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(54) Title: NEUROTROPHIC FACTOR

(57) Abstract

Neurotrophic factor compositions are provided, obtainable from porcine lung tissue. Neurotrophic factor is active on parasympathetic ganglion neurons, enhancing survival of the neurons and increasing choline acetyltransferase activity.

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NEUROTROPHIC FACTOR

INTRODUCTION

Technical Field

The invention relates to neurotrophic factors useful in treating neurodegenerative diseases that affect the ciliary ganglion and parasympathetic ganglia. The factors find use in maintaining viability of parasympathetic neurons and enhancing acetylcholine formation in certain eye diseases.

Background

Glaucoma is a disease characterized by an increase in intraocular pressure (IOP), cupping of the optic nerve head, loss of visual field and parasympathetic neuropathy, but the cause of glaucoma is unknown. Pathological changes (neuropathy) in the parasympathetic (ciliary and pterygopalatine) innervation of the ciliary muscle may be a cause of glaucoma; the cause of the pathological changes is unknown.

In a number of conditions where nerves are damaged or destroyed, the target organ atrophies or exhibits abnormal functions. The survival of the neurons is essential for the maintenance of normal target organ function. Moreover, a mutually beneficial, reciprocal relationship exists between the target tissue and neuron. While the target tissue supplies neurotrophic factors (NTFs) for neurons, the neurons also provide trophic support and regulate the function of the target tissue. Indeed, in many cases and in clinical disorders, when the neurons die there is atrophy and loss of function of the target tissue.

Neurotrophic factors are believed to be both elaborated by target tissues and released by wound-repair cells and taken up into nerve terminals or at nerve cell bodies. Some factors may be transported in a retrograde fashion to nerve cell bodies. Neurons are dependent on neurotrophic factors (NTFs) not only for survival and function, but also for wound-repair, and many NTFs are thought to exist, each directed against a specific neuronal type or in some cases, several neuronal types. While the NTFs are usually produced in extraordinarily small

quantities by target tissues, such as ciliary muscle and iris, or by wound repair cells, several NTFs have been at least purified and shown to possess survival-promoting effects *in vitro*.

In view of the fact that there seem to be a large number of different neurotrophic growth factors, which appear to have different characteristics and different properties somewhat analogous to the family of interleukins, it is of great interest to be able to identify all of the naturally occurring NTFs, characterize them as to their physiological activities, either individually or in combination, and to determine their utility in treating a wide variety of symptoms, syndromes, and diseases. NTFs as a class of compounds are known to exert a variety of actions on nerves. It therefore would be of interest to identify neurotrophic factors that could directly and specifically stimulate the ciliary ganglion as an effective and novel means of treating glaucoma and other diseases that are related to pathological changes in the parasympathetic innervation of the ciliary muscle.

Relevant Literature

Levi-Montalcini, Science (1987) 237:1154-1162 provides an excellent review of nerve growth factor. Articles concerned with NTFs, including isolation, purification, and bioassays, include Barde *et al.*, EMBO. J. (1982) 1:549-553; Gospodarowicz *et al.*, Proc. Natl. Acad. Sci. USA (1984) 81:6963-6967; Barbin, J. Neurochem. (1984) 43:1468-1478; Weber *et al.*, ibid. (1985) 1541-1547; Fukada, Proc. Natl. Acad. Sci. USA (1985) 82:8795-8799; Manthorpe *et al.*, Developmental Brain Research (1986) 25:191-198; Wallace and Johnson, Brain Research (1986) 375:92-101; Gurney *et al.*, Science (1986) 234:566-574; Watters and Hendry, J. Neurochem. (1987) 49:705-713; Unsicker, Proc. Natl. Acad. Sci. USA, (1987) 84:5459-5463; Wallace and Johnson, Brain Research (1987) 411:351-363; Dal Toso *et al.*, J. Neurosci. (1988) 8:733-745; McManaman, J. Biol. Chem. (1988) 263:5890-5897; and Anderson *et al.*, Nature (1988) 332:360-361. Also of interest is EPA Serial No. 0 233 838, entitled "Neurite-Promoting Factor and Process for the Manufacture Thereof".

Other neurotrophic factors that have been described include the ciliary neurotrophic factor (CNTF), Lin *et al.*, Science (1989) 246:1023-1025;

neurotrophin-3, which is a neurotrophic factor related to NGF and BDNF, Maison-pierre *et al.*, Science (1990) 247:1446-1451; interleukin 3, Masahiro *et al.*, Neuron (1990) 2:429-436; rat ciliary neurotrophic factor, Stockli *et al.*, Nature (1989) 342:920-923; and brain-derived neurotrophic factor, Leibrock *et al.*, Nature (1989) 341:149-152; acidic and basic fibroblast growth factors, Unsicker *et al.* (1987) Proc. Natl. Acad. Sci. 84:5459-5463); purpurin, Berman *et al.* (1987) 51:135-142; activin, Schubert *et al.* (1990) Nature 344:868-870; leukemia inhibitory factor, Yamamori *et al.* (1989) Science 246: 1412-1416; and neurotrophin-4, Hallbook *et al.*, (1991) Neuron 6:845-858. These factors are all distinct from pig lung-derived NTF in that they have different amino acid sequences, molecular weights and isoelectric points, for example.

Neurotrophic factors maintain the survival of neurons whose axons have been severed (Yip *et al.*, (1984) J. Neurosci. 4:2986-2992), or whose nerve axons or terminals have been destroyed by chemical toxins or autoimmune phenomena (Johnson, (1978) Brain Res. 141:105-118; Peterson and Crain, (1982) Science 217:377-379). Neurotrophic factors also increase protein and RNA synthesis (Angeletti *et al.*, (1965) Biochim. Biophys. Acta 95:111-120), induce the formation of new axons, increase the arborization of existing nerve terminals (Purves *et al.*, (1988) Nature 336:123-128), act trans-synaptically (Schafer *et al.*, (1983) J. Neurosci. 3:1501-1510), cause hypertrophy of neurons, prevent naturally occurring developmental neuronal death, and increase the levels of neurotransmitter synthesizing enzymes and neuromodulatory neuropeptides in neuron cell bodies and nerve terminals (Lindsay and Harmer, (1989) Nature 337:362-364). Also see Thoenen and Barde, (1980) Physiol. Rev. 60:1284-1335; Dreyfus, (1989) Trends in Pharmacological Sci. 10:145-149.

SUMMARY OF THE INVENTION

Novel neurotrophic factor compositions are provided in substantially pure form, finding use as pharmaceutical compositions in therapy. Methods of preparation of NTFs include purification from natural sources and synthesis by recombinant means. NTF is characterized as being produced by tissue having parasympathetic innervation, and at least one of the following properties: capable

of maintaining viable embryonic chicken ciliary neurons *in vitro*, increasing choline acetyltransferase activity in parasympathetic ciliary neurons in culture and having a substantially equivalent amino acid sequence to at least a portion of a polypeptide isolatable from mammalian lung tissue and exhibiting at least one of the previously indicated properties. The compositions find use in treating disorders which may be associated with ocular function, neuronal viability, maintenance of normal target organ function following nerve damage or degeneration, as well as other symptoms and diseases associated with ciliary ganglion and parasympathetic neuron function.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a flow diagram of the sequential events in the purification and characterization of NTF. The reference to other figures at particular steps indicates where the results of a particular step are shown.

Figure 2 shows the results from Preparative Isoelectrofocusing of NTF for the Determination of the Isoelectric Point. The open circles show the results of 35 μL NTF/well on neurons surviving per well. The closed squared show the pH of each fraction.

Figure 3 shows the results of Molecular Sieving Chromatography using S-100 HR Sephacryl of non-heparin binding neurotrophic activity.

Figure 4 shows the results of Phenyl Hydrophobic Interaction HPLC.

Figure 5 shows the results of Ether Hydrophobic Interaction HPLC of neurotrophic factor.

Figure 6 shows the results of SDS Electrophoresis of purified pig lung-derived neurotrophic factor (coomassie blue-stained). Purified NTF from Phenyl Hydrophobic Interaction HPLC was subjected to SDS Electrophoresis under reducing conditions (50mM dithrothreitol) in a 4-20% acrylamide gel. Lanes 2 and 3 represent purified NTF; Lane 5 shows protein standards. The identities of the protein standards (BioRad) from top to bottom are; rabbit muscle phosphorylase B, 97,400 D; bovine serum albumin, 66,200 D; chicken ovalbumin, 42,699; bovine carbonic anhydrase, 31,000 D; soybean trypsin inhibitor, 21,500

D; and hen egg white lysozyme, 14,400D. Lane 6 shows a carbonic anhydrase protein standard (31 kD). The NTF bands stained with Coomassie blue as shown at 31.5 kD (arrow). Note that protein having the same 31.5 kD molecular weight also was stained by rabbit antiserum raised against a 20 amino acid NTF peptide fragment in Western blots (see Figure 9).

Figure 7 shows SDS Electrophoresis of purified pig lung-derived neurotrophic factor (silver-stained). Purified NTF as described in Figure 6 was subjected to SDS electrophoresis under reducing conditions (50mM dithiotreitol) in a 4-20% acrylamide gel. Lane 7 shows protein standards. The identities of the protein standards (BioRad) from top to bottom are; rabbit muscle phosphorylase B, 97,400 D; bovine serum albumin, 66,200 D; chicken ovalbumin, 42,699; bovine carbonic anhydrase, 31,000 D; soybean trypsin inhibitor, 21,500 D; and hen egg white lysozyme, 14,400D. Lanes 8 and 9 are purified NTF. The silver-stained material in the 50-68 kD area was an artifact found in all the lanes and was due to contamination with the operators skin keratin (See Ochs, 1983 Anal. Biochem. 135:470) for an explanation of this artifact). The silver staining showed a single band of NTF at 31.5 kD (arrow).

Figure 8 shows the serum antibody titer of rabbits immunized with fragment #3 peptide (ELISA). For the sequence of fragment #3, see Table 2. The open triangles represent normal rabbit serum; the open circles represent rabbit #3498; the open squares represent rabbit #3499; the closed circles represent rabbit #3500; and the closed squares represent rabbit #3501. The ELISA plate antigen is fragment #3 peptide PL(250μg/ml). The detection system is goat anti-rabbit peroxidase-conjugated IgG.

Figure 9 shows a western blot of the 25,000 X G pig-lung supernatant preparation subject to SDS Electrophoresis: detection of the NTF with rabbit polyclonal antiserum raised against NTF peptide fragment #3. Thirty micrograms of protein of the 25,000 X G supernatant preparation of pig-lung were subjected to SDS electrophoresis under reducing conditions on a 4-20% acrylamide gel. The gel was electroblotted to PVDF paper. The NTF was detected with the rabbit polyclonal antiserum raised against NTF peptide fragment #3 and goat anti-rabbit Ig G conjugated to peroxidase. The protein standards were stained with

Coomassie blue. The antiserum recognized a doublet band at the 31.5 kD position (arrow) in the pig-lung supernatant. This is the same MW as the purified NTF seen after Coomassie blue staining. There was also some immunoreactivity at the dye front of the gei, possible representing small molecular weight fragments. There was no apparent staining of a protein in the range of the 60,187 MW heterogenous ribonucleoprotein L protein that has some homology with NTF.

Figure 10 shows a western blot of the 25,000 X G pig-lung supernatant preparation subjected to analytical isoelectric focusing: detection of the NTF with rabbit polyclonal antiserum raised against NTF fragment #3. Two hundred micrograms of protein of the 25,000 X G supernatant preparation of pig-lung and 25 micrograms of the 20 amino acid NTF peptide fragment/polylysine-conjugate were subjected to analytical isoelectric focusing. The gel was electroblotted to PVDF paper. The NTF was detected with the rabbit polyclonal antiserum raised against the 20 amino acid NTF peptide fragment, and visualized with goat anti-rabbit Ig G conjugated to peroxidase. The pI markers were protein standards run simultaneously, but stained with Coomassie blue. The identities of the pI markers (Sigma) are: human hemoglobin A_0, pI 7.1; human carbonic anhydrase, pI 6.6; bovine carbonic anhydrase, pI 5.4. The antiserum recognized 4-5 bands with isoelectric points in the pH 5.4 to 6.7 range (arrow) in the pig-lung supernatant preparation. This is the same approximate isoelectric point of this NTF as determined by preparative isoelectric focusing (see Figure 2). The antiserum also recognized the 20 amino acid NTF peptide fragment/polylysine-conjugate immunogen in the high pH area, due to the basic nature of the lysine.

Figure 11 shows the effect of rabbit antiserum against NTF peptide fragment #3 on NTF activity in a 25,000 X G pig-lung supernatant preparation. The open circles show the results with normal rabbit serum, the closed circles rabbit #3499 antiserum.

Figure 12 shows the effect of rabbit antiserum against NTF peptide fragment #3 on NTF 25,000 X G pig lung preparation and ciliary neurotrophic factor (CNTF) (recombinant) activity. The closed circles show CNTF + rabbit antiserum; the open circles NTF + rabbit antiserum; the closed squares NTF + normal rabbit serum.

Figure 13 shows the anatomy of the ciliary ganglion innervation and the aqueous humor outflow tract in the eye.

Figure 14 shows the expected effect of NTF on ciliary nerves innervating iris and ciliary muscle.

BRIEF DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Compositions comprising polypeptides, derivatives, fragments or analogs thereof and formulations containing such compositions are provided which affect the maintenance of the ciliary ganglion, ciliary muscle and ciliary body. The subject polypeptides are collectively referred to as NTF and are related to a naturally occurring polypeptide isolated from pig lung which has a pI in the range of 5.5-6.8 by preparative isoelectric focusing, is acid labile and does not bind to heparin. The pig lung NTF exhibits a single silver stained band on SDS gel electrophoresis and has an apparent molecular weight of about 31.5 kD as determined by SDS gel electrophoresis under reducing conditions using proteins of known molecular weight as the standards.

As a therapeutic, NTF offers several advantages since it has direct effects on the parasympathetic neurons that innervate the eye (see Figure 13 for a diagram showing the anatomy of ciliary ganglion innervation and the aqueous humor outflow tract in the eye.) The parasympathetic neurons originate primarily in the ciliary ganglion and secondarily in the pterygopalatine ganglion and accessory ganglion cells. The ciliary ganglion neurons innervate the ciliary muscle/body and iris and are known to be responsible for regulation of aqueous humor outflow and accommodation. Outflow facility is mediated by ciliary muscle contraction. Contraction of the ciliary muscle deforms the trabecular meshwork, which decreases outflow resistance and decreases intraocular pressure. Ciliary neurons also innervate the trabecular meshwork, but the exact function of this parasympathetic innervation in the trabecular meshwork is not understood. The pterygopalatine ganglion, another source of parasympathetic innervation, innervates choroidal blood vessels: pterygopalatine innervation can also be found in the trabecular meshwork, but its role there is not understood. For a review of

this information, see Stone and Laties (1987) in "Glaucoma Update III," G.K. Krieglstein, Ed., Springer-Verlag, Berlin, pp. 1-16.

One of the effects of NTF is to increase the activity of choline acetyltransferase activity in target tissues such as ciliary neurons. Choline acetyltransferase is the neurotransmitter synthesizing enzyme for acetylcholine. The enzyme is localized and can be measured in both the parasympathetic neuron cell bodies and in the target tissues, where the enzyme is found in the nerve terminals. Choline acetyltransferase is a specific marker for parasympathetic nerves in the anterior segment of the eye and is commonly used to indicate the presence and level of cholinergic innervation.

Acetylcholine is the primary neurotransmitter used for neurotransmission in the parasympathetic nerves and acetylcholine released from parasympathetic nerves acts on muscarinic acetylcholine receptors that are located on the ciliary muscle and iris. Longitudinal fibers of the ciliary muscle are responsible for changes in outflow facility, whereas circular fibers of the ciliary muscle are responsible for changes in accommodation. Muscarinic receptors are also found in the trabecular meshwork (WoldeMussie *et al.*, (1990) Invest. Ophthalmol. Vis. Sci. 31:338), presumably at a postjunctional site of parasympathetic innervation, but their role there is not understood.

Presently available anti-glaucoma drugs include adrenergic antagonists, parasympathomimetics, sympathomimetics, hyperosmotics, or carbonic anhydrase inhibitors. The mechanism of action of parasympathomimetics such as pilocarpine is to directly increase the outflow of aqueous humor and cause accommodation, while anti-acetylcholinesterases act indirectly to increase outflow and cause accommodation by increasing the availability of acetylcholine for postjunctional muscarinic acetylcholine receptors. See Kaufman (1984) In "Glaucoma: Applied pharmacology in medical treatment. S.M. Drance and A.H. Neufeld, Eds., Grune and Stratton, New York, pp. 395-427. However, these drugs and others used to treat glaucoma, have short durations of action and must be administered at least once, and usually several times daily. Also, they have a variety of adverse side effects. See Bienfang *et al.* (1990) New England J. Med. 323:956-967. By way of comparison, NTF has direct effects on the ciliary ganglion neurons and axons,

and indirect effects on the ciliary muscle and trabecular meshwork. It is a theory of the invention that NTF therefore can maintain the survival of parasympathetic neurons in the ciliary and pterygopalatine ganglia, increase the level of choline acetyltransferase, increase the number of axons innervating the ciliary muscle, and restore innervation of ciliary muscle. Since neurons have long term influences on the structural and functional integrity of target tissue and on the physiological, pharmacological and biochemical properties of target tissue (Hasegawa *et al.* (1982) Trends in Pharmacol. Sci., August, pp. 340-342), the restoration of parasympathetic function would have restorative effects on the ciliary muscle and trabecular meshwork. The restoration of parasympathetic innervation by the NTF would act to normalize the number of acetylcholine receptors and ionic channels, improve the contractile properties, and improve structural and functional integrity of ciliary muscle and trabecular meshwork. Some or all of these actions may improve the ability of the ciliary muscle to decrease outflow resistance in glaucoma. NTF offers the advantage that parasympathetic innervation of ciliary muscle and trabecular meshwork would increase, and ciliary muscle function would improve. These effects, which would involve the synthesis of new protein in both nerve and muscle, would be long lasting (i.e. from several days to weeks in duration). Thus, this factor will benefit the glaucoma patient by treating the parasympathetic neuropathy directly by its effect on the ciliary neurons and indirectly through trans-junctional actions of the ciliary neurons.

To determine the complete amino acid composition and sequence analysis of naturally occurring pig lung NTF any of a number of methods known in the art may be used , such as using an Applied Biosystems Gas Phase Sequenator on tryptic fragments. To verify the novelty of the sequence obtained, a library search can be conducted on the protein databases of, for example, the National Biomedical Research Foundation (NBRF) and nucleotide sequence databases (for example, GENBANK), using respectively the FASTP and FASTA programs described by Lipman and Pearson, Science, (1985), 227:1435-1441 and Pearson and Lipman, Proc. Nat'l. Acad. Sci. (USA), (1988), 85:2444-2448. Using amino acid sequencing of tryptic fragments, the following amino acid sequences have been identified as present in native NTF.

F D S V Q S A Q R

I Q T V K

L N V F K N D Q D T W D Y T N P N L S G

D N A G D Q

For the purpose of this application, NTF is defined as including any protein or polypeptide which has substantial homology with the amino acid sequence of pig lung NTF isolated as described in Example 1 and coded for by the cDNA clones deposited on November 19, 1992 with the ATCC under accession numbers _____, but excluding any protein or polypeptide which does not have at least one biological activity of NTF. Thus, the polypeptides of this invention include congeners of NTF, namely compounds having at least one biological activity corresponding to that of NTF and having at least one amino acid sequence having substantially the same amino acid sequence as native pig lung NTF.

A congener may be of greater or lesser amino acid number than NTF. Biological activity includes immunological cross reactivity with naturally occurring NTF or binding to an NTF receptor molecule with high affinity. By immunological cross reactivity is meant that an antibody induced by a novel polypeptide of this invention will bind with intact NTF at least when NTF is in a denatured state. By high affinity is meant a dissociation constant (K_d) of at least about 10^{-7}M. By NTF receptor is meant a binding side on the surface of a cell which specifically binds NTF with high affinity, the binding being saturable and non-inhibited by structurally unrelated polypeptides. Some of the polypeptides may also retain the cell growth modulatory activity of naturally occurring NTF but the activity may be different, usually reduced.

Ordinarily, NTF polypeptides will be about from 40 to 100% homologous to the amino acid sequence of naturally occurring pig lung NTF, preferably substantially (at least 80 to 90% homologous). Homology is determined by optimizing residue matches by introducing gaps as required but without considering conservative substitutions as matches. This definition is intended to include natural allelic variations in NTF sequence.

Pre-NTF is a species of NTF included within the above definition of NTF. Pre-NTF is characterized by a presence in the molecule of signal (or leader) amino acid sequence which serves post-translationly to direct a protein to a site inside or outside of the cell. Derivatives of NTF included herein are amino acid sequence mutants, glycosylation variants and covalent or aggregative conjugates with other chemical moieties. Mutant NTF derivatives include predetermined, i.e. site specific, mutations of NTF or its fragments. Mutant NTF is defined as a polypeptide otherwise falling within the homology definition for NTF as set forth above but which has an amino acid sequence different from that of NTF as found in nature, whether by way of deletion, substitution or insertion.

The present invention also includes polypeptide fragments of at least eight amino acids comprising an amino acid sequence having at least five amino acids, either consecutive or non-consecutive, that correspond to an amino acid sequence in the complete sequence of NTF. Also included are polypeptides of at least eight amino acids having at least five amino acids that differ from the complete sequence of NTF by no more than three, usually no more than one amino acid. That difference can be the insertion of an amino acid, the deletion of an amino acid or the substitution of one amino acid for another, particularly a conservative substitution. Usually the polypeptide will contain at least ten, more usually at least twelve, consecutive amino acids that correspond to the sequence of NTF and differ by no more than one amino acid from the native sequence of NTF. For purposes of the subject invention, the various amino acids can be divided into a number of sub-classes. The following table indicates the sub-classes:

Aliphatic

Neutral

Non-Polar	G A P Z L I
Polar	S T C M N Q
Acidic	D E
Basic	K R
Aromatic	F H Y W

By conservative substitution, it is meant that amino acids from the same sub-class (i.e. either neutral aliphatic, acidic aliphatic, basic aliphatic or aromatic), more particularly the same polarity, will be substituted for each other. Desirably, amino acids of 2-4 carbon atoms or 5-6 carbon atoms will define monomial groupings in the aliphatic sub-class. Any of these polypeptides may be joined to other compounds, such as antigens, receptors, labels or the like.

Other neurotrophic factors that share a conserved amino acid sequence homology with NTF may be identified using a method similar to that used for NGF and homologous proteins. For example, nerve growth (NGF) (Levi-Montalcini, Science (1987) 237:1154-1162) and brain-derived neurotrophic factor (BDNF) (Leibrock *et al.* (1989) Nature 341:149-152) are proteins of approximately 120 amino acids that share about 50% amino acid homology. The most highly conserved regions are those that flank the six cysteine residues. Oligonucleotides corresponding to those regions have been used in polymerase chain reactions to find a third member of the family, called neurotrophin-3 (Maisonpierre, *et al.* (1990) Science 247:1446-1451). Neurotrophin-3 has 119 amino acids, has the six conserved cysteine residues, has a similar isoelectric point (pH 9.5) and is approximately 58% homologous in its amino acid sequence to NGF and BDNF. Thus, a similar strategy can be used to identify other neurotrophic factors related to the NTF. Included within the subject invention are polypeptides which share a conserved amino acid sequence homology with NTF and share at least one biological function with NTF, such as binding to an NTF receptor with high affinity.

The polypeptides will have at least one biologically active sequence, for example immunological or epitopic, and may have more than one biologically active sequence, where such sequence can compete with a naturally occurring product for the biological property. NTF-like material, including NTF fragments, mutants of the polypeptide, as well as fusion peptides comprising NTF or a functional portion thereof, having one or more biological activity of the intact NTF including ability to maintain neuronal viability, receptor binding activity, neurologic activity, neuritic outgrowth (generates axons from neuronal body), ability to modulate choline acetylcholine transferase activity in parasympathetic

neurons, is included within the subject application. NTF may be an agonist or an antagonist of native NTF or a partial agonist or antagonist. Thus, for example, NTF which does not have the ability to maintain neuronal viability or to increase choline acetylcholine transferase activity, as is seen with nature NTF, but binds to an NTF receptor with sufficient affinity to affect binding of native NTF, is included.

The subject composition may be obtained from any suitable source, particularly mammal, for example, primate (including human), bovine, equine, porcine, canine, feline, etc. The tissue used as a source of NTF may be any tissue that has parasympathetic innervation, such as lung tissue. In the subject invention, porcine lung NTF is exemplified. Presence of NTF in a tissue of interest may be determined by bioassay, for example, evaluating the effect of a cell free extract on neuronal viability; where synergistic or antagonistic agents may be present in the cell-free extract, an antibody which binds specifically to NTF may be used, for example.

The subject NTF may be isolated by combining a variety of techniques, including molecular sieving, anion and cation exchange chromatography, hydrophobic chromatography, heparin affinity chromatography and SDS-PAGE. The NTF may be extracted from a tissue of interest by an initial homogenization in a variety of low ionic strength (1 to 50 mM) buffers (e.g. tris-HCl, phosphate) at approximately pH 7.0. After separating the liquid from the particulate matter to obtain a cell free extract and dilipidating the liquid phase, the liquid phase may be separated using a variety of chromatographic resins at low, medium, or high pressures, as appropriate for the resin type. The types of anion exchange resins that can be used are, for example, DEAE, Fast Q, Mono Q; the types of cation exchange resins that can be used for, for example, SP, Fast S, Mono S, using either gradients or batch elutions of NaCl. The anion exchange buffers should have a pH of about 6.8 to 7.2, which is higher than the pI of the subject NTF, whereas the cation exchange buffers should have a pH of about 5.6 to 5.9, which is lower than the pI of the NTF.

The NTF may be fractionated using a variety of molecular sieving resins (e.g. low pressure resins: Pharmacia G-50, G-75 or G-100 Sephadex;

S100, S200, S300, or S400 Sephacryl; or high pressure columns: Waters Protein-Pak 125, Zorbax G-250), which fractionate approximately in the 20,000 to 40,000 Dalton range. Heparin binding proteins, including heparin binding growth factors, can be removed by using ready-made heparin-affinity resins or columns which may be obtained from a number of sources (for example, Pharmacia, Biorad) or home-made heparin-affinity columns (for example, Pierce). The subject NTF does not bind to heparin and can be collected in the material which passes through the heparin columns. The NTF also can be purified using hydrophobic interaction chromatography (HIC) (for example, Pharmacia phenyl-sepharose; Toyosota phenyl-5PW and ether-5PW). Typically, the NTF preparation is brought to either 2 M ammonium sulfate for phenyl HIC or 2 M for ether HIC, at pH 7.0, and eluted with a descending gradient of ammonium sulfate. The order of the aforementioned types of chromatography can be changed and still result in purified NTF.

Final purification and concentration can be achieved by SDS poly-acrylamide gel electrophoresis (SDS PAGE), using either straight percentage acrylamide gels or gels constructed of various acrylamide gradients. Western blotting of the NTF can be used to prepare the subject NTF for chemical analysis using either PVDF (Immobilon) or nitrocellulose paper. The subject NTF can be stained in gels or on electroblotted paper using a variety of dyes such as Coomassie Blue, ponceau S, or silver. Native NTF migrates in SDS gels as a 31,500 molecular weight protein. The methods used to determine physicochemical properties such as apparent molecular weight, isoelectric points, and the like are known to those skilled in the art.

Rather than isolate NTF from natural sources, the factor or a congener thereof may be prepared by recombinant techniques. For screening purposes, the subject factor may be used to prepare monoclonal antibodies in accordance with conventional ways. See for example, U.S. Patent Nos. 4,716,111; 4,716,117 and 4,713,325, and references cited therein. Using conventional techniques described by Stratagene, or others, the cells may be lysed and the mRNA from a tissue of interest which codes for NTF isolated, the mRNA

reverse transcribed, and the resulting single-stranded (ss) DNA used as a template to prepare double-stranded (ds) DNA and the dsDNA gene isolated.

Another technique is to isolate a piece of the DNA from a cell in the tissue of interest and using a probe, appropriately degenerate, comprising a region of the most conserved sequences in the gene of interest, identify sequences encoding NTF in the host cell genome. Genes encoding NTF can also be isolated using a piece of cDNA from a cell in the tissue of interest and using a probe, appropriately degenerate, constructed based upon the amino acid sequence of NTF, identify sequences encoding NTF in the genome of cells in the tissue of interest. Both DNA and RNA probes can be used.

Genes can also be isolated using probes obtained in heterologous hybridization experiments. These probes can be restriction fragments derived from NTF encoding DNA isolated from, for example, pig lung tissue; restriction fragments can easily be selected and isolated using a restriction map. On the other hand, synthetic oligonucleotide probes can be made based upon amino acid sequence information and either used directly or used in a PCR reaction to generate a larger probe fragment.

The DNA obtained by any of the above means may be inserted into an appropriate expression library such as a λgTll library, which is transfected into *E. coli* cells. The resulting fused proteins may then be screened with antibodies (monoclonal or polyclonal) prepared using the subject NTF or a fragment thereof as antigen. The various plagues may then be screened with the subject antibody for proteins specifically binding to the subject monoclonal antibodies. Those clones may be used as probes for identifying specific mRNA using Northern analysis and the resulting mRNA reverse transcribed, cloned and sequenced to identify the specific sequence.

The term homologous when applied to nucleic acid sequences intends nucleic acid sequences capable of hybridizing to each other at a stringency of at least about 25°C below the T_m (T_m is the temperature at which about half the nucleic acid strands are denatured). Homology is determined under stringency conditions of 2% - 6% SSC, 0.1% - 0.5% SDS and a temperature range of 37°C - 68°C; more preferably 2% SSC, 0.5% SDS and 37°C. In referring to "NTF

encoding DNA homologous sequences" it is intended to include the native NTF DNA coding sequence itself. Most generally used hybridization protocols may be used with NTF DNA derived probes. See Molecular Cloning: A Laboratory Manual, second edition (1989), Sambrook *et al.*, Cold Spring Harbor Laboratories Press, Cold Spring Harbor, N.Y., for examples of such protocols.

Hybridization probes based on the NTF DNA sequence find use for many purposes, including the isolation of sequences from a genetic library. Probes may be either single or double stranded, either RNA or DNA. Probes may be produced by *in vitro* or *in vivo* synthesis. Probes may also be produced by a combination of *in vitro* and *in vivo* synthesis. Methods of *in vitro* probe synthesis include organic chemical synthesis processes or enzymatically mediated synthesis, e.g., by means of SP6 RNA polymerase and a plasmid containing the NTF DNA sequence under the transcriptional control of an SP6 specific promoter. Usually, probes will have a specific complementary sequence of at least 12 nucleotides, more usually at least 14 nucleotides and preferably greater than 50 nucleotides and, more preferably having the entire NTF DNA sequence.

Probes may have either complete or partial homology with NTF DNA. Probes containing partial homology to NTF DNA will usually have less than 20% mismatch with NTF DNA and preferably less than 10% mismatch with NTF DNA. Probes may be modified by conjugation to a variety of labels which allow for detection of duplex formation between the probe and its complementary target. Labels include radioactive isotopes, ligands, e.g., biotin, enzymes, fluorescers and the like. A wide variety of protocols for labeling probes and detecting duplexes formed between probes and their target hybridization sequences have been described in the literature. See for example, Berger and Kimmel, editors, Guide to Molecular Cloning Techniques (1987) Academic Press Inc., San Diego, CA. Probe sequences may be joined to a variety of other nucleic acid sequences. Among these other nucleic acid sequences are vectors such as plasmids, cosmids, phages, and the like. By joining the probe sequence to a vector sequence, probes may be conveniently created, expanded, stored, and modified.

The amino acid sequence of native NTF or fragments thereof can be used to isolate corresponding genes, using the so-called "reverse genetics" approach in which oligonucleotide probes are designed based on the amino acid sequence (e.g. Sambrook *et al.*, Molecular Cloning 2nd ed. (1989) supra. These oligonucleotide probes, which will be degenerate, due to the degeneracy of the genetic code, are subsequently used to screen a genomic library or a cDNA library, in a suitable vector. By use of these amino acid sequences the genes can be isolated by methods known in the art.

In addition the subject NTF may be sequenced, either partially or completely, so that the sequence of the NTF protein can be compared to the sequence encoded by the mRNA. Alternatively, the DNA sequences encoding NTF can be synthesized using conventional techniques such as PCR or by synthesis of overlapping single strands which may be ligated together to define the desired coding sequences. The termini can be designed to provide restriction sites or one or both termini may be restricted for ligation to complementary ends of an expression vector.

It is highly desirable that the three-dimensional structure of the expression product be retained, particularly that portion of the structure which may be responsible for binding to a receptor moiety on a target cell. Convenient restriction sites may be designed into the DNA sequence of interest; when possible, the restriction site(s) leaves the amino acid sequence of the expression product unaltered. However, in some cases, incorporation of new restriction sites may yield an altered amino acid sequence.

Once a DNA sequence encoding the NTF or congener thereof, has been isolated the DNA sequence may then be used for expression in any convenient host, either prokaryotic or eukaryotic. There are an ample number of expression systems in the literature; see for example, Sambrook *et al.* (1989) supra. For expression of the sequence, where necessary an initial methionine is provided. Where the DNA sequence encoding NTF is to be expressed in a host which recognizes the wild type transcriptional and translational regulatory regions of the native NTF gene, the entire gene with its wild type 5' and 3'-regulatory regions may be introduced into an appropriate expression vector. However, where

the DNA encoding sequence encoding NTF is to be expressed in a host cell which does not recognize the naturally occurring wild type transcriptional and translational regulatory regions or where the native gene encoding NTF is not being used, further manipulation may be required. The noncoding 5'- region upstream from the structural gene may be removed by endonuclease restriction, Bal31 restriction, or the like. Alternatively, where a convenient restriction site is present near the 5' terminus of the structural gene, the structural gene may be restricted and an adaptor employed for linking the structural gene to the promoter region, where the adapter provides for the lost nucleotides of the structural gene.

The DNA may be introduced into the host cell in accordance with known techniques, such as transformation, transfection by contact in the cells of the virus, microinjection of the DNA into the cells, calcium precipitation, and the like. A variety of 3'- transcriptional regulatory regions are known and may be inserted downstream from the stop codons.

Cloned DNA can be screened by Southern analysis using oligonucleotides that code for any of the tryptic fragments that were sequenced from the purified pig lung NTF. A number of different oligonucleotides would be constructed due to the degeneracy of the DNA code for amino acids. Cloned cDNA that has been transcribed into mRNA can be screened by Northern analysis. In addition, cloned DNA could be screened using antibodies to any of the tryptic fragments. By this method, colonies of the cDNA in lambda phage such as lambda GT11 can be screened after growing the phage as plaques on lawns of bacteria such as *E. coli* and replica transfer of some of the expressed protein to nitrocellulose paper. A Western analysis is then performed on the nitrocellulose paper. For example, goat antisera raised against the 20 amino acid fragment were used to screen the pig lung cDNA library. Expressed protein derived from the cDNA in plasmid or other expression systems is then screened in the same manner by replica transfer of some of the bacterial colony to nitrocellulose paper and subsequent Western analysis using antibody to the NTF. The ability of the antibodies to detect the expressed protein is dependent upon expression of the protein in the proper reading frame. References for these Southern, Northern, and Western analysis methods can be found in "Molecular Cloning: A Laboratory

Manual" Sambrook, Fritsch, and Maniatas, Cold Spring Harbor Laboratory Press, 2nd edition, 1989.

A bioassay such as the ciliary ganglion bioassay can be used to screen for expressed protein if the protein has biological activity. The greatest likelihood that the expressed NTF will have biological activity is if it is expressed in a vector that glycosylates and folds the NTF into an appropriate three-dimensional structure. In the ciliary ganglion bioassay, a conditioned cell medium or an extract of lysed cells is incubated directly with dissociated embryonic chicken ciliary nerve cells that are attached to a layer of rat tail collagen in 48- or 96-well plates. The biological activity detected in this assay is survival - promoting activity. Control wells contain the dissociated ciliary neurons and non-conditioned cell tissue culture medium instead of the conditioned cell medium or cell lysate. The ciliary neurons will survive in the presence of, but die in the absence of, active NTF. After 3-7 days, the vital dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is added. Viable ciliary neurons form a blue crystal in a spoke-well shape and can be counted using an inverted phase objective on a microscope. Other measures of biological activity could also be assessed in the ciliary ganglion bioassay, such as changes in choline acetyltransferase activity (see Wallace *et al.* Brain Research, (1986) 375:92-101 and Wallace *et al.* Brain Research (1987) 411:351-363).

The subject compositions have a number of physiological activities, as evidenced by *in vitro* bioassays, including maintaining viable embryonic chicken ciliary neurons *in vitro* and increasing choline acetyltransferase activity in parasympathetic ciliary neurons in culture. The increase in choline acetyltransferase activity can be enhanced by the presence of KCl in from about 5 to 50 mM KCl, with the activity increasing linearly within that range. A number of other salts do not show the same stimulation. The subject compositions have a ED_{50} of at least 25ng/ml, preferably at least about 50ng/ml based on the bioassay described in the experimental section. In addition, the purification achieves at least a 15,000-fold increase in activity over the pig lung 25,000 x g supernatant.

Antibodies against the NTF can be made using either synthetic peptides derived from the amino acid sequences of tryptic fragments or using the

native NTF as the immunogen. This could be done, for example, by immunization of various animals with the synthetic peptide or native NTF mixed with Freund's complete adjuvant followed by boosting with the synthetic peptide or native NTF in incomplete Freund's adjuvant. As an alternative immunogen, the synthetic peptide can be conjugated to a polylysine to make it more immunogenic, according to the method of Tam (1988) Proc. Natl. Acd. Sci. 85: 5409-5413. Other adjuvants such as keyhole limpet hemocyanin can be used also. The synthetic peptides may constitute conserved, functional, immunogenic, or structural domains of the NTF that may react with other neurotrophic factors with similar homology.

The development of antibodies against the NTF will have a number of uses. Antibodies can be used to identify the NTF in Western blots; immunochemically stain the NTF tissues to identify cell types producing, binding, or internalizing the NTF; develop diagnostic assays for the NTF; block the effects of the NTF; immunoaffinity purify the NTF; and to identify other cross-reacting neurotrophic factors. In addition, anti-idiotype antibodies may be developed that mimic or block the actions of the NTF; and be useful therapeutically or diagnostically.

The subject compositions can find use in the treatment of glaucoma in which there is inadequate functioning of ciliary ganglion and parasympathetic nerve cells or of the tissues innervated by the ciliary ganglia. By correcting the underlying parasympathetic neuropathy present in glaucomatous patients, ciliary nerve function may be restored, thereby improving ciliary muscle function. This enhanced cholinergic tone in the ciliary muscle provides for a reduction in intraocular pressure.

A number of clinical studies have demonstrated that a significant percentage of glaucomatous patients and patients with ocular hypertension have a parasympathetic neuropathy. Fifty eight percent of 120 patients with closed-angle glaucoma were found to have systematic parasympathetic neuropathy (i.e. altered cardiovascular function) versus 6.8% of 75 sex and age-matched control subjects (Clark and Mapstone (1985) Research Clinical Forums 7:45-50; Mapstone and Clark, (1985) Trans. Ophthalmol. Soc. U.K. 104:265-269). Thirty seven percent

of 67 patients with open-angle glaucoma were found to have systemic parasympathetic neuropathy (i.e. altered cardiovascular function) versus 3% of 76 age and sex-matched control subjects (Clark and Mapstone (1986) Documenta Ophthalmologica 64:179-185). Pupil cycle time was significantly prolonged in patients with closed-angle glaucoma as compared to control patients (Clark and Mapstone (1986) Can J. Ophthalmol. 21:88-91). A group of 78 patients with ocular hypertension showed significantly greater pupillary constriction after receiving methacholine compared to 47 age- and sex-matched control subjects (Clark and Mapstone (1987) Invest. Ophthalmol. Vis. Sci. 28:1732-1735), indicating that there was supersensitivity due to parasympathetic denervation. Forty two percent of 189 patients with ocular hypertension were found to have systemic parasympathetic neuropathy (i.e. altered cardiovascular function) versus 3% of 76 age and sex-matched control subjects (Clark and Mapstone (1985b) The Lancet 2:185-187). Ninety-five percent of 20 patients with untreated primary open angle glaucoma had cholinergic supersensitivity of the iris (indicating ciliary nerve degeneration) compared to 30% of 20 control subjects (Jordan *et al.* (1988) Eye 2:233-237). Significantly increased pupillary constriction in response to methacholine (also an indication of ciliary nerve degeneration) was present in both closed-angle (44 patients) and open-angle glaucoma patients (20 patients) compared to 40 age- and sex-matched control subjects (Clark (1989) Eye 3:349-254). In addition, several other studies have shown using histological techniques that degenerative changes actually occur in ciliary ganglion cells in glaucomatous patients (Kapoor and Sood, (1975) British Journal of Ophthalmology 59:573-576; Faschinger and Kleinert (1986) Klinische Monatsclatter Fur Augenheilkunde 189:400-401; Faschinger *et al.* (1988) Forschritte Der Ophthalmologie 85:54-56) supporting the systemic findings. These results indicate that glaucomatous patients have both a systemic and ocular parasympathetic neuropathy indicating the possibility that parasympathetic innervation (i.e. ciliary and pterygopalatine) may be an effective treatment in glaucoma.

The subject formulation for therapy is prepared in a physiologically acceptable medium such as saline, PBS, or a balanced salt solution. The subject compositions are administered at different levels, depending on the disorder, the

severity of the disorder, the solution, the frequency of treatment, and the like. Levels of treatment will generally be in the range of analogous factors, e.g. about 0.1-5 mg/kg. Administration is by injection, transdermal or transcleral delivery or other suitable method known to those of skilled in the art.

NTF preferably will be administered by a parenteral route. The results of clinical studies indicate that glaucoma is associated with not only with an ocular (ciliary) parasympathetic neuropathy (Clark and Mapstone, 1987; Jordan *et al.*, 1988; Clark, 1989), but also with a systemic (e.g. cardiovascular) parasympathetic neuropathy (Clark and Mapstone, 1985a, b; Mapstone and Clark, 1985; Clark and Mapstone, 1986a). NTF can be administered systemically as an intramuscular injection initially, but it also can be administered systemically for example by subcutaneous implantation in the skin of a controlled-release pump. The pump can be a biodegradable polymer or other device. In addition, NTF can be administered systemically via a colon-targeted drug delivery system. The colon is a preferential site of protein absorption due to decreased concentrations of degradatory enzymes and long resonance time (10-24 hours). NTF delivery could also be linked to a carrier molecule such as vitamin B12, since vitamin B12 is absorbed via a receptor-mediated process in the colon. Liposomes may also be used to deliver the NTF, either nasally, parenterally, intravenously, or by another route. Congeners of NTF that are smaller than the native pig NTF can be also be administered topically or orally if they are sufficiently small to penetrate the corneal barrier or gastrointestinal barriers. Penetration can be enhanced by the use of lipophilic agents absorption enhancers such as bile salts or non-ionic surface active agents.

The complete amino acid sequence of the subject can be reduced into fragments that have utility. These fragments may be agonists, or antagonists for the subject NTF receptor. In addition, immunogenic domains of the subject NTF can be obtained that are useful in devising diagnostic assays for NTF. Domains that are present not only in the subject NTF, but also in other neurotrophic factors that may be in the same gene family and possess amino acid homology to the NTF can also be obtained.

NTF which does not have survival-promoting activity on ciliary neurons, for example, a non-glycosylated NTF as is made in *E. coli*, may have usefulness in a number of ways. The expressed protein may have utility as an antagonist of biologically active NTF protein if receptor binding properties are present. The expressed protein may have utility as an immunogen for preparation of antibodies to the NTF. The expressed protein will have utility in determining whether glycosylation is necessary for biological activity (survival-promoting activity on ciliary neurons). The expressed protein may have usefulness in identification and isolation of the NTF receptor on ciliary neurons. Identification of the receptor can be performed by chemically crosslinking the expressed, radiolabelled protein with the receptor on ciliary neurons, with subsequent analysis by SDS electrophoresis. Isolation of the receptor could be accomplished by construction of an affinity column with the expressed protein, with subsequent chromatographic elution of bound receptor.

The recombinant expression of biologically active NTF will have utility as a drug to treat glaucoma and possibly other neurodegenerative disorders. cDNA clones would be useful in the preparation of single stranded DNA for site directed mutagenesis. Site directed mutagenesis could be used to determine the shortest DNA sequence necessary for expression of biologically active NTF, for creation of a mutant with biological activity greater than the native protein, and for creation of receptor antagonists.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1

Purification of Porcine Lung Neurotrophic Factor

In Figure 1 is shown a flow diagram that represents the sequential events in the purification and characterization of the NTF. The protocol represents a fifteen-fold scaled up version of the purification procedure described in U.S. Patent No. 5,166,317 and was designed to produce sufficient NTF for

chemical analysis. The order of some of the chromatography steps is changed so as to allow the use of fast flow resins and minimize elution times.

Preparation of Pig Lung Supernatant Fraction

The supernatant fraction was prepared as described in Example 1 with the addition of the following step. Following the recentrifugation step to remove cryoprecipitated material, the supernatant fraction was filtered through a 5 μm membrane (Nalgene) and diluted to a final concentration of 4 mg protein/ml and then was adjusted to pH 7 and 0.07 M NaCl (conductivity equal to 7.5-7.7 mMHO) for anion exchange chromatography.

Low-Pressure Anion Exchange Chromatography

The 25,000 xg pig lung supernatant fraction obtained as described above (typically 10 L) was applied to a process scale column (Pharmacia T113/60 bioprocess) containing Q resin (Pharmacia 11.3 tm diameter x 60 cm height; 5 L total bed volume) which was equilibrated in 10 mM Tris buffer plus 0.07 M NaCl, pH 7.0 The column was washed with approximately 8 L of the same buffer. The NTF was batched eluted with 10 mM Tris-buffer plus 0.5 M NaCl/pH 7.0. The loading and diluting flow rates were 9.7 ml/m in. The protein concentration of the eluted fraction was typically 4 mg protein/ml. All low pressure chromatography was carried out at 4°C.

Low Pressure Cation Exchange Chromatography

The active fast Q faction was diluted approximately ten-fold with 10mM sodium phosphate (United States Biochemicals, Molecular Biology Grade) buffer plus 0.15 M NaCl/pH 5.7 to obtain material (approximately 30 L) with a conductivity of 16 mMHO and a protein concentration of 0.4 mg/ml. The final pH was adjusted as necessary with HCl to pH 5.7. This material was applied at a flow rate of 30 ml/min to a fast S column (Pharmacia XK50/30) (5.0 cm diameter x 15 cm height; 300 ml bed volume) that had been equilibrated with 10 mM sodium phosphate buffer plus 0.15 M NaCl/pH 5.7. The column was washed at a flow rate of 15 ml/min with the same buffer. The NTF was batch-eluted with 10

mM sodium phosphate buffer plus 0.75 M NaCl/pH 5.7 at a flow rate of 15 ml/min to obtain a fraction with a protein concentration of approximately 0.8 mg/ml.

Low Pressure Heparin Affinity Chromatography

A heparin affinity column was used to remove heparin binding growth factors (for example acidic and basic FGFs). This distinguishes this NTF from the ones described in the papers of Wallace *et al.* (1986) and(1987) supra in which a heparin affinity column was not used. This previous work did not remove heparin binding growth factors such as FGFs which are present in the pig lung and elute from the heparin affinity column with 2 M NaCl. The active fast S fraction was diluted approximately two-fold with 10 mM sodium phosphate buffer, pH 7.0 and brought to pH 7.0 with 2 N NaOH and a conductivity of 30-40 mMHO. This material was loaded onto a column (Pharmacia C10/20) (1.0 cm diameter x 20 cm height; 13 ml bed volume) containing heparin affi-gel (BioRad) that had been equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The material that did not bind to the heparin affi-gel column was concentrated to 1 mg protein/ml over a 5,000 molecular weight membrane (Amicon YM-5) using an Amicon concentrating device (Model 8400).

Preparative Isoelectric Focusing

After removal of heparin binding proteins from the pig lung preparation in the previous step, some of the active NTF preparation was dialyzed in 10,000 molecular weight cut-off tubing and then analyzed by preparative isoelectric focusing using a BioRad Rotofer device. Fractions were tested for NTF activity using the ciliary neuron bioassay. The results of this experiment are shown in Figure 2 and indicate that the isoelectric of the active NTF molecule is approximately 6.2 to 6.8 using this method.

Molecular Sieving Chromatography

The active concentrated NTF material that did not bind to the heparin affinity column was loaded onto a column (Pharmacia C26/100) containing

S-100 Sephacryl HR (2.6 cm diameter x 94 cm height; 500 ml bed volume) which had been equilibrated with 10 mM sodium phosphate buffer plus 0.5 M NaCl/pH 7.0. The volume loaded was 25 ml, equal to 5% of the bed volume. The flow rate was 1.3 ml/min and 4.1 ml fractions were collected. Gel filtration protein standards (BioRad) were used to characterize the molecular sieving properties of the column. The active fractions elute between 20,000 D and 40,000 D. See Figure 3.

Phenyl Hydrophobic Interaction Chromatography

A Waters 840 system was used and protein absorbance was monitored at OD_{280} The active fractions from the S-100 Sephacryl HR column were pooled and diluted 1:2 with 10 mM sodium phosphate buffer plus 2 M ammonium sulphate (BioRad)/pH 7.0. The pH was adjusted to 7.0 with NaOH. This material was loaded onto a phenyl HPLC column (Biored phenyl-5-PW; 75 mM diameter x 7.5 cm height) which have been equilibrated in 10 mM sodium phosphate buffer plus 2 M ammonium sulphate/pH 7.0. The column was washed with the same buffer. No NTF activity was found in the material that passed directly through the column or in the wash. The NTF was eluted with a linear gradient from 100% 2 M ammonium sulphate and 10 mM sodium phosphate buffer, pH 7.0 to 100% 10 mM sodium phosphate buffer, pH 7.0 over 60 minutes. 1 ml fractions were collected. For analysis of NTF activity the fractions were first dialyzed in 10,000 molecular weight tubing (Spectraphor) against water. The active fractions elute between 0.025 M and 0.125 M ammonium sulphate (see Figure 4).

Ether Hydrophobic Interaction Chromatography

The dialyzed, active fractions from the phenyl hydrophobic interaction column were pooled and diluted 1:2 with 10 mM sodium phosphate buffer plus 4 M ammonium sulphate (BioRad)/pH 7.0. This material was loaded onto an ether HPLC column (ether-5-PW; 75 mM diameter x 7.5 cm height) which had been equilibrated in 10 mM sodium phosphate buffer plus 2 M ammonium sulphate, pH 7.0. The NTF was eluted with a linear gradient from

100% 2 M ammonium sulphate in 10 mM sodium phosphate buffer, pH 7.0 to 100% 10 mM sodium phosphate buffer, pH 7.0 over 60 minutes. 1 ml fractions were collected. For analysis of NTF activity the fractions were first dialyzed in 10,000 molecular weight tubing (Spectraphor) against water. The active fractions elute between 0.45 M and 0.75 M ammonium sulphate (see Figure #5).

SDS-PAGE, and Chemical Analysis

Active fractions from the ether hydrophobic interaction column were pooled, concentrated, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) Nature 227, 680-685. The NTF had a MW of 31,500 and was pure according to both Coomassie blue and silver-stained gels. See Figure 6 for the Coomassie blue stained gel photograph and Figure 7 for the silver stained gel photograph.

For the purpose of obtaining the amino acid sequence, the 31,500 MW NTF was electroblotted onto PVDF paper according to Matsudaira (1987) J. Biol. Chem. 262:10035-10038. Initial attempts to sequence the 31,500 MW NTF indicated that the NTF has a blocked N-terminus. In order to generate fragments of the NTF for sequencing, the 31,500 MW NTF was electroblotted onto nitrocellulose and tryptic fragments were generated according to Aebersold *et al.* (1987) Proc. Natl. Acad. Sci. 84:6970-6974. The tryptic fragments sere separated by reversed phase HPLC and four fragments were sequenced using an Applied Biosystems Gas Phase Sequenator.

Table 1

Amino acid sequences of NTF tryptic fragments

1. FDSVQSAQR
2. IQTVK
3. LNVFKNDQDTWDYTNPNLSG
4. DNAGDQ

The sequence of the four tryptic fragments were compared against the NBRF.PIR database. Fragments #1, 3, and 4 matched identically to a heterogenous nuclear ribonucleoprotein (hnRNP), L form, (Pinol-Roma *et al.*

(1989) J. Cell Biol. 109:2575-2587) and are all contained within approximately one-half of the hnRNP sequence. The hnRNP has a MW of 60,187 D (560 amino acids) and is believed to play a role in processing pre-RNAs to mRNAs (Pinol-Roma *et al.* (1989) J. Cell Biol. 109:2575-2587). However, as will be seen in further work in this application, rabbit polyclonal antisera raised against the 20 amino acid NTF peptide fragment #3 recognized a 31,500 D MW protein (288 amino acids calculated) in the pig lung and not a 60,187 D MW protein in pig lung, at least within the limits of detection of the immunological staining. Since the antisera also blocked the NTF activity, there was no question that the 20 amino acid NTF peptide fragment #3 was contained in the NTF molecule. Thus, the hnRNP was probably not the NTF, but was likely to be a precursor or otherwise related to the NTF, possibly as a result of alternative splicing or proteolytic processing, both of which are known to be involved in the synthesis of growth factors (See Neurath (1989) Trends in Biochem. Sci. July 14, 1989: 268-271; Bach *et al.* (1990) Mol. Endocrin. 4:899-904). Fragment #2 matched identically with two known proteins: a yeast DNA binding protein (Ju *et al.* (1990) Mol. Cell. Biol. 10:5226-5234) and a murine class II major histocompatibility complex protein termed Mb (Cho *et al.* (1991) Nature 353:573-576). The amino acid sequences of the tryptic fragments of the subject NTF do not share homology with any known neurotrophic factors, including nerve growth factor, ciliary neuronotrophic factor, brain-derived neurotrophic factor, neurotrophin-3, and acidic and basic fibroblast growth factors.

For determination of the amino acid composition, the 31,500 MW NTF was electroblotted onto PVDP membranes (Millipore) according to Matsudaira (1987) J. Biol. Chem. 262, 10035-10038. The amino acid composition of the NTF is shown in Table 2, below.

Table 2

Amino Acid Composition of the Pig-Lung-Derived Neurotrophic Factor

MW/#11: 31.5kD (286 amino acids/molecule)

amino acid	amino acid	mole%	amino acids

ASX(ASN,ASP)	N,D	10.0	29
GLX(GLU,GLN)	E,Q	12.9	37
SER	S	5.2	15
GLY	G	10.7	31
HIS	H	1.6	5
ARG	R	4.2	12
THR	T	4.1	12
ALA	A	7.3	21
PRO	P	4.7	13
TYR	Y	3.3	9
VAL	V	6.3	18
MET	M	3.8	11
ILE	I	5.7	16
LEU	L	8.5	24
PHE	F	4.5	13
LYS	K	7.2	21

Example 2

Polyclonal antibodies against the NTF

The 20 amino acid fragment #3 was synthesized onto an octavalent polylysine backbone according to the method of Tam (1988) Proc. Natl. Acad. Sci. 85:5409-5413, thereby making a multiple antigen peptide having a polylysine core with eight synthetic 20 amino acid peptide arms. This was done in order to make the peptide fragment more immunogenic. Four rabbits were immunized and boosted with the 20 amino acid peptide fragment in order to raise polyclonal antibodies against the NTF, according to the animal protocol. All four rabbits produced titers in an ELISA assay against the 20 amino acid fragment. (See Figure 8.) In a Western blot, the antisera stained a protein, actually a doublet, of

31,500 MW of the 25,000 X G pig lung supernatant fraction subjected to SDS electrophoresis. (See Figure 9.)

The antiserum recognized 4-5 bands on a Western blot of the 25,000 X G pig lung supernatant fraction subjected to analytical isoelectrofocusing; these bands were in the 5.4-6.7 pH range and may represent heterogeneity of the NTF. (See Figure 10.) The rabbit anti-peptide antisera was used at 1:250 and the goat anti-rabbit IgG conjugated to peroxidase was used at 1:500. The MW (31.5 kD) and isoelectric point (5.4-6.7) of the proteins identified by antisera staining of Western blots agree closely with the MW (31.5 kD) and isoelectric point of approximately 5.5-6.8 determined by gel filtration, SDS electrophoresis, and preparative isoelectrofocusing. Significantly, the antisera also neutralized NTF activity in a 25,000 X G pig lung supernatant preparation. See Figures 11 and 12. However, the antisera did not neutralize the activity of recombinant ciliary neuronotrophic factor (rCNTF (Regeneron)), another molecule that has neurotrophic actions (survival-promoting activity) on ciliary neurons. See Figure 12. These experiments substantially prove that the molecule in the pig lung that was responsible for neurotrophic activity was the 31.5 kD protein that was purified.

Example 3

Bioassay of Pig Lung Neurotrophic Factor

Fractions derived from all chromatographic columns and the preparative isoelectric focusing column were assayed on embryonic day 8 chicken ciliary neurons. The neurons were incubated on a single layer of rat tail collagen in Eagle's minimum essential medium containing 10% fetal bovine serum, antibiotics, and the chromatographic fraction 35μL. The total volume in the well was 350 μL. The tissue culture plates were then placed in a 95% air, 5% $C0_2$ environment at 37°C. After 3-10 days in culture, surviving neurons were quantitated using the vital dye MTT (3-[4,5-dimethylthiazon-2-yl]-2-5-dipenyltetrazolium bromide (see Manthorpe *et al.*, Dev. Brain Res. (1986) 25:191-198.) MTT was taken up and converted into a large blue crystal if the neuron was alive. The living neurons were counted using a phase contrast objective on the

microscope, 36 hrs after adding the MTT. Negative control wells contained the same tissue culture medium, but instead of a chromatography fraction, they received the buffer (e.g., Tris buffer) used to elute the fractions from the chromatography column. Using this procedure, all of the control neurons were dead after several days, whereas the neurons in those wells that received chromatographic fractions containing the factor were alive.

Example 4

Cloning of NTF Gene

Screening of the pig lung cDNA library

A bacteriophage lambda GT11 pig lung cDNA library was purchased from Clontech. *E. coli* strain Y1090r- were infected with the bacteriophage GT11 library and plated onto agarose petri dishes. Goat polyclonal antisera against a synthetic 20 amino acid fragment of the neurotrophic factor was used to immunoscreen the plaques on nitrocellulose filter overlays of the agarose dishes. The synthetic fragment used to raise the goat antisera had the sequence LNVFKNDQDTWDYTNPNLSG, the same as tryptic fragment #3 that had been sequenced after purification of the NTF from pig lung. The goat antisera had a titer of greater than 1/5,000 against this 20 amino acid fragment in an ELISA. This resulted in the identification of nine positive plaques. Of these, clones were established from plaques 1, 3, 5, and 6. Two references that were used for this work were "Molecular Cloning: A Laboratory Manual" Sambrook, Fritsch, and Maniatas, Cold Spring Harbor Laboratory Press, 2nd edition, 1989; and Clontech "Library protocols handbook 1: General procedures for the hybridization of lambda phage libraries with DNA probes."

Determination of insert size

After isolation of lambda DNA, *EcoR* 1 restriction endonuclease (Stratagene) was used to cut the insert from each clone. The size of the insert was determined by DNA electrophoresis. Clone 1 had a size of approximately 2000

bases. Clone 3 had a size of approximately 1300 bases. Clone 5 had an insert size of 719 bases and clone 6 had an insert size of approximately 700 bases.

Subcloning

For the purpose of sequencing, the clones from the lambda GT11 library were first subcloned into pBluescript II (Stratagene) phagemid according to the Stratagene instruction manual. pBluescript phagemids with inserts (recombinants) were white colonies, whereas non-recombinant colonies were blue. The white colonies were isolated and constituted clones that were used for DNA sequence analysis.

Use of cloned RNA transcripts and cDNA clones

The RNA transcripts synthesized from the pig lung cDNA inserts in pBluescript can be used for Southern and Northern analysis, and S1 nuclease analysis of gene copy number. In addition, the RNA transcripts can be translated into protein either using an *in vitro* translation system, or by injection into Xenopus oocytes.

DNA sequencing

The cDNA insert in pBluescript has utility for determination of the size of the gene by DNA electrophoresis. Single stranded DNA can be prepared from pBluescript and sequenced by the dideoxy method using primers that are specific for pBluescript. The DNA sequence can be compared by computer against known DNA sequences in DNA databanks. In addition, the DNA sequence can be translated into the corresponding amino acid sequence and the amino acid sequence can be compared against known sequences in protein databanks. These data bank searches are useful for determining the possible relationship between the cloned gene and other cloned genes.

To date, one strand of clone 5 that had been subcloned from the lambda GT11 into pBluescript has been sequenced and shown to contain 719 bases. However, the DNA did not code for any of the tryptic fragments obtained from the purified NTF.

Example 5

Preparation of Recombinant NTF

Cloned DNA is screened by Southern analysis using oligonucleotides that code for any of the tryptic fragments that are sequenced from the purified pig lung NTF. A number of different oligonucleotides are constructed due to the degeneracy of the DNA code for amino acids. Cloned cDNA that has been transcribed into mRNA is screened by Northern analysis. In addition, cloned DNA is screened using antibodies to any of the tryptic fragments. By this method, colonies of the cDNA in lambda GT11 are screened after growing the phage as plaques on lawns of *E. coli* and replica transfer of some of the expressed protein in nitrocellulose paper. A Western analysis is then performed on the nitrocellulose paper.

Goat antisera was raised against the 20 amino acid fragment and were used to screen the pig lung cDNA library described above. Expressed protein derived from the cDNA in plasmid or other expression systems is screened in the same manner by replica transfer of some of the bacterial colony to nitrocellulose paper and subsequent Western analysis using antibody to the NTF. The ability of the antibodies to detect the expressed protein is dependent upon expression of the protein in the proper reading frame.

If the protein expressed by pBluescript does not have biological activity, the cDNA insert in pBluescript is further subcloned into yeast, baculovirus, or mammalian recombinant expression systems in order to express a protein which is glycosylated and folded into a three-dimensional structure with biological activity. For these procedures, reference is made to Murray, Methods in Molecular Biology, Volume 7, Gene transfer and expression protocols, Human Press, (1991).

Example 6

In Vivo Testing of NTF On Glaucoma and On Parasympathetic Nerves

The rationale for the use of this NTF in the treatment of glaucoma in humans is based on the strategic role for ciliary nerve in the regulation of intraocular pressure and the clinical evidence that a parasympathetic neuropathy

exists in glaucomatous patients that could cause or contribute to the disorder. The NTF will first be tested in animals. Unfortunately, there is no animal model of glaucoma. Thus, even though there are some proposed animal models of glaucoma (see Lauber, (1987) J. Neurosci. 3:77-100), it is generally agreed that these models do not exhibit characteristics similar enough to glaucoma in humans to justify using them as models. For this reason, in the pharmaceutical industry much experimentation on anti-glaucoma drugs is carried out using normal (normotensive) animals, generally rabbits, cats and monkeys. (For example, see Potter and Burke, (1988) J. Ocular Pharmacol. 4:19-18; Colansanti and Wilkinson, (1988) J. Ocular Pharmacol 4:221-230; Bar-llan *et al.*, (1989) J. Ocular Pharmacol. 5:99-110; and Ellis *et al.*, (1991) Invest. Ophalmol. Vis. Sci. 32:520-522). Therefore, in the following studies, the effect of NTF on intraocular pressure is studied in normotensive rabbits. The effects of NTF is also studied on ciliary nerves in embryonic chickens. These studies in chickens precede the rabbit studies. NTF is chronically administered to chickens or rabbits over several days or weeks. The effects of NTF, because they will primarily involve morphological changes are seen only after several injections.

Effect of NTF on the survival and/or function of ciliary neurons *in vivo*.

The experiments below address the effects of NTF on ciliary nerves including the ability of NTF (1) to maintain the survival of ciliary neurons during the period of naturally occurring neuronal death during embryonic development, (2) to increase the size of ciliary neurons, (3) to increase the amount of innervation of the ciliary muscle and iris, and (4) to increase the levels of choline acetyltransferase activity in the ciliary ganglion, ciliary muscle and iris, and (5) stimulate the regeneration of parasympathetic nerves. These experiments are first carried out using embryonic chicken ciliary ganglia, for the following reasons. The chicken ciliary ganglion is very similar anatomically and functionally to the human ciliary ganglion (Pilar, G. (1984) In "Peripheral Neuropathy," volume 1, 2nd edition, P.J. Dyck, P.K. Thomas, E.H. Lambert, and R. Bunge, eds., W. B. Saunders, Philadelphia, pp. 231-24), and based on the effects of other neurotrophic factors, information derived from the study of the effects of the NTF

on chicken ciliary neurons are applicable to ciliary neurons of other animal species.

Small amounts 3-6 μg/day/chick of the NTF are administered to the embryo via the chorionic-allantoic membrane; the NTF reaches the ciliary ganglion after being absorbed into the chick's bloodstream. Indeed, the NTF was isolated based on its trophic effects on embryonic chicken ciliary ganglia *in vitro* and it is theorized that it will produce similar results *in vivo*.

The effect of the NTF on ciliary neurons in embryonic chickens.

A well accepted experimental way of determining the ability of neurotrophic factors to maintain the survival of neurons *in vivo* is to administer the neurotrophic factor during the period in which target neurons undergo naturally occurring death (Berg D.K. (1982) In "Neuronal Development," N.C. Spitzer, ed., Plenum Press, New York, pp. 297-331). Naturally occurring neuronal death is a natural phenomenon which happens to many neuronal types in which approximately 50% of the number of neurons in a given population die during an early stage of development because of competition for a limited supply of neurotrophic factor (Berg, 1982 supra). This has been well characterized in the chicken ciliary ganglion; from embryonic day 8 until embryonic day 14, there is an approximately 50% loss of neurons in the ciliary ganglion. Neuronal survival can be measured by either counting the actual number of neurons that are living or by measuring the levels of choline acetyltransferase activity. Basic fibroblast growth factor, a protein that has trophic effects on a wide variety of cell types, has been shown to prevent naturally occurring neuronal death in the embryonic chick ciliary ganglion (Dreyer, D. *et al.* (1989) Neuroscience Letters 99:35-38).

Immunoaffinity purified NTF from pig lungs or recombinant NTF is administered daily (3 μg NTF/day/chick) to the chorionic-allantoic membrane of five chicken embryos during the period of naturally occurring neuronal death between embryonic day 8 and 14 (7 NTF administrations). (Note that Dreyer *et. al.* (1989) supra) used 3 μg/chick/day in their study of basic FGF.) The dose is raised to 6 μg/day/chick if an effect is not seen with 3 μg NTF/chick/day. The NTF reaches the ciliary ganglia after being taken up into the vascular system.

Five control embryos are given the vehicle only. The eggs are candled before injection to make sure that the eggs contain viable embryos.

Determination of ciliary ganglion neurons number and size in embryonic chickens.

After day 14, some of the ciliary ganglion are removed, fixed, and stained with toluidine blue; the size and number of neurons is determined by light microscopy according to the method of Abercrombie (1946). The percentage of ciliary neurons treated with NTF is compared to the number of untreated ciliary neurons that normally die during the period of naturally occurring neuronal death.

Determination of choline acetyltransferase activity in embryonic chickens.

After day 14, the ciliary ganglia from other chicks are removed and assayed for choline acetyltransferase (ChAT) activity. (See Wallace et al., (1986), (1987) *supra*.) The iris and ciliary muscle are also removed and assayed for ChAT activity using the same method; the results are expressed as ChAT activity per milligram wet muscle weight. Higher levels of ChAT activity are expected in both the ciliary ganglion and in the iris and ciliary muscle, which contain the ciliary nerve terminals, in chicks treated with NTF. The choline acetyltransferase activity in the ciliary muscle and iris is actually in the prejunctional ciliary nerve terminals which are broken off when the ciliary muscle and iris are dissected. As stated earlier, the reason that the iris, in addition to the ciliary muscle, is examined is that the ciliary ganglion also innervates the iris. The iris muscle regulates pupil size.

Histochemical staining of ciliary nerves in iris and ciliary muscles in embryonic chickens.

After day 14, the iris and ciliary muscle are removed, frozen, and stained with bromoindoxyl acetate dye combined with silver-gold in order to visualize nerves. This combined reagent simultaneously stains cholinesterase, found at ciliary nerve terminal/neuromuscular junctions, and the ciliary nerve axons, respectively (Pestronk, A. *et al.* (1978) Muscle and Nerve 1:70-74). This produces a transparent blue stain of the cholinesterase and a brown/grey stain of the nerves, and is ideal for quantitating nerve terminal arborization (branching) at neuromuscular junctions (Pestronk and Drachman, (1978) supra). All neuromuscular junctions are specifically stained in each histological section using

this method and both coarse and fine axon branches are seen. Quantitative measurements are made by light microscopy using a computer-based system for 3-D serial section reconstruction of nerves (Eutectics Electronics, Inc.). Using this Eutectics system, the following are quantitated for the ciliary nerve terminals: branch angle distribution, branch segment length, branch order, branch diameter, soma area, interminal distance, end plate length, and number of terminal branch points. This will provide a quantitative assessment of the effect of the NTF on ciliary nerves. An increase is expected in all of these parameters in the NTF-treated chickens (and rabbits). As an example, the number of terminal branch points and the length of the endplate should increase, as seen in figure 14. An endplate is the area where the nerve forms a synapse with the muscle. This neuronal tracing analysis is a common way of quantitating nerve innervation. (See Purves, D. and and Lichtman, J.W. (1985) "Principles of Neural Development," Sinhauer Associates, Inc. Publishers, Sunderland, Massachusetts; Shields, M.B. (1987) "Textbook of Glaucoma," 2nd edition, Williams and Wilkins, Baltimore).

Another way of visualizing the effect of NTF on ciliary nerve innervation is to stain with antisera to choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of acetylcholine and a specific marker of ciliary nerves. By immunohistochemistry an increase in the density of innervation of the ciliary muscle and iris is expected. This density change is quantitated using the computerized neuronal tracing system.

The effect of NTF on ciliary nerves and intraocular pressure in rabbits.

Whereas embryonic chickens provide a good model on which to perform the certain NTF experiments, these animals are not suitable for studying the effect of the NTF on intraocular pressure (IOP) since they are *in ovo*. Therefore, the effect of the NTF on IOP pressure is performed using rabbits. The rabbit is a frequently used animal for the study of anti-glaucoma drugs. (See for example Ellis *et. al.*, (1991) supra; Yoshitome, T. *et al.* (1991) Invest. Ophthalmol. Vis. Sci. 32:523-528).

The effect of NTF on intraocular pressure in rabbits.

For these experiments, the rabbits are first anesthetized with an i.m. injection of ketamine (33 mg/kg) and Rompum (3 mg/kg), and the NTF is injected

intracamerally (into the anterior eye chamber) through a 30 gauge needle inserted through the cornea. The anterior chamber contains the aqueous humor which bathes the iris and ciliary muscle, so that substances injected into the anterior chamber gain access to these tissues. The anterior chamber injection is an accepted way of injecting substance into the eye for both animal and human studies, and does not harm the rabbits. The intracameral route is not proposed for the ultimate treatment of humans and is used here only to conserve the NTF. The route for human treatment could be intramuscular, intravenous, or oral, depending upon further studies on the NTF's absorption. One μg NTF/10 μl PBS/3 kg rabbit is injected into the anterior eye chamber of three rabbits twice weekly for three weeks. Three control rabbits receive the vehicle only. If no effect of the NTF is seen on intraocular pressure after three weeks, the dose is increased to 5 μg NTF/10 μl PBS/3 kg rabbit for another two weeks.

Intraocular pressure is determined daily in both eyes using a tonometer (Bio-Rad applantion pneuma tonometer, modular one complete). Baseline IOP is taken over a one week period on all rabbits before treatment with NTF. Measurements are taken during the same time of the day to minimize the effects of fluctuations in IOP that are known to occur during the day. The intracameral injection per se does not have an effect on IOP. The injection site, which is at the periphery of the cornea, typically closes within seconds after the needle is removed so that little or no fluid is lost. The rabbits are sacrificed according to an approved animal protocol guideline after the three or five week period, as described above, and the iris and ciliary muscles are further processed for histochemistry and immunocytochemistry as described below.

It is possible that the NTF will not have any effect on resting intraocular pressure in normal rabbits since their ciliary nerves may be supplied with a sufficient supply of trophic substances to maintain ciliary nerve function as it relates to IOP. Although a dramatic effect on ciliary nerve arborization and levels of ChAT activity is expected, a negative result would not be considered evidence against use of NTF to treat glaucoma in humans, since a substantial percentage of human patients have a parasympathetic neuropathy that cannot be tested in a normal rabbit model.

The effect of NTF on ChAT activity and on ciliary nerve innervation of the iris and ciliary muscles in rabbits.

A limited number of similar experiments previously described for the embryonic chickens are performed in rabbits in order to show that the same basic effects that are seen in embryonic chickens are seen in rabbits. The effect of the NTF on ChAT activity in iris and ciliary muscle, ciliary nerve innervation (histochemical and immunocytochemical staining) in rabbit ciliary muscle and iris is determined, as described above for the chickens, on the rabbits after their three to five week IOP observation period. The effect of the NTF activity on ciliary ganglion neuron number and size, and the effect of the NTF on ChAT activity in the rabbit ciliary ganglion is not determined because of the small size of the rabbit ciliary ganglion and difficulty in dissecting it from the rabbit.

The effect of NTF on parasympathetic (ciliary) nerve regeneration in rabbits.

In order to examine nerve regeneration *in vivo*, the parasympathetic nerve terminals in the eye are destroyed using the specific cholinergeric neurotoxin AF64A (Research Biochemicals Inc., Natick, MA). AF64A (ethylcholine aziridinium) is structurally related to choline and irreversibly inhibits high affinity choline transport into cholinergic nerve terminals, which leads to destruction of the cholinergic nerve terminals by an unknown mechanism (Smith, (1988). It has been used to destroy the nerve terminals of parasympathetic nerves in the eye (Kessler, (1985). 2-3 kg rabbits are used. The rabbits are first anesthetized with an i.m. injection of ketamine (33 mg/kg) and Rompum (3 mg/kg). Unilateral injections of AF64A (10 nmoles in 10 μl) are made into the anterior chamber of six rabbits on two consecutive days. The dose is adjusted if no effect (no loss of light reflex) is seen. Vehicle is injected into three control rabbits.

Destruction of the parasympathetic (ciliary) nerve terminals is monitored by measuring pupil size. Although the iris is not involved in regulation of intraocular pressure, it is innervated by the ciliary ganglion. Pupil diameter is a classical noninvasive, pharmacological method of determining the state of parasympathetic innervation of the eye (Armaly, M.F. *et al.* (1963) Arch. Int. Pharmacodyn. 145:89-96; Colansanti, B. K. *et al.* (1982) J. Autonom. Nerv. Sys. 5:111-118). The onset of ciliary nerve terminal destruction is seen when the

rabbit eyes do not constrict their pupils in response to a brief flash of light from a pen light (loss of light reflex); the pupils remain dilated due to the patent sympathetic innervation. The light reflex is monitored every several hours over the first day and then daily thereafter. A micrometer is used to measure pupil diameter in rabbits placed in an approved restraining device. In addition, the intraocular pressure is measured using the tonometer. The loss of light reflex occurs in approximately 1-5 days and indicates that there has been destruction of the ciliary nerve terminals. At the time of loss of light reflex, NTF (1 μg/10 μl) is injected intracamerally daily, into three out of the six rabbits that have received the AF64A, for up to 14 days into the anterior chamber. Vehicle (sterile phosphate buffered saline) is injected into the three other rabbits that also received AF64A.

The NTF enhances (speeds up) the regeneration of the ciliary nerves to the iris, thereby returning the light reflex. Regeneration of the ciliary nerves in the untreated (vehicle) may occur, but this should occur at a much slower rate. Rabbits are monitored daily. Thus, there is a fewer numbers of days for the return of the light reflex in rabbits that are treated with the NTF as compared to the untreated rabbits. This experiment is stopped either when there is evidence of ciliary nerve regeneration (return of the light reflex) or 14 days after the last injection of NTF. At that time the animals are sacrificed, and ChAT activity is determined and immunohistochemistry (ChAT staining) is performed on parts of the iris and ciliary muscle.

Immunoassay of NTF.

The purpose of developing an immunoassay is to have a method of quantitating recombinantly expressed NTF in culture supernatants and quantitating NTF in a general survey of various tissues. An NTF capture assay is set up using purified mouse monoclonal or sheep polyclonal antibody. Antibody is bound to the bottom of wells of a 96-well plate; the wells are incubated for several hours and then washed with PBS; nonspecific protein binding sites are blocked with BSA; the wells are incubated for 2 hours to overnight and then are washed with PBS. A known amount of ^{125}I-NTF peptide fragment #3 is added to the well and the experimental sample with an unknown quantity of unlabelled NTF is added;

the wells are incubated for several hours and the plate is washed; wells are individually counted in a gamma counter. An inhibition in the amount of bound ^{125}I-NTF peptide fragment #3 signifies the presence of the NTF, assuming that the whole NTF competes with the ^{125}I-NTF peptide fragment #3. The amount of NTF present is calculated from the IC_{50}. Note that the residue #13 of NTF peptide fragment #3 is a tyrosine and can be ^{125}Iodinated using iodo-gen™ iodination reagent (Pierce). Alternatively, the primary amines of NTF peptide fragment #3 could be ^{125}Iodinated with the Bolton-Hunter reagent (Pierce). NTF levels are measured in lung, heart, kidney, intestine, eye, skeletal muscle, and brain of the rabbits that received the vehicle control injections for the IOP experiment.

In accordance with the subject invention, novel compositions are provided for treatment of ocular disorders associated with ciliary ganglionic nerve cell degeneration. The amino acid sequences of the tryptic fragments of the subject NTF do not share homology with any known neurotrophic factors. In addition, the apparent molecular weight (31,500), isoelectric point (5.5-6.8), inability to bind heparin (heparin affinity chromatography is used in the purification of the NTF), preparative tissue source (pig lung), and neuronal specificity (specific for parasympathetic neurons) make the subject NTF different from other known neurotrophic factors. The subject compositions are highly purified and can be obtained by extraction of mammalian lung tissue or produced by recombinant means. The subject compositions provide an alternative treatment for various disorders of the eye which are treated only with difficulty today or for which no useful treatment exists.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit of scope of the appended claims.

WHAT IS CLAIMED IS:

1. A composition comprising a neurotrophic factor which is characterized as (a) having a pI in the range of 5.6-7.0; (b) having a molecular weight as determined by SDS gel electrophoresis of about 31.5kD; (c) being obtainable from mammalian lung tissue; (d) being capable of maintaining viable embryonic ciliary neurons *in vitro*, as compared to the absence of said composition; (e) being capable of increasing choline acetyltransferase activity in parasympathetic ciliary neurons in vitro, said increasing being responsive to changes in potassium ion concentration; and (f) not binding heparin.

2. A composition according to Claim 1, wherein said composition is concentrated in said neurotrophic factor by at least one of anionic, cationic and molecular sieving HPLC columns.

3. A composition according to Claim 1, wherein said composition is at least substantially pure.

4. A composition according to Claim 1, wherein said neurotrophic factor is isolated from pig lung.

5. A composition according to Claim 1, wherein said ED_{50} is at least 50 ng/ml as measured *in vitro* using embryonic day 8 chicken ciliary neurons.

6. A composition according to Claim 3, wherein said composition provides a single silver-stained band at approximately 31.5 kD using SDS electrophoresis.

7. A method for increasing parasympathetic activity in a parasympathetic nerve cell, said method comprising:

contacting said parasympathetic nerve cell with a composition comprising a neurotrophic factor whereby said parasympathetic activity is increased.

8. The method according to Claim 7, wherein said nerve cell is *in vivo*.

9. The method according to Claim 7, wherein said parasympathetic activity comprises survival of parasympathetic neurons.

10. The method of Claim 9, wherein said neurons are *in vitro*.

11. The method according to Claim 10, wherein said cell is a ciliary ganglion neurons and said activity enhancing amount is sufficient to prevent death of said ciliary ganglion neurons.

12. The method according to Claim 10, wherein said activity enhancing amount is 0.1 mg/kg to 5mg/kg body weight of said neurotrophic factor.

13. A method for enhancing choline acetyltransferase activity in a cell's said method comprising:

contacting said cell with a choline acetyl transferase activity enhancing amount of a composition comprising a neurotrophic factor whereby said parasympathetic activity is increased.

14. A method according to Claim 13, wherein said cell is a ciliary ganglion nerve cell.

15. A method according to Claim 13, wherein said cell is in vivo and the amount administered is 0.1 mg/kg to 5 mg/kg body weight.

16. Antibodies specific for a polypeptide according to Claim 1.

17. The antibodies according to Claim 16, wherein said antibodies are polyclonal antibodies.

18. A recombinant DNA sequence or a naturally occurring mutant DNA sequence thereof, wherein said DNA sequence comprises a nucleotide sequence which encodes a mammalian neurotrophic factor, and wherein said nucleotide sequence hybridizes under stringency conditions corresponding to a wash of 2.0 SSC, 0.5% SDS at 37°C to porcine lung cDNA having ATCC accession number [add numbers], said DNA sequence being free from DNA encoding additional protein normally produced in porcine lung cells.

19. A mammalian neurotrophic factor encoded at least in part by a cDNA clone having ATCC Accession No. _____.

20. A cDNA clone having ATCC Accession No. _____.

21. A mammalian neurotrophic factor having substantial homology with a polypeptide encoded by a cDNA clone having ATCC Accession No. _____.

22. The polypeptide according to Claim 21, wherein said polypeptide is a congener of a neurotrophic factor which is characterized as (a) having a pI in the range of 5.6-7.0; (b) having a molecular weight as determined by SDS gel electrophoresis of about 31.5kD; (c) being obtainable from mammalian lung tissue; (d) being capable of maintaining viable embryonic ciliary neurons *in vitro*, as compared to the absence of said composition; (e) being capable of increasing choline acetyltransferase activity in parasympathetic ciliary neurons in vitro, said increasing being responsive to changes in potassium ion concentration; and (f) not binding heparin.

23. The polypeptide according to Claim 19 or 22, wherein said polypeptide comprises one or more amino acid sequences selected from the group consisting of:

F D S V Q S A Q R;

I Q T V K;

L N V F K N D Q D T W D Y T N P N L S G; and

D N A G D Q.

24. An antibody, the antigen combining site of which binds to a protein antigen having an amino acid sequence LNVFKNDQDTWDYTNPNLSG.

25. An antibody according to Claim 24, wherein said antibody blocks ciliary ganglion survival-promoting activity of a mammalian neurotrophic factor.

26. The antibody according to Claim 24 or Claim 25, wherein said antibody is a polyclonal antibody.

[received by the International Bureau on 6 May 1994 (06.05.94); original claims 18, 19,20 and 21 cancelled; original claims 13,22 and 23 amended; other claims unchanged (3 pages)]

contacting said parasympathetic nerve cell with a composition comprising a neurotrophic factor whereby said parasympathetic activity is increased.

8. The method according to Claim 7, wherein said nerve cell is *in vivo*.

9. The method according to Claim 7, wherein said parasympathetic activity comprises survival of parasympathetic neurons.

10. The method of claim 9, wherein said neurons are *in vitro*.

11. The method according to Claim 10, wherein said cell is a ciliary ganglion neurons and said activity enhancing amount is sufficient to prevent death of said ciliary ganglion neurons.

12. The method according to Claim 10, wherein said activity enhancing amount is 0.1 mg/kg to 5mg/kg body weight of said neurotrophic factor.

13. A method for enhancing choline acetyltransferase activity in a cell said method comprising:

contacting said cell with a choline acetyl transferase activity enhancing amount of a composition comprising a neurotrophic factor whereby said parasympathetic activity is increased.

14. A method according to Claim 13. wherein said cell is a ciliary ganglion nerve cell.

15. A method according to Claim 13, wherein said cell is in vivo and the amount administered is 0.1 mg/kg to 5 mg/kg body weight.

16. Antibodies specific for a polypeptide according to Claim 1.

17. The antibodies according to Claim 16, wherein said antibodies are polyclonal antibodies.

22. A polypeptide comprising a congener of a neurotrophic factor which is characterized as (a) having a pI in the range of 5.6-7.0; (b) having a molecular weight as determined by SDS gel electrophoresis of about 31.5kD; (c) being obtainable from mammalian lung tissue; (d) being capable of maintaining viable embryonic ciliary neurons *in vitro*, as compared to the absence of said composition; (e) being capable of increasing choline acetyltransferase activity in parasympathetic ciliary neurons in vitro, said increasing being responsive to changes in potassium ion concentration and (f) not binding heparin.

23. A polypeptide comprising one or more amino acid sequences selected from the group consisting of:

F D S V Q S A Q R;

I Q T V K;

L N V F K N D Q D T W D Y T N P N L S G; and

D N A G D Q.

24. An antibody, the antigen combining site of which binds to a protein antigen having an amino acid sequence LNVFKNDQDTWDYTNPNLSG.

25. An antibody according to Claim 24, wherein said antibody blocks ciliary ganglion survival-promoting activity of a mammalian neurotrophic factor.

26. The antibody according to Claim 24 or Claim 25, wherein said antibody is a polyclonal antibody.

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PURIFICATION, ISOELECTRIC POINT DETERMINATION,
AND PARTIAL SEQUENCING OF THE NTF
HOMOGENIZATION OF PIG LUNGS
PREPARATION OF A 25,000 X G SUPERNATANT FRACTION
LOW PRESSURE ANION-EXCHANGE (FAST Q) CHROMATOGRAPHY (Batch elution)
LOW PRESSURE CATION-EXCHANGE (FAST S) CHROMATOGRAPHY (Batch elution)
LOW PRESSURE HEPARIN AFFINITY CHROMATOGRAPHY (Batch elution)
(Collect the non-heparin binding fraction)
PREPARATIVE ISOELECTRIC FOCUSING FOR DETERMINATION
OF THE ISOELECTRIC POINT (Figure 2)
LOW PRESSURE MOLECULAR SIEVING (S-100 SEPHACRYL)
CHROMATOGRAPHY (Figure 3)
PHENYL HYDROPHOBIC INTERACTION HPLC (Figure 4)
ETHER HYDROPHOBIC INTERACTION HPLC (Figure 5)
SDS ELECTROPHORESIS
(Figure 6 is coomassie blue stained and figure 7 is silver stained)
ELECTROBLOTTING TO PVDF OR NITROCELLULOSE PAPER FOR SEQUENCING
DETERMINATION OF THE AMINO ACID COMPOSITION (Table 2)
TRYPTIC DIGESTION
HPLC SEPARATION OF TRYPTIC FRAGMENTS
AMINO ACID SEQUENCING OF THE TRYPTIC FRAGMENTS (Table 1)
SYNTHESIS OF NTF PEPTIDE FRAGMENT #3 ONTO A POLYLYSINE BACKBONE
PRODUCTION OF RABBIT POLYCLONAL ANTISERA AGAINST NTF
PEPTIDE FRAGMENT #3 THAT RECOGNIZES THE NATIVE 31.5 MW
NTF MOLECULE AND NEUTRALIZES NTF ACTIVITY
ELISA TO DETERMINE RABBIT SERUM ANTIBODY TITER (Figure 8)
WESTERN BLOTS OF THE 25,000 XG PIG LUNG SUPERNATANT FRACTION
SUBJECTED TO SDS ELECTROPHORESIS AND ANALYTICAL ISOELECTROFOCUSING
(Figures 9 and 10)
BIOASSAYS THAT SHOW THE NEUTRALIZING EFFECT OF THE RABBIT
ANTISERUM ON NTF ACTIVITY (Figures 11 and 12)

FIG. 1

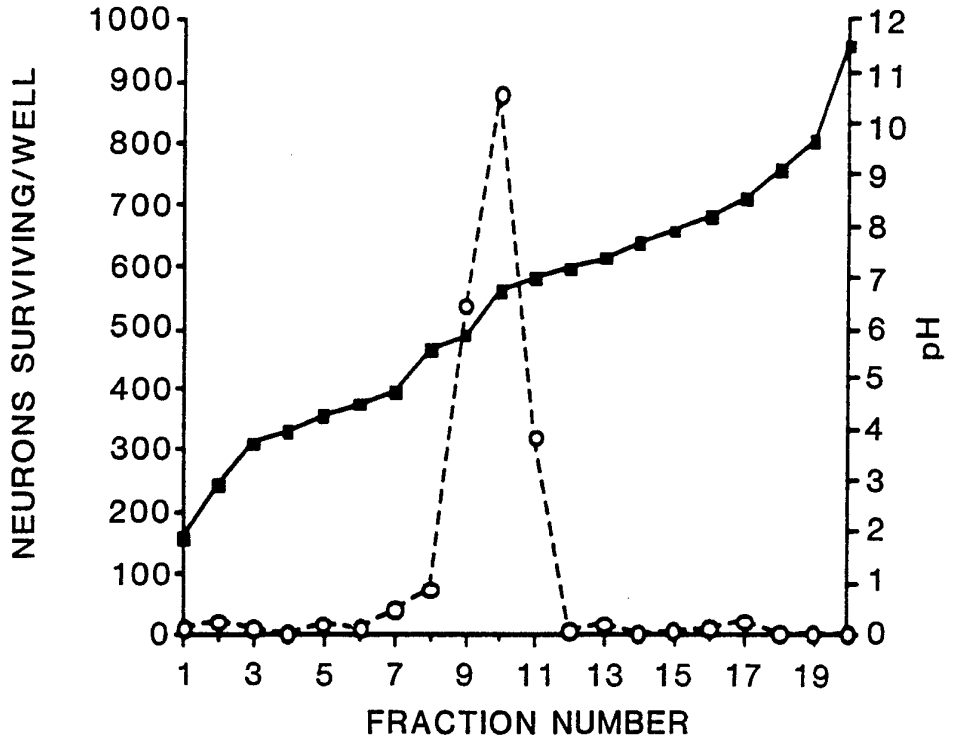
NEURONS SURVIVING/WELL
1000
900
800
700
600
500
400
300
200
100
0
12
11
10
9
8
7
6
5
4
3
2
1
0
pH
1 3 5 7 9 11 13 15 17 19
FRACTION NUMBER

FIG. 2

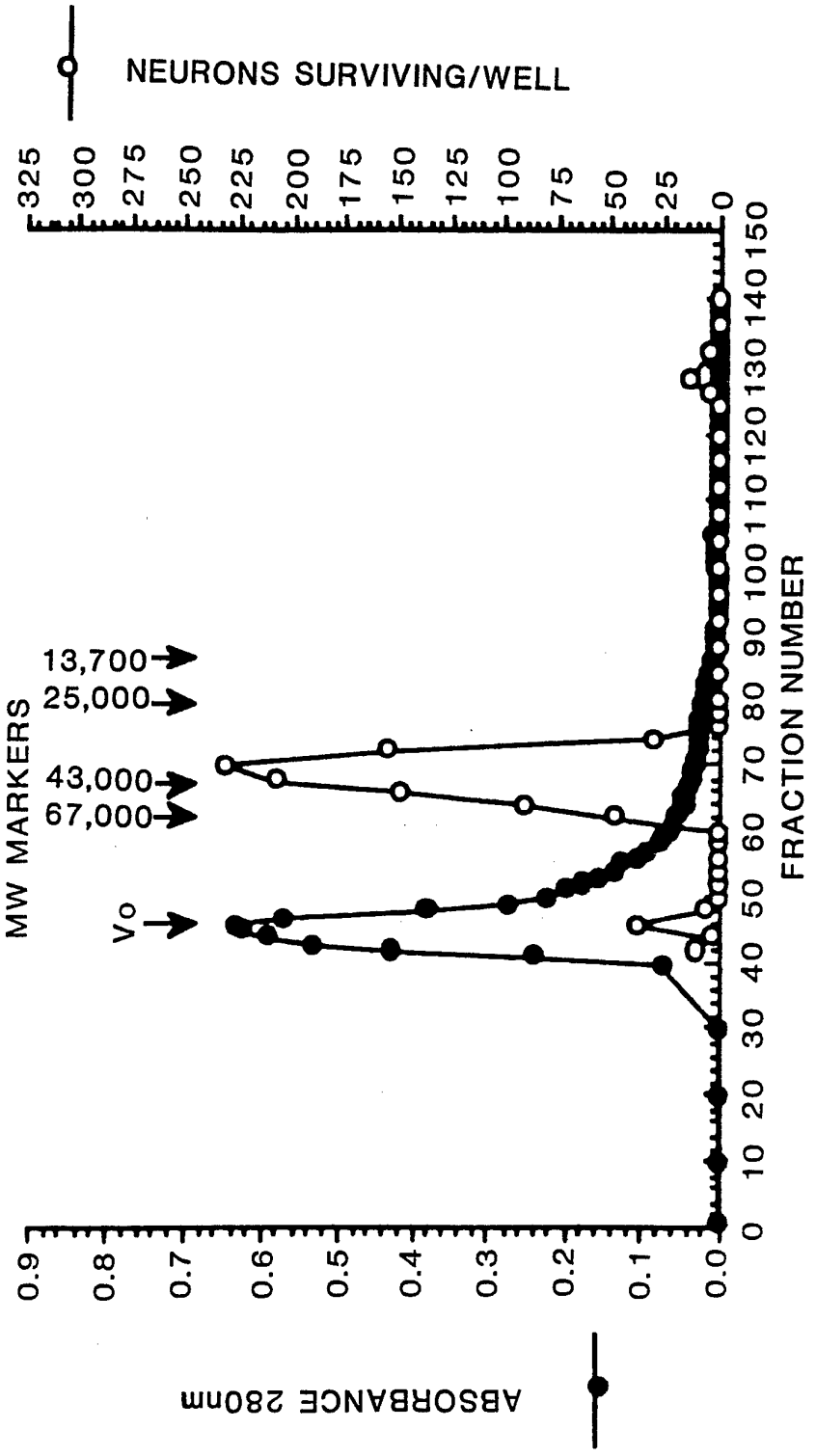
NEURONS SURVIVING/WELL
325 300 275 250 225 200 175 150 125 100 75 50 25 0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
FRACTION NUMBER
MW MARKERS
13,700
25,000
43,000
67,000
Vo
0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
ABSORBANCE 280nm

FIG. 3

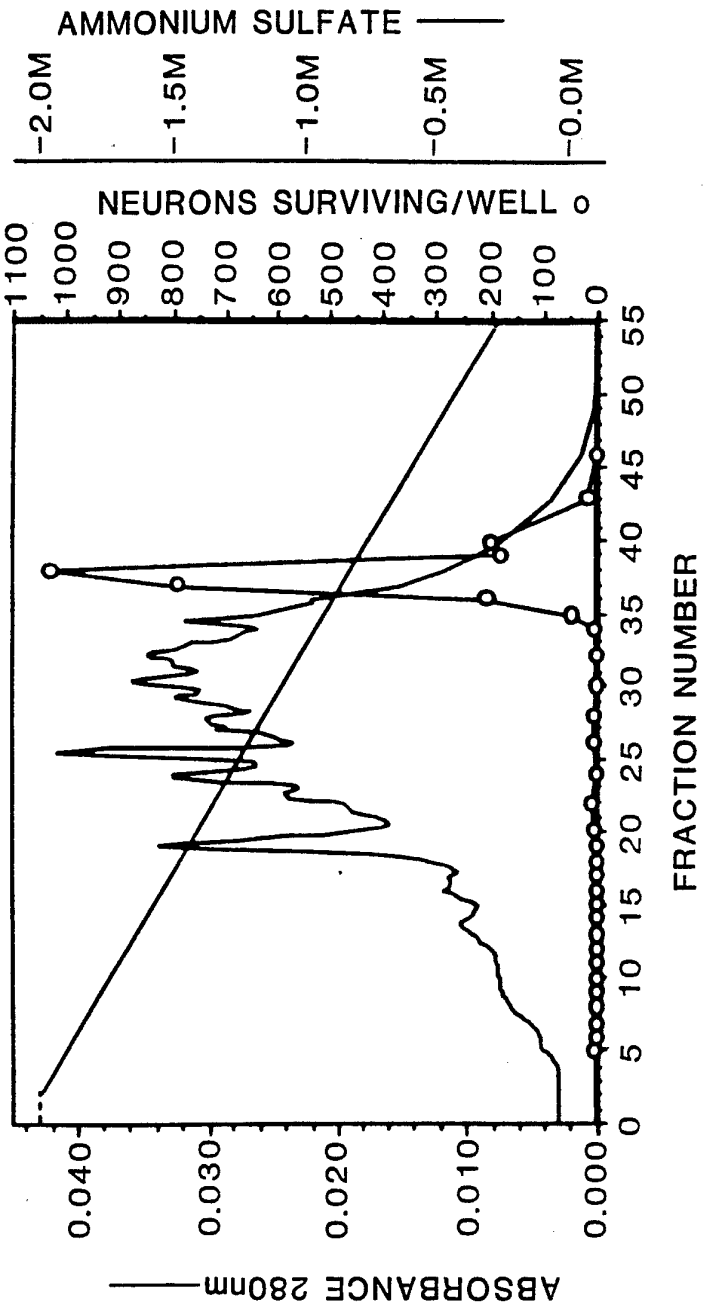
AMMONIUM SULFATE
2.0M
1.5M
1.0M
0.5M
0.0M
NEURONS SURVIVING/WELL o
1100
1000
900
800
700
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40 45 50 55
FRACTION NUMBER
0.040
0.030
0.020
0.010
0.000
ABSORBANCE 280nm

FIG. 4

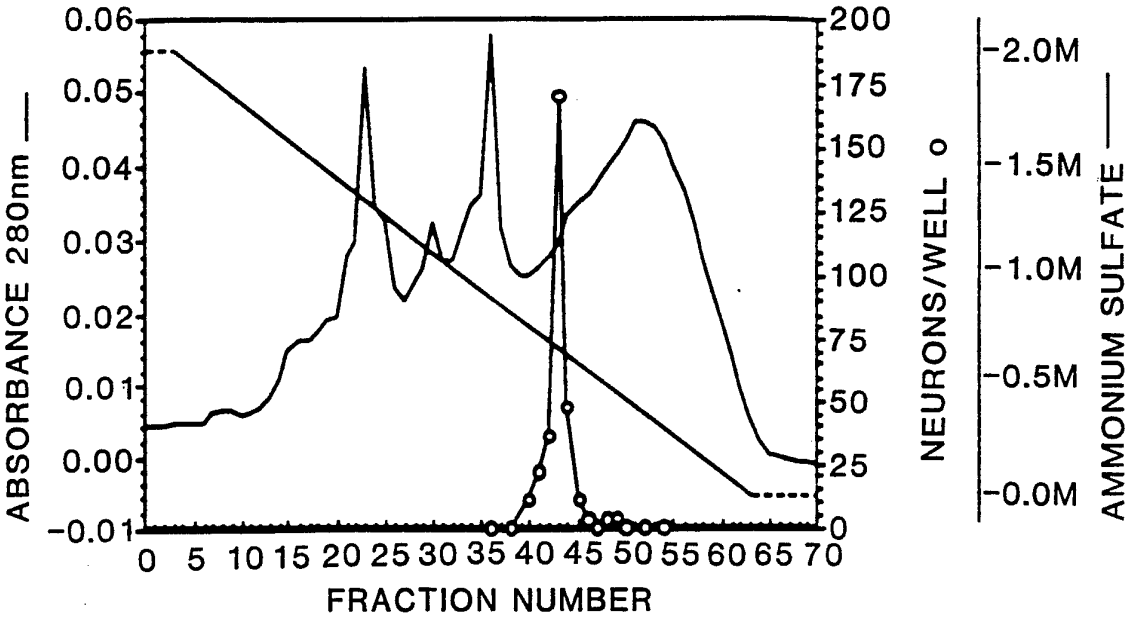
ABSORBANCE 280nm
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
FRACTION NUMBER
200
175
150
125
100
75
50
25
0
NEURONS/WELL o
-2.0M
-1.5M
-1.0M
-0.5M
-0.0M
AMMONIUM SULFATE

FIG. 5

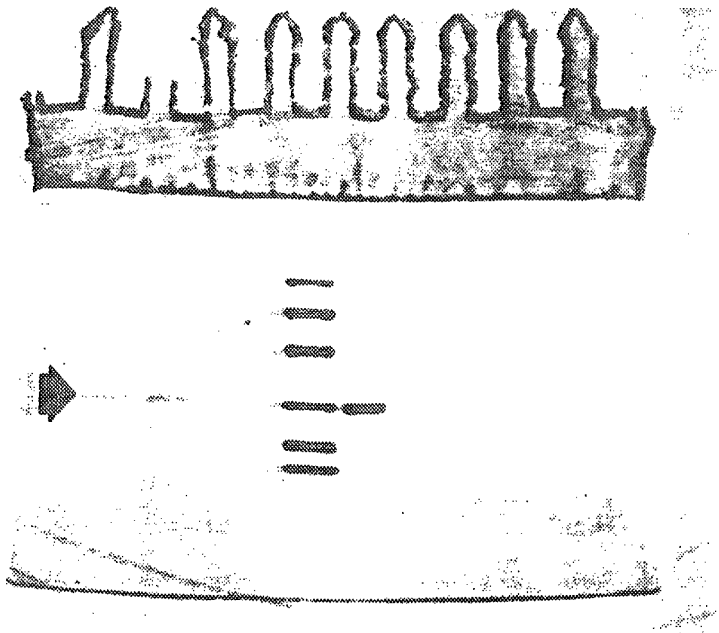

FIG. 6

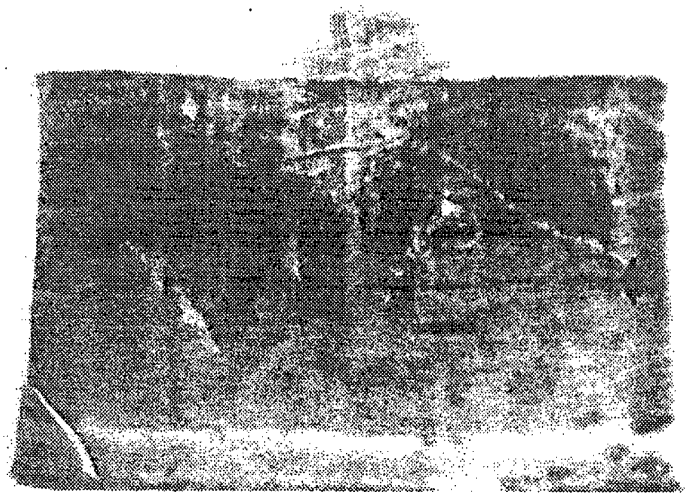

FIG. 7

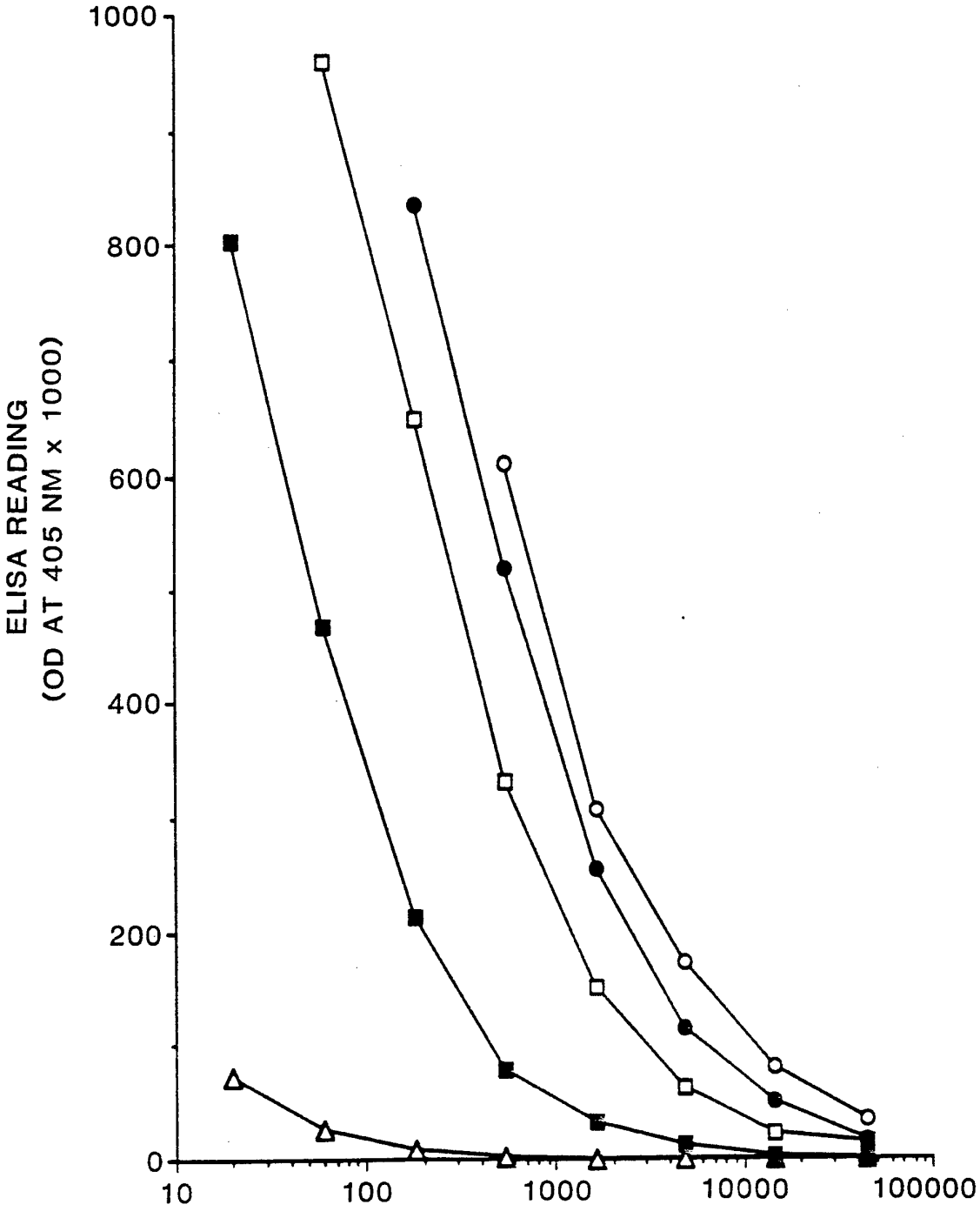
1000
800
600
400
200
0
ELISA READING
(OD AT 405 NM x 1000)
10
100
1000
10000
100000
RABBIT NORMAL SERUM OR ANTISERUM DILUTIONN

FIG. 8

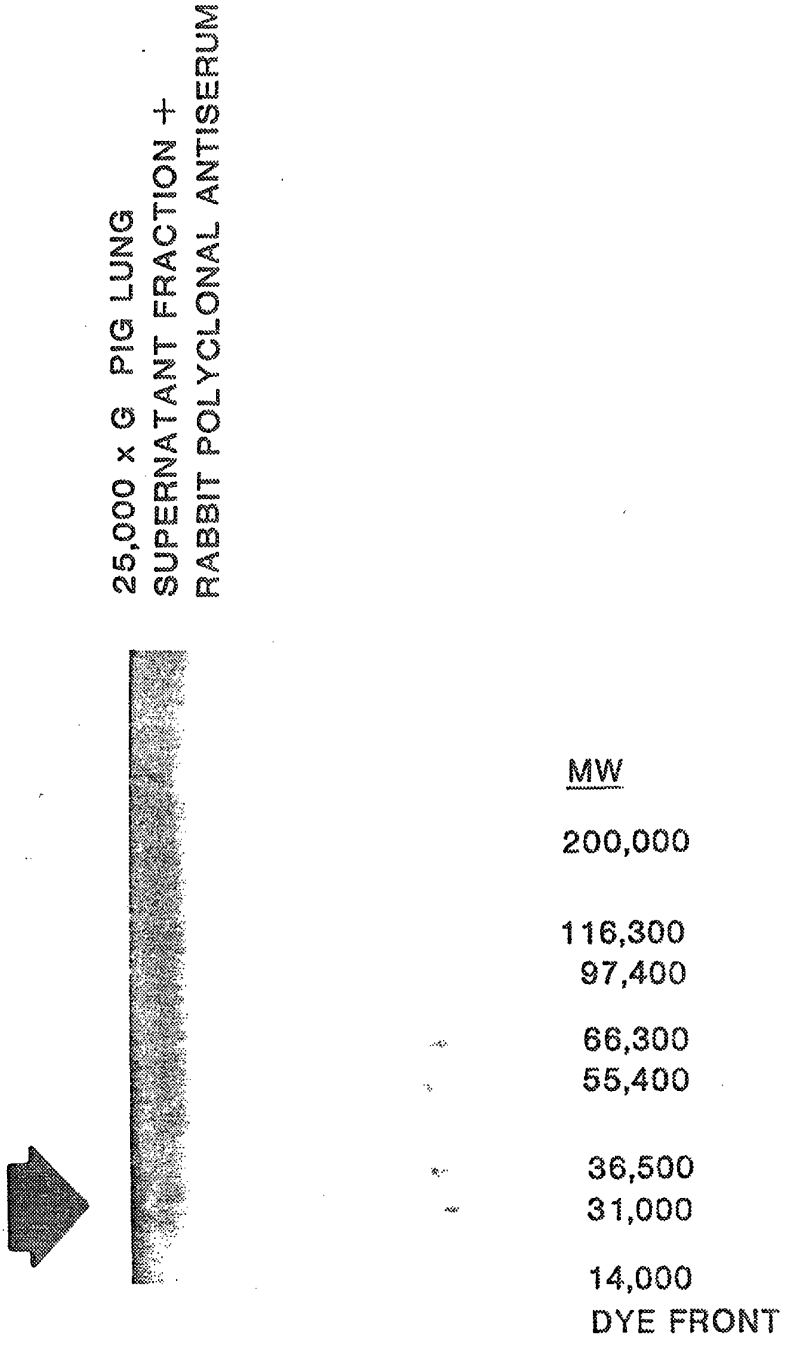
25,000 x G PIG LUNG
SUPERNATANT FRACTION +
RABBIT POLYCLONAL ANTISERUM
MW
200,000
116,300
97,400
66,300
55,400
36,500
31,000
14,000
DYE FRONT

FIG. 9

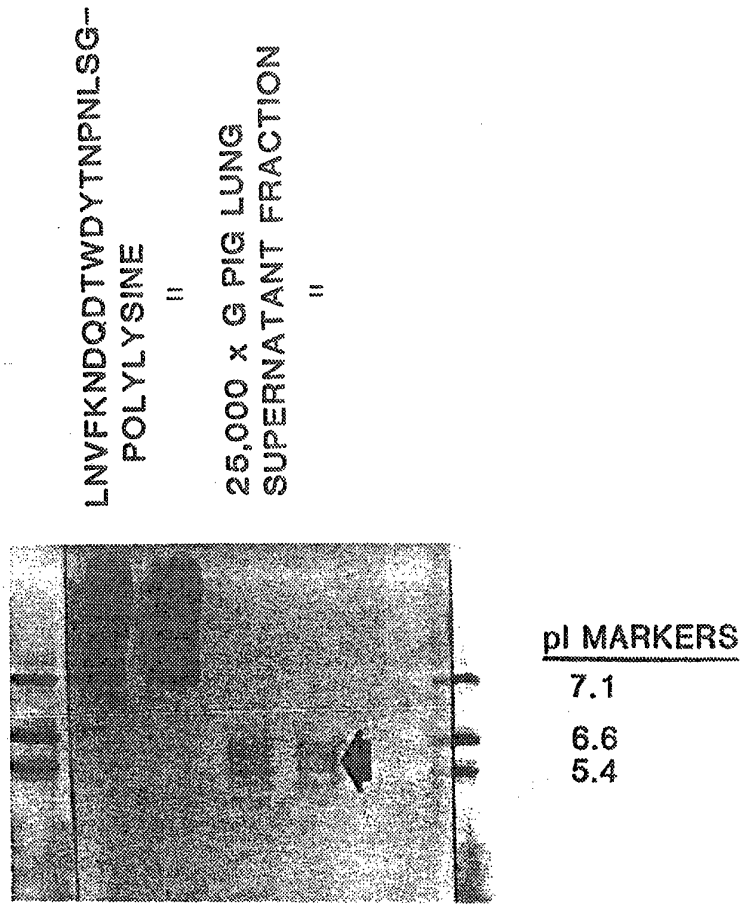
LNVFKNDQDTWDYTNPNLSG--
POLYLYSINE
=
25,000 x G PIG LUNG
SUPERNATANT FRACTION
=
pI MARKERS
7.1
6.6
5.4

FIG. 10

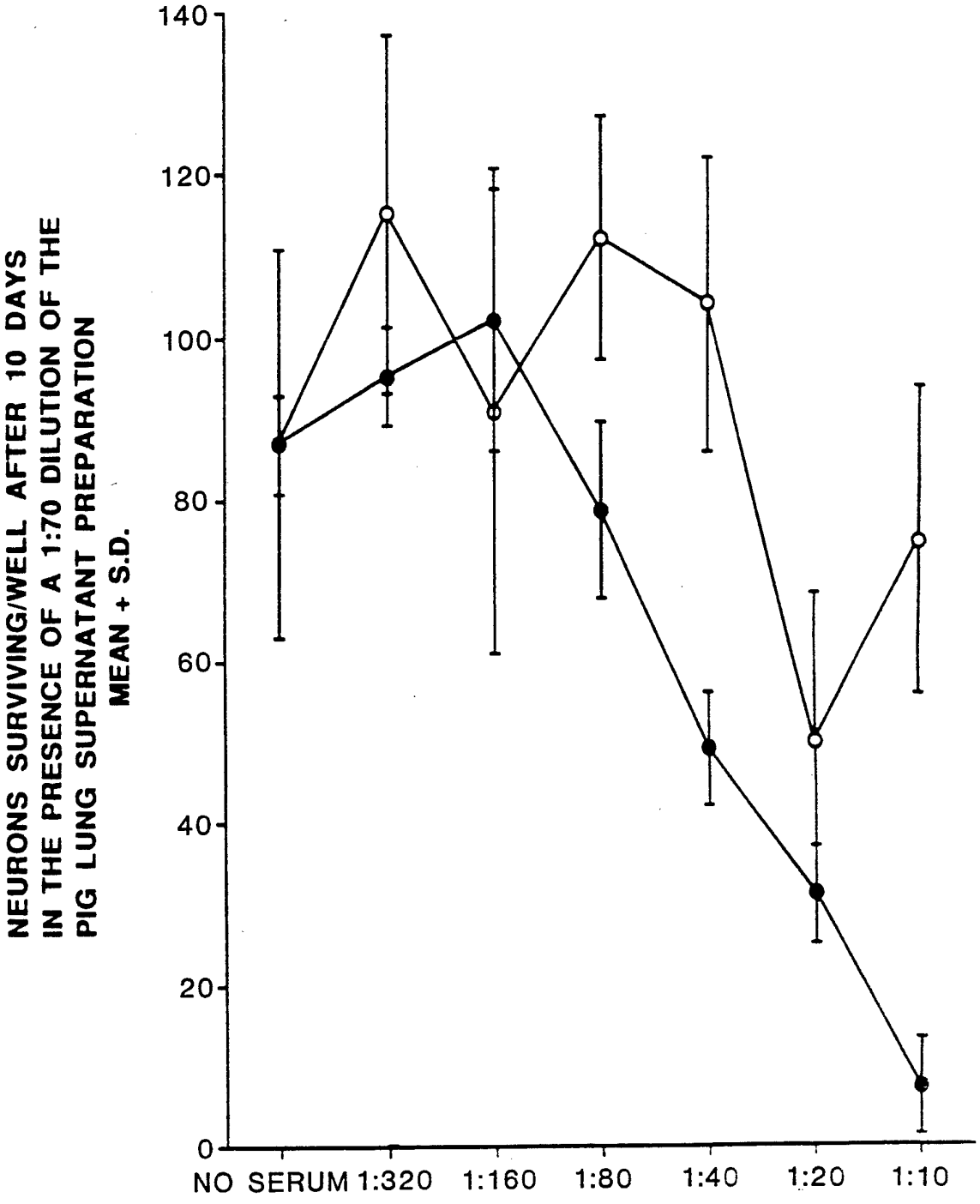
NEURONS SURVIVING/WELL AFTER 10 DAYS
IN THE PRESENCE OF A 1:70 DILUTION OF THE
PIG LUNG SUPERNATANT PREPARATION
MEAN + S.D.
140
120
100
80
60
40
20
0
NO SERUM
1:320
1:160
1:80
1:40
1:20
1:10
RABBIT NORMAL SERUM OR ANTISERUM DILUTION

FIG. 11

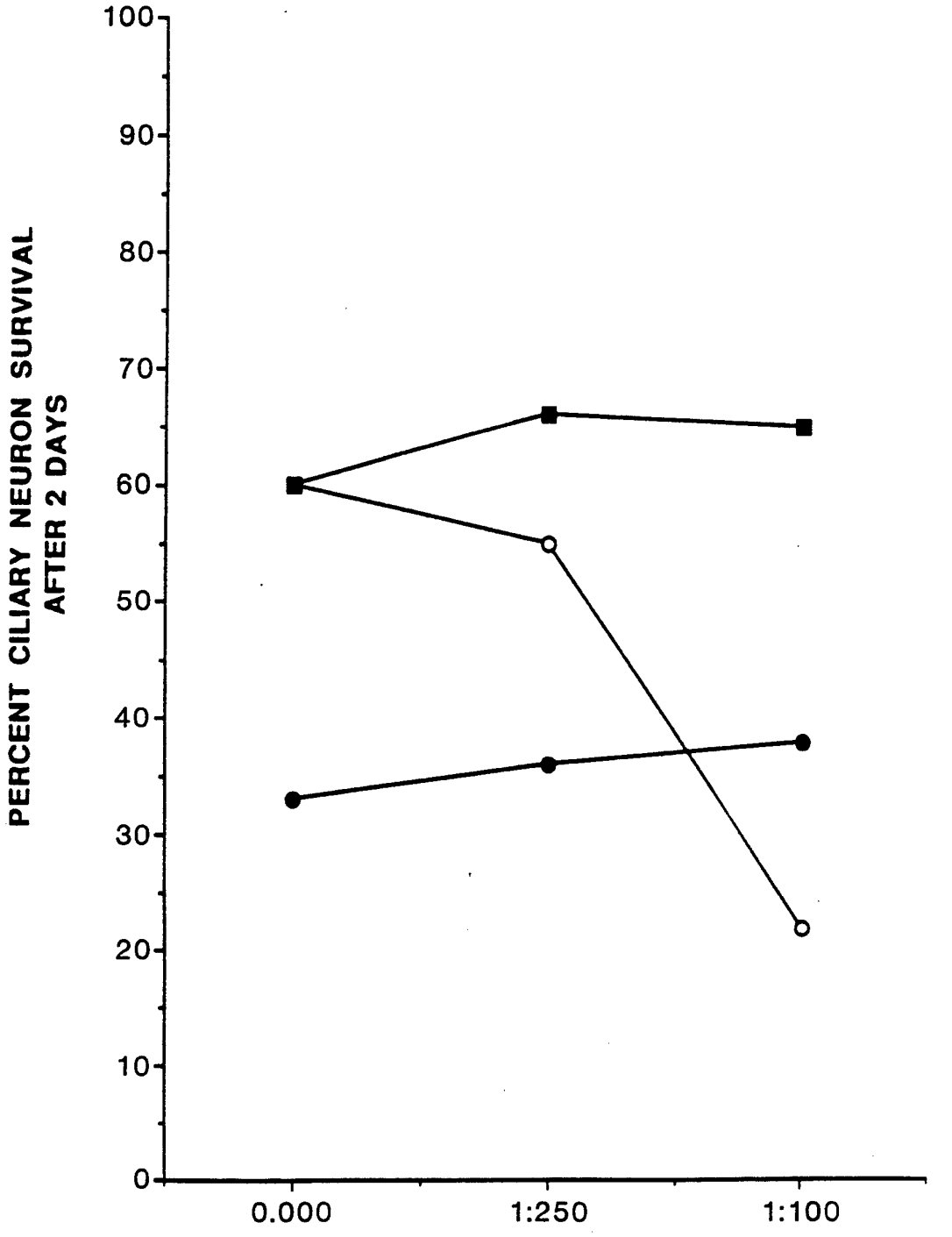
PERCENT CILIARY NEURON SURVIVAL AFTER 2 DAYS
100
90
80
70
60
50
40
30
20
10
0
0.000
1:250
1:100
RABBIT NORMAL SERUM OR ANTISERUM DILUTION

FIG. 12

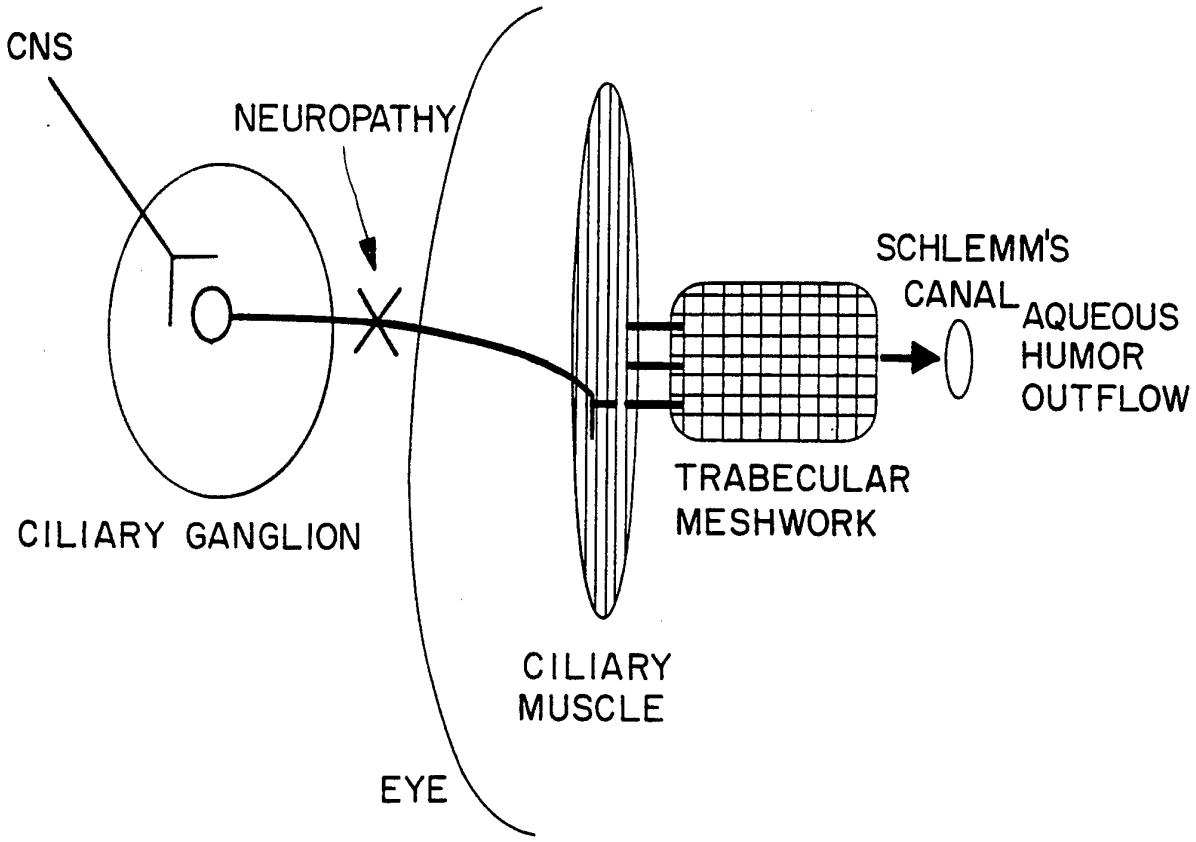
CNS
NEUROPATHY
SCHLEMM'S
CANAL
AQUEOUS
HUMOR
OUTFLOW
TRABECULAR
MESHWORK
CILIARY GANGLION
CILIARY
MUSCLE
EYE

FIG. 13

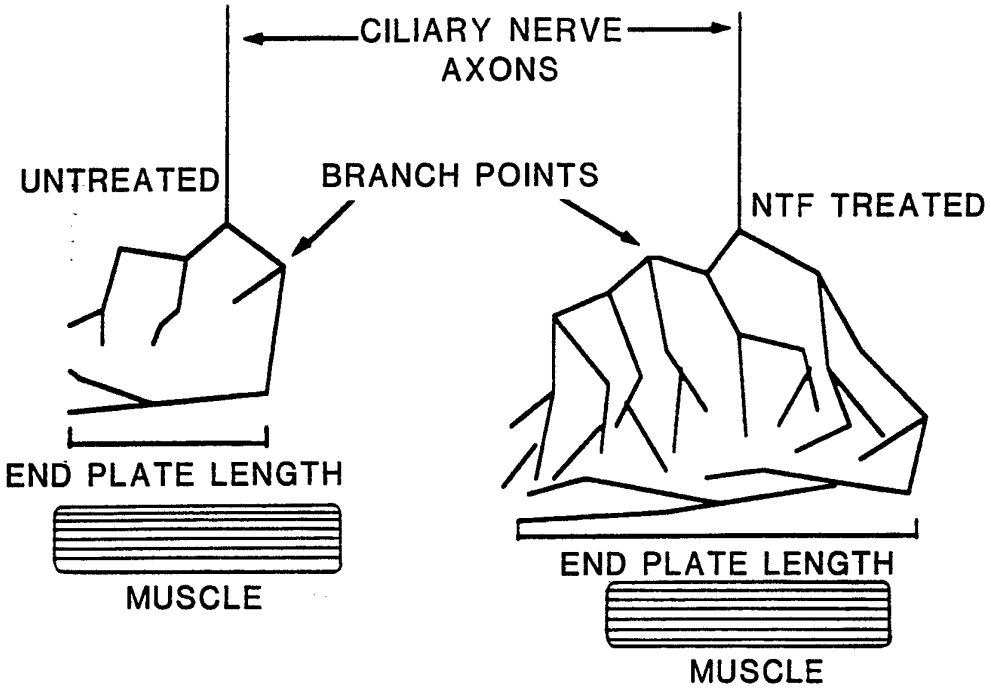
CILIARY NERVE AXONS
UNTREATED
BRANCH POINTS
NTF TREATED
END PLATE LENGTH
MUSCLE
END PLATE LENGTH
MUSCLE

FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/10117

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; A61K37/02; C12P21/02; C07K15/00
C12P21/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; C12P ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BRAIN RESEARCH vol. 411, no. 2, 19 May 1987, pages 351 - 363 WALLACE, T. & JOHNSON JR., E. 'Partial purification of a parasympathetic neurotrophic factor in pig lung' cited in the application	7-15
A	see the whole document	1-26

X	BRAIN RESEARCH vol. 375, no. 1, 4 June 1986, pages 92 - 101 WALLACE, T. & JOHNSON ,E. 'Characterization of choline acetyltransferase-sustaining and survival-promoting activities for parasympathetic neurons in pig lung' cited in the application see the whole document	7-15

	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 AUGUST 1993	01. 09. 93.
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	SCIENCE vol. 246, 24 November 1989, LANCASTER, PA US pages 1023 - 1025 LIN, L.-F. ET AL. 'Purification, cloning, and expression of ciliary neurotrophic factor (CNTF)' cited in the application see the whole document	7-12
X	THE JOURNAL OF CELLULAR BIOLOGY vol. 109, no. 6, December 1989, pages 2575 - 2587 PIÑOL-ROMA, S. ET AL. 'A novel heterogenous nuclear RNP protein with a unique distribution on nascent transcripts' cited in the application see figure 11	23-24
E	US,A,5 166 317 (WALLACE, T. ET AL.) 24 November 1992 cited in the application see the whole document	1-15,22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/10117

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 7-9, 13-15 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant s protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9210117
SA 68038

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 16/08/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5166317	24-11-92	None	