IDENTIFICATION AND PARTIAL CHARACTERIZATION OF A NEURITE-OUTGROWTH STIMULATING FACTOR FROM SEMINAL VESICLE OF RAT

A Thesis Presented to the
University of Manitoba

In Partial Fulfillment of the Requirements
for the Degree of Masters of Science

by

© Peter S. McPherson
Department of Physiology
Faculty of Medicine

September, 1988

ISBN 0-315-48026-2

IDENTIFICATION AND PARTIAL CHARACTERIZATION OF A NEURITE-OUTGROWTH STIMULATING FACTOR FROM SEMINAL VESICLE OF RAT

by

PETER S. McPHERSON

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I wish to express my gratitude to my thesis supervisor, Dr. K.W. Cheng, who introduced me to the joys of research by allowing me the freedom to explore the many questions which arose during this study. I also thank him for the financial support and for allowing me to order and use laboratory equipment and perishables at my discretion.

I also wish to thank the other members of my thesis advisory committee, Dr. R.P.C. Shiu and Dr. J.D. Geiger, for the critical analysis of this thesis, and for many helpful discussions over the course of this study.

I am indebted to the many professors, research associates, post-doctoral fellows, and students of the physiology department, for helpful discussions, and for introducing me to many new techniques. Included are Dr. Liam Murphy, Dr. M.L. Duckworth, Dr. I. Worsely, Dr. M. Robertson, Dr. P. Murphey, Dr. G. Wagner, Dr. B. Matusik, Dr. F. Croze, Dr. S. DeToledo, and Mr. G. Dimattia among many others.

The support of my lab mates, Elaneor Ling and Aziz Ghahary has been deeply appreciated.

Finally, I would like to thank my wife Sandra for her unending support. She has been constantly present to ease the dejection, and share the joy, and I look forward to acknowledging her on every accomplishment I am lucky enough to enjoy for many years to come.

LIST OF FIGURES

LIST OF TABLES

Page

LIST OF ABBREVIATIONS

Units of Measurement

mA = milliampere

ml = milliliter

mg = milligram

mM = millimolar

M = molar

g = gram

µl = microliter

µg = microgram

µCi = microCurrie

cm = centimeter

Kb = kilobase

KDa = kilodaltons

nM = nanometers

General Terms

BDNF = brain-derived neurotrophic factor

BSA = bovine serum albumin

CAM = cell adhesion molecule

CAT = choline acetyltransferase

cDNA = complementary deoxyribonucleic acid

CG = ciliary ganglia

CM = conditioned medium

CNS = central nervous system

CNTF = ciliary neurotrophic factor

cpm = counts per minute

DEAE = diethylaminoethyl

DG = diglyceride

DMEM = Delbeco's Modified Eagles Medium

DRG = dorsal root ganglia

ECM = extra cellular matrix

EDTA = ethylenediamine-tetraacetic acid

EGF = epidermal growth factor

FA = fatty acid

FCS = fetal calf serum

FGF = fibroblast growth factor

GAP-43 = growth associated protein-43

GIT = guanidine thiocyanate

HPLC = high performance liquid chromatography

KDa = kilodaltons

MOPS = 3-(N-morpholino)propanesulfonic acid

mRNA = messanger ribonucleic acid

M.W. = molecular weight

NC = nitrocellulose

NGF = nerve growth factor

NILE = nerve growth factor inducible large external glycoprotein

NTFs = neurotrophic fators

PAGE = polyacrylamide gel electrophoresis

PBS = phosphate buffered saline

PC = phosphatidyl choline

PE = phosphatudyl ethanolamine

PGE_1 = prostoglandin E_1

PHI = phosphohexose isomerase

PNPF = poly-ornithine-binding neurite promoting factor

PNS = peripheral nervous system

PORN = poly-ornithine

RBP = retinol binding protein

RP = retinol pupurin

rpm = rotations per minute

SCG = superior cervical ganglia

SD = Sprauge-Dawley

SDS = sodium dodecyl sulfate

SEM = standard error of means

SV = seminal vesicle

SHB = Southern hybridization buffer

SM = sphingomyelin

SSC = sodium-sodium citrate

TCA = trichloroacetic acid

TE = Tris-ethylenediamine-tetraacetic acid

TG = triglyceride

t.l.c. = thin layer chromatography

Vo = void

ABSTRACT

Extracts of rat seminal vesicles stimulate rapid (t1/2=2 hrs) outgrowth of neurites from neuroblastoma x glioma hybrid NG108-15 cells without effecting cell proliferation. The neurite outgrowth effect is dose dependent with a half-maximal activity at 63.7 $\pm$ 4.5 µg of protein/ml of medium. Upon ultrafiltration, the retenate fraction of M.W.>10 KDa stimulates substantial neurite outgrowth in cultures containing 5-10% FCS, although most significantly in 5% FCS. No neurite outgrowth activity is observed in the filtrate fraction of M.W.<10 KDa. Extracts of other organs, including the prostate, kidney, heart, lung, spleen, and skeletal muscle, stimulate neurite outgrowth variously from 0 to 12%, as compared to 33% of that of the seminal vesicle. The activity is heat and acid labile, but alkaline stable. The active principle does not bind to plastic surfaces of culture plates or surfaces coated with poly-L-lysine. Upon gel filtration on Sepharose CL-4B, the activity is excluded in the void volume, indicating a M.W.> 2000 KDa. The activity equilibrates at a buoyant density of 1.04-1.06 grams/ml upon centrifugation on a linear sucrose gradient. However, this partially purified seminal vesicle factor, though active in stimulating neurite outgrowth is unable to alter the expression of GAP-43, which appears to play an important role in neural development and is highly expressed during neurite outgrowth. Further purification of this factor is in prog-

ress to study its biological role(s) in the maturation and differentiation of developing neurons.

INTRODUCTION

The generation of specialized cells from non-specialized ancestor cells is one of the most important questions in the studies of development. Neurons are highly evolved cells with specific, specialized functions, and the evolution from an early somatic cell to a mature neuron is inherently complex. Differentiation of a cell type evolves progressively from complex interactions between genetic instructions, information from the cell cytoplasm, and influences from the extracellular enviroment (Purves and Lichtman, 1985). One important generalization is that, during development, a gradual restriction of expression of certain genes causes the differentiating cells to become more and more specialized (Purves and Lichtman, 1985). To what extent neuronal development unfolds according to genetic instructions, and to what extent it is a result of epigenetic influences from the neuronal environment, has become an area of intense study.

In 1920, Harrison first demonstrated the extrinsic influences for neuronal development when dorsal root ganglia, deprived of their peripheral target, were hypoplastic, whereas peripherally overloaded ganglia were hyperplastic (Hamburger, 1980). Hamburger and Levi-Montalcini (1949) showed that neuronal death in developing sensory ganglia was a normal phenomenon, and that removal of target tissue for these neurons accentuated the process. Moreover, some of the naturally occuring neuronal cell death which occurs during development could be prevented

by increasing the amount of target tissues available to the innervating neurons (Hollyday and Hamburger, 1976). These observations have given rise to the view that there is a competition among neurons for something at the level of the target (Patterson and Purves, 1982), possibly a survival factor, produced in limited quantities.

A role for extrinsic factor(s) on the development of neurons was confirmed upon the discovery of nerve growth factor (NGF). Beuker (1948) observed that dorsal root ganglia innervating a transplant of mouse sarcoma 180, which had been grafted onto 3 day chick embryos, became enlarged. Levi-Monatalcini (1952) extended these findings and determined that sympathetic and sensory, but not motor fibers, innervated the tumor, and that sensory and sympathetic ganglia enlarged in the presence of sarcoma 180, even when direct contact was prevented. These findings suggested that a humoral factor was produced by this tumor (Levi-Montalcini, 1952). When sensory or sympathetic ganglia were placed in culture in the presence of fragments of sarcoma 180, the ganglia produced a dense halo of nerve fibers (Levi-Montalcini et al., 1954). This observation led to the development of an in vitro bioassay, which was essential for the purification of NGF (Cohen et. al., 1954). NGF remains the only neurotrophic factor characterized to date with an established physiological role (for reviews see Thoenen and Barde, 1980; Levi-Montalcini, 1982).

It was subsequently shown that daily injections of

NGF into neonatal rodents resulted in a six- to nine-fold increase in the size of the sympathetic ganglia (Levi-Montalcini and Booker, 1960). On the other hand, generation of an antiserum to NGF (Cohen, 1960) allowed in vivo studies on deletion of NGF. In neonatal rats, daily injections of the antiserum produced a massive destruction of adrenergic neurons in the para- and pre-vertebral chain ganglia (for reviews see Levi-Montalcini and Angeletti, 1966; Thoenen and Barde, 1980). However, nerve cells of the sympathetic ganglionic complexes positioned close to their end organs (including neurons projecting to the genital system) were not vulnerable to the NGF antiserum (Levi-Montalcini, 1982). Recent evidence has pointed to a physiological role for NGF in the central nervous system as well (for review see Thoenen et al., 1987a). For example, neurons of the septo-hipocampal system were shown to express NGF receptors (Richardson et al., 1986), to increase choline acetyltransferase production upon NGF treatment (Gnahn et al., 1983), and to display, within the hippocampus, a stong correlation between the density of cholinergic innervation and the amount of NGF present (Whittemore et al., 1986).

Naturally occuring cell death has been documented for almost every population of neurons studied (Oppenheim, 1981). It appears that every neuron is faced with the problem of finding appropriate targets. Only sensory and sympathetic peripheral neurons, and possibly some central neurons, however, have been shown to depend on NGF for

their normal development. More recently, numerous tissue culture experiments have shown that NGF is only one of a large number of factors which affect the development and survival of embryonic neurons (Thoenen and Edgar, 1985). It is possible that such factors also have an important role in vivo in guiding neuronal development.

Purification and characterization of new neurotrophic factors (NTF's) depends on the development of defined bioassay systems (Barde et al., 1983). These bioassays allow for the quantitation of neurotrophic activities within and between preparations. In this review I categorize the various neurotrophic activities on the basis of the types of bioassays used in a method similar to Berg (1984). The four categories are: 1) neuronal survival factors, 2) soluble neurite outgrowth stimulating factors, 3) substratum-bound neurite outgrowth stimulating factors, 4) and neurotransmitter specifying factors. Special emphisis are given to factors which have been purified or partially purified.

1) Neuronal survival factors

A) Peripheral nervous system

The second NTF to be purified came 20 years after the purification of NGF. Thoenen and collaborators (Barde et al., 1978) described a factor from culture medium conditioned by C6-glioma cells which supported the survival and neurite outgrowth of chick dorsal root ganglion (DRG)

neurons in culture. These effects were not blocked by NGF antibodies. The observation of an activity in rat brain extracts with similar effects on DRG neurons (Lindsay and Tarbit, 1979) led to the purification by Barde et al. (1982) of the "brain derived neurotrophic factor" (BDNF).

BDNF has been purified by a factor of 1.4×10^6 to achieve homogeneity (Thoenen et al., 1987b). The physiochemical characteristics of BDNF (12,300 Da; pI 10.0) are reminiscent of the NGF β-sub-unit (13,200 Da; pI 9.3), but there is no immunological cross-reactivity between the two (Thoenen et al., 1987b). Also, in contrast to NGF, sympathetic neurons are not responsive to BDNF; and BDNF supportes the survival of sensory neurons of ectodermal placodal origin (eg. vestibular ganglion) (Davies et. al., 1986). BDNF has been demonstrated to support survival in cultures of specific populations of neurons which project to the brain. However, the source of BDNF and the physiological relevance of this neurotrophic factor remain to be defined (Thoenen et al., 1987b).

A factor supporting the survival of chick parasympathetic ciliary ganglion (CG) neurons, ciliary neurotrophic factor (CNTF), was purified by Barbin et al. (1984) from chick intraocular tissue, the target tissue of CG neurons. CNTF activity had previously been reported in conditioned medium (CM) from heart cells (Helfand et. al., 1976), extracts of skeletal muscle (Bennett and Nurcombe, 1979), chick embryo (Tuttle et al., 1980), and adult rat sciatic nerve (Williams et al., 1984). CNTF was purified 400-fold,

determined to have a M.W. of 20,300 Da and pI of 5.0, and was is inactivated by NGF antibodies (Barbin et al., 1984). CNTF, purified on the basis of CG neuron survival, was unable to support chick dorsal root ganglion neurons, but supported chick and rat sympathetic ganglia and chick sensory ganglia older than embryonic day 8 (Barbin et al., 1984).

Extracts from neuroblastoma cell lines C1300, N2a, and IMR-32 contain CNTF or CNTF-like activity (Heymanns and Unsicker, 1987). The neuroblastoma NTF affects identical targets of sympathetic and sensory ganglia, has similar heat and protease sensitivity, and has a similar M.W. of 20,000 Da as CNTF (Heymanns and Unsicker, 1987).

A clearly different factor supporting survival of chick neural retinal cells (Schubert and LeCorbiere, 1985) and ciliary ganglion neurons (Schubert et al., 1986) was termed retinal purpurin (RP). Conditioned culture medium of chick neural retina cells contain macromolecular complexes of several proteoglycans and proteins called adherons, which directly promote cell-cell and cell-substratum adhesion in many experimental systems (Schubert et al., 1983). RP was dissociated from the adheron complex, purified, and shown to have a high degree of homology in amino acid sequence with the human serum retinol-binding protein (RBP) (Schubert et al., 1986), which is produced by the liver. Monoclonal antibodies against RP did not block CNTF stimulated CG survival, and CNTF did not support survival of neural retina cells (Heymanns and Unsic-

ker, 1987).

Wallace and Johnson (1986) surveyed a large number of tissues of the pig and found that extracts from lung were the most able to stimulate choline acetyltransferase (CAT) activity and support survival of chick CG neurons. The active factor was purified 3500-fold. It has a M.W. of 3 KDa upon gel permeation chromatography. It is heat and protease stable, and unlike CNTF, promotes survival of parasympathetic CG neurons (Wallace and Johnson, 1987).

NGF from the mouse submaxillary gland has been the most thoroughly studied, and remains as the best characterized factor effecting the survival of sympathetic neurons. Recently, NGF was found in high concentrations in various parts of the male genital tract, including prostate glands of the guinea pig (Harper et al., 1979), rabbit and bull (Harper and Thoenen, 1980), bull seminal plasma (Harper and Thoenen, 1980) and bull seminal vesicle (Hofmann and Unsiker, 1982). Hofmann and Unsiker (1987) partially purified, from bovine seminal vesicle, a protein factor which was slightly larger and more acidic than NGF. It effected almost the same spectrum of target tissues, but was not recognized by anti-NGF antibodies. Its role as a new sympathetic neurotrophic factor awaits its purification and characterization (Hofmann and Unsiker, 1987).

B) Central nervous system

NGF has recently been shown to act as a neurotrophic agent for some groups of neurons in the central nervous

system, though many groups are not affected by NGF (Thoenen et al., 1987a). Recently, Gurney et al. (1986a) purified a factor from the mouse salivary gland, utilizing monoclonal antibodies against a 56 KDa protein, which was released by denervated muscle in organ culture. They cloned successfully the cDNA for the 56 KDa antigen by using an oligonucleotide probe synthesized according to the amino acid sequence of the purified protein (Gurney et al., 1986a). Suprisingly, the cloned cDNA product of the 56 KDa factor supported the survival of chick spinal neurons without effecting neurite outgrowth. Subsequently, the 56 KDa factor was identified as a potent lymphokine (Gurney et al., 1986b), and was therefore named neuroleukin (Gurney et al., 1986a). Neuroleukin promoted the survival of a population of sensory neurons which were not affected by NGF, but did not affect sympathetic or parasympathetic neurons (Gurney et al., 1986a). More recently, Chaput et al. (1987) cloned the glycolysis enzyme phosphohexose isomerase (PHI) from the pig, and found it to be 90% homologous with neuroleukin, with minor differences reflecting species and organ variation. Neuroleukin was since found to have PHI activity. The ability of PHI to act as a neurotrophic agent remains to be clarified.

Epidermal growth factor (EGF) (Carpenter and Cohen, 1979) and acidic and basic fibroblast growth factor (FGF) (Baird et. al., 1986), have recently been shown to have a trophic role for neurons in vitro. Dissociated neuronal cell cultures derived from the subneocortical telencepha-

lon of neonatal rats showed a 15-fold increase in the number of surviving cells in the presence of EGF. EGF also stimulated an increase in the number of neurites and the degree of neurite branching (Morisson et al., 1987). Conditioned medium of astrocytes grown in the presence of EGF did not effect the survival of telencephalic neurons in culture. This provided evidence that EGF acted directly on the neurons and not through stimulation of the small percentage of contaminating astrocytes in the telencephalon cultures (Morisson et al., 1987).

Basic FGF was found to increase neuronal survival and neurite extension in a highly purified population of fetal rat hippocampal neurons under well defined serum-free culture conditions (Walicke et al., 1986). Basic FGF stimulated a 4-fold increase in survival over seven days as compared to untreated cultures. More recently, basic FGF was also shown to support survival of neurons in cultures derived from many brain regions of E18 fetal rats, including entorhinal cortex, frontal cortex, parietal cortex, occipital cortex, striatum, septum and thalamus (Walicke, 1988). The proportion of neurons supported by basic FGF varied among neuronal populations, suggesting the existence of subpopulations of responsive neurons (Walicke, 1988).

Similarily, acidic FGF was observed to increase the survival of hippocampal neurons as significantly as basic FGF. Acidic FGF was also shown to increase the survival of entorhinal cortex, parietal cortex, and septal

neurons, though not as significantly as basic FGF (Walick, 1988). The significance of the role for EGF, basic FGF, and acidic FGF in normal neuronal development remains speculative at this time.

Muller et al. (1984) described a factor from astroglial conditioned medium which stimulated the survival in serum-free culture of dissociated neurons from embryonic rat hippocampus. Unlike other polypeptide neurotrophic factors, the astroglial factor was small, having a M.W. of 500 daltons as estimated by gel permeation, and was heat and protease stable. The exact identification of this factor remains to be investigated.

2) Soluble neurite outgrowth stimulating factors

The stimulation of outgrowth of axons and dendrites (neurites) from neurons in culture has traditionally been used to assay for the effects of NGF (Berg, 1984). Similar assays have revealed a variety of substances, unrelated to NGF, that stimulate neurite outgrowth. These factors fall into two categories: substances which are soluble in the culture medium, and substances which adhere to the culture substratum (Berg, 1984).

Klingman (1982) purified, from bovine brain, a protein factor which stimulated neurite outgrowth from cerebral cortex neurons of day-7 chick embryos, using heat treatment, DEAE-cellulose chromatography, and gel filtration. Analysis of the amino acid sequence of the factor, subsequently purified by reversed-phase HPLC, revealed

that it was a disulfide-bonded form of the brain protein S100 (Klingman and Marshak, 1984). S100 protein is an extremely acidic polypeptide of 10,507 daltons, present mainly in the cytoplasm of glial cells, and the nuclei of neurons (Isobe and Okuyama, 1978), and is homologous to the Ca^{++}-binding proteins troponin C and parvalbumin (Isobe and Okuyama, 1978). Recently, Klingman and Hsieh (1987) showed that this factor stimulated neurite outgrowth from the mouse neuroblastoma cell line Neuro-2a. Further studies using this cell line should help clarify the mechanism of action of this disulfide-linked S100 protein in stimulating neurite outgrowth.

Growth factors EGF, acidic FGF, and basic FGF all have roles as neuronal survival factors. However, EGF was recently shown to not only promote survival in primary cultures of subneocortical telencephalic neurons of neonatal rats, but also to stimulate neurite outgrowth from these neurons (Morisson et al., 1987). Similarily, basic FGF is not only a survival factor for neurons from the hippocampus, entorhinal cortex, frontal cortex, parietal cortex, striatum, and septum (as described), but it also stimulates significant neurite outgrowth from neurons of the hippocampus, entorhinal cortex, and parietal cortex (Walicke, 1988).

BDNF has previously been described as a survival factor for neonate DRG neurons. It also stimulates neurite outgrowth from these neurons (Barde et al., 1982). Lindsay (1988) used dissociated cultures of DRG

neurons from 3-4 month old adult rats to determine that BDNF and NGF enhance axonal regeneration, but are not neccesary for survival of these adult neurons.

It has been established that many non-neuronal cells release factors which stimulate survival and neurite outgrowth of neuronal cells. Monard et al. (1973) observed that culture medium conditioned by C6-glioma cells stimulated neurite outgrowth from NB-2a neuroblastoma cells. This factor was purified and shown to be a 43 KDa protease inhibitor (Guenther et al., 1985). These investigators further determined that various proteases on the cell membrane could affect the turnover of membrane proteins needed for neurite outgrowth of neuroblastomas, and that the glial derived protease inhibitors could regulate protease activity (Guenther et al., 1985).

Factors other then proteins, have also been discovered to stimulate neurite outgrowth. Borg et al., (1987) isolated a long-chain fatty alcohol, n-hexacosanol, from a Far-Eastern medicinal plant, Hyrophila erecta , and found that it stimulated neurite outgrowth by 4- to 6-fold in cultures of cerebral hemispheres of E13 rats. It is known that long-chain fatty alcohols are produced in rat sciatic nerve, and this production increases maximally during nerve regeneration and development (Natarajan et al., 1984). The physiological significance of n-hexacosanol in stimulation of neurite outgrowth is not yet known.

Granule cells, from cultures of early postnatal rat cerebellum extended neurites minutes after plating, and

most cells possessed neurites after 24 hours in culture (Pearce et al., 1987). Neurite outgrowth was blocked by kynurenate, a broad spectrum glutamate receptor antagonist, as well as by the selective N-methyl-D-aspartate receptor antagonist, D-2-amino-5-phosphonovalerate (Pearce et al., 1987). Exogenous glutamate prevented the inhibition of neurite outgrowth caused by kynurenate. The observations that cerebellar granule cells in culture release glutamate (Gallo et al., 1982), and that granule cells have N-methyl-D-aspartate receptors (Garthwaite et al., 1986), has led to the conclusion that glutamate, released in an autocrine fashion, may control the development of these neurons, through the activation of N-methyl-D-aspartate recptors by other unknown genetic or epigenetic factors (Pearce et al., 1987).

3) Substratum-bound neurite outgrowth stimualting factors

A) The role of cell surface macromolecules in stimulating neurite outgrowth

During development, interactions between growth cones and neuronal enviroments influence the direction and rate of process outgrowth. During neurite outgrowth, growth cones come in contact with neuronal and non-neuronal surfaces, (Tomaselli et. al., 1986), and the role of macromolecules present on cell surfaces in directing neurite outgrowth has recently come under investigation.

Neurite outgrowth of CNS neurons (retinal, cerebral

cortex, hippocampus) was supported by glial cells (astrocytes and Schwann cells) but not by non-glial cells (eg. fibroblasts) (Fallon, 1985). This effect of neurite outgrowth is not observed in CM from glial or non-glial cultures.

CG neurons regenerated neurites rapidly when plated on surfaces coated with laminin or fibronectin, and this outgrowth was blocked by two monoclonal antibodies which prevented the adhesion of neurons to these extracellular (ECM) components (Tomaselli et al., 1986). CG neurons also extended neurites on surfaces of cultured Schwann cells and astrocytes, but this neurite outgrowth was not blocked by the two monoclonal antibodies. This indicates that laminin and fibronectin are not involved in the stimulation of neurite outgrowth in this situation (Tomaselli, 1986). The molecular mechanism(s) by which macromolecules on non-neuronal surfaces guide neurite outgrowth, remain yet unknown.

Interactions between neural membranes, as evidenced by nerve bundling (axon fasiculation) are important events in the development of the nervous system (Rutishauser et al., 1978). Two types of cell adhesion molecules (CAMs) have been described: 1) neural-CAM (N-CAM), 2) and nerve growth factor-inducible large external glycoprotein (NILE) (Lagenaur and Lemmon, 1987). Immunological evidence indicated that NILE is similar or identical to other cell adhesion molecules which have been purified, namely L1, neuron-glial CAM, G4, and 8D9 (Langenaur and Lemmon,

1987). Recently, purified 8D9 was shown to contain an activity that promotes the attachment of neurons and outgrowth of neurites from E10 chick embryo tectal cells, and postnatal day 5 mouse cerebellar cells (Langenaur and Lemmon, 1987). The finding that L1 (8D9) became strongly expressed on Schwann cells in the distal stumps of transected sciatic nerve led to the speculation that this expression represents the establishment of a pathway for axon regeneration (Langenaur and Lemmon, 1987).

Neural-cadherin (N-cadherin) is a Ca^{++}-dependant cell-cell adhesion molecule found in both neuronal and non-neuronal tissues (Hatta et al., 1988). Recent immunological evidence indicated that N-cadherin, or the functionally similar neural Ca^{++}-dependant CAM (N-cal-CAM), may play a role in the formation of neurites from chick ciliary ganglion neurons grown on myotubes (Bixby et al., 1988). Matsunaga et al. (1988) observed that neural retina explants from early chick embryos, when placed on monolayer cultures of Neuro-2a cells, did not extend neurites. However, neural retina explants showed vigorous neurite outgrowth when plated on Neuro-2a cells which expressed N-cadherin after previous transfection with chicken N-cadherin cDNA (Matsunaga et al., 1988). This observation strongly suggests that this molecule plays a role in axonal development.

Rauvala and Pihlaskari (1987) isolated, from detergent solubilized young rat brain, an adhesive protein which stimulated neurite outgrowth from rat embryo brain

cells in culture. The protein factor had a molecular mass of 30 KDa (p30) under reducing conditions. This factor may be related to the 43 KDa neurite outgrowth factor (Guenther et al., 1985), which was identified as a protease inhibitor, as both proteins show similar affinities for heparin and affi-gel blue (Rauvalar and Pilaskari, 1987). The observation that the level of p30 in rat brain tissue was higher in perinatal than adult rats implicated a possible developmental role for p30 in neuronal growth (Rauvala and Pihlaskari, 1987).

B) The role of extracellular matrix molecules in stimulating neurite outgrowth

Neurite elongation in developing or regenerating neurons is likely influenced by not only intrinsic neuronal signal, but also signals from the environment. These extrinsic signals may be soluble or cell-membrane associated as previously discussed. Extracellular matrix (ECM) components, derived from cell surfaces, extracellular spaces, or basement membranes (Davis et al., 1985a) also play an important role in neurite elongation. This is of particular interest because of the correlation between the presence of ECM and regenerative capacity in the PNS, and the lack of both in the CNS (Ard et al., 1987).

The role of ECM components in neurite outgrowth has only recently been elucidated. Originally, substratum bound neurite outgrowth factors were observed in CM from many cell types such as glial, endothelial, and

muscle (Adler et al., 1981). These factors were found to adhere to polyornithine (PORN) coated culture dishes and stimulated neurite outgrowth from a wide variety of neurons. They were therfore termed polyornithine-binding neurite promoting factors (PNPFs) (Adler et al., 1981). Initial characterization of PNPFs in CMs from rat Schwannoma (Manthrope et al., 1981), bovine corneal endothelial (Lander et al., 1982), and mouse heart (Coughlin et al., 1981) revealed them to be large (one million or more daltons), acidic glycoproteins. Because of their large molecular weight, involvement in cell-substratum interactions, and synthesis from a variety of cell types, the PNPFs were thought to be related to various ECM proteins, such as fibronectin, laminin, or proteoglycans (Davis et al., 1985a). An ECM nature for the PNPFs was enhanced by the discovery that various ECM components had neurite outgrowth activity. Carbonetto et al. (1983) determined that fibronectin, and collagens type I, III, IV, but not glucosylaminoglycans, when bound to the substratum, stimulated neurite outgrowth from DRG neurons. Fibronectin was also found to stimulate neurite outgrowth from chick neural retina (Akers et al., 1981), as well as sympathetic ganglia (Rogers et al., 1983). Laminin was first shown to stimulate neurite outgrowth in human fetal sensory neurons (Baron-Van Evercooren et al., 1982), and was subsequently found to induce neurite outgrowth from a wide variety of chick and mouse, central and peripheral neurons (Manthrope et al., 1983). A hierarchy of neurite promoting potency

of the various ECM components has thus emerged, which in descending order is laminin, fibronectin, and collagens (Davis et al., 1985a).

The impressive potency of purified laminin suggested that laminin may be the active component of the CM PNPFs. Anti-laminin antibodies, however, blocked laminin-induced neurite outgrowth, but not neurite outgrowth induced by CMs from RN22 Schwannoma, rat astroglia, rat C6 glioma or mouse Schwann cell (Manthrope et al., 1983). Davis et al. (1985b) purified the rat Schwannoma PNPF, and found that it co-purified with laminin, having a similar pattern on SDS-PAGE and immunological cross-reactivity. The neurite outgrowth stimulating abilities of rat Schwannoma PNPF and laminin were very similar with respect to potency and range of action, and parameters of neurite outgrowth such as time of neurite outgrowth initiation, and total neurite length (Davis et al., 1985c).

The PNPF from bovine corneal endothelial cells co-purified with laminin (Lander et al, 1985a). Also, antibodies against laminin immunoprecipitated the neurite outgrowth promoting activity in the CM of bovine corneal endothelial cells, as well as a variety of other cell types (Lander et al., 1985b). Hayashi et al. (1987) recently purified a neurite outgrowth factor from chiken gizzard muscle and identified it as a laminin like molecule.

Therefore, it appears likely that laminin is a key component of the PNPFs from many CMs, even though antibodies which prevent laminin from promoting neurite out-

growth are not effective on all of the PNPFs (Lander et al., 1985b; Davis et al., 1985b; Hayashi et al., 1987). Lander et al. (1985b) proposed that there may be structural differences between neurite outgrowth-laminin and the laminin used to prepare antibodies, or that proteoglycans associated with the laminins (Davis et al., 1987; Lander et al., 1985b) restrict antibody access to certain antigenic sites. It is also doubtful that the associated proteoglycans promote neurite outgrowth them-selves, as they can be dissociated free of the laminin component, which remains active in promoting neurite outgrowth activity (Lander et al., 1985b). The role of laminin in neurite outgrowth is far from clear, and so is the exact relationship among the PNPFs, laminin, fibronectin, and various proteoglycans.

4) Neurotransmitter specifying factors

One of the primary choices a developing neuron makes is which neurotransmitter to produce, thereby determining the effects it will have on post-synaptic target cells (Patterson, 1978). When quail neural tube plus crest cells, which normally would give rise to adrenergic sympathetic and adrenal medullary cells, were transplanted into the "vagal" neural tube level of chick embryos, the quail cells populated the enteric ganglia of the chick gut (Le Douarin et al., 1975). The quail cells that migrated to this abnormal site no longer showed the formaldehyde induced flourescence which is indicative of catecholamines,

and their electrical stimulation produced cholinergically driven, gut-muscle contraction (Le Douarin et al., 1975). This result is consistent with the conclusion that at least some of the adrenergic population have become cholinergic, and stresses the importance of the environment in neurotransmitter development.

Patterson and Chun (1974) used tissue culture methods to show the effects of the environment on neurotransmitter development. Cultures of dissociated superior cervical ganglia (SCG) produced catecholamines and barely detectable levels of acetylcholine (ACh). When placed in co-culture with rat C6-glioma cells (Patterson and Chun, 1974) or medium conditioned by newborn rat heart cells, or other non-neuronal cells (Patterson and Chun, 1977), SCG ganglion cells showed a 100- to 1000-fold increase in ACh production. Weber (1981) reported a 1500-fold purification of the active factor, and further characterized (Weber et al., 1985) this partially purified preparation for its stokes' radius, sedimentation coefficient, partial specific volume, and a molecular weight of 21 KDa. Recently, Fukuda (1985) purified the cholinergic stimulating factor 100,000-fold to apparent homogeneity, and determined a molecular weight of 45 KDa. However, treatment with endo-B-N-acetylglucosaminidase F, which causes deglycosylation, reduced its molecular weight to 22 KDa (Fukuda, 1985). It was further proposed (Fukuda, 1985) that the factor was the same as that described by Weber (Weber, 1981; Weber et al., 1985), and that it was a glycoprotein with six glyco-

sylation sites. The mechanisms, by which this factor affects transmitter development in sympathetic neurons, and its role in vivo are under investigation.

Several extracellular matrix components have been shown to promote adrenergic properties in various neurons. The ECM glycoprotein, fibronectin, was found to stimulate adrenergic properties in quail neural crest cells in culture (Siebier-Blum et al., 1981). Substratum bound laminin was able to stimulate an initial increase in Vmax, and a later increase in levels of tyrosine hydroxylase in young (late fetus and calf) bovine adrenal chromaffin cells (Acheson et al., 1986). The adrenergic stimulating effect appeared to be developmentally regulated, as no change in tyrosine hydroxylase occured in adult adrenal chromaffin cells exposed to laminin (Acheson et al., 1986). In addition, increased cell contact elevated the specific activity of tyrosine hydroxylase in bovine adrenal chromaffin cells, without affecting acetyl cholinesterase or lactate dehydrogenase (Acheson and Thoenen, 1983). Similarly, Zurn and Murdy (1986) showed that increased cell density of cultured superior cervical ganglia (SCG) increased catecholamine production. It was proposed that increased cell contact during tissue (adrenal medulla, SCG) formation and development may be a signal to induce cell maturation of appropriate neuronal properties.

The majority of neurotrophic agents so far characterized are macromolecules, although small molecules do play a role in nerve development. Ascorbate, the co-factor

for the enzyme dopamine-B-hydroxlase, increased the epinephrine and norepinephrine content of adrenal chromaffin cells in culture (Wilson and Kirshner, 1983). Recently, Zurn and Murdy (1986) determined that culture medium conditioned by liver cells (LCM) increased catecholamine production by 4-fold in cultures of SCG neurons, and that the active factor in the LCM had a molecular weight of less then 500 daltons (Zurn, 1987). The factor was heat and pronase stable, but could not replace NGF as a neuronal survival factor, and was not ascorbic acid (Zurn, 1987).

Increased cell-cell contact enhances catecholamine production in both adrenal chromaffin and SCG cells (Zurn and Murdy, 1986; Acheson and Thoenen, 1983). Wong and Kessler (1987) showed that treatment of SCG neurons with membranes derived from adult spinal cord or sympathetic neurons, led to an increase in the expression of substance P and in choline acetyltransferase activity. The active membrane factor was extracted by octyl glucoside, and fractionated on Sephadex G-75 at 29 KDa (Wong and Kessler, 1987). Further purification is neccessary for the elucidation of the precise biological and chemical nature of this factor.

Obviously, a wide variety of factors do exist which affect the growth and development of neurons. Purification, antibody production, and molecular cloning of these factors will allow researchers to determine the physiological significance and biochemical nature of these fac-

tors. Neurotrophic factors may then be used clinically to stimulate or enhance nerve regeneration in diseases, or after trauma.

5) Neuron cytoskeleton and GAP-43

The cells of the vertebrate nevous system show a great variety of forms, as illustrated by the hundreds of distinguishable cell types (Lasek, 1981). Since the CNS is virtually devoid of extracellular connective tissue, neurohistologists have pointed to a role for the cytoskeleton of neurons and glia in determining neuronal form (Lasek, 1981). In order to develop a complex, yet reproducible neuronal circuitry, cytoskeletons must be both stable and plastic. Plasticity is best exemplified by axon outgrowth (Lasek, 1981).

Virtually all neurons extend axons during development. Axonal growth becomes much more limited in a mature nervous system. It is conceiveable that some of the proteins involved in axon outgrowth may be expressed transiently during neuronal differentiation (Skene, 1984). These developmentally expressed proteins would have to be re-induced for the process of neurite regeneration after nerve injury (Skene, 1984).

Induction of specific neuronal proteins (as determined by 2-D gel electrophoresis) was found during axonal regeneration in toad retinal ganglia cells (Skene and Willard, 1981a) and various mammalian PNS nerves (Skene and Willard, 1981b). These various growth-associated pro-

teins (GAPs) are members of a group of the most rapidly moving proteins transported into axons (Skene, 1984). In the optic nerve, the most prominent increase was of an acidic protein of molecular weight estimated between 43 and 49 KDa (GAP-43, GAP-49) (Skene, 1984). It was found that this protein (GAP-43) became associated with the nerve terminal membrane after axonal transport, was produced at its highest level during axon outgrowth, and declined during synaptogenesis (Benowitz and Routtenberg, 1987).

In the developing visual pathway in rabbit, levels of GAP-43 were highest during the first postnatal week, and then decreased dramatically (Skene and Willard, 1981b). A similar pattern was observed in the visual system of the hampster (Moya et al., 1987). Jacobson et al. (1986) showed that GAP-43 was prominent among the total proteins of neonatal rat brain, indicating that this protein was a common feature of many neurons. Levels of GAP-43 in rat brain were found to be highest in the first postnatal week, when axon outgrowth and synaptogenisis was still occuring, and then declined by 90% over the next few weeks (Jacobson et al., 1986). However, in the mature mammalian CNS pathways, which fail to regenerate, axotomy did not cause any increase in GAP-43 expression (Benowitz and Routtenberg, 1987). These observations have led to the hypothesis that the GAP's, of which GAP-43 is the most prominent, are critical for axonal outgrowth during development or regeneration (Benowitz and Routtenberg, 1987).

Recently, a cDNA for GAP-43 was cloned, using antibody screening of an expression library, and the DNA sequence of the clone was reported (Karns et al., 1987). The protein product had a predicted molecular size of 24 KDa, and it was hypothesized that GAP-43 runs anomalously on SDS-PAGE, which led to the molecular weight estimate of approximently 43 KDa (Karns et al., 1987). It became appearent that GAP-43 was identical to several proteins isolated independently (Benowitz and Routtenberg, 1987). GAP-43 is the phosphoprotein pp46, described from growth cones of fetal rat brain (Katz et al., 1985), is identical to F1 protein, a membrane enriched protein-kinase C substrate from rat hippocampus (Nelson and Routtenberg, 1985), and the same as protein B50, a membrane bound, brain specific, phosphoprotein (Aloyo et al., 1983).

Thus, GAP-43 appears to play an important role in such diverse phenomena as neural development, axonal regeneration, and synaptic modulation (Benowitz and Routtenberg, 1987). Immunological studies of specific antibody staining for GAP-43 revealed a wide distribution of the protein throughout the mammalian brain, emphasizing the importance of the molecule (Benowitz et al., 1988). Given the apparant involvement of GAP-43 in a wide array of neural processes, future studies on the structure, physiological significance, and regulation of this protein, are likely to yield a wealth of information for the understanding of some of the most basic phenomena of the nervous sytem (Benowitz and Routtenberg, 1987).

6) Rationale of the investigation

A role for extrinsic influences in neuronal development was observed as early as 1920 (Hamburger, 1980). NGF was the first molecule known to play such a role. The physiological significance of NGF was demonstrated with the discovery that anti-NGF antibodies caused immunosympathectomy (Thoenen and Barde, 1980). However, it was observed that the sympathetic ganglia, which send fibers to the genital system, were not vulnerable to NGF (Levi-Montalcini, 1982).

NGF was found in high concentrations in various regions of the male genital tract, including prostate glands of the guinea pig (Harper et al., 1979), rabbit and bull (Harper and Thoenen, 1980), and bull seminal vesicle (Hofmann and Unsicker, 1982). Biological activities of all these molecules were inhibited by anti-NGF antibodies. However, Hofmann and Unsicker (1987) described a protein from bovine seminal vesicle which had similar physical properties as NGF, and acted on the same spectrum of tissues, but was not inhibited by antisera to NGF. Clearly, the male genital tract, and especially the seminal vesicle contain neurotrophic factors of importance. It is therefore possible that other factors may exist in the seminal vesicle which are unlike NGF.

7) Aims of the present study

A) To determine if the rat seminal vesicle contains a

neurotrophic activity, unrelated to NGF.

B) To develop a rapid bioassay for the activity based on stimulation of neurite outgrowth on neuroblastoma x glioma hybrid NG108-15 cells, which are insensitive to NGF.

C) To characterize the active factor from seminal vesicle extracts.

D) To probe the possible relationship between the activity of the seminal vesicle factor and the expression of the GAP-43 mRNA as a marker for neurite outgrowth.

MATERIALS AND METHODS

1) Materials

Tissue culture supplies including Dulbeco's modified Eagle's medium (DMEM), penicillin-streptomycin, fetal calf serum (FCS), and L-glutamate, were purchased from GIBCO Canada (Calgary, Alberta). Tissue culture dishes and multiwells were from Corning Co. (Corning, New York, USA). Centricon and centriprep concentrators were from Amicon (Danvers, Massaachusetts, USA). Sepharose CL-4B was from Pharmacia (Uppsala, Sweden). Phenol, chloroform, agarose, sodium dodecyl sulfate, sucrose, and T4 polynucleotide kinase were from BRL (Bethesda, Maryland, USA). Standards for thin layer chromatography including phosphatidylcholine and sphingomyelin were from Sendary Research Labs (London, Ontario). Elutip-d and dot blot minifold apparatus were from Schleicher and Schuell (New Hampshire, USA), 3MM paper was from Whatman (Maidstone, England), and nitrocellulose paper was from Bio-Rad, Canada (Mississagua, Ontario). Gamma ^{32}P-ATP was purchased from New England Nuclear (Boston, Massachusetts, USA), and x-ray film was from Eastman Kodak (Rochester, New York, USA). All other reagents were purchased from Fisher Scientific Company (Winnipeg, Manitoba), including ethidium bromide and guanidium thiocyanate from Fisher Biotech, or from Sigma Chemical Company (St. Louis, Missouri, USA).

2) Methods

A) Tissue culture methods

a) Hybrid NG108-15 cell line

The neuroblastoma x glioma hybrid cell line NG108-15 was a gift from Dr. B. Shrier, laboratory of Developmental Biology, National Institute For Health, Bethesda, MD., USA. NG108-15 cells were maintained in medium D, containing 90% DMEM, 10% FCS, 1 x 10^{-4}M hypoxanthine, 1 x 10^{-6}M aminopterin, 1.6 x 10^{-5}M thymidine, 100 i.u./ml penicillin, 100µg/ml streptomycin, and 2mM L-glutamate. Cells were grown in Corning T-75 polystyrene culture flasks at 37°C in a humidified atmosphere of 10% CO_2-90% air. For routine passage or assay, cells were dislodged, collected by centrifugation (500xg for 5 minutes), and resuspended in appropriate volumes of prewarmed (37°C) medium. Cell number was determined by a heamocytometer. Only cells which had been passaged at least 15 and not more than 25 times were used for experiments.

b) Bioassay for neurite outgrowth activity

The bioassay for neurite outgrowth activity was based on the ability of various fractions to stimulate neurite outgrowth from NG108-15 cells when cultured in medium D, but containing only 5% FCS. For assays, hybrid NG108-15 cells were plated in Corning 24 well polystyrene plates at 15,000 cells/well in 1.5 ml of medium. After 2 hours in

culture, aliquots of tissue extracts or chromatographed eluants were introduced into the hybrid cells. After an additional 6 hours of culture, cells were fixed in 10% gluteraldehyde and stained with trypan blue. The percentage of cells bearing neurite(s) of length greater than one cell diameter was determined at 200X magnification with the aid of an ocular graticule. A unit (1U) was defined as the amount of material neccessary to stimulate 1/2-maximal neurite outgrowth .

c) Culture of cells for RNA extraction

NG108-15 cells were plated in $50cm^2$ Corning polystyrene dishes at a concentration of 1.5×10^6 cells/dish in 10ml of medium D, containing 5% FCS. After 2 hours of incubation, plates received either a) 1ml of PBS buffer or b) 250 µg of protein, in 1 ml of PBS, from active fractions of the Sepharose CL-4B chromatography. After an additional 6 hours of culture, RNA was isolated from 16 dishes each of both control and neurite outgrowth induced cells.

B) Analytical procedures

a) Protein determination

The distribution of proteins after gel permeation was monitored with a LKB 4050 spectrophotometer by absorbance at 278 nm. For more accurate determination of protein concentrations, the method of Bradford (1976) was used.

b) Concentration of protein samples

Large and small volumes of tissue extracts or pools of eluants after chromatography were concentrated by centriprep-10 concentrators and centricon-10 microconcentrators, respectively. Both concentrators have retention limits of 10 KDa.

c) DNA assay

The DNA assay was based on the method of Howard (1974). Cells were dissolved from the culture dish and pelleted by centrifugation. The dish was rinsed with 2 ml of 1% SDS and 1ml of 0.2% BSA in PBS, the rinses were combined and added to the cell pellet. After 15 minutes, 3 ml of 20% TCA was added, and the TCA precipitate was pelleted. The pellet was washed with 1 ml of cold 10% TCA, and then resuspended in 0.6 ml of hot 5% TCA, boiled for 10 minutes, centrifuged, and the supernatant collected. The procedure was repeated, and the supernatants were pooled. Aliquots of 500 µl of supernatants, or DNA standards dissolved in 5% TCA, were added to 500 µl of diphenylamine reagent (1.5 grams diphenylamine, 50 ml acetic acid, 1.5 ml H_2SO_4, 0.5 ml of 2% acetaldehyde solution) and 10 µl of 59.5% perchloric acid. The samples were allowed to react overnight, and were quantitated by measuring absorbance at 600 nm.

d) Polyacrylamide gel electrophoresis

Analytical PAGE was performed on a 10% seperating gel

and a 4% stacking gel at pH 8.9 (Ozeta and Market, 1979). The electrophoresis was carried out at a current of 20 mA. At the end of electrophoresis, the gel was stained with silver nitrate by the method of Morrissey (1981).

e) Thin layer chromatography for lipids

Lipids in an aliquot of 6 ml fraction after Sepharose CL-4B chromatography, containing 180 µg protein and 26.5 units of activity, were extracted by the Bligh and Dyer method (Bligh and Dyer, 1959) with slight modifications. In a seperation funnel, 15 ml of methanol and 7.5 ml of chloroform were added to the 6 ml fraction. The mixture was allowed to sit for 30 seconds, and then 7.5 ml of chloroform and 7.5 ml of a 1M KCl solution were added, to a final proportion of chloroform/methanol/water (2:2:1.8). The bottom chloroform layer, containing the lipids, was drained into a flask, and dried on a rotary flash evaporator. The lipid residue was then redissolved in chloroform and transferred to a tube, where chloroform was then evaporated to a minimal volume under a stream of nitrogen.

The sample and lipid standards (in chloroform) were spotted onto a thin layer chromatography (t.l.c.) silica gel plate. The plate was chromatographed in chloroform/methanol/water/acetic acid (35:15:2:1) until the solvent was 10 cm from the origin. The plates were then removed, dried, and rechromatographed in heptane/isopropyl ether/ aetic acid (15:10:1) (Arthur et al., 1986). The plates were stained with a combination of iodine spray for double

bonds and Dittmer stain for phosphorous, followed by baking at 80°C to visualize the lipid bands.

C) Characterization of neurite outgrowth activity

a) Tissue extraction

Rats were sacrificed by decapitation and the prostates, including seminal vesicles were removed. The coagulating gland was teased away and the seminal fluid removed before the seminal vesicle was frozen on dry ice.

For extraction, tissues were homogenized in 5 volumes of cold PBS buffer using a Polytron, stirred for 1 hour, sonicated with 5x15 second bursts, and centrifuged at 15K for 30 minutes. The resulting supernatant was dialysed extensively overnight against PBS buffer, and used for bioassay. All steps were carried out on ice or at 4°C.

b) Gel permeation on Sepharose CL-4B

For column chromatography, seminal vesicles from adult rats were homogenized in PBS buffer containing 1% β-mercaptoethanol, followed by centrifugation at 15K for 30 minutes. The extract was then sonicated, and centrifuged again at 40K for 1 hour. The clear supernatant was then applied onto a column of Sepharose CL-4B equilibrated in PBS buffer containing 0.1% β-mercaptoethanol, and eluted with the same buffer. For neurite outgrowth assay, fractions were concentrated on centricon-10 microconcentrators, and added to the NG108-15 cells.

c) Sucrose density gradient centrifugation

A linear sucrose density gradient ranging from 1.02 to 1.14 g/ml was prepared by placing 6 ml of a 50% sucrose solution in 10mM $NaPO_4$, pH 7.0 in the reservoir chamber of a Hoefer gradient mixer, and an equal volume of a 10% solution in the mixing chamber. A piece of tygon tubing was connected from the outlet port of the mixing chamber, through a LKB microperistaltic pump, to a long needle which delivered the mixed sucrose solution to the bottom of a Beckman Ti-75 ultracentrifuge tube.

A sample of the concentrated active fractions after the Sepharose CL-4B chromatography was gently layered on top of the gradient, the centrifuge tube was sealed, and spun at 200,000xg for 15 hours. After centrifugation, the gradient was fractionated into 1ml fractions using a peristaltic pump and an LKB fraction collector. The sucrose content of each the fraction was monitored based on refractive index, read on a Bausch and Lamb ABBE-3L refractometer. For neurite outgrowth assay, fractions were washed free of sucrose, concentrated on centricon-10 microconcentrators, and added to the NG108-15 cells in culture.

D) Northern and dot blot analysis

a) RNA extraction

RNA was isolated from rat tissues or NG108-15 cells by the method of Chirgwin et al. (1979). Briefly, tissues were homogenized at 5:1 (w:v) in cold 4.0M guanidinium

thiocyanate (GIT), and cultured cells were washed on the dish with culture medium, dislodged and pelleted by centrifugation, and the resuspended cells in cold GIT were then disrupted by passing through a 21 gauge needle, followed by an 18 gauge needle. Cell or tissue homogenates (approximatly 7ml) were layerd onto 4ml of 5.7M $CsCl_2$ in 3M sodium acetate, pH 6.0, in a Ti-75 ultracentrifuge tube, and spun at 25,000 rpm for 20 hours. The reulting pellet was dissolved in 400 µl of TE buffer (10mM Tris, pH 7.4 containing 0.1mM EDTA). To the mixture, 400 µl of salt-saturated phenol-chloroform (1:1) was added, vortexed, and spun in a microcentifuge for 7 minutes at $4^{o}C$. After centrifugation, RNA was removed in the aqueous layer and precipitated overnight at $-20^{o}C$ by adding 2 volumes of 0.25M $NaCl_2$ in ethanol. After centrifugation, the pelleted RNA was washed with 70% ethanol, dried under vaccum, and redissolved in a minimal volume of TE buffer. RNA content was measured by absorbance at 260 nm.

b) Agarose-formaldehyde gel electrophoresis

Agarose-formaldehyde gel electrophoresis was performed according to the method of Lehrach et al. (1977). Gel of 1.2% was obtained by dissolving 3.6 grams of agarose in 216 ml of water upon boiling. While the mixture was being cooled to $60^{o}C$ with constant stirring, 54 ml of formaldehyde, 30 ml of 10X MOPS [0.2M 3-(N-morpholino)propanesulfonic acid, 0.05M sodium acetate, 0.01M EDTA] buffer, and 18 µl of a 1.0% ethidium bromide

solution were added. The mixture was then poured into a horizontal gel electrophoresis apparatus and allowed to cool and solidify.

To a sample of 50 µg of total RNA in 11 µl of TE buffer, 20 µl of formamide, 7 µl of formaldehyde, and 2 µl of 10X MOPS were added. After heating the sample mixture at 65°C for 20 minutes, 2 µl of loading buffer containing glycerol and bromophenol blue were added. Electrophoresis was carried out at 40 volts for 18 hours in 1X MOPS running buffer.

c) Blotting RNA to nitrocellulose

After electrophoresis, RNA was transfered from the agarose gel to nitrocellulose (NC) paper as described by Davis et al. (1986). After washing in 20X SSC (3M NaCl, 0.3M sodium citrate, pH 7.0), the agarose gel was placed upside-down onto a filter paper, and a piece of NC paper soaked in 20X SSC, was placed over the gel with 3 pieces of 3MM paper equilibrated in 20X SSC, followed by a stack of dry paper towels. After 24 hours, transfer of RNA to the paper was monitored under u.v. light. The NC paper was air dried , and the RNA was baked onto the paper at 80°C under vaccum for 2 hours.

d) Dot blots

Dot blot analysis of RNA samples was performed as described by Davis et al. (1986) with slight modifications. Samples of RNA in 6X SSC containing 5% formaldehyde

were heated to 65°C for 30 minutes and applied onto NC paper (equilibrated in 6X SSC) through the wells of a Schleicher and Schuell minifold dot blot apparatus under vaccum. After rinsing 3 times with 6X SSC, The NC paper was air dried and then baked under vacuum at 80°C for 2 hours.

e) End labelling of oligonucleotide probe

A 48-mere synthetic oligonucleotide was purchased from the Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alberta, and labelled with gamma ^{32}P-ATP as described by Davis et al., (1986). The sequence of the synthetic oligonucleotide is:

5'-cctgccttgctgggctcctcagccttagggctggtggctggggctgca-3'

This sequence is the reverse complement of the mRNA produced by DNA encoding the neuronal growth associated protein-43 (GAP-43). The 48-mere corresponds to bases 339-387 of the cDNA reported by Karns et al., 1987.

For labelling of the oligonucleotide, 14 µl of water, 50 µCi of gamma ^{32}P-ATP, and 2 µl of T4 polynucleotide kinase was added to 640 ng of GAP-43 oligonucleotide in 3 µl of 10x kinase buffer. The mixture was placed at 37°C for 45 minutes. The reaction was stopped by placing the reaction vessel at 65°C for 15 minutes and free gamma ^{32}P-ATP was separated from the labelled oligonucleotide by washing 2x on Schleicher and Schuell Elutip-d columns as described by Davis et al. (1986).

f) Hybridization

Hybridization of the ^{32}P-labelled oligonucleotide with RNA on NC paper was performed as described by Davis et al. (1986) with slight modifications. The baked NC filter was wetted in 6X SSC and placed in a Seal-a-Meal bag containing 10 ml of Southern hybridization buffer (SHB), consisting of 6X SSC, 1X Denhart's solution (10 grams polyvinylpyrrolidone, 10 grams BSA, and 10 grams of Ficoll 400 in 500 ml water), 0.1mg/ml of sonicated, single stranded, salmon sperm DNA, 1 mg/ml SDS, and 1 mg/ml sodium pyrophosphate. The bag was sealed and placed in a water bath at 55^{o}C. After 3-4 hours, the SHB was drained, 10 ml of fresh SHB containing approximently 10 x 10^{6} counts of ^{32}P-labelled GAP-43 oligonucleotide probe was added, the bag was resealed and placed at 55^{o}C for approximately 15 hours. After hybridization, the NC paper was washed free of unbound radioactivity in 1X SSC containing 0.1% SDS at 55^{o}C, and autoradiographed.

g) Quantitation of hybridization

For more accurate measurement of radioactivity, dots on NC paper containing hybridized ^{32}P-labelled GAP-43 probe were cut out of the NC paper, placed in vials with 5 ml of scinti-versa scintilation fluid, and counted in an LKB gamma counter.

RESULTS

1) Conditions for assaying neurite outgrowth activity using the hybrid NG108-15 cell line

For successful isolation and characterization of biologically active principles, a simple and specific bioassay is essential. Extracts of adult rat seminal vesicle (SV) were observed to be able to stimulate neurite outgrowth from neuroblastoma x glioma hybrid NG108-15 cells. A bioassay based on the ability of stimulating neurite outgrowth from these cells was developed to monitor the activity in crude SV extracts and eluants from fractionationed SV extracts.

The NG108-15 cell line was derived from a Sendai-virus-induced fusion of a mouse neuroblastoma cell line (N18 TG-2) and a rat glioma cell line (C6BU-1). This cell line has been shown to have a high basal expression of choline acetyltransferase (Hamprecht, 1977) and to form functional synapses with striated muscles *in vitro* (Nelson et al., 1976). The cells have also been documented to possess receptors for neuroactive compounds, including muscarinic acetylcholine, serotonin, neurotensin, adenosine, PGE_1, alpha-2-adrenergic, and opiate (Nierenberg et al., 1983). In adapting this NG108-15 cell line for monitoring neurite outgrowth activity, preliminary experiments were carried out to establish a simple and reproducable bioassay.

A) Extraction of seminal vesicles

Whole prostates were removed from adult Sprauge-Dawely rats. Seminal vesicles were dissected out, and the coagulating glands were removed by gently teasing them from the vesicle. The seminal fluid was then squeezed out. SV tissue was rinsed and homogenized in PBS, and centrifuged at 20,000xg for 30 minutes. The resulting supernatant was sonicated and recentrifuged at 100,000xg for 60 minutes. The clear supernatant was then applied directly for bioassay, or was taken for further characterization and fractionation procedures.

B) Morphological assay of neurite outgrowth using NG108-15 cells

NG108-15 cells were plated at 15,000 cells/well in DMEM containing 5% FCS. After 2 hours incubation, aliquots of tissue samples or BSA solution (control) were applied. After an additional 6 hours incubation, cells were fixed in 10% gluteraldehyde and stained with trypan blue. The number of cells bearing neurite(s) longer then one cell diameter was determined and expressed as % of the total number of cells present. Figure 1C shows that cells grown under control conditions in DMEM with 5% FCS for 2 hours had a rounded morphology, and tended to grow in small clumps. After an additional 6 hours of culture (Fig. 1D), cells began to unclump, with some cells (<5%) flattening on the plate and extending axons or dendrites (neurites).

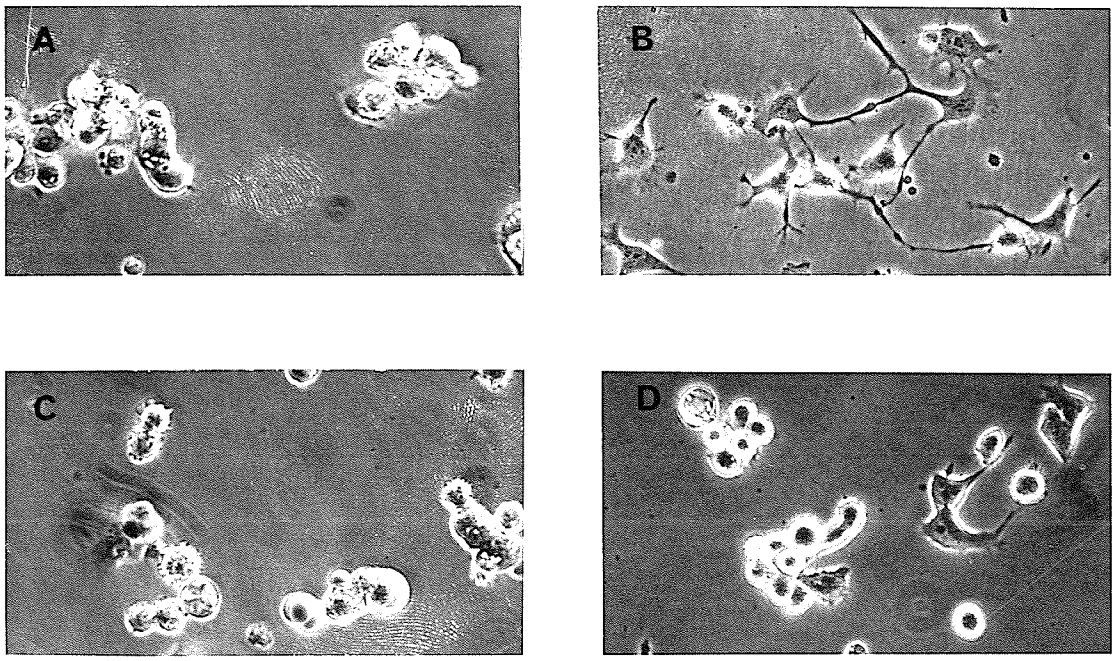

Figure 1. Phase-contrast photomicrography of NG108-15 cells. NG108-15 cells were plated in DMEM containing 5% FCS at 15,000 cells/well. After 2 hours incubation (1A and 1C), cells were treated with BSA (1D) or with SV extracts or fractions (1B). After an additional 6 hours incubation, cells were fixed in 10% gluteraldehyde, stained with trypan blue, and the number of cells bearing neurite(s) greater then one cell diameter was determined.

However, after incubation in the presence of SV extracts (Fig. 1B), most cells became flattened on the plate. They grew in singlets, and a good portion of the cells (35-45%) extended 1 or more long neurites.

C) Time course of neurite outgrowth

The outgrowth of neurites from SV stimulated NG108-15 cells was relatively rapid. Figure 2 shows the time course of neurite outgrowth of NG108-15 cultures in the presence of SV extracts. Half-maximal neurite outgrowth activity was achieved within 2 hours after addition of the extract, and recorded its maximal outgrowth by 6 hours. For assays of neurite outgrowth, 6 hour incubations were therefore routinely used.

D) Effect of serum on assaying neurite outgrowth activity

The effect of serum on neurite outgrowth activity is shown in Figure 3. Extracts of SV were separated by ultrafiltration into fractions of molecular weight greater and less than 10 KDa by Centricon-10 microconcentrators upon centrifugation. Equal volumes of each fraction were assayed for neurite outgrowth activity by addition to the NG108-15 cells plated in the presence of 5% FCS or 10% FCS. Neurite outgrowth activity was found only in the fraction of molecular weight >10 KDa (Fig 3). This fraction was able to stimulate neurite outgrowth of NG108-15 cells even in the presence of 10% FCS, but the activity was significantly higher in cultures containing 5% FCS.

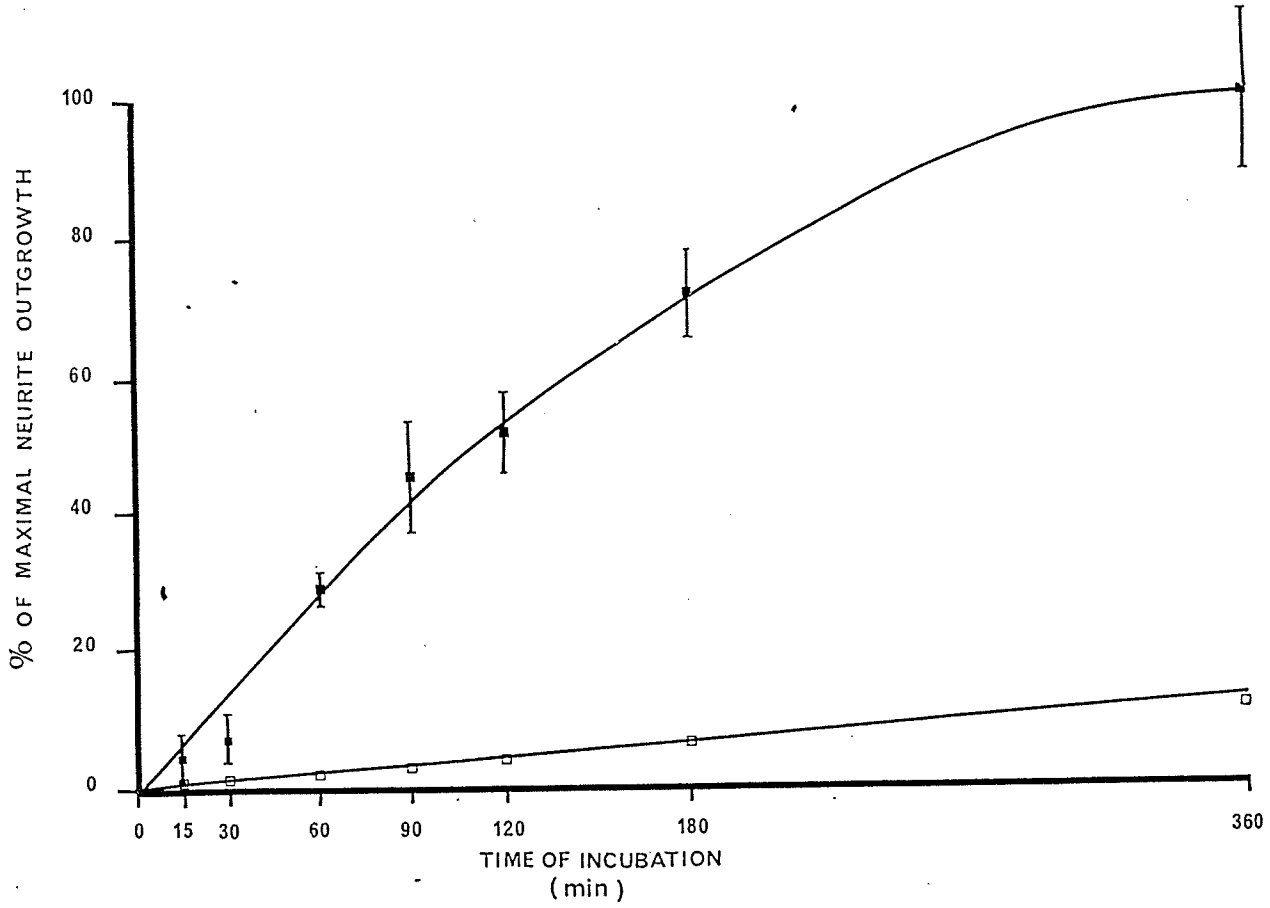

Figure 2. Time course of neurite outgrowth. Cells were plated under standard assay conditions, and 250 µg of seminal vesicle extract was added to each well. Cells were fixed after 15, 30, 60, 120, 180, and 360 minutes, and the number of cells with neurite(s) greater than one cell diameter was determined. Activity is expressed as percent of cells bearing neurite(s) greater then one cell diameter after 360 minutes. Data represents means ± SEM of three experiments. (■), 250 µg of seminal vesicle extract applied; (□), BSA control.

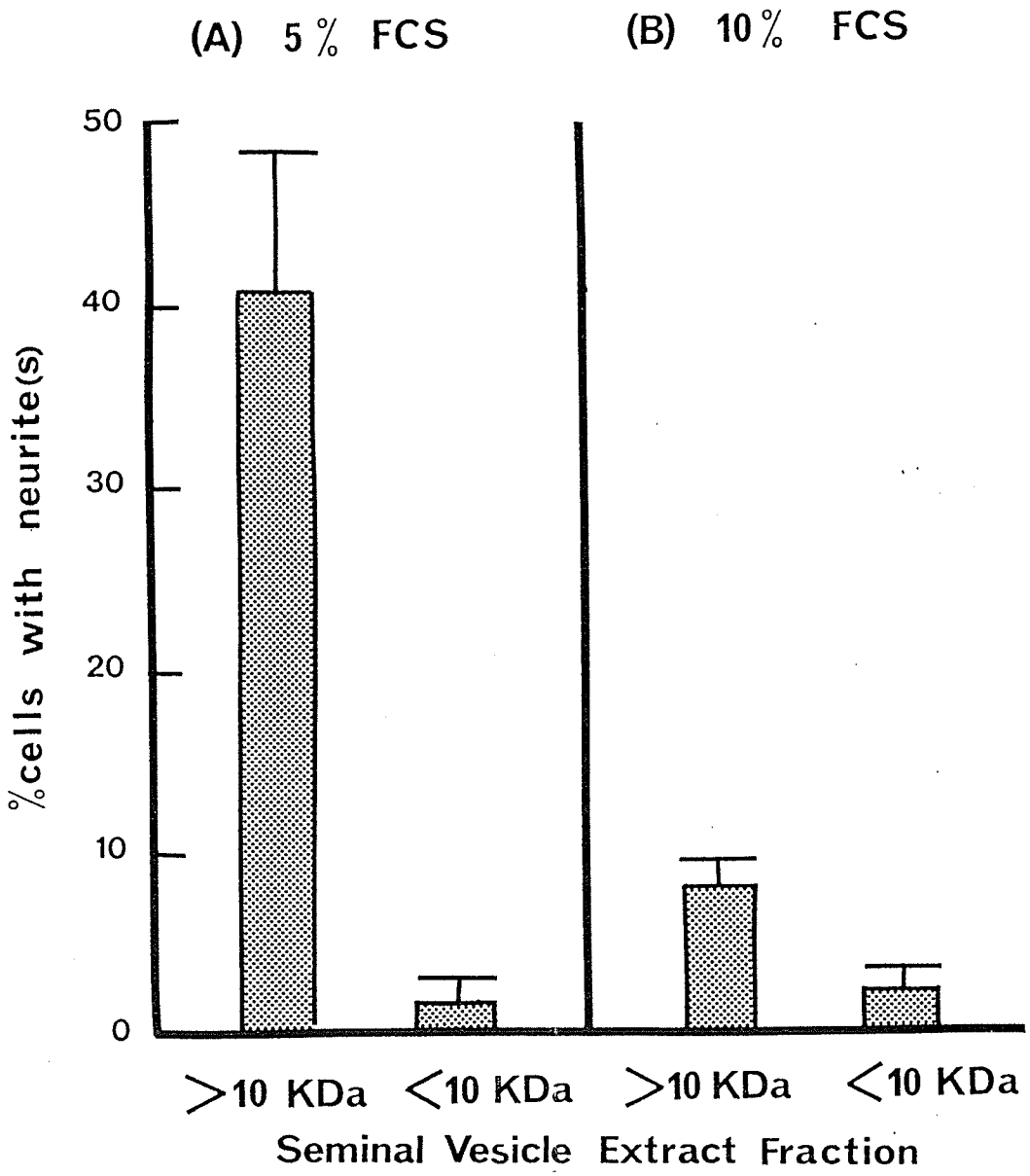

Figure 3. Effect of serum on assaying neurite outgrowth activity using NG108-15 cells. Extracts of SV at protein concentrations of 250 µg were separated into fractions of molecular weight >10 KDa or <10 KDa by centricon-10 micro-concentrators under centrifugation. Equal volumes of each fraction were bioassayed on cells plated in (A) 5% FCS or (B) 10% FCS. Data represents means ± SEM of three experiments

Thereafter, cultures of NG108-15 cells in DMEM containing 5% FCS were routinely used for the neurite outgrowth assays.

E) Effect of seminal vesicle extract on cell proliferation

The SV extracts stimulated neurite outgrowth, but did not affect cell proliferation as shown in Figure 4. Plated NG108-15 cells received 250 µg of an active fraction of the SV extract after CL-4B column chromatography (Figure 8). After 24, 48, or 72 hours of incubation, cells were harvested and their DNA content was determined as a marker of cell proliferation. Under these conditions, cells doubled approximatly every 24 hours, but no significant difference in growth was observed between SV-treated and untreated control cells. NG108-15 cells treated with the SV active fraction did show significant neurite outgrowth.

The extraction of SV and bioassay of the neurite outgrowth activity using NG108-15 cells in culture is summarized in Fig. 5.

2) Characterization of the neurite outgrowth stimulating factor

A) Binding affinity of the neurite outgrowth stimulating factor

Neurite outgrowth stimulating factors have been found to fall into two categories; soluble, and substratum-binding. The results of experiments designed to determine

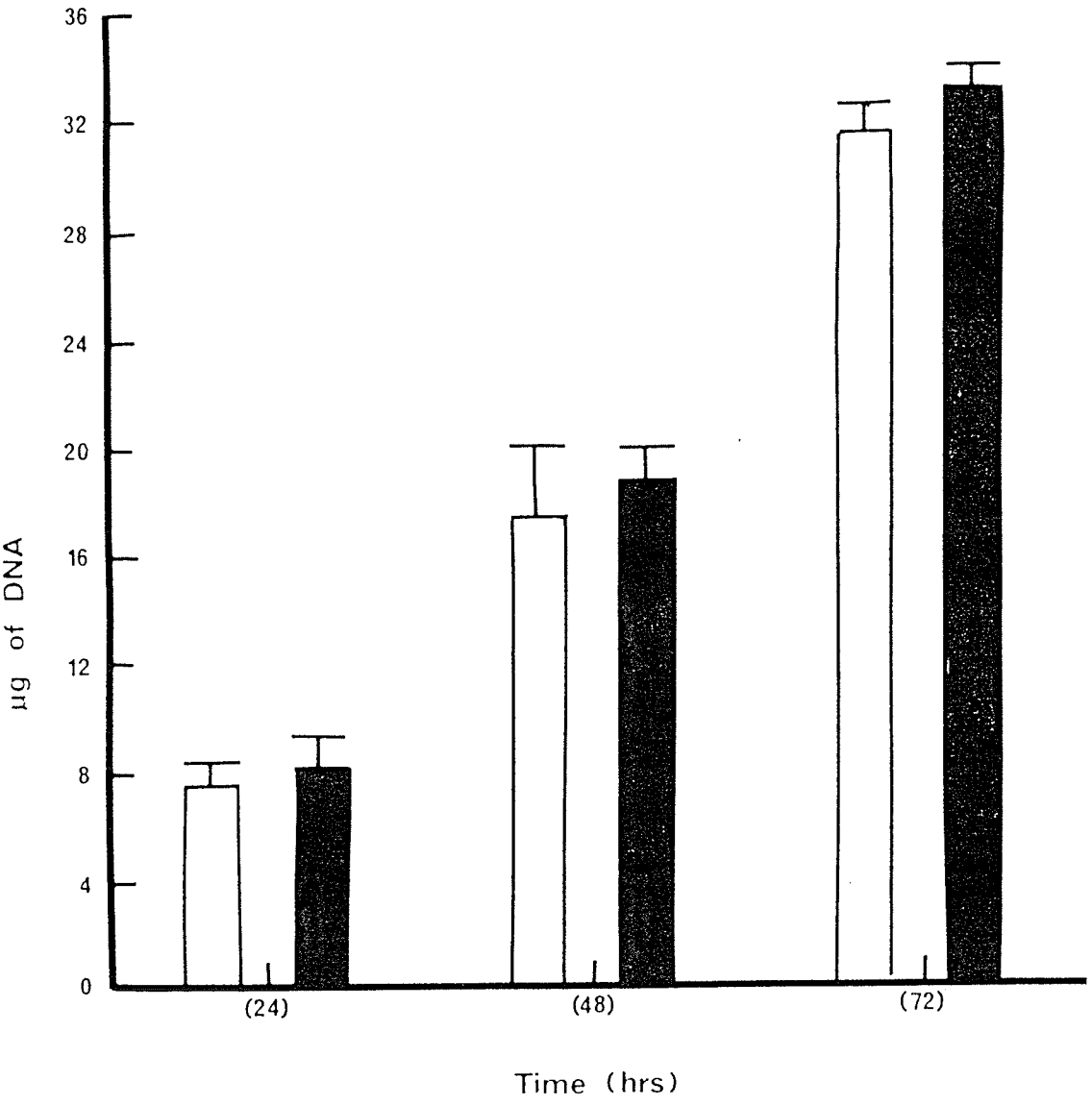

Figure 4. Effect of seminal vesicle extracts on cell proliferation. NG108-15 cells were plated at 15,000 cells/ml in DMEM containing 5% FCS. Cells received 25 µg/ml of an active SV fraction after Sepharose CL-4B column chromatography (Figure 8). Cells were harvested after 24, 48, or 72 hours incubation, and their DNA content was assayed. Data represent means ± SEM of 4 or 6 experiments. (□),PBS control; (■),SV fraction.

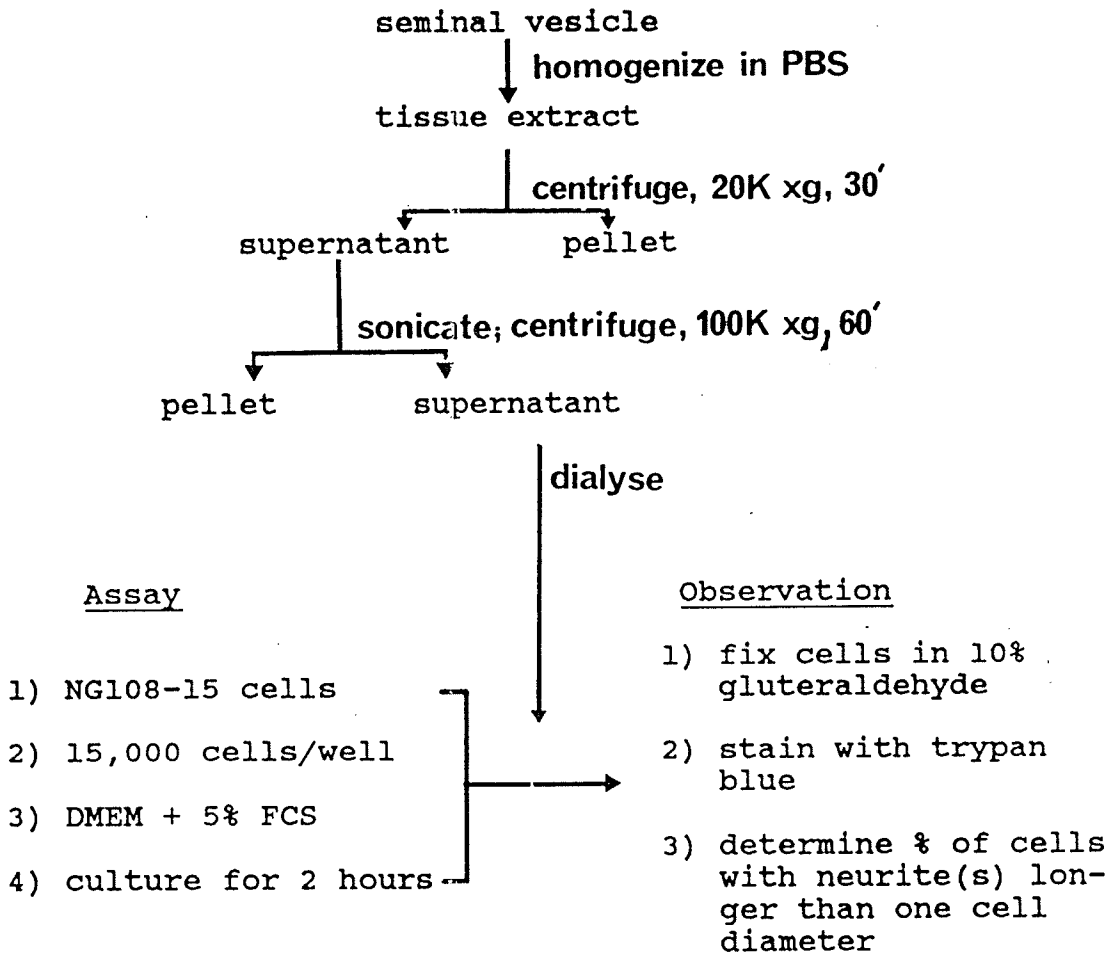

Figure 5. Summary of extraction and bioassay of the seminal vesicle neurite outgrowth activity.

Table 1. Ability of seminal vesicle neurite outgrowth stimulating factor to bind to poly-L-lysine coated plastic surfaces. Tissue culture dishes were coated with a 0.2% solution of high molecular weight or regular poly-L-lysine for 6 hours at 4°C, followed by a PBS rinse. An active seminal vesicle fraction (30 µg) after Sepharose CL-4B chromatography (Figure 8) was added to the dishes for 16 hours at 37°C, followed by washing with PBS. Cells were then plated into the dishes in DMEM containing 5% FCS at 15,000 cells/well. After 8 hours of culture, cells were fixed and counted.

Substrate surface	SV Active fraction (30 µg)	% cells with neurites
plastic	+	11.9 ± 0.9
	-	5.5 ± 1.0
poly-L-lysine (HMW)	+	17.1 ± 1.7
	-	Most cells died (remaining cells had rounded morphology)
poly-L-lysine (regular)	+	13.2 ± 1.2
	-	Most cells died (remaining cells had rounded morphollogy)

if the neurite outgrowth stimulating factor (NOSF) binds to plastic or poly-cationic coated plastic surfaces (poly-L-lysine) of tissue culture dishes are shown in Table 1. Tissue culture dishes were coated with a 0.2% solution of regular or high molecular weight (HMW) poly-L-lysine (Sigma, Co., St. Louis) for 6 hours at 4°C, followed by an extensive rinse in PBS. Aliquots of 30 µg of an active fraction after Sepharose CL-4B column chromatography (Figure 8), in 270 µl, were added to the regular or poly-L-lysine coated dishes for 16 hours at 37°C , followed by washing with PBS. Cells were then plated onto the dishes in DMEM containing 5% FCS. Little neurite outgrowth activity was observed in cells cultured on plastic surfaces coated with the SV active fraction. Cells grown on poly-L-lysine coated surfaces were found to be dead 2 hours after plating. Cells grown on dishes coated with poly-L-lysine, followed by treatment of SV active fraction survived and showed a small degree of neurite outgrowth. No significant difference in neurite outgrowth was observed between cells cultured in dishes previously treated or non-treated with poly-L-lysine.

Affinity of NOSF for heparin was examined by affinity chromatography on heparin-sepharose (Figure 6). Seminal vesicle extracts were dialysed against 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl, and applied onto a heparin-sepharose column equilibrated in the same buffer. After extensive washing, bound proteins were eluted stepwise, first with 1.0 M and then 3.0 M NaCl in

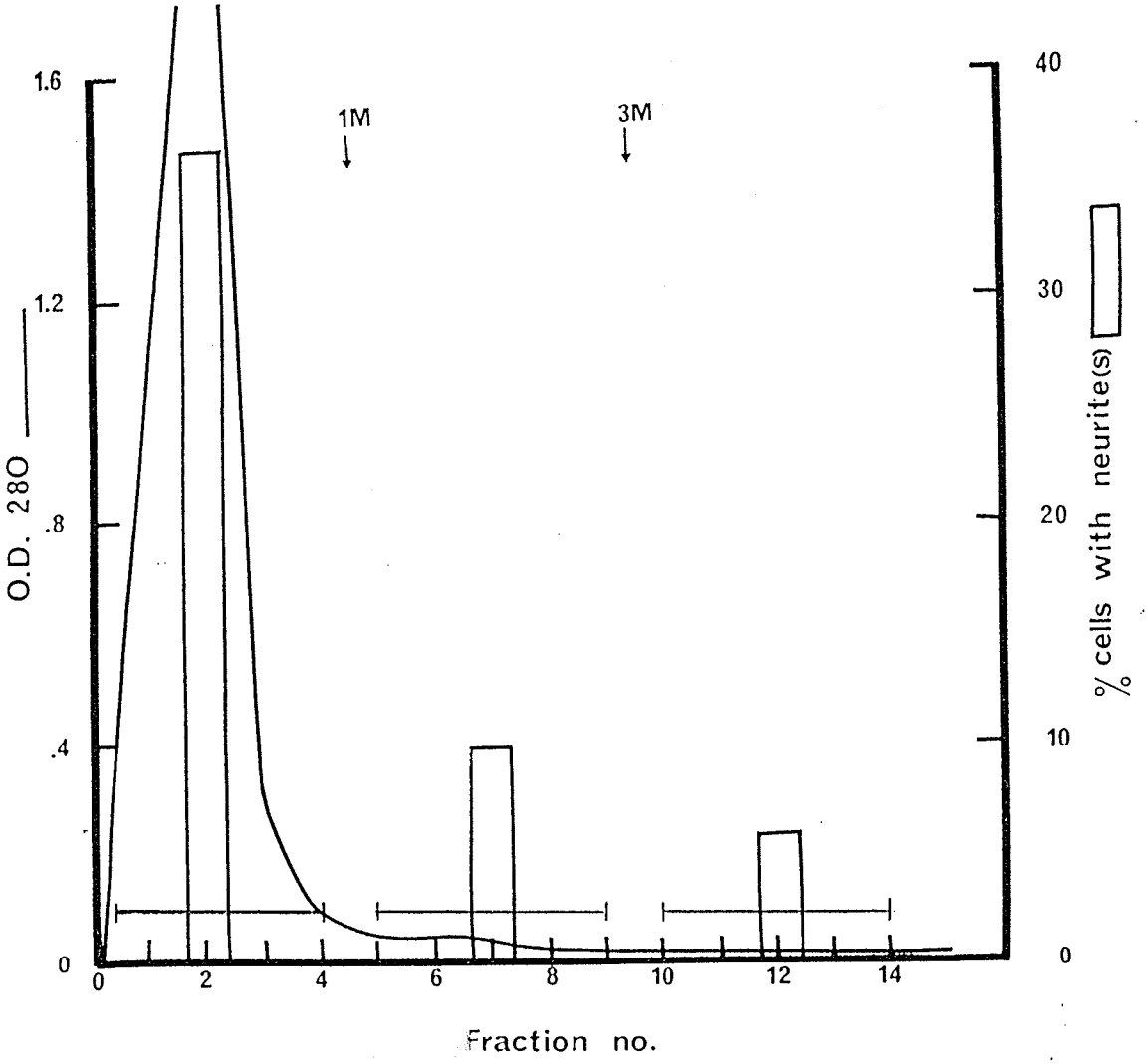

Figure 6. Heparin-sepharose affinity chromatography of the neurite outgrowth stimulating factor. Seminal vesicle extracts were dialysed against 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl, and then applied to a heparin-sepharose column equilibrated in the same buffer. After washing, bound proteins were eluted stepwise with 1M and 3M NaCl in 10 mM sodium phosphate buffer. Fractions 1-4, 5-9 and 10-14 were pooled and concentrated on centricon-10 microconcentrators and assayed for neurite outgrowth activity. Samples of 200 µg of protein were used for the assay. Protein distribution was monitored by O.D. 280.

10 mM sodium phosphate buffer, and the fractions were assayed for neurite outgrowth activity. The bulk of proteins, including NOSF did not bind to the column, and were eluted in the unadsorbed fractions, indicating that NOSF does not have an affinity for heparin.

B) Stability of the neurite outgrowth stimulating factor

Table 2 shows that the NOSF was relatively stable at pH values that ranged from 6-8. Aliquots of seminal vesicle extract were dialysed against various buffers at different pH values as indicated for 8 hours. After dialysis, the pH of the samples was readjusted to 7.0 by dialysis for another 8 hours. Aliquots of 250 µg of protein were applied for neurite outgrowth assay. The activity remained relatively stable at pH values from 6.0 to 10.0, but was labile under acidic conditions.

For heat stability, aliquots of the seminal vesicle extract were sealed in microcentrifuge tubes and placed in a water bath at various temperatures as indicated. After an incubation of 30 minutes, allowing for the transfer of heat to the SV extract, samples were spun and aliquots of 250 µg of protein each were applied for neurite outgrowth assay. The activity was stable up to 55^{o}C for 30 minutes, and was partially or totally destroyed at higher temperatures as shown in Table 3.

C) Tissue distribution of neurite outgrowth stimulating activity

Table 2. Effect of pH on the neurite outgrowth stimulating factor. Aliquots of the rat SV extract were dialysed for 8 hours against the following buffers: 100mM glycine-HCl, pH 4.0; 200mM sodium phosphate, pH 6.0; 200mM sodium phosphate, pH 7.0; 200mM sodium phosphate, pH 8.0; 100mM sodium phosphate-sodium bicarbonate, pH 10.0. After dialysis, the pH of the samples were readjusted to 7.0 by dialysis for another 8 hours. Aliquots of 250 µg of protein from each sample were assayed for neurite outgrowth activity. Data represents Means ± SEM of three experiments.

pH	%cells with neurite(s)
4.0	11.7 ± 2.5
6.0	30.8 ± 3.0
7.0	33.4 ± 1.1
8.0	33.4 ± 3.4
10.0	28.6 ± 2.8

Table 3. Effect of temperature on the neurite outgrowth stimulating factor. Aliquots of the SV extract were sealed in microcentrifuge tubes and placed in water baths at 37, 55, 70, 82, and 100°C for 30 minutes. After incubation, samples were spun at 10,000 rpm for 5 minutes, and aliquots of 250 µg of protein from each sample were assayed for neurite outgrowth activity. Data represents Means ± SEM of three experiments.

temperature (°C)	%cells with neurite(s)
37	36.9 ± 3.1
55	43.5 ± 2.4
70	15.1 ± 13.2
82	0.6 ± 0.7
100	2.3 ± 0.9

To determine the seminal vesicle specificity of the activity, tissues of coagulating gland, various lobes of the prostate, other peripheral organs, including lung, muscle, kidney, spleen, and heart were dissected from adult male SD rats, extracted with PBS, and centrifuged. To assay neurite outgrowth activity, samples of various extracts at protein concentration of 200 µg were added to the cultured NG108-15 cells. Figure 7 shows that tissues, other then the SV, were able to stimulate neurite outgrowth variably from 2-12% only. The neurite outgrowth stimulating activity was significantly higher in SV, being 35%.

3) Partial purification of the neurite outgrowth stimulating factor

A) Gel permeation on Sepharose CL-4B

To further characterize the NOSF, the SV extracts were fractionated on a Sepharose CL-4B column (Figure 8). SV tissue extracts were fractionated by this column into one small peak, corresponding to Vo, followed by two large peaks of protein. Various fractions were monitored by the neurite outgrowth assay, and the activity was found in the eluants of the Vo peak, corresponding to a molecular weight of approximantly 2000 KDa or greater. Fractions 70-110 were pooled (CL-4B active fraction), and concentrated for the next step of purification.

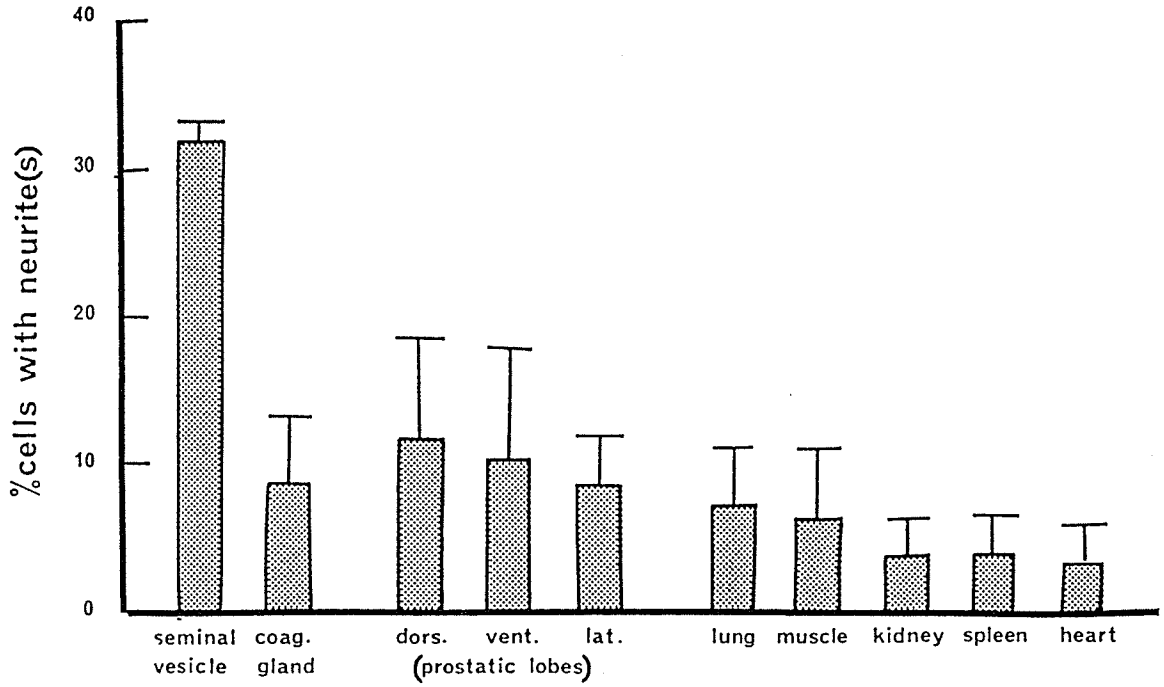

Figure 7. Tissue distribution of the neurite outgrowth activity. Tissues were dissected from adult male SD rats, and extracted in PBS, followed by centrifugation. Samples of 200 µg of protein from each tissue extract was applied to the neurite outgrowth assay. Data represents means ± SEM of 3 or 4 experiments.

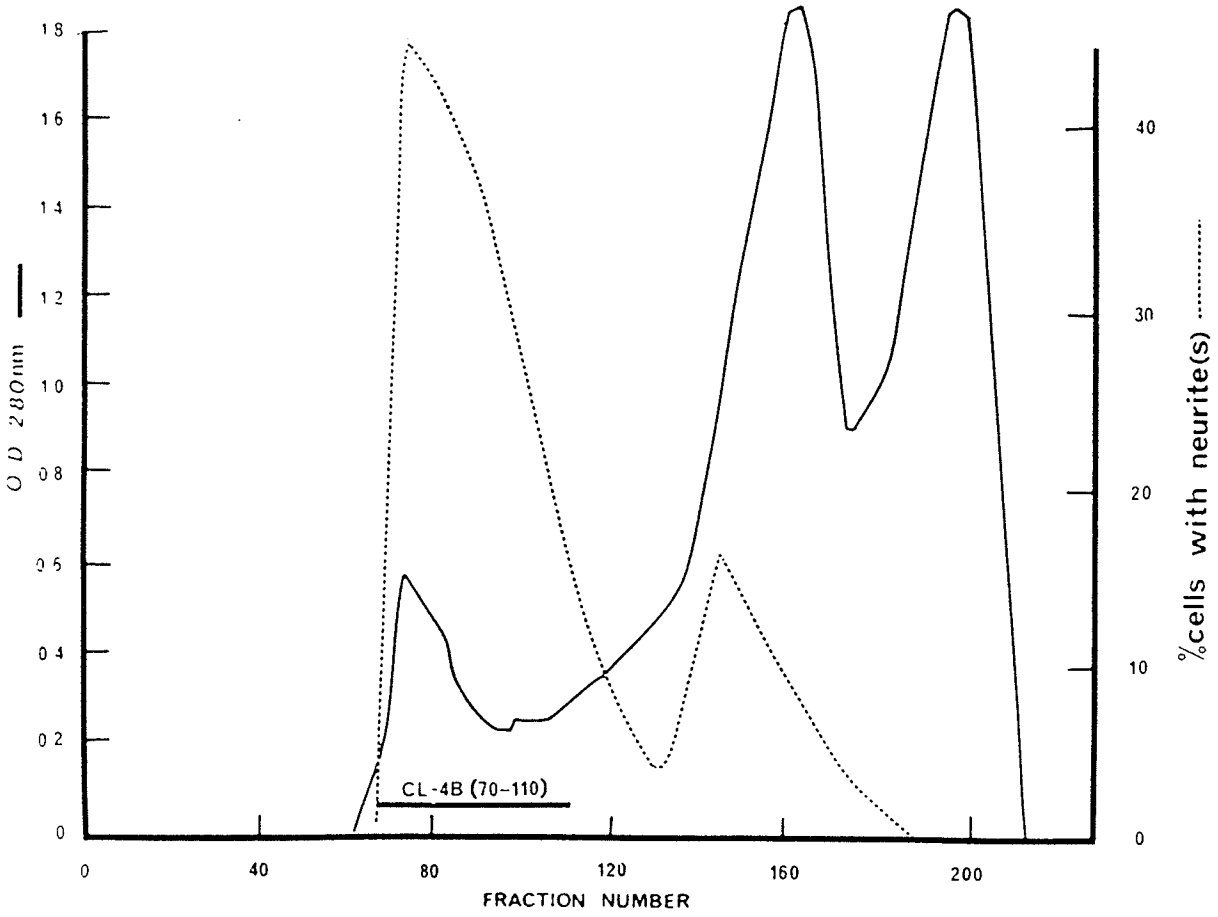

Figure 8. Gel permeation on Sepharose CL-4B. The SV tissue was homogenized in PBS buffer containing 1% β-mercaptoethanol, followed by centrifugation at 15K for one hour. The extract was then sonicated and centrifuged again at 40K for one hour. The clear supernatant was then applied onto a column (2.1 x 110cm) of Sepharose CL-4B (Pharmacia), equilibrated in PBS buffer containing 0.1% β-mercaptoethanol, and eluted with the same buffer. Fractions were concentrated on Centricon 10 microconcentrators, and assayed for neurite outgrowth activity. Protein content of the fractions was monitored by O.D. at 278 nm. (——), protein; (·····), neurite outgrowth activity.

B) Sucrose density gradient centrifugation

A linear sucrose density gradient ranging from 1.02-1.14 grams/ml was prepared in a ultracentrifuge tube. A sample of the concentrated active fractions after the Sepharose CL-4B column chromatography was separated into density fractions by centrifugation, and the fractions were assayed for neurite outgrowth activity. The majority of the NOSF was concentrated in sucrose solution corresponding to a density of 1.06-1.08 grams/ml, with activity trailing off to a density as high as 1.10 grams/ml (Fig. 9).

C) Summary of purification

Table 4 summarizes the partial purification of the neurite outgrowth stimulating factor from the SV extracts. The activity recovery after gel filtration on the Sepharose CL-4B column was greater than 90%, and that of the sucrose density gradient centrifugation was 70%. The two steps achieved a sixty-fold purification over the crude material.

4) Characterization of partially purified neurite outgrowth stimulating factor

A) Polyacrylamide gel electrophoresis

Figure 10 shows the protein profile upon SDS-PAGE analysis of the fractions of the SV neurite outgrowth factor at different steps of purification. Gel permeation

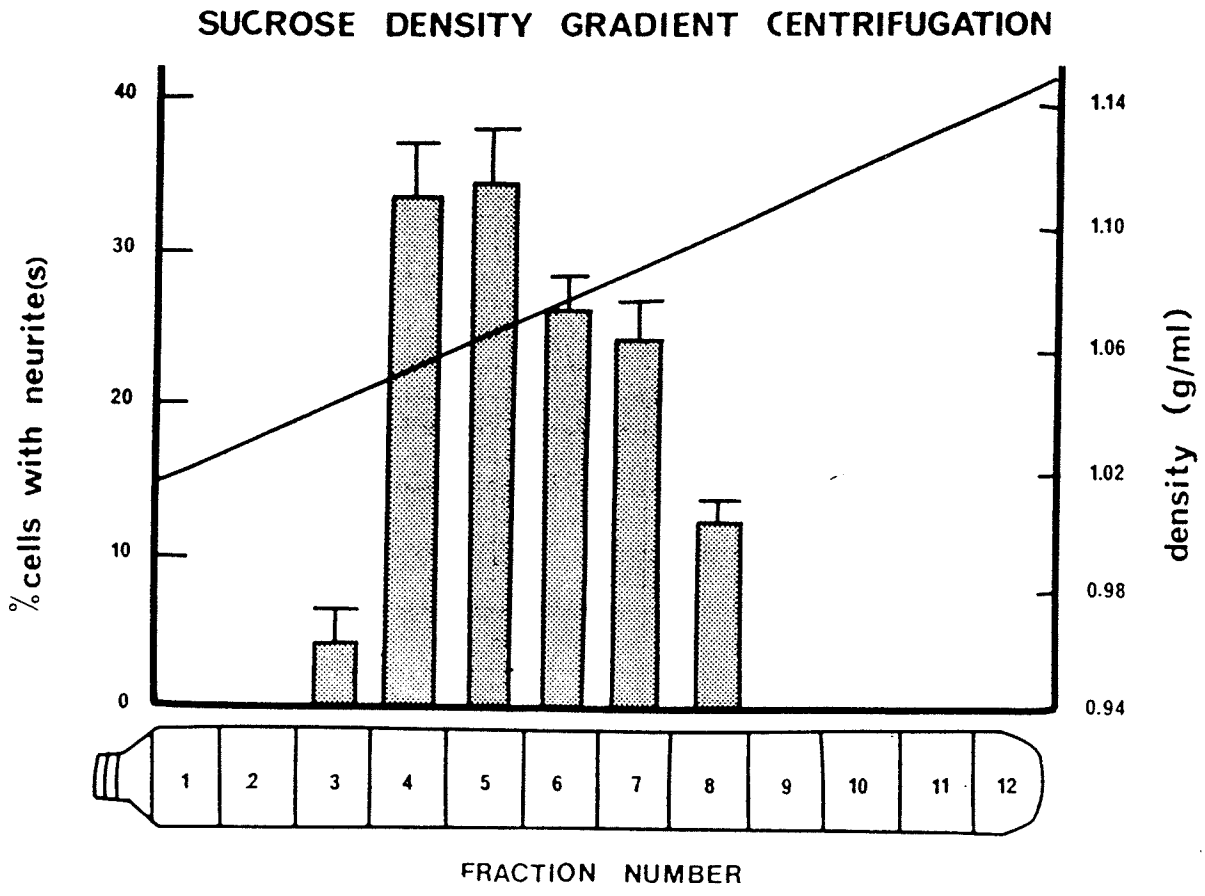

Figure 9. Sucrose density gradient centrifugation. Sucrose density gradients were produced in ultrancentrifuge tubes as described in Materials and Methods. After centrifugation, the fractions were washed free of sucrose, and assayed for neurite outgrowth activity (▩). The density gradient of the sucrose solution after centrifugation was measured to be 1.02 to 1.15 grams/ml (——).

Table 4. Summary of purification.

Purification step	Half-maximal activity[a] (µg/ml)	Purification (folds)	Activity recovery
Crude homogenate	63.7 ± 4.5	1.0	100.0
Gel permeation on Sepharose (CL-4B)[b]	6.6 ± 2.0	9.7	90.5
Sucrose density gradient[c]	1.1 ± 0.4	57.9	68.0

a) Half-maximal activity for each sample was determined from dose response curves at various dilutions of protein

b) Gel permeation sample: Fractions 70-110 from the Sepharose CL-4B column were pooled, concentrated, and assayed for neurite outgrowth promoting activity.

c) Sucrose density gradient centrifugation: Fractions corresponding to a density of 1.04-1.06 grams/ml of sucrose were washed free of sucrose, and assayed for neurite outgrowth promoting activity.

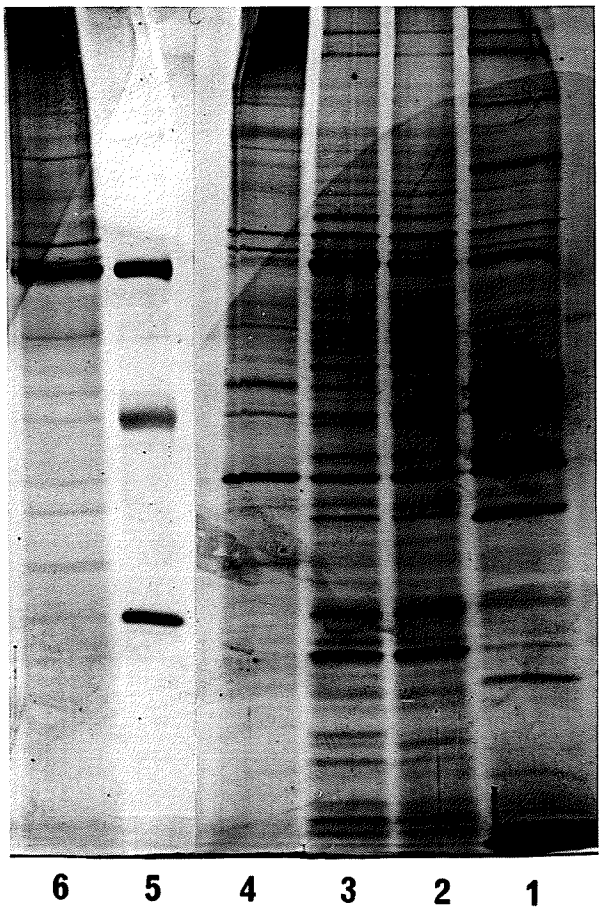

Figure 10. SDS-PAGE of SV-NOSF at various steps of purification. Samples of 5 µg of protein were analysed by 10% polyacrylamide gel: (1), seminal vesicle tissue homogenized in SDS sample buffer; (2), supernatant of low speed centrifugation of SV extract; (3), supernatant of high speed centrifugation; (4), Sepharose CL-4B fraction (Figure 8); (5), molecular weight markers of BSA (66 KDa), egg albumin (45 KDa) and carbonic anhydrase (29 KDa); (6), fractions of sucrose density gradient, 1.04-1.06 grams/ml. After electrophoresis, gel was stained with silver nitrate.

on Sepharose CL-4B and sucrose density gradient centrifugation steps removed a great deal of contaminating proteins from the samples, especially low molecular weight contaminants. However, the preparation of the rat SV NOSF remained quite crude, with at least 30 protein bands (Figure 10). An attempt to interpret which band corresponds to the neurite outgrowth activity would be premature.

B) Thin layer chromatography

Due to the bouyant nature of the neurite outgrowth activity (1.06-1.08 grams/ml), the lipid content of the Sepharose CL-4B active fraction (Figure 8) was examined by thin layer chromatography (t.l.c.) (Figure 11). The high molecular weight fraction after gel permeation was analysed to contain triglyceride (TG), fatty acids (FA), diglyceride (DG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomeylin (SM). This pattern of lipids is characteristic of membrane vesicles (Dr. G. Arthur, personal communication), as these lipids are the major components of cell membranes (Lehninger, 1972), and this would explain the appearently very large M.W. of the NOSF upon gel filtration on Sepharose CL-4B (Figure 8). Taking the bouyant density of the activity into account, it is possible that the neurite outgrowth factor is a component of, or is associated with, lipid vesicles, or is a separate protein component incompletely extracted from a membrane bound domain.

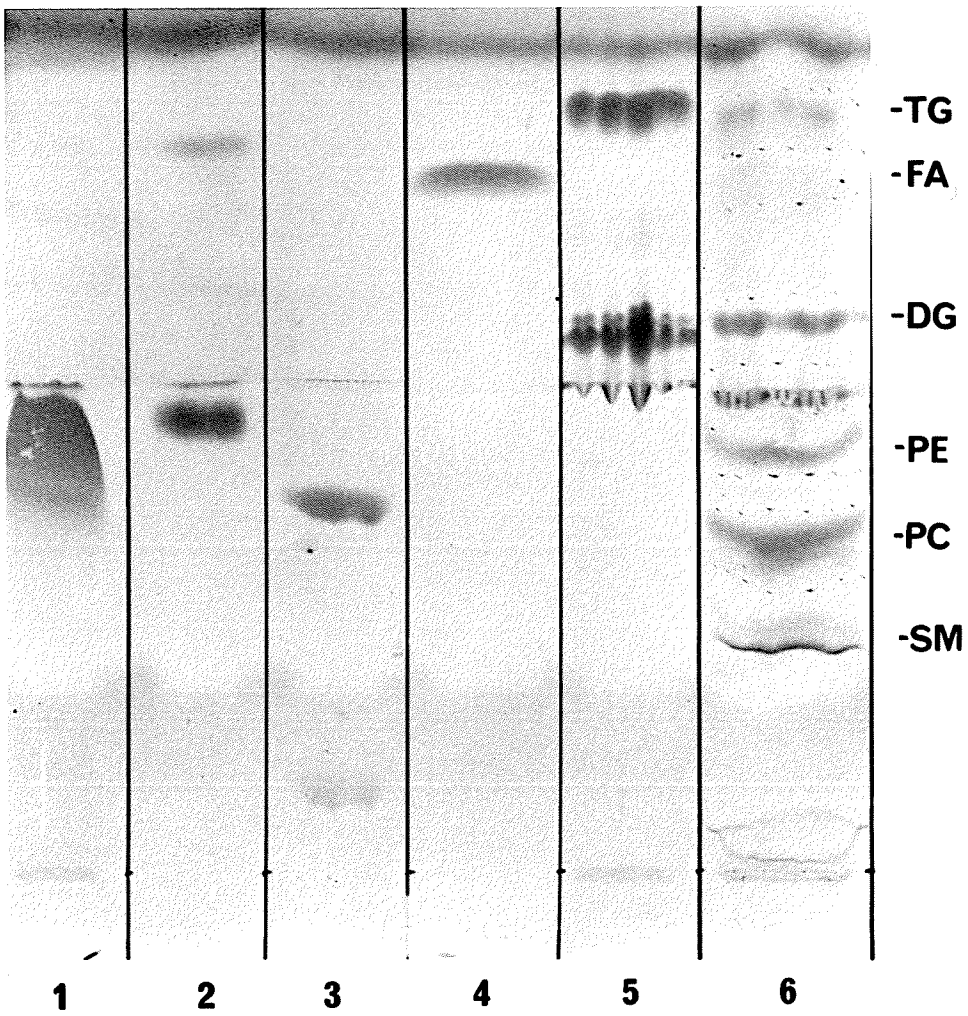

Figure 11 Thin layer chromatography of the total lipid content of the Sepharose CL-4B active fraction. Total lipids were extracted from the active fraction (Figure 8) and seperated by thin layer chromatography as described in "Materials and Methods". 1), phosphatidylserine; 2), phosphatidylethanolamine; 3), phosphatidylcholine; 4), fatty acid; 5), tri- and di-glycerides; 6), the Sepharose CL-4B active fraction.

C) Northern blot analysis of growth associated protein-43 in NG108-15 hybrid cells

GAP-43 is a neuronal growth associated protein, and is widely distributed throughout the mammalian brain. It is hypothesized that GAP-43 and other growth associated proteins are critical for axonal outgrowth during development and regeneration.

To test the functional specificity of the chemically synthesized GAP-43 probe, Northern blot analysis of total RNA from neonatal and adult rat brain was carried out as described in Figure 12(A). The presence of a RNA transcript just below the 18S ribosomal RNA (Figure 12), indicated a 1.2 Kb transcript of GAP-43 as described by Karns et al. (1987). The level of GAP-43 was much higher in neonatal than adult rat brains, similar to the findings of Jacobson et al. (1986).

Total RNA was then isolated from NG108-15 cells grown in medium D containing 10% FCS. Figure 12(B) shows that NG108-15 cells also expressed a RNA transcript for GAP-43, but the level in these actively proliferating cells was much lower (approximatly 8.5-fold) than that of the neonate brain.

To examine the effect of SV-NOSF on the levels of GAP-43 transcript in NG108-15 cells, RNA was isolated from cells cultured in medium D containing 10% FCS, cells in DMEM containing 5% FCS and cells in DMEM containing 5% FCS in the presence of SV-NOSF. Figure 13 shows that the

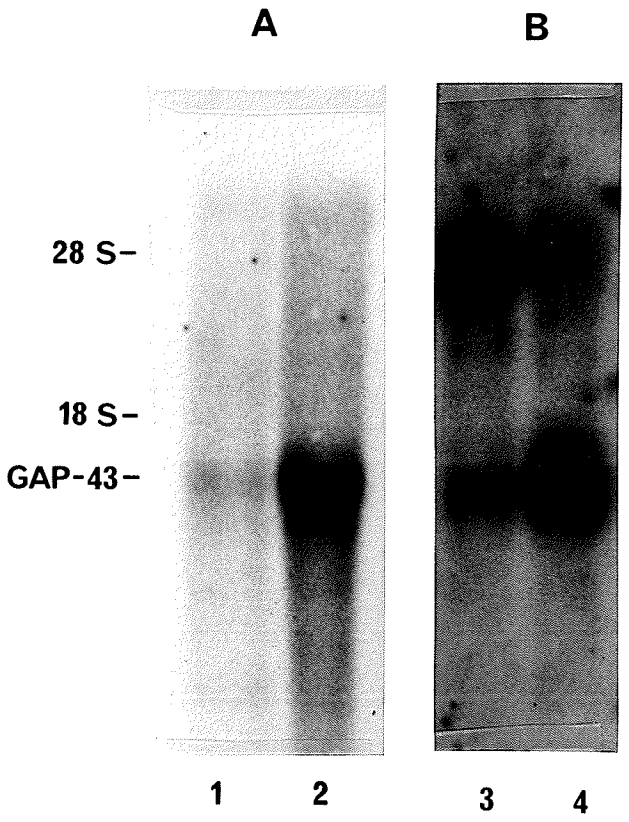

Figure 12. Northern blot analysis of GAP-43 mRNA. Total RNA was isolated from cultured NG108-15 cells, and whole brain of adult and 1-day neonate rats. Hybridizations were performed at 55°C and samples of 50 µg of total RNA was used for analysis. (1), adult rat; (2), neonate rat; (3), NG108-15 cells in medium D; (4), neonate rat.

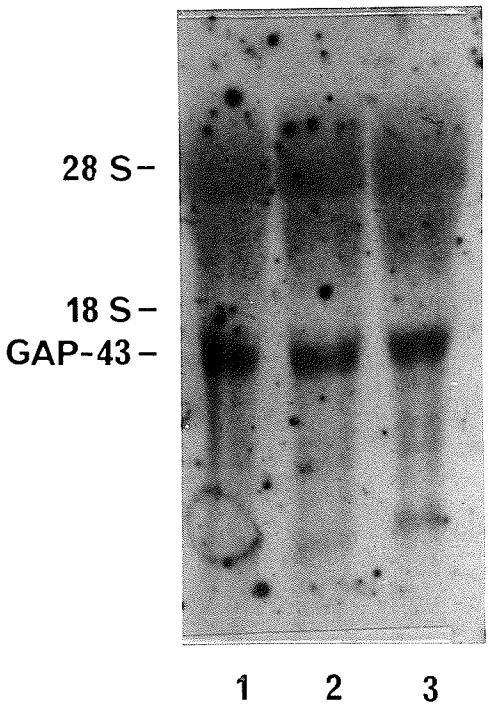

Figure 13. Northern blot analysis of total mRNA from NG108-15 cells. Total RNA was isolated from NG108-15 cells in growth medium D containing 10% FCS (1), cells replated in DMEM containing 5% FCS (2) and cells replated in DMEM containing 5% FCS in the presence of Sepharose CL-4B active fraction (3). Samples of 50 µg of total RNA was used, and the blot was hybridized at 55°C.

levels of GAP-43 mRNA transcript did not appear to be significantly different in NG108-15 cells grown in various culture conditions.

In order to quantify more accuretly any changes in levels of GAP-43 mRNA, dot blot analysis was performed. Total RNA, isolated from NG108-15 cells under various culture conditions, was blotted at 5 or 1 µg and hybridized with the GAP-43 DNA probe (Figure 14).

After hybridization, radioactive dots were cut off from the NC paper, soaked in 5 mls of scinti-versa, and counted by the liquid scintilation-counter (Table 5). In two separate experiments, levels of GAP-43 RNA transcripts were higher in cells grown in medium D containing 5% FCS then cells grown in the presence of 10% FCS. However, no significant difference in the level of GAP-43 mRNA was observed between untreated cells in 5% FCS and cells with neurite outgrowth induced by SV-NOSF (Table 5).

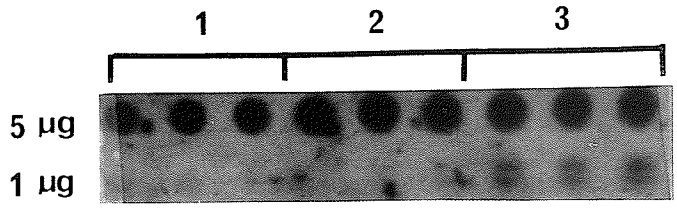

Figure 14. Dot blot analysis of total mRNA from NG108-15 cells. Total RNA was isolated from cells in growth medium D containing 10% FCS (1), cells replated in DMEM containing 5% FCS (2) and cells replated in DMEM containing 5% FCS in the presence of Sepharose CL-4B active fraction (3). Samples of 5 or 1 µg of total RNA were dotted onto nitrocellulose, and hybridized at 55°C with ^{32}P-labelled GAP-43 oligonucleotide probe.

Table 5. Quantitation of dot blot analysis. Dots of RNA, after hybridization to ^{32}P-labelled GAP-43 oligonucleotide probe were cut off from the NC paper, soaked in 5 ml of scinti-versa, and counted in a scintilation-counter. Data represent Means ± SEM in triplicates.

	Culture conditions	µg of RNA	cpm
Experiment 1	A) Medium D (10% FCS)	5.0	2174 ± 119.8
		1.0	906 ± 80.5
	B) DMEM (5% FCS)	5.0	2786 ± 151.3
		1.0	1235 ± 64.8
	C) DMEM (5% FCS) + CL-4B fraction	5.0	2571 ± 55.1
		1.0	1322 ± 127.1
Expt. 2	A) Medium D (10% FCS)	5.0	502 ± 20.0
		2.5	402 ± 25.0
	B) DMEM (5% FCS)	5.0	608 ± 30.4
		2.5	424 ± 20.4
	C) DMEM (5% FCS) + CL-4B fraction	5.0	638 ± 52.9
		2.5	444 ± 37.1

DISCUSSION

1) Conditions for assaying the neurite outgrowth activity using the hybrid NG108-15 cells line

It was observed that extracts of adult rat seminal vesicle were able to stimulate neurite outgrowth from hybrid NG108-15 cells. As an initial step in the purification of the active factor in the extract, a specific bioassay was established and optimized to characterize the active principle. Neurite outgrowth, in the presence of seminal vesicle extracts, was significantly higher in cells cultured in medium containg 5% FCS than in 10% FCS (Figure 3). This is expected since neuroblastoma cells have been shown to develop processes in low-serum or serum-free cultures, but not in the presence of higher serum concentrations (Seeds et al., 1970). One possible reason for this is the presence of serum growth factors or factors affecting the attachment of cells to plates. Therefore, a bioassay system of culturing NG108-15 cells in medium containg 5% FCS was established and performed routinely. This allowed maximal neurite outgrowth in induced cells, while preventing outgrowth in non-induced control cells.

The induction of neurite outgrowth by seminal vesicle extracts was relatively rapid (Figure 2). Cells were routinely incubated with seminal vesicle extracts for 6 hours to produce maximal neurite outgrowth. 'Rapid onset' neurite formation has been observed in NG108-15 cells

grown on laminin (Smalheiser and Schwartz, 1987), and in other neuroblastomas in the presence of haemin (Ishii and Maniatis, 1978); whereas neurite outgrowth from neuroblastoma is slower in the presence of other inducing agents (Ishii and Maniatis, 1978), or from primary neuronal cultures (Klingman, 1982).

It was interesting to observe that seminal vesicle extracts stimulated neurite outgrowth without effecting the growth rate of the cells (Figure 4). In this regard, this neurite outgrowth stimulating effect of the seminal vesicle was similar to that of a glial cell protease inhibitor which also stimulated neurite outgrowth from neuroblastoma cells without effecting cell growth (Monard et al., 1973; Guenther et al., 1985).

2) Characterization of the neurite outgrowth stimulating factor

The fact that the seminal vesicle neurite outgrowth stimulating factor did not bind to poly-cationic surfaces or tissue culture plastic plates (Table 1) distinguishes it from poly-cationic neurite promoting factors (PNPFs) from a variety of conditioned media (Manthrope et al., 1981; Lander et al., 1982; Coughlin et al., 1981). Recently, laminin has been shown to be a key component of the various PNPFs (Davis et al., 1985c; Lander et al., 1985b). The lack of substratum-adhesion of the seminal vesicle factor indicates that it is not related to the PNPFs or to laminin (Baron-Van Evercooren, 1982). The neurite out-

growth activity from seminal vesicle did not bind to heparin-sepharose in buffer containing 0.5M NaCl (Fig. 6). Acidic- and basic-FGF both bind strongly to heparin-sepharose in buffer containing NaCl as high as 1.6 M; whereas, EGF does not have affinity for heparin-sepharose (Folkman, 1986). However, EGF is stable to boiling (Cohen, 1962), unlike the seminal vesicle factor which was heat labile (Table 3).

The neurite outgrowth activity, at least in the rat, was relatively specific to the seminal vesicle, although slight activity was observed in the prostate (Figure 7). NGF is found in high concentrations in various parts of the male genital tract, including the prostate gland of the guinea pig (Harper et al., 1979), rabbit and bull (Harper and Thoenen, 1980), bull seminal plasma (Harper and Thoenen, 1980) and bull seminal vesicle (Hofmann and Unsicker, 1982). Hofmann and Unsiker (1987) partially purified from bovine seminal vesicle a protein factor, which is similar to NGF and affects almost the same spectrum of target tissues. However, NGF is not able to stimulate neurite outgrowth from mouse neuroblastoma cells (Bottenstein, 1981), and purified NGF (Sigma) did not stimulate neurite outgrowth from NG108-15 cells (data not shown).

3) Partial purification of the neurite outgrowth stimulating factor

Fractionation of seminal vesicle extracts by gel

filtration on Sepharose CL-4B revealed the neurite outgrowth stimulating activity in the void volume, corresponding to a M.W. of 2000 KDa (Figure 8). PNPFs from various CM are also known to correspond to large M.W. fractions of 1 million or more daltons (Manthrope et al., 1981; Lander et al., 1982; Coughlin et al., 1982). However, the seminal vesicle factor differs from these compounds as it did not bind to polycationic substrates. In addition, PNPFs have been shown to be co-purified with the ECM component laminin, which equilibrated in the $CsCl_2$ gradient at a density of approximently 1.35 grams/ml (Engel and Furthmayr, 1987), as did the PNPFs (Lander et al., 1982); whereas, the seminal vesicle factor equilibrated at a density of 1.06-1.08 grams/ml (Figure 9).

The bouyant density of 1.06-1.08 observed for the seminal vesicle factor corresponds to that of a class of lipoproteins, the low-density lipoproteins, which have densities in the range of 1.019-1.063 grams/ml and a molecular weight of 2300 KDa (Gotto et al., 1986). The low-density lipoproteins contain 40-50% cholesterol (Gotto et al., 1986); however, the active fractions of the seminal vesicle extract after gel filtration upon Sepharose CL-4B (Fig. 8) contained no cholesterol upon analysis of lipid content (Figure 11). Interestingly, these active fractions showed instead lipids characteristic of membranes (Lehninger, 1972) and were likely due to non-specific membrane vesicles, which were created upon extraction of tissues in aqueous buffers (Dr. G. Arthur,

personal communication). Furthermore, such vesicles have bouyant densities similar to that observed for the seminal vesicle neurite outgrowth factor (Dr. G. Arthur, personal communication). Artificial liposomes composed of mixtures of phosphatidylcholine and phosphatidylserine were shown to stimulate neurite outgrowth in a mouse neuroblastoma cell line (Chen et al., 1976). However, it is fair to point out that the seminal vesicle neurite outgrowth factor was specific to the seminal vesicle and was labile to heat. It is therefore unlikely that the active principle is due to non-specific vesicles of lipids acting alone to cause neurite outgrowth. It is possible that a protein, tightly bound in a membrane domain, is extracted from the seminal vesicle tissue in association with membrane lipids, and that the lipid vesicle acts as a carrier for the protein to penetrate the NG108-15 cell membranes, as described for artificial liposomes (Chen et al., 1976).

4) Analysis of growth associated protein-43 in NG108-15 cells

Northern blot analysis of total RNA from rat brain, probed with an oligonucleotide of the reverse complement of GAP-43 mRNA, revealed the presence of GAP-43 mRNA corresponding to a transcript size of 1.2 Kb (Figure 12). The transcript size of 1.2 Kb is identical to that observed recently for GAP-43 from rat dorsal root ganglia and brain (Karns et al., 1987). Higher levels of GAP-43 in neonatal than adult brain tissues (figure 12), agrees with

previous findings (Jacobson et al., 1986) that GAP-43 protein is important in some aspects of brain development.

GAP-43 mRNA was expressed in actively growing NG108-15 cells (Figure 13). The level of GAP-43 message under these conditions was much lower than what was seen in neonate rat brains on the basis of total RNA, and corresponded more closely to levels seen in the adult brain. Sub-culturing NG108-15 cells from medium containing 10% FCS to medium containing 5% FCS led to a slight increase in the level of GAP-43 mRNA (Figure 14, Figure 15, Table 5). However, no change was observed in GAP-43 levels after an increase in neurite outgrowth of 30-40%, stimulated by incubating with the partially purified SV neurite outgrowth fraction (Table 5). Recently, Karns et al. (1987) demonstrated that GAP-43 mRNA was expressed in the pheochromocytoma cell line PC12, and that the level of the message increased dramatically upon morphological differentiation induced by NGF or cAMP. From our findings, however, the expression of GAP-43 mRNA in NG108-15 cells did not appear to be regulated during neurite outgrowth. Alternatively, it is possible that GAP-43 levels changed transiently during the outgrowth process over a certain time frame that was not appropriately examined in this study. It appears that culturing of NG108-15 cells in medium containing lower concentrations of FCS caused a slight increase in GAP-43 message (Table 5), suggesting that serum growth factors are inhibitory to GAP-43 expression. However, the role of GAP-43 in the process of neur-

ite outgrowth in NG108-15 cells remains unclear.

5) Possible functions of the seminal vesicle neurite outgrowth factor

The presence of NGF and a NGF-like molecule in the seminal vesicle and other prostatic tissues has been documented by several investigators (Harper et al., 1979; Harper and Thoenen, 1980; Hofmann and Unsicker, 1982; Hofmann and Unsicker, 1987). It is possible that the presence of large concentrations of NGF in male reproductive tissues explains the observation that sympathetic ganglia, which send fibers to these tissues, are not vulnerable to immunosympathectomy created by injections of NGF antibodies into neonatal rats (Levi-Montalcini, 1982). However, the effects of these factors were neutralized by NGF antibodies in culture (Harper et al., 1979; Harper and Thoenen, 1980; Hofman and Unsicker, 1982), and the factor purified by Hofman and Unsicker (1987) may actually be NGF. It is therefore possible that a factor, unrelated to NGF, which plays a role in the maintenance of sympathetic tracts in the presence of NGF antibodies may also be present in the seminal vesicle. Evidence for the factor described in this report to play such a role will await for its purification and further studies.

Clearly, the seminal vesicle factor has the ability to induce a more differentiated phenotype in neuroblastoma cells. Neuroblastoma tumors may arise from any dividing neuronal cells (Amano et al., 1972). The seminal vesicle

factor, acting in an endocrine or paracrine manner, may be important in inducing a more differentiated phenotype in neuroblasts during embryonic and early postnatal development. Its production in adult tissues could prevent regression of mature neurons to an undifferentiated form. However, one must stress the conjectural nature of any attempt to assign a functional role to the seminal vesicle factor. Deineation of its physiological role must await its purification and biochemical characterization of the factor will allow further studies to delineate its physiological role.

REFERENCES

Acheson, A.L. and H. Thoenen. 1983. Cell contact-mediated regulation of tyrosine hydroxlase synthesis in cultured bovine adrenal chromaffin cells. J. Cell Biol. 97:925-928.

Acheson, A.L., D. Edgar, R. Timpl and H. Thoenen. 1986. Laminin increases both levels and activity of tyrosine hydroxylase in calf adrenal chromaffin cells. J. Cell. Biol. 102:151-159.

Ackers, R.M., D.F. Mosher and J.E. Lilien. 1981. Promotion of retinal neurite outgrowth by substum-bound fibronectin. Dev. Biol. 86:179-188.

Adler, R., M. Manthrope, S.D. Skaper and S. Varon. 1981. Polyornithine-attached neurite-promoting factors (PNPFs). culture sources and responsive neurons. Brain Res. 206:129-144.

Aloyo, V.J., H. Zwiers and W.H. Gispen. 1983. Phosphorylation of B-50 protein by calcium-activated phospholipid dependant protein kinase and B-50 protein kinase. J. Neurochem. 41:649-653.

Amano, A., E. Richelson and M. Nirenberg. 1972. Neurotransmitter synthesis by neuroblastoma clones. Proc. Natl. Acad. Sci. USA. 69:258-263.

Ard, M.D., R.P. Bunge and M.B. Bunge. 1987. Comparison of the Schwann cell surface, Schwann cell extracellular matrix as promotors of neurite growth. J.

Neurocytology. 16:539-555.

Arthur, G., L. Page, T. Mock and P.C. Choy. 1986. The catabolism of plasmenycholine in the guinea-pig heart. Biochem. J. 236:475-480.

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

Barbin, G., M. Manthrope and S. Varon. 1984. Purification of the chick eye ciliary neurotrophic factor. J. Neurochem. 43:1468-1478.

Barde, Y-A., D. Edgar and H. Thoenen. 1982. Purification of a new neurotrophic factor from mammalian brain. EMBO. J. 1:549-553.

Barde, Y-A., D. Edgar and H. Thoenen. 1983. New neurotrophic factors. Ann. Rev. Physiol. 45:601-612.

Barde, Y-A., R.M. Lindsay, D. Monard and H. Thoenen. 1978. New factor released by cultured glioma cells supporting survival and growth of sensory neurons. Nature. 274:818.

Baron-Van Evercooren, A., H.K. Kleinman, S. Ohno, P. Marangos, J.P. Schwartz and M.E. Dubois-Dalcq. 1982. Nerve growth factor, laminin, and fibronectin promote neurite growth in human fetal sensory ganglia cultures. J. Neurosci. Res. 8:179-193.

Bennet, M.R. and V. Nurcombe. 1979. The survival and development of cholinergic neurons in skeletal muscle conditioned medium. Brain Res. 173:543-548.

Benowitz, L.I. and A. Routtenberg. 1987. A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism, and synaptic plasticity. Trends Neurosci. 10:527-532.

Benowitz, L.I., P.J. Apostolides, N. Perrone-Bizzazero, S.P. Finklestein and H. Zwiers. 1988. Anatomical distribution of growth-associated protein GAP-43/B-50 in the adult rat brain. J. Neurosci. 8:339-352.

Berg, D.K. 1984. New neuronal growth factors. Ann. Rev. Neurosci. 7:149-170.

Beuker, E.D. 1948. Implantation of tumors in the hind limb field of the embryonic chick and the developmental response of the lumbrosacral nervous system. Anat. Rec. 102:369-390.

Bixby, J.L., R.S. Pratt, J. Lilien and L.F. Reichardt. 1988. Neurite outgrowth on muscle cell surfaces involves extracellular matrix receptors as well as Ca^{2+}-dependant- and independant-cell adhesion molecules. Proc. Natl. Acad. Sci. USA. 84:2555-2559.

Bligh, E.G. and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Bioc-

hem. Physiol. 37:911-917.

Bottenstein, J.E. 1981. Differentiated properties of neuneuronal cell lines. In Functionally Differentiated cell lines. Alan R. Liss, Inc. New York, NY. pp 154-184

Borg, J., T.H. Hietter, M. Henry, G. Schmitt and B. Luu. 1987. Neurotrophic effects of naturally occuring long-chain fatty alcohols on cultured CNS neurons. FEBS Lett. 213:406-410.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utalizing the principle of protein-dye binding. Anal. Biochem. 72:248-253.

Carbonetto, S., M.W. Gruver and D.C. Turner. 1983. Nerve fiber growth in cultures on fibronectin, colagen, and glycosaminoglycan substrates. J. Neurosci. 3:2324-2335.

Carpenter, G. and S. Cohen. 1979. Epidermal growth factor. Ann. Rev. Biochem. 48:193-216.

Chaput, M., V. Class, D. Portetelle, I. Cludts, A. Cravader, A. Burny, H. Gras and A. Tartar. 1987. The neurotrophic factor neuroleukin is 90% homologous with phosphohexose isomerase. Nature. 332:454-455.

Chen, J.S., A.D. Fa, A. DiLuzio and P. Callisane. 1976. Liposome induced morphollogical differentiation of murine neuroblastoma. Nature. 263:604-606.

Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald and W.J.

Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry. 18:5294-5299.

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

Cohen, S. 1962. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J. Biol. Chem. 237:1555-1562.

Cohen, S., R. Levi-Montalcini and V. Hamburger. 1954. A nerve growth stimulating factor isolated from sarcoma 37 and 180. Proc. Natl. Acad. Sci. USA. 40:1014-1018.

Coughlin, M.D., E.M. Bloom and I.R. Black. 1981. Characterization of a neuronal growth factor from mouse heart-cell-conditioned medium. Dev. Biol. 82:56-68.

Davies, A.M., H. Thoenen and Y-A. Barde. 1986. The response of chick sensory neurons to brain-derived neurotrophic factor. J. Neurosci. 6:1897-1904.

Davis, G.E., F.G. Klier, E. Engvall, C. Cornbrookes, S. Varon and M. Manthrope. 1987. Association of laminin with heparin and chondroitin sulfate-bearing proteoglycans in neurite-promoting factor complexes from rat Schwannoma cells. Neuroc-

hem. Res. 12:909-921.

Davis, G.E., S. Varo, E. Engval, and M. Manthrope. 1985a. Substrtum binding neurite-promoting factors: relationship to laminin. Trends Neurosci. 8:528-532.

Davis, G.E., M. Manthrope, E. Engvall and S. Varon. 1985b. Isolation and characterization of rat Schwannoma neurite-promoting factor: evidence that the factor contains laminin. J. Neurosci. 5:2662-2671.

Davis, G.E., M. Manthrope and S. Varon. 1985c. Parameters of neurite growth from ciliary ganglion neurons in vitro: influence of laminin, Schwannoma polyornithine-binding neurite promoting factor and ciliary neurotrophic factor. Dev. Brain Res. 17:75-84.

Davis, L.G., M.D. Dibner and J.F. Battey. 1986. Basic Methods in Molecular Biology. Elsevier Science Publishing Co. Inc. New York. 388 pp.

Engel, J. and H. Furthmayr. 1987. Electron microscopy and other physical methods for the characterization of extra cellular matrix components; laminin, fibronectin, collagen IV, collagen VI, and proteoglycans. Methods in Enzymology. 145:3-78.

Fallon, J.R. 1985. Preferential outgrowth of central nervous system neurites on astrocyte and Schwann cells as compared with non-glial cells in vitro. J. Cell Biol. 100:198-207.

Folkman, J. 1986. How is blood vessel growth regulated in normal and neoplastic tissue? Cancer Res. 46:467-473.

Fukuda, K. 1985. Purification and partial characterization of a cholinergic neuronal differentiation factor. Proc. Natl. Acad. Sci. USA. 82:8795-8799.

Gallo, V., M.T. Ciotti, A. Coletti, F. Alosis and G. Levi. 1982. Selective release of glutamate from cerebellar granule cells differentiating in culture. Proc. Natl. Acad. Sci. USA. 79:7919-7932.

Garthwaite, J., G. Garthwaite and F. Hajos. 1986. Amino acid neurotoxicity: relationship to neuronal depolarization in rat cerebellar slices Neuroscience. 18:449-460.

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

Gotto, A.M., H.J. Pownall and R.J. Havel. 1986. Introduction to the plasma lipoproteins. Methods in Enzymology. 128:3-41.

Guenther, J., H. Nick and D. Monard. 1985. A glial derived neurite promoting-factor with protease inhibatory activity. EMBO. J. 4:1963-1966.

Gurney, M.E., S.P. Heinrich, M.R. Lee and H.S. Yin. 1986a. Molecular cloning and expression of neuroleukin, a neurotrophic factor for spinal and

sensory neurons. Science. 234:566-574.

Gurney, M.E., B.R. Apatoff, G.T. Spear, M.J. Baumel, J.P. Antel, M.B. Binia and A.T. Reder. 1986b. Neuro leukin: a lymphokine product of lectin-stimulated T cells. Science. 234:574-581.

Hamburger, V. 1980. Trophic interactions in neurogenisis: a personal historical account. Ann. Rev. Neurosci. 3:269-277.

Hamburger, V. and R. Levi-Montalcini. 1949. Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J. Exp. Zool. 111:457-501.

Hamprecht, B. 1977. Structural, electrophysiological, biochemical, and pharmacological properties of neuroblastoma-glioma cell hybrids in culture. Int. Rev. Cytol. 49:99-170.

Harper, G.P. and H. Thoenen. 1980. Nerve growth factor: biological significance, measurment, and distribution. J. Neurochem. 34:5-16.

Harper, G.P., Y-A. Barde, G. Burnstock, J.R. Carstairs, M.E. Dennison, K. Suda and C.A. Vernon. 1979. Guinea pig prostate is a rich source of nerve growth factor. Nature. 279:160-162.

Hatta, K., A. Nose, A. Nagafuchi and M. Takeichi. 1988. Cloning and expression of cDNA encoding a neural calcium-dependant cell adhesion molecule: its identity in the cadherin gene family. J. Cell

Biol. 106:873-881.

Hayashi, Y., H. Taniura and N. Miki. 1987. A proteolytic 140-KDa fragement of neurite outgrowth factor and its cell-binding activity. Neurosci. Lett. 79:321-325.

Helfand, S.L., G.A. Smith and N.K. Wessells. 1976. Survival and development in culture of dissociated parasympathetic neurons from ciliary ganglia. Dev. Biol. 50:541-547.

Hofmann, H.D. and K. Unsicker. 1982. The seminal vesicle of the bull: a new and very rich source of nerve growth factor. Eur. J. Biochem. 128:421-426.

Hofmann, H.D. and K. Unsicker. 1987. Characterization and partial purification of a novel neurotrophic factor from bovine seminal vesicle. J. Neurochem. 48:1425-1433.

Hollyday, M. and V. Hamburger. 1976. Reduction of the naturally occuring motorneuron loss by enlargement of the periphery. J. Comp. Neurol. 170:311-320.

Howard, E. 1974. Hormonal effects on the growth and DNA content of the developing brain. In Biochemistry of the Developing Brain. Vol. 2 (E. Hirivich and J. Dekker eds.).

Hymanns, J. and K. Unsiker. 1987. Neuroblastoma cells contain a trophic factor sharing biological and molecular properties with ciliary neurotrophic factor. Proc. Natl. Acad. Sci. 84:7758-7762.

Ishii, D.N. and G.M. Maniatis. 1978. Haemin promotes rapid neurite outgrowth in cultured mouse neuroblastoma cells. Nature. 274:372-373.

Isobe, T. and T. Okuyama. 1978. The amino-acid sequence of S-100 protein (PAP-Ib protein) and its relationship to calcium binding proteins. Eur. J. Biochem. 89:379-388.

Jacobson, R.D., I. Varag and J.H.P. Skene. 1986. A protein associated with axon growth, GAP-43 is widely distributed and developmentally regulated in rat CNS. J. Neurosci. 6:1843-1855.

Karns, L.R., S-C. Ng, J.A. Freeman and M.C. Fishman. 1987. Cloning of complementary DNA for GAP-43, a neuronal growth related protein. Science. 236:597-600.

Katz, F., L. Ellis and K.H. Pfenninger. 1985. Nerve growth cones isolated from fetal rat brain. III calcium-dependant protein phosphorylation. J. Neurosci. 6:1402-1411.

Klingman, D. 1982. Isolation of a protein from bovine brain which promotes neurite extension from chick embryo cerebral cortex neurons in defined medium. Brain Res. 250:93-100.

Klingman, D. and L-S. Hsieh. 1987. Neurite extesion factor induces rapid morphologicall differentiation of mouse neuroblastoma cells in defined medium. Dev. Brain Res. 33:296-300.

Klingman, D. and D.R. Marshak. 1984. Purification and characterization of a neurite extension factor

from bovine brain. Proc. Natl. Acad. Sci. USA. 82:7136-7139.

Lagenaur, C. and V. Lemmon. 1987. An L-1 like molecule,the 8D9 antigen, is a potent substrate for neurite extension. Proc. Natl. Acad. Sci. USA. 84:7753-7757.

Lander, A.D., D.K. Fujii, D. Gospodarowicz and L.F. Reichart. 1982. Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparan sulfate proteoglycan. J. Cell Biol. 94:574-585.

Lander, A.D., D.K. Fujii and L.F. Reichardt. 1985a. Purification of a factor that promotes neurite outgrowth: isolation of laminin and associated molecules. J. Cell Biol. 101:893-913.

Lander, A.D., D.K. Fujii and L.F. Reichardt. 1985b. Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned medium. Proc. Natl. Acad. Sci. USA. 82:2183-2187.

Lasek, R.J.. 1981. Dynamic ordering of neuronal cytoskeletons. Neurosci. Res. Prog. Bull. 19:7-32.

LeDouarin, N.M., D. Renaud, M.A. Teillet and G.H. LeDouarin. 1975. Cholinergic differentiation of presumptive adrenergic neuroblasts in intraspecific chimeras after heterotropic transplants. Proc. Natl. Acad. Sci. USA. 72:728-732.

Lehninger, A.L. 1972. Biochemistry. Worth Publishers Inc., New York, N.Y. pp 189-215.

Lehrach, H., D. Diamond, J.M. Wazney and H. Boedtker. 1977. RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry. 16:4743-4751.

Levi-Montalcini, R. 1952. Effects of mouse tumor transplantation on the nervous sytem. Ann. NY. Acad. Sci. 55:330-343.

Levi-Montalcini, R. 1982. Developmental neurobiology and the natural history of nerve growth factor. Ann. Rev. Neurosci. 5:341-362.

Levi-Montalcini, R. and P.H. Angeletti. 1966. Immunosympathectomy. Pharmaco. Rev. 18:619-628.

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

Levi-Montalcini, R., R. Myer and V. Hamburger. 1954. In vitro experiments on the effects of mouse sarcoma on the sensory and sympathetic ganglia of the chick embryo. Cancer Res. 14:49-57.

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

Lindsay, R.M. and J. Tarbit. 1979. Developmentally regulated induction of neurite outgrowth from immature chick sensory neurons (DRG) by homogenates

of avian or mammalian heart, liver, and brain. Neurosci. Lett. 12:195-200.

Manthrope, M., E. Engvall, E, Ruoslahti, F.M. Longo, G.E. Davis and S. Varon. 1983. Laminin promotes neurite regeneration from cultured peripheral and central neurons. J. Cell Biol. 97:1882-1890.

Manthrope, M., S. Varon and R. Adler. 1981. Neurite-promoting factor in conditioned medium from RN22 Schwannoma cultures: bioassay, fractionation, and properties. J. Neurochem. 37:759-767.

Matsunaga, M., K. Hatta, A. Nagafuchi and M. Takeichi. 1988. Guidance of optic nerve fibers by N-cadherin adhesion molecules. Nature. 334:62-64.

Monard, D., F. Solomon, M. Rentsch and R. Gysin. 1973. Glial-induced morphollogical differentiation in neuroblastoma cells. Proc. Natl. Acad. Sci. USA. 70:1894-1897.

Morrison, R.S., H.I. Kornblum, F.M. Leslie and R.A. Bradshaw. 1987. Trophic stimulation of cultured neurons from neonatal rat brain by epidermal growth factor. Science. 238:72-75.

Morrissey, J.H. 1981. Silver stain for proteins in polyacrylamide gels: a modified proceedure with enhanced uniform sensitivity. Anal. Biochem. 117:307-310.

Moya, K.L., L.I. Benowitz, S. Jhaveri and G.E. Schneider. 1987. Enhanced visualization of axonally transported proteins in the immature CNS by suppres-

sion of systemic labeling. Dev. Brain Res. 31:183-191.

Muller, H.W., S. Beckh and W. Seifert. 1984. Neurotrophic factor for central neurons. Proc. Natl. Acad. Sci. USA. 81:1248-1252.

Natarajan, V., H.H.O. Schmid and P.S. Sastry. 1984. Biosynthesis of long-chain alcohols by developing and regenerating rat sciatic nerve. J. Neurochem. 43:328-334.

Nelson, P., C. Christian and M. Nierenberg. 1976. Synapse formation between clonal neuroblastoma x glioma hybrid cells and striated muscle cells. Proc. Natl. Acad. Sci. USA. 73:123-127.

Nelson, R.B. and A. Routtenberg. 1985. Characterization of protein F1 (47KDa, 4.5 pI): a kinase C substrate directly related to neural plasticity. Exp. Neurol. 89:213-224.

Nierenberg, N., S. Wilson, H. Higashida, A. Potter, K. Krueger, N. Busis, R. Ray, J.G. Keinmer and M. Adler. 1983. Modulation of synapse formation by cyclic adenosine monophosphate. Science. 222:794-799.

Ogita, Z-I. and C.L. Market. 1979. A miniaturized system for electrophoresis on polyacrylamide gels. Anal. Biochem. 99:233-241.

Oppenheim, R.W. 1981. Neuronal cell death and some related regressive phenomena during neurogenisis: In Studies in Developmental Neurobilogy: Essays in

Honor of Victor Hamburger. (W.M. Cowan ed.). Oxford University Press. New York, NY. pp. 74-133.

Patterson, P.H. 1978. Environmental determination of autonomic neurotransmitter function. Ann. Rev. Neurosci. 1:1-17.

Patterson, P.H. and L.L.Y. Chun. 1974. The influence of non-neuronal cells on catecholamine and acetylcholine sythesis and accumulation in cultures of dissociated sympathetic neurons. Proc. Natl. Acad. Sci. USA. 71:3607-3610.

Patterson, P.H. and L.L.Y. Chun. 1977. The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons Dev. Biol. 56:263-280.

Patterson, P.H. and D. Purves. 1982. Neuronal death in normal development. In Readings in Developmental Neurobiology. (P.H. Patterson and D. Purves, eds) Cold Spring Harbour Laboratory. pp. 217-277.

Pearce, I.A., M.A. Cambray-Deakin and R.D. Burgoyne. 1987. Glutamate acting on NMDA receptors stimulates neurite outgrowth from cerebellar granule cells. FEBS Lett. 223:143-147.

Purves, D. and J.W. Lichtman. 1985. Principles of Neural Development. Sinaur and Associates Inc. Sunderland, MA. 433pp.

Rauvala, H. and R. Pihlaskari. 1987. Isolation and some

characteristics of an adhesion factor of brain that enhances neurite outgrowth in central neurons. J. Biol. Chem. 262:16,625-16,635.

Richardson, P.M., V.M.K. Verg Issa and R.J. Riopelle. 1986. Distribution of neuronal receptors for nerve growth factor in the rat. J. Neurosci. 6:2312-2321.

Rogers, S.L., P.C. Letourneau, S.L. Palm, J. McCarthy and L.T. Furcht. 1983. Neurite expression by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev. Biol. 98:212-220.

Rutishauser, U., W.E. Gall and G.M. Edelman. 1978. Adhesion among neural cells of the chick embryo. lV role of the cell surface molecule CAM in the formation of neurite bundles in cultures of spinal ganglia. J. Cell Biol. 79:382-393.

Schubert, O. and M. LaCorbiere. 1985. Isolation of an adhesion-mediating protein from chick neural retina adherons. J. Cell Biol. 101:1071-1077.

Schubert, O., M. LaCorbiere and F. Esch. 1986. A chick neural retina adhesion and survival molecule is a retinol-binding protein. J. Cell Biol. 102:2295-2301.

Schubert, O., M. LaCorbiere, F.G. Klier and G. Birdwell. 1983. The structure and function of myoblast adheron. Cold Spring Harbour Symp. Quant. Biol. 48:539-549.

Seeds, N.W., A.G. Gilman, T. Amano and M.W. Nirenberg. 1970. Regulation of axon formation by clonal lines of a neural tumor. Proc. Natl. Acad. Sci. USA. 66:160-167.

Sieber-Blum, M., E. Sieber and K.M. Yamada. 1981. Cellular fibronectin promotes adrenergic differentiation of quail neural crest cells in vitro. Exp. Cell Res. 133:285-295.

Skene, J.H.P. 1984. Growth-associated proteins and the curious dichotomies of nerve regeneration. Cell. 37:697-700.

Skene, J.H.P. and M. Willard. 1981a. Changes in axonally transported proteins during axon regeneration in toad retinal ganglion neurons. J. Cell Biol. 89:86-95.

Skene, J.H.P. and M. Willard. 1981b. Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous sytem. J. Cell Biol. 89:96-103.

Smalheiser, N.P. and N.B. Schwartz. 1987. Kinetic analysis of 'rapid onset' neurite formation in NG108-15 cells reveals a dual role for substratum bound laminin. Dev. Brain Res. 34:111-121.

Thoenen, H. and Y-A. Barde. 1980. Physiology of nerve growth factor. Physiol. Rev. 60:1284-1335.

Thoenen, H. and D. Edgar. 1985. Neurotrophic factors. Science. 229:238-242.

Thoenen, H., Y-A. Barde, A.M. Davies and J.E. Johnson.

1978b. Neurotrophic factors and neuronal death. In Selective Neuronal Death. (G. Beck and M. O'Connor eds). Ciba Foundation Symposium 126. John Wiley and Sons. Toronto. pp. 82-95.

Thoenen, H., C. Bundtlow and R. Heumann. 1987a. The physiological function of nerve growth factor in the central nervous sytem: comparison with the periphery. Rev. Physiol. Biochem. Pharmacol. 109:145-178.

Tomaselli, K.J., L.F. Reichardt and J.L. Bixby. 1986. Distinct molecular interactions mediate neuronal process outgrowth on non-neuronal cell surfaces and extracellular matrices. J. Cell Biol. 103:2659-2672.

Tuttle, T.K., J.B. Suszkiw and M. Ard. 1980. Long term survival and development of dissociated parasympathetic neurons in culture. Brain Res. 183:161-180.

Walicke, A.P. 1988. Basic and acid fibroblast growth factors have trophic effects on neurons from multiple CNS regions. J. Neurosci. 8:2618-2627.

Walicke, A.P., W.M. Cowan, N. Ueno, A. Baird and R. Guillemin. 1986. Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extesion. Proc. Natl. Acad. Sci. USA. 83:3012-3016.

Wallace, T.L. and E.M. Johnson. 1986. Characterization of choline-acetyltransferase-sustaining and survi-

val promoting activities for parasympathetic neurons in pig brain. Brain Res. 375:92-101.

Wallace, T.L. and E.M. Johnson. 1987. Partial purification of a parasympathetic neurotrophic factor in pig lung. Brain Res. 411:351-363.

Weber, M.J. 1981. A diffusible factor responsible for the determination of cholinergic functions in cultured sympathetic neurons. J. Biol. Chem. 7:3447-3453.

Weber, M.J., B. Raynaud and C. Delteil. 1985. Molecular properties of a cholinergic differentiation factor from muscle-conditioned medium. J. Neurochem. 45:1541-1547.

Whittemore, S.R., T. Ebendal, L.Larkfors, L. Olson, A. Seiger, I. Stromberg and H. Person. 1986. Development and regional expression of β-nerve growth factor messanger RNA and protein in the rat central neurvous system. Proc. Natl. Acad. Sci. USA. 83:817-821.

Williams, L.R.,M. Manthrope, G. Barbin, M. Nieto-Sampedro, C.W. Cotman and S. Varon. 1984. High ciliary neurotrophic specific activity in rat peripheral nerve. Int. J. Devl. Neurosci. 2:177-180

Wilson, S.P. and N. Kishner. 1983. Effects of ascorbic acid, dexamethasone, and insulin on the catecholamine and opioid peptide stores of cultured adrenal medullary chromaffin cells. J. Neurosci. 3:1971-1978.

Wong, W. and J.A. Kessler. 1987. Solubilization of a membrane factor that stimulates levels of substance P and choline acetyltransferase in sympathetic neurons. Proc. Natl. Acad. Sci. USA. 84:8726-8729.

Zurn, A.D.. 1987. A new low-molecular-weight component promoting adrenergic development in cultured chick sympathetic neurons. J. Neurosci. 7:3566-3573.

Zurn, A.D. and F. Murdy. 1986. Conditions increasing the adrenergic properties of dissociated chick superior cervical ganglion neurons grown in long term culture. Dev. Biol. 117:365-379.