernd and Greene, 1984; Hosang and

Shooter, 1987) with no evidence for internalization for cells displaying only low-affinity binding sites (Green et al., 1986;Radeke et al., 1987). The actual function of the low-affinity binding site is speculative. A small induction of the protein NILE by NGF has been observed in cultured Schwann cells (Seilheimer and Schachner, 1987) on which only low-affinity binding sites have been detected (Carbonetto and Stach, 1982; Zimmerman and Sutter, 1983; Taniuchi et al., 1988). Taniuchi et al., (1986) propose that the tremendous induction of low-affinity receptors on Schwann cells in the distal stump of injured peripheral nerves might serve to loosely bind and concentrate NGF and other homologous trophic factors (Rodriguez-Tébar et al., 1990) for presentation to regenerating axon tips. Little has been proposed for its function on intact neurons. Perhaps the abundance of low-affinity receptors on NGF-responsive neurons serves to convert the quest of NGF for its high-affinity receptors from a three dimensional search into a two dimensional search in the plane of the membrane, similar to the role proposed for the interleukin-2 low-affinity binding site (Wang and Smith, 1987).

Following endocytosis both NGF and its receptor are retrogradely transported back to the body (Hendry et al., 1974a,b; Stoekel et al., 1974; Yip and Johnson, 1983; Korsching and Theonen, 1983; Richardson and Riopelle, 1984; Abrahamson et al., 1987; Raivich and Kreutzberg, 1987; Johnson,E.M., et al., 1987) presumably as a complex, although this is not known. The

importance of this retrograde supply of trophic factor is demonstrated by the degenerative changes, including death, that results from surgical or pharmacological interruption of axonal transport reminiscent of that observed with NGF deprivation (Thoenen and Barde, 1980). To explore the role of the internalized NGF and its receptor, the intracellular pathways have been traced and their significance determined using biochemical perturbations of various subcellular compartments. Two components of the internalized NGF pathway have been described - one which is directed along with its receptor to lysosomes for degradation (Bernd and Greene, 1983; Hogue-Angeletti et al., 1982; Pioro et al., 1989) while the other appears to involve recycling of NGF to the extracellular milieu and possibly its receptor back to the cell surface (Eleveth and Bradshaw, 1988). The internalization process of bound NGF is very rapid with estimates of >80% of radioactivity specifically associated with PC12 cells being found within internal compartments 15 minutes after exposure to ^{125}I-NGF (Bernd and Greene, 1983). This would necessitate a large cytoplasmic pool of recycled and/or newly synthesized receptors for replenishment of surface receptors. In vitro radioautographic visualization of NGF high-affinity binding sites on frozen tissue sections have revealed such a pool (Richardson et al., 1986; Raivich et al., 1985).

It has been postulated that one of the purposes of ligand-receptor internalization would be to transport receptors to the

nucleus or to deliver ligand to pre-existing nuclear receptors the activation of which may result in a cascade of nuclear events regulating gene transcription (Burwen and Jones, 1987). The existance of nuclear NGF receptors is unresolved. While some have described their presence (Yankner and Shooter, 1979; Bernd and Greene, 1983), others have refuted the results of Yankner and Shooter stating that cytoskeletal contaminants in the nuclear preparations actually contained the supposed nuclear receptors (Schecter and Bothwell, 1982), or failed to visualize them ultrastructurally (Rohrer et al., 1982; Hogue-Angeletti et al., 1982). The failure to detect nuclear receptors ultrastructurally could be related to the exceedingly high concentrations of ^{125}I-NGF (1-4 nM) used. At high concentrations of ^{125}I-NGF the probability that the internal grain represents ^{125}I-NGF bound to a low-affinity site that is internalized as a result of constituitive recycling of membrane (Pastan and Willingham, 1985) increases. Additionally at these extremely high doses of ^{125}I-NGF, binding of ^{125}I-NGF to high-affinity receptors of homologous neurotrophic factors such as BDNF can occur (Rodriguez-Tébar et al., 1990), and if these receptors are internalized with NGF bound to them the intracellular route for the complex might differ from that of the NGFR-NGF complex. When a more physiological dose ^{125}I-NGF was used (200pM) (Bernd and Greene, 1983), a small but significant number of internalized silver grains were found at the nuclear membrane . At this concentration a considerably higher

proportion of ^{125}I-NGF molecules selectively bind to high-affinity sites (Sutter et al., 1979) and presumably gain entry into the cell by specific internalization of the high-affinity receptor-ligand complex. Perhaps the different modes of entry into the cell result in different intracellular routing pathways. It is not known whether the putative nuclear binding sites subserve a function, but evidence for lack of a functional internal receptor stems from initial work done investigating signal transduction mechanisms of the NGF response described below.

4.2 SIGNAL TRANSDUCTION

To determine whether the intracellular pool of cytoplasmic and putative nuclear receptors were involved in the signal by which NGF mediates its actions on the cell, NGF or its antibody was introduced into the cytoplasmic and nuclear compartments of PC12 cells thereby circumventing surface membrane receptors (Heumann et al., 1981; Seeley et al., 1983). From the lack of morphological or biochemical response it was concluded that freely accessible NGF does not act on cytoplasmic or nuclear target sites. Moreover, blockade of lysosomal enzymes or inactivation of lysosomal activity by altering pH do not change normal NGF effects on PC12 cells, thus making it unlikely that a proteolytic degradation fragment released from lysosomes is an active component of the response (Layer and Shooter, 1985; Heumann et al., 1984; Eleveth and Bradshaw, 1988). Therefore,

NGF must act via a second messenger(s) activated following surface membrane interactions, the exact nature of which remains unknown.

Previous studies have systematically eliminated cAMP, calcium influx or sodium transport as likely candidates for the NGF-receptor transducer, however do not exclude the possibility of a secondary role for these second messengers in certain actions of NGF (reviewed in Fujita et al., 1989; Thoenen et al., 1987). More recent evidence indicates that signal transduction might effect itself by the coupling of a ras gene product or G protein with the low-affinity binding site. Neurite outgrowth on PC12 cells, similar to that observed by extracellular exposure to NGF can be induced by direct injection of ras proteins into the cells (Bar-Sagi and Feramisco, 1985) and suppressed by injected ras protein antibodies in the presence of NGF (Hagag et al., 1986). Furthermore, ras proteins have also been shown to mimic NGF effects on embryonic survival and neurite outgrowth (Borasio et al., 1989). G proteins due to their close sequence homology to ras gene products (Barbacid 1987) and role in high-affinity receptor formation and transmembrane signalling (Freissmuth et al., 1989) are also be implicated as a possible effector. Isolation of high-affinity binding sites sufficient for sequencing or protein analysis would aid in resolving this mystery of signal transduction.

5.0 ROLE OF NGF IN SENSORY NEURONS

Specific properties of primary sensory neurons such as an axonal process with both a central and peripheral branch, containment within ganglia and easy accessibility make them ideal candidates for studies aimed at defining and elucidating neurotrophic and neurotropic concepts. The main areas in which a role for NGF in sensory neurons has been postulated include:

(i) cell viability;

(ii) chemotropic guidance of fibers into their target regions;

(iii) maintenance and regulation of differentiated phenotype, morphological properties and cellular function;

(iv) and neuronal plasticity.

5.1 CELL VIABILITY

Early in development when sensory neurons are still actively growing and differentiating, interactions between peripheral target derived trophic factors and the nerve cell body play a critical role in determining its fate (Hollyday and Hamburger, 1976). The initial implication of NGF in this role stems from in vitro work on dissociated chick embryonic sensory ganglia (E_8-E_{11}), which fail to survive unless nanogram quantities

of NGF are included in the medium (Levi-Montalcini and Angeletti, 1963). Subsequent in vivo experiments confirmed this essential role. Deprivation of NGF by either administration of NGF antiserum to rat fetuses (Aloe et al., 1981) or autoimmunization of pregnant rats against endogenous NGF results in massive neuronal loss (up to 80%) in the dorsal root ganglia (Johnson,E.M., et al.,1980; Johnson,E.M., et al., 1986). The effect appears to be specific for those sensory neurons derived from the neural crest and does not influence death of placodally-derived sensory neurons such as the nodose neurons (Pearson et al., 1983; Davies and Lindsay, 1985; Rohrer et al., 1988). During the developmental period of normally occurring cell death, the restricted amount of trophic support limits the number of surviving neurons. If exogenous NGF is administered at this time point it can retard or prevent this phenomenon in vivo (Hamburger et al., 1981; Hamburger and Yip, 1984).

The neuronal dependency on NGF for survival diminishes with age (Johnson,E.M., et al., 1980; Barde et al., 1980; Millareulo et al., 1988). An hypothesis put forth to explain the diminished requirement for peripherally-derived NGF in neonates is the presence of a centrally-derived source of trophic support. Although earlier studies revealing an NGF-reversible loss of 50% of sensory neurons in neonate DRG following dorsal rhizotomy (Yip and Johnson, 1984) strongly supported this hypothesis, recent experiments show that no neuronal loss occurs in neonates in response to dorsal rhizotomy for up to 30 days after injury

(Himes and Tessler, 1989). The results of the latter study cast serious doubt on the contribution of centrally-derived trophic factors to neuronal survival. Chronic deprivation of NGF in adult rats and guinea pigs by exposure to NGF antibodies did not produce DRG neuronal death nor increase it following crush injury (Schwartz et al., 1982; Rich et al., 1984). These findings are supported by in vitro studies indicating that adult rat sensory neurons do not require NGF for survival (Lindsay, 1988). Yet, pharmacological adminstration of NGF to the transected peripheral nerve stump of neonate and adult rats prevented normal injury-induced neuronal loss for up to six weeks post-injury (Yip et al., 1984; Rich et al., 1987; Otto et al., 1987). It is interesting that the percentage of neuronal cell loss prevented by the concomitant administration of NGF at the time of injury roughly equals the percentage of adult neurons that do not survive the in vitro plating procedure (Lindsay, 1988) and might represent a population more susceptible to death following injury that was not detected in the antibody studies. Thus, even though adult sensory neurons in vitro (≈ 80% of total survive the plating procedure) no longer require NGF for survival extrapolation of this to the entire population might not be valid.

5.2 NEUROTROPISM

The ability of NGF to promote survival and neurite outgrowth

of embryonic ganglia coupled with its chemotropic properties both in vivo and in vitro (Levi-Montalcini and Angeletti, 1968; Levi-Montalcini, 1976; Gundersen and Barrett, 1979; Gundersen and Barrett, 1980; Campenot, 1982) led to the hypothesis that NGF might play a role in initiation of outgrowth and guidance of developing neurites into their proper target regions. This type of a neurotropic function for NGF was disproved. Innervation of the mouse whisker pad by trigeminal ganglion neurons during development was used as the model (Davies and Lumsden, 1984; Davies et al., 1987). First, it was shown that the initial outgrowth of trigeminal fibres does not require NGF (Davies and Lumsden, 1984). In fact, NGF mRNA and protein in the target are detected only at the time of target innervation (Davies et al., 1987). With regard to receptors, a low basal rate of NGFR message expression in the neuronal component of the ganglion is present before innervation and this is upregulated during target innervation (Wyatt et al., 1990), at which point NGF receptors can be detected radioautographically with ^{125}I-NGF (Davies and Lumsden, 1984). NGF receptor message is also expressed during development in the dermal mesenchymal portion of the target region where large numbers of trigeminal axons terminate (Wyatt et al., 1990). It has been postulated that these receptors might serve to concentrate NGF synthesized there and in the overlying epithelium (Bandtlow et al., 1987) so that it is available to incoming axons, as has been proposed for the role of NGF low-affinity receptors that are induced in the distal stump of an

injured peripheral nerve (Taniuchi et al., 1986). Finally, the tight temporal link between target innervation, responsiveness to NGF, synthesis of NGF and display of neuronal NGF receptors, raises the question whether innervation of the target triggers transcription of the NGF gene. Rohrer et al., (1988) by removal of portions of the neural crest and neural tube in three day old chick embryos, thereby removing the innervating source, demonstrated that initiation of NGF mRNA synthesis proceeds in complete absence of innervation. What triggers the initial transcription of NGF or its receptor remains unknown.

5.3 NEURAL FUNCTION AND PLASTICITY

The role of NGF in sensory neurons is not limited to neuronal survival during development. NGF regulates a number of properties in developing and mature neurons such as peptide and enzyme synthesis, perikaryal and axonal volume, and action potential duration (Otten et al., 1980; Kessler and Black, 1980; Rich et al., 1984; Csillik et al., 1985; Chalazonitis et al., 1987; Lindsay and Harmar, 1989; Matheson et al., 1989).

Substance P regulation is the best characterized of the NGF-regulated peptides in vivo, and serves as a paradigm for other peptide molecules although calcitonin gene-related peptide (CGRP) synthesis has recently been shown to be regulated by NGF in vitro (Lindsay and Harmar, 1989). Substance P synthesis is regulated by NGF throughout development into adulthood.

Administration of NGF in neonates or adults increases levels of substance P (Kessler and Black, 1980; Otten et al., 1980; Geodert et al., 1981), or counteracts its loss following injury (Fitzgerald et al., 1985). Increases in the substance P content in sensory neurons are also observed when sympathetic fibres competing for the same source of NGF are eliminated (Kessler et al., 1983). Conversely, antibodies to NGF decrease substance P levels (Ross et al., 1981, Schwartz et al., 1982).

The exact cellular location of the endogenous source of NGF for adult sensory neurons is unresolved (Bandtlow et al., 1987). NGF mRNA is expressed within nerve (Shelton and Reichardt, 1984), but localization by in situ hybridization has been unsuccessful due to the extremely low copy numbers of NGF mRNA (≈1 in every 10 million poly A+RNA) even in the densely innervated target regions (Bandtlow et al., 1987). However in neonate rat sciatic nerve where NGF mRNA levels are 10-15 times higher than in adults virtually all Schwann cells in the sciatic nerve irrespective of ensheathment around an NGF-responsive axon expressed message for NGF as detected by in situ hybridization. In addition, the epithelium and adjacent mesenchyme (region densely innervated by sensory neurons) in the whisker pad showed a strong hybridization signal (Bandtlow et al., 1987). Thus both the endoneurial cells and the target region are potential sources of endogenous NGF. The endogenous NGF protein levels in adult rat sciatic nerve have been calculated to be in the 100pM range (Heumann et al., 1987) sufficient for interaction

with high-affinity receptors. The high-affinity NGF receptors that would bind the endogenous NGF are localized on approximately 40% of adult rat primary sensory neurons as visualized following incubation of DRG sections in picomolar concentrations of ^{125}I-NGF (Richardson et al., 1986) or specific retrograde transport of ^{125}I-NGF back to the cell body (Richardson and Riopelle, 1984). The rat NGF-responsive neurons are presumably homologous to the dorsomedial population of chick primary sensory neurons that have also been shown to display specific NGF binding sites (Raivich et al., 1985, 1987). Binding is seen over the dorsal root entry zone and specific high-affinity uptake and retrograde transport of ^{125}I-NGF by both central and peripheral NGF-responsive processes of the sciatic nerve in adult rat has been demonstrated (Richardson et al., 1986; Richardson and Riopelle, 1984).

Following peripheral nerve injury there is a ubiquitous induction of mRNA for both NGF and its low-affinity binding site by Schwann cells in the distal stump and tip of the proximal stump associated with degenerating axons (Taniuchi et al., 1986,1988; Raivich et al., 1987; Heumann et al., 1987). The induction of NGF protein and low-affinity binding site during Wallerian degeneration of the distal stump appears to be differentially regulated (Heumann et al., 1987b). The time course of NGF mRNA induction correlates with activated macrophage invasion (Heumann et al., 1988) which along with the macrophage-secreted interleukin-1 have been shown to increase

NGF transcription and message stabilization in rat sciatic nerve segments and cultured fibroblasts in vitro (Heumann et al., 1987b; Lindholm et al., 1987, 1988). In contrast, mRNA for the low-affinity NGF binding site was not affected by the presence of activated macrophages (Heumann et al., 1987b), but rather appears to be regulated by axonal-glial contact, falling to basal levels upon contact with regenerating fibers (Raivich et al., 1987; Taniuchi et al., 1988).What is the significance of this induction of NGF synthesis ? The tremendous source of NGF in the distal stump of an injured nerve could play a role in reversing many of the NGF-regulated retrograde axotomy responses of sensory neurons such as atrophy and peptide loss as evidenced by the ability of exogenous NGF to counteract these changes following transection of the sciatic nerve (Rich et al., 1984, 1987; Fitzgerald et al., 1985).

Whether the NGF-laden substrate actually influences the regeneration of the injured fibers is debatable. Regeneration of sensory neurons can occur in the absence of Wallerian degeneration (Lunn et al., 1989) and is not dependant on NGF (Diamond et al., 1987). A role for NGF in neural plasticity however, may involve its ability to influence collateral innervation of denervated regions by NGF-responsive neurons. Collateral sprouting by sensory neurons into denervated regions could be prevented either by autoimmunization of adult guinea pigs to endogenous NGF or injection of antibodies into the area (Owen et al., 1989; Diamond et al ., 1987) and may be indicative

of an ongoing role for NGF in regulation of terminal fields.

The major role of NGF in adult sensory neurons appears to be one of maintaining differentiated phenotype, such as peptide expression and neuronal size. But, virtually nothing is known of the cellular mechanisms underlying the role of NGF in intact and injured sensory neurons in vivo, especially with respect to the ability of a neuron to bind NGF with high-affinity. In addition, this subpopulation of NGF-responsive neurons is uncharacterized in vivo. The remaining chapters of this thesis will attempt to broaden our understanding of how NGF influences this population of neurons. A cellular approach will be taken to first identify and characterize the population of NGF-sensitive neurons, then study how these neurons change in their responsiveness to NGF and various other markers of neural injury following sciatic nerve transection which interrupts the normal retrograde transport of NGF. Finally, the ability of exogenous NGF to counteract these injury-induced changes will be examined. Hopefully the results will give new insights into how trophic molecules exert their effects and their potential role in restoration of function following injury.

CHAPTER 2

QUANTITATIVE RADIOAUTOGRAPHY FOR NGF RECEPTORS

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1. INTRODUCTION

All of the known physiological actions of nerve growth factor (NGF) are mediated through receptors found in the cell membrane of target neurons and half-maximally saturated by picomolar concentrations of NGF. More abundant binding sites with much lower affinity serve unknown functions. Radioautography following incubation of tissue sections with radioiodinated NGF has been used to survey the cellular distribution and binding properties of NGF receptors in the nervous system (Richardson et al, 1986; Raivich and Kreutzberg, 1987; Riopelle et al, 1987). This review describes the technique used in this laboratory and discusses some of its potentialities and pitfalls.

2. PREPARATION OF RADIOLIGAND

NGF is prepared by a standard protocol (Mobley, 1976) that exploits a change in pI of NGF following dissociation from the 7s complex. β-NGF is eluted from the second column by a gradient of 0-0.4 M NaCl in 0.05 M Tris-HCl buffer pH 9.0 (Chapman et al, 1981), dialyzed against 0.2% acetic acid, concentrated to > 1 mg/ml, and stored at < -20°C in small aliquots.

Radioiodination is effected in the radioisotope shipping vial by mixing 2 mCi carrier-free $Na^{125}I$ (20 μl), 0.3 ug lactoperoxidase (10 μl), 10 μg NGF, and 0.003% H202 (10 μl). An additional 10 μl of H202 is added after 30 minutes. 0.1 M sodium phosphate buffer (pH 7.4) is used to dilute all reagents and to bring the final volume to 75 μl. The reaction is allowed to proceed at room temperature for 1 hour. A small aliquot is removed for precipitation with 10% trichloroacetic acid showing, typically, that the reaction efficiency (^{125}I bound to protein) is 85-95% and the specific activity of the ^{125}I-NGF is 100-200 $\mu Ci/\mu g$.

To separate ^{125}I-NGF from free ^{125}I and reagents, the reaction mixture is loaded onto a small cation-exchange cartridge (Waters CM Sep-Pak), equilibrated with 0.05 M acetate buffer (pH 4.0). The cartridge is washed with 20 ml of 0.05 M Tris-HCl buffer (pH 7.4) and ^{125}I-NGF is eluted by 0.05 M Tris-HCl (ph 9.0) containing 1 M NaCl and 1 mg/ml bovine serum albumin. This separation procedure has diminished non-specific labelling on tissue sections by at least 50% in comparison with earlier studies (Richardson et al, 1986; Riopelle et al, 1987) when ^{125}I-NGF was prepared with dialysis as the last step (Sutter et al, 1979). Best results are obtained when ^{125}I-NGF is used on the day of preparation.

The radioligand can be further purified by HPLC on a

cation-exchange column (mono-S, Pharmacia). Although not yet used routinely, this step permits accurate calibration of the specific activity of the radioligand and opens the possibility of separating free, monoiodinated, and diiodinated NGF.

3. PREPARATION OF SECTIONS

After systemic perfusion per aorta with cold phosphate buffered saline but no fixative, tissues of interest are quickly removed, frozen in N-methyl butane and stored at -85°C. Cryostat sections, 5-10μm thick are mounted on cold gelatin-coated slide, allowed to dry for 2 hours at -24°c in the cryostat, and stored with dessicant at -85°C until use (usually in the next few days). The resolution in radioautography is, of course, related to the thickness of sections because approximately one-half of the silver grains will lie within a circle with diameter equal to this thickness (Salpeter et al, 1969). Where absolute values of bound radioligand are required, radioautographic standards are prepared from brain paste mixed with known quantities of (125)-iodine (Unnerstall et al, 1982)

4. INCUBATION

Slides are removed from the freezer, briefly warmed on a hot plate to ensure good adherence, and incubated, for 90 minutes at room temperature in a solution of radioligand diluted

in phosphate-buffered saline (0.1 M, pH 7.4), containing magnesium chloride (0.5 mM), cytochrome C (1 mg/ml), phenylmethylsulphonylfluoride (0.5 mM) and leupeptin (4 µg/ml). The incubation procedure was developed empirically without rigorous optimization of each condition. The concentration of radioiodinated NGF is 40-60 pM for best ratio of neuronal to background labelling but can be varied outside that range to examine binding properties. Sections are rinsed in 5-6 changes of cold buffer for 3 minutes and fixed for 10 minutes in mixed aldehydes (3% formaldehyde, 1% glutaraldehyde). For neurotransmitter receptors, great care is needed to prevent dissociation of radioligand from receptor during washing and other post-incubation procedures (Wamsley et al, 1983). For NGF, such precautions are less critical because ligand and receptor dissociate very slowly (Sutter et al, 1979). Sections are rinsed, defatted in a graded series of alcohols and xylene, rehydrated, dried with a stream of cool filtered air, and left overnight at 4°C with dessicant. The sections are then dipped in radiosensitive emulsion (Kodak NTB2), exposed in light-proof boxes at 4°C for 2-4 days, and developed (Kodak D-19). Sections are counterstained with filtered thionin with care to avoid precipitated or overstaining that might interfere with measurements of grain density. After coverslipping, the sections can be surveyed with darkfield microscopy (Figure 1) or quantified with oil-immersion light microscopy. A scanning condenser (Mårtensson) is very useful for darkfield

photomicroscopy at low magnification.

In radioautographs of rat lumbar dorsal root ganglia (DRG) used for quantification, grain densities over heavily labelled neurons are 10-20 times greater than densities over non-neuronal regions and 2-15% of the neuronal cross-sectional area is covered by grains. Longer exposure times and high grain densities tend to be better for photography.

5. QUANTIFICATION

A computer-assisted image-analysis system has been adapted to measure cross-sectional areas and grain densities of large numbers of neurons. For this purpose, slides are examined under oil-immersion light microscopy and images from a charge-coupled device are transferred in real time to a frame-grabber (Coreco) in an IBM-XT computer. Sections are examined without knowledge of the conditions before or during incubation. Images of single neurons with nucleoli are digitized in a 512-480 matrix at 7 bits/pixel and subsequently analyzed by a programme written by W.G. Tatton (Playfair Neurosciences Unit, Toronto) and further modified in this laboratory. The window within which a pixel is considered to be labelled is adjusted interactively so that the area per grain is appropriate and consistent. The circumference of the selected neurons is traced and the cross-sectional area and percentage of area covered by grains is measured. Where

cytoplasmic staining is light in DRG preparations, the position of satellite cells provides a guide to the periphery of the neurons. To estimate non-specific labelling, measurements are made in regions of tissue not containing labelled neurons.

Because the area covered by grains is measured rather than the actual number of grains, a correction is made for grain overlap.

If $x = da$

where d = grains/unit area,

a = area of each grain

and f = fraction of neuronal area covered by grains,

then $f = x - x^2 + x^3 - x^4 + x^5 \ldots$

$f + fx = x$

$x = f/(1-f)$

The corrected parameter x is linearly related to the grain density and concentration of bound radioligand.

6. DISTRIBUTION OF NGF RECEPTORS

Most of our studies have been on the rat where NGF binds to the cell bodies of virtually all sympathetic neurons, some primary sensory neurons, large cholinergic neurons in the forebrain, and several clusters of neurons in the brainstem (Richardson et al, 1986; Figure 1). With the current radioautographic technique, NGF receptors that are known to be present on axons in peripheral nerves and the hippocampus cannot be visualized. However, NGF-binding sites are visible on the central axons of sensory neurons in the spinal cord and brainstem. Reasons for the failure to see some axonal receptors are unknown but might include masking by endogenous NGF or low receptor density on small structures. It is also unclear why NGF binds predominantly to perikarya whereas neurotransmitter binding can seldom be localized to individual cell bodies (Kuhar, 1987). Differences in the subcellular distribution of receptors for growth factors and neurotransmitters might reflect differences in their turnover rates.

In general, the distribution of perikaryal NGF receptors in the rat is similar whether they are mapped by receptor radioautography or immunohistochemistry with monoclonal antibodies to the low-affinity receptor (Taniuchi et al, 1986). The latter technique is perhaps more sensitive and is more readily adapted for electron microscopy. However, in the

striatum, cholinergic neurons that bind ^{125}I-NGF at high affinity are not usually detected in immunohistochemical studies (Hefti et al, 1986; Eckenstein, 1987). This discrepancy in results with the two techniques raises the possibility that the ratio of high- to low-affinity binding sites differs among neuronal populations.

The binding of NGF varies among primary sensory neurons in the same DRG (Figure 2). To quantify such variation, cross-sectional areas and grain densities are measured for several hundred representative neurons with visible nucleoli. In scatter diagrams where the two parameters are used as coordinates for points representing individual neurons (Figure 2, lower left), the density of binding is shown to be heterogeneous for all sizes of neurons. Individual neurons can usually be classified as "labelled" or "unlabelled" if they are large but not if they are small. The ambiguity for small neurons probably arises for technical reasons rather than from an extensive overlap between populations. Because emissions from the tissue often strike points in the emulsion which do not overlie their source, the measured grain density for any small neuron could be falsely high or falsely low: thinner sections might better resolve the two categories. In histograms of grain density (Figure 2, lower right), the data again suggest the existence of two neuronal populations with and without high-affinity receptors. The width of the peak for the "labelled"

population can be attributed to heterogeneity among neurons and emission scatter. The fact that the mean density for "unlabelled" neurons slightly exceeds background density could be artefactual or could indicate that all sensory neurons have a few low-affinity binding sites. However, analysis of the concentration dependence of binding to the most lightly labelled DRG neurons clearly shows them to lack high-affinity site (Verge, unpublished observations). The population of sensory neurons that is responsive to physiological concentrations of NGF can probably be better defined by correlative immunohistochemistry with a panel of antibodies (Dodd et al, 1983).

7. ANALYSIS OF BINDING PROPERTIES

The binding properties of receptors in a group of neurons can be examined by incubation of adjacent tissue sections with a range of concentrations of NGF. For this purpose, radioligand is varied in amount or used at a single low concentration with different amounts of unlabelled NGF. The first strategy is cumbersome, requiring at least two exposure times and two sets of tissue standards, but proved useful in the basal forebrain (Riopelle et al, 1987) where the lightly labeled neurons in displacement studies are difficult to distinguish from unlabelled neurons. In DRG, the second method with radioiodinated NGF as a tracer is highly satisfactory. The four

graphs in Figure 3 all show the same data obtained from measuring neuronal grain densities in sections of rat L5 DRG incubated with radioiodinated NGF at 20 pM and unlabelled NGF at 0-2560 pM. In each section, representative neurons were counted from the most heavily labelled 5-10% of the entire population. The upper two graphs show displacement profiles in which binding of radioligand is half-maximally displaced by unlabelled NGF at 100-200 pM. Note the non-sigmoid shape of the curve and the non-linearity on logit-log plotting which are inconsistent with the presumption of a single binding site (Akera & Cheng, 1977). When total (neuronal) and non-specific (background) binding of NGF (both labelled and unlabelled) are plotted against the concentration of NGF (labelled plus unlabelled) in the incubation medium (lower left), specific binding is seen to be unsaturated even at 2.5 nM. Scatchard plotting (lower right) with curve-fitting (Minnemann et al, 1979) indicates two binding sites, one of which has a dissociation-equilibrium constant (Kd) of approximately 50 pM and Bmax of 15 fmol/mg.

The number of high-affinity receptors in a heavily labelled perikaryon of average size is calculated to be in the order of 300,000. This high density, much greater that on intact fetal chick sensory neurons, reflects a large cytoplasmic pool of receptors, probably freshly synthesized and awaiting axonal transport but possibly transported retrogradely as part of ligand-receptor complexes. NGF receptors may be stored in

relative abundance because they are continually internalized with NGF on an equimolar basis and not locally recycled (Hosang & Shooter, 1987).

The existence of two binding sites and the characteristics of the high-affinity site on adult rat neurons are fully compatible with data regarding NGF receptors on fetal chick neurons in tissue culture (Sutter et al, 1979; Godfrey & Shooter, 1987). Also, the binding properties of the high-affinity NGF receptor match the concentration of NGF in the sciatic nerve (Heumann et al, 1987). Although it is still unknown what molecular configuration or associated molecule converts the NGF receptor to its high-affinity state (Green & Greene, 1986; Johnson et al, 1986; Radeke et al, 1986), the necessary conditions can apparently be satisfied in the cytoplasm without insertion of the receptor into the axolemma.

Why substantial binding at low affinity is seen in these radioautographs is difficult to explain. Given that the concentration of ^{125}I-NGF is 40 pM and Kd for the low affinity receptor is 2 nM, only 2% of low-affinity sites should be occupied at equilibrium and, after a 3-minute rinse, almost all of the radioligand that was bound to low-affinity sites should have dissociated (Sutter et al, 1979). The results cannot be entirely attributed to heterogeneity of the radioligand because the ratios of high- to low-affinity binding differ for neurons

and Schwann cells (Figure 4). Understanding of slowly dissociating low-affinity binding demands a more comprehensive theoretical basis for kinetic and steady-state binding than simple equilibrium between ligand, receptor and ligand-receptor complex (Godfrey & Shooter, 1987; Wiley & Cunningham, 1981). In one model that can be expressed mathematically, some receptor-ligand complexes are assumed to be sequestered to a slowly dissociating form. If r is the ratio of the rate constants for sequestration and subsequent hydrolysis of the complex, and hydrolysis is much slower than dissociation of the unsequestered complex, then Scatchard plotting will yield an apparent steady-state constant of Kd/(1+r). Even though low-affinity binding is of uncertain biological importance and perhaps partially artefactual, it must be taken into account to estimate accurately the density and affinity of high-affinity receptors.

8. SUMMARY

Radioautography with radioiodinated NGF is currently the only method for confident mapping of the high-affinity NGF receptor in tissues. Quantitative radioautographic studies confirm the classical binding properties (Sutter et al, 1979) are valid for adult mammalian neurons and permit developmental and pathological changes to be analyzed (Raivich et al, 1985; Verge et al, 1986; Raivich et al, 1987). Radioautography complements other techniques for studying the NGF receptor and

offers cardinal potentiality to quantify the binding sites of identified cells in tissues.

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FIGURE 1. Darkfield photomicrographs of cryostat sections that have been incubated with 50 pM ^{125}I-NGF show labelled neurons in the basal forebrain (upper left, magnification x 20), striatum (upper right, magnification x 14), and medullary reticular formation (below, magnification x 14). All sections are from rats. In the basal forebrain and striatum, the distribution of neurons with NGF receptors closely resembles the distribution of cholinergic neurons. Note also binding to fibres in the spinal tract of the trigeminal nucleus.

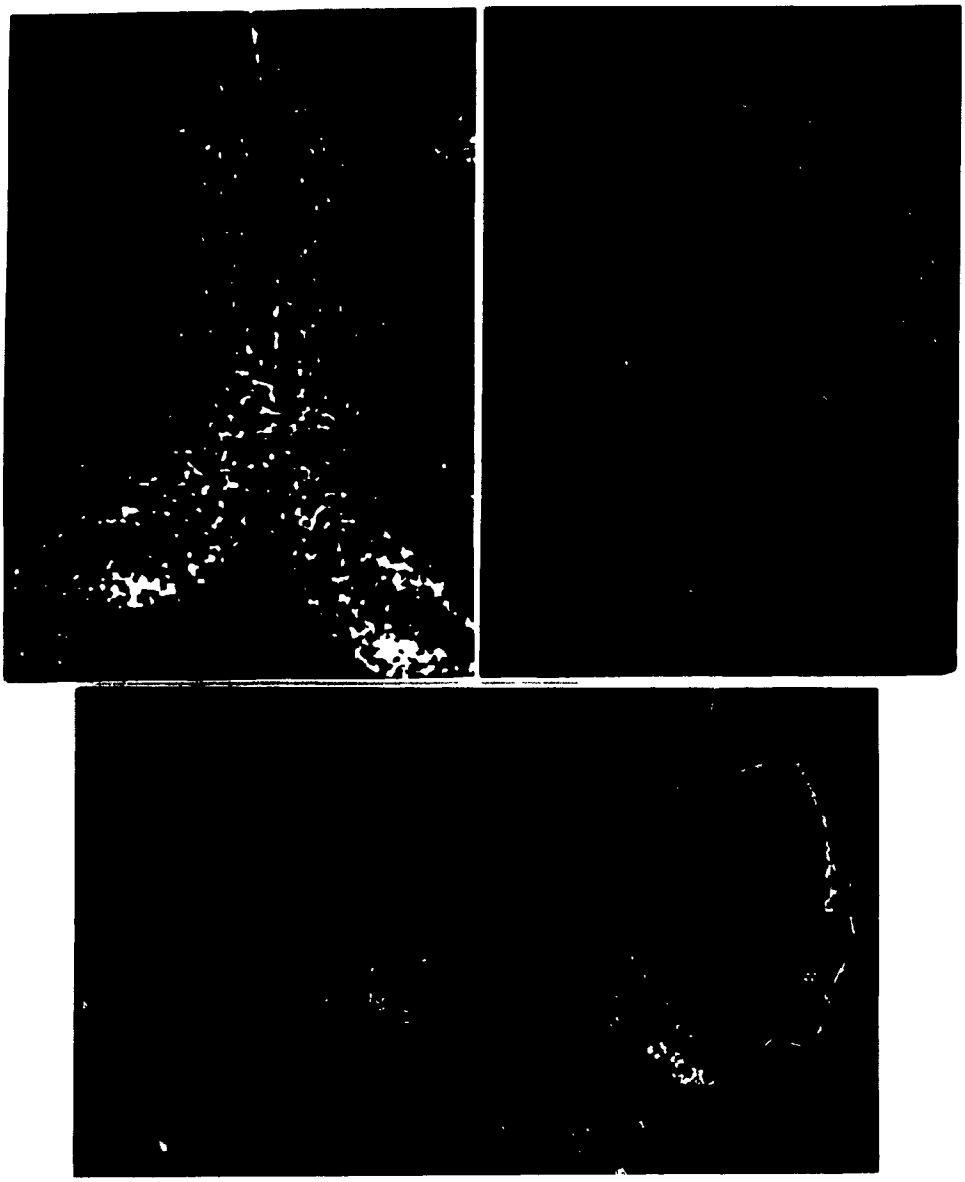


FIGURE 1

FIGURE 2. In light and darkfield photomicrographs of the same area of a radioautograph of a rat L5 DRG (above, magnification x 240), it can be seen that some neurons are heavily labelled whereas others are at background levels. Most neurons in the field could readily be categorized as "labelled" or "unlabelled". Two neuronal populations can also be discerned in the scattergram (n = 245 neurons) and histogram (n = 385 neurons) showing data from other animals (below). The two curves in the histogram were fitted by a maximum likelihood programme (Lawson, 1979). The concentration of ^{125}I-NGF was 20-28 pM in all 3 studies.

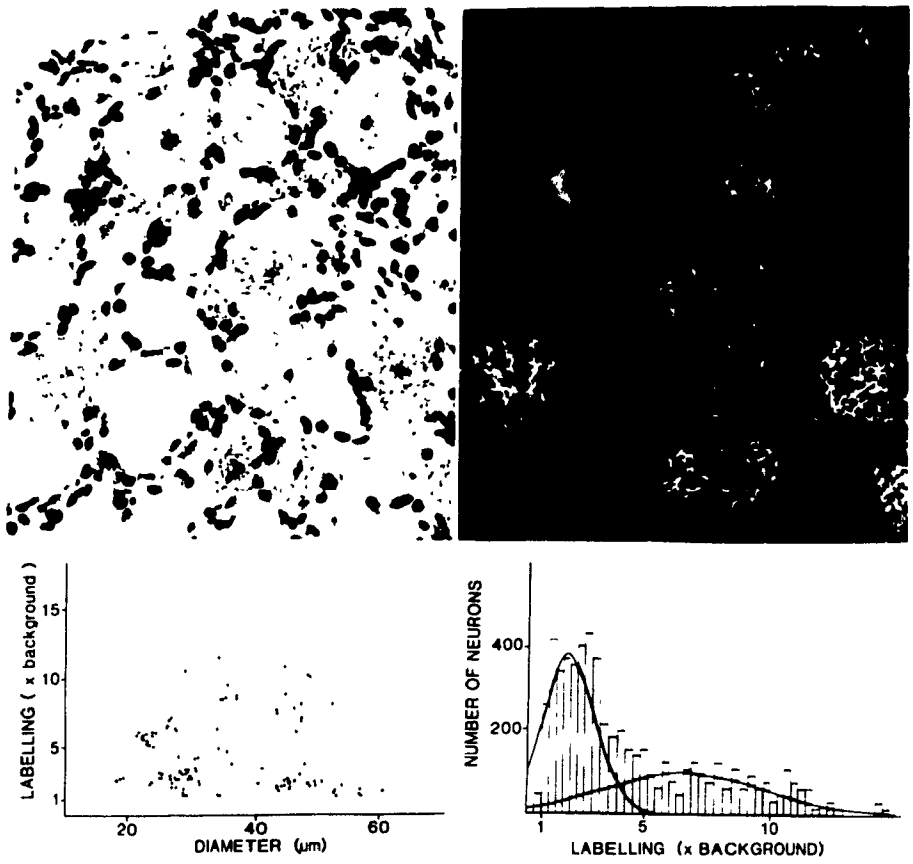

LABELLING (x background)
15
10
5
1
20
40
60
DIAMETER (µm)
NUMBER OF NEURONS
400
200
1
5
10
LABELLING (x BACKGROUND)

FIGURE 2

FIGURE 3. Series of adjacent sections of 3 rat L5 DRG were incubated with 20 pM radioiodinated NGF plus 0-2560 pM unlabelled NGF. 10-20 of the most heavily labelled neurons in 3 sections were analyzed for each concentration of unlabelled NGF. The same data are presented in four different manners. In the upper two diagrams, the binding of labelled NGF is plotted: "Labelling index" refers to the percentage of cross-sectional area covered by grains with correction factor applied. In the lower two diagrams, the binding of labelled plus unlabelled NGF is used. (m ± s.e.m.)

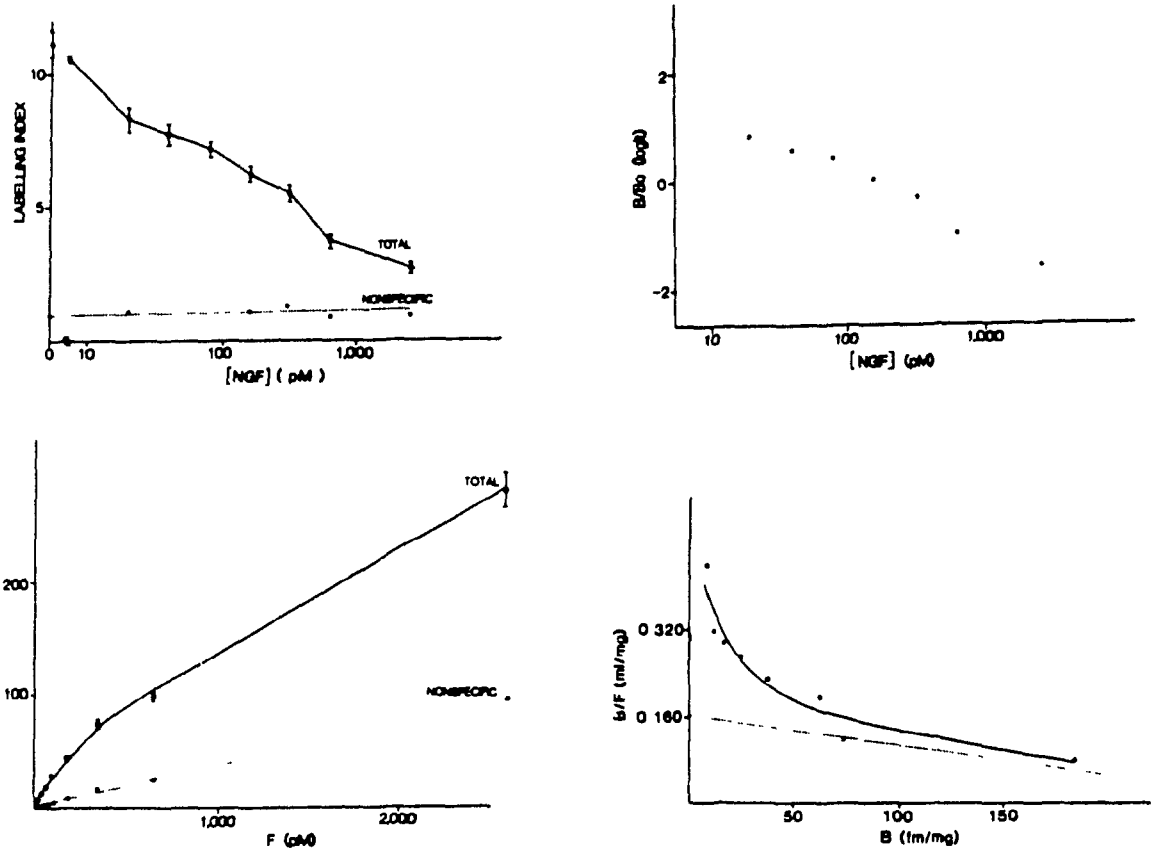
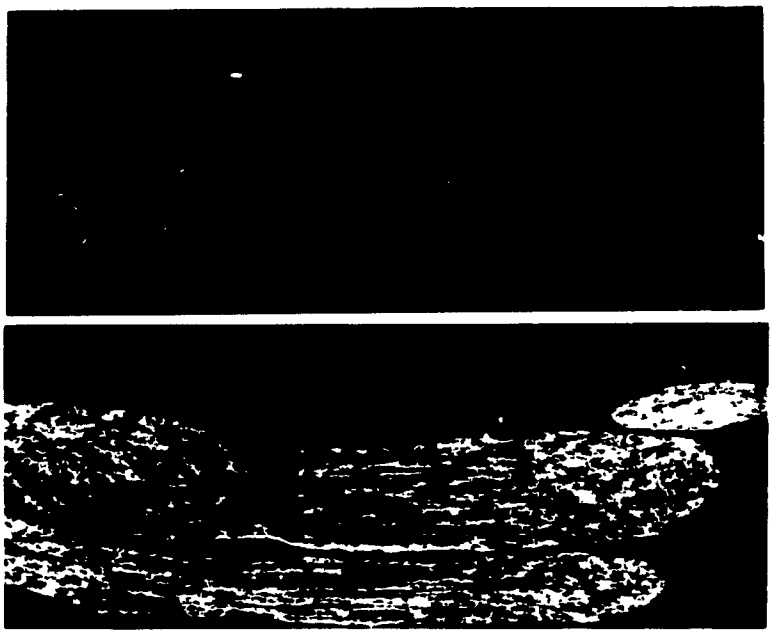

LABELLING INDEX
10
5
0
10
100
1,000
TOTAL
NONSPECIFIC
[NGF] (pM)
B/Bo (log)
2
0
-2
10
100
1,000
[NGF] (pM)
B (fm/mg)
200
100
TOTAL
NONSPECIFIC
1,000
2,000
F (pM)
B/F (ml/mg)
0 320
0 160
50
100
150
B (fm/mg)

FIGURE 3

FIGURE 4. Darkfield photomicrographs (magnification x 21) show ^{125}I-NGF binding to a normal rat sciatic nerve (above) and the distal stump of a nerve cut 5 days previously (middle). Note that binding sites on Schwann cells are induced by Wallerian degeneration (Taniuchi et al, 1986) and that the displacement profile for Schwann cells differs from that for neuronal binding.



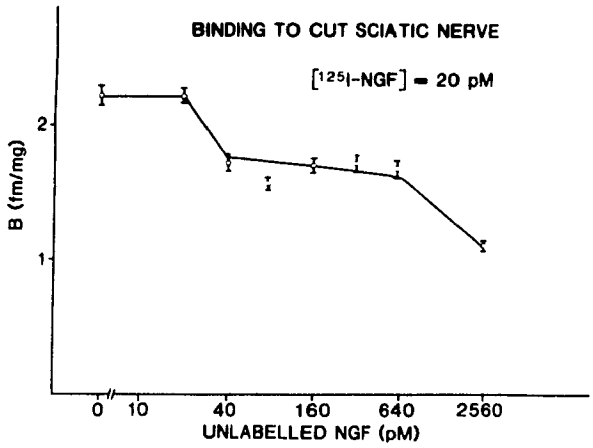

BINDING TO CUT SCIATIC NERVE
[125I-NGF] = 20 pM
B (fm/mg)
2
1
0
10
40
160
640
2560
UNLABELLED NGF (pM)

FIGURE 4

CHAPTER 3

HISTOCHEMICAL CHARACTERIZATION OF SENSORY NEURONS WITH HIGH-AFFINITY RECEPTORS FOR NERVE GROWTH FACTOR

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Running title: sensory neurons with NGF receptors

Key words: NGF receptor, neuropeptide, acid phosphatase, sensory neuron, co-localization

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SUMMARY

Approximately one half of the neurons in lumbar dorsal root ganglion of adult rats display high-affinity receptors for NGF (nerve growth factor). To ascertain which types of sensory neurons are potentially responsive to NGF, adjacent cryostat sections of rat dorsal root ganglia were processed either for NGF-receptor radioautography or by one of four histochemical procedures. Histograms of densities of neuronal labelling by radioiodinated NGF were examined for subpopulations of lumbar sensory neurons with thiamine monophosphatase enzyme activity or with immunoreactivity for CGRP (calcitonin gene-related peptide), substance P, or somatostatin. Virtually all neurons with strong CGRP immunoreactivity had high-affinity NGF binding sites although some neurons with faintly positive CGRP immunoreactivity lacked such NGF binding. A subpopulation of large neurons, approximately 5% of total, had dense labelling by ^{125}I-NGF, but were not stained by this immunohistochemical technique for CGRP. Of the three major populations of small neurons, those with substance P immunoreactivity were consistently and heavily labelled by radioiodinated NGF whereas those with somatostatin immunoreactivity or thiamine monophosphatase activity were not specifically labelled by radioautography.

For these primary sensory neurons in mature rats, the genes for substance P and CGRP seem to be strongly expressed only in neurons capable of responding to NGF. On the other hand, neurons containing somatostatin and thiamine monophosphatase invariably lack high-affinity NGF receptors.

INTRODUCTION

Primary sensory neurons with cell bodies in dorsal root ganglia (DRG) are homogeneous in their origin from the neural crest (Le Douarin, 1980) yet heterogeneous in their connectivity and expression of neuropeptides, enzymes, and cell-surface antigens (Hokfelt et al., 1976; Nagy & Hunt, 1982; Dodd et al., 1983; Lee et al., 1985; Goedert & Hunt, 1987; Ju et al., 1987). Previous studies indicate that approximately one half of the lumbar sensory neurons in adult rats can bind NGF (nerve growth factor) with high affinity (Verge et al., 1989), can internalize NGF (Richardson & Riopelle, 1984), and are therefore potentially responsive to endogenous NGF. Whether or not NGF is essential for the survival of mature sensory neurons (Rich et al., 1987; Lindsay, 1988), it influences their structural and functional differentiation in several ways (Goedert et al., 1981; Schwartz et al., 1982; Rich et al., 1987; Verge et al., 1989). The present study investigates correlation in location of the high-affinity NGF receptor and four recognized markers of sensory neurons - calcitonin gene-related peptide (CGRP), substance P, somatostatin, and thiamine monophosphatase in rat lumbar dorsal root ganglia.

In studies involving several target cells, two classes of NGF receptor have been described with half-maximal saturation at nanomolar and picomolar concentrations of NGF (Sutter et al.,

1979; Hosang & Shooter, 1985). Monoclonal antibodies (Ross et al., 1984; Chandler et al., 1984) and cDNA probes (Johnson et al., 1986; adeke et al., 1987) identify a receptor that binds NGF with low affinity. Until the basis for the two different binding affinities is clarified (Hempstead et al., 1989), observations from immunohistochemistry and in situ hybridization must be interpreted cautiously. In this study, radioautography of tissue sections incubated with radiolabelled NGF has been used to identify neurons bearing high-affinity NGF receptors.

METHODS

All experiments were performed on adult female Sprague-Dawley rats weighing approximately 200 g. With animals deeply anaesthetized by intraperitoneal injection of pentobarbital, the L_4 & L_5 DRG (fourth and fifth lumbar dorsal root ganglia) were removed and quickly frozen in isopentane at -50°C. Cryostat sections 5 μm thick were mounted on gelatin-coated slides for NGF-receptor radioautography, neuropeptide immunohistochemistry, or enzyme histochemistry.

For receptor radioautography, βNGF from mouse submandibular glands was radioiodinated by the lactoperoxidase method (Richardson et al., 1989). Sections were incubated for 90 minutes at room temperature in a 50 pM solution of ^{125}I-NGF in phosphate-buffered saline (0.1M, pH 7.4) with magnesium chloride (0.5 mM), cytochrome C (1 mg/ml), phenylmethylsuphonylfluoride

(0.5 mM), and leupeptin (4 μg/ml). They were rinsed in several changes of cold buffer over 3 minutes, fixed for 15 minutes in 2% paraformaldehyde/2% glutaraldehyde, defatted in alcohols and xylene, rehydrated, and dried with cool filtered air. Sections were dipped in Kodak NTB2 emulsion, exposed in the dark at 4°C for 4 days, developed, and stained with toluidine blue. Further details are provided in previous publications (Richardson et al., 1989; Verge et al., 1989).

For immunohistochemistry, sections were immersed immediately after mounting in a fixative of 2% paraformaldehyde and 15% saturated picric acid solution in 0.1M phosphate buffer for 10 minutes. Sections were rinsed in Tris-buffered saline, incubated in 0.15% hydrogen peroxide for 30 minutes, and further rinsed in buffered saline containing 100 mM 1-lysine for 30 minutes. They were then incubated with 10% normal goat serum for 30 minutes, rinsed, and incubated overnight at 4°C with primary antisera diluted in buffered saline with 1% goat serum and 0.1% Triton-X. Three rabbit antisera characterized for immunohistochemistry were used: anti-substance P (Immunonuclear) at 1:750, anti-CGRP (Peninsula Laboratories) at 1:1000, and anti-somatostatin-28 (S309) prepared by one of us (R.B.) at 1:2000. The next day, sections were incubated with biotinylated goat anti-rabbit immunoglobulins and avidin-biotin-peroxidase reagent (Vectastain ABC). Sections were then rinsed and preincubated in a buffered solution of 50 mM diaminobenzidine with 0.02% nickel ammonium sulfate and 0.025%

cobalt chloride for 15 minutes (Adams, 1981). Hydrogen peroxide was added slowly to a final concentration of 0.003% and the incubation continued for 6 minutes. Sections were rinsed dried, dehydrated, and mounted with a coverslip. Control studies included pre-absorption of the somatostatin antibody with 10^{-6} M somatostatin-28 and, in all cases, omission of the primary antibody or substitution with normal rabbit serum.

For thiamine monophosphatase localization, a published protocol (Knyihár-Csillik et al., 1986) was slightly modified. Immediately after mounting, sections were fixed for 10 minutes in 7.5% paraformaldehyde in Tris-HCl buffer, pH 7.2, rinsed for 30 minutes in 0.1M Tris-HCl buffer, pH 5.0, and incubated for 45 minutes at 37°C in a filtered solution prepared from 10 ml 1.25% thiamine monophosphate chloride, 10 ml 0.1M Tris-maleate, pH 5.0, 20 ml 0.19% lead nitrate and 10 ml distilled water. Following incubation, sections were rinsed for 10 minutes with Tris-maleate buffer, pH 5.0, developed for 5-7 minutes with 2% sodium sulphide in the same buffer, rinsed in distilled water, counterstained with thionin, dehydrated in alcohols, cleared in xylene, and mounted with a coverslip. In control experiments, pre-incubation of the sections with 0.2 mM sodium fluoride (Knyihár-Csillik et al., 1986) completely abolished thiamine monophosphatase reaction product.

For co-localization, montages were prepared from photomicrographs of adjacent sections processed for radioautography or histochemistry. All immunohistochemical

studies involved the same ganglion and same batch of radioautographs: the thiamine monophosphatase study was done with another ganglion. Under oil immersion light microscopy, neurons with histochemical reaction product were carefully selected from given regions, identified in the corresponding radioautographs, and quantified if a nucleolus was seen in one of the two sections. Digitized images of radioautographs obtained with a CCD (charge-coupled device) camera were analyzed with computer assistance (Richardson et al., 1989) to obtain the cross-sectional area of individual neurons and the fraction of area covered by silver grains (f). To correct for grain overlap and obtain numbers linearly related to grain densities, the function f/(1-f) was used (Richardson et al., 1989). Specific labelling refers to the total labelling minus background labelling averaged over several areas of the ganglion without neuronal cell bodies. Diameters were calculated on the assumption that neurons are spherical. The diameters in co-localization studies are, on average, slightly less than true values because a nucleolus was not necessarily present in the radioautographic section. Four groups of ^{125}I-NGF-labelled neurons were analyzed, each containing one of the three peptides or acid phosphatase. In addition, all neurons with visible nucleoli were analyzed in several sections to represent the total population. Histograms of labelling densities were fitted to a single normal curve or to two normal curves by a maximum-likelihood algorithm (Lawson, 1979).

RESULTS

In immunohistochemistry with each of the three antisera, punctate staining was seen over the cell bodies of some but not all neurons (Figs. 1 & 2). In control sections incubated with no primary antiserum, normal rabbit serum, or pre-absorbed antiserum, this type of staining was not present. For control and experimental preparations, nucleolar staining and diffuse cytoplasmic staining were sometimes seen but could readily be distinguished from the punctate reaction product which was accepted as immunopositive.

The results of neuropeptide immunohistochemistry, phosphatase histochemistry, and NGF receptor radioautography were, by themselves, consistent with published observations. For all five markers, positive neurons were scattered through the ganglia with no obvious predilection for any subregion. In sections where every single neuron was counted (n = 148-260), 44%, 18%, 10%, and 31% were respectively positive for CGRP, substance P, somatostatin, and thiamine monophosphatase. The latter three markers were restricted to small neurons, usually less than 30 μm in diameter: CGRP was found in small and medium-sized neurons. In receptor radioautographs, sensory neurons were heterogeneous in grain density with a slight tendency to light labelling among the large neurons. A histogram of grain densities for 441 neurons representing the entire neuronal

population could be fitted to two overlapping normal curves representing 49% and 51% of the neurons and with mean specific densities of 2.5 and 0 times background levels (Fig. 3).

A total of 158 neurons with CGRP immunoreactivity was identified and quantified in radioautographs of adjacent sections. The histogram of labelling densities for this neuronal subpopulation was roughly fitted to a normal curve with mean specific labelling of 4.3 times background levels. However, a slight skewing to the left or relative abundance of neurons with near background labelling was apparent. In 12 such neurons that would be classified as positive for CGRP but negative for the NGF receptor, the punctate immunoreaction product was extremely faint and the neuron size was small. In addition to the CGRP-immunopositive subpopulation, approximately 5% of all neurons had high grain densities on radioautography but no visible CGRP immunostaining: the latter were large neurons.

All neurons (74/74) with substance P immunoreactivity were labelled above background levels by receptor radioautography. Specific grain densities were again unimodal in distribution with mean of 3.6 times background (Fig. 3). Neurons labelled by ^{125}I-NGF and not by antiserum to substance P could readily be detected in all regions of the ganglia.

Radioautographic data were quantified for 47 somatostatin-immunoreactive neurons. None were heavily labelled and the mean grain density was at background (Fig. 3). The two

subpopulations of small neurons with immunoreactivity for substance P and somatostatin were similar in cross-sectional area but strikingly different in labelling by ^{125}I-NGF.

Forty-five neurons with thiamine monophosphatase activity were identified and quantified on the adjacent section which had been incubated with ^{125}I-NGF. This subpopulation, like the somatostatin subpopulation, had only background levels of labelling with ^{125}I-NGF (Fig. 4).

DISCUSSION

Technical considerations

Under the described conditions for radioautography, it can be assumed that high-affinity NGF receptors are present on heavily labelled neurons. The statement is justified by earlier analyses of neuronal binding in serial sections incubated with different concentrations of ligand (Verge et al., 1989). Although the radioautographic technique distinguishes populations of neurons with and without the high-affinity NGF receptor, individual neurons with intermediate labelling cannot be categorized because of overlap in labelling densities for the two neuronal populations (Fig. 3). In this study, CGRP, substance P, and somatostatin were identified by immunohistochemistry alone: other investigators have provided evidence that the appropriate peptide is synthesized in immunopositive but not immunonegative neurons (Goedert & Hunt,

1987; Mulderry et al., 1988). No attempt was made to distinguish α- and β-CGRP both of which are found in sensory neurons (Amara et al., 1985; Mulderry et al., 1988).

For technical reasons, tissue was not fixed by perfusion and histochemistry and NGF-receptor radioautography were not performed on the same section. Reduced sensitivity does not appear to have been a major problem because the percentages of positive neurons for the four markers are comparable to published observations on perfused tissue without colchicine pretreatment (Hökfelt et al., 1976; Nagy & Hunt, 1982; Lee et al., 1985). It remains unknown whether unlabelled neurons have no marker or subthreshold concentrations of marker. A few errors may have occurred in the matching of neurons in adjacent sections, 5 μm thick, but are unlikely to have influenced the conclusions.

Correlation for individual peptides

CGRP serves as a fair but imperfect marker for the NGF receptor, false positive for very weakly immunoreactive neurons and false negative for large receptor-bearing neurons. If colchicine were used to enhance immunoreactivity, the former population might be higher and the latter population might be r (Ju et al., 1987). The faintly CGRP-immunopositive neurons lacking NGF receptors are probably somatostatin-immunopositive neurons: the latter have been previously shown to

contain CGRP immunoreactivity (Ju et al., 1987). Despite discrepancies in detail, the data are consistent in general with recent observations that NGF can increase CGRP gene expression in mature rat sensory neurons (Lindsay & Harmar, 1989).

The finding of high-affinity NGF receptors in neurons with substance P immunoreactivity was predictable from previous studies in mature mammals, showing that substance P is up-regulated by NGF and down-regulated by anti-NGF antisera (Goedert et al., 1981; Schwartz et al., 1982; Fitzgerald et al., 1985; Lindsay & Harmar, 1989). The correlation between substance P and the high-affinity NGF receptor is also consistent with observations that almost all substance P-containing neurons also contain CGRP (Lee et al., 1985). An unknown factor(s) in addition to NGF probably regulates the gene for substance P because some receptor-bearing neurons have little or no peptide.

From the present findings, it is concluded that mature somatostatin-containing sensory neurons lack functional NGF receptors and are normally unable to respond to endogenous or exogenous NGF. In three previous investigations, NGF and anti-NGF did not influence the somatostatin content of sensory ganglia (Ross et al., 1981; Otten & Lorez, 1983; Maclean et al., 1988). In a fourth study when antiserum to NGF was injected into rat fetuses at 16 days of gestation, somatostatin immunoreactivity was later reduced in both dorsal root ganglia and the dorsal horn of the spinal cord (Goedert et al., 1984).

The contradiction between the latter observation and present results could reflect a difference between immature and mature neurons.

The present observations that neurons with thiamine monophosphatase activity lack high-affinity NGF binding sites is difficult to reconcile with earlier reports that exogenous NGF can counteract the transganglionic decrease of acid phosphatase that normally follows nerve injury (Csillik et al., 1985; Fitzgerald et al., 1985). In one of these experiments, a very high dose of NGF was needed; in the other case, the deficiency in acid phosphatase was partially prevented. However, the absence of NGF receptors on neurons with thiamine monophosphatase activity and presence on neurons with substance P immunoreactivity is consistent with a previous conclusion (Nagy & Hunt, 1982) that the two populations are separate.

The medium and large neurons without high-affinity NGF receptors have not been explicitly studied but presumably include those with immunoreactivity for the monoclonal antibody RT97 (Lawson et al., 1984) and carbonic anhydrase enzyme activity (Wong et al., 1987; Robertson & Grant, 1989). Rat DRG neurons lacking NGF receptors are probably homologous to large, early developing, ventrolateral neurons in chick spinal ganglia thought to lose receptors during development (Hamburger et al., 1981; Dimberg et al., 1987; Raivich et al., 1987).

Functions of the NGF receptor in vivo

With the exception of faintly CGRP-immunopositive neurons, subpopulations of neurons with each of the 3 neuropeptides or enzyme can be neatly classified as positive or negative for high-affinity NGF binding sites. These correlations support the categorizing of mature sensory neurons as NGF-responsive or unresponsive and suggest functions for endogenous NGF. NGF has been shown to increase the expression of some neuropeptide genes (Lindsay & Harmar, 1989) in sensory neurons from adult rats. Other possible actions of NGF on these neurons include the regulation of ion channels (Chalazonitis et al., 1987), neurotransmitter receptors (Mandelzys & Cooper, 1988), and reactions to injury (Diamond et al., 1987; Rich et al., 1987; Verge et al., 1989).

Because sensory axons are exposed to NGF in the endoneurium (Richardson & Ebendal, 1982; Shelton & Reichardt, 1984), the neuropeptide phenotype of mature sensory neurons must be determined by presence or absence of the NGF receptor rather than ligand. Which sensory neurons bear high-affinity NGF receptors is, in turn, established by unknown events during development (Rohrer & Barde, 1982; Davies et al., 1987; Ruvich et al., 1987).

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FIGURE 1: Photomicrographs of 2 pairs of rat DRG processed for CGRP immunohistochemistry (left) or NGF-receptor radioautography (right) (magnification x640). Note that most immunopositive neurons are heavily labelled with ^{125}I-NGF (solid arrows). However, one small faintly CGRP-positive neuron does not bind ^{125}I-NGF (open arrow) and one large neuron with many NGF binding sites is immunonegative (star).

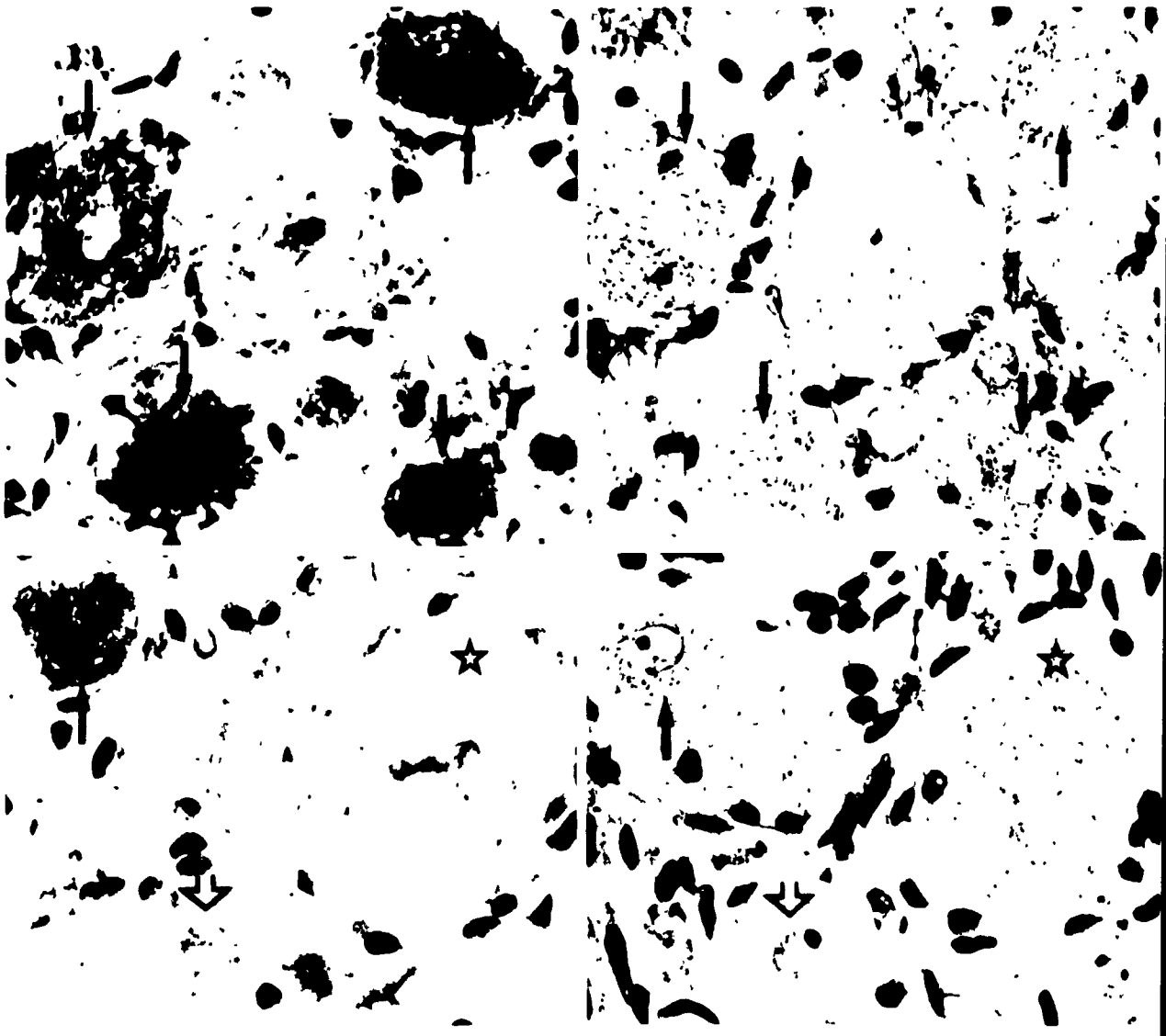

FIGURE 1

FIGURE 2: Photomicrographs of 2 pairs of rat DRG processed for immunohistochemistry (left) or NGF-receptor radioautography (right). Two neurons with substance P immunoreactivity (arrows) are also heavily labelled with ^{125}I-NGF (top). Two neurons with somatostatin-immunoreactivity (arrows) are virtually unlabelled by radioautography (below) (magnification x640).

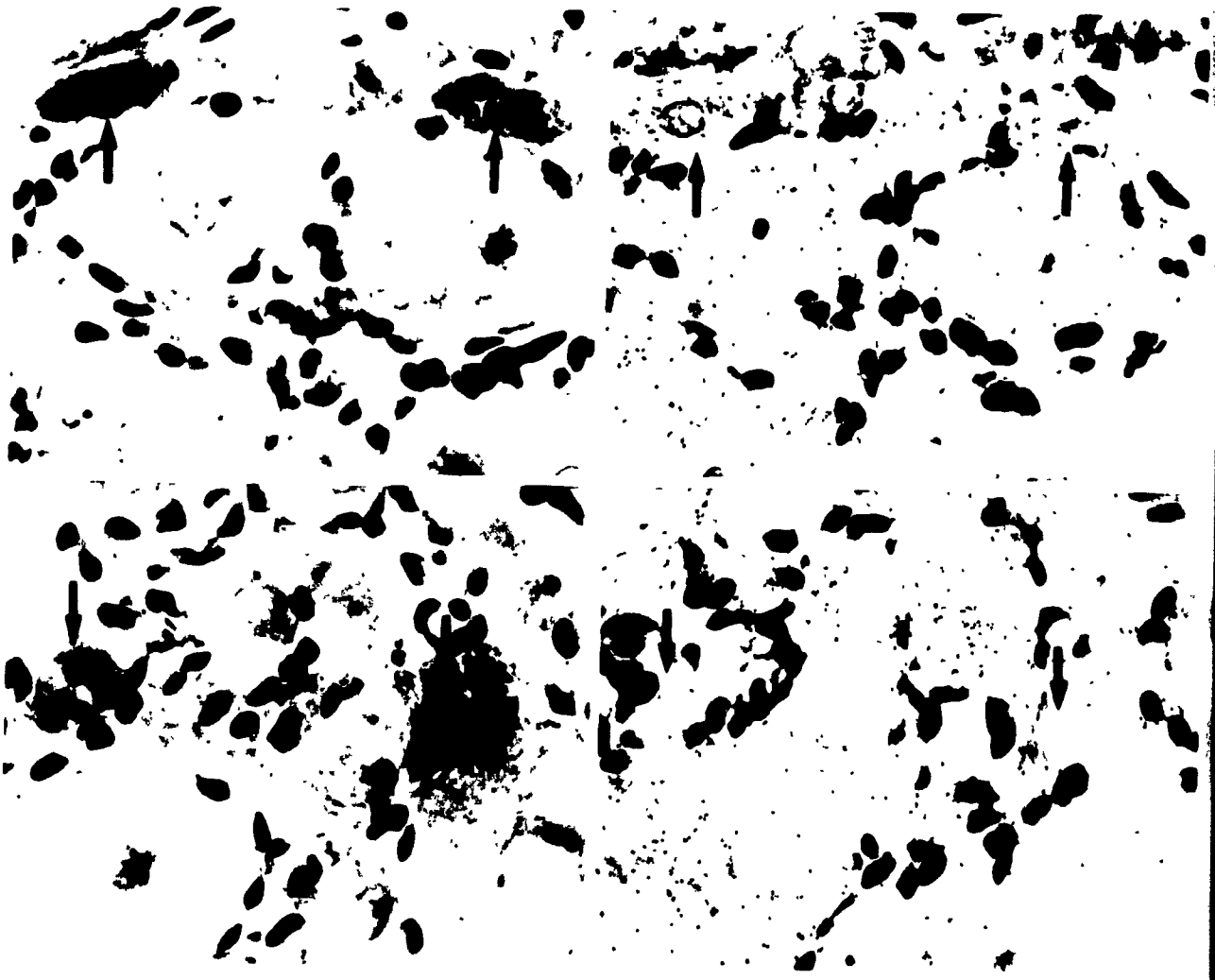

FIGURE 2

FIGURE 3: Scatter diagrams and histograms of radioautographic labelling densities for a random sample (411 neurons) representing the entire L_5 DRG and samples of neurons immunoreactive for CGRP (158), substance P (74), and somatostatin (47). For the lower 3 histograms, neurons were identified by immunohistochemistry and quantified in the adjacent section (5 μm thick) processed for radioautography. Specific labelling (total labelling minus non-specific labelling) is expressed as a multiple of non-specific labelling over non-neuronal areas of the DRG.

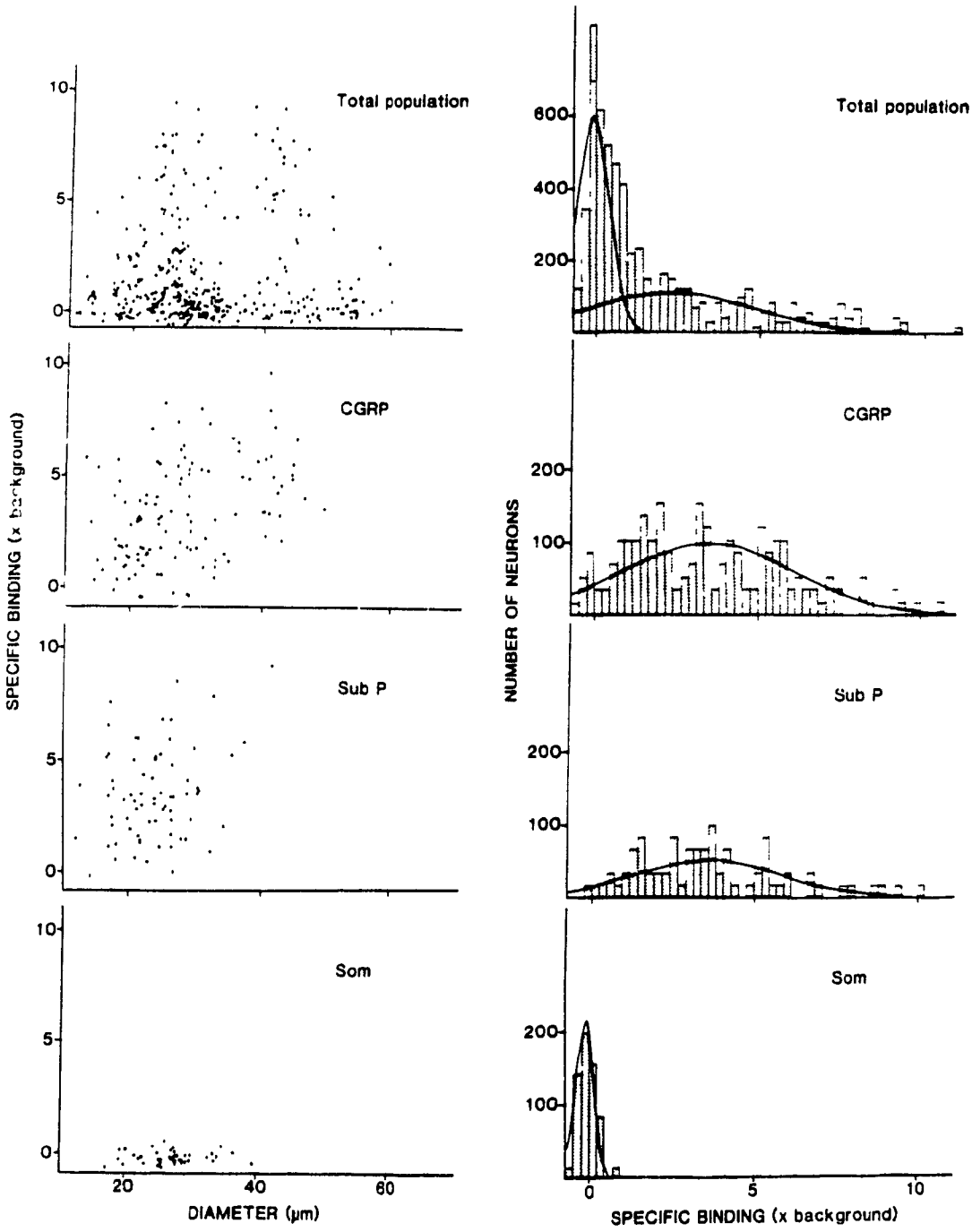
Total population
CGRP
Sub P
Som
SPECIFIC BINDING (x background)
DIAMETER (μm)
NUMBER OF NEURONS
SPECIFIC BINDING (x background)

FIGURE 3

FIGURE 4: Photomicrographs of adjacent sections processed for thiamine monophosphatase enzyme activity (left) and NGF receptor radioautography (right). Note that the 2 neurons (arrows) with thiamine monophosphatase reaction product are not labelled by ^{125}I-NGF (magnification x 1050). Scatter diagrams show specific ^{125}I-NGF labelling and diameter for 405 representative neurons from the entire ganglion (left) and 45 neurons with thiamine monophosphatase activity (right).

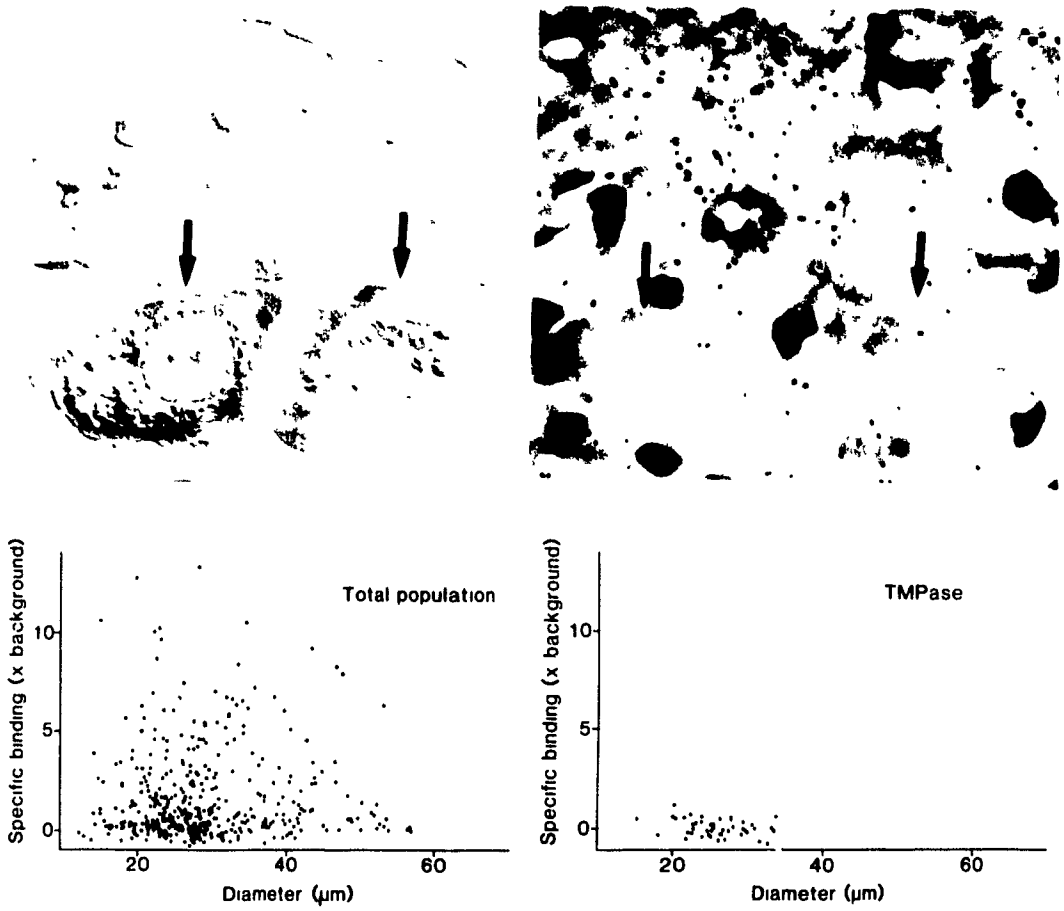
Total population
TMPase
Specific binding (x background)
10
5
0
20
40
60
Diameter (μm)

FIGURE 4

CHAPTER 4

NERVE GROWTH FACTOR RECEPTORS ON NORMAL AND INJURED SENSORY NEURONS

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SUMMARY

The density and binding properties of receptors for nerve growth factor (NGF) in normal and injured sensory neurons have been analyzed by quantitative radioautography following incubation of tissue sections with radioiodinated NGF. The technique is designed to study binding sites that are half-maximally saturated by picomolar concentrations of NGF: additional sites of lower affinity have not been emphasized. In normal adult rats, approximately half of lumbar sensory neurons have high-affinity receptors for NGF. One month after the sciatic nerve is cut, the mean number of high-affinity sites on heavily labeled neurons in the fifth lumbar dorsal root ganglion falls to less than 20% of normal values because of reduced receptor density and cell volume. Neurons with high-affinity receptors are more liable to atrophy after injury than those lacking such receptors. Receptors are lost not only in the cell bodies of sensory neurons but also on their peripheral and central processes. Delayed administration of NGF to the sciatic nerve 3 weeks after it is cut restores the receptor density to normal values and partially restores the neuronal cell volume.

As part of the response to axonal injury and possibly because the cell body is deprived of NGF, fewer high-affinity receptors are displayed by sensory neurons. For at least 3 weeks after nerve transection, neurons that are atrophic and depleted of NGF receptors can be resuscitated by exogenous NGF.

INTRODUCTION

Nerve growth factor (NGF) is selectively internalized by some primary sensory neurons in adult mammals and influences their survival and function (Goedert et al, 1981; Richardson et al, 1984; EM Johnson et al, 1986). High-affinity receptors, half-maximally saturated by picomolar concentrations of NGF, are thought to internalize NGF and mediate its biological actions (Sutter et al, 1979). More abundant low-affinity binding sites of uncertain biological importance are also found on many neurons with high-affinity NGF receptors. The relationship between 2 receptors of different affinity is not as well understood for NGF (Hosang & Shooter, 1985; Green & Greene, 1986) as for some other growth factors (Robb & Greene, 1987). Because of this ambiguity, studies with monoclonal antibodies to the low-affinity receptor (Chandler et al, 1984; Ross et al, 1984; Taniuchi et al, 1988) or probes for the encoding mRNA (D Johnson et al, 1986; Heuer et al, 1987; Radeke et al, 1987) cannot be assumed to apply to the high-affinity receptor. However, high-affinity NGF binding sites can be unequivocally identified and quantified by receptor radioautography on tissue sections (Richardson et al, 1986; 1988; Raivich & Kreutzberg, 1987).

The retrograde changes in nerve cell bodies following peripheral nerve injury can be broadly classified as

degenerative or regenerative. The latter category is exemplified by an activated growth state which is demonstrable in both peripheral and central axons of sensory neurons following peripheral axotomy (McQuarrie et al, 1977; Richardson & Issa, 1984; Richardson & Verge, 1987). Degenerative neuronal reactions include atrophy and death (Aldskogius et al, 1985) plus decreased synthesis of several proteins (Rotter et al, 1977; Tessler et al, 1985; Hoffman et al, 1987). To explore the possible implication of NGF in neuronal responses to injury, high-affinity NGF receptors in sensory neurons were examined by quantitative receptor radioautography. Two specific questions were asked. First, does the number and/or affinity of receptors increase or decrease after peripheral nerve injury to correlate respectively with axonal regeneration or neuronal degeneration? Second, are changes in the NGF receptor, like some other reactions in injured sensory neurons (Fitzgerald et al, 1985; Rich et al, 1987), counteracted by exogenous NGF?

MATERIALS AND METHODS

Surgical techniques

All experiments were performed on adult female Sprague-Dawley rats weighing approximately 200 gm. Before nerve cutting, nerve injection, or perfusion, the rats were anesthetized by intraperitoneal injection of pentobarbital (50 μg/gm). The standard procedure for 53 rats was to cut the right

sciatic nerve, including the branch to biceps femoris, and to leave the left sciatic nerve intact. In 3 of these rats, the sciatic nerve was re-exposed 3 weeks after cutting, and NGF was infused to the nerve stump via silicone tubing attached to an osmotic pump (Alza). NGF was infused at 250 ng/hr (1 μg/hr) in 0.1 M PBS containing BSA (1 mg/ml) and penicillin (100 U/ml). Rats were killed for radioautography 9 d after the pumps were inserted.

Preparation of NGF and ^{125}I-NGF

β-NGF was purified from male mouse submandibular glands by cation-exchange chromatography (Mobley et al, 1976; Chapman et al, 1981) and radioiodinated by the lactoperoxidase method (Sutter et al, 1979), with final separation of radioligand from free iodine and lactoperoxidase (Richardson et al, 1988) on a cartridge (Accell CM, Waters). ^{125}I-NGF with a specific activity of 80-200 μCi/μg was usually used within 24 hr of preparation.

Radioautographic studies

One to eight weeks after right sciatic nerve transection, rats were perfused with cold 0.1 M PBS, pH 7.4, and the fifth lumbar dorsal root ganglia (L_5 DRG) were removed and quickly frozen in isopentane. Sections 6-10 μm thick were thaw-mounted on gelatin-coated slides and stored 1-3 d at -80°C. They were then incubated for 90 min at 20°C with 20-40 pM ^{125}I-NGF in 0.1 M PBS, pH 7.4, with magnesium chloride (0.5 mM), cytochrome C (1 mg/ml), leupeptin (4 μg/ml), and phenylmethylsulfonylfluoride (0.5 mM) (Richardson et al, 1986). For studies of the

concentration dependence of binding, unlabeled NGF was added at 0-2560 pM concentration. Following incubation with ^{125}I-NGF, the sections were rinsed for 3 min in several changes of cold PBS, fixed by immersion for 10 min in 4% paraformaldehyde, rinsed briefly in distilled water, and dried with cool filtered air. In earlier experiments, the sections were further fixed the next day in hot paraformaldehyde vapor for 2 hr at 80°C. In later experiments, fixative containing 2% paraformaldehyde and 2% glutaraldehyde was substituted for 4% paraformaldehyde, and the treatment with hot paraformaldehyde vapor was omitted. Sections were defatted, dipped in radiosensitive emulsion (Kodak NTB2), exposed at 4°C in lightproof boxes for 1-6 d, developed and counterstained with thionin. Radioautographic standards, prepared from sections of brain homogenates with known amounts of ^{125}I-NGF, were fixed, defatted, dipped, exposed, and developed in the same way.

For quantification of radioautographic results, slides were examined by light microscopy under oil immersion (Fig. 1), and digitized images from a charge-coupled device were analyzed interactively by computer with software developed by W.G. Tatton (Playfair Neurosciences Unit, Toronto). Only neurons with visible nucleoli were selected for quantification. The cross-sectional area and fraction (f) of area covered by grains were recorded and the correction f/(1-f) was applied to yield a parameter linearly related to grain count (Richardson et al, 1988). Absolute amounts of ^{125}I-NGF bound to tissue (fmol/mg)

were obtained by comparison with the radioautographic standards on the assumption that the specific density of neurons is 1 gm/cc. Diameters and volumes were calculated on the assumption that neurons are spherical. All radioautographs were examined without knowledge of laterality or concentration of radioligand. Scatchard plots were fitted to the expectations of 2 binding sites by an iterative program to minimize least squares (Minneman et al, 1979). Histograms of labeling densities were fitted to 2 normal curves by a maximum likelihood program (Lawson, 1979).

For rats killed 7 weeks after transection of the right sciatic nerve, radioautographs were prepared from tranverse sections of the spinal cord through the entry zone of the L_5 roots.

Neuronal counts

The number of neurons in the right and left L_5 DRG was estimated in 4 rats with right sciatic nerve transection 4 weeks previously. In these animals, the nerve to the biceps femoris was spared. The rats were perfused with 4% paraformaldehyde, and cryostat sections, 20 μm thick, were cut serially through the L_5 DRG and stained with thionir. All neurons with visible nucleoli were counted in every fifth section, and the error due to split nucleoli was ignored.

Retrograde transport studies

In 13 rats, the right sciatic nerve was crushed in midthigh and the left nerve was intact. Both sciatic nerves were

injected at the level of the crush site with a micropipette containing 11 ng ^{125}I-NGF in 1 μl saline either 0, 4, or 12 d after crush. Rats were killed 12 hr after injection, and accumulation of gamma emission activity in L_4 and L_5 DRG was measured.

In 33 rats, the right sciatic nerve was cut in midthigh, and, 9-10 d later, 1 μl of a solution containing 1.5-12 ng of ^{125}I-NGF was injected by micropipette into each sciatic nerve at the level of the nerve to the biceps femoris. Accumulation of gamma emission activity in L_4 and L_5 DRG was measured 18 hr later following death. The data were fitted to a hyperbola (Barlow, 1983) to estimate the uptake capacity.

RESULTS

Receptor radioautography for normal DRG

Two types of quantitative information were extracted from the radioautographs. Binding properties were analyzed for heavily labeled neurons at several concentrations of ^{125}I-NGF, and variation among neurons in labeling density was surveyed for a single concentration.

To study binding properties, radioautographs were prepared from adjacent sections of DRG incubated with 20 pM ^{125}I-NGF and 0-2560 pM unlabeled NGF. At each concentration, neurons were selected from the most heavily labeled 10% and the concentration of bound NGF (radiolabeled plus unlabeled) was calculated. In 2

such analyses, Scatchard plots indicated heterogeneous binding with 2 sites half-maximally saturated at picomolar (23,49) or nanomolar concentrations (Fig. 2). The binding capacity for the high-affinity receptor was approximately 15 fmol/mg or 2-300,000 receptors per medium-sized neuron. Low-affinity binding was not analyzed in detail. At the concentrations of ^{125}I-NGF used for routine radioautography, high-affinity binding constitutes approximately half of the specific binding to heavily labeled neurons.

The concentration dependence of binding to the most lightly labeled neurons was examined in radioautographs of sections incubated with several concentrations of ^{125}I-NGF (data not shown). At most concentrations, specific binding to these lightly labeled neurons was approximately 1/10 that to heavily labeled neurons in the same section. By Scatchard plotting, no high-affinity receptors were detected on this subpopulation of neurons, and the density of low-affinity sites was relatively low. Because the dissociation-equilibrium constant for this scanty binding cannot be reliably determined, it remains unclear whether lightly labeled neurons possess true low-affinity receptors.

To examine labeling for a representative sample of neurons, random sections were selected from several simultaneously processed radioautographs of L_5 DRG. Cross-sectional areas and corrected grain densities were recorded for all neurons with visible nucleoli with special effort to detect inconspicuous,

small, lightly labeled neurons. In such radioautographs, nonspecific binding over non-neuronal areas of the ganglia was approximately 1 fmol/mg, and specific binding over neurons ranged from 0 to 20 times background levels. No strong correlation between cell diameter and labeling was detected (Fig. 3). Histograms of labeling densities could be fitted to 2 normal curves designated as "labeled" and "unlabeled" populations (Fig. 4). Because the 2 populations overlap in labeling density, not all individual neurons could be classified as "labeled" or "unlabeled". In 3 analyses of normal ganglia ([^{125}I-NGF]=20-40 pM, n=245-448 neurons), the "labeled" populations were estimated to contain 43, 60, and 65% of all neurons and to have mean densities of specific labeling 5.7, 5.4, and 4.2 times background. Corresponding densities of specific labeling for the "unlabeled" population were 1.1, 1.1, and 0.5 times background.

Changes in radioautographs following sciatic nerve transection

Four weeks after transection of the right sciatic nerve, the numbers of neurons in L_5 DRG on the left and right were estimated to be 5406 ± 462 and 4333 ± 402, respectively (mean ± s.e.m., n=4). The mean ratio of counts in right and left DRG was 0.80 ± 0.01. These numbers were used to convert percentages to absolute numbers in the histograms of labeling density (Fig. 3). In receptor radioautographs for 48 pairs of ganglia examined 1-8 weeks after unilateral sciatic nerve injury, the binding of ^{125}I-NGF was consistently diminished on the side of the

cut nerve (Fig. 1). Quantitative data were obtained for 19 pairs of ganglia. For concentrations of 20-40 pM ^{125}I-NGF in the incubation medium and time intervals of 3-6 weeks after cutting, mean specific binding was significantly reduced to 58-88% of normal values. Small, medium, and large neurons were all subnormally labeled, and the absolute number of small lightly labeled neurons was above normal (Fig. 3). In histograms of labeling densities (Fig. 4, middle column), the mean density for the "labeled" population was reduced by one-third, whereas that for the "unlabeled" population was unchanged.

The change in density of high-affinity receptors is best appreciated in Scatchard plots because labeling at a single concentration reflects a mixture of low- and high-affinity binding. By Scatchard analysis, the most heavily labeled 10% of neurons were still seen to have 2 binding sites after injury (Fig. 3). High-affinity binding was reduced to one-third of normal; low-affinity and nonspecific binding were apparently unchanged.

Reduction in perikaryal size after peripheral nerve transection was also quantified (Table 1). For all time intervals, atrophy was more severe for the most heavily labeled third of neurons than for the most lightly labeled third. One month after injury, the mean volume was decreased by one-half and one-quarter for the former and latter populations, respectively.

In radioautographs of the spinal cord through the L_5 root

entry zone, NGF binding in superficial laminae of the dorsal horn was diminished ipsilateral to the nerve transection (Fig. 5). The pattern and degree of depletion of the NGF receptor match that for substance P (Tessler et al., 1985) and fluoride-resistant acid phosphatase (Fitzgerald et al., 1985).

NGF infusion

In this experimental group, radioautographs were compared for 3 sets of ganglia - left and right DRG from animals with simple sciatic nerve transection plus DRG associated with cut, NGF-infused nerves. On dark-field inspection of radioautographs, binding was, as expected, diminished after simple transection but appeared nearly normal when NGF had also been infused. For the "labeled" population, the mean specific binding was reduced from 5.4 to 3.6 times background by nerve transection and restored to 4.6 times background by NGF (Fig. 4, middle column). Nonspecific binding and binding for the "unlabeled" population were uninfluenced by NGF. The mean diameter fell from 35.1 ± 0.5 to 30.5 ± 0.4 μm after nerve cutting and returned to 32.7 ± 0.4 μm after NGF infusion (mean ± s.e.m., n=448-548). Examination of the most heavily and lightly labeled thirds of the neuronal population showed that NGF counteracted atrophy and decreased labeling for the former but not the latter group (Table 2). In summary, delayed infusion of NGF substantially reverses the atrophy and reduction in receptor density that follow sciatic nerve transection.

Retrograde transport studies

Uptake of ^{125}I-NGF from crushed and normal sciatic nerves did not differ significantly at any of the 3 times after injury (Fig. 6).

Ten days after cutting of the sciatic nerve, the maximal accumulation of ^{125}I-NGF in L_5 DRG following intraneural injection was calculated to fall from 29 to 18 pg (Fig. 7). Because very little NGF would be expected to diffuse to midthigh after intraneural injection at the nip (Richardson and Riopelle, 1984), the effective length of nerve exposed to ^{125}I-NGF should be the same for cut and normal nerves. Therefore, the reduced uptake indicates fewer receptor-bearing axons and/or fewer receptors per unit length of axon.

DISCUSSION

NGF receptors on normal sensory neurons

The binding sites within neuronal perikarya are here called receptors even though most of them are not inserted in the cell membrane and are therefore not in position to interact with endogenous NGF. It is assumed that most perikaryal receptors are newly synthesized and awaiting anterograde transport, although some may have been retrogradely transported with their ligand (Raivich and Kreutzberg, 1987).

For radioautographs of normal L_5 DRG, histograms of neuronal labeling densities are bimodal, fitting 2 normal curves and

suggesting the existence of 2 populations of sensory neurons. Scatchard analysis of the concentration dependence of binding shows that heavily labeled neurons have saturable binding with dissociation-equilibrium constant of 15-50 pM plus additional binding of lower affinity. For lightly labeled neurons, Scatchard analysis indicates an absence or very small number of high-affinity receptors. Several tissue culture and retrograde transport studies also suggest that significant numbers of rat and chick DRG neurons are unresponsive to NGF at physiological concentration (Richardson and Riopelle, 1984; Davies and Lindsay, 1985; Davies et al., 1986). For lumbar DRG in adult rats, approximately half of the sensory neurons have high-affinity NGF receptors. This population of NGF-responsive sensory neurons has not been described in terms of function and other markers (Dodd et al., 1983).

Because of technical considerations, no conclusions have been drawn from the radioautographs regarding low-affinity receptors. For picomolar concentrations of radioligand, only a small percentage of low-affinity sites should be occupied, and this binding should be displaced after washing for 3 min (Sutter et al., 1979). The persistence of low-affinity binding under the conditions for radioautography could indicate nonphysiological sequestration of NGF by intracellular receptors artificially exposed to NGF. Nevertheless, low-affinity binding can and must be taken into account to obtain accurate values for the functionally important high-affinity receptor.

Regulation of the NGF receptor

Following peripheral nerve injury, the number of high-affinity receptors on some sensory neurons falls by more than 80% through a combination of atrophy and reduced density. This disappearance of perikaryal NGF receptors after nerve injury does not merely reflect translocation from cell body to axon because the receptors on peripheral and central axons are also decreased. The receptor loss is not a simple consequence of selective neuronal death because it is reversed by delayed administration of NGF. Finally, the injury-induced loss of high-affinity NGF receptors exceeds any general reduction in ganglionic proteins or axonally transported proteins (Perry and Wilson, 1981). In response to peripheral nerve injury, either high-affinity receptors are selectively down-regulated or neurons with such receptors are exceptionally perturbed.

The molecular mechanisms leading to a reduction in high-affinity NGF receptors are unknown. In the absence of a reliable cDNA probe for the high-affinity receptor, the defect cannot be defined as translational or posttranslational. Perhaps, the NGF receptor is co-regulated with substance P and neurofilament, which are also reduced in injured sensory neurons (Tessler et al., 1985; Hoffman et al., 1987) and induced by NGF (Goedert et al., 1981; Fitzgerald et al., 1985; Dickson et al., 1986). Because several neuronal receptors are down-regulated in response to axonal injury (Rotter et al., 1977; Fumagalli and de Renzis, 1980; Helke et al., 1984), the change in NGF receptors

might reflect a general impairment in receptor preparation (Goldberg and Ambron, 1986). The different responses to nerve injury of high-affinity neuronal receptors and low-affinity Schwann cell receptors (Raivich and Kreutzberg, 1987; Taniuchi et al., 1988) attest to some complexity in the regulation of NGF receptors.

Infusion of NGF restores high-affinity sites that disappear on neurons with cut axons. This evidence that NGF can regulate its functional high-affinity receptor in adult mammalian neurons corroborates previous studies in pheochromocytoma cells (Landreth and Shooter, 1980; Bernd and Greene, 1984). The general concept that growth factors induce synthesis of their own receptors receives support from studies of epidermal growth factor (Carpenter, 1987) and interleukin-2 (Waldmann, 1986).

Neuronal responses to injury

Sensory neurons with high-affinity NGF receptors are selectively vulnerable to atrophy following peripheral nerve injury (Table 2). However, lack of NGF is not the only cause of neuronal atrophy because NGF fails to restore cell volume completely (Table 2; Rich et al., 1987). These and other neuronal counts (Arvidsson et al., 1986) suggest that approximately 20% of the neurons in rat L_5 DRG have died 1 month after sciatic nerve transection. Although some sensory neurons indubitably die after peripheral nerve injury (Aldskogius et al., 1985), neuronal death could be overestimated in cryostat sections, where shrunken neurons can become difficult to

identify. For cholinergic forebrain neurons (Hagg et al., 1988) and sensory neurons, biochemical and morphological changes that can mimic neuronal death are reversible by delayed infusion of NGF.

The changes in neuronal NGF receptors following peripheral nerve injury are clearly degenerative rather than regenerative. The data provide no suggestion of a supersensitivity to NGF that might contribute to the regenerative propensity of injured sensory neurons (Richardson and Issa, 1984; Richardson and Verge, 1987). Rather, regressive changes are probably accentuated by the diminution of NGF binding, possibly through a vicious cycle with reduction in perikaryal NGF and decreased receptor synthesis. These and other findings suggest that some degenerative changes in injured sensory neurons are caused by a reduction in retrograde axonal transport of NGF: such responses are mitigated by exogenous NGF and presumably by NGF in the distal stump (Heumann et al., 1987) or a crushed or sutured nerve.

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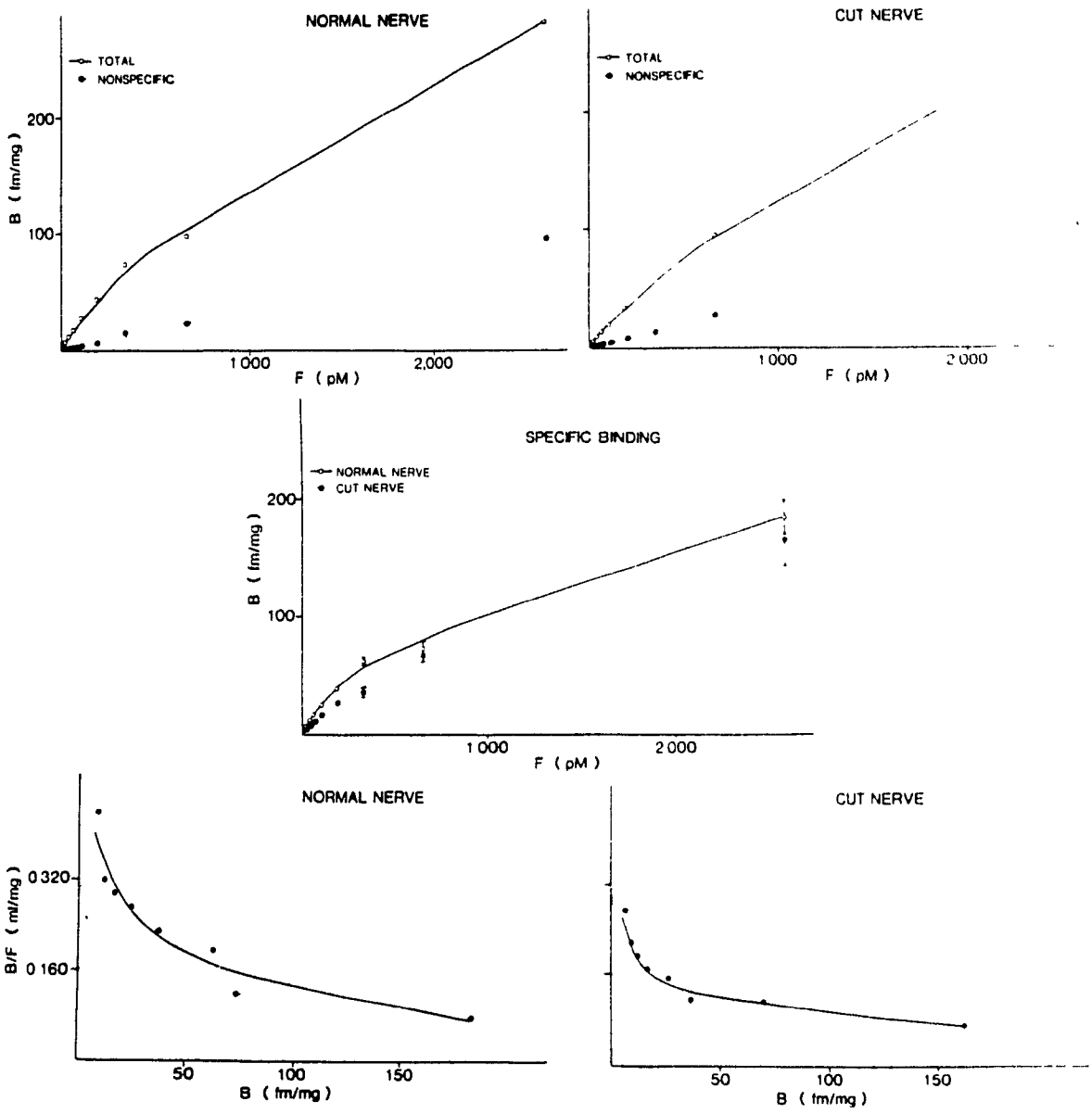
FIGURE 1: Photomicrographs of sections of L_5 DRG incubated with ^{125}I-NGF and prepared for radioautography. **Top:** Dark-field photograph of normal DRG (40 pM ^{125}I-NGF). x75. **Middle:** Dark-field photograph of the contralateral DRG associated with sciatic nerve transection 3 weeks earlier. The reduction in binding results from a decreased receptor density plus marked atrophy of neurons with many receptors (40 pM ^{125}I-NGF). x75. **Bottom:** Oil-immersion photograph showing 2 neurons in a normal DRG (20 pM ^{125}I-NGF). x1100.

FIGURE 1

FIGURE 2 : Concentration dependence of binding to neurons in left and right L_5 DRG removed 3 weeks after cutting of the right sciatic nerve. Sections were incubated with 20 pM ^{125}I-NGF and 0-2560 pM unlabeled NGF. For each concentration, the binding of labeled plus unlabeled NGF was calculated for representative neurons among the most heavily labeled 10%. **Top:** Total binding over neurons and non-neuronal area (means ± SEM, n = 30-60). **Middle:** Specific binding compared for the 2 sides. **Bottom:** Scatchard plotting with curve-fitting on the assumption of 2 binding sites (Minneman et al, 1979). For high-affinity sites in the left DRG, Bmax = 15 fmol/mg and Kd = 50 pM. For the right DRG, Bmax = 5 fmol/mg and Kd = 15 pM.


NORMAL NERVE
TOTAL
NONSPECIFIC
B (fm/mg)
200
100
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F (pM)
CUT NERVE
TOTAL
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1 000
2 000
F (pM)
SPECIFIC BINDING
NORMAL NERVE
CUT NERVE
B (fm/mg)
200
100
1 000
2 000
F (pM)
NORMAL NERVE
B/F (ml/mg)
0 320
0 160
50
100
150
B (fm/mg)
CUT NERVE
50
100
150
B (fm/mg)

FIGURE 2

FIGURE 3: Data from NGF-receptor radioautographs for the same pairs of L_5 DRG as in Figure 2. **Top:** Scatter diagrams in which each point represents a single neuron (n = 400). In the normal DRG, clusters of neurons with dense and light labeling are separable for large neurons but merge for small neurons. Note that binding and size both tend to decrease following nerve injury. **Below:** Neurons were classified as small (<30 μm), medium (30-40 μm), or large (>40 μm), and histograms of binding densities were prepared for each population. After nerve injury, very few of the large or medium neurons have labeling more than 5 times background. The absolute number of small lightly labeled neurons is increased.

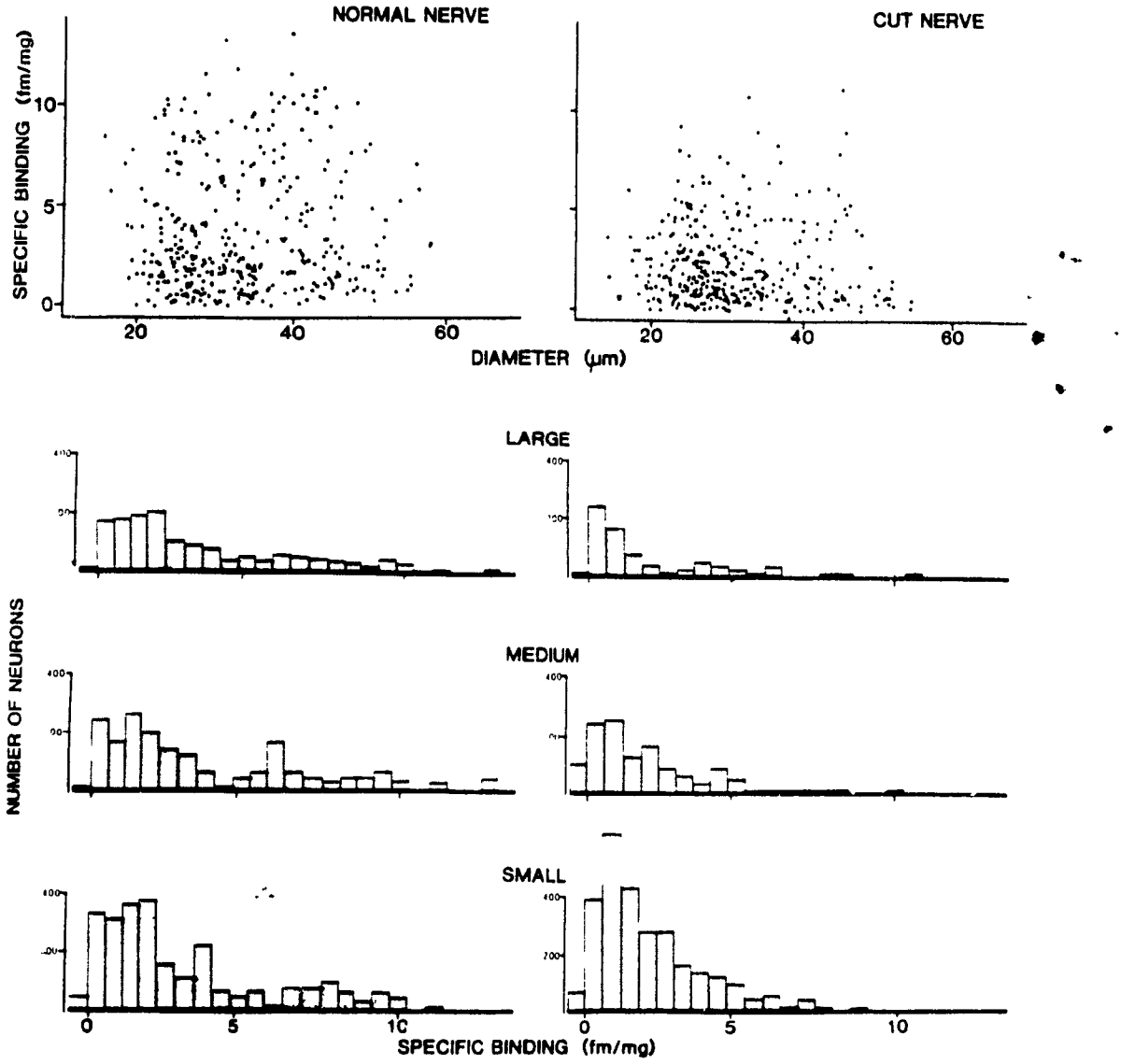
NORMAL NERVE
CUT NERVE
SPECIFIC BINDING (fm/mg)
10
5
0
20
40
60
DIAMETER (μm)
LARGE
MEDIUM
SMALL
NUMBER OF NEURONS
0
5
10
SPECIFIC BINDING (fm/mg)

FIGURE 3

FIGURE 4: Quantitative data for radioautographs of 3 groups of DRG in which the sciatic nerve was either intact, cut, or cut and later infused with NGF. In the scatter diagrams (**left**), specific binding is expressed relative to nonspecific binding over non-neuronal areas of normal ganglia. Note the diminution of neuronal size and ^{125}I-NGF labeling following nerve cut with partial restoration by infusion of NGF (n = 448). Histograms of the density of binding (**middle**) were fitted to 2 normal curves by a maximum likelihood programme (Lawson, 1979). The 3 "labeled" populations constituted 60-64% of neurons and had binding densities of 5.4 ± 0.4, 3.6 ± 0.2, and 4.6 ± 0.3 times background for the normal, cut, and NGF groups (means ± SEM, n = 448-548). **Right:** neurons are classified as small, medium, or large with diameters <30 μm, 30-40 μm, and >40 μm, respectively, and also subdivided into those with specific binding less or greater than 4 times nonspecific binding. The latter partition seems appropriate at least for large neurons in normal ganglia. Notice that the bin for large heavily labeled neurons is very small following nerve transection and increases after NGF infusion.

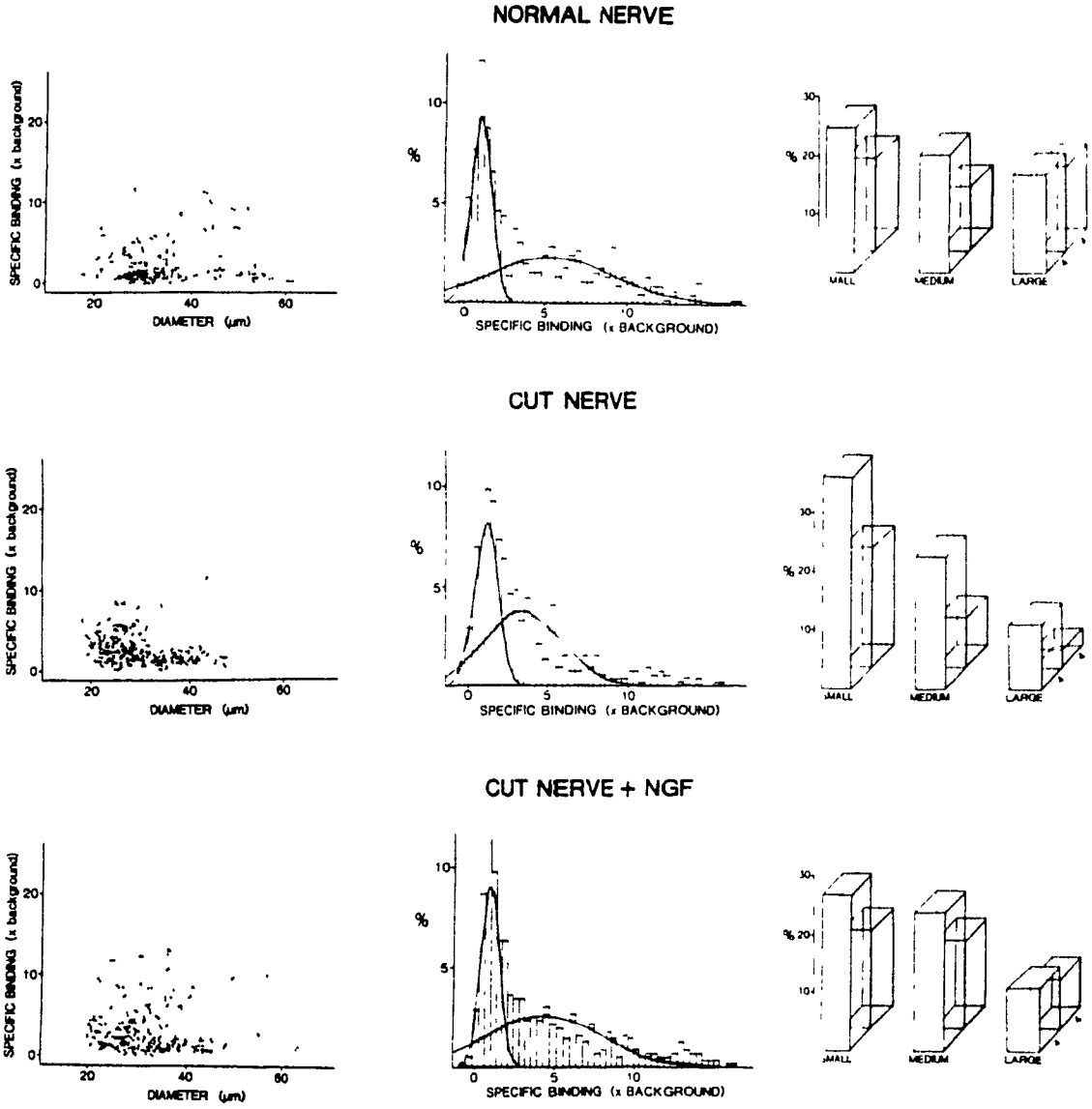
NORMAL NERVE
SPECIFIC BINDING (x background)
DIAMETER (µm)
SPECIFIC BINDING (x BACKGROUND)
%
SMALL
MEDIUM
LARGE
CUT NERVE
CUT NERVE + NGF

FIGURE 4

FIGURE 5: Radioautography of the spinal cord at L_5 root entry level in a rat sacrificed 7 weeks after transection of the right sciatic nerve. The dorsal spinal roots are outlined. NGF binding to fibers in the superficial dorsal horn is diminished on the right side compared with the normal left side. Also, abnormal speckled binding is seen within the right L_5 dorsal spinal root, presumably NGF receptors expressed by Schwann cells associated with degenerated central processes ([^{125}I-NGF] = 50 pM). x40.

FIGURE 5

FIGURE 6: Accumulation of ^{125}I-NGF in L_4 and L_5 DRG 12 hr after injection (1 μl, 11 ng) into normal sciatic nerve or nerve that had been crushed 0-12 d earlier (means ± SEM, n = 4-5 for each point).

FIGURE 7: Accumulation of ^{125}I-NGF in L_4 and L_5 DRG 18 hr after injection into normal sciatic nerve or nerve transected 9-10 d earlier (n = 7-10 for each point).

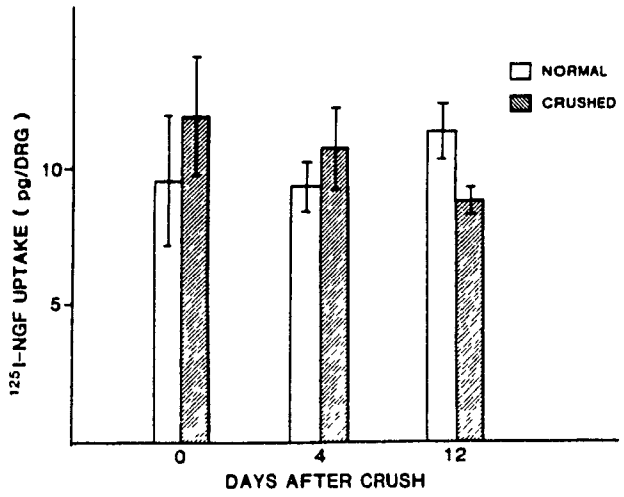

FIGURE 6

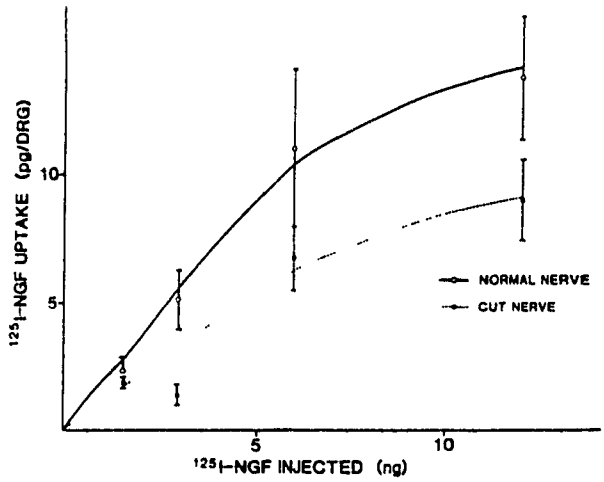

FIGURE 7

TABLE I

Changes in perikaryal volume after nerve injury

Days after nerve cut	Lightly labeled neurons	Heavily labeled neurons
18	.85 (32.6/38.5)	.67 (20.9/31.2)
18	.97 (22.2/22.9)	.72 (18.2/25.3)
30	.73 (23.1/31.6)	.54 (15.7/29.1)
39	.77 (23.9/31.0)	.54 (15.1/28.2)

For this table the most heavily and lightly labeled thirds of the neuronal population were selected from each group. The values for DRG associated with cut sciatic nerves are expressed as fractions of the values for control DRG. Atrophy is consistently more severe for heavily than lightly labeled neurons. (Volumes/10^3 in μm^3, means ± s.e.m., n = 80-179).

TABLE II

Recovery of size and binding sites following administration of NGF to the cut sciatic nerve.

	VOLUME		LABELING	
Sciatic nerve	Lightly labeled	Heavily labeled	Lightly labeled	Heavily labeled
Normal	31.6±2.4	29.1±1.9	0.6±.03	8.4±0.2
Cut	23.1±1.4	15.7±0.9	0.7±.03	6.9±0.3
NGF-treated	25.0±1.8	24.0±1.8	0.6±.03	8.9±0.3

The most lightly and heavily labeled thirds of the neuronal population were analysed separately. NGF pumps were inserted 21 days after cutting of the sciatic nerve and rats were sacrificed after a further 9 days. Note that NGF significantly counteracts the effects of nerve cutting for the heavily labeled group only ($p<0.0001$ by the Student's t test for both labeling and volume). Specific labeling is expressed as a ratio of non-specific labeling for normal ganglia. (Volumes/10^3 in μm^3, means ± s.e.m., n=145-180).

CHAPTER 5

CORRELATION BETWEEN GAP43 AND NERVE GROWTH FACTOR RECEPTORS IN RAT SENSORY NEURONS

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SUMMARY

In mature rat sensory neurons, expression of the gene for the growth-associated protein, GAP43, was studied by in situ hybridization with a cDNA probe. Among neurons in normal lumbar dorsal root ganglia, labelling for GAP43 mRNA was heterogeneous, approximately one half of the neurons being densely labelled. To characterize the latter population, individual neurons were examined in adjacent sections processed either for GAP43 hybridization or NGF-receptor radioautography. Virtually all neurons with high-affinity NGF binding sites had high basal levels of GAP43 mRNA and most GAP43 positive neurons bore NGF receptors. Another NGF-responsive population, sympathetic neurons in the superior cervical ganglion, also had high basal concentrations of GAP43 mRNA. Further co-localization studies in dorsal root ganglia were performed with immunohistochemistry for somatostatin and enzyme histochemistry for acid phosphatase. The latter two groups of sensory neurons have been previously shown to lack high-affinity receptors and were here shown to have low basal concentrations of GAP43 mRNA. From this and earlier studies, it can be assumed that substance P-immunoreactive neurons and strongly positive CGRP neurons synthesize GAP43 at high basal rate. One week following peripheral nerve transection, almost all neurons had high concentrations of GAP43 mRNA without correlation with NGF

binding. Intrathecal infusion of NGF after the sciatic nerve was cut did not strongly influence this post-traumatic elevation in GAP43 mRNA.

In normal dorsal root ganglia, neurons that have high-affinity NGF binding sites and are therefore potentially responsive to NGF also have high basal rates of synthesis of GAP43. As an early response to nerve injury, synthesis of GAP43 is increased in sensory neurons whether or not they are NGF-responsive. The synthesis of GAP43 in sensory neurons appears to be regulated by NGF under normal conditions but by some alternative signal soon after axonal injury.

INTRODUCTION

Growth-associated protein GAP43 (also known as B50, F1, pp 46 & pp 57 and neuromodulin) is widely considered to be involved in neuronal mechanisms underlying axonal growth, axonal regeneration and synaptic plasticity (Benowitz & Routtenberg, 1987). Through molecular mechanisms that have not yet been fully elaborated (Gispen, 1986; Alexander et al, 1988), GAP43 is thought to contribute directly to the outgrowth of neuronal processes (Zuber et al, 1989). While first recognized for its increased synthesis and axonal transport in neurons responding to peripheral nerve injury (Skene, 1984; Bisby, 1988), GAP43 and its mRNA are also detectable in some regions of the normal brain (Benowitz et al, 1988; Neve et al, 1988). More GAP43 immunoreactivity has been detected in unmyelinated than myelinated axons both in normal rat sciatic nerves and in the proximal stump of transected nerves (Tetzlaff et al, 1989) and intense immunohistochemical staining has been observed in varicose axons innervating the vasa vasorum of the sciatic nerve and in unmyelinated axons of the myenteric plexus (McGuire et al, 1988; Sharkey et al, 1989). Thus, GAP43 normally appears to be heterogeneously distributed in both the peripheral and central nervous system.

The signals following nerve injury that trigger an increased synthesis of GAP43 (Bisby, 1988) and a regenerative

propensity (McQuarrie & Grafstein, 1973; Richardson & Verge, 1986; Molander et al, 1988) in nerve cell bodies are not well understood. With respect to nerve growth factor (NGF) as a possible signal, the observations are paradoxical: both regenerative behaviour and its marker, GAP43 are induced in vitro by NGF (Karns et al, 1987; Federoff et al, 1988) yet also induced in vivo by axotomizing lesions that restrict the normal supply of NGF to neurons. However, it has not been proven that all sensory neurons respond in the same way to axotomy: only some sensory neurons have high-affinity NGF receptors (Verge et al, 1989 a,b) and it might be speculated that injured NGF-responsive and NGF-unresponsive neurons differ in GAP43 synthesis and conditioning responses.

In experiments to be described, expression of the GAP43 gene was studied in normal and injured rat primary sensory neurons by in situ hybridization with a cDNA probe. Emphasis was placed on heterogeneity among sensory neurons in GAP43 mRNA content and on possible correlations between GAP43 and the high-affinity NGF receptor.

MATERIALS and METHODS

Animal surgery

Experiments were performed on 26 adult female Sprague-Dawley rats weighing approximately 200 g. In some rats, the right sciatic nerve was transected with microsurgical technique

under pentobarbital anesthesia (50 mg/kg intraperitoneally). To expose the sciatic nerve at its origin from the fourth and fifth lumbar (L_4 & L_5) spinal nerves, a small amount of bone was rongeured from the iliac crest. The sciatic nerve was cut and a 5 mm segment was removed. In some instances, β NGF from male mouse submandibular glands (Mobley et al, 1976; Chapman et al, 1979) was infused into the lumbar subarachnoid space at 125 ng/hour. For this purpose, Silastic tubing, 0.012 inches in diameter (Dow-Corning) was connected to an osmotic pump (Alza model 2001) and passed through the dura and arachnoid at the lumbosacral junction so that a 1.5 cm segment lay intrathecally. The pump was secured to the lumbar fascia. Tissues were analyzed from normal rats and rats that had undergone sciatic nerve transection one week previously with or without subsequent NGF infusion. Rats were deeply anesthetized with pentobarbital and perfused per aorta with cold phosphate-buffered saline (pH 7.4). The L_5 DRG and/or superior cervical ganglion were removed, quickly frozen in isopentane and stored at -80°C for several days. In rats with cut sciatic nerves, the right and left L_5 DRG were frozen in the same block of Tissue-Tek (Miles Laboratories) so that they could be processed on the same slide under identical conditions.

Northern blotting

Total cytoplasmic RNA was prepared from 4 pooled DRG (L_4, L_5) according to a standard technique (Schibler et al, 1980).

Equal amounts of RNA were fractionated by electrophoresis on 1.2% agarose gels in the presence of 1M formaldehyde (Maniatis et al, 1982) and blotted onto nitrocellulose by capillary transfer. The filters were covered for 2 hours at 43°C in prehybridization solution containing 50% formamide, 5x Denhardt's solution, salmon sperm DNA and tRNA (250 μg/ml), DTT (100 mM), 0.2% SDS, 75 mM NaCl, 25 mM EDTA in 25 mM PIPES buffer, pH 6.8. Hybridization for 12-14 h again at 43°C was performed using the same solution with the addition of a ^{35}S-labelled GAP43 cDNA probe (2 x 10^6 cpm/ml, 10-20 ng/ml) which was generated by random oligonucleotide primed labelling (Feinberg & Vogelstein, 1983) of an isolated cDNA insert (Basi et al, 1987). Post-hybridization washes were carried out with 4x, 2x, 1x, 0.5x and 0.1x SSC at 45°C. Dried filters were exposed to Kodak X-omat x-ray film for 1 to 2 weeks and developed.

In situ hybridization

Cryostat sections, 5 μm thick were thaw mounted on gelatin-coated slides, post-fixed in phosphate-buffered 4% paraformaldehyde and treated for prehybridization and hybridization as previously described (Miller et al, 1987). After fixation, the sections were washed in PBS, treated with proteinase K (10 ng/ml) for 8 minutes at room temperature, fixed again for 5 minutes in 4% paraformaldehyde and dehydrated in 70%, 90% and 100% ethanol in 0.3M sodium acetate. After brief drying, the sections were prehybridized, hybridized and washed

with the same solutions used for Northern blotting. Sections were dipped in Kodak NTB_2 emulsion, diluted 1:1 in water, exposed in the dark for 2-3 weeks and developed. Control slides were hybridized with ^{35}S-labelled plasmid DNA rather than GAP43 cDNA.

NGF-receptor radioautography

Unfixed cryostat sections adjacent to those used for GAP43 hybridization were incubated for 90 minutes at room temperature with 50 pM ^{125}I-NGF, post-fixed, defatted, dipped in emulsion, exposed in the dark for 4 days, developed, and stained. As described previously (Verge et al, 1989a), this technique is designed to detect high-affinity NGF binding with half-maximal saturation by picomolar concentrations of NGF.

Histochemistry

Histochemical procedures for detecting somatostatin immunoreactivity and acid phosphatase enzyme activity in post-fixed tissue sections have been previously described in detail (Verge et al, 1989b). For immunohistochemistry, sections were post-fixed in buffered formaldehyde/picric acid, pretreated with hydrogen peroxide to deplete endogenous peroxidase, incubated overnight with antisomatostatin-28 (S309 serum generously provided by Dr. Robert Benoit) and processed with avidin-biotin reagents and horseradish peroxidase histochemistry. For acid phosphatase localization, sections were post-fixed, rinsed, incubated in a solution containing thiamine monophosphate

chloride and lead nitrate and developed with a sodium sulphide solution.

Image Analysis

To correlate GAP43 hybridization and high-affinity NGF binding sites, montages were prepared from adjacent sections and each neuron with a visible nucleolus in one of the two sections was identified and numbered in both sections (Verge et al, 1989b). Then, NGF-receptor radioautography and GAP43 hybridization were quantified by computer-assisted image analysis (Richardson et al, 1989) of individual neurons viewed under oil-immersion light microscopy. By tracing of the periphery of identified neurons, the cross-sectional area and fraction of area covered by silver grains were measured. To obtain the fraction of cytoplasm covered by silver grains (f) nuclear labelling and area were subtracted from total area and labelling. The fraction f/(1-f) designated "labelling density" was used to correct for grain overlap (Richardson et al, 1989).

Areas were obtained for the same neurons in both preparations and corrected for 8% linear shrinkage in receptor radioautographs: the greater of the two measurements was taken as the true area and the diameter was calculated on the assumption that neurons were spherical. To avoid negative numbers and permit logarithmic conversion, total labelling was used without subtraction of background labelling. Labelling densities were normalized by reference to background labelling

obtained by measuring grain densities over several areas of the ganglia devoid of nerve cell bodies. Statistical analyses were performed by computer according to published algorithms (Press et al, 1988).

RESULTS

Northern blots of DRG (Fig. 1) showed that the probe was recognizing a single mRNA species of 1.4 kb (Basi et al, 1987) and that the mRNA in DRG increased substantially following sciatic nerve transection. For the superior cervical ganglion, where most neurons bear high-affinity NGF binding sites (Richardson et al, 1986), in situ hybridization yielded strong labelling over virtually all neurons (Fig. 1). Hybridization of radiolabelled plasmid DNA to L_5 DRG gave no specific labelling.

Normal DRG

Labelling with GAP43 cDNA was extremely heterogeneous among neurons in normal L_5 DRG (Fig. 2) ranging from less than background to almost 100 times background (Fig. 3) in most preparations. Histograms of labelling densities (Fig. 3) for preparations from five normal DRG did not suggest a single neuronal population but rather the existence of two normally distributed populations (on a logarithmic scale) with mean labelling densities in the order of 2-3 times background and 5-20 times background. Labelling tended to be denser in small

rather than large neurons (Fig. 4) although many small neurons were lightly labelled and some large neurons were heavily labelled.

As in earlier studies, histograms of the binding of ^{125}I-NGF to L_5 DRG neurons could be fitted to two normal curves with 50-60% and 40-50% of the neurons (Verge et al, 1989a; 1989b). Evidence has been presented that these two curves represent neurons without and with high-affinity NGF receptors (Verge et al, 1989b). Inspection of groups of sensory neurons suggested a correlation between labelling with NGF and GAP43 mRNA (Fig. 2). To verify this impression, detailed quantitative analysis was performed on pairs of adjacent sections from 4 normal DRG. One ganglion was from an unoperated rat and three were left L_5 DRG removed one week after right sciatic nerve transection. No difference was noted between the true normal ganglion and other three control ganglia. In log-log plots, GAP43 labelling densities were obviously correlated to NGF labelling densities (Pearson coefficient = 0.34-0.50, $p<0.00001$). When the thirds of total neuronal population with lightest and heaviest NGF labelling were selected to represent neurons without and with high-affinity receptors (Table 1), mean densities of GAP43 labelling for the receptor-bearing populations were 2.3-4.4 times greater than those for the receptor-lacking populations ($p < 0.0001$ by the Student's t test). Most neurons could be placed into one of two clusters (Fig. 3) - lightly labelled or heavily labelled by both procedures. Virtually all neurons with dense

^{125}I-NGF labelling also had high concentrations of GAP43 mRNA and most neurons with light NGF labelling had little GAP43 mRNA. However, 3-7% of all neurons fell in the subpopulation defined arbitrarily by NGF labelling in the lower third and GAP labelling in the upper third.

In another L_5 DRG, GAP43 hybridization was examined for neurons with somatostatin immunoreactivity and acid phosphatase enzyme activity: both groups have previously been shown to lack high-affinity NGF receptors (Verge et al, 1989b). Somatostatin-positive and phosphatase-positive neurons were invariably small and lightly labelled in GAP43 hybridization (Fig. 4).

Nerve transection and NGF infusion

In rats sacrificed one week after right sciatic nerve transection, labelling by GAP43 hybridization was consistently greater in the right than left L_5 DRG. This observation was made for four rats by inspection under dark-field illumination of pairs of sections, mounted on single slides (Fig. 5). In two rats, NGF labelling densities and GAP43 labelling densities were quantified for both right and left L_5 DRG. In both cases, the mean NGF labelling density was significantly reduced (by 25% and 18%) and the mean GAP43 labelling was significantly increased (to 2.0 and 2.7 times normal). In histograms of GAP43 labelling densities in DRG associated with a cut sciatic nerve (Fig. 5), most values fell within a single normal curve except for 5-10% of the total which remained at lower values. When GAP43

labelling densities were plotted against NGF labelling densities in scatter diagrams for individual neurons or as three-dimensional histograms, no correlation between the two labelling densities was detected. Mean GAP43 labelling densities for the thirds of the population most heavily and lightly labelled by NGF were almost identical. In short, one week after sciatic nerve transection, the concentrations of GAP43 mRNA were homogeneously high in almost all sensory neurons.

Three animals were examined one week after right sciatic nerve transection followed by continuous infusion of NGF into the subarachnoid space. Intrathecal infusion of NGF counteracted the loss of NGF-binding sites that usually follow nerve transection more reliably than application of NGF to the cut nerve stump (Verge et al, 1989a). In the contralateral L_5 DRG associated with an intact sciatic nerve, most neurons fell into one of two clusters either heavily labelled or lightly labelled by both procedures (Fig. 6). NGF infusion seemed to accentuate both GAP43 and NGF labelling in the heavily labelled subpopulation, so that, in 3-D histograms, the two clusters of neurons were more widely separated than in normal ganglia. Following sciatic nerve transection and continuous infusion of NGF, the distribution of GAP43 labelling densities in the ipsilateral L_5 DRG conformed to a single peak with uniformly high labelling: the pattern was similar to that after simple transection.

DISCUSSION

Technical considerations

The presence of a single band on Northern blots and the absence of any hybridization with labelled plasmid vector indicate that the in situ hybridization is indeed recognizing GAP43 mRNA. It is not known whether changes in GAP43 mRNA reflect changes in transcription or in mRNA stability.

The procedures for quantification currently used allow comparison of labelling by GAP43 cDNA among groups of neurons in the same section or in different sections on the same slide. Because of slide-to-slide variability due to temperature dependence of the GAP43 hybridization, probe concentration, and other unknown factors, we have not compared labelling densities from sections processed on different slides. Neither have we attempted to convert labelling densities to absolute values of cytoplasmic mRNA per cell. This difficult task would require rigorous control of conditions plus standardizing hybridizations with known amounts of GAP43 mRNA.

Correlation between GAP43 and NGF binding in normal ganglia

Among normal rat DRG neurons, expression of the GAP43 gene is strongly correlated with deployment of the high-affinity NGF receptor. Given that NGF can influence GAP43 synthesis in pheochromocytoma cells (Karns et al, 1987; Federoff et al,

1988), it seems plausible to attribute the high basal synthesis of GAP43 in sympathetic neurons and some sensory neurons to continuous stimulation by endogenous NGF. An alternative possibility is that periodic mechanical injury to the exposed cutaneous terminals of some primary sensory neurons stimulates a mild manifestation of the cell body response to axotomy: degenerated terminals have been observed in the subepidermal C fibre network of normal rats (Jackson & Diamond, 1984). However, this hypothesis seems highly unsatisfactory for sympathetic neurons in the superior cervical ganglia where GAP43 is abundantly synthesized even though their axon terminals are minimally exposed to chronic injury. We favour the suggestion that NGF is causally involved in the high basal rates of GAP43 synthesis by some neurons.

Virtually all substance P-immunoreactive neurons and most CGRP-immunoreactive neurons have high-affinity NGF binding sites (Verge et al, 1989b) and neurons with significant high-affinity binding invariably are labelled by GAP43 cDNA (Figure 3). Therefore it can be assumed that neurons with substance P immunoreactivity and strong CGRP immunoreactivity synthesize GAP43 at high basal level. In most normal DRG, a few small neurons were found with little NGF binding but relatively high GAP43 hybridization. Histochemical studies indicate that neurons in this latter class contain neither somatostatin nor acid phosphatase. Small sensory neurons with somatostatin immunoreactivity or acid phosphatase enzyme activity have been

previously shown to lack high-affinity NGF binding sites and are here directly shown to have low basal levels of GAP43 mRNA. The few neurons that appear positive for GAP43 and negative for the NGF receptor could represent a true subpopulation or falsely high GAP43 labelling of small cross-sectional areas.

One speculation is that high basal concentration of GAP43 confers upon some DRG neurons a capacity for collateral sprouting. After injury to a peripheral nerve, some functionally defined subclasses of sensory axons from an adjacent nerve can grow into the denervated territory (Jackson & Diamond, 1984). The fact that such sprouting is blocked by antiserum to NGF (Diamond et al, 1987) suggests the involvement of NGF and participation of NGF-receptor-bearing neurons: the latter population is shown here also to express the GAP43 gene. By similar conjecture, the outgrowth of uninjured sympathetic neurons in response to partial denervation (Kennedy & Sakuta, 1984; Kessler, 1985) might also depend upon the presence of GAP43 within neurons and some local stimulus from denervated Schwann cells or target cells. On the other hand, uninjured motoneurons are also capable of sprouting (Brown et al, 1981) yet have uniformly low basal levels of GAP43 (Tetzlaff & Bisby, 1988). The possible contribution of GAP43 to sprouting might be clarified by better histochemical definition of sensory axons that are capable of collateral sprouting. The possibility that GAP43 has some unrecognized function in a subpopulation of sensory neurons must also be entertained.

Changes after injury

One week following peripheral nerve transection, the concentration of GAP43 mRNA is increased in most L_5 DRG neurons. The few neurons with low GAP43 labelling after injury are assumed to project into the dorsal cutaneous nerve (Diamond et al, 1987) and not the sciatic nerve. Although sensory neurons differ in some of their degenerative responses to axonal injury (Aldskogius & Risling, 1983), the present observations indicate that synthesis of GAP43 is induced to a high level in all or almost all sensory neurons shortly after their axons are cut. It remains to be proved that sensory nerve cell bodies are conditioned to the same extent or maintain high levels of GAP43 for the same period of time.

One week after nerve transection, GAP43 synthesis is increased in neurons with or without high-affinity NGF receptors, and under conditions of reduced retrograde supply of NGF, or continuous infusion of NGF. These observations indicate that some signal other than NGF or lack of NGF is responsible for the early post-traumatic induction of GAP43. Both increased axonal transport of GAP43 and a propensity for regeneration result directly or indirectly from the loss of some retrograde signal normally arising distal to the site of axonal injury (Benowitz et al, 1983; Richardson & Verge, 1986; Bisby, 1988). Because NGF counteracts some of the changes of axotomized sensory neurons (Fitzgerald et al, 1985; Rich et al, 1987; Verge

et al., 1989a) but not the increase in GAP43, it seems necessary to invoke at least two molecular signals to explain the multiple nerve cell responses to injury.

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TABLE 1

MEAN GAP43 LABELLING DENSITY (X BACKGROUND)

Animal Number	Total Population	Lightly labelled by NGF	Heavily labelled by NGF
1 (normal)	13.2 ± 0.9	5.3 ± 0.6	23.1 ± 1.5
2 (normal)	9.8 ± 0.6	4.8 ± 0.6	18.5 ± 1.1
3 (normal)	19.0 ± 1.3	8.3 ± 1.3	36.7 ± 2.5
4 (normal)	5.0 ± 0.3	3.4 ± 0.3	8.0 ± 0.6
3 (cut)	38.4 ± 2.0	43.6 ± 3.8	40.9 ± 3.5
4 (cut)	13.5 ± 0.5	13.4 ± 0.8	13.6 ± 0.9

GAP43 labelling densities were calculated for the thirds of normal neurons most lightly and heavily labelled by NGF in normal L_5 DRG from 4 different rats. Also included is the comparable information for 2 ganglia associated with a sciatic nerve transection, 1 week previously. Data from animal 1 and animal 4 are displayed in Fig. 2 and Fig. 5 respectively (m ± s.e.m.; n = 243-327).

FIGURE 1: Darkfield photomicrograph (**left**) of GAP43 hybridization to rat superior cervical ganglion (magnification x55). Northern blot preparation (**right**) of total cytoplasmic RNA from control L_5 DRG (con) and DRG associated with axonal transection 5 days previously (ax). The filter was hybridized with ^{35}S-GAP43 cDNA which recognizes a single mRNA of 1.4 kilobases and much greater manifestation following injury.

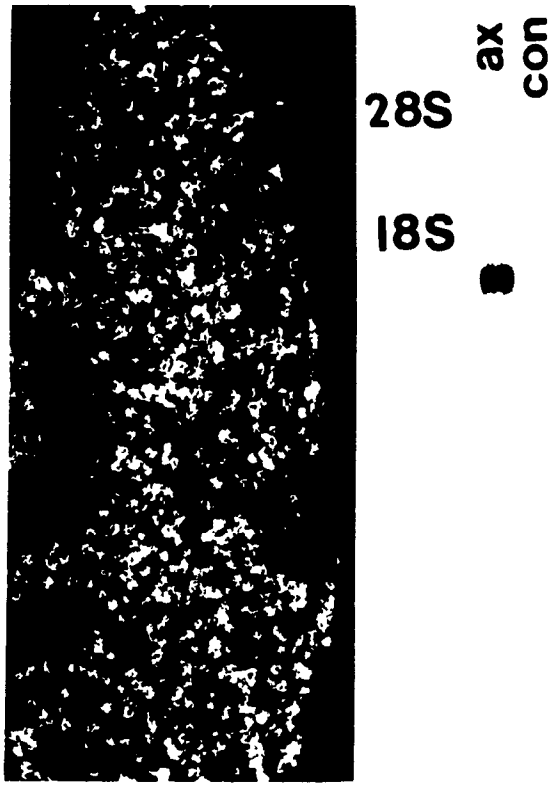


FIGURE 1

FIGURE 2: Photomicrographs of adjacent sections, 5 μm thick, from L_5 DRG processed for GAP43 hybridization (**left**) or NGF-receptor radioautography (**right**). Note that 7 neurons are heavily labelled and 3 large neurons are lightly labelled with each of the two markers (magnification x700).

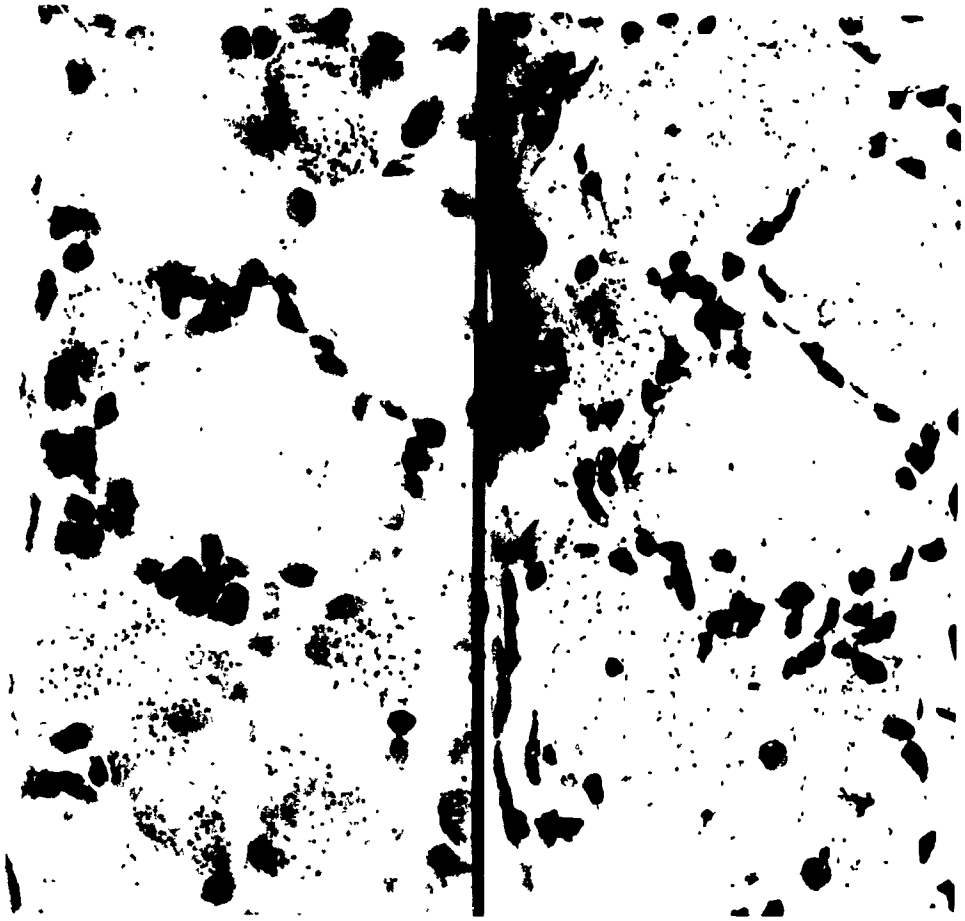
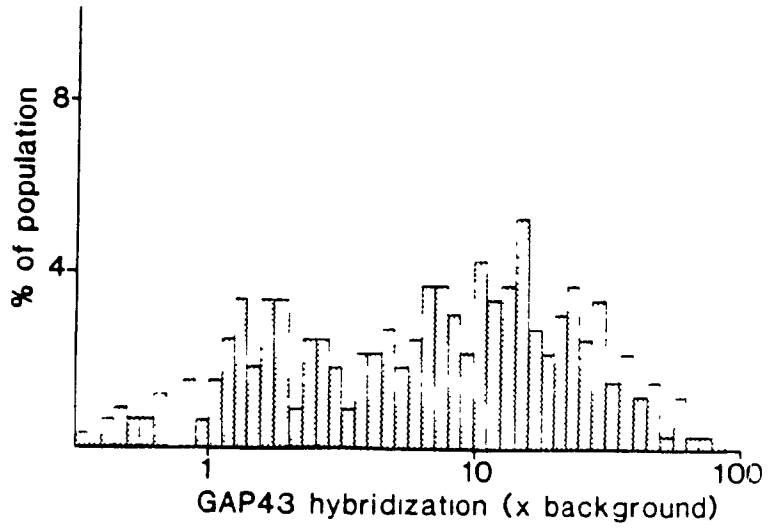
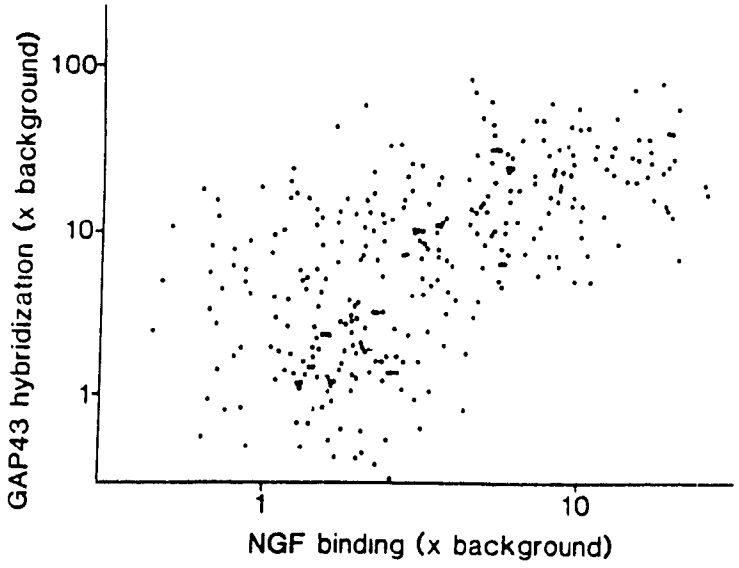


FIGURE 2

FIGURE 3: The 3 diagrams all present data from 322 neurons identified and quantified for binding of ^{125}I-NGF and hybridization with ^{35}S-GAP43 cDNA. The histogram of GAP43 labelling densities (**upper**) shows a bimodal distribution compatible with the existence of two populations. The scatter diagram (**middle**) and three-dimensional histogram (**lower**) are derived from labelling densities for individual neurons in the two preparations. Note that most neurons fall within two clusters, "labelled" or "unlabelled" by the two markers.


% of population
8
4
1
10
100
GAP43 hybridization (x background)


GAP43 hybridization (x background)
100
10
1
1
10
NGF binding (x background)

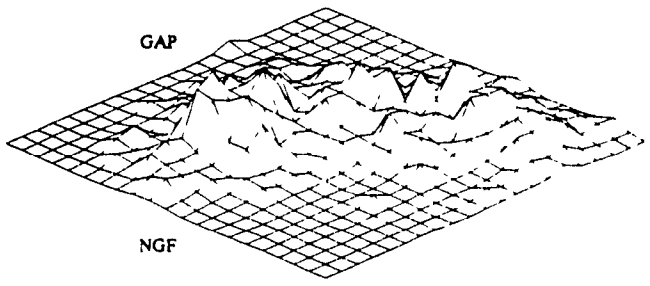

GAP
NGF

FIGURE 3

FIGURE 4: Upper left, Photomicrographs of adjacent sections of L_5 DRG processed to show thiamine monophosphatase activity (left) or GAP43 mRNA (right). Note that the two neurons with thiamine monophosphatase activity have little GAP43 mRNA (magnification x810).

Lower left, Photomicrographs of adjacent sections processed for somatostatin immunohistochemistry (left) or GAP43 hybridization (right). Note that the somatostatin-positive neuron is GAP43-negative (magnification x810).

Upper right, Scatter diagram in which GAP43 labelling density is plotted against diameter for 223 neurons representing the entire L_5 DRG. There are wide ranges of size and labelling density with little correlation between the two measurements.

Middle right: Individual neurons were identified to have acid phosphatase activity and quantified for GAP43 hybridization on the adjacent section. Note that all the neurons with thiamine phosphatase reaction product are small with little GAP43 labelling (n = 48).

Lower right, Quantification of GAP43 labelling density and size for neurons seen in the adjacent section to have somatostatin immunoreactivity. Somatostatin positive neurons are small with GAP43 labelling near background (n = 14).


TMPase
Som

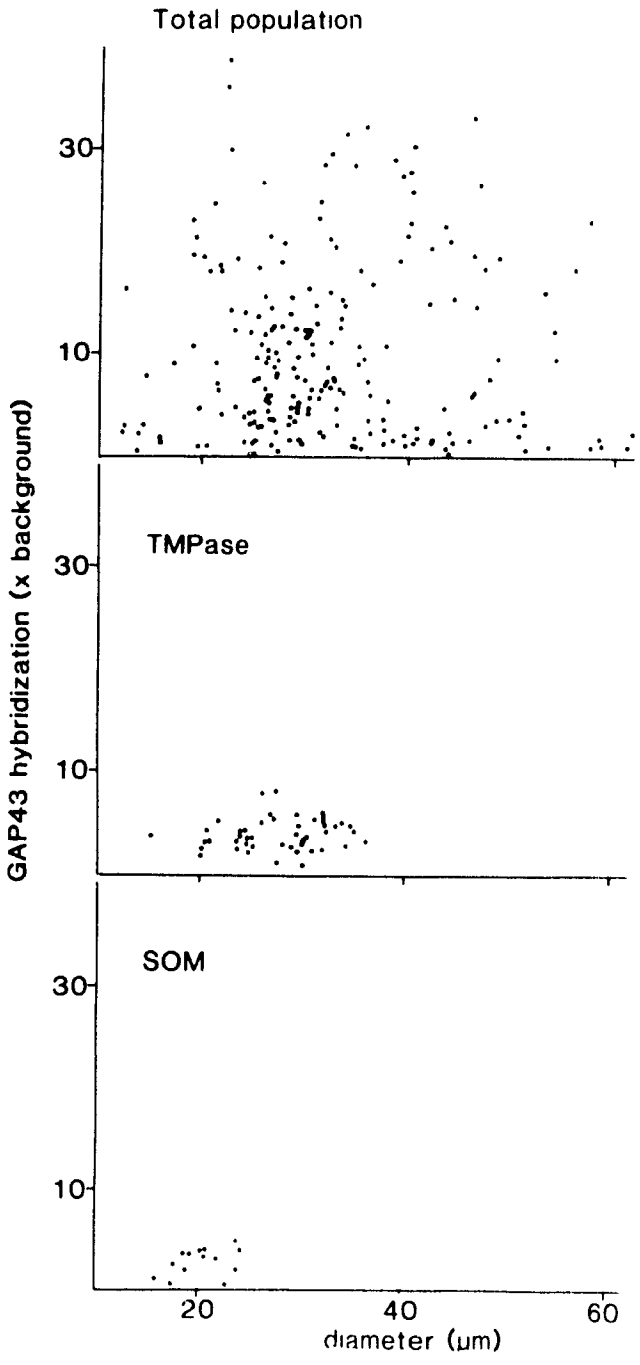

Total population
30
10
TMPase
30
10
SOM
30
10
GAP43 hybridization (x background)
20
40
60
diameter (µm)

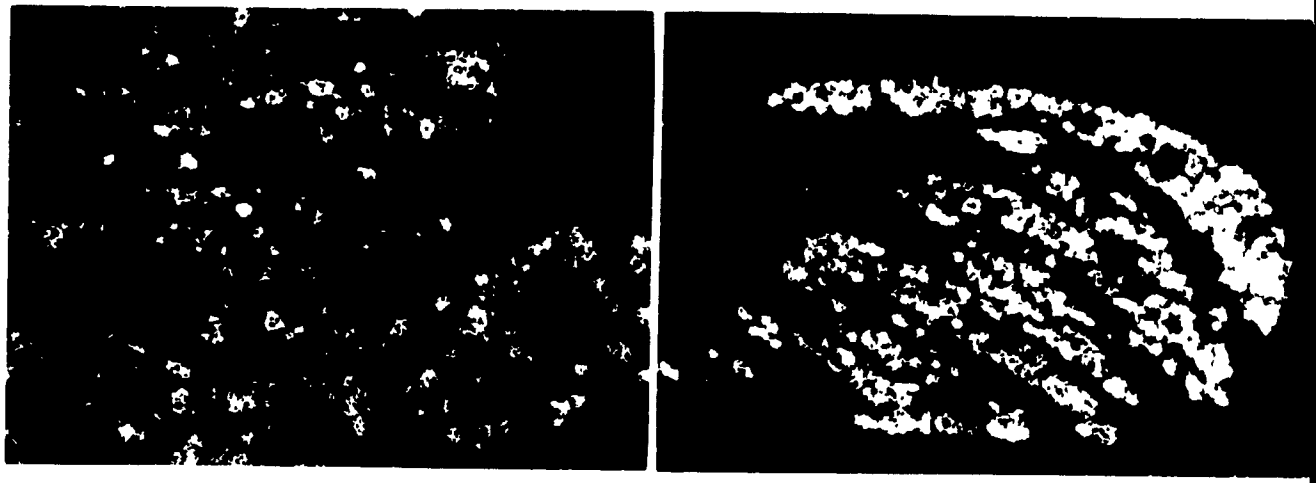
FIGURE 4

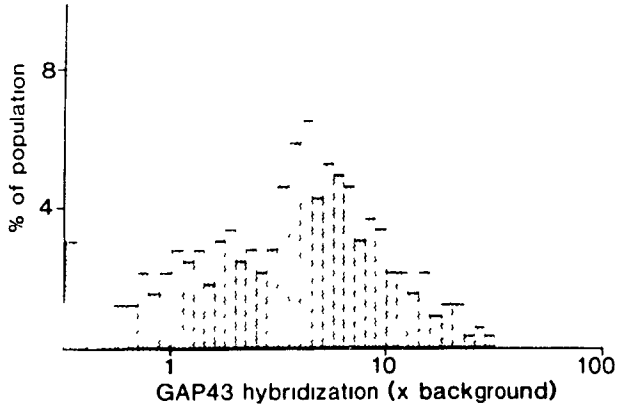
FIGURE 5: One week after sciatic nerve transection the left and right L_5 DRG were removed. Data on the left and right are for the left and right DRG respectively.

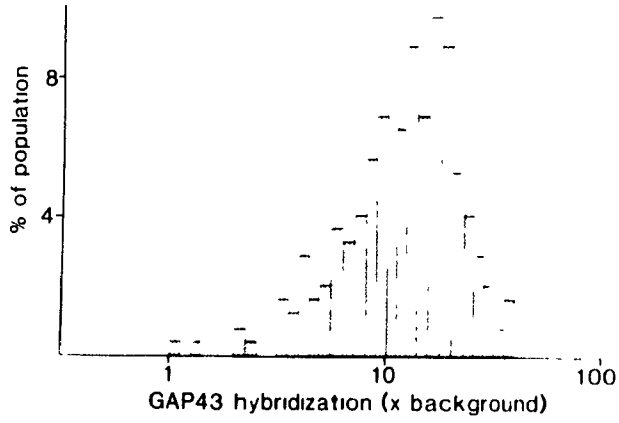
Upper, Dark-field photomicrographs of sections after GAP43 in situ hybridization. Note the heterogeneous labelling in the normal ganglia and the increased homogeneous labelling associated with nerve transection (magnification x55).

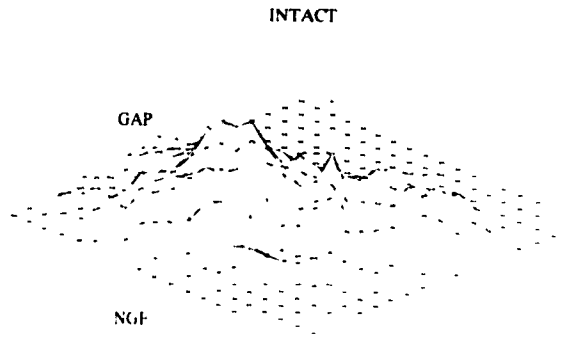
Middle, Histograms of GAP43 labelling densities showing a bimodal distribution in the left DRG and a single densely labelled peak in the right DRG.

Lower, 3-dimensional histograms of NGF and GAP43 labelling. In the normal ganglion (n = 327), note the correlation between the two labels: after nerve transection (n = 248) this correlation is lost and GAP43 labelling densities appear to be uniformly high.




% of population
8
4
1
10
100
GAP43 hybridization (x background)


% of population
8
4
1
10
100
GAP43 hybridization (x background)


INTACT
GAP
NGF

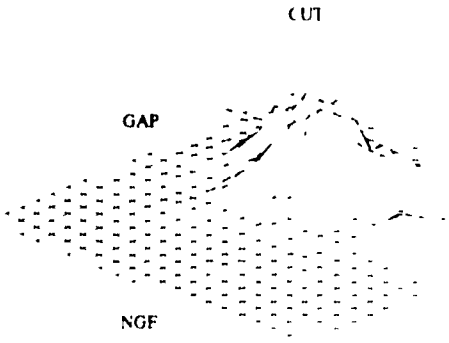

CUT
GAP
NGF

FIGURE 5

FIGURE 6: Data for left and right L_5 DRG removed one week after right sciatic nerve transection followed by continuous infusion of NGF.

Upper, Dark-field photomicrographs of sections after GAP43 in situ hybridization. Labelling appears to be supranormal but is still heterogeneous in the left DRG and homogeneously high in the right DRG (magnification x55).

Middle, Histograms of GAP43 labelling densities showing a bimodal distribution (left) and unimodal distribution (right). The patterns are similar to those in Figure 5.

Lower, 3-dimensional histograms of GAP43 and NGF labelling showing once again a correlation between the two indices when the sciatic nerve is intact (n = 309) and a uniform increase of GAP43 labelling after the nerve has been cut (n = 347).

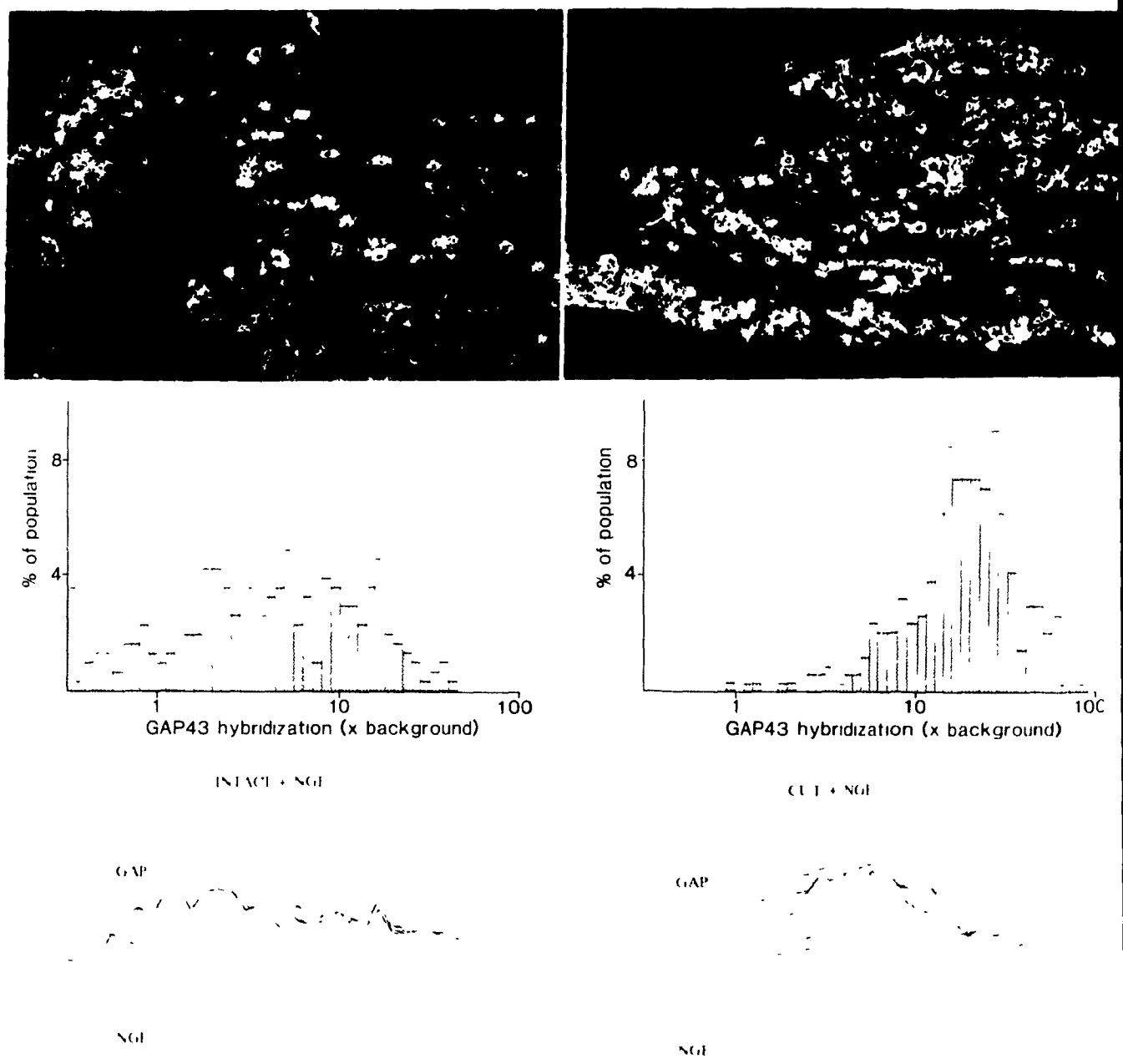

% of population
8
4
1
10
100
GAP43 hybridization (x background)
% of population
8
4
1
10
100
GAP43 hybridization (x background)

FIGURE 6

CHAPTER 6

INFLUENCE OF NERVE GROWTH FACTOR ON NEUROFILAMENT GENE EXPRESSION IN MATURE PRIMARY SENSORY NEURONS

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SUMMARY

To analyze the possible influence of nerve growth factor (NGF) on neurofilament synthesis in primary sensory neurons, adjacent cryostat sections of lumbar DRG (dorsal root ganglia) from adult rats were processed for either NGF-receptor radioautography or in situ hybridization with a neurofilament cDNA probe. Labelling by both procedures was quantified with computer assistance for approximately three hundred neurons in each of selected ganglia. For uninjured neurons, no correlation was detected between NGF binding and neurofilament mRNA, even after infusion of NGF into the lumbar subarachnoid space for one week. One or three weeks after sciatic nerve transection, neurofilament labelling densities in large DRG neurons were sharply reduced and the normal bimodal pattern in frequency histograms had become unimodal. Intrathecal infusion of NGF counteracted this injury-induced reduction of neurofilament mRNA but only in neurons with high-affinity NGF receptors. To explain the effects of NGF on axotomized neurons and the normal diversity of neurofilament gene expression among neurons with NGF receptors, we postulate that NGF permits NGF-sensitive DRG neurons to respond differentially to a second factor stimulating neurofilament synthesis.

INTRODUCTION

The number of neurofilaments within axons is believed to control their diameter and thereby their conduction velocity: neurofilament number and axonal diameter are closely correlated with each other under normal and abnormal conditions (Friede and Samorajski, 1970; Weiss and Mayr, 1971; Hoffman et al, 1984). Given an additional correlation between conduction velocity and size of the nerve cell body (Harper and Lawson, 1985; Lee et al, 1986), it is appropriate that large neurons have more neurofilaments than small neurons (Sharp et al, 1982). From analyses of perikaryal size, cytoplasmic appearance, and neurofilament immunohistochemistry, rodent primary sensory neurons have been subdivided into small dark neurons with unmyelinated axons and large light neurons with myelinated axons (Andres, 1971; Duce and Keen, 1977; Lawson, 1979; Rambourg et al, 1983; Lawson et al, 1984). The tinctorial properties of large light neurons are probably related to the presence of many perikaryal neurofilaments. It is intriguing to speculate that a few molecules influencing expression of the neurofilament gene might control such fundamental properties as neuronal size and conduction velocity. However, nothing is yet known about the molecules that regulate neurofilament content of DRG (dorsal root ganglion) neurons.

NGF (nerve growth factor) increases expression of the genes

for all three neurofilament subunits in cloned pheochromocytoma (PC-12) cells (Dickson et al, 1986; Lindenbaum et al, 1986). The hypertrophy of primary sensory neurons following NGF injection into chick embryos (Straznicky and Rush, 1985; Dimberg et al, 1987) may reflect induction of neurofilament synthesis by NGF. The fact that axons of adult rat sensory neurons undergo atrophy following administration of NGF antiserum suggests a regulatory role for endogenous NGF in axonal caliber (Matheson et al, 1989). In mature rats, approximately 40% of lumbar sensory neurons have high-affinity NGF receptors and are potentially responsive to NGF (Verge et al, 1989a,b). We were therefore interested to determine whether endogenous NGF is involved in controlling neurofilament synthesis in normal mature DRG neurons.

Several properties of NGF-sensitive sensory neurons, notably the concentration of substance P are maintained by endogenous NGF (Lindsay and Harmar, 1989), falling when NGF is depleted by antibodies (Schwartz et al, 1982) or injury (Jessell et al, 1979) and augmented by infusion of exogenous NGF (Goedert et al, 1981; Fitzgerald et al, 1985). Following peripheral nerve transection, nerve cell body size, axonal diameter, neurofilament synthesis and axonal transport of neurofilament are all reduced in sensory neurons (Cavanaugh, 1951; Aitken and Thomas, 1962; Carlson et al, 1979; Hoffman et al, 1987; Wong and Oblinger, 1987; Oblinger and Lasek, 1988; Goldstein et al, 1988). NGF can partially prevent or restore this injury-induced

atrophy (Rich et al, 1987; Verge et al, 1989a). We hypothesized that the down-regulation of neurofilament synthesis in axotomized sensory neurons with functional NGF receptors might also be counteracted by infusion of NGF.

MATERIALS AND METHODS

Operations were performed on 18 female Sprague-Dawley rats, weighing approximately 200 gm, with general anesthesia (pentobarbitol 50 mg/kg intraperitoneal) and aseptic microsurgical conditions. The right sciatic nerve was transected at its origin from the L_4 and L_5 spinal nerves and a 5 mm segment of nerve was excised. In 9 rats, β-NGF was infused at 1μl/h by a subcutaneous osmotic pump (Alza #2001) connected to silicon tubing (outer diameter 0.6 mm) inserted intrathecally at the lumbosacral junction to a length of 2 cm. β-NGF, prepared from mouse submandibular glands (Mobley et al, 1976; Chapman et al, 1981), was diluted to 125 ng/μl in phosphate-buffered saline containing rat serum albumin (1 mg/ml), penicillin and streptomycin (100 units/ml). Four experimental groups were studied with simple nerve transection one or three weeks before death, and with transection one or four weeks before death plus infusion of NGF in the last week. At death, rats were deeply anesthetized and perfused per aorta with buffered saline. The fourth and fifth lumbar dorsal root ganglia (L_4 and L_5 DRG) were removed and the right and left ganglia at each level were quickly mounted on the same cork and frozen in isopentane cooled to -60°C. Cryostat sections 5 μm thick were thaw-mounted on gelatin-coated slides and stored at -80°C for not more than one week. Adjacent sections, each with a

pair of DRG, were processed either for NGF-receptor radioautography or neurofilament in situ hybridization.

NGF-receptor radioautography was performed according to a published protocol (Richardson et al, 1989; Verge et al, 1989a). β-NGF was radioiodinated by the lactoperoxidase method (Sutter et al, 1979; Richardson et al, 1989) to a specific activity of approximately 100 μCi/μg. Slides were incubated for 90 minutes in 40-50 pM ^{125}I-NGF in phosphate-buffered saline containing cytochrome C (1 mg/ml) to diminish non-specific labelling and protease inhibitors (4 μg/ml leupeptin and 0.5mM PMSF). Slides were washed for 3 minutes in several changes of cold buffer, fixed in mixed aldehydes, dehydrated, defatted, rehydrated and dried. They were then dipped in radiosensitive emulsion (Kodak NTB2), exposed in the dark for 4-7 days and developed (Kodak D-19).

The ^{35}S-radiolabeled probe used for hybridization was generated from isolated cDNA insert of the medium neurofilament subunit (NFM) (Julien et al, 1986) according to the random hexonucleotide priming procedure (Feinberg and Vogelstein, 1983). The cDNA clone was generously provided by Dr. J-P. Julien, McGill University. The technique for in situ hybridization was essentially that of Miller et al (1987). Sections were post-fixed for 20 minutes in 4% formaldehyde, washed in buffered saline, treated for 8 minutes with proteinase K (10 ng/ml) at room temperature, rinsed and fixed an additional 5 minutes in 4% paraformaldehyde. Sections were then dehydrated

in 70%, 90%, and 100% ethanol containing 0.3 M sodium acetate, briefly dried and pre-hybridized for 2-3 hours at 43°C with a solution containing 50% formamide, 5x Denhardt's solution, salmon sperm DNA and tRNA (250μg/ml), 0.2% SDS and 100mM DTT. The same solution was used for overnight (12-14 hours) hybridization at 43°C with the addition of ^{35}S-labelled NFM cDNA probe (2 x 10^6 cpm/ml, 10-20 ng/ml). Post-hybridization washes were done at 45°C with 4x,2x,1x, and 0.5x for 30 minutes each and 0.1x SSC for 5 minutes. Sections were processed for radioautography by dipping in Kodak NTB2 emulsion 1:1 in distilled water, exposed in the dark at 4°C for 6-14 days, developed in D-19 solution and stained with toluidine blue. Control sections were hybridized with ^{35}S-labelled plasmid DNA instead of neurofilament cDNA.

Quantitative analysis was performed for 10 ganglia in 5 animals subjected to right sciatic nerve transection with or without NGF infusion. For this purpose, montages were prepared from photomicrographs (magnification 450x) of adjacent sections processed for NGF-receptor radioautography and NFM cDNA hybridization. Neurons with visible nucleolus in one of the two sections were identified and numbered on both montages and analyzed quantitatively under oil-immersion light microscopy with computer assistance (n=224-360 per preparation). Image analysis software was kindly provided by Dr. W.G. Tatton, University of Toronto. For individual neurons, the cross-sectional area and percentage of cytoplasmic area covered by

silver grains were measured in both histological preparations. Volumes were calculated from the larger of the two cross-sectional areas in adjacent sections with correction for shrinkage and assumption that the neurons are spherical. Labelling indices included a correction for grain overlap and were normalized against background labelling over areas of the ganglia without nerve cell bodies. Data were analyzed statistically according to published computer programs (Press et al, 1988). Where appropriate, histograms were fitted to a double Gaussian curve by the Levenberg-Marquardt algorithm.

RESULTS

Normal ganglia

In left L_5 DRG with or without NGF infusion, labelling of DRG neurons with the NFM cDNA probe was heterogeneous (Figure 1). Background labelling was 0.0015-0.0024 of area and neuronal labelling indices ranged from below background to more than 100 times background. Frequency histograms of labelling indices (Figure 2) could be fitted to two normal curves with 0.62 ± 0.04 and 0.38 ± 0.04 of neurons and mean labelling indices of 3.4 ± 0.6 and 34.9 ± 4.7 (logarithmic scale, mean ± s.e.m., 5 plots, 250-346 neurons per plot). No specific labelling was observed for sections hybridized with radiolabelled plasmid.

Frequency histograms of cell volume could also be fitted to two normal curves with 0.67 ± 0.04 and 0.33 ± 0.04 of neurons and mean volumes of 14,000 ± 700 μm^3 and 50,000 ± 4000 μm^3 (logarithmic scale, mean ± s.e.m., 5 plots, 250-346 neurons per plot, data not shown).

In scatter diagrams or three-dimensional histograms (Figure 2) to show the frequency distribution of neurons according to volume and NFM mRNA labelling indices (both on a logarithmic scale), a strong correlation between the two measurements was detected (Pearson coefficient = 0.64-0.71, $p<0.0001$ by Student's t test for the parameter z, 5 plots). Most of the neurons belong to one of two populations - with small volume and light

NFM mRNA labelling or large volume and heavy NFM mRNA labelling.

No correlation was detected between labelling indices in NGF-receptor radioautography and NFM in situ hybridization (Figures 1 & 2). By this analysis, most neurons could be placed into four clusters with light or heavy labelling by the two procedures. For neurons with little NGF binding, the subpopulations with high and low NFM mRNA concentration were distinct: for neurons with high NGF binding, the two populations were less clearly separated and the total range of NFM mRNA labelling was diminished.

Administration of NGF seemed to accentuate the difference between neuronal populations that were heavily and lightly labelled by the NFM probe (Figure 3), although this impression was not quantitatively documented. In otherwise normal ganglia, the percentage of neurons in the two populations was not appreciably changed by exogenous NGF and the values quoted in the first paragraph are for left DRG with or without NGF infusion. After administration of NGF, the correlation between NFM mRNA labelling index and neuronal volume was still strong and the four populations of neurons with low or high NGF binding and low or high NFM mRNA labelling were more evident (Figures 4 & 5).

Changes after injury

As expected from previous reports (Hoffman et al, 1987; Wong and Oblinger, 1987; Goldstein et al, 1988; Verge et al,

1989a), both cytoplasmic NFM mRNA and NGF binding in DRG neurons fell substantially after sciatic nerve transection. Diminished NFM mRNA labelling in the right as compared to left DRG was apparent upon dark-field inspection of all 9 pairs of ganglia removed 1 or 3 weeks after simple sciatic nerve transection (Figure 3). The right:left ratio of mean labelling indices for NFM mRNA was 0.29 at 1 week and 0.21 at 3 weeks (quantification for 3 rats, 224-352 neurons). The loss of total cytoplasmic NFM mRNA was even greater because neuronal volume also fell after nerve injury (to 0.91 of normal at 1 week and 0.65 of normal at 3 weeks). In frequency histograms of NFM mRNA labelling indices, the normal bimodal pattern disappeared and the data fitted reasonably well to a single normal curve (data not shown). Three weeks after nerve transection, both NFM mRNA labelling and NGF binding had unimodal distributions so that in 3-dimensional plots of frequency vs NGF and neurofilament labelling (Figure 5), most neurons belonged to a rather homogeneous population with subnormal labelling on both axes.

On darkfield inspection of NFM cDNA hybridizations to DRG, the loss of labelling after sciatic nerve transection was mitigated in 6 of 9 rats infused with NGF (Figure 3). This visual result was similar whether NGF was infused from 0-1 weeks or 3-4 weeks after nerve injury. In one of the 3 non-responding rats that was killed four weeks after nerve transection, NGF infusion must have been technically unsatisfactory because NGF binding was not restored. Why

neurofilament mRNA did not respond to NGF in 2 other animals is unknown because they were killed at 1 week when NGF binding has not fallen enough to be used for monitoring pump adequacy. That NGF counteracts NFM mRNA loss after nerve injury was best substantiated by pattern shifts in 3-dimensional histograms of frequency vs NGF binding and NFM mRNA labelling indices. Infusion of NGF restored NFM mRNA labelling (Figure 5) or prevented its loss (Figure 4) in approximately one-half of neurons with high NGF binding but did not significantly alter the changes in neurons with low NGF binding. For the one third of neurons most heavily labelled by NGF (Figure 6), the right:left ratio of mean NFM mRNA labelling indices fell to 0.27 three weeks after right sciatic nerve transection and was restored to 0.83 after a further week of NGF infusion (quantification for 2 rats, 224-297 neurons).

DISCUSSION

Technical considerations

The dual quantification of NGF binding and NFM mRNA labelling for hundreds of neurons permits correlations and conclusions that are not evident on visual inspection, independent consideration of parameters, or global analysis of the DRG. Side-to-side comparisons of absolute values between ganglia of the same animal seem valid when each of the pair is mounted on the same slide and treated by identical histological

procedures. Because of presumed variability in the conditions for in situ hybridization, no conclusions have been based on comparison of mean labelling indices between sections mounted on different slides. However, consistent changes in pattern of 3-dimensional frequency histograms seem to be a reliable indication of real change, not attributable to variability in hybridization conditions.

The present data concern only the medium neurofilament subunit, yielding no direct information about the light and heavy subunits.

Classification of sensory neurons

Among normal rat DRG neurons, the frequency distributions of NFM mRNA concentration and volume are bimodal and strongly correlated with each other. These observations support the concept of a fundamental division between small dark and large light neurons, NFM mRNA serving as a marker for the latter population. NFM mRNA is not absent in small sensory neurons, merely scarce. The data are entirely consistent with the suggestion that neurofilament content is an important determinant of neuronal size and axonal diameter. It also appears that the difference in neurofilament content between the two populations of neurons is determined by different rates of neurofilament synthesis rather than other possible explanations.

In normal DRG, NFM mRNA concentration was not correlated with NGF binding. Neurons with or without high-affinity NGF

receptors could be large or small and could have high or low concentration of NFM mRNA. It can therefore be assumed that some NGF-receptor bearing sensory neurons have unmyelinated axons and some have myelinated axons (Harper & Lawson, 1985; Lee et al, 1986). This result stands in contrast to studies correlating NGF-binding with neuropeptides where close co-localization or absence of co-localization was the rule (Verge et al, 1989b). It can be concluded that small dark and large light neurons in rat DRG are not homologous to small dorsomedial NGF-responsive and large ventrolateral NGF-unresponsive neurons in chick DRG (Raivich et al, 1987). The unknown factors governing expression of the genes for the NGF receptor and neurofilament seem to be largely independent of one another.

Regulation of the neurofilament gene

This and other studies (Hoffman et al, 1987; Wong & Oblinger, 1987; Goldstein et al, 1988; Tetzlaff et al, 1988) show that neurofilament protein and mRNA have fallen markedly in neurons one week after their peripheral axons have been cut. The reduction in NFM mRNA is followed by reduction in perikaryal volume, more evident at three weeks than one week. The axotomy-induced change in NFM mRNA is obvious in large light neurons that normally synthesize neurofilament abundantly. We cannot say with confidence whether NFM synthesis is also reduced in small neurons that normally have little NFM mRNA. Why neurofilament mRNA falls after axonal injury has not been

rigorously established. Probably, reduced retrograde transport of NGF causes the change in neurofilament mRNA in NGF-receptor-bearing neurons, some analogous growth factor being implicated for other neurons. Alternatively, it is possible that loss of a single common factor is responsible for the post-injury reduction of neurofilament mRNA in both NGF-sensitive and NGF-insensitive neurons, being overcome by exogenous NGF for only that neuronal subpopulation with functional NGF receptors.

In controlling neurofilament gene expression, NGF appears to have more limited influence on sensory neurons in vivo than on clonal cell lines in vitro. For adult rat DRG neurons, sensitivity to NGF is neither necessary nor sufficient for high levels of neurofilament gene expression. Some neurons with high concentrations of NFM mRNA do not bear high-affinity NGF receptors and some neurons with NGF receptors have low concentrations of NFM mRNA. However, NGF is relevant to NFM synthesis in sensory neurons with NGF receptors because it counteracts post-axotomy down-regulation of NFM mRNA (Figures 4,5 & 6). One speculative explanation of these results is that NGF (or other trophic factors for NGF-insensitive DRG neurons) permits neurons to respond differentially to some unknown factor that stimulates neurofilament synthesis. Axotcmy, by depriving cells of retrogradely transported trophic factor(s), abolishes the permissive state, reducing expression of the neurofilament gene to a basal level in all DRG neurons. Application of NGF restores the permissive state and a normal range of

neurofilament mRNA concentration in NGF-sensitive neurons.

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FIGURE 1: Photomicrographs showing adjacent sections of normal rat L_5 DRG processed for in situ hybridization with a NFM cDNA probe (left) or NGF-receptor radioautography (right). NFM mRNA labelling is denser for large than small neurons but shows little relationship to NGF binding. (Magnification x530)

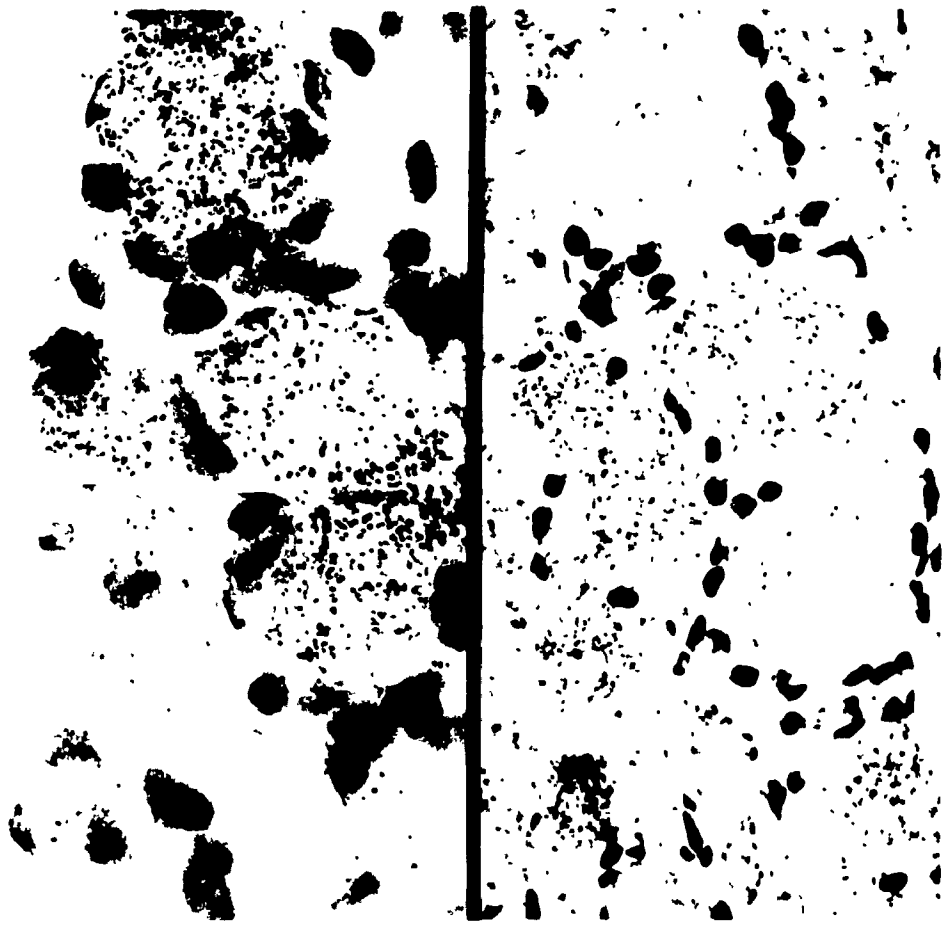

FIGURE 1

FIGURE 2: Quantification of NFM mRNA labelling in an uninjured L_5 DRG of a rat infused intrathecally for one week with NGF (n=296). **Top:** Frequency histogram showing percentage of neurons versus NFM mRNA labelling indices. The data can be fitted to two normal curves with mean labelling of 5.4 X background and 50 X background.

Middle: 3-dimensional histogram with frequency plotted against neurofilament labelling and volume. The neurons are distributed into two major pools with small volume and little NFM mRNA or large volume and abundant mRNA. Volume 6000-180,000 μm^3, NFM mRNA labelling index 0.6-180 X background, both on logarithmic scale.

Bottom: 3-dimensional histogram with frequency plotted against NFM mRNA labelling and NGF-binding. Little correlation is seen between the two labelling indices. NGF labelling index 0.32-32 X background, NFM labelling index 0.6-180 X background, both on logarithmic scale.

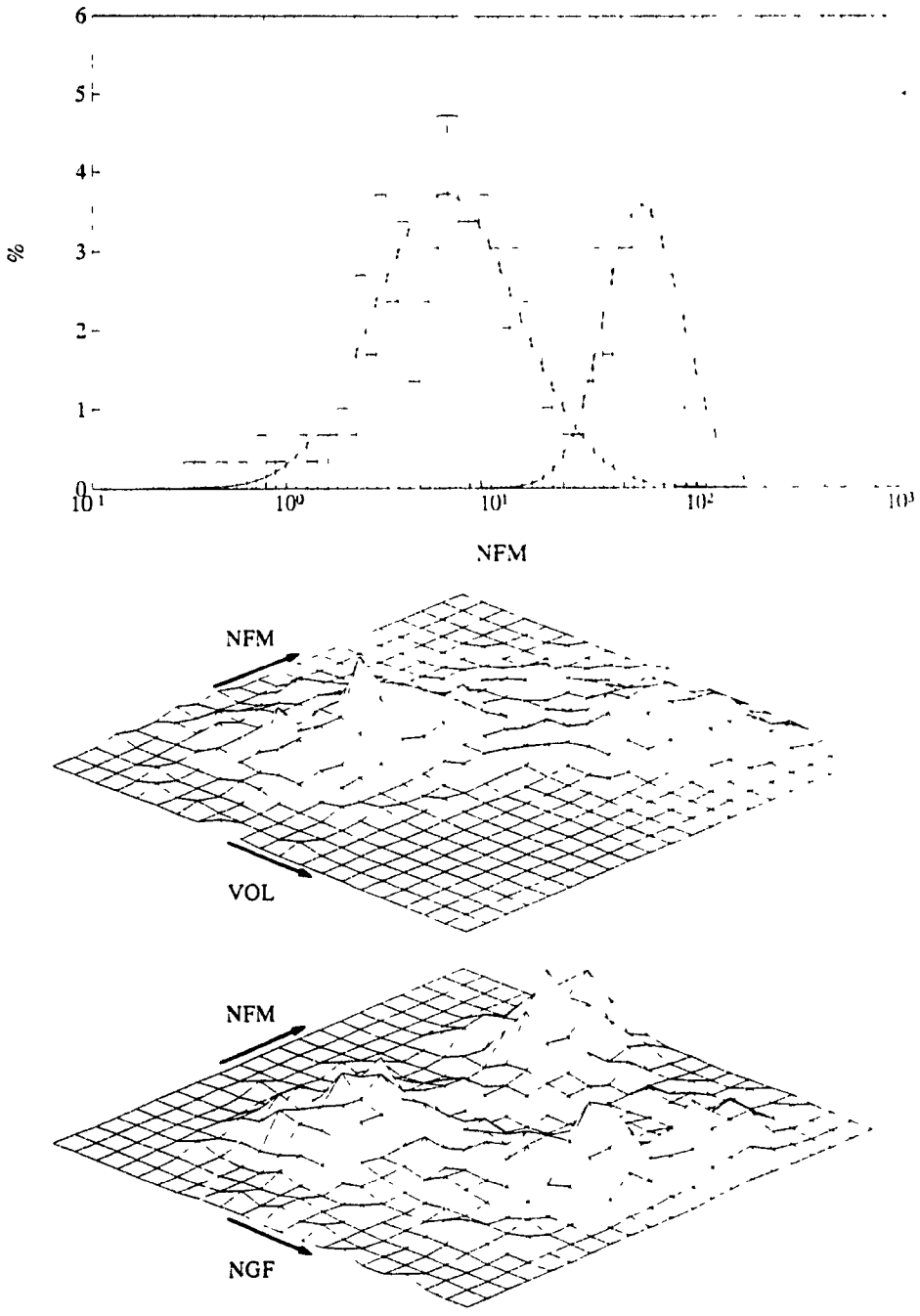

FIGURE 2

FIGURE 3: Dark-field photomicrographs showing NFM in situ hybridization for left and right L_5 DRG from 2 rats. In both rats, the right sciatic nerve was cut one week before sacrifice: for one rat, NGF was infused intrathecally during this week. L_5 DRG are associated with normal sciatic nerve (**upper**), cut sciatic nerve (**lower**), and NGF infusion (**right**) or no infusion (**left**). NFM mRNA is depleted by peripheral nerve injury but more heavily labelled neurons persist when injury is followed by NGF infusion (magnification x60).

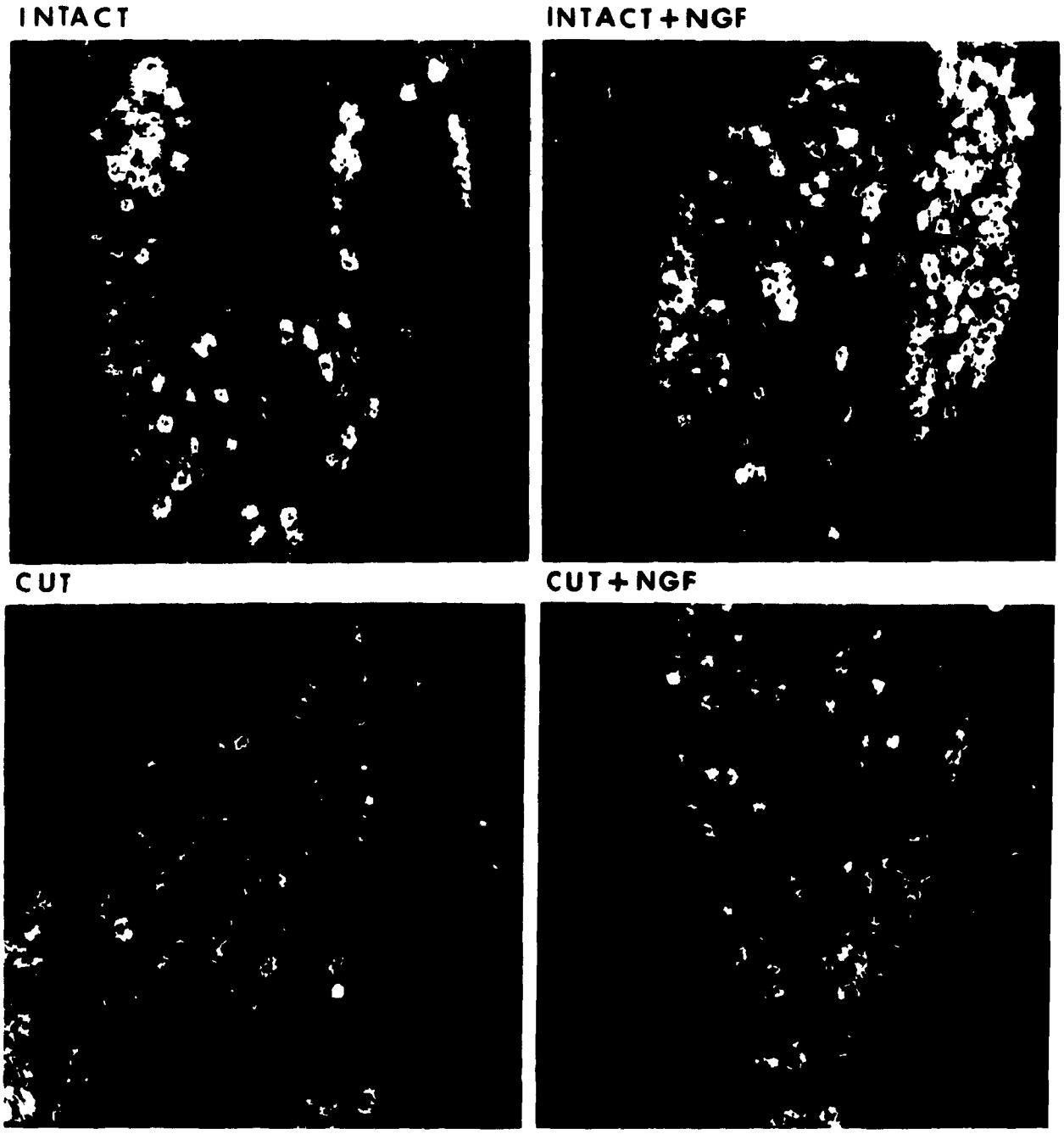

FIGURE 3

FIGURE 4: Three-dimensional histograms with neuronal frequency plotted against NFM and NGF labelling indices. Sciatic nerve transection and/or initiation of NGF infusion were one week before sacrifice. Normal nerve, no NGF infusion **(upper left)**, cut nerve, no NGF infusion **(lower left)**, normal nerve, NGF infusion **(upper right)**, cut nerve, NGF infusion **(lower right)**. With normal sciatic nerve, NGF and NFM labelling indices are not correlated with each other. One week after sciatic nerve transection, NFM mRNA is low in all neurons but NGF binding has changed little from normal. Injury-induced loss of NFM mRNA is prevented by NGF infusion but only for neurons with high NGF binding. The region of each histogram outlined with dark lines arbitrarily designates neurons considered to be very heavily labelled by both procedures. NGF labelling 0.6-18X background, NFM labelling 0.3-100X background, both on logarithmic scale (n = 231-360).

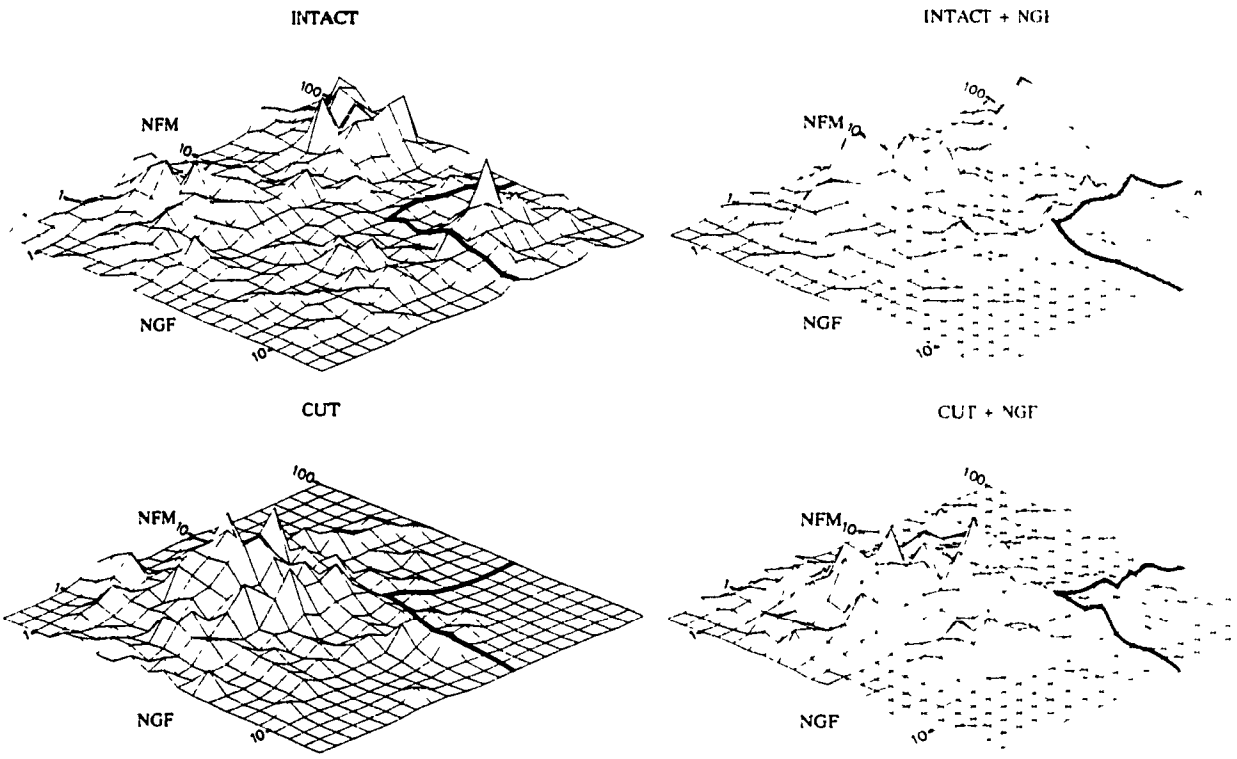

FIGURE 4

FIGURE 5: Three-dimensional histograms with neuronal frequency plotted against NFM mRNA and NGF labelling indices to show the effects of delayed infusion of NGF. Normal nerve, no NGF infusion (**upper left**), nerve cut 3 weeks before sacrifice, no NGF infusion (**lower left**), normal nerve, NGF infusion for one week (**upper right**), nerve cut 4 weeks before sacrifice, NGF infusion for last week (**lower right**). With normal sciatic nerve, neurons can be placed into four subpopulations with high or low labelling by both procedures. Three weeks after simple sciatic nerve transection, NGF binding and NFM mRNA are reduced for neurons with or without high-affinity NGF receptors. NFM mRNA is restored by delayed infusion of NGF for some neurons with high NGF binding, and therefore high-affinity NGF receptors. However, NGF does not counteract the loss of NFM mRNA in the subpopulation that normally lacks NGF receptors yet has abundant neurofilament mRNA. Again, the outlined regions contain neurons heavily labelled by both markers. NGF labelling 0.32-32 X background, neurofilament labelling 0.6-180 X background, both on logarithmic scale (n=224-297).

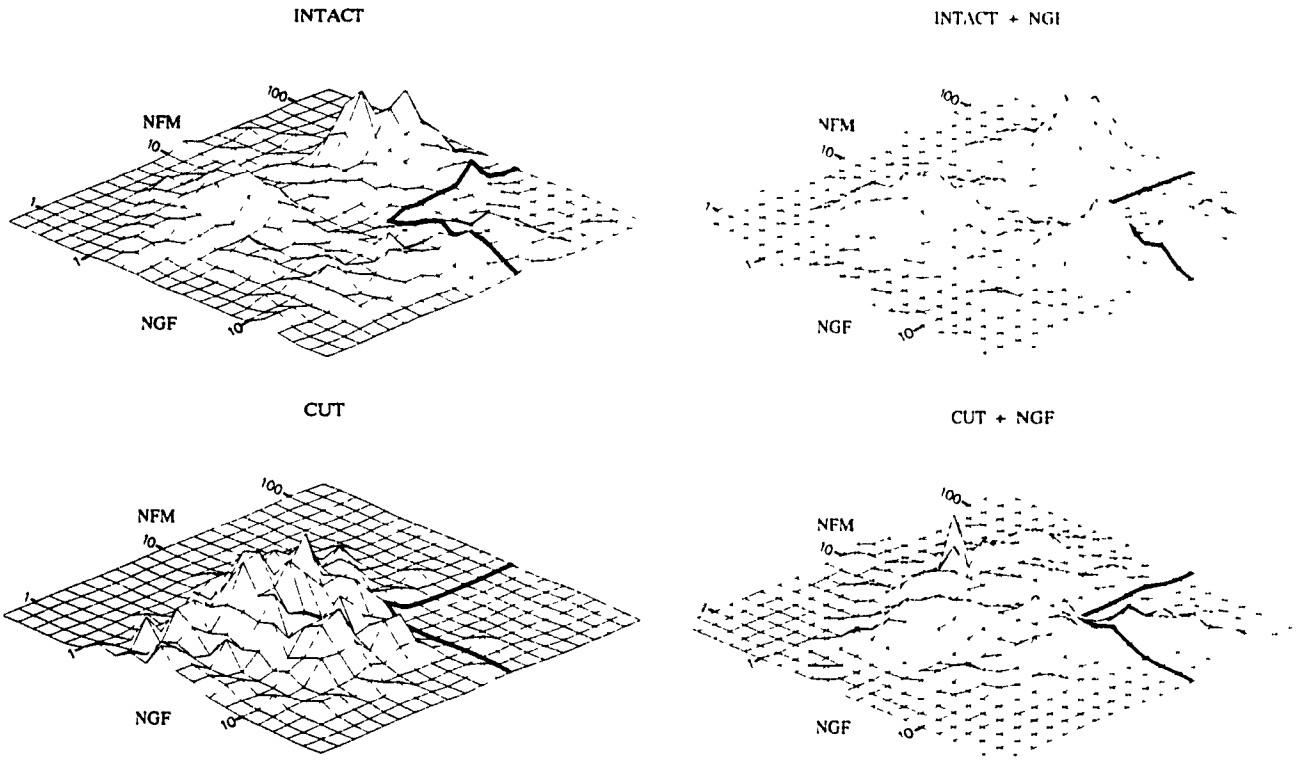

FIGURE 5

FIGURE 6: Frequency histograms of NFM labelling indices (x background) for the one third of neurons in figure 5 with densest binding of NGF. Intact NGF-receptor-positive neurons are heterogenous in their content of NFM mRNA (**upper panel**). Three weeks following injury few of such neurons have more than ten times background labelling for NFM mRNA (**lower left**). A population of neurons rich in NFM mRNA re-appears following delayed infusion of NGF (**lower right**) (n=75-99).

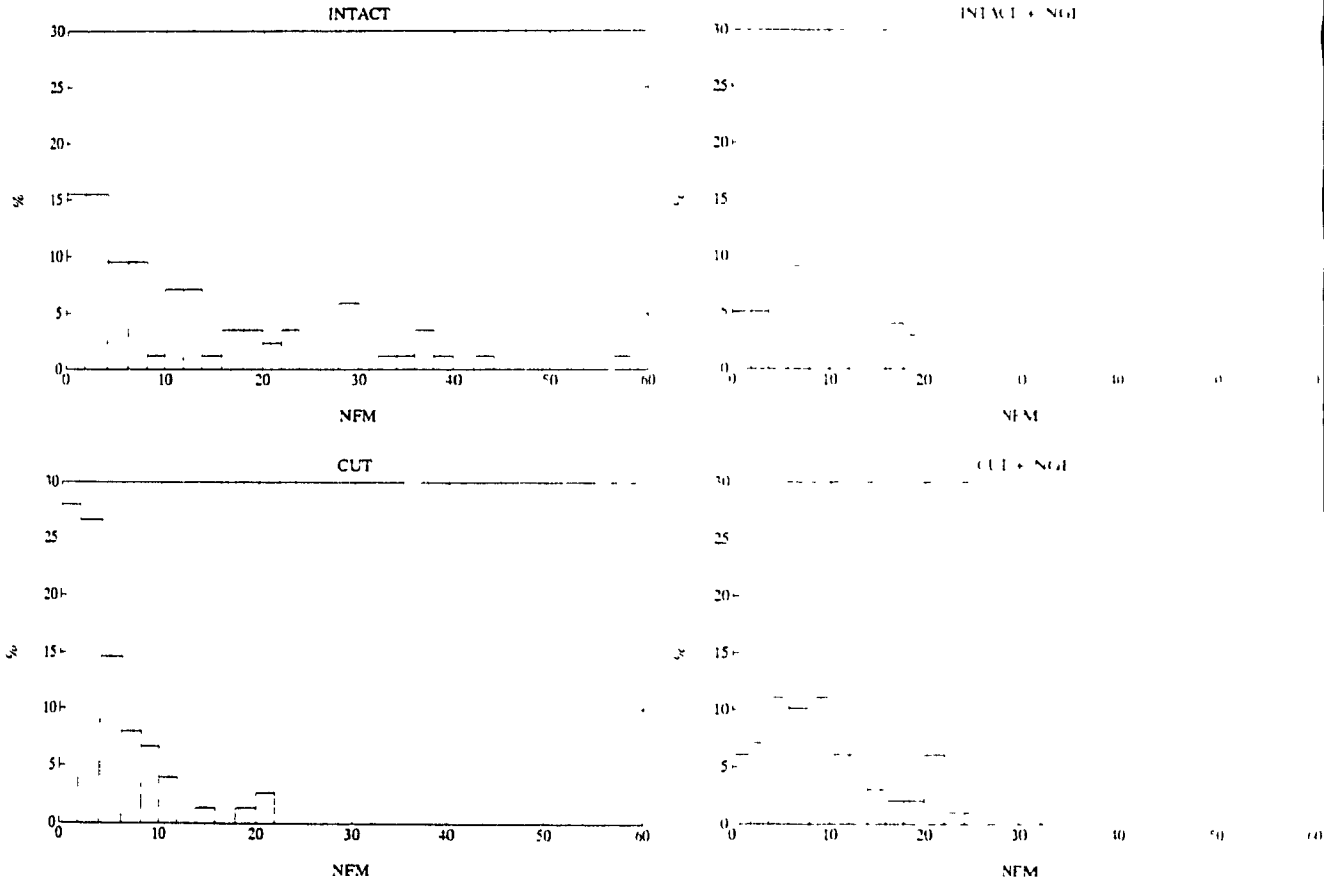

FIGURE 6

CHAPTER 7 - SUMMARY AND CONCLUSIONS

TABLE OF CONTENTS

1.0 SUMMARY

The cellular mechanisms underlying the role of trophic molecules in normal and injured neurons in vivo are not well understood. Nerve growth factor (NGF) is the best characterized of the neurotrophic molecules and has served as a model in defining mechanisms of trophic action. Speculation on a role for NGF following axonal injury stems from its growth promoting properties observed in tissue culture as well as its ability to counteract some of the retrograde consequences of axotomy in vivo (Fitzgerald et al., 1985; Rich et al., 1987; Hagg et al., 1989). NGF mediates its effects on survival and maintenance of selected neuronal populations via interactions with specific membrane bound receptors. To gain insights into the cellular function of NGF in normal and injured mature rat primary sensory neurons, a quantitative receptor radioautographic technique was developed that detects high-affinity NGF binding sites and thus identifies neurons potentially responsive to endogenous NGF. This approach yields both morphometric data, and information on the distribution, binding properties and densities of NGF binding sites. Two other approaches employing monoclonal antibodies (Ross et al., 1984; Chandler et al., 1984) or cDNA probes (Johnson,D. et al, 1986; Radeke et al., 1987) which identify the low-affinity NGF receptor of yet unknown physiological significance, are used by various laboratories to

identify cells expressing NGF binding sites. However, it has recently been shown that at least two neurotrophic proteins, BDNF & NGF, can bind to the same low-affinity receptor and thus this receptor might be a common binding protein used by a variety of neurotrophic substances in the same gene family to which NGF and BDNF belong (Rodriguez-Tébar et al., 1990). The process by which the respective high-affinity receptors are formed is unknown. Nonetheless, the high-affinity state of the receptors appears to be the way by which a neuron discriminates between the two ligands and selectively responds (Rodriguez-Tébar et al., 1990). So, until the relationship between high- and low-affinity NGF receptors is clarified and because most if not all of the physiological responses to NGF are mediated by membrane bound high-affinity receptors, the receptor radioautographic technique used in this thesis is the only approach that detects high-affinity NGF binding sites at a cellular level. The technique optimizes visualization of NGF binding sites by using thin cryosections for better resolution of labelling over small neurons (Salpeter et al., 1969), incubating the sections in concentrations of radioiodinated NGF close to the Kd of the high-affinity NGF receptor (20-40 picomolar) to minimize nonspecific labelling and increase percentage bound to high-affinity sites (Sutter et al., 1979), and finally wash times only long enough for dissociation from the low-affinity site (Sutter et al., 1979). The papers presented in Chapters 3-6 used quantitative NGF receptor

radioautography alone or in conjunction with adjacent section histochemistry to :

(i) identify and characterize the NGF-responsive population in intact mature sensory neurons;

(ii) note the changes following injury in NGF binding, neuronal size, and two markers of neural injury - the mRNA levels for growth-associated protein (GAP43) and the medium neurofilament subunit (NFM);

(iii) study the ability of exogenous NGF to counteract these axotomy-induced changes.

The major findings of these studies are summarized below. Please note that NGF binding sites visualized within neuronal perikarya will herein be referred to as receptors even though some of them have not yet been inserted into the membrane and thus are not in a position to interact with endogenous NGF. It is assumed that most perikaryal receptors are newly synthesized and awaiting anterograde transport, although the retrograde transport of NGF receptors presumably with their ligand has been demonstrated (Raivich and Kreutzberg, 1987).

1.1 INTACT SENSORY NEURONS - IDENTIFICATION AND CHARACTERIZATION OF THE NGF-RESPONSIVE POPULATION

Visual inspection of radioautographs of ^{125}I-NGF binding to

sections of mature rat dorsal root ganglion (DRG) revealed heterogenous neuronal labelling in agreement with receptor mapping studies for rat (Richardson et al., 1986) and chick (Raivich et al., 1985). Histograms of individual neuronal labelling densities, as determined by quantitative radioautographic analysis, were bimodal, fitting two normal curves. This suggests the existence of two populations of sensory neurons - those 'unlabelled' or 'labelled' by the technique. Approximately 40-50% of the neurons fall within the latter category and presumably represent the NGF-responsive neurons. However, because the two populations overlap, not all neurons can be unequivocally classified as belonging to one category. Scatchard analysis of the concentration dependence of ^{125}I-NGF binding for heavily labelled neurons indicated heterogenous binding, consisting of saturable high-affinity binding with a dissociation-equilibrium constant of 15-50 pM and additional binding of lower affinity. The binding capacity for the high-affinity receptor was approximately 15 fmol/mg or 2-300,000 receptors per medium-sized neuron. For those neurons with only background levels of labelling, Scatchard analysis did not reveal the presence of a high-affinity binding component.

Unlike the chick (Raivich et al., 1985,1987), rat primary sensory neurons with high-affinity receptors are neither topographically distributed nor correlated with neuronal size (Richardson et al., 1986). However, a role for NGF in regulation of neuronal size is suggested by the cellular hypertrophy of

embryonic sensory neurons observed following NGF injections (Straznicky and Rush, 1985; Dimberg et al., 1987) and the ability of NGF to regulate synthesis of all three neurofilament subunits in cloned pheochromocytoma cells (Dickson et al., 1986; Lindenbaum et al., 1988). Therefore, message levels for the medium neurofilament subunit were examined in relation to the ability of the neuron to bind NGF. Quantitative radioautographic analysis of adjacent sections processed for NGF high-affinity binding or NFM in situ hybridization revealed a complete lack of correlation between the two labelling indices, yet showed a strong positive correlation between neuronal volume and expression of NFM mRNA. Infusion of NGF into the lumbar subarachnoid space for 1 week did not alter these relationships. Thus, neurons with or without NGF receptors range from small to large in size and express low to high levels of NFM mRNA. In contrast, histochemical characterization of the NGF-sensitive subpopulation employing several peptidergic and one enzymatic marker of sensory neurons, showed close colocalization or absence of colocalization with NGF binding to be the rule. Histograms of the neuronal labelling densities of bound ^{125}I-NGF were examined for subpopulations of sensory neurons identified in the adjacent section as containing the enzyme thiamine monophosphatase (TMPase) or immunoreactivity for calcitonin gene-related peptide (CGRP), substance P, or somatostatin. CGRP serves as a good marker for the NGF-responsive population. Virtually all neurons with strong CGRP immunoreactivity bind NGF

with high-affinity, although some small neurons with faint CGRP immunoreactivity lacked such NGF binding. In addition, a subpopulation of large neurons, approximately 5% of the total, have high levels of ^{125}I-NGF binding but were not immunoreactive for CGRP. Of the three histochemical markers used to identify the principal populations of small sensory neurons only substance P-positive neurons were found to be consistently and heavily labelled by ^{125}I-NGF, while those containing somatostatin or TMPase activity did not specifically bind NGF. The finding that virtually all substance P neurons display high-affinity NGF receptors is consistent with the observation that they also contain CGRP (Lee et al., 1985). Thus, strongly positive CGRP and substance P neurons are capable of responding to NGF, whereas those containing somatostatin or TMPase activity lack high-affinity NGF receptors. Finally, a strong positive correlation was found between GAP43 expression and the display of NGF receptors in intact sensory neurons. GAP43 is a protein believed to be involved in neuronal mechanisms underlying axonal growth (Benowitz and Routtenberg, 1987; Zuber et al., 1989). Examination of adjacent section NGF receptor radioautography and GAP43 in situ hybridization revealed labelling to be heterogenous with approximately one half of the neurons densely labelled by either technique. Virtually all neurons with high-affinity NGF binding sites had high basal levels of GAP43 mRNA and most GAP43 mRNA-positive neurons displayed NGF receptors. Moreover, adjacent section colocalization studies of GAP43

hybridization and one of two markers for neurons that lack NGF receptors, somatostatin immunoreactivity or TMPase enzyme activity, show these neurons to have low basal concentrations of GAP43 mRNA. To verify the correlation of GAP43 expression with NGF-responsiveness, another population of NGF receptor-positive neurons, the sympathetic neurons in the superior cervical ganglion was examined. Once again all neurons binding NGF with high-affinity had high basal rates of GAP43 synthesis. The strong correlation between NGF binding and GAP43 mRNA suggests a regulatory role for NGF in GAP43 synthesis in normal sensory and sympathetic neurons.

1.2 CHANGES AFTER INJURY

To study changes after injury, the right sciatic nerve was transected. Binding of ^{125}I-NGF to pairs of ganglia 1-8 weeks following axotomy, was consistently diminished on the side of the injured nerve. Four weeks after the sciatic nerve is cut, approximately 20% of neurons die, and the mean number of binding sites on heavily labelled neurons in the L_5 dorsal root ganglion falls to less than 20% of normal values due to reduced receptor density and cell volume. Small, medium and large neurons were all subnormally labelled with the absolute number of small neurons increasing following injury as a result of neuronal atrophy. By Scatchard analysis the heavily labelled neurons were still seen to bind with both low- and high-affinity. However,

high-affinity binding was reduced to one-third of normal while low-affinity and nonspecific binding were apparently unchanged. The loss of perikaryal NGF receptors following injury does not merely reflect translocation of receptors into peripheral or central axonal processes as receptors on both of these processes were also reduced.

Neurofilament mRNA labelling densities in lesioned sensory neurons were greatly diminished and the normal bimodal pattern in frequency histograms of labelling densities became unimodal. This reduction is proceeded by a decrease in perikaryal volume more evident at 3 weeks than 1 week after injury. The axotomy-induced reduction in NFM message expression was very apparent in large neurons with or without NGF receptors which normally synthesize NFM abundantly. However, it was more difficult to determine if small neurons undergo a similar response due to their extremely low basal levels of expression.

One week following nerve transection, all injured sensory neurons expressed high levels of GAP43 mRNA irrespective of whether they were NGF-responsive, while expression in uninjured contralateral ganglia remained heterogenous and correlated with NGF binding. Frequency histograms of GAP43 hybridization labelling densities for axotomized neurons became unimodal in this early response to injury, conforming to a single normal distribution with uniformly high labelling. Although NGF is implicated in the expression of GAP43 in intact sensory neurons with NGF receptors, once injured the retrograde supply of NGF to

these neurons is severely reduced, and all the injured neurons appear to have elevated levels of GAP43 expression. Consequently, an alternative signal must be accountable for the posttraumatic levels of GAP43 expression, the nature of which is unknown.

1.3 INFUSION OF NGF - ABILITY TO COUNTERACT AXOTOMY-INDUCED CHANGES

Infusion of NGF for one week either intrathecally into the subarachnoid space or directly onto the proximal stump of the injured nerve, at the time of injury or three weeks after, counteracted many of the axotomy-induced changes described in the previous section. The reduction in NGF receptor densities and neuronal atrophy for neurons within the "labelled" injured population were substantially reversed by exogenous NGF and indicate that NGF can regulate synthesis of its receptor and possibly cytoskeletal element(s) contributing to neuronal size. Infusion of NGF did not influence nonspecific binding nor alter the volume of, or binding to neurons within the "unlabelled" population.

Injury-induced down regulation of NFM mRNA expression was counteracted in NGF-infused rats, primarily for the medium to large neurons with high-affinity NGF receptors which abundantly expressed NFM before injury. The inability of infused NGF to

significantly increase NFM mRNA levels in small intact or small injured NGF receptor-positive neurons implies that NGF might permit neurons to respond differentially to some unknown factor that stimulates NFM synthesis. An analogous trophic factor(s) might play a similar role for the NGF non-responsive population of sensory neurons, as exogenous NGF did not notably alter the reduction in NFM mRNA or neuronal volume of axotomized neurons that do not specifically bind NGF, a subset of which expressed NFM at high levels before nerve transection.

Intrathecal infusion of NGF at the time of injury did not appear to alter the changes in GAP43 expression that occur following axotomy alone. GAP43 expression remained homogeneously high for the entire population of infused injured sensory neurons, without correlation with NGF binding. However, because GAP43 is already abundantly expressed in intact NGF-responsive neurons the possibility that the exogenous NGF is overriding the signal which effects high levels of GAP43 expression in this subpopulation after injury can not be excluded.

2.0 CONCLUSIONS

In conclusion, the strong correlation observed between the display of high-affinity NGF receptors and the presence or absence of markers for distinct subpopulations of intact mature sensory neurons support a functional role for endogenous NGF in these neurons. Following injury, the changes within the

population of NGF-responsive neurons are mainly degenerative with many of the NGF-regulated axotomy responses possibly accentuated by a vicious cycle where restricted supply of NGF to the cell body results in diminished synthesis of receptors which in turn further reduces the supply of NGF to the neuron. The ability of exogenous NGF to counteract some of the axotomy-induced degenerative changes, such as loss of receptors, atrophy, and downregulation of NFM mRNA for those neurons with NGF receptors, strongly promotes a role for endogenous NGF within the distal stump of an injured nerve (Heumann et al.,1987) in restoration of function following regeneration.

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