Title
Expression of the Beta-nerve growth factor gene

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Publication Date
1985

Peer reviewed|Thesis/dissertation

Expression of the Beta-Nerve Growth Factor Gene

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

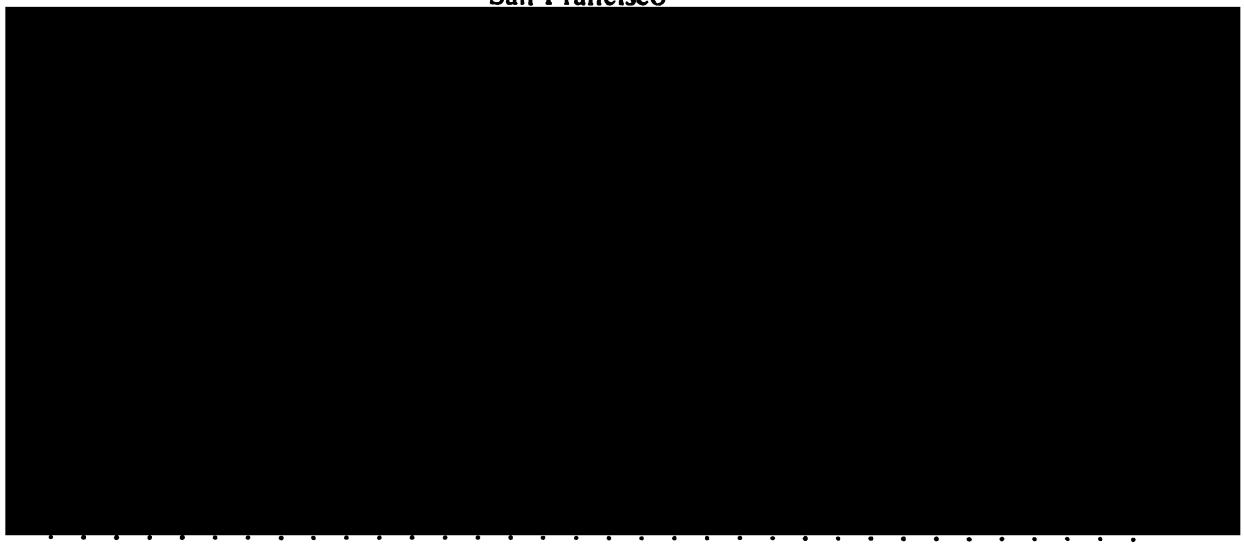
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Louis Reichardt for his contributions to this thesis and his support over the past years. I appreciate the opportunities he has provided to allow me to become an effective, independent investigator. I look forward to his continued advice and support as a colleague in the future.

There are many faculty members at UCSF which have made great contributions to my education and experience here. Dr. Jennifer Lavail has provided invaluable advice and assistance over the years, and I thank her for her willingness to share her time. Dr. Regis Kelly has also generously given of his time and expertise, and was a large help in completing this thesis. Dr. Patrick O'Farrell freely shared his great wealth of knowledge and more than once saved me large amounts of time and frustration. Thanks also to Dr. Zach Hall for his encouragement and Dr. Bill Rutter for providing the cDNA clone which made this work possible.

Laboratory colleagues have greatly aided in my development as a scientist. Dr. Bill Matthew has given advice, inspiration and friendship from the very beginning of my time here. Drs. Arthur Lander and Anne Calof have always provided spirited intellectual discussion, and Dr. Ralph Greenspan has taught me the value of careful thought. Special thanks go to Drs. Karen Valentino, Janet Winter, and Kevin Tomaselli for their scientific advice, moral support, and their invaluable friendship.

My friends and colleagues at UCSF have been a bastion of support, both scientific and personal. Barry Drees gave freely of his time

and expertise. Special thanks are due to Sheri Strickland, Dr. Kathy Buckley, Dr. Jeff Miller, Dr. Steve Carlson, and Dr. Gary Reiness, who have been the most valued of friends, and extremely helpful.

Many people outside UCSF have also contributed greatly to this thesis. Drs. Becky Akers and Nathan Chu have provided both scientific and personal advice and encouragement beyond measure. There is a large group of people who have to a large degree made the completion of this work possible, even if they are not aware of it. In no partricular order they are Harry Page, Joan Shimakawa, Kathy Fujimoto, Bo Barnes, Brian Hansell, Jenny Belser, Jim Lord, Keith Miller, Ron Pruitt, and Terry Kreuger.

My family has been an enormous source of support and encouragement. My parents have supported me without reservation, and their inspiration and encouragement have been priceless.

EXPRESSION OF THE BETA-NERVE GROWTH FACTOR GENE

David L. Shelton

ABSTRACT

Beta-nerve growth factor (NGF) is a protein necessary for the normal development and maintenance of sympathetic and sensory neurons in vivo and in vitro. Evidence has accumulated which indicates that NGF is required at the growing tips of the axons of these neurons and when present there, is bound, internalized, and transported retrogradely to the cell body. This has led to the hypothesis that NGF is produced by the target tissues of the neurons which require it, but so far this has been impossible to demonstrate.

An assay based on RNA blot hybridization capable of detecting 10 femtograms of mRNA encoding NGF (NGF mRNA) or one part in 10^9 by weight has been developed. Tissues with varying densities of sympathetic innervation were surveyed for their content of NGF mRNA as an indication of their level of NGF synthesis. Tissues which have a dense sympathetic innervation have relatively high levels of NGF mRNA. Those tissues with a paucity of sympathetic innervation have much lower levels. Using norepinephrine content as a measure of sympathetic innervation density, a strong positive correlation between NGF mRNA content and sympathetic innervation density was noted. Although levels of NGF mRNA are very low in all tissues, there appears to be more than enough NGF mRNA to account for the NGF which is found in target organs. NGF mRNA was also found in elements of the adult peripheral nervous system, sensory and sympathetic ganglia and sciatic nerve.

There is mounting evidence that NGF can affect certain subpopula-

tions of CNS neurons. The CNS was found to contain high levels of NGF mRNA, and there were forty-fold differences between different regions. The highest concentrations of NGF mRNA are found in cortex and hippocampus, areas which are the targets for the CNS neurons which respond to NGF. This suggests that endogenous NGF may play a role in normal adult CNS function.

A rat iris denervated *in vivo* or grown *in vitro* rapidly accumulates NGF. The mechanism causing this increase is unknown. The NGF mRNA content of irises was assayed after these treatments to see if this rise in NGF content was caused by an increase in NGF mRNA. Denervation *in vivo* had no effect on the NGF mRNA content of the iris, and so the increase in NGF after denervation must be due to another cause. Irises grown *in vitro* do undergo a large, rapid rise in NGF mRNA content, as do irises which have been subjected to trauma *in vivo*. This suggests that the increase in NGF content *in vitro* is due at least partially to an increase in synthesis.

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INTRODUCTION

During development, a single cell divides repeatedly and its progeny give rise to all of the differentiated tissues of an organism. Nature and nurture both may play a role in the control of this process. A cell's final differentiated fate may be affected by both its developmental history, and interactions with the environment. Both of these mechanisms acting in concert are required to produce a normal adult.

In its differentiated form, the vertebrate nervous system is one of the most complex organ systems known, and cellular interactions with the environment are known to be important in the control of its development. One class of these interactions occurs between a neuron and its target. Neurons can affect the differentiation of their targets. For example, muscle fibers undergo a spatial redistribution of their acetylcholine sensitivity in response to innervation(Diamond and Miledi, 1962). Conversely, target tissue can influence the development of the innervating neurons. Contact with the appropriate target is required to insure the survival and function of at least some neurons. For example, spinal motoneurons and primary sensory neurons both undergo a period of naturally occurring cell death (Hamburger and Levi-Montalcini, 1949; Hamburger, 1975). The extent of this cell death can be increased by reducing the size of the peripheral targets of these neurons, leading to fewer surviving neurons in the adult. Experimentally increasing the size of the periphery causes an increase in the number of neurons surviving in the adult by decreasing the amount of cell death (Hamburger, 1958). It has been postulated that this

survival effect is mediated by diffusible trophic substances produced by the target (Hamburger and Levi-Montalcini, 1949). The apparent control of neuron survival by the periphery can be explained as a competition among the neurons for a limiting amount of this trophic factor.

There are three substances that seem to act as such trophic factors and which have been at least partially characterized. Each of these affects specific subpopulations of neurons, although there is some overlap between the types affected by each factor. The first discovered and by far the most well characterized of these trophic substances is beta-nerve growth factor (NGF). It acts upon neurons of the sympathetic and sensory nervous systems, and there is also increasing evidence that this protein has some effects on parasympathetic neurons (Yankner and Shooter, 1982).

The parasympathetic neurons, however, exhibit a more dramatic response to another of these factors, the so-called ciliary neurono-trophic factor (CNTF). The activity of this factor was first demonstrated by its ability to support the survival of chick ciliary ganglion neurons in culture (Helfand et al., 1976). A rich source of this factor is the anterior half of the embryonic chicken eye, which contains the tissues innervated by the responsive ciliary ganglion neurons. The factor has recently been purified from this source and characterized as a dimer of two peptide chains, each 20kd in size and pI=5.0 (Barbin et al., 1984). It has been reported that this purified factor also support sensory and sympathetic neurons in vitro (Barbin et al., 1984).

The most recently discovered of the three known factors affects the primary sensory neurons of the dorsal root ganglia (DRG). These neurons undergo a transient dependence on NGF during embryogenesis but later in development, DRG neurons no longer seem to require NGF for survival in culture or in vivo (Gorin and Johnson, 1979; Aloe et al., 1981). Chick sensory neurons instead become dependent on another factor which can be found in glioma conditioned medium and in brain extract. Using pig brain as a source, a factor has now been purified which supports the in vitro survival of sensory neurons obtained from ten day chicken embryos (Barde et al., 1982). This so called brain derived neuronotrophic factor (BDNF) is a protein with a molecular weight of 12 kD and pI > 10.1.

A BRIEF HISTORY OF NGF

The effects of NGF were first reported by Bueker (1948) while investigating the control of sensory and motor system development by the periphery. It was known that the grafting of an extra limb bud onto a chicken embryo gave rise to an increase in the survival of sensory and motor neurons (Hamburger and Levi-Montalcini, 1949) The specificity of this response was investigated by transplanting different types of tissue into chicken embryos. Bueker observed an increase in the size of sensory ganglia after transplantation of certain mouse sarcomas into the body wall. Levi-Montalcini and Hamburger(1951) extended the work by discovering that the sympathetic ganglia were affected even more than sensory ganglia and moreover that the effect was not limited to the ganglia actually innervating the tumor. This led them to propose that the effect was due to a

diffusible substance released by the tumor. This theory was substantiated by showing that the sarcoma still caused hyperplasia of sympathetic ganglia when placed on the chorioallantoic membrane, at a distance from the embryo (Levi-Montalcini and Hamburger, 1953). Further studies designed to prove the humoral nature of NGF gave rise to the standard bioassay for NGF-like activity, i.e.; in vitro fiber outgrowth from chick sensory or sympathetic ganglia explanted with pieces or extracts of possible NGF sources (Levi-Montalcini et al., 1954).

While working to characterize the factor from sarcoma, Cohen (1959) used snake venom as a source for phosphodiesterase and found that samples incubated with this crude fraction actually had more activity than the untreated sarcoma extract. He then assayed the venom directly and found that it was a much richer source of NGF than the mouse sarcoma. Snake venom, then, was used as the source for the first purification of NGF (Cohen, 1959). This prompted a search for other possible sources and led to the discovery of male mouse salivary glands as a tissue remarkably rich in NGF (Cohen, 1960). The purification of NGF from this source led to the characterization of the molecule. It has since been demonstrated that NGF is actually synthesized in the mouse salivary gland and does not accumulate there from some other source(Berger and Shooter, 1977; 1978).

The application of an antiserum specific to NGF from mouse salivary gland provided the first really convincing demonstration that NGF plays a role in the normal development of some neurons (Cohen, 1960). This antiserum could produce the destruction of the sympathetic nervous system when injected into young animals,

thus demonstrating an NGF requirement for sympathetic neurons in vivo (Levi-Montalcini and Booker, 1960b). By using prenatal exposure to antibodies, sensory neurons have also been shown to require endogenous NGF for survival, although at an earlier stage of development (Gorin and Johnson, 1979).

The mechanism by which NGF acts upon neurons has been the object of much work. In 1973, the presence of specific receptors for NGF on the cells of the rabbit superior cervical ganglion (SCG) was demonstrated (Bannerjee et al., 1973). Since then, receptors for NGF have been demonstrated on a variety of cell types, all of which are neurons or of neural crest origin (Herrup and Shooter, 1973; Fabricant et al., 1977; Schecter and Bothwell, 1980; Zimmermann et al., 1978; Frazier et al., 1974). Binding to these receptors leads to the internalization of NGF, which in neurons, is followed by retrograde transport to the cell body (Dumas et al., 1979), where many of its biological effects occur (Paravicini, et al., 1975; Hendry, 1977). NGF also has local effects. Culture experiments have shown that NGF is required at the growing tips of neurites in order to maintain their growth (Campenot, 1977; Gunderson, 1985). Similar experiments have shown that NGF applied only to the tips of neurites is sufficient to maintain the cell body (Campenot, 1977).

Recently, Hamburger and Yip (1984) have shown that NGF is almost certainly the molecule which mediates the very phenomenon which Bueker was studying when he first observed the effect of the factor i.e., the effect of the periphery on the development of the sensory system of chicks. They found that application of NGF can substitute for the

presence of peripheral tissue in maintaining the normal population of DRG neurons.

THE NGF MOLECULE

Initially, NGF was defined as a biological activity which could be obtained from a number of sources. The male mouse salivary gland is the richest source known (400 to 500 ug per pair of glands) and the activity as purified from this source is by far the most well characterized (Yankner and Shooter, 1982). As a result of this characterization, the term NGF is used to refer to substances with some molecular homology to mouse NGF in addition to the originally defining bioactivity (Harper and Thoenen, 1980).

In homogenates of the salivary gland and in saliva, activity is contained in a complex of molecular weight 130 kd (Varon et al., 1968). Because of its sedimentation properties, this complex has been termed 7S NGF. It is composed of three different subunits, alpha, beta and gamma. In the complex the subunits are in the stoichiometry $\alpha_2\beta_1\gamma_2$. All of the nerve growth promoting activity resides in the beta subunit, which is itself a dimer (Varon et al., 1968). As isolated, the complex also contains one or two atoms of zinc, which greatly increase the stability of the complex (Bothwell and Shooter, 1978).

Beta NGF

Beta-NGF is composed of two identical highly basic peptide chains (MW= 13,259 d, pI=9.3)which are non-covalently linked (Greene at al., 1971). The affinity of this interaction is very high with an dissociation constant of less than 10^{-11} M^{-1} (Moore and Shooter, 1975). This implies

that the dimer would be the physiologically relevant form. From studies in which the dimer was covalently crosslinked with dimethyl-suberimidate, it is clear that the dimer is physiologically active (Stach and Shooter, 1974; Pulliam et al., 1975). However, studies with NGF crosslinked to Sephadex beads while in the monomeric form have also yielded evidence of biological activity of the monomer (Frazier et al., 1973a).

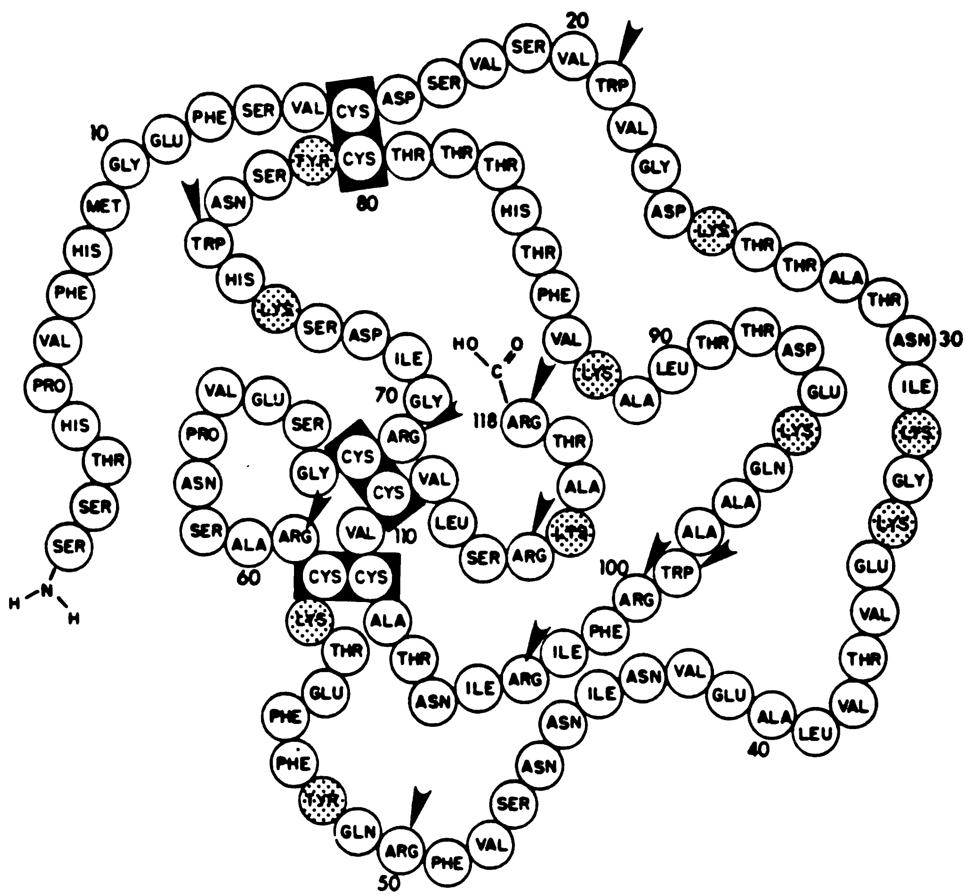
The molecule has been completely sequenced (Hogue-Angeletti and Bradshaw, 1971)(figure 1). A weak sequence homology was found to insulin, relaxin and the insulin-like growth factors and one intrachain disulfide bond was conserved in all molecules. There is as yet no information on the three dimensional structure of NGF, although it has been crystallized (Wlodaver et al., 1975).

A number of studies that have attempted to define the receptor binding site of the molecule by modifications of specific amino acid residues, unfortunately have not yet led to a clear answer. Modifications of one or both tyrosine residues (Herrup and Shooter, 1973; Sutter et al., 1979) or most, if not all, of the lysine residues (Stach and Shooter, 1974; Pulliam et al., 1975; Bradshaw et al., 1977) has no effect on the biological activity. In contrast, complete modification of the arginine residues does lead to inactivation (Bradshaw et al., 1977). There are three tryptophan residues in the NGF peptide which can be derivatized at different rates by n-bromosuccinimide (Hogue-Angeletti, 1970; Frazier et al., 1973b). As the tryptophan residues are progressively oxidized, there is a progressive loss of biological activity, leading to a completely inactivated molecule when two

FIGURE 1

Amino acid sequence of beta-NGF.

Sequence determined by and figure taken from Hogue-Angeletti and Bradshaw (1971). Amino acid residues which can be modified with no effect on biological activity (lysine and tyrosine) are stippled. Arginine and tryptophan residues, which can not be modified without affecting activity, are indicated by arrowheads.


20
SER VAL
VAL TRP
SER
ASP
CYS
VAL
SER
PHE
GLU
10
GLY
TYR CYS THR THR THR
SER
ASN
80
VAL
GLY
MET
HIS
ASP LYS THR THR ALA THR
TRP
HIS
THR
PHE
HIS
LYS SER ASP
PHE
VAL
90
THR THR
ASN 30
HO
O
C
ILE
LEU
ASP
LYS
ALA
GLU
ILE
PRO
VAL GLU
70 GLY
118 ARG
LYS
LYS
HIS
SER
ARG
THR
PRO
CYS
GLN
THR
GLY
CYS
VAL
ALA
ALA
GLY
ASN
LEU
LYS
SER
SER ALA ARG VAL 110
ALA
SER ARG
LYS
SER
100
TRP
GLU
60
CYS CYS
ARG
H N H
LYS
ALA
PHE
VAL
THR
THR
ILE
ARG
ILE
ASN
VAL
THR
GLU
ASN
ILE
GLU
ALA
LEU
VAL
PHE
ASN
40
PHE
ASN
TYR
SER
GLN ARG PHE VAL
50

residues per chain are derivatized (Hogue-Angeletti, 1970; Bradshaw et al., 1973b). It should be noted that the region of the insulin molecule corresponding to the region around tryptophan 21 has been implicated in the binding of insulin to its receptor (Pullen et al., 1976)

Limited proteolytic modifications can occur during various procedures used to isolate beta-NGF. Both the carboxy-terminal arginine residue and an amino-terminal octapeptide may be removed during isolation, depending on the technique used (Moore et al., 1974; Mobley et al, 1976). Neither of these cleavages has any observable effect on biological activity (Moore et al.,1974; Mobley et al., 1976). These modifications do lead to molecular heterogeneity of the protein, however, and NGF which is isolated by methods which lead to these cleavages are generally referred to in terms of their sedimentation coefficient and called 2.5S NGF, reserving beta-NGF to refer to the homogenous, unmodified protein.

THE GAMMA SUBUNIT

The gamma subunit of NGF has no known nerve growth promoting activity, but is an arginine specific esteropeptidase which can be inhibited by diisopropyl fluorophosphate (Greene et al., 1968). It has been cloned and sequenced and this information shows that the gamma subunit is homologous to trypsin, the EGF binding protein, and other kallikreins (Howles et al., 1984). Some evidence suggests that it may participate in the processing of a precursor to mature beta-NGF (Berger and Shooter, 1977; 1978)(see Synthesis and Processing).

After catalyzing the cleavage, the gamma-subunit would then stay bound to the substrate, much as trypsin stays bound to trypsin inhibitor (Hunt etal., 1974). Evidence supporting this includes the fact that the proteolytic activity of the gamma-subunit is completely inhibited in the 7S NGF complex (Bothwell and Shooter, 1978). However, when the the carboxyterminal arginine residue is removed from beta-NGF, it is no longer capable of forming a complex with the gamma-subunit (Moore et al., 1974), and this carboxyterminal residue is not the site where the gamma-subunit apparently cleaves the beta-NGF precursor (Berger and Shooter, 1977; Darling et al., 1983).

The gamma-subunit is composed of two peptide chains of unequal size, with some attached carbohydrate (Stach et al., 1976). Like the beta-subunit, the gamma-subunit also undergoes several types of limited proteolyses which give rise to heterogeneity (Greene et al., 1969). Although these different forms do not seem to differ in their activity as assayed with artificial substrates, they do differ in their ability to reassociate with the alpha and beta-subunits to form the 7S complex (Greene et al., 1969; Server and Shooter, 1976).

THE ALPHA SUBUNIT

The alpha-subunit of 7S NGF is similar to the gamma-subunit in size, peptide composition and heterogeneity. It lacks, however, the serine protease activity of the gamma-subunit. Recently, a cDNA corresponding to the alpha-subunit has been cloned and sequenced (Evans and Richards, 1985). This has shown that the alpha-subunit is highly homologous to the gamma-subunit except for a small deletion

which causes a glutamine to replace an arginine in a region which is thought to be critical for cleavage of the zymogen peptide and subsequent serine protease activation. This may explain the lack of protease activity of the alpha-subunit. The only known biological activity of alpha-subunit is to combine with the beta- and gamma-subunits to form 7S NGF.

SNAKE VENOM NGF

NGF has been found in the venom of all three classes of poisonous snakes, Crotalidae, Viperidae, and Elapidae (Hogue-Angeletti, 1971; Hogue-Angeletti and Bradshaw, 1977). None of the molecules responsible for this activity have been as well characterized as the mouse submaxillary gland protein, but some interesting information about snake venom NGF does exist.

The most well characterized of the venom NGFs is that purified from the cobra, Naja naja. This apparently pure preparation is similar in size to the mouse beta-NGF and is also composed of two apparently identical, non-covalently linked peptides (Hogue-Angeletti, 1976). Partial amino acid sequence analysis indicates approximately 60% homology with the mouse protein and identical positioning of the half cistinyl residues indicates probable conservation of disulfide linkages. The proteins as isolated from the two sources are also similar in having no carbohydrate associated with the protein.

There are also significant differences between the two proteins. Cobra NGF is much less basic than the mouse protein with a pI of 6.75 and cannot associate with the alpha and gamma-subunits from mouse

to form a larger complex (Server et al., 1976). Further, there is no evidence for the existence of a higher molecular weight form in venom. In addition, the biological activity is subtly different. Using the biological assay of neurite formation by explanted chick sensory ganglia, the snake NGF is effective over the same concentration range as the mouse protein, but the maximum response obtained is only about 50% of that obtained with mouse NGF. (Server et al., 1976). Similarly, although the snake protein will compete for binding to the NGF receptor, it is only capable of displacing 80% of the ^{125}I-NGF bound to chick DRG cells.

NGF has also been isolated from the venom of Russell's viper and is reported to have somewhat different properties than either mouse or cobra NGF. It apparently has a molecular weight of about 35 kd and no evidence for a dimeric structure is known. Also, it contains approximately 20% carbohydrate (Pearce et al., 1972). It has also been reported that NGF as partially purified from three other venoms (two crotalids and another viper) also contain carbohydrate (Hogue-Angeletti and Bradshaw, 1977). Although these appear to be major differences, immunological cross-reactivity indicates that NGF from mouse, cobra, viper and the active proteins partially purified from other snake venoms are at least somewhat similar (Hogue-Angeletti, 1971). Some of the apparent differences may be due to lack of purity of some of the venom preparations.

NGF ASSAYS

The classic assay for NGF activity relies upon the formation of a "halo" of neurites by chick embryo sensory or sympathetic

ganglia in vitro (Levi-Montalcini et al., 1954). This assay was used for many years, and to a certain extent is still used as a final measure of the specificity of the more recent assays. The first major improvement upon this assay also relied upon the biological activity of NGF but used dissociated neuronal cultures (Greene, 1974). By counting the number of process bearing neurons in such cultures, a more quantititative and sensitive assay of NGF activity could be obtained. This assay could be made somewhat more convenient by using cultures of the PC12 cell line instead of the primary cultures of chick neurons (Greene, 1977). However the counting involved in both assays is time consuming and laborious.

Attempts to avoid the tedium involved in bio-assays and further increase sensitivity led to the use of immunological assays. Competitive radioimmunoassays (RIA) were developed and widely used to measure the level of NGF in tissues and serum from many sources (Johnson et al., 1971; Hendry and Iversen, 1973). NGF levels as measured with these assays, however, were often much higher than the corresponding bioactivity. This discrepancy was partially due due to nonspecific antisera caused by highly immunogenic contaminants in most preparations of NGF. A more serious difficulty with competitive RIAs was discovered when it was shown that there were substances present in biological samples which could bind to NGF. By competing with the antibodies for the binding of the radioactive NGF, these substances could interfere with the assay and give erroneously high levels of NGF (Suda et al., 1978). This problem was overcome with the introduction of a two-site, non-competitive RIA, in which

these binding substances did not interfere (Suda et al., 1978). Results obtained with this assay gave good agreement with results of bioassays but were not more sensitive. A large increase in sensitivity was achieved by replacing the radioactively labelled antibodies with enzyme tagged antibodies and using a fluorescence assay to detect the enzyme (Korsching and Thoenen, 1983). The use of this two-site enzyme linked immunosorbent assay (ELISA) has allowed the unambiguous detection of NGF in some physiologically relevant sources (see page 26).

CLONING

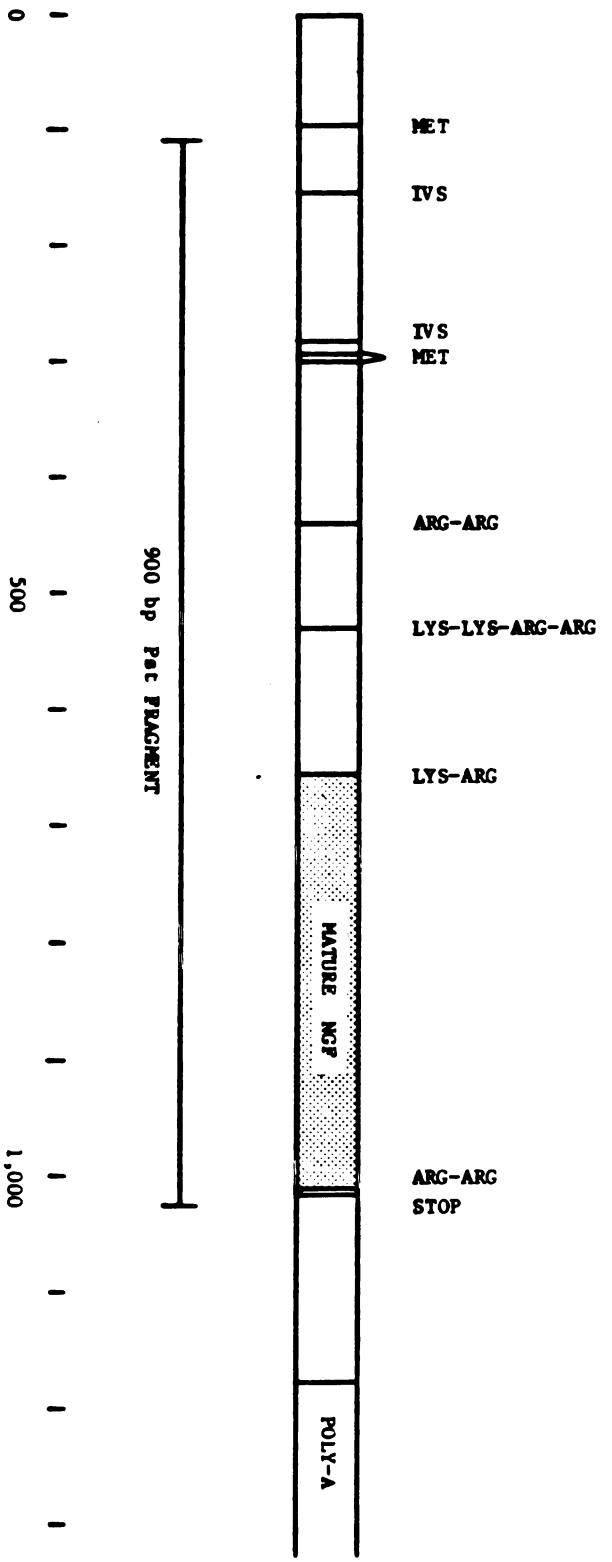
Recently, two independent groups have isolated cDNA clones corresponding to the mRNA encoding beta-NGF (NGF mRNA) from the mouse salivary gland (Scott et al., 1983; Ullrich et al., 1983) Salient features of the mRNA are illustrated in figure 2. The NGF mRNA is polyadenylated and approximately 1350 bases in length. There are three possible translation initiation methionines, all in the same open reading frame, which translates to the amino acid sequence of beta-NGF. Depending on which methionine is actually used to initiate translation, the primary translation product predicted from the sequence will be either 34 kd or approximately 27 kd. There are four sets of dibasic residues which are likely to correspond to proteolytic processing sites.

Evidence indicates that there is only a single copy of the NGF gene per haploid genome and clones containing this gene have been isolated from a human genomic library (Ullrich et al., 1983). Comparison with the mouse cDNA clone indcate greater than 90%

FIGURE 2

Structure of the mRNA encoding NGF (NGF mRNA).

Information derived from the nucleotide sequence after Scott et al. (1983) and Ullrich et al. (1983). Shown are the locations of the three possible translation initiation methionines (MET), dibasic residues, intervening sequences (IVS), beta-NGF sequence (stipple), and a 900 bp Pst I fragment used as the probe for all studies. Scale at bottom is in base pairs.


0
500
1,000
900 bp Pst FRAGMENT
MET
IVS
IVS
MET
ARG-ARG
LYS-LYS-ARG-ARG
LYS-ARG
MATURE NGF
ARG-ARG
STOP
POLY-A

homology of nucleic acid sequence between the species. This genomic clone has also revealed that the NGF mRNA is made up of at least three exons. The exon or exons corresponding to the 5' end of the mRNA has still not been identified in the genome.

SYNTHESIS AND PROCESSING

Information gained from the nucleic acid sequence has extended our knowledge of the post-translational processing of beta-NGF. Berger and Shooter (1977; 1978) have demonstrated that NGF is synthesized in the submaxillary gland by incubating slices of the gland in media containing ^{35}S-cysteine and then immunoprecipitating proteins cross-reacting with anti-NGF. When labelling occurred over several hours, the immunoprecipitated product comigrated with authentic beta-NGF on SDS-polyacrylamide gels. Brief periods of labelling revealed the presence of a slower migrating species of approximately 22 kd. Pulse-chase experiments demonstrated that this molecule shared a precursor product relationship with beta-NGF. Further, incubation of the precursor with the gamma-subunit of NGF gave rise to a protein which then comigrated with beta-NGF (Berger and Shooter, 1977; 1978).

The above experiments never gave any evidence of the larger primary translation product predicted by the NGF mRNA sequence. This might be explained by either an extremely rapid conversion of this peptide to the smaller 22 kd fragment, to poor recognition of the primary translation product by the antibody used, or both. Recent experiments using different antisera have found evidence for the existence of a larger precursor and suggest that a lack of immunological cross-reactivity is at least part of the reason it was not identified

in the earlier experiments (Darling et al., 1983). The size of this peptide argues that translation is initiated at the first methionine codon in the mRNA.

BIOLOGICAL EFFECTS OF NGF ON PERIPHERAL NEURONS

In the past thiry-five years, there has been a great deal of work done in an attempt to define the biological effects of NGF. Because of the initial observations of Hamburger and Levi-Montalcini (1951;1953), most of this work has justifiably been centered on effects of NGF on sympathetic and sensory neurons.

The initial observations of the massive hyperplasia which treatment with NGF causes in neonatal sympathetic ganglia have now been repeated many times. Early workers noted an increase in neuronal numbers and mitotic index in addition to increased neuronal size in these animals (Levi-Montalcini and Booker, 1960a). This led to the hypothesis that NGF was affecting the number of neurons born in the ganglia (Levi-Montalcini and Booker, 1960a). Later work has shown that this interpretation is incorrect, and that the increase in neuronal number reflects a decrease in the amount of normally occurring cell death (Levi-Montalcini et al., 1975; Hendry and Campbell, 1976; Kessler et al., 1979; Hamburger et al., 1981). The increase in cell size appears to be a direct consequence of NGF action on the neurons (Hendry and Campbell, 1976). The increase in mitotic index is, however, confined to the non-neuronal cells of the ganglia (Hendry, 1977), and these satellite cells are almost certainly not responding to NGF directly, but instead to the increase in neuronal number and size (Pannese, 1969).

There are also several well characterized biochemical effects of administration of NGF to neonates. Two enzymes required for the synthesis of the sympathetic neurotransmitter, norepinephrine, have been examined in detail. Both dopamine beta hydroxylase (DBH) and the rate limiting tyrosine hydroxylase (TH) undergo an increase in activity after treatment with NGF (MacDonnell et al., 1977b; Otten et al., 1977). This increase is partially dependent on translation, and by immunotitration, it has been shown that there is an absolute increase in the number of molecules of TH (MacDonnel et al., 1977b). Another enzyme which undergoes an increase in activity in response to NGF is ornithine decarboxylase (ODC) (MacDonnell et al., 1977a). ODC is the rate limiting enzyme in the synthesis of polyamines, which are known to play a major role in the regulation of protein synthesis at both the transcriptional and translational level (Janne et al., 1978). Increases in ODC are commonly associated with induction of growth and differentiation in many cell types (Janne et al., 1978; Russell and Snyder, 1968).

Administration of NGF also has marked effects on primary sensory neurons. The effects are similar to those observed in sympathetic ganglia: a decrease in naturally occurring cell death leading to an increase in neuronal cell numbers (Hamburger et al., 1981) and an increase in neuronal size (Levi-Montalcini and Hamburger, 1951). Also, there is an increase in the content of substance P (Kessler and Black, 1980), the putative neurotransmitter for a subset of sensory neurons.

Although the results presented above show that NGF can produce

dramatic effects in certain neurons when administered exogenously, they do not show that NGF functions in the development of a normal animal. This has been demonstrated by the effects of antiserum to NGF on young animals. The results of these experiments give convincing proof that the presence of endogenous NGF is critical in controlling the early development and differentiation of sympathetic and sensory neurons.

Injection of newborn mammals with antibodies to NGF causes an almost complete destruction of the sympathetic nervous system (Levi-Montalcini and Booker, 1960b). The mechanism of this "immunosympathectomy" involves a blocking of endogenous NGF and is not due to complement mediated cytotoxicity. This was demonstrated by showing that injections of NGF given up to forty-eight hours after administration of the anti-serum are capable of rescuing the neurons (Ennis et al., 1979; Goedert et al., 1980) and by showing that immunosympathectomy occurs in complement deficient mice (Ennis et al., 1979). Exposure to anti-NGF prenatally causes the destruction of the primary sensory neurons derived from neural crest (Gorin and Johnson et al., 1979; Aloe et al., 1981; Pearson et al., 1983).

The effect of NGF antiserum is critically dependent on the time of its administration. With increasing age, the number of sympathetic neurons destroyed by a single administration of anti-NGF decreases (Angeletti et al., 1971). In the adult, there is no cell death observed, but only a transient decrease in the activity of norepinephrine synthetic enzymes, TH and DBH (Goedert et al., 1978). Likewise, sensory neurons also respond to anti-NGF much less dramatically

with increasing age. In order to get significant effects on the survival of these cells, anti-NGF must be administered prenatally. (Aloe et al., 1981; Gorin and Johnson, 1979) The technical difficulties involved in administering NGF prenatally delayed the demonstration of the NGF-dependence of sensory neurons *in vivo*.

The age dependence of anti-NGF effects is reflected in differing responses to the administration of exogenous NGF at different ages. Clearly, NGF given after the period of naturally occurring cell death can not rescue the neurons which have already died, so the effect on neuronal cell number decreases with increasing age (Angeletti et al., 1971). Other effects of NGF, such as the increase in ganglion volume (Angeletti et al., 1971), and the induction of ODC (MacDonnel et al., 1977a; Thoenen et al., 1979) are not as robust in older animals. However, induction of the noradrenergic transmitter enzymes does not seem to decrease with age (Thoenen et al., 1979).

Many of these phenomena which are seen *in vivo* also have been investigated *in vitro*. For instance, both sensory and sympathetic neurons from embryonic birds and mammals require the addition of NGF to their culture medium to survive and and differentiate (Levi-Montalcini and Hamburger, 1953; Levi-Montalcini and Angeletti, 1963). Similar to the age dependence of this requirement seen *in vivo*, neurons removed from older animals show less dependence on NGF(Yankner and Shooter, 1982). Although this dependence on NGF would seem to make explants and dissociated neuronal cultures a useful system for studying the mechanism of action of NGF, in fact it adds a serious drawback. Since the cells are dependent on NGF for survival,

control cultures without added NGF are, in fact, dying. This severely compromises the utility of these *in vitro* systems for many studies.

Although sensory and sympathetic neurons are by far the most studied and well accepted targets for NGF's action, recent evidence indicates that other cell types may respond to NGF, at least in a limited manner. Adrenal medullary cells are similar to sympathetic neurons in being derived from the neural crest. In embryos treated with NGF, these cells extend processes and become neuron-like (Aloe and Levi-Montalcini, 1979). Further, prenatal administration of anti-NGF causes the degeneration of these cells. *In vitro* experiments indicate that glucocorticoids prevent the differentiating actions of NGF on these cells (Unsicker et al., 1978). As there are high endogenous levels of glucocorticoids in the adrenal, they may control the developmental path taken by these cells *in vivo*.

There are two transformed cell lines derived from a neural crest tumor which also respond to NGF. One of these, the PC12 line, was derived from a pheochromocytoma and has been of great use in studying the mechanism of action of NGF. Grown without NGF, these cells exhibit many characteristics of the chromaffin cells from which they are derived. Upon the addition of NGF, these cells become much more like neurons, slowing their mitotic rate, extending long neurite-like processes (Greene and Tischler, 1976), increasing their electrical excitability, and becoming more sensitive to acetylcholine (ACh) (Dichter et al., 1977). They also undergo an increase in the activity of choline acetyltransferase (CAT) (Schubert et al.,

1977) and acetylcholinesterase (AChE) (Rieger et al., 1980). Unlike sympathetic cells, PC12 cells do not increase the level of TH upon exposure to NGF (Greene and Tischler, 1976). In another cell line derived from pheochromocytoma, the PC12, NGF does induce TH but does not cause neurite extension (Goodman and Heschmann, 1978). Both of these cell lines are particularly useful because they do not require NGF for survival, so it is possible to have viable NGF-free control cultures.

There is also growing evidence that cholinergic parasympathetic neurons respond to NGF. Although no evidence exists which suggests that these cells require NGF for survival at any stage of their development, either *in vivo* or *in vitro*, it has now been shown that the presence of NGF in cultures of these neurons increases the rate of neurite outgrowth (Collins, 1984). Experiments done *in vivo* have strongly suggest that these cells possess a retrograde transport system for NGF (Max et al., 1978).

BIOLOGICAL SOURCES OF NGF

The effects of experimental administration of NGF antiserum provide direct evidence that NGF is required during development. This implies that there must be an endogenous source of NGF. Evidence exists that indicates that the immediate source is the peripheral targets of the neurons which respond to NGF. The experiments which most directly demonstrate this are those which interrupt the connection of a neuron to its target organ. Thus, in the neonate, axotomy or pharmacological block of axonal transport have

effects similar to treatment with anti-NGF (Hendry et al., 1975; Hendry and Campbell, 1976; Hendry, 1977; Thoenen et al., 1978). The similarity extends to the ability of NGF to reverse the effects of axotomy. These results show that removing a neuron's connection to the periphery has an effect analogous to removing its supply of NGF, and that these effects can be reversed by treatment with NGF.

The trophic model for NGF action predicts that NGF should be present in the targets of neurons which respond to NGF, but this has been difficult to demonstrate. Neurons are known to respond to levels of NGF in the ng/ml range in vitro, so similar or even lower concentrations could be physiologically important. NGF-like bioactivity has been detected in explants of some sympathetic targets (Harper, et al., 1980a; Ebendal et al., 1980), however extracts of these same tissues removed directly from the animal never contained detectable NGF (Harper et al., 1980b). The development of the extremely sensitive two-site ELISA has allowed the detection of NGF in normal sympathetic targets such as iris and heart, where it is present at several ng/g of wet weight (Korsching and Thoenen, 1983). There is less NGF present in tissues which are not heavily innervated by sympathetic neurons. There are levels of tens of ng/g in the sympathetic ganglia themselves.

The fact that treatment with drugs which block axonal transport has a similar effect to actual severance of the axon suggests that NGF from the target may have effects in the cell body which are mediated by retrograde axonal transport. It is now known that NGF present at the terminal field of sympathetic or sensory (or parasym-

pathetic) neurons can be bound, internalized and transported to the neuronal cell body (Hendry et al., 1974; Stockel et al., 1975; Johnson et al., 1978; Dumas et al., 1979; Max et al., 1978). At least part of the NGF reaches the cell body intact (Johnson et al., 1978), and NGF administered in this way causes similar effects on transmitter synthetic enzymes as systemically administered NGF (Paravicini et al., 1975). The ability to transport NGF is neuronal type specific, i.e. sensory and sympathetic neurons, which respond to NGF, are capable of transporting NGF, while spinal motoneurons, with no apparent response to NGF, do not display this transport (Stoeckel et al., 1975). This specificity is apparently due to the specificity of the initial binding to the neuronal membranes Stockel et al., 1975).

It has recently been demonstrated that endogenous NGF is also likely to undergo retrograde axonal transport, as there is an accumulation of NGF distal to a nerve crush and a decrease in NGF proximal to the crush site (Thoenen et al., 1983). This decrease extends to the ganglion, which undergoes a drastic reduction in its normally large content of NGF after destruction of sympathetic terminals with 6-hydroxydopamine (6OHDA) or treatment with colchicine (Korsching and Thoenen, 1985a). This is further evidence that the neurons obtain their supply of NGF via retrograde transport from a source in the periphery. How peripheral target organs obtain their supply of NGF remains unknown. It is possible that they accumulate NGF from some systemic source or the tissue could synthesize it locally. Experiments designed to answer this question are

the foundation of this doctoral dissertation and are described in detail in Chapter 2.

CELLULAR MECHANISM OF NGF ACTION

The binding of NGF to neuronal membranes,the first step in retrograde transport, is due to the presence of specific cell surface receptors for NGF. These receptors are present on sensory and sympathetic neurons (Bannerjee et al., 1973; Herrup and Shooter, 1973) and several cell lines from tumors of neural crest derived tissue (Fabricant et al., 1977; Herrup and Thoenen, 1979; Ross et al., 1984). On neurons, there are at least two types of receptors, as distinguished by their apparent affinity for NGF (K_d=$2X10^{-11}$ and $2X10^{-9}$)(Sutter et al., 1979). There are also two different types of receptors present on PC12 cells, but they both have the same affinity ($2X10^{-10}$), and differ in their kinetic parameters, being referred to as fast and slow (Schechter and Bothwell, 1981). Certain melanoma cell lines have very large numbers of receptors on their surface (Fabricant et al., 1977.) These are of low affinity, but their numbers have made the cells useful for the isolation and purification of the receptor (Puma et al., 1984; Ross et al., 1984). It remains to be elucidated what relationship these various receptors from different cell types have to each other, both structurally and functionally.

It is quite clear that binding to the receptor is the first step in NGF action. It is known that NGF is internalized and almost certainly acts through a second messenger(Heumann et al., 1981). However, despite this knowledge and the list of phenomena which

are known to occur after binding, there is not a clear understanding of the mechanism by which NGF exerts its effects.

Studies using NGF labelled with either ^{125}I or the enzyme horseradish peroxidase (HRP) have shown that internalized labelled NGF is contained within membrane bound compartments which eventually fuse with secondary lysosomes (Schwab, 1977; Schwab and Thoenen, 1977). There is no evidence for the presence of NGF existing free in either the cytoplasm or in the nucleus. In fact it has been shown that cells which have had NGF injected into their cytoplasm do not exhibit any of the normal responses to NGF application (Heumann et al., 1981). Furthermore, injection of antibodies to NGF into the cytoplasm does not prevent the cell from responding normally to externally applied NGF (Heumann et al., 1981). This is strong evidence that the effects of NGF are mediated through a second messenger. Although some studies have implicated cAMP as the second messenger (Nikodijevic et al., 1975; Schubert and Whitlock, 1977), other experiments have not observed this (Frazier et al., 1973c; Otten et al., 1978). This discrepancy has now been partially explained by the observation that cAMP can potentiate some of the effects of NGF (Near and Wagner, 1985).

There are a number of effects which occur quickly after the application of NGF to cells in culture. Within seconds, a transient change in surface morphology occurs in PC12 cells (Connolly et al., 1979). The microvilli which are present on NGF-free cells are replaced by large ruffles of membrane. These then recede, leaving a relatively smooth cell surface. Large blebs appear on the surface

between one and four hours post treatment, and microvilli begin to reappear by six to seven hours.

Within the first few minutes of NGF application there are also measurable changes in some biochemical parameters. There is an increase in the transport of sugars, amino acids, and uridine into the cell (Horii and Varon, 1977). All of these processes are Na^+-dependent, and it has been proposed that NGF directly influences Na^+ transport and thereby exerts many of its effects, including survival (Skaper and Varon, 1983). Events occurring hours to days after application of NGF include neurite extension and associated changes in several cytoskeletal proteins, as well as the previously mentioned changes in several enzymes (ODC, TH, and DBH) (MacDonnell et al., 1977a; 1977b)

NGF IN THE IRIS

The iris is a five layered tissue derived in part from neuroectoderm (Zinn, 1972). The rat (and mouse) iris has been particularly useful for many studies because it is a thin sheet of essentially transparent tissue in albino animals. This allows it to survive as an explant (Silberstein et al., 1971) and to be used as a whole mount for many anatomical studies (Malmfors, 1965). It is innervated by three well characterized types of neurons, sympathetic, parasympathetic, and sensory (Zinn, 1972).

The sensory nerves originate in the trigeminal ganglion, and travel in the ophthalmic branch of the trigeminal nerve. After leaving the cranial cavity through the anterior lacerated foramen, the axons destined to innervate the eye branch off as the nasociliary

nerve, and then enter the eye with the short and long ciliary nerves. The endings in the iris are poorly characterized, but are very responsive to mechanical stimuli (Tower, 1940) and at least a subset of them contain substance P (Cuello et al., 1978).

The cell bodies of the cholinergic parasympathetic nerves lie in the ciliary ganglion, which lies on the dorsal aspect of the inferior rectus muscle in the rat. The axons then enter the eye with the short ciliary nerves, and innervate primarily the ciliary body and the sphincter muscle of the iris (Zinn, 1972).

The sympathetic fibers originate in the superior cervical ganglion and follow the internal carotid artery to the carotid plexus. They then run along with the ophthalmic branch of the trigeminal nerve and with the nasociliary nerve before branching to run partly through the ciliary ganglion and into the eye via the short ciliary nerves and partly with the long ciliary nerves. In the iris, they innervate the vascular smooth muscle, melanocytes, and the dilator muscle of the iris (Zinn, 1972).

There is also a poorly characterized plexus of fibers in the iris which contain enkephalin (Bjorklund et al.,1984). The location of the cell bodies of origin of these fibers is unknown, they do not enter the eye with any of the above described nerves. This implies that the cell bodies are either intrinsic to the eye or that the fibers enter the eye with the optic nerve.

The anterior chamber of the eye is one of the most useful sites in the body for studies utilizing tissue transplantation. It is readily accessible, and the transplanted tissue is easily inspected

through the transparent cornea. Tissue grafts become vascularized readily, and the iris can serve as a source of innervation (Olson and Malmfors, 1970). Conversely, grafts of nervous tissue have easy access to the iris as a target. Further, the anterior chamber is an immunologically privileged site, so cross species transplants are possible (Olson and Malmfors, 1970).

Many of these advantages were utilized in an elegant series of experiments by Olson and Malmfors (1970) investigating the dynamics of the sympathetic innervation of the iris. They found that an iris transplanted into the anterior chamber readily became innervated by sprouts from the host's iris. However, if a sympathetic ganglion was transplanted to the anterior chamber, it was able to sprout into the host's iris only if this iris had been previously deprived of its normal sympathetic innervation. These results show that density of sympathetic innervation in the iris is not limited by the ability of the sympathetic system to increase the extent of its terminal field, but rather by the inability of the iris to accept or support any more than the normal innervation. One interpretation of this finding is that the sympathetic fibers are competing for some limiting factor produced by the iris.

In a more recent series of experiments, the interactions of the sympathetic and sensory projections to the iris have been investigated (Kessler et al., 1983). Upon removal of the sympathetic fibers, there was an increase in the density of sensory innervaton as measured by the level of substance P. This result could also be interpreted in terms of competition between sensory and sympathetic

innervation. Interestingly, the sensory sprouting in these experiments could be blocked by application of anti-NGF, and it has been shown that sensory and sympathetic neurons compete for endogenous NGF (Korsching and Thoenen, 1985b).

The amount of NGF which is present in the rat iris has been shown to depend on the state of innervation. Using the semiquantitative bioassay, Ebendal and coworkers (1980; 1983) demonstrated that there is a transient increase in the level of NGF activity when irises are surgically deprived of their sensory and sympathetic innervation, or, to a lesser extent, when deprived of only their sympathetic innervation. There was also a large increase in NGF activity when irises were placed into culture either in vitro or in oculo. The increase observed after growth in oculo disappeared concomitantly with reinnervation.

These findings have since been extended using the quantitative two site ELISA assay to show that the increase in iris NGF following chemical sympathectomy with 6-hydroxydopamine is approximately two fold (Korsching and Thoenen, 1985a). The increase in NGF during organ culture of the iris in vitro was, however, about two hundred fold (Barth et al., 1984). It is possible that this large increase in NGF content following explant also occurs in other sympathetic targets and explains the previously mentioned difference in levels of explanted tissues and these same organs assayed directly from the animal.

There are several mechanisms which might contribute to these increases. Perhaps most obviously, these treatments might cause an increase in the synthesis of NGF, thereby leading to higher levels

in the tissue. Another possible way to increase the NGF content of a tissue is to decrease the turnover of the molecule. This could be accomplished by reducing degradation or removal of NGF. Any or all of hese mechanisms might combine to cause the increase observed after denervation or explant. In order to determine whether the iris increases the synthesis of NGF after denervation, the levels of NGF mRNA in normal and denervated irises were measured. The levels of NGF mRNA were also determined in irises which had been cultured in order to determine if the response to explant is similar to *in vivo* denervation. These experiments are described in Chapter 4.

NGF IN THE CENTRAL NERVOUS SYSTEM

Early investigations of possible NGF effects in the central nervous system (CNS) centered on the noradrenergic neurons of the locus coeruleus (LC). However, injections of NGF and anti-NGF have failed to have any observable effect on these neurons (Konkol et al., 1978). Also there is no apparent effect of NGF or anti-NGF on these neurons *in vitro* (Olson et al., 1979). There is also no demonstrable retrograde transport of NGF by these neurons (Schwab et al., 1979). In contrast, when the regeneration of fibers from these neurons has been examined, treatment with NGF does give a marked increase in the biochemical and morphological parameters of regrowth (Bjorklund and Stenevi, 1981). NGF also effects the sprouting of these fibers into an iris transplanted into the diencephalon (Stenevi et al., 1974).

In one attempt to demonstrate retrograde transport by these neurons, Schwab et a l., (1979) injected ^{125}I-NGF into the hippocampal

formation. They observed no transport by the noradrenergic fibers from the LC or the dopaminergic fibers of the substantia nigra, but did notice an apparently specific transport back to the cells of the septal nuclei and the nucleus of the diagonal band of Broca. These cells give rise to a large projection to the hippocampus Lewis and Shute, 1967) which is partly cholinergic (Rye et al., 1984). There is now a growing body of evidence that NGF does indeed affect certain cholinergic neurons in the CNS. The cholinergic cells in the rat basal forebrain which correspond to the nucleus basalis of Meynert in higher mammals project to the entire cortical mantle (Shute and Lewis, 1967; Lewis and Shute, 1967). These cells now have also been shown to have a retrograde transport system for NGF (Seiler and Schwab, 1984). The presence of this tranport system suggests the existence of NGF receptors on these cells and indeed there is direct evidence that both chick and mammalian brain contain such receptors (Frazier et al., 1974; Zimmermann et al., 1978). In addition, NGF has been reported to be present in brain (Korsching and Thoenen, 1984).

It has now been shown that the neurons of these cholinergic basal forebrain nuclei (CBFN) do respond to NGF, although in a somewhat more limited way than do sympathetic and sensory neurons. The addition of NGF either <u>in vitro</u> (Honegger and Lenoir, 1982; Hefti et al., 1985) or <u>in vivo</u> (Gnahn et al., 1983; Hefti et al., 1984) causes an increase in the level of CAT. NGF has no apparent effect on neuron survival, process extension or the level of AChE <u>in vitro</u> (Honegger and Lenoir, 1982; Hefti et al., 1985). The effect of NGF on CAT

activity can be blocked by simultaneous administration of anti-NGF. The antibody given without NGF does not appear to lower the basal level of CAT activity *in vitro* (Hefti et al., 1985) or *in vivo* (Gnahn et al., 1983; Hefti et al., 1984).

NGF has also been reported to have effects on the regeneration of the visual system of the lower vertebrates. Injection of NGF into the eye after optic nerve crush speeds several parameters of regeneration in both fish (Turner et al., 1980) and newts (Turner and Glaze, 1977). In addition, it has been reported that goldfish brain contains a relatively large amount (i.e., detectable by bioassay) of NGF activity (Benowitz and Greene, 1979).

In the iris, there is a large increase in NGF content after removal of the neurons which respond to NGF. There is suggestive evidence that this same phenomenon may occur when the cholinergic projection to the hippocampus or cortex is removed. After lesion of the fimbria-fornix, the sympathetic innervation of the external transverse hippocampal artery crosses the pia and invades the neuropil of the hippocampus (Loy et al., 1980). It is unknown if any synapses are formed, but these "sympathohippocampal" fibers project to a restricted region of the hippocampus, limited to, but not entirely encompassing the original septal projection. Lesions of any other of the projections to the hippocampus do not induce this sprouting. Similar denervation of the neocortex by lesion of the CBFN leads to similar sympathetic sprouting into the neuropil of the denervated cortex (Crutcher, 1981). It has also been shown that neonatal (Gage et al., 1984) or adult (Bjorklund and Stenevi,

1981) sympathetic ganglia when transplanted to a cavity made in the retrosplenial cortex such that they are in contact with the hippocampus, will send fibers into the hippocampus only if the septal projection to the hippocampus is removed. Given the known dependence of sympathetic process outgrowth on NGF, it is possible that these results are due to an NGF increase in the hippocampus following the lesion to the NGF responsive septal projection.

There is now ample evidence that NGF can have effects on the neurons of the CNS, and circumstantial evidence that there may be a source of NGF present in vivo, at least after denervation. In order to determine if NGF is synthesized in the brain, its content of NGF mRNA has been measured. Further, different areas of the brain have been assayed to find out if the spatial distribution of NGF mRNA can be related to the distribution of the cells which respond to NGF. These results are presented in Chapter 3.

CHAPTER 2

DEMONSTRATION OF NGF mRNA IN SYMPATHETIC TARGET ORGANS

INTRODUCTION

Several lines of evidence suggest that NGF is a target-derived trophic factor for peripheral sympathetic and sensory neurons. However, the ultimate source for the NGF in peripheral target tissues remains unknown. It seems possible that the NGF is synthesized locally or is accumulated from some systemic source. Using a recently cloned cDNA as a probe, an assay using RNA blot hybridization which can detect <10 femtograms of NGF mRNA has been developed. In this chapter, it is shown that many mammalian sympathetic and sensory effector organs do express the NGF gene and, therefore, have the ability to synthesize NGF. The level of gene expression, although low, appears to be sufficient to account for the NGF found in sympathetic effector organs.

MATERIALS AND METHODS

Sources

Sprague-Dawley rats, New Zealand White rabbits, Swiss-Webster mice, dogs, and cow eyes were obtained from local suppliers. Nitrocellulose paper (0.45 um, BA85) was obtained from Schleicher & Schuell; restriction enzymes, Klenow fragment, and primer, from New England Biolabs; oligo(dt)-cellulose, from Collaborative Research; and [^{32}P]CTP (3000 Ci/mol; 1 Ci=37 GBq), from Amersham. All other chemicals were reagent grade.

Probe Preparation

For initial work we used a double stranded probe prepared from the NGF cDNA described (Scott et al., 1983). The 0.9 kilobase (kb) Pst I fragment complementary to the coding sequence and to the 5' untranslated region was labeled by nick-translation to a specific activity of ca. 2 x 10^9 cpm/ug (Rigby et al., 1977). To create a single-stranded cDNA probe, this fragment was cloned into the Pst I site of phage M13 mp8 (Messing and Vieira, 1982) Labeled probe was synthesized on a phage DNA template hybridized with a 15-base primer. Phage DNA (700 ng; 80 ng of NGF insert) was boiled for 2 min. with a 10-fold molar excess of primer in 4.0 ul of 20 mM Tris chloride, pH 7.4/10 mM $MgCl_2$/1 mM dithiothreitol/ 0.2 mM $CaCl_2$ and slowly cooled to room temperature. The final reaction volume was 20 ul of the same solution containing 200 uCi of [^{32}P]dCTP (dried under vacuum) and 250 uM each of unlabeled dATP, dGTP, and TTP. Synthesis, initiated by the addition

of 10 units of Klenow fragment of Escherichia coli DNA polymerase I, proceeded for 5 min at 22°C and 90 min at 37°C. After unincorporated dCTP (<10%) was removed by gel filtration through Sephadex G-50, DNA was precipitated with 2 vol of ethanol, dissolved in water, and diluted with an equal vol of 100mM NaOH/2 mM EDTA. The sample was boiled for 2 min, chilled on ice, and loaded directly on a 1.5% "low melting temperature" agarose gel equilibrated with 40 mM Tris acetate, pH 7.5/2 mM EDTA. After electrophoresis, the labeled probe band was excised, melted at 68°C, and added directly to the hybridization solution.

RNA Preparation and Assay

Animal tissues were homogenized in 5 M guanidinium thiocyanate/ 5% 2-mercaptoethanol/10mM EDTA/50 mM Na Hepes, pH 7.4. RNA was isolated either by sedimentation through 5.7 M CsCl and extraction with phenol/chloroform (Chirgwin et al., 1979) or by precipitation with five volumes of 4 M LiCl and extraction with phenol/chloroform (Cathala et al., 1983). All solutions used after the initial homogenization were treated with 0.05% diethyl pyrocarbonate and were autoclaved. All glassware was baked for 4 hr at 200°C. Poly(A)$^+$ RNA was selected by two cycles of chromatography an oligo(dT)-cellulose (Aviv and Leder, 1972). Selected RNA was denatured with formaldehyde, separated on formaldehyde-containing 1.5% agarose gels (Lehrach et al., 1977), and transferred to nitrocellulose paper with 3 M NaCl/0.3 M Na Citrate, pH 7.0, without presoaking the gel (Thomas, 1980). Blots were then treated with a prehybridization solution that contained 50% deionized formamide,

0.1% $NaDodSO_4$, denatured salmon sperm DNA at 200 ug/ml, 5x Denhardt's solution (1 x Denhardt's is 0.02% each of Ficoll 400, polyvinyl-pyrrolidone, and bovine serum albumin), 0.75 M NaCl, 5 mM EDTA, and 50 mM sodium phosphate (pH 7.7) for at least 8 hr at 50°C. Hybridization was carried out for 48 hr at 50°C in a solution of the same composition except with Denhardt's reduced to 1X and either 2.5 ng of double-stranded probe or 8 ng of single-stranded probe per ml. The filter was then washed four times with 0.3 M Na citrate, pH 7.0 and four times with 15 mM NaCl/0.1% $NaDodSO_4$/ 1.5 mM Na citrate, pH 7.0, both at room temperature, and twice with the latter solution at 60°C. After drying, the paper was exposed to preflashed Kodak XAR-5 film with a DuPont Cronex intensifying screen at -80°C. The resulting autoradiogram was used to locate hybridizing bands on the filter, which were excised, dissolved in 1 ml of 2-ethoxyethanol, and assayed for radioactiviy in Aquasol (New England Nuclear) on a scintillation counter. Hind III fragments of phage lambda DNA were end-labelled with [^{32}P]dCTP, denatured with formaldehyde, and used as molecular weight standards. Serial dilution of male mouse submaxillary gland poly(A)$^+$ RNA and standard curves of phage DNA from the M13 subclone were run on every gel.

RESULTS

Sequence Homology of Mouse NGF cDNA with the NGF Gene in Other Mammals

In order to test the sequence homology of the mouse cDNA with NGF sequences from other mammals, genomic DNA was digested with HindIII and analyzed by the techniqueof Southern (1975). After hybridization to the mouse single-stranded probe and autoradiography, single bands of approximately equal intensity were found for mouse, rat, rabbit, dog, and cow DNA (Fig 3). The hybridization and washes were done under stringent conditions (see Fig. 3 legend), and when transfers were washed sequentially with 0.015 M NaCl/ 0.0015 M Na citrate, pH 7.0, at increasing temperatures, the bands hybridizing to DNA of various species decreased and disappeared approximately coincidentally (data not shown). Thus, the mouse cDNA is highly homologous to the NGF sequences of genomic DNA from each of these mammals and, therefore, is expected to hybridize to their NGF mRNAs.

Development of a Sensitive Assay for NGF mRNA.

Since NGF is present in minute or undetectable quantities in targets of sympathetic neurons (Korsching and Thoenen, 1983), we expected to detect correspondingly low levels of NGF mRNA. Therefore, we felt it was necessary to show that any hybridizing RNA was of the appropriate size and attempted to develop a more sensitive assay with RNA blot transfers.

At the time this work was initiated, no assay of RNA transferred to nitrocellulose claimed a sensitivity of <0.5 pg of specific mRNA

FIGURE 3

Hybridization of NGF (^{32}P)cDNA to genomic DNA.

High molecular weight DNA samples were prepared as described (Kunkel et al., 1977), digested with HindIII, and analyzed by electrophoresis, transfer, and hybridization to a single-stranded ^{32}PDNA probe. Samples (100ng) were run on 0.8% agarose gels and transfered to nitrocellulose (Southern, 1975). Pretreatment and hybridization were done at 42°C, and the final washes were performed at 50°C in conditions otherwise the same as for RNA assays. The positions and sizes in kilobase pairs of HindIII fragments of lambda DNA are indicated by arrowheads and numbers.
Lanes: a, mouse; b, rat; c, rabbit; d, cow; e, dog.


a
b
c
d
e
23
9.4
6.6
4.4
2.3
2.0

(Gal et al., 1983). Using serial dilution of a rich source of NGF mRNA, poly(A)$^+$ RNA from the male mouse submaxillary gland, we examined several parameters of this assay. We tried both nick-translated and single-stranded probes, various conditions of electrophoresis, transfer, hybridization, and washing in attempts to increase sensitivity.

A standard curve obtained with our optimized method with a single-stranded probe is shown in Fig. 5. The radioactivity varied linearly with applied RNA or DNa over at least 3 orders of magnitude. The absolute detection limit of DNA varied somewhat but was always <10 fg of hybridizing sequence. Assuming equal hybridization efficiency to NGF mRNA, this corresponds to about 12,000 mRNA molecules. Each gel lane had a capacity of at least 10 ug of poly(A)$^+$ RNA, giving the ability to detect <1 part in 10^9 by weight. By this assay, NGF mRNA appeared to be 0.08 $\pm$ 0.03% of the total poly(A)$^+$ RNA in the male mouse salivary gland.

Levels of NGF mRNA in Different Tissues.

To determine if NGF mRNA was present in sympathetically innervated tissues, poly(A)$^+$ RNA was prepared from major sympathetic targets and assayed as described. In addition, we measured NGF mRNA in tissues with sparse sympathetic innervation and in nerves and ganglia containing elements of the sensory and sympathetic nervous systems.

A summary of NGF mRNA levels in tissues tested is shown in Fig. 6, and an example of an autoradiogram obtained with this technique is shown in Fig. 7. All tissues in which NGF mRNA was

FIGURE 4

NGF DNA and RNA standard curves.

Two dilution series were performed, one with male mouse submaxillary gland poly(A)$^+$ RNA (■) and one with M13 phage DNA containing the 900 base Pst I fragment of the NGF cDNA clone (●). Each sample was counted for at least 40 min. The background has been subtracted from each point.

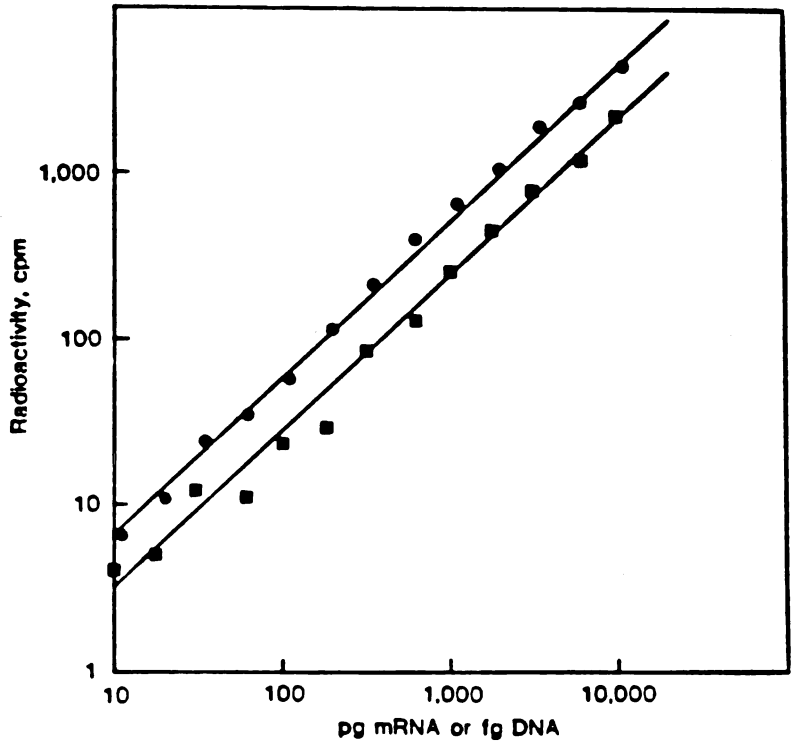

1,000
100
10
1
Radioactivity, cpm
10
100
1,000
10,000
pg mRNA or fg DNA

FIGURE 5

NGF mRNA content of various mammalian tissues.

Unless otherwise specified, all tissues are from rabbits. Bars represent either single determinations or the means of all preparations for a given organ. Individual points represent individual determinations. Different symbols mark different preparations, while identical symbols represent independent determinations done on the same preparation of RNA. Note the change in scale between A (rabbit, dog and cow) and B (rat).

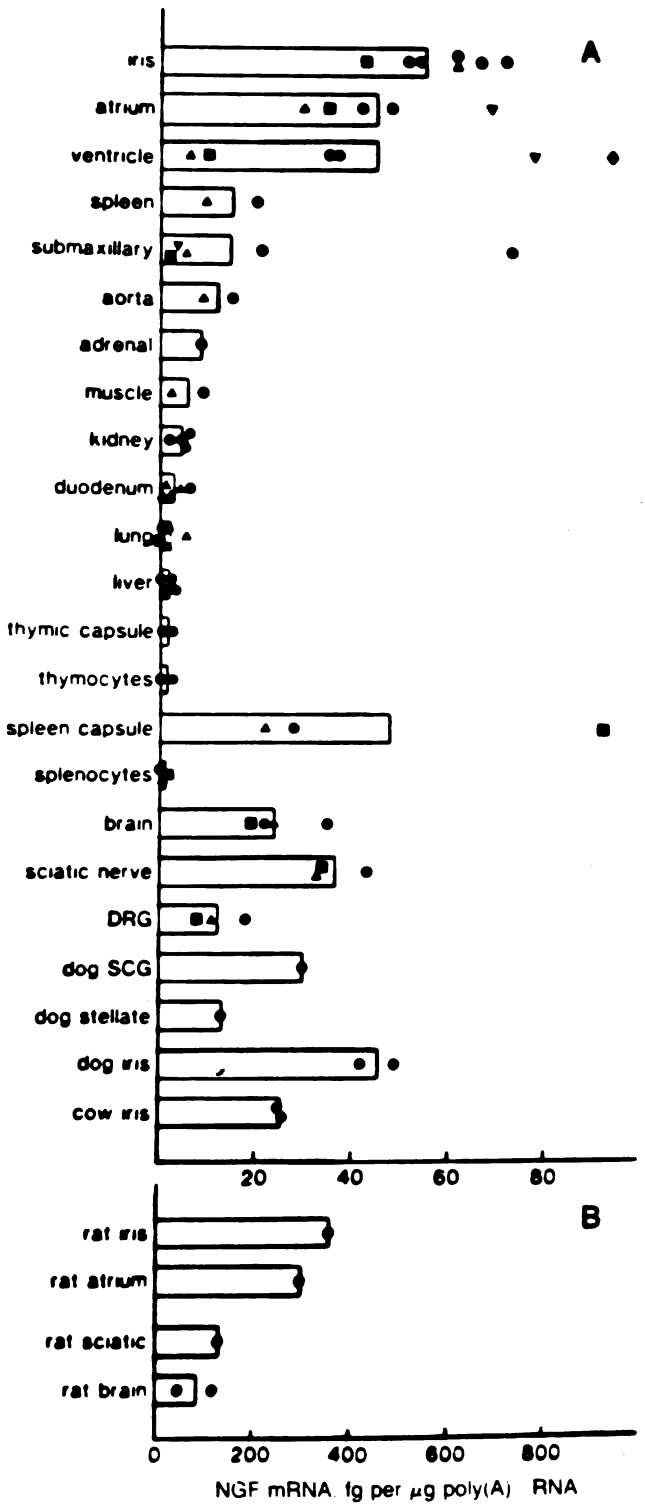
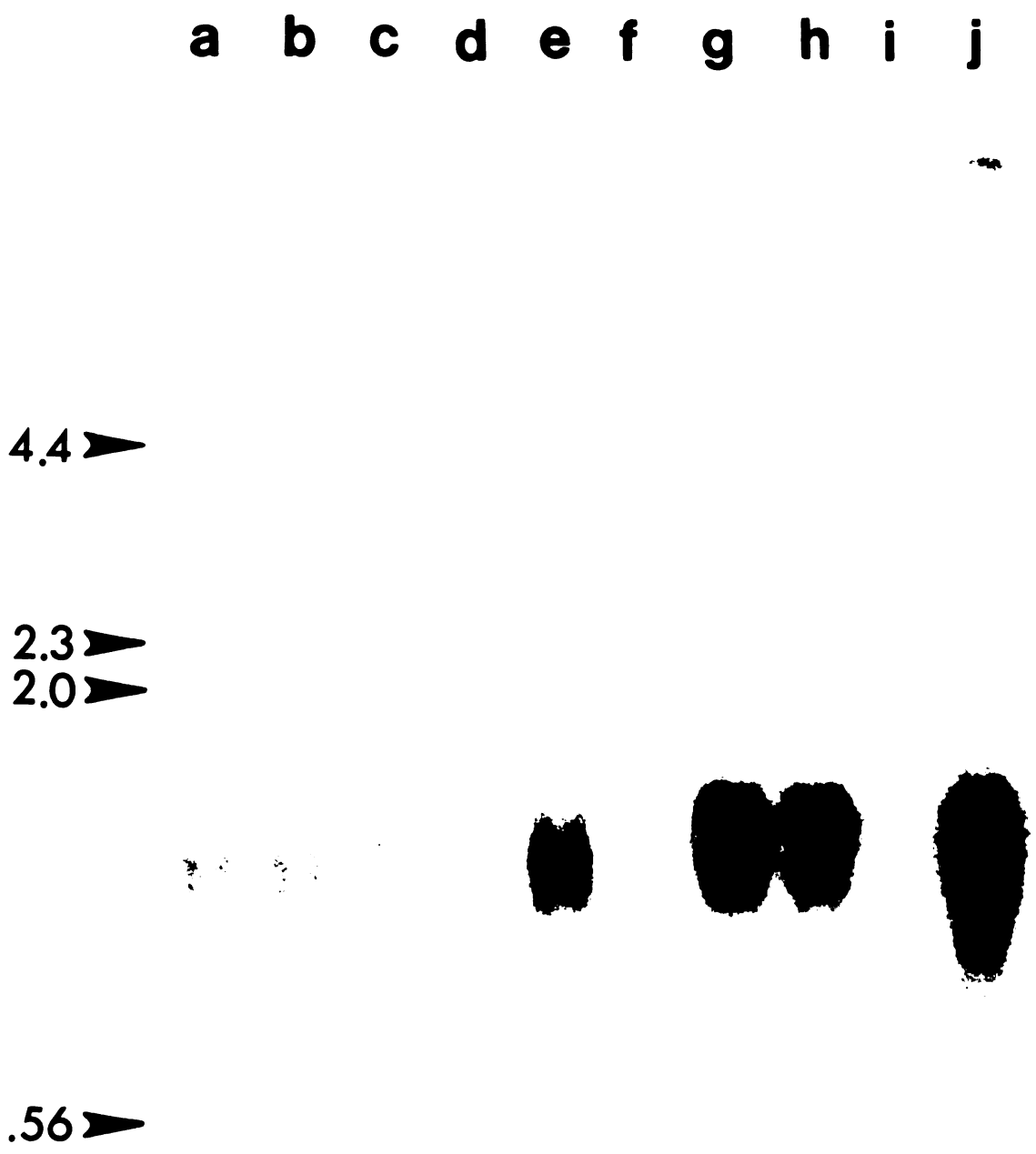

A
iris
atrium
ventricle
spleen
submaxillary
aorta
adrenal
muscle
kidney
duodenum
lung
liver
thymic capsule
thymocytes
spleen capsule
splenocytes
brain
sciatic nerve
DRG
dog SCG
dog stellate
dog iris
cow iris
20
40
60
80
B
rat iris
rat atrium
rat sciatic
rat brain
0
200
400
600
800
NGF mRNA: fg per μg poly(A) RNA

FIGURE 6

Autoradiogram of an RNA blot hybridization.

Poly(A)$^+$ RNA samples are from the rabbit unless otherwise specified. The positions of standards are indicated by arrows, and numbers are sizes in kb. Figure in parentheses after tissue indicates amount of RNA applied to gel. Lanes: a, cow iris (2ug); b, dog iris (1.5ug); c, dorsal root ganglion (2.6ug); d, kidney (5ug); e, iris (2ug); f, duodenum (5ug); g, heart ventricle (2.6ug); h, heart atrium (2.6ug); i, lung (5ug); j, spleen capsule (5ug).


a b c d e f g h i j
4.4
2.3
2.0
.56

detected in mouse, rabbit, dog and cow showed a single hybridizing band of ca. 1.3 kb, the same size as NGF mRNA in the male mouse submaxillary gland. All rat tissues tested had higher levels of NGF mRNA of this molecular size than in the same tissues from the other species. Also, in some but not all tissues from the rat, an additional hybridizing band was detected, with a molecular size of 1.7 kb. This band was always less intense than the 1.3-kb band, accounting for no more than 20% of the total amount of hybridization and was not included in the data in Fig. 3.

There is a correlation between the level of NGF mRNA and the level of sympathetic innervation. Iris had the highest level of NGF mRNA of any tissue in all species tested (with the exception of male mouse submaxillary gland and rabbit prostate gland, two exocrine tissues with anomalously high NGF levels). Other tissues with heavy sympathetic innervation, such as heart and spleen, also contained comparatively high levels of NGF mRNA. Tissues with little sympathetic innervation, such as skeletal muscle and thymus, had much lower levels of transcription.

In addition, some tissues were subdivided into regions with different levels of sympathetic innervation. For example, heart atrium and ventricle, both of which receive a dense sympathetic innervation, were both shown to contain comparatively high levels of NGF mRNA. The innervated spleen capsule was shown to contain essentially all of the NGF mRNA in the spleen and had at least a 10-fold higher level of NGF mRNA than the uninnervated splenocytes. The thymus receives very little sympathetic innervation, and neither

thymocytes nor thymic capsule had high levels of NGF mRNA.

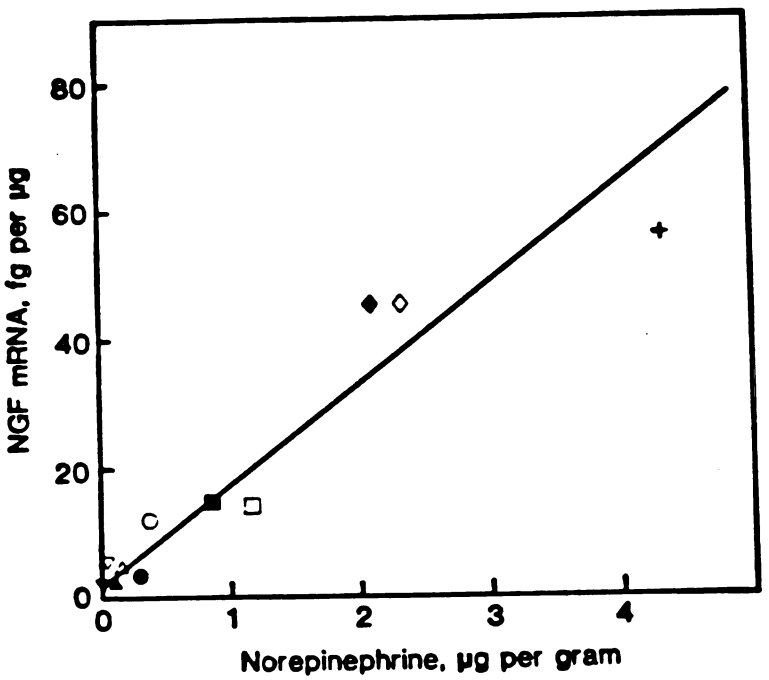
To determine how closely the level of sympathetic innervation correlated with the level of NGF mRNA in different tissues, we compared the NGF mRNA level to the previously reported norepinephrine content for each tissue (Holzbauer and Sharman, 1972). There was a strong positive correlation between these two values (Fig. 8).

Elements of the peripheral nervous system- sympathetic ganglia from dog and dorsal root ganglia and sciatic nerve from rabbit- also contained readily detectable NGF mRNA. Surprisingly, brain, which has only a sparse sympathetic innervation of the vasculature, had a relatively high level of NGF mRNA. This will be further discussed in Chapter 3.

FIGURE 7

Correlation of NGF mRNA content with norepinephrine content of organs of the rabbit.

Norepinephrine (NE) values are from Holzbauer and Sharman(1972) and expressed as ug per gram of tissue we weight. The line is drawn from a linear least squares fit to the experimental data (r^2=0.95). In a few organs, the NE content has not been determined in the rabbit. In those cases, levels of rabbit NGF mRNA are plotted against means of NE levels in the same organs of other species, which are listed in parentheses. ▼, lung; ▽ muscle, (rat, dog, cat, and cow); ▲ liver; △ kidney; ● duodenum; ○ aorta; ■ spleen; □ salivary gland (rat, dog, cat, and cow); ◆ heart ventricle; ◇ heart atrium; + iris (rat and cat).


80
60
40
20
0
NGF mRNA, fg per µg
0
1
2
3
4
Norepinephrine, µg per gram

DISCUSSION

Detection of NGF mRNA.

To determine whether sympathetic (and sensory) effector organs are capable of synthesizing NGF, we developed an assay based on the RNA blot hybridization technique which is capable of detecting extremely low levels of NGF mRNA. This assay appears to detect authentic NGF mRNA for the following reasons. First, the hybridization and washing conditions were very stringent. Second, the major bands of RNA that hybridized under these conditions comigrated with authentic NGF mRNA from male mouse submaxillary and rabbit prostate glands. Finally, under conditions of similar stringency, Southern blots of genomic DNA from the species used showed single hybridizing bands, implying that there is only one gene capable of hybridizing under these conditions. Although we have not used pure NGF mRNA to calibrate this assay, the values obtained for male mouse submaxillary gland mRNA agree closely with estimates obtained from the frequency of occurrence of NGF cDNA in a submaxillary gland cDNA library (Scott et al., 1983) and with the relative rate of synthesis of the protein in the same gland (Berger and Shooter, 1978). The standard curves obtained using serial dilutions of DNA and RNA are linear over at least 3 orders of magnitude; therefore, the assay should give reliable estimates for the relative amounts of NGF mRNA in samples of poly(A)$^+$RNA.

NGF mRNA in Sympathetic Effector Organs.

The major result in this paper is the unambiguous detection of NGF mRNA in sympathetic effector organs, thereby extending earlier work with sensitive antigen assays that demonstrated the presence of NGF in some of these same tissues, retrograde transport of endogenous NGF, and substantial accumulation of NGF in the sympathetic ganglion (Korsching and Thoenen, 1983; Thoenen et al., 1983). In the rat atrium, there is about 1.0 ng/g (wet weight) (Korsching and Thoenen, 1983). Assuming 10% of the atrium wet weight is protein, NGF is ca. 1×10^{8} of the total protein. In contrast, NGF mRNA is ca. 3×10^{-7} of the total poly(A)$^{+}$ RNA in this tissue. Thus, the proportion of mRNA encoding NGF appears to be about 30-fold higher than the proportion of NGF. This may reflect the loss of NGF due to secretion. NGF mRNA levels in sympathetic effector organs seem to be high enough to direct the synthesis of all the NGF found in these tissues. Since NGF cannot be detected in normal serum (Korsching and Thoenen, 1983) all the NGF in a target is probably synthesized locally.

Our results suggest that there is a correlation of NGF mRNA content with sympathetic innervation in a variety of tissues. To clarify this relationship, we sought some parameter that would indicate relative innervation density in the various organs. In most tissues of the body, norepinephrine is localized in the varicosities of sympathetic nerves and, therefore, provides an assay for the level of sympathetic innervation. The striking correlation between norepinephrine content and NGF mRNA content

(Fig. 8) is unlikely to reflect simply a direct relationship per se because the adrenal gland, which has a high level of norepinephrine not due to sympathetic innervation (480 ug/g, compare with figure 7) (Holzbauer and Sharman, 1972) has a relatively low level of NGF mRNA (figure 5). It seems most plausible that both the NGF mRNA content and the norepinephrine content parallel the density of sympathetic innervation.

The strong correlation between NGF mRNA levels and estimated densities of sympathetic innervation in sympathtic targets suggests that the level of NGF synthesis may limit the density of this innervation in adult tissues. In support of this possibilty, systemic application of NGF is known to induce a general hypertrophy in sympathetic innervation (Levi-Montalcini and Hamburger, 1953). Furthermore, anti-NGF has been shown to inhibit, and NGF to promote, the growth of sympathetic fibers into transplanted sympathetic targets (Bjerre et al., 1974). It should be interesting to see whether increases in expression of the NGF gene precede increases in sympathetic innervation during development. While NGF is likely to be important, it is clearly not the only factor important in regulating innervation (Black and Mytilineou, 1976).

It is perhaps surprising that the level of NGF mRNA correlates so well with the density of sympathetic innervation, since virtually all of these tissues are also innervated by sensory neurons, some of which also have a high-affinity retrograde transport system for NGF (Dumas et al., 1979). If neurons efficiently remove secreted NGF, the two neuronal populations should compete for available NGF.

In this case, levels of NGF mRNA should correlate with the combined innervation density of these two neuronal types. Unfortunately, there is no way to measure the innervation density of the subset of sensory neurons that transports NGF.

The cell types that synthesize NGF in sympathetic targets are currently not known. Although we have not measured the amount of poly(A)$^+$RNA per cell in these tissues, conventional estimates are on the order of 2 x 10^5 poly(A)$^+$ RNA molecules per cell (Robins et al., 1982). This suggests that only a small percentage of cells can contain NGF mRNA at any one time, even in a richly innervated tissue such as the rat atrium, where the NGF mRNA is about 3 parts in 10^7 of the poly(A)$^+$ RNA. It is unknown whether every cell expresses the NGF gene a small fraction of the time or whether a small percentage of cells makes NGF all of the time. There is evidence suggesting that many cell types make NGF in vitro (Harper et al., 1980a).

NGF mRNA in Other Elements of the Perpheral Nervous System.

Comparatively large amounts of NGF mRNa were found in peripheral elements of the nervous system. Sensory ganglia, sympathetic ganglia, and sciatic nerve appear capable of synthesizing some NGF in adult animals. Since these tissues have a small mass compared to targets, this does not mean that sympathetic or sensory neurons normally derive much of their NGF content from the nerve or ganglia. Axotomy results in a large decrease in the level of NGF in the sympathetic ganglion (Thoenen et al., 1983), providing direct evidence that the bulk of this trophic factor is not derived from

the vicinity of the ganglion. However, local synthesis of NGF in the nerve or ganglion may partially explain why treatments such as axotomy and chemical sympathectomy do not have irreversible effects on adult sympathetic neurons, even though they prevent retrograde transport from targets (Hendry and Campbell, 1976).

NGF mRNA in the Brain.

Mammalian brains have surprisingly high levels of mRNA encoding NGF (Fig. 3), considering that antibodies to this trophic factor have not been reported to have effects on neurons in the central nervous system (Gorin and Johnson, 1980). This NGF mRNA cannot reflect sympathetic innervation of the pineal, since this gland was removed from the brain tissue. It is also not likely to be associated exclusively with the vasculature. The norepinephrine content of brain is low, comparable to that of duodenum, and very little of that content is due to sympathetic innervation (Holzbauer and Sharman, 1972). If all of the brain norepinephrine were due to sympathetic innervation, the results in Fig. 8 would predict ca. 7 fg of NGF mRNA per ug of poly(A)$^+$ RNA. In fact, 4-fold more NGF mRNA is seen. Moreover, we have detected large differences in the level of NGF mRNA in different brain regions (Chapter 3).

Other NGF Transcripts.

In several tissues of the rat, but not other mammalian species examined, [^{32}P] NGF probes reproducibly hybridize to transcripts of two different sizes. While the smaller transcript of 1.3 kb is the same size as the NGF mRNA detected in tissues of other species, the larger one of 1.7 kb (never >20% of the smaller band) does not

correspond to any NGF transcript detected elsewhere. Similar amounts of this band are seen whether or not poly(A)$^+$ RNA is purified, so it not likely to be ribosomal RNA. While it seems most likely to us that the larger RNA also represents a transcript of the NGF gene, further analysis will be needed to determine definitely the relationship between this transcript and the more common NGF mRNA.

CHAPTER 3

SPATIAL DISTRIBUTION OF NGF mRNA IN THE CENTRAL NERVOUS SYSTEM

INTRODUCTION

NGF acts as a target derived trophic factor for a subset of neurons in the peripheral nervous system (PNS). There is a rapidly growing body of evidence which shows that NGF affects at least a subpopulation of central nervous system (CNS) neurons, the cholinergic cells of the basal forebrain nuclei. Treatment of these cells either in vitro (Honegger and Lenoir, 1982; Hefti et al., 1985) or in vivo (Gnahn et al., 1983; Hefti et al., 1984) leads to an increase in their choline acetyltransferase (CAT) activity. There is indirect evidence that it may also be acting as a target derived trophic factor for these neurons, the relevant target structures being the hippocampus and the cerebral cortex. Exogenously supplied NGF can be retrogradely transported from these targets to the cell bodies of these cholinergic neurons (Schwab et al., 1979; Seiler and Schwab, 1984). There is circumstantial evidence for an accumulation of NGF-like activity in the cortex and hippocampus after their cholinergic projection is removed (Loy et al., 1980; Crutcher, 1981; Gage et al., 1984; Bjorklund and Stenevi, 1981). This may be similar to the accumulation of NGF seen in the iris after the removal of its sensory and sympathetic innervation (Ebendal et al., 1980). However, there is very little known about the existence of endogenous NGF in the CNS. As described in Chapter 2, high levels of NGF mRNA were found in the brain. In order to determine its localization, various brain regions have been assayed for their content of NGF mRNA. There are wide regional variations in content of NGF message, the levels in different regions differing by up to forty-fold. This finding supports the

view that NGF is fulfilling some endogenous function in the brain. Furthermore, the high concentrations of NGF mRNA found in the cerebral cortex and hippocampus is consistent with NGF being a target derived trophic factor for cholinergic neurons in the basal forebrain.

MATERIALS AND METHODS

Adult Sprague-Dawley rats obtained from Bantin-Kingman were used for all experiments. Initial experiments showed that there were no obvious differences between males and females in the level of NGF mRNA in brain regions, so data from both sexes have been pooled. Rats were sacrificed by cervical dislocation, and the brain and spinal cord quickly removed to ice cold saline. The following regions were then dissected from the brains while they were maintained on ice; olfactory bulb, cerebellum, medulla, pons, midbrain, diencephalon, the septal area, striatum, hippocampus and cortex. The cortex was further divided along the rhinal fissure, that part dorsal of the fissure being called neocortex and that part ventral, pyriform-entorhinal. As the regions were dissected, they were homogenized in the solution described in Chapter 2 by trituration with a 3cc syringe and 20ga hypodermic needle. The tubes with homogenization buffer and syringes were previously tared so that total wet weight of each region could be determined. RNA preparation, poly(A)$^+$ selection, and NGF mRNA assay were as described in Chapter 2 except that amount of NGF mRNA was determined by densitometry of autoradiograms of the nitrocellulose transfers and comparison to standard curves of male mouse submaxillary gland poly(A)$^+$ RNA and cloned cDNA which were always run on each gel.

RESULTS

When poly(A)$^+$ RNA was prepared from different regions of the rat CNS and assayed as described, NGF mRNA was unambiguously detectable in each area examined. A standard curve obtained with the assay is shown in figure 8. A typical autoradiogram obtained after an overnight exposure is shown in figure 9. For each region the major band recognized in the assay comigrated with NGF mRNA from male mouse submaxillary gland with an apparent size of about 1.3 kb. The previously described 1.7 kb band could be detected in all brain regions which had very dense 1.3 kb bands. In several measurements, this more slowly migrating band averaged about 15% of the hybridization signal of the 1.3 kb band, but was not included during densitometry. Although NGF mRNA was present in every region, the level of NGF mRNA varied over a forty fold range between different areas, as shown in figure 10.

In general, cortical areas had the highest concentration of NGF mRNA, at least 100 fg/ug. The level was highest in hippocampus (archaecortex) and followed in order by pyriform-entorhinal and the neocortex. Areas of the hindbrain and diencephalon had levels over 50 fg/ug, whereas other areas examined had less than 50 fg/ug. The cerebellum had the lowest observed level, 5 fg/ug.

Figure 11 shows the data replotted to indicate each region's contribution to the total CNS content of NGF mRNA. Given the high level and relatively large size, cortical areas contain over 75% of the NGF mRNA in the CNS, with neocortex alone accounting for over 40%.

FIGURE 8

Standard curve obtained using densitometry of autoradiogram.

Example of standard curve of NGF mRNA assay obtained by densitometry of autoradiogram. Autoradiographic density was obtained by an integration of a scan covering the band of interest. Line was drawn by eye. There is 0.08% NGF mRNA in submaxillary gland poly $(A)^+$RNA (Chapter 2).

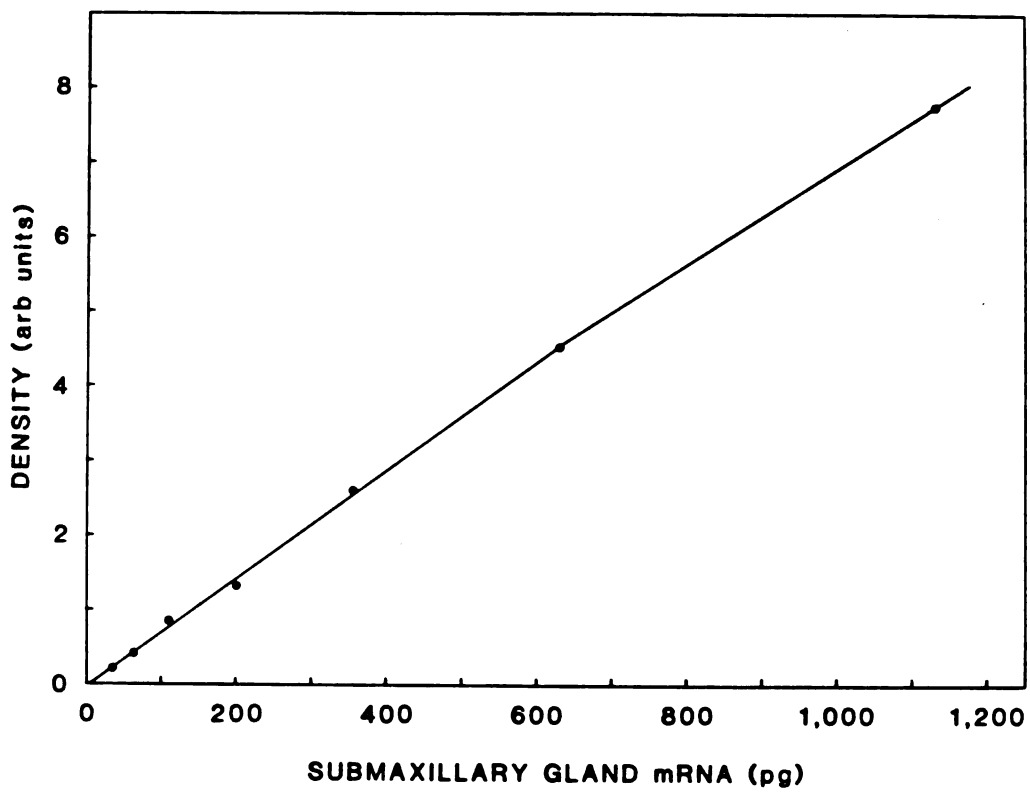

DENSITY (arb units)
0
2
4
6
8
0
200
400
600
800
1,000
1,200
SUBMAXILLARY GLAND mRNA (pg)

FIGURE 9

Autoradiogram of RNA blot hybridization to regions of the rat CNS.

Poly(A)$^+$ RNA from different areas of the rat CNS was assayed as described. Arrows and numbers indicate the molecular size of the hybridizing bands. Region run in each lane with amount of poly(A)$^+$ RNA in parentheses: A, spinal cord (6.0ug); B, medulla (6.1ug); C, pons (7.9ug); D, midbrain (10.2ug); E, diencephalon (10.4ug): F, cerebellum (19.9ug); G, septal area (4.1ug); H, striatum (8.2ug); I, hippocampus (7.4ug); J, pyriform-entorhinal (9.1ug); K, neocortex (13.8ug); L, olfactory bulb (11.6ug).

A B C D E F G H I J K L

FIGURE 10

Levels of NGF mRNA in regions of the CNS.

The amount of NGF mRNA per microgram of poly(A)$^+$RNA is indicated in femtograms. Bars are the means of determinations done on two independent dissections and RNA preparations. The vertical lines are the ranges of those determinations.

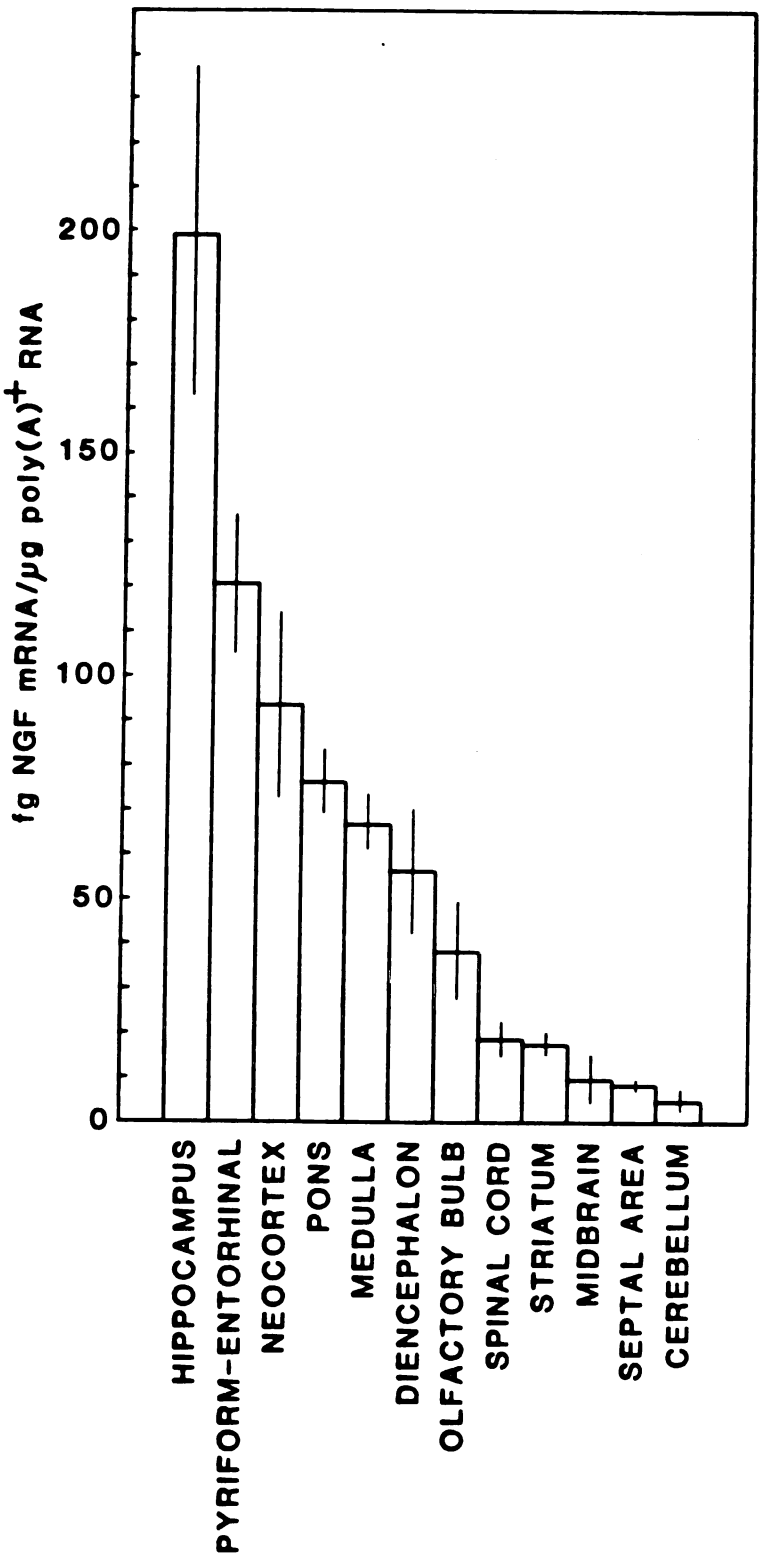
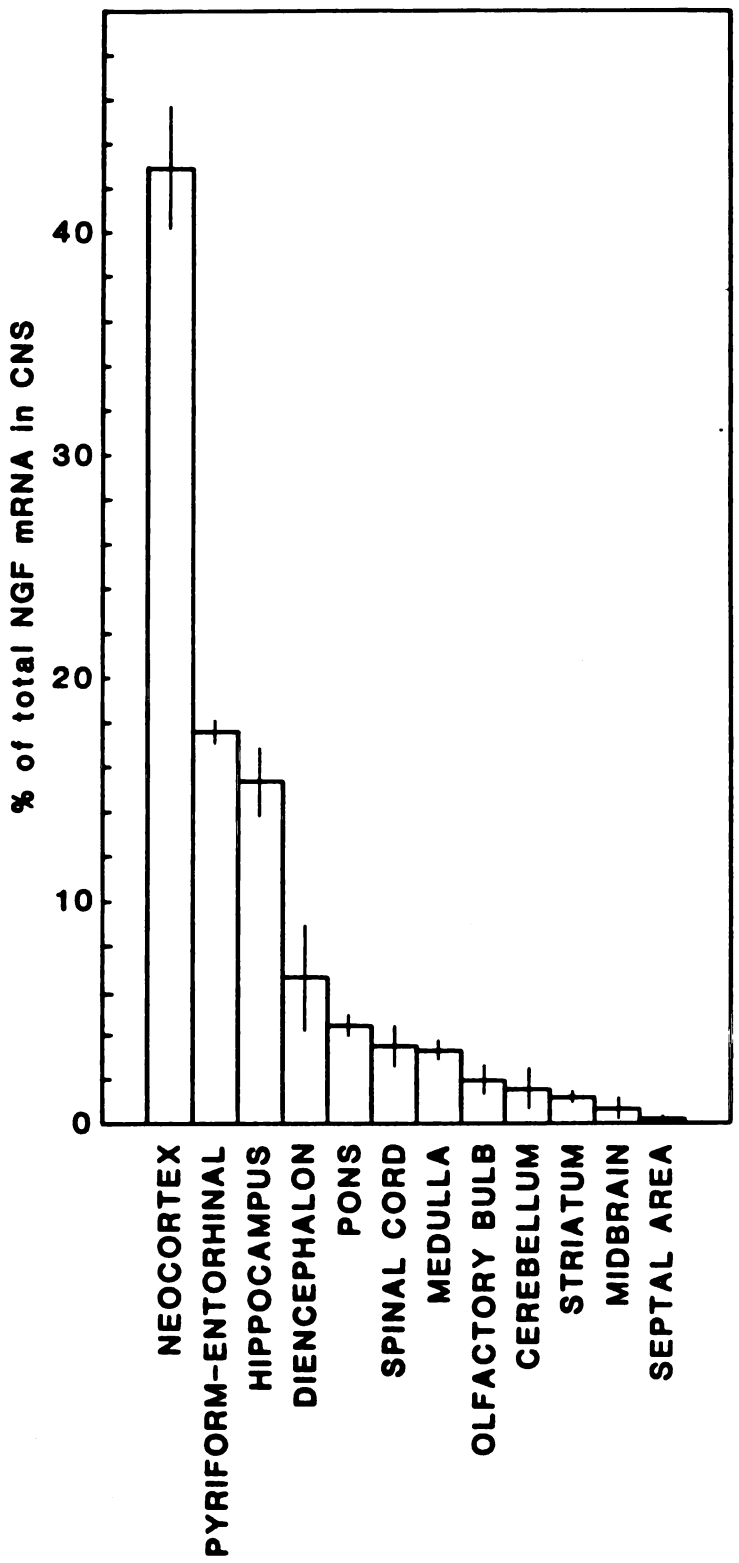

fg NGF mRNA/μg poly(A)+ RNA
200
150
100
50
0
HIPPOCAMPUS
PYRIFORM-ENTORHINAL
NEOCORTEX
PONS
MEDULLA
DIENCEPHALON
OLFACTORY BULB
SPINAL CORD
STRIATUM
MIDBRAIN
SEPTAL AREA
CEREBELLUM

FIGURE 11

Contribution of regions to the total NGF mRNA content of the CNS.

Content of each region of the CNS is displayed as a percentage of the total content of NGF mRNA in the entire CNS. Bars represent the means, and vertical lines the ranges of two independent determinations. In the two determinations, the total CNS content was 3.4 pg and 2.3 pg.


% of total NGF mRNA in CNS
0
10
20
30
40
NEOCORTEX
PYRIFORM-ENTORHINAL
HIPPOCAMPUS
DIENCEPHALON
PONS
SPINAL CORD
MEDULLA
OLFACTORY BULB
CEREBELLUM
STRIATUM
MIDBRAIN
SEPTAL AREA

DISCUSSION

The data presented show that there is expression of the gene for beta-NGF in rat brain, and moreover that the level of this expression varies widely in different brain regions.

Although there have been investigations into the possible effects of NGF on neurons of the CNS for a number of years (Stenevi et al., 1974; Konkol et al., 1978; Olson et al., 1979; Scwab et al., 1979), the evidence in the literature that the mammalian CNS might have access to NGF *in vivo* is minimal. It has been reported in abstract form that NGF is detectable in the brain by the use of a highly sensitive two-site ELISA (Korsching and Thoenen, 1984), and we have previously demonstrated the presence of NGF mRNA in the brains of rat and rabbit (Chapter 2). However, there has been no information in the literature regarding the regional localization of NGF or its mRNA. In order to better understand the recent discovery of cholinergic cells in the basal forebrain which respond to NGF (Gnahn et al., 1983; Hefti et al., 1984), a description of this regional localization was undertaken.

The regional distribution of NGF mRNA is consistent with the hypothesis that NGF may be acting as a target derived trophic factor for certain cholinergic neurons in the basal forebrain. The neurons of the septal nuclei and the nucleus of the diagonal band send a massive cholinergic projection to the hippocampus (Lewis and Shute, 1967), and the neurons of the nucleus basalis have a widespread projection to the cortex (Shute and Lewis, 1967; Lewis and Shute, 1967). These cholinergic neurons respond to NGF

by increasing choline acetyltransferase (CAT) activity (Gnahn et al., 1983; Hefti et al.,1984) and are also capable of retrogradely transporting exogenous NGF which is administered to their terminal field in either the hippocampus or cortex (Schwab et al., 1979; Seiler and Schwab, 1984). We have found that the hippocampus and cerebral cortex have especially high levels of NGF mRNA and so may be serving as an endogenous source of NGF for these neurons in vivo. The level of NGF mRNA in the hippocampus is about two-thirds of that found in the rat heart atrium, which has a dense sympathetic innervation (Chapter 2).

Indirect evidence that this may be occurring comes from lesion studies. Removal of the sensory and sympathetic innervation from the iris leads to an accumulation of NGF in the iris. After lesions of the cholinergic basal forebrain nuclei (CBFN) there is evidence suggestive of an accumulation of NGF in their targets, the hippocampus and the cortex. After such a lesion, sympathetic fibers innervating the vasculature sprout into the neuropil of the hippocampus and cortex (Loy et al, 1981; Crutcher, 1981). Also, a sympathetic ganglion transplanted into a cavity in the retrosplenial cortex sends processes into the adjacent hippocampus only if the cholinergic input to the hippocampus is removed (Gage et al, 1984; Bjorkland and Stenevi, 1981). This process outgrowth by sympathetic neurons is similar to that seen in the presence of NGF (Levi-Montalcini and Hamburger, 1954). It is possible that the sympathetic sprouting responses are manifestations of an accumulation of NGF in the hippocampus and cortex following removal of the input from the

CBFN. This would be similar to the accumulation of NGF seen in the iris after removal of the sympathetic and sensory innervation (Ebendal et al., 1980).

If NGF is acting as a target derived trophic molecule for the cells of the CBFN, it is seems to be playing a more limited role than in the PNS. NGF has major effects on the survival and process outgrowth of sensory and sympathetic neurons, but there are data which indicate that NGF has no effect on the survival or process outgrowth of CBFN neurons (Hefti et al., 1985; Gnahn et al., 1983). Thus, there is no difference in number of surviving neurons, number of neurons with processes, or number of cholinergic neurons in parallel cultures of CBFN neurons grown with and without added NGF and/or anti-NGF (Hefti et al., 1985). Also, although anti-NGF is capable of blocking the increase in CAT activity due to exogenous NGF, it has no effect on the basal level of CAT in the absence of added NGF (Hefti et al., 1985; Gnahn et al., 1983). This is an important finding, for one possible interpretation of this result is that endogenous NGF does not play a role in mediating the interactions of these cells in the brain, and the observed induction of CAT activity is not relevant _in vivo_. An alternative explanation could be that endogenous NGF does function in the regulation of CAT activity _in vivo_, but that the endogenous NGF can not be blocked by antibodies. It is possible that antibodies can not block the activity endogenous NGF because it is present in an extracellular compartment to which they do not have access.

Another reason that antibodies might not block an endogenous

"NGF" is that the endogenous "NGF" might be different from the protein isolated from the mouse salivary gland. NGF is known to go through a complex post-translational processing (Darling et al., 1983) and it is possible that this processing is different in brain. Any differently processed "NGF" would have to share some antigenic determinants with NGF or it would not have been detectable by ELISA (Korsching and Thoenen, 1984), but the binding of antibodies to the shared antigenic determinants may not block function. Clearly, submaxillary gland NGF must have some functional homology to this "brain NGF" or it would not affect the CNS neurons. It is also possible that there could be alternative splicing of NGF mRNA. The hybridizing RNA from brain does comigrate with salivary gland NGF mRNA, but this only shows that they are a similar size. It is possible that different splicing schemes could give rise to two different mRNAs of the same size. These mRNAs could share a region of identical sequence and so give rise to the hybridization signal observed. To determine why antibodies to NGF have no apparent effect on the brain in vivo, a more thorough analysis of the NGF antigen and mRNA which are found in the CNS will be necessary.

It has recently been demonstrated that sensory neurons are capable of retrogradely transporting NGF via their central processes in the spinal cord (Richardson and Riopelle, 1984; Johnson and Yip, 1985). It is interesting that the spinal cord does have low, but significant quantities of NGF mRNA, so it is presumably synthesizing NGF in vivo and may be serving as an endogenous source for the spinal ganglia. The NGF mRNA which is present in the pons and

medulla may supply the cranial ganglia, which are known to have NGF receptors (Raivich et al., 1985). The pons and medulla have much higher levels of NGF mRNA than the spinal cord, so it is also possible that these hindbrain areas are serving as a source of NGF for other populations of neurons than the cranial ganglia.

It is not known what cell types in the CNS contain the NGF mRNA detected. It has been reported that cultured astrocytes produce an NGF-like activity (Lindsay, 1979). Furthermore, the sympathetic sprouting caused by the cholinergic denervation of the hippocampus occurrs in the absence of either hippocampal pyramidal cells or granule cells (Peterson and Loy, 1983). This suggests that it may be non-neuronal cells which are the source of NGF mRNA.

The NGF mRNA which is present in the non-cortical areas of the brain does not correspond to the projection field of any neurons which are known to respond to NGF. Given the subtlety of the response to NGF in the neurons of the CBFN however, it is possible that there are other populations of responsive neurons in the CNS which remain unknown. The knowledge of the spatial localization of NGF mRNA in the brain may prove helpful in their discovery.

CHAPTER 4

CONTROL OF THE LEVEL OF NGF mRNA IN THE RAT IRIS BY DENERVATION AND BY GROWTH IN VITRO

INTRODUCTION

It is well established that there is an increase in the NGF content of the rat iris in response to sensory and/or sympathetic denervation *in vivo* (Ebendal et al., 1980; 1983) or culture *in vitro* (Ebendal et al., 1980; Barth et al., 1984). There are several mechanisms which might contribute to this increase. Perhaps most obviously, these treatments might cause an increase in the synthesis of NGF, thereby leading to higher levels in the tissue. An increase in synthesis might be caused by an increase in NGF gene expression, by increasing NGF mRNA stability, or by increasing the efficiency of translational or of post-translational processing. Alternatively, the NGF content of a tissue may be increased by decreasing the degradation or removal of NGF. A combination of these mechanisms might also cause the increased levels of NGF after denervation or explant.

Using the previously developed assay for detecting NGF mRNA, rat irises were assayed after denervation *in situ* and explanting into culture. There is no change in NGF mRNA levels after denervation *in vivo*, suggesting that some other mechanism(s) must be important in the observed increase. However, irises cultured *in vitro* do show a rapid, large increase in NGF mRNA.

MATERIALS AND METHODS

Materials

Sprague Dawley rats were obtained from Bantin-Kingman and New Zealand white rabbits were obtained from Nitabell. Enzymes were purchased from New England Biolabs, alpha ^{32}P-dCTP (3000 Ci/mmole) was from Amersham, and all other chemicals were reagent grade or the best commercially available.

Denervation Procedures

Rats were anesthetised with ketamine HCl (100 mg/kg, IM) and pentobarbital (40 mg/kg, IP). Sympathetic denervation was performed by the unilateral removal of the superior cervical ganglion (SCG) after a midline incision. Combined sensory and sympathetic denervation was achieved by the intracranial electrocoagulation of the ophthalmic branch of the trigeminal nerve under visual control as described (Ueda et al., 1982). Eyelids were sutured after this procedure to avoid corneal erosion. Complete retrobulbar denervation was accomplished by exposing the retroorbital region (Malmfors and Nilsson, 1964). The ciliary ganglion was removed and the long and short ciliary nerves and the optic nerve were cut. In order to chemically sympathectomize animals, they were injected with 6-hydroxydopamine (6-OHDA)(200 mg/kg, IP) which was dissolved in normal saline with 0.1% ascorbate immediately before use.

Anterior Chamber Manipulations

Rats were anaesthetized as above, and a tracheotomy was performed to aid respiration. Anaesthesia was maintained with pentobarbital for the six hour duration of the experiment. For flushing the iris with culture fluid, two cannulae were implanted into the anterior chamber,

with their openings at opposite sides of the chamber to provide a flow across the iris from the inflow cannula to the exit cannula. The cannulae were made of PE10 polyetylene tubing which had been trimmed to a bevel. In order to avoid increases in intraocular pressure during implantation, the cornea was first pierced with a 25 ga needle, which was then removed. The cannula was then inserted into the hole left by the needle, taking care not to injure the iris. After the two cannulae were implanted from opposite sides of the eye, the flow of culture medium was started at a rate of 200 ul/hr with a syringe pump. The free end of the exit cannula was kept 15cm above the eye to maintain normal intraocular pressure. Sham animals had two cannulae implanted which were sealed approximately one cm from the inserted end. These animals were also kept under anaesthesia for six hours and had a tracheotomy performed.

For anterior chamber injections, a solution of drug or vehicle (saline) was diluted 1:1 with a 4% solution of low melting temperature agarose in saline at 40°C. 0.2ul of this solution was drawn into a 25 ga needle which was attached to a microliter syringe. After a few moments at room temperature to allow the agarose to solidify, the needle was inserted into a previously pierced hole in the cornea of an anaesthetized rat and the agarose plug expelled with gentle pressure. The needle was then withdrawn, leaving the plug in the anterior chamber. Tetrodotoxin (TTX) dose was 1 ug per eye.

Organ Culture

Rat irises with attached ciliary bodies were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose and no pyruvate (DME-H21)

in 8% CO_2, 92% air at 37°C with additives as described in individual experiments. 20mM Na-Hepes (pH 7.4) was added in some experiments.

NGF mRNA ASSAY

RNA was prepared from irises by a modification of the method of Cheley and Anderson (1984). Individual irises were homogenized in 200 ul of 7.6 M GuHCl, 0.1 M KAcetate pH 5.0, 0.01% blue dextran by repeated trituration through a 23 ga needle. RNA was precipitated by addition of 120 ul of ethanol and storage at -20°C for at least twelve hours. RNA was pelleted by centrifugation for ten minutes at 13,000Xg, and the resultant pellet was washed twice with 70% ethanol. The pellet was then resuspended directly in sample buffer for formaldehyde gels (Lehrach et al., 1977) and heated to 70°C for ten minutes. These samples were then separated on 1.2% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized to ^{32}P-labelled single-stranded cDNA probe for mouse NGF as described earlier (Chapter 2). The amount of NGF mRNA was then determined by densitometry of autoradiograms of the nitrocellulose transfers and comparison to standard curves of male mouse submaxillary gland poly A^+ RNA and cloned cDNA which were always run on each gel.

RESULTS

Denervation in vivo

In order to determine if the NGF mRNA content of the rat iris increased in response to denervation, the sympathetic innervation to the iris was interrupted by surgically removing the ipsilateral superior cervical ganglion. This procedure always led to immediate ptosis on the operated side. As shown in Figure 12, analysis of these irises from one to fourteen days after surgery revealed no significant change in NGF mRNA content compared to those from the control side. There is an increase in the NGF content of irises ten days after a sympathetic denervation (Ebendal et al., 1980).

Sympathetic denervation was also achieved by the use of 6-OHDA. Animals injected with 200 mg/kg developed piloerection and extreme exophthalmia within five minutes. By the time of sacrifice at eighteen hours post-injection, there was pronounced bilateral ptosis. The NGF mRNA content of these irises was not significantly increased from age-matched controls (Figure 13). In fact there was a decrease, which, although slight, was statistically significant. ($p=0.02$, Mann-Whitney U test). This is in contrast to the approximately two-fold increase in NGF antigen seen eighteen hours after 6-OHDA treatment (Korshing and Thoenen, 1985).

After intracranial electrocoagulation of the ophthalmic branch of the trigeminal nerve, a complete severance of all nerves leaving the anterior lacerated foramen was observed postmortem in all animals at all postoperative times studied. Immediately before sacrifice, absence of the corneal blink reflex on the operated side was verified in all animals used. As shown in Figure 14, there was no significant

FIGURE 12

NGF mRNA content of irises after surgical sympathetic denervation.

The content of NGF mRNA in individual irises at various times after removal of the superior cervical ganglion. Bars are the means of the three individual determinations shown by the dots. C is the iris from the control, unoperated side of the animal. L is the iris which has had its sympathetic ganglion removed. No statistically significant differences between control and lesion side by the Mann-Whitney U test.

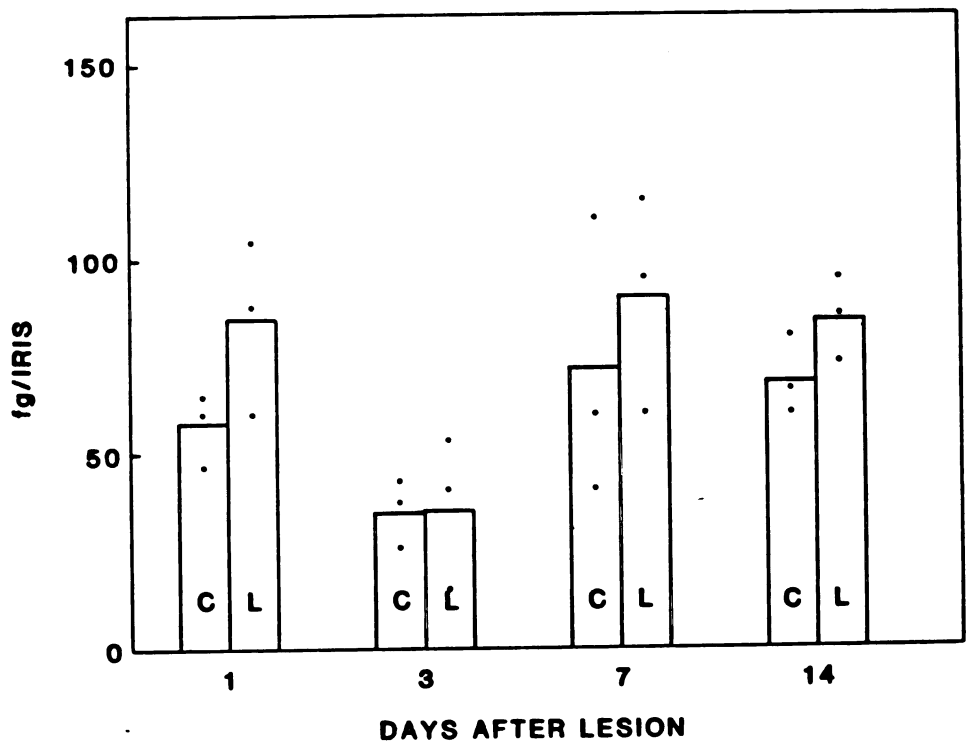

150
100
50
0
fg/IRIS
C
L
C
L
C
L
C
L
1
3
7
14
DAYS AFTER LESION

FIGURE 13

NGF mRNA content of irises after chemical sympathetic denervation

The content of NGF mRNA in irises from animals treated with 200mg/kg 6-OHDA (6OHDA) or vehicle alone (CON) eighteen hours before. Bars represent the means of the six determinations shown by the individual points. There is a significant decrease in the content after treatment with 6-OHDA ($p=0.02$, Mann-Whitney U test).

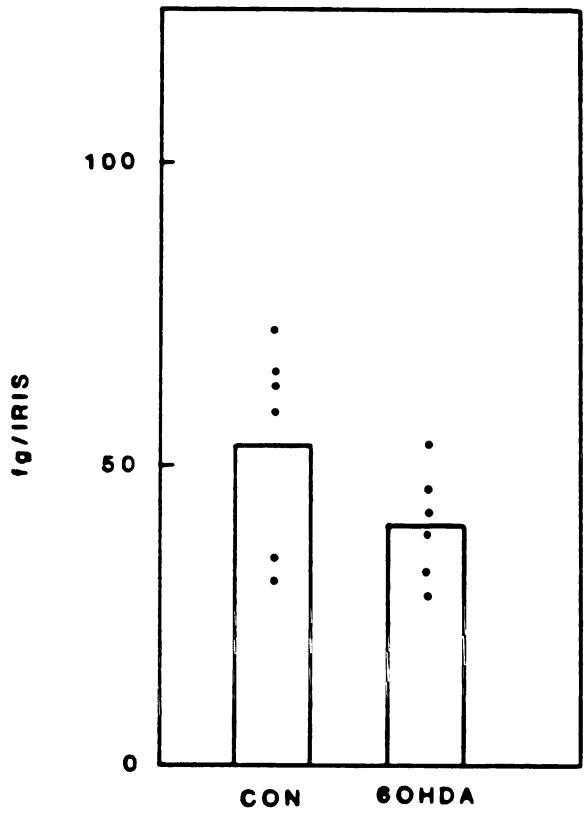
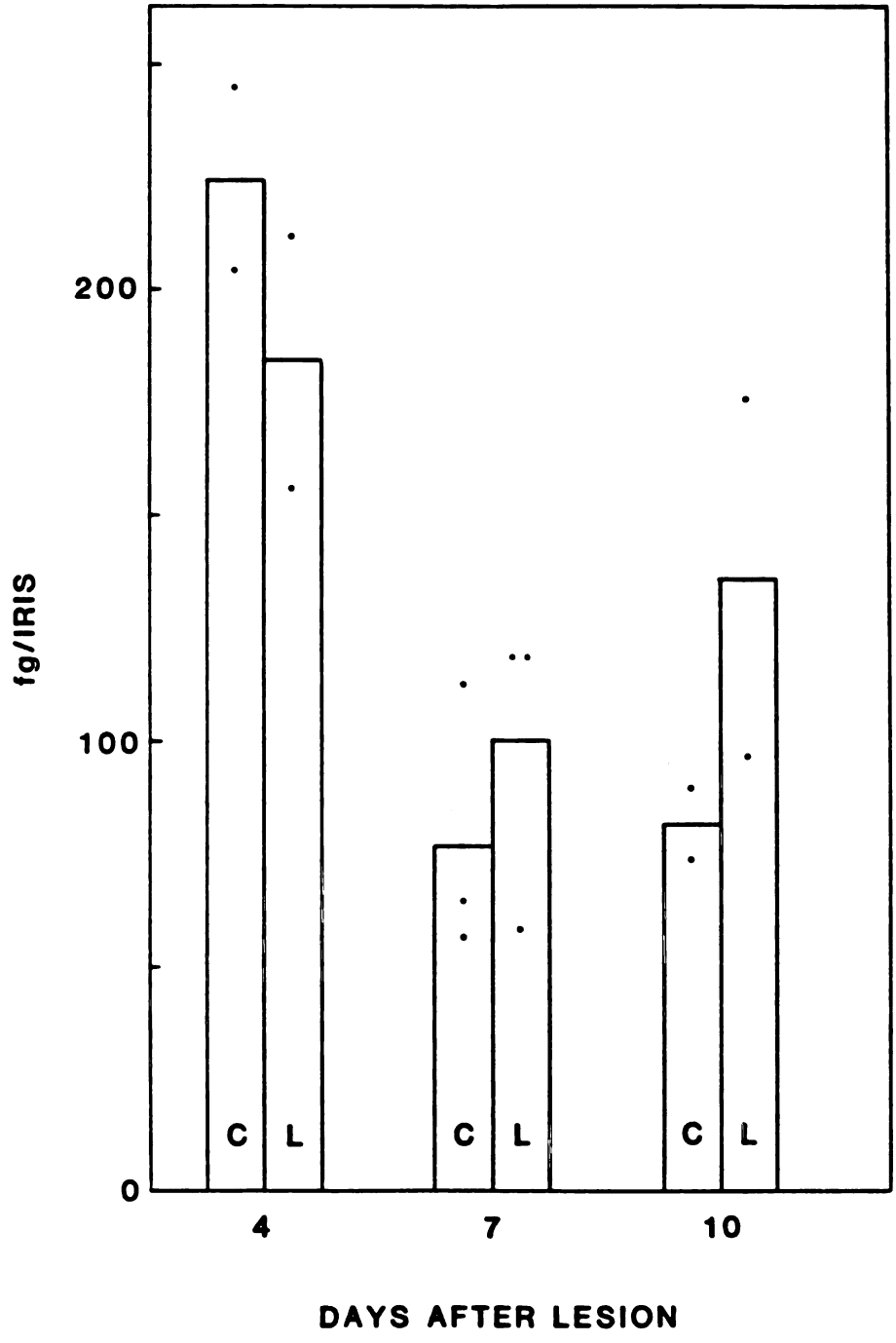

100
50
0
fg/IRIS
CON
6OHDA

FIGURE 14

Content of NGF mRNA in irises after sympathetic and sensory denervation.

The content of NGF mRNA in individual irises at various times after electrocoagulation of the ophthalmic branch of the trigeminal nerve. Bars are the means of the individual determination shown by the dots. C is the iris from the control, unoperated side of the animal. L is the iris which had the combined denervation. No statistically significant differences between the control and lesion sides by the Mann-Whitney U test.


200
100
0
fg/IRIS
C
L
C
L
C
L
4
7
10
DAYS AFTER LESION

difference in NGF mRNA content between irises from operated and control sides at between four and ten days after surgery. Increases in the NGF content are seen in irises ten days after this operation (Ebendal et al., 1980; 1983). Irises from both sides had elevated levels of NGF mRNA compared to unoperated controls at four days after surgery (compare to zero time in figure 16)

After the retrobulbar section of the optic nerve and the long and short ciliary nerves, there was an extreme response on the operated side. The anterior chamber became cloudy, and the cornea began to ulcerate. There was a somewhat variable amount of bleeding into the anterior and posterior chambers and into the vitreous humor. By approximately one week after surgery, the cornea became vascularized and the intraocular bleeding was severe. The corneal blink reflex remained absent at all times studied, but a blink reflex could usually be elicited by brushing of the eyelash on the operated side. There was a significant increase in NGF mRNA content of the iris on the operated side between thirty-six hours and ten days after this treatment (figure 15)($p=0.05$ Mann-Whitney U test. control vs operated at each time). The increase was variable, but averaged about five fold at each post-operative time examined.

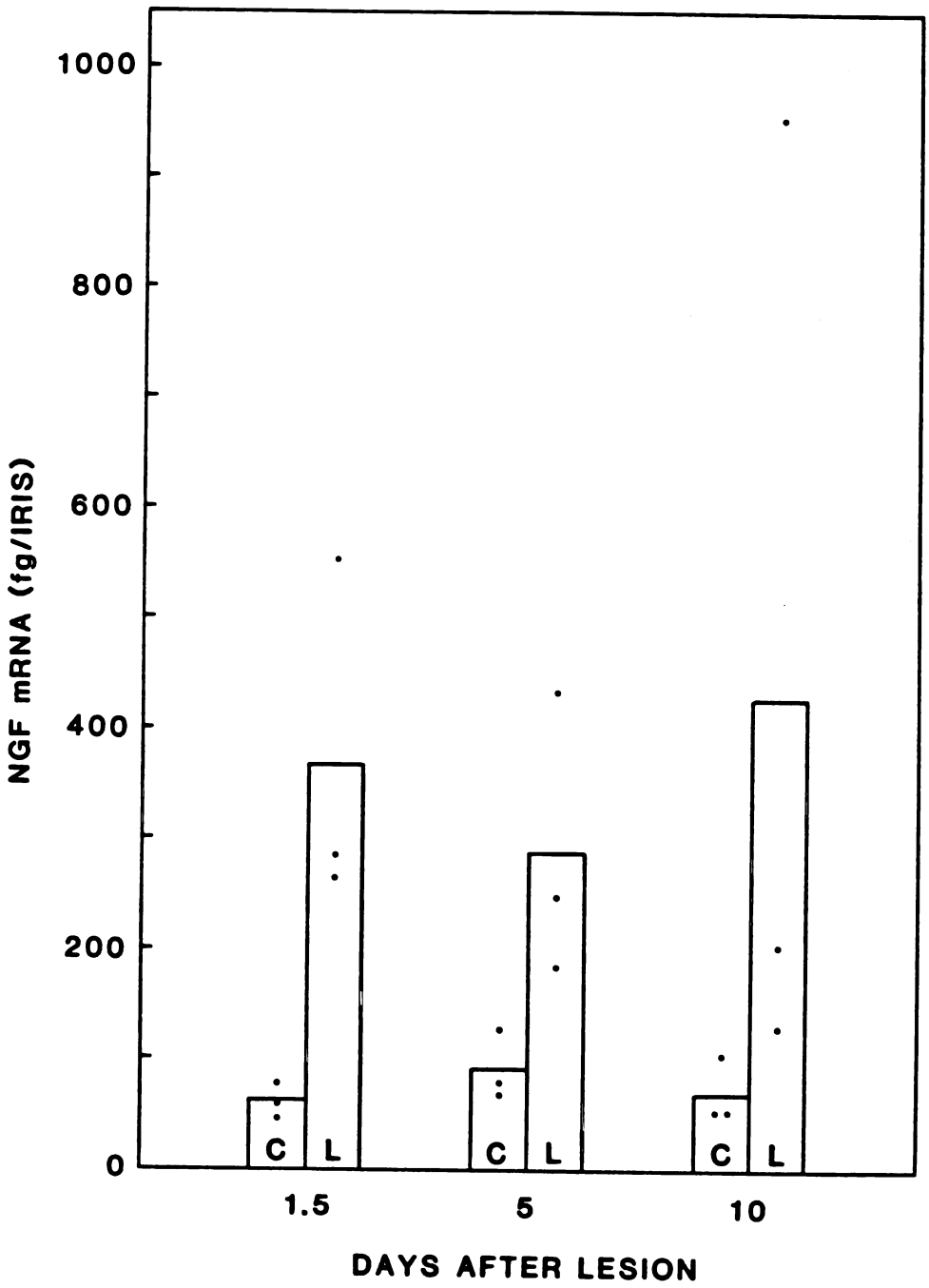
Irises placed in vitro

When rat irises were cultured under the conditions described, they maintained a normal gross morphology for at least three days. They tended not to adhere to the culture dish except occasionally at a small, limited area of contact. When this occurred, there were cells which migrated out of the explant and onto the surface of the culture dish. If irises were dark adapted for several hours in the incubator

FIGURE 15

Content of NGF mRNA in irises after complete retrobulbar denervation.

The content of NGF mRNA in individual irises at various times after the complete severance of all nerves leading to the eye. Bars are the means of the individual determination shown by the dots. C is the iris from the control, unoperated side of the animal. L is the iris which was denervated. There is a statistically significant increase in NGF mRNA in the operated iris at all times after surgery ($p=0.05$ Mann-Whitney U test).


1000
800
600
400
200
0
NGF mRNA (fg/IRIS)
C
L
C
L
C
L
1.5
5
10
DAYS AFTER LESION

and then exposed to bright light, there was a vigorous contraction. Since the irises were irregularly folded, it was not possible to determine whether this was due to a contraction of the sphincter, dilator or both. Irises which were killed by freezing did not display this behavior.

When RNA from these irises was analyzed with Northern blots, a large, rapid increase in NGF mRNA was observed. The data from one such experiment is shown in figure 16 and the corresponding gel is shown in figure 17. The increase was readily detectable after one hour in culture (five-fold in the experiment shown in figure 16) and by six to twelve hours reached a maximum increase which varied in different experiments from six to twenty fold. The increase in NGF mRNA appeared to start at the time of explantation, with no observable lag. The maximum level was maintained until twenty-four hours, after which there was a slow decline in levels of NGF mRNA. Even at the longest times studied (72 hours) the level of NGF mRNA was still much higher than in a freshly dissected iris(fifteen-fold in the experiment shown in figure 16). Experiments done in parallel have shown that the total RNA content of an iris falls to forty to fifty percent of a freshly dissected iris over one to two days *in vitro* (data not shown). As shown in figure 16, the 1.7 kb band of hybridizing RNA increases and decreases in parallel with the 1.3 kb NGF mRNA.

In an attempt to define what might be causing the induction of NGF mRNA observed after placing irises *in vitro*, a variety of different culture conditions and additives were surveyed. The induction occurred in DME-H21 with 10% horse, calf, or rat serum

FIGURE 16

Content of NGF mRNA in irises cultured in vitro.

The amount of hybridizing RNA migrating with the indicated size in individual irises after various times in culture. Irises were harvested after 0, 1, 3, 6, 12, 24, 48, and 72 hours in culture. Lines are drawn through the means of the three indvidual determinations indicated by points. Points represent RNA of the following size; (●) 1.3 kb. (▲) 1.7 kb. Individual points for the 1,7 kb RNA at zero time have been omitted for clarity. The increase above the content at time zero is statistically significant at all times after explant for each band. ($p=0.05$, Mann-Whitney U test).

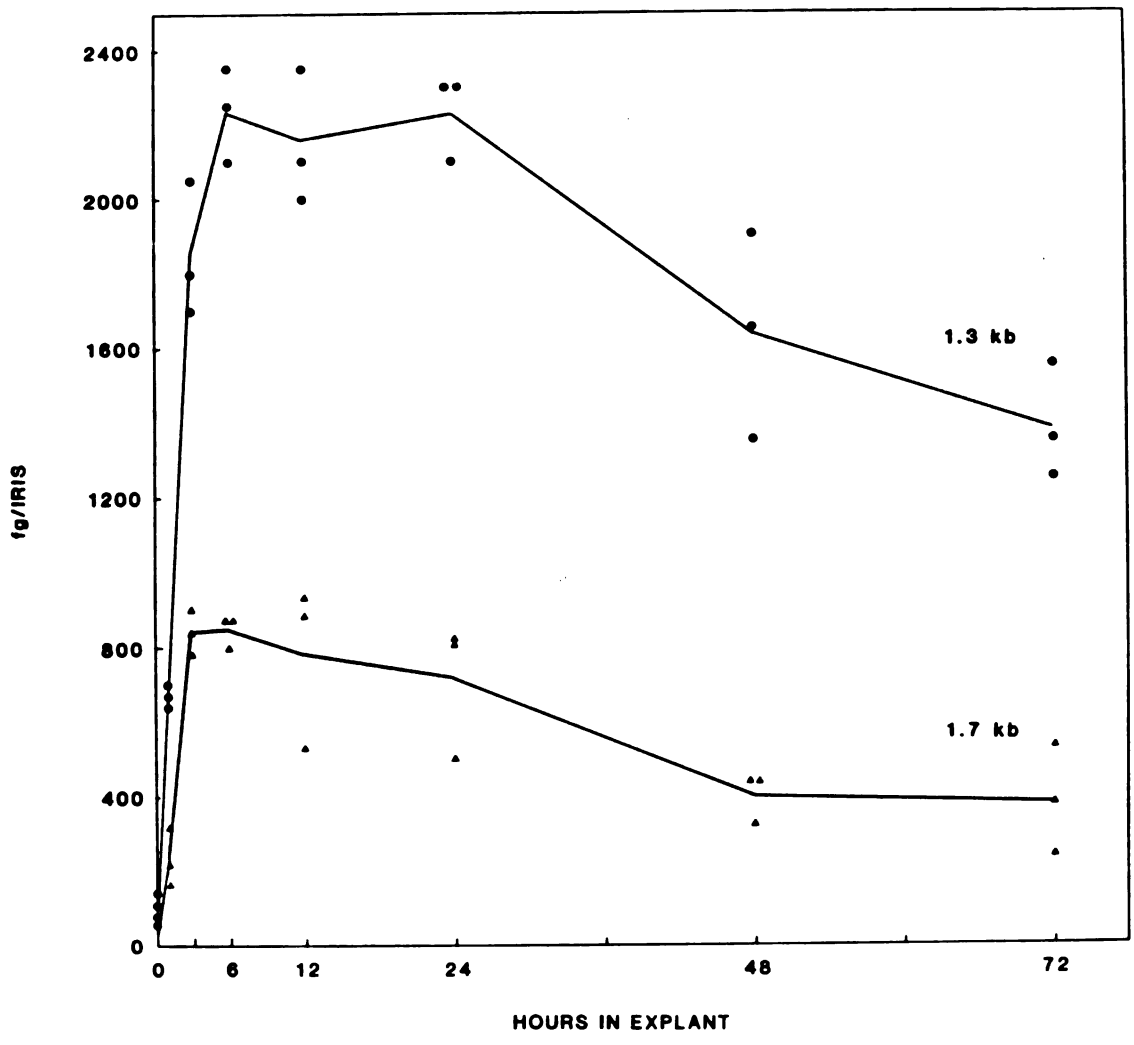
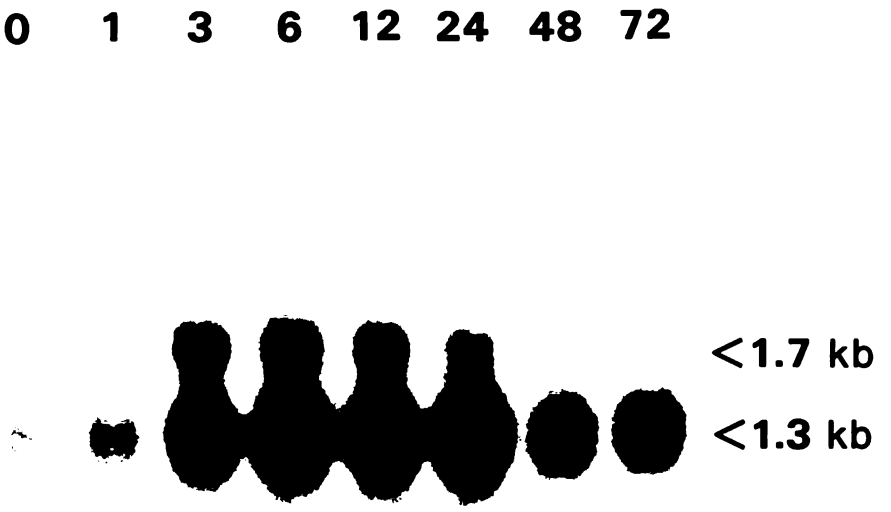

2400
2000
1600
1200
800
400
0
fg/IRIS
1.3 kb
1.7 kb
0
6
12
24
48
72
HOURS IN EXPLANT

FIGURE 17

Autoradiogram of RNA blot hybridization of iris RNA after explant.

Autoradiogram of RNA transfer after hybridization ^{32}PDNA single-strande probe. Each lane contains the RNA from a single iris after various times *in vitro*. Lanes from left to right contain RNA from an iris: zero, one hour, three hours, six hours, twelve hours, twenty-four hours, two days, or three days after explant. Main hybridizing band is 1.3kb. In darker lanes notice the existence of band at slightly higher molecular weight (1.7kb).


0 1 3 6 12 24 48 72
<1.7 kb
<1.3 kb

or in DME-H21 which was completely serum free (Figure 18). Addition of NGF at lug/ml or rabbit antiserum to NGF at 10% or ascorbate at 2.8 mM did not change the *in vitro* induction (data not shown).

Because of preliminary results which indicated that aqueous humor could influence the level of NGF mRNA when added to cultures, an experiment was performed to ascertain the effect of aqueous humor *in vivo*. In order to test this, two cannulae were placed into the anterior chamber and used to continuously wash the iris with DME-H21 *in vivo*. During the implantation of these polyethylene cannulae, most of the aqueous humor initially present in the anterior chamber was lost, as judged by leakage and the decrease in volume of the anterior chamber. This loss occurred both in the experimental case and when the plugged cannulae used for the sham experiments were implanted. The cornea quickly resealed around the polyethylene tubing, and the anterior chamber appeared to regain its original volume within thirty to sixty minutes. After six hours of continuously flushing the anterior chamber, the iris was removed and assayed for content of NGF mRNA. As seen in Figure 19, irises which were treated in this way had an increase of five- to seven-fold compared to the control side. This was similar to that seen after six hours *in vitro* (compare with figure 18). Irises from sham animals, which had cannulae implanted but were not perfused, showed a similar increase compared to their control side. Increases in both perfused and sham animals were statisically significant ($p=0.05$, Mann-Whitney U test).

In order to test the effect of complete blockage of Na^+-dependent action potentials in the iris, TTX was injected into the anterior chamber. To avoid losing all of the injected TTX in the outflow

FIGURE 18

Effect of various culture media on induction of NGF mRNA in vitro.

Left side of figure shows time course of increase in NGF mRNA content of irises cultured in DME H-21 with no serum or other additives. Right side of figure show the increase obtained in parallel cultures after ten hours growth in DME H-21 with serum from the indicated species added to ten percent. Bars show the means of two determinations. Vertical lines indicate the range.

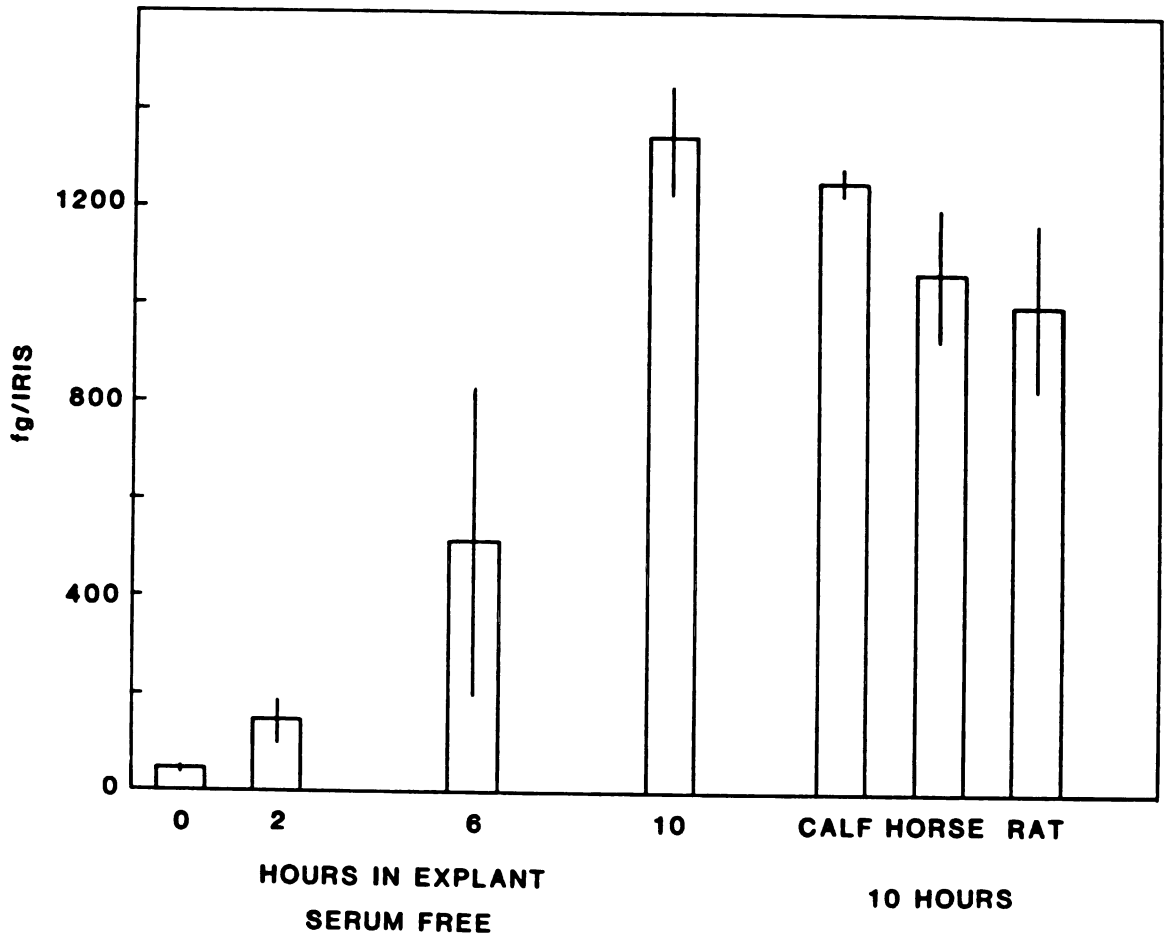
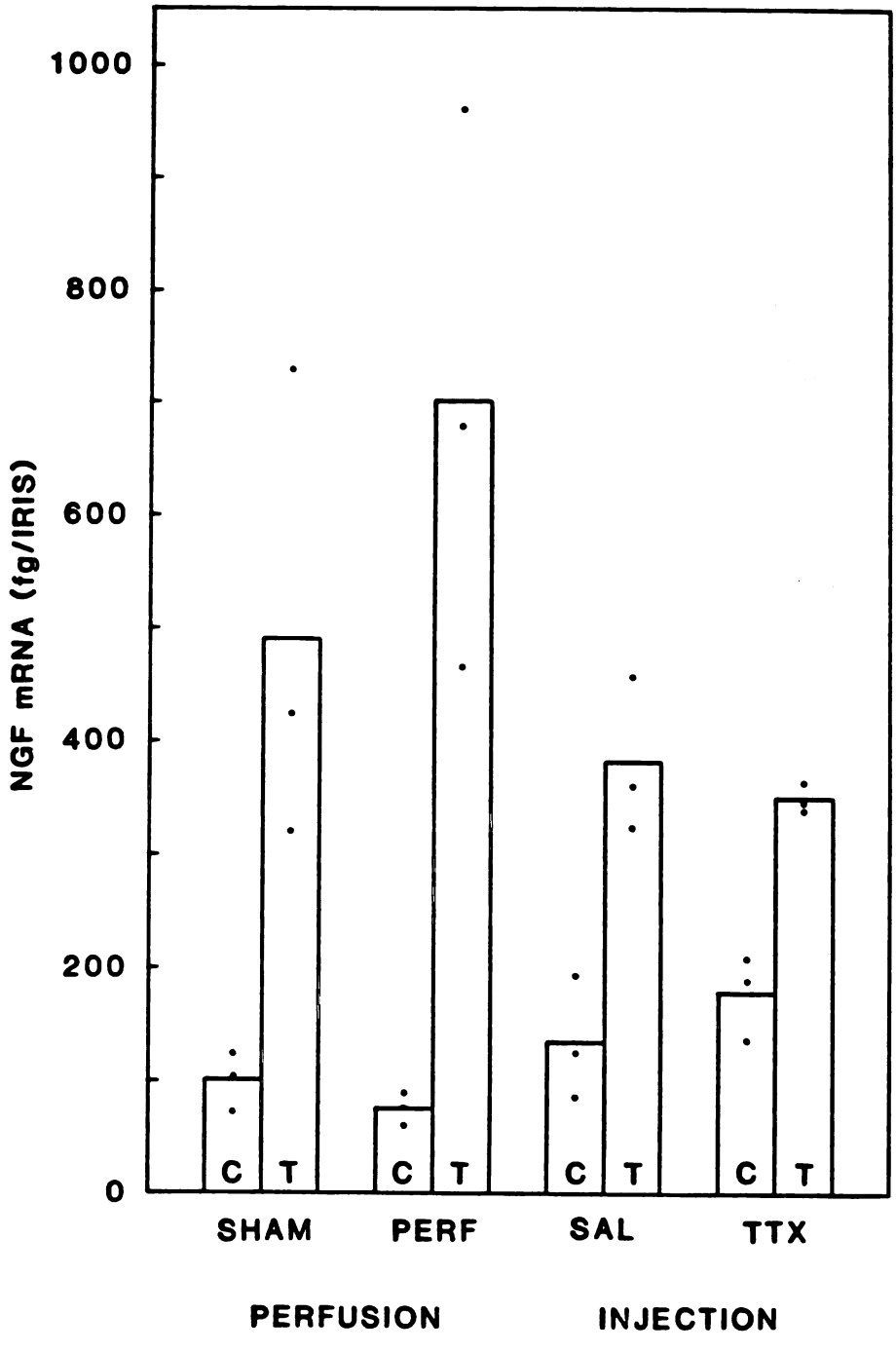

fg/IRIS
1200
800
400
0
0
2
6
10
CALF HORSE RAT
HOURS IN EXPLANT
SERUM FREE
10 HOURS

FIGURE 19

Content of NGF mRNA in irises subjected to anterior chamber insult.

The content of NGF mRNA was determined in irises removed from eyes which were subjected to either cannulae implantation (PERFUSION) or injection (INJECTION) six hours previously. Anterior chambers implanted with cannulae were either continuously perfused with DME H-21 (PERF) or not (SHAM). Anterior chambers were injected with 0.9% saline (SAL) or saline containing 1 ug tetrodotoxin (TTX). In all cases, the contralateral, untreated eye served as a control (C) for the eye which had been subjected to trauma (T). All treated irises have a statisically significant increase over the control ($p=0.05$, Mann-Whitney U test)


1000
800
600
400
200
0
NGF mRNA (fg/IRIS)
C
T
C
T
C
T
C
T
SHAM
PERF
SAL
TTX
PERFUSION
INJECTION

which followed needle withdrawal, the TTX was delivered in a plug of solidified low melting temperature agarose. Injection with TTX resulted in immediate relaxation of the iris, which lasted the six hours until sacrifice. As seen in figure 19, there was a three- to four-fold increase in NGF mRNA in the treated iris compared to the control side. In this case also, however, sham animals, which received injections of saline in agarose, showed an equivalent increase when compared to the untreated contralateral side. Both these increases were significant ($p=0.05$, Mann-Whitney U test). The NGF mRNA content of the control irises in this experiment, though high, are not outside the range seen in untreated animals.

DISCUSSION

The results in this chapter show that denervation *in situ* of the rat iris does not increase the synthesis of NGF by increasing the level of NGF mRNA. Further, we demonstrate that placing the iris into culture medium does cause an increase in NGF mRNA, and that this response is not due to denervation.

In vivo denervation

Each of the denervation techniques used has been shown in other studies to increase the content of NGF in the iris. Surgically denervated irises have been examined using an NGF bioassay, and although the bioassays used are only semi-quantitative, the increase in content appears to be at least an order of magnitude (Ebendal et al., 1980). Irises from animals treated with 6-OHDA have been shown to undergo a two to four fold increase in their content of NGF antigen with a two-site ELISA (Korsching and Thoenen, 1985).

Since we find no increase in NGF mRNA after denervation, the observed increase in content must be due to some other factor. We felt it important to be certain of the efficacy of our denervation procedures. The surgical procedure for removing the sympathetic innervation is very simple and straightforward, as is the injection of 6-OHDA. The trigeminal nerve lesion, although more difficult, can be done under direct visual observation, and the ablation can be examined carefully after sacrifice. All of the procedures have easily visible physiological symptoms, which were verified in each animal, confirming the lesion.

Although there is no increase in NGF mRNA, it is possible

that there is an increase in synthesis of NGF, but that the regulation is occurring at the level of translational efficiency. Alternatively, there might be an increase in synthesis which reflects an increase in the efficiency of the processing of the initial translation product. It is also possible that there is no increase in the synthesis rate of NGF and that the increase in content after denervation is due to a decrease in breakdown. These hypotheses can not be ruled out without further experimentation.

All of the above hypotheses involve the assumption that the observed change in NGF content is due to a change in some metabolic process of the iris in response to denervation. It is known that denervation can induce such changes in the iris, for example, receptor supersensitivity (Bourgon et al., 1978). However, it is possible that the change in NGF content is not due to denervation caused changes in the target tissue, but instead is a direct consequence of the loss of the nerve plexus. Loss of the innervation of the iris obviously removes the neuronal retrograde transport system, which is known to take up and remove NGF with high affinity and capacity (Dumas et al., 1979).

For the removal of the retrograde transport system alone to cause an increase in the content of NGF, the transport system must be important in establishing the level of NGF in the normal target. If this is the case, at least a portion of the NGF in a tissue must be extracellular, so that it would be accessible to the nerve. There is some evidence that some NGF is extracellular in normal tissue, as Korsching and Thoenen (1983) have noted that

brief saline rinses of tissue, and especially the iris, lead to a loss of NGF. It is not difficult to imagine that NGF, a highly basic and notoriously 'sticky' protein, in the presence of the predominantly acidic extracellular matrix, could achieve a relatively high extracellular concentration. In fact, it has recently been demonstrated that NGF can be stably adsorbed onto anionic substrates for at least five days in vitro and is capable of promoting neurite outgrowth when present only in this adsorbed form (Gunderson, 1985). Thus, at this time, it seems that removal of the retrograde transport system can explain the build up of NGF after denervation without postulating denervation induced changes in the metabolic processes of the iris tissue. Consistent with this hypothesis, it has recently been shown that treatment with cochicine leads to an increase in the NGF content of the iris (Korsching and Thoenen, 1985a). Further, this explanation predicts that the increase in NGF seen after denervation will be extracellular.

Irises cultured in vitro

Although we have found that denervation in vivo does not cause any change in the level of NGF mRNA, culturing the iris in vitro does cause a rapid and large increase in the level of specific message. This increase does not seem to require any special culture medium or serum requirements (Figure 18). There is no discernable lag in its initiation (Figure 16) and it does not reflect a general increase in total message level of the tissue. While it is clear that an iris cultured in vitro has undergone a complete denervation, the results obtained in vivo (Figures 12-14) argue that the observed

change in NGF mRNA level are not due solely to this denervation. Further, the difference in response to denervation *in vivo* and growth *in vitro* raises serious questions about the utility of the explant procedure as a model for denervation.

We also show that the large increase observed upon explant can be mimicked in the fully innervated iris *in situ*. As a result of a variety of insults to the anterior chamber, a large increase in NGF mRNA is induced (Figure 19). This shows that denervation is not a necessary prerequisite for the induction of NGF mRNA.

The mechanism of this injury induced increase is not known. Although care was taken not to injure the iris, it is possible that this is due to some type of wound response, either because of undetectable damage to the iris itself or because of the injury to the cornea. Alternatively, the induction might be caused by the release and subsequent rapid replacement of the aqueous humor. Whatever the cause of the increase seen after these implantations, it may help to explain the sympathetic hyperinnervation of the iris which has been reported to occur after transplantation of tissue into the anterior chamber (Olson and Malmfors, 1970). This transient effect does not depend on the type of tissue transplanted.

Retrobulbar lesions

The only denervation procedure which resulted in a significant increase in NGF mRNA was the complete retrobulbar section of all nerves entering the eye. This caused a major traumatic response in the eye, similar to that described after lesions of the trigeminal nerve (Moses and Feldman, 1969). In our hands, the response following

the retrobulbar procedure was always much greater than any observed after the trigeminal lesions. Although it is possible that the increase in NGF mRNA was due to the denervation per se, this explanation seems unlikely. It seems more probable that the trauma following the surgical procedure led to the increase in NGF mRNA. The much less damaging procedures of cannula placement or anterior chamber injection cause a large, reproducible increase in NGF mRNA without denervation. Furthermore, the other denervation procedures used, albeit giving less extensive denervations, give no increase in NGF mRNA. The retrobulbar procedure does remove input to the eye that is not removed by the sympathetic and sensory denervation procedures. Specifically, the parasympathetic innervation of the iris from the ciliary ganglion is eliminated. It is possible that other, unknown projections are also differentially affected by the different procedures and their removal contributes to the observed increase in NGF mRNA. Whatever the mechanism behind this increase, the increase in NGF content observed after chemical (Korsching and Thoenen, 1985) or surgical (Ebendal et al., 1980; 1983) sympathetic and sensory denervation of the iris is not due to an increase in the level of NGF mRNA.

CHAPTER 5

SUMMARY

SUMMARY

NGF is clearly established as a target derived trophic factor for the sympathetic and sensory nervous systems. Despite this knowledge, many gaps exist in our present understanding of the cellular interactions which are mediated by NGF. The results presented here narrow some of the gaps but also raise new questions concerning the functions of NGF.

There was overwhelming evidence that sympathetic and sensory neurons obtained NGF from their peripheral fields of innervation even before the technical innovation of a two-site ELISA (Korsching and Thoenen, 1983) allowed the detection of NGF in these peripheral target organs. When a developing sympathetic or sensory neuron is deprived of contact with its peripheral target, the naturally occurring cell death is greatly augmented (Hamburger and Yip, 1984; Hendry, 1975), and when extra tissue is supplied, cell death is decreased (Hamburger, 1958). Treatment with anti-NGF antibodies mimicks the effect of removing the periphery, and treatment with NGF gives similar results as increasing the periphery (Levi-Montalcini and Booker, 1966a; 1966b). Furthermore, treatment with NGF can block the increase in cell death caused by removal of peripheral tissue (Hamburger and Yip, 1984; Hendry, 1975). By far the most parsimonious explanation of these results is that NGF is the substance which mediates the effect of the periphery on neuronal survival. The more recent findings that NGF is present in sympathetic target organs and that endogenous NGF is retrogradely transported *in vivo* (Thoenen et al., 1983) leave little doubt that sympathetic

neurons obtain a supply of NGF from their peripheral field, including their target organs, and perhaps the nerves. Logically, one of the next questions to arise concerns how the peripheral target tissues obtain the NGF which they supply to the neurons. It might be synthesized locally, or be accumulated from a systemic source. One prerequisite for the synthesis of NGF is the presence of NGF mRNA. In an attempt to determine if sympathetic target organs are capable of NGF production or to find a possible central source, many tissues were assayed to determine their content of NGF mRNA.

The results of this study clearly show that sympathetic targets do contain NGF mRNA. The content of NGF mRNA bears a striking positive relationship to the density of symapthetic innervation. Furthermore, there appears to be enough NGF mRNA in peripheral tissues to account for their entire content of NGF. In fact, NGF mRNA is thirty-fold higher as a percentage of total mRNA than NGF is as a percentage of total protein (Chapter 2). This is consistent with NGF being a secreted protein with rapid turnover. The finding that there is a direct relationship between one measure of a tissue's ability to synthesize NGF and the density of the sympathetic innervation of that tissue suggests that the production of NGF might be controlling the innervation density. It is known from transplantation studies that there is something which limits the amount of sympathetic innervation a tissue such as iris can support (Olson and Malmfors, 1970). Similar experiments examining apparent competition between the sensory and sympathetic fibers of the iris found results which suggest that NGF may be the factor limiting innervation density

(Kessler et al., 1983). There is also direct evidence that sympathetic and sensory fibers compete for NGF in the periphery (Korsching and Thoenen, 1985b). The finding that there is a correlation between the innervation density and NGF mRNA level of a tissue is certainly consistent with the idea that NGF production at least partially controls the extent of innervation. However, other factors are also known to be involved (Black and Mytilineou, 1976).

There were several unexpected findings in this work. Perhaps most surprisingly, NGF mRNA was present in peripheral nerves and ganglia. It remains unknown which cells in the ganglia and nerve contain the NGF mRNA, although there is evidence which suggests that the nonneuronal cells from chick dorsal root ganglia synthesize an NGF-like activity in vitro (Varon et al., 1974). Since the mass of tissue which a given ganglion innervates is much larger than the mass of the ganglion itself, the presence of the NGF mRNA in the ganglion does not necessarily suggest that the NGF which might be produced by the ganglion is quantitatively important. As dicussed, there is evidence which shows that neurons obtain NGF from their targets, not locally. However, local production may be partially responsible for the ability of adult sympathetic neurons to survive treatments which interrupt their connection to the periphery.

The knowledge that target organs synthesize the NGF which they supply to neurons allows one to investigate the control of that synthesis. Sensory or sympathetic denervation of the iris leads to a large increase in the NGF content of this tissue. Culturing the iris as an explant also gives rise to an accumulation of NGF

(Ebendal et al., 1980; Barth et al., 1984). Irises were analyzed for their content of NGF mRNA after *in vivo* denervation or growth *in vitro* to determine if an increase in mRNA could account for the observed increase in NGF content following these procedures. There was no change seen in the NGF mRNA content observed after denervation. Thus the increase in NGF content observed after denervation must be due to some other mechanism. There could be an increase in synthetic rate regulated by translation or post-tanslational processing. There might not be any change in the rate of production of NGF, for a decrease in the rate of breakdown or removal of NGF will also lead to an increase in content. The loss of the neuronal retrograde transport system after denervation might be responsible for the accumulation seen in the iris.

In contrast to the result after denervation *in vivo*, there was a large, rapid increase in NGF mRNA in the rat iris *in vitro*. This increase could be up to twenty-fold, and was maximal by six to twelve hours after explant. The level of NGF mRNA was also found to rapidly increase in response to minor trauma of the anterior chamber. The mechanism or mechanisms which underly the increase in NGF mRNA following these two treatments are totally unknown. These observations do provide two easy, quick and reproducible techniques for altering the content of NGF mRNA of the iris, and so may be useful in gaining a further understanding of the ways in which NGF expression is controlled *in vivo*.

The discovery of relatively high levels of NGF mRNA in the brain adds to the growing body of evidence which indicates that NGF

may play a functional role in the CNS as well as the PNS. The neurons of the cholinergic basal forebrain nuclei (CBFN) project to the entire cortical mantle and hippocampus (Shute and Lewis, 1967; Lewis and Shute, 1967) and respond to exogenous NGF with an increase in the activity of choline acetyltransferase (CAT) (Gnahn et al., 1984; Hefti et al., 1985). These neurons can retrogradely transport ^{125}I NGF which is injected into their terminal field in the cortex or hippocampus (Schwab et al., 1979; Seiler and Schwab, 1984). This suggests that NGF may be acting as a target derived trophic factor for the CBFN neurons of the CNS. The regional localization of NGF mRNA determined in a survey of various areas of the rat CNS supports this hypothesis. While NGF mRNA was detectable in every region of the CNS, the highest concentrations of NGF mRNA were found in the targets of these cholinergic cells, the hippocampus and cerebral cortex. The hippocampus and cerebral cortex, then, seem to be capable of supplying NGF to the terminals of the CBFN cells. The NGF could then be retrogradely transported by these cells and cause its physiological effects.

There is a problem with this model, however, in that treatment with antibodies to NGF causes no change in the level of CAT activity in the CBFN (Hefti et al., 1985; Gnahn et al., 1984). This might mean that there is no endogenous NGF to be blocked in vivo and so there is no effect. Alternatively, there could be problems with the endogenous NGF being inaccessible to antibodies so that blockage did not occur. It is also possible that the NGF in the brain is a slightly different molecule than salivary gland NGF, due to differences in mRNA splicing or post-translational processing. This might give a

"brain NGF" which shared some functional similarity to NGF and some antigenic determinants with NGF, but its function might not be blocked with antibodies to NGF. Further characterization of the NGF-like molecule in the brain may explain this apparent discrepancy.

There are a number of regions in the brain (medulla, pons, and diencephalon) which do not correspond to the terminal fields of neurons which are known to respond to NGF and yet have high levels of NGF mRNA. It may be that this knowledge will lead to the discovery of more cell types in the CNS which respond to NGF.

The method developed to measure NGF mRNA is by far the most sensitive assay known for measuring a specific transcript. It should have wide application in research. One intriguing finding which results from the sensitivity of the assay (1 part in 10^9) is that no tissue examined has had an undetectable level of NGF mRNA. Several tissues have very low levels, on the order of 1-2 parts in 10^9, but the NGF mRNA is detectable. It is possible that this is due to unavoidable contamination of samples with vascular tissue. It may also be that some fundamental limit of the control of gene expression has been detected. A cell may not be able to turn off expression of a gene to an absolute zero level. This might be better determined with RNA from a pure cell line, where tissue contamination could be avoided. This type of experiment has been performed, and no growth hormone mRNA could be detected in a liver cell line (Ivarie et al., 1983). The assay used however, would not have detected the low levels observed with the described assay. (sensitivity 1 part 10^8 of total RNA).

The results described have answered several questions concerning NGF. It is now known that the NGF in an animal is primarily made in the targets of the neurons which respond to NGF. On the basis of NGF mRNA content, it appears that peripheral nerves and ganglia are capable of synthesizing NGF. The increase in NGF content observed in denervated irises is not due to an increase in the level of NGF mRNA. In contrast, the increase in NGF content observed in cultured irises appears to be at least partly due to an increase in NGF mRNA levels. The rat CNS appears capable of making NGF, and this capability is concentrated in the cortex and hippocampus, which contain the terminal fields of neurons which are known to respond to NGF (Gnahn et al., 1983; Hefti et al., 1985).

The results have also raised new questions regarding NGF. Does a cause and effect relationship underlie the strong correlation between NGF mRNA and innervation density? Does the NGF mRNA present in peripheral nerves and ganglia have any physiological function? What causes the increase in NGF content in the denervated iris? What is the mechanism which causes the induction of NGF mRNA in irises grown in culture or traumatized in vivo? Does endogenous NGF function as a target derived trophic factor in the CNS? Is this NGF identical to mouse submaxillary gland NGF? Are there as yet unidentified cells in the CNS which respond to NGF?

It is becoming clearer that NGF is only one of a family of trophic factors which mediate neuron target interactions. It is important to try to understand the complex functions of NGF for, as by far the best characterized of the known trophic factors,

principles learned in the study of NGF will undoubtedly guide the direction of research on the others.

APPENDIX

ISOLATION OF CLONES CONTAINING CHICKEN GENOMIC DNA HIGHLY HOMOLOGOUS TO MOUSE NGF cDNA

INTRODUCTION

The presence of vast quantities of NGF in the male mouse submaxillary gland has been of immense assistance in the investigation of the biological importance of NGF. It has allowed the relatively simple isolation of large amounts of NGF (7-10 mgs/100 mice) (Mobley et al., 1976), which simplifies the production of the antisera to NGF (Cohen, 1960) which have been so important in elucidating NGF's physiological role (Levi-Montalcini and Booker, 1960a). Easy access to the purified protein was also necessary for deducing the sequence of the protein (Hogue-Angeletti and Bradshaw, 1971). The fact that NGF is actually synthesized in the submaxillary gland (Berger and Shooter, 1977; 1978) made it useful as a source of mRNA for the construction of the cDNA libraries used to isolate a clone containing the sequence of NGF mRNA (Scott et al., 1983; Ullrich et al., 1983). The production of specific antibodies and the cDNA clone have led to the development of sensitive assays for NGF (Korsching and Thoenen, 1983) and NGF mRNA (Chapter 2) in mammalian tissue.

However, in many respects, mammals are not ideal for the study of development. Mammalian embryos are difficult to experimentally manipulate whereas both avian and amphibian embryos are easily accessible for manipulation (Hamburger, 1960). Unfortunately, the assays for NGF and its message are not applicable to species other than mammals. The cross reactivity between species is limited with the available NGF antisera (Hogue-Angeletti, 1971) and the nucleic acid sequence homology is unknown outside mammals (Chapter 2).

Although the presence of NGF-like activity has been reported in the explanted chick iris (Ebendal et al., 1982; and unpublished observations), there is no known source of NGF equivalent to the male mouse submaxillary gland in avian or amphibian tissues. The use of molecular cloning techniques offers a possible way to obtain both nucleic acid and antibody probes which will be useful for the investigation of the developmental role of NGF in non-mammalian species. With this goal, two clones contining chicken genomic DNA which have sequences highly homologous to mouse NGF mRNA have been isolated.

MATERIALS AND METHODS

Materials

White leghorn chickens were obtained from local suppliers. Restriction enzymes and other DNA modifying enzymes were from Nes England Biolabs or Boehringer-Mannheim. DNA from lambda strain EMBL 4 (Frischauf et al., 1983), *in vitro* packaging reaction mix, and host strain E. coli (NM539) were obtained from Promega Biotec.

DNA

High molecular weight genomic DNA was isolated from newly hatched chick brain (Kunkel et al., 1977). DNA was digested with restriction enzymes as per manufacturers instructions. The method of Southern (1975) was used to identify restriction fragments cross-hybridizing with the NGF cDNA from mouse. Hind III fragments of lambda DNA were end-labelled and used as molecular weight standards.

Library Construction and Screening

Five hundred micrograms of chicken genomic DNA was cut with a two-fold excess of Bam Hl and then separated by size on a 10-40% linear sucrose gradient. The gradients contained 1 M NaCl, 10 mM Tris-Cl, pH 8.0, and 1 mM EDTA and were poured in tubes for a Beckman SW27 rotor (37 mls). The gradients were centrifuged for 24 hrs and 24,000 rpm at 22°C and then eluted from the bottom in seventy-five 0.5 ml fractions. The first fifty of these fractions were analyzed on a 0.8% agarose gel and hybridizing sequences were located (Southern, 1975). Fraction 18, which corresponds to a molecular weight of about 24 kb, was precipitated with ethanol,

dissolved in H_2O, and ligated to Bam Hl cut lambda EMBL4 DNA prepared as described by the supplier. The ligation mix was packaged *in vitro* and used to infect E. coli NM539 as described by the supplier. For screening, the phage were plated at a density of approximately 10,000 plaques per 150 mm petri dish. Yield was about 80,000 clones. Hybridizing clones were identified (Benton and Davis, 1977) and isolated by three rounds of plaque purification. The final plating yielded all positive plaques. A single plaque of each initial clone was then picked, grown by the plate lysate method and DNA was prepared (Maniatis et al., 1982).

Hybridization

Hybridization of Southern blots and plaque lifts were exactly as described in Chapter 2 with the modifications for DNA described in the legend to figure 3.

RESULTS AND DISCUSSION

Somewhat surprisingly, Southern analysis revealed strongly hybridizing bands in restriction digests of chicken genomic DNA (Figure 20). The hybridization signal was equivalent in density to that from rat genomic DNA run in parallel (data not shown). Digestion with Bam Hl yielded a hybridizing fragment of about 10.6 kb. Partial digestion of genomic DNA with Bam Hl showed that this fragment was contained in a larger Bam Hl fragment of about 24 kb (data not shown, but see figure 21). Since these fragments were both within the size range possible to insert into lambda EMBL 4 (Frischauf et al., 1983) a partial digest was fractionated using sucrose gradient sedimentation. An analysis of the first twenty-five fractions from the gradient is shown in figure 21. There were hybridizing bands apparent with molecular sizes of about 24 kb and 11 kb. The resolution achieved by this separation was not sufficient to completely separate these bands, so a fraction containing a majority of the larger band was chosen for the construction of the library.

When the resultant library was screened for clones hybridizing to the NGF cDNA from mouse, two clones were positive. These were subsequently purified and DNA was prepared. After preliminary mapping of the inserts, it will now be possible to subclone the chicken sequence homologous to the mouse NGF cDNA into an M13 vector (Messing and Vieira, 1982), which will allow the synthesis of the same type of single-stranded probe described in Chapter 2 in addition to the sequencing of the DNA. With the probe available,

FIGURE 20

Southern analysis of chicken genomic DNA

DNA was prepared as described, cut with restriction enzymes, and separated on a 0.8% agarose gel. It was then transferred to nitrocellulose, and hybridized as described in legend to Figure 3. Each sample lane contains 0.5 ug of DNA cut with the following enzymes: A) Bam Hl, B) Bgl II, C) Eco Rl, D) Sal 1, E) Xho 1. Numbers and arrows indicate position of molecular weight markers (S) in kb.

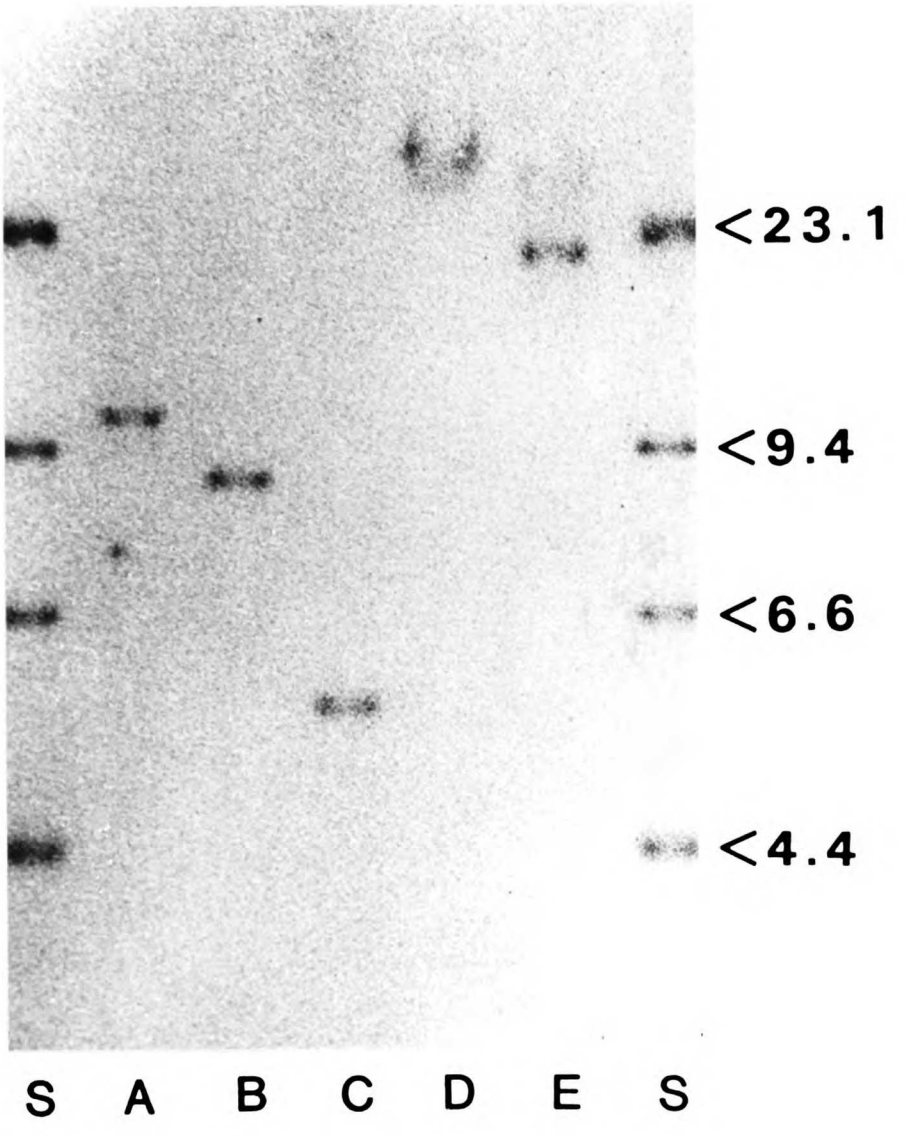
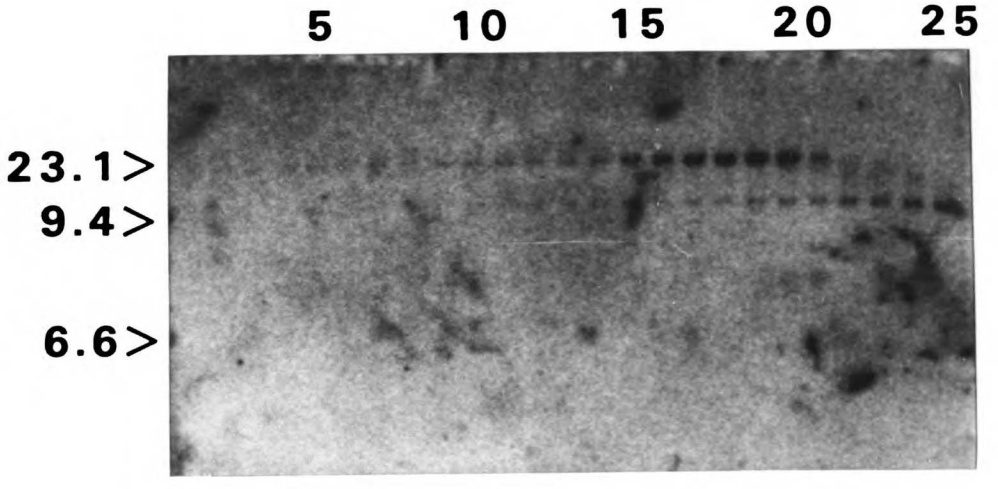

<23.1
<9.4
<6.6
<4.4
S A B C D E S

FIGURE 21

Southern analysis of size fractions obtained by sucrose gradient sedimentation.

A 10 ul aliquot of each 0.5 ml fraction obtained from the elution of the sucrose gradient was run on a 0.8% agarose gel and transferred and hybridized as per figure 20. Numbers indicate the fraction number, and numbers with arrows indicate the position of molecular weight markers and their size in kb. Fraction 18 was used in construction of the library.


5
10
15
20
25
23.1>
9.4>
6.6>

various tissues of the chicken can be screened for their content of NGF mRNA in order to find the best source from which to obtain RNA for the construction of a cDNA library. With a cDNA clone available, sequence comparison between mouse and chicken NGF will be possible. This may lead to a better understanding of regions of the molecule involved in receptor binding. It should also be possible to use the cDNA insert in an expression vector to produce substantial quantities of protein with the correct primary sequence for chicken NGF. Although this protein may not be processed identically to the native chicken protein, it may serve as a useful immunogen for the production of antibodies which cross-react with native chicken NGF (Ullmann, 1984). These antibodies should be useful for developing a sensitive assay for chicken NGF and potentially as reagents for blocking the function of endogenous NGF in the easily accessible chick embryo.

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