Comparison of the properties of cholinergic differentiation factors and examination of their possible role in vivo

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My thesis involved a joint project between Dr. S. Landis' and Dr. P. H. Patterson's laboratories. One reason the collaboration was fruitful was that both Paul and Story made great efforts to accomodate a sometimes stubborn and headstrong student. I am particularly grateful to Story for her advice and support, both personal and professional, throughout my thesis training. Certainly much less would have been accomplished without her willingness to do everything necessary to get the experiments done. I thank Paul for his enthusiasm, advice and the several contributions he made to the experimental design. The developmental biology course that Paul taught is still my favorite course.

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I would also like to thank members of Story's and Paul's laboratory who were there when I needed them. Hiro Nawa, Sigrun Korsching, Nagesh Mahanthapa and Jossette Carnahan all helped make the laboratory a fun place. Bob Schotzinger, Paul Henion and Mike Dobrea showed me how much real men drink. Finally, I would like to thank my wife, Seema, for her support and encouragement in my more depressed moments, and for her willingness to put up with my irregular hours.

Comparison of the properties of cholinergic differentiation factors and examination of their possible role in vivo

We have compared the immunological, biochemical, and biological properties of three previously described cholinergic factors, cholinergic differentiation factor/Leukemia inhibitory factor (CDF/LIF), ciliary neurotrophic factor (CNTF), and membrane-associatedneurotransmitter-inducing substance (MANS). CDF/LIF differs from CNTF and MANS in that it does not have any ciliary neurotrophic activity. Further, antibodies generated against the N-terminal sequence of CDF/LIF do not precipitate cholinergic activity from sciatic nerve (CNTF) or spinal cord (MANS) preparations indicating that CDF/LIF is a distinct molecule. MANS preparations contain a 24 kD molecule immunologically related to CNTF and CNTF antisera also, immunoprecipitate the cholinergic differentiation activity present in MANS fractions. CNTF, like CDF/LIF, affects neuropeptide expression. Neuropeptide Y levels are reduced and vasoactive intestinal peptide, somatostatin and Substance P levels are elevated in a dose-dependent fashion. Unlike CDF/LIF, the effects of CNTF on cholineacetyltransferase and peptide induction are not antagonized by depolarization. In addition, CNTF, in contrast to CDF/LIF, does not modulate peptide levels in DRG neuronal cultures. Thus, at least two distinct factors, CNTF and CDF/LIF, exist and have distinct but overlapping functions.

We have investigated the possible role of CDF (cholinergic differentiation factor from skeletal muscle), CDF/LIF CNTF, and MANS in mediating the target-directed noradrenergic to cholinergic switch that characterizes sweat gland innervation. Sweat gland extracts contain a cholinergic and peptidergic differentiation activity for cultured sympathetic neurons. Extracts from tabby mice (which lack sweat glands) and noradrenergic sympathetic targets have significantly reduced cholinergic differentiation activity. Expression of the differentiation activity in footpads occurs at a time period appropriate for a role for this factor(s) *in vivo*. Comparison with other differentiation molecules suggests that it is distinct from MANS, CDF and the heparin binding cholinergic factor. Immunological and biochemical analysis indicate that the major cholinergic-inducing activity is not LIF but is a CNTF-like molecule. Western blots, northern blot and *in situ* hybridization analysis fail to detect CNTF or CNTF message in footpads. The possible relationship between CNTF, LIF and sweat gland cholinergic differentiation factor(s) is discussed.

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Table of Contents

Introduction

The development of the peripheral nervous system in vertebrates involves differentiation, migration to an appropriate location, projection to an appropriate target, synaptogenesis and expression of the appropriate neurotransmitters. Making the appropriate choices at each of these developmental steps is critical to the construction of an appropriately functioning nervous system. Our increasing awareness of the sheer number of neurons in the vertebrate nervous system, the diversity of neuronal phenotypes distinguishable on morphological, biochemical and electrophysiological criteria, and the plasticity of the nervous system has led to an appreciation of the magnitude of the problem that a developing organism faces in specifying the appropriate developmental fate of every neuron.

Consider the question of how a developing organism regulates the neurotransmitter(s) a particular neuron will express. There are an increasing number of neurotransmitter candidate molecules: about a dozen known classical neurotransmitters and over 30 putative purine and peptidergic neurotransmitters (for review see Hokfelt *et al.*, 1980, and references therein). Furthermore, evidence has accumulated to show that neurons contain more than one and often two or even three different neurotransmitters (see Hokfelt *et al.*, 1980); most often a classical neurotransmitter is colocalized with a peptidergic neurotransmitter(s). Given this large number of neurotransmitter candidates and the possibility that any single neuron can express two, three or more of these molecules, the transmitter phenotypes a neuron could potentially express is extremely large. For example, if any three neurotransmitters of the 50 neurotransmitter candidates can be expressed in a neuron, the number of combinatorial phenotypes is over 110,000. While there are useful generalizations as to the combinations of classical transmitters and peptides produced, the numerous exceptions do not lead to any significant reduction in the number of possible combinatorial phenotypes. The problem becomes even more complex if one takes into account evidence that the neurotransmitters and peptides produced by a single neuron are differentially regulated both on a short term and a long term basis (see below). One would then not only have to specify in some manner the expression of a particular subset of transmitter and peptide molecules for every neuron, but also exercise temporal regulation over their expression. An example from the peripheral nervous system may make the magnitude of the problem clearer. In the rat, the stellate ganglion in the sympathetic

chain develops from cells which migrate from the neural crest. Precursor cells undergo mitosis to give rise to approximately 20,000 neurons in the adult. These neurons subsequently send projections to a number of different targets. Within the stellate ganglion, one can identify cells which express various permutations and combinations of at least five different neurotransmitters. The cell bodies of the neurons expressing any one subset of neurotransmitters are not distributed in any organized fashion, but rather seem to be randomly distributed throughout the ganglion (Lundberg *et al.*, 1979, 1982, 1983, Henion *et al.*, unpublished results). However, the nerve fibers innervating a specific target seem to express a characteristic subset of neurotransmitters. For example, the sympathetic fibers innervating blood vessels contain TH (tyrosine hydroxylase), the rate-limiting enzyme for catecholamine synthesis and NPY (neuropeptide Y), while those that innervate the sweat glands do not; instead, they contain ChAT (Choline acetyltransferase) and VIP (Vasoactive intestinal peptide), and the fibers that innervate the piloerector muscles in the hairy skin contain TH but not NPY. It is also important to note that these targets are not necessarily spatially segregated. Blood vessels lying adjacent to the sweat glands are innervated by fibers arising from the same ganglion and projecting along the same nerve trunk as fibers innervating the sweat glands, and yet the transmitter profiles are distinct. Thus, neurons expressing a subset of classical transmitter and peptide molecules, randomly distributed within the ganglion, project to their appropriate target even when the targets are not widely segregated. Furthermore, the lack of inappropriate projections suggests that the organism matches neurons expressing the appropriate subset of purine, peptidergic and classical neurotransmitters to targets possessing the corresponding set of receptors. Multiply this kind of specification from one ganglion to the whole nervous system and one begins to appreciate the degree of regulation involved.

Several possible developmental strategies could regulate which two or three of the 50 or so transmitter candidates a neuron will express. One possible mechanism is prespecification by lineage. The classical transmitters, synthesizing enzymes, purines and peptides that every neuron in the nervous system will express could be specified by its lineage. Loss of that particular neuron would lead to an irreparable defect of that cell and its progeny. Studies in invertebrate development do in fact tend to support this view (Kenyon, 1985, Doe and Goodman,1985; Davidson, 1990). Indeed, in the C. elegans the lineage of every neural crest derived cell has been worked out (Sulston *et al.*, 1983). In vertebrates, however, it has become increasingly clear that the expression of the transmitter and peptide

molecules cannot be specified only on the basis of a cell's lineage history. Given the estimated number of neurons in the central nervous system (10^{11} neurons) and the 50 or so neurotransmitters, the possible permutations that would have to be coded for would far exceed the coding capacity of DNA. Thus, in vertebrates at least, specifying the lineage of every neuron is not feasible. Further clear examples of environmental regulation of transmitters expressed by a neuron are available (see below).

Another strategy that does not require as extensive a control on the lineage of the cell is one similar to that which specifies the lineage and development of the hemopoietic system. For example, in the hemopoietic system a stem cell can give rise to macrophages, granulocytes, erythrocytes, lymphocytes and platelets by generating a series of committed progenitor cells which are restricted to a particular sublineage (Nicola and Johnson, 1982, Ogawa *et al.*, 1983). These committed progenitor cells give rise to a large number of identical cells (clonal amplification), which then undergo terminal differentiation. By analogy, in the nervous system a pleuripotent precursor would undergo a series of increasingly restricted developmental choices to give rise to committed or restricted precursors until the final division would lead to a committed postmitotic neuron. Such a model of neuropoiesis (see Anderson, 1989 and references therein) has been successfully invoked to explain segregation of the melanocytic, sensory and sympathetic lineages in the development of the peripheral nervous system (for review see Anderson, 1989, Le Douarin 1986). The development of neurotransmitter subtypes in the stellate ganglion by analogy would be explained on the basis of a pluripotential cell present in the neural crest, which would undergo restriction in developmental fate to perhaps a restricted neuron or glial precursor The neuron precursor would undergo subsequent developmental restrictions to give rise to different neuron precursors, each of which is only capable of giving rise to neurons that express a particular subset of purine, peptide and classical transmitters. These specific neuron precursors would then divide to give rise to a large or small number of similar cells (clonal amplification). These cells would then undergo terminal differentiation to give rise to the differentiated transmitter phenotype. A more detailed analysis of the development of the stellate ganglion, however, makes it clear that such a developmental strategy does not completely explain the development of this ganglion. The cells expressing a particular neuropeptide, say the VIP positive cells, are not arranged in clusters showing a common developmental origin at any stage in development. Further, the expression of at least some of the different

transmitter and peptide molecules occurs well after the neuronal precursors have undergone their terminal division. Finally, external manipulations both in young and adult animals can alter the expression of classical transmitters and peptides in postmitotic neurons (see below). Thus, a hemopoietic model cannot completely explain the acquisition of different transmitter profiles by subsets of neurons seen in sympathetic ganglia such as the stellate (Lundberg *et al.*, 1979, 1982, 1983) or chromaffin cells (Unsicker *et al.*, 1980, Soinila and Eranko, 1980) or the phenotypic plasticity that has been observed in several developmental systems (see below).

Another possible mechanism to regulate the temporal and spatial pattern of neurotransmitter expression would be to use extracellular signals to instruct a cell to switch on a particular neurotransmitter or group of neurotransmitters. Using environmental signals as a mechanism to generate diverse groups of neurons expressing different neurotransmitter profiles is particularly attractive for several reasons. Such a mechanism explains the remarkable diversity in neurotransmitter phenotypes seen in the developing and adult organism without the necessity of an extremely tight control on lineage. It is also attractive, in part, because the same external signals that regulate the expression of subsets of peptides and classical transmitters could modulate the short-term changes in levels of neurotransmitters and their synthesizing enzymes that have been described (see Schultzberg *et al.*, 1978, Fischer Colbrie *et al.*, 1988, Zigmond *et al.*, 1989, Sachs *et al.*, Neuroscience abstract, 1990). Using environmental signals can also bypass the problem of matching neurons expressing the appropriate subset of purine, peptide and classical transmitters to their appropriate targets with the appropriate receptors; one simply presumes that the specification of the appropriate neurotransmitter profile occurs after the neuron has innervated its target, i.e., at least in some cases the target tissue specifies the transmitter phenotype. Thus, one can postulate the existence of several factors which specify a single or some particular subset of the 50 neurotransmitter candidates. Spatial and temporal control of expression of a few of these factors, which would act on a number of neurons irrespective of their lineage history with certain caveats, would enable one to generate nearly all the different combinatorial phenotypes of neurotransmitters that have been described. In the simplest model, one would predict that individual factors would specify the induction of a particular purine, peptide or classical neurotransmitter molecule, and, therefore, there would be 50 or so factors, a number which, while large, is certainly not improbable. Neurons would respond to

the factors to which they were exposed during development to express appropriate peptidergic and classical transmitter molecules. It is, however, not strictly necessary that there be 50 or so modulators. One can imagine a situation where fewer factors code for subsets of neurotransmitters that are commonly co-expressed, for example a VIP and ChAT-inducing factor, or a TH and NPY factor. The transmitter phenotype could also be regulated by factors that inhibit the constitutive expression of a particular neurotransmitter. In this hypothesis the development of the stellate ganglion would be simpler to explain. The neurons in the stellate ganglion would be identical in terms of their developmental potential and would project along pathways to specific targets. Enroute to their target or soon after reaching the target, the neurons would react to environmental signals expressed in a restricted spatial pattern to express or repress the expression to some subset of the possible neurotransmitter phenotype that the cell is capable of expressing. This hypothesis does not imply that lineage does not play any role in determining at least some of the phenotypic diversity seen in vertebrates; rather, it envisages an additional layer of regulation which becomes necessary, due to the fact that within particular sublineages of neurons (sympathetic, sensory, etc.) additional subtypes are present which express different transmitter phenotypes.

A hypothesis which postulates such an environmental regulation of the neurotransmitter phenotype would lead to several predictions. 1) Postmitotic neurons or their proximate precursors should be plastic with respect to their neurotransmitter phenotype. 2) It should be possible to identify extracellular signals that can differentially modulate, either up or down regulate, the levels of peptides and other neurotransmitters. In addition, one could also postulate that, at least in some instances, extracellular signals would be present at or made by the target. 3) Individual factors should act on neurons of different lineages. This is an important prediction, since only if we had general factors which acted on a reasonable subset of neurons would there be any advantage (as far as reduction in coding requirements) in devising a system that worked in this manner.

Evidence has accumulated in support for all three of these predictions. I will discuss examples from both vertebrate and invertebrate literature for each of these points.

Postmitotic neurons or their immediate precursors are plastic.

An important prediction for a hypothesis postulating that external factors regulate the expression of peptide and classical neurotransmitters is that neurons should be capable of switching their neurotransmitter status. Several examples of neurons changing neurotransmitter status *in vitro* and *in vivo* have been described. I will describe a few relevant examples. For a more complete review, see Landis, 1988.

Plasticity in invertebrates

Given that, to a large extent lineage determines the development of invertebrates (Kenyon, 1985, Doe and Goodman, 1985, review by Davidson, 1990), it seems surprising to find examples of plasticity. However, plasticity in identified neurons has been described in at least three different invertebrate systems: snails, hydra and the moth (Gesser and Larsson, 1985, Koizume and Bode, 1986, Tublitz and Sylwester, 1990). These examples are of particular importance in that they represent the best examples of plasticity in identified populations of neurons *in vivo.*

In Hydra, Koizumi and Bode showed that the FMR amide immunoreactive cells (FLI+) arise from FLI- neurons when these neurons are displaced to the tentacle, hypostome or peduncle from the body column. Furthermore, when the peduncle with FLI+ neurons is grafted to the body column, it is converted to basal disc or body column tissue and the FLI disappears. Since this appearance and loss of FLI is always position dependent it is clear that neurons in the mature nerve net of the hydra can modulate their neuropeptide phenotype.

In the Lymanea stagnalis, seasonal changes in neuropeptide content have been described in a particular population of neurosecretory cells: the light green cells. In the spring, these cells contain Enk-LI, in the fall, gastrin/CCK- LI, and in winter, immunoreactivity for both these peptides. In another group of neurons, the caudodorsal cells, which secrete an ovulation hormone, age-dependent changes in peptide immunoreactivity can be detected: In the young snail these cells are immunoreactive for both ENK and gastrin-CCK. In the adult, however, the same cells display only gastrin-CCK immunoreactivity.

In the Manduca sexta, Tublitz and Sylwester have identified a group of four neurosecretory neurons that in the larva express high levels of CAP (cardioactive peptide) activity. When these same neurons are assayed in the pupa or the adult, no

CAP activity can be detected, but now these cells contain high levels of bursicon, another peptide hormone. Thus, functionally mature cells *in vivo* are capable of altering their neurotransmitter phenotype.

The vertebrate nervous systems.

A large body of evidence for plasticity has accumulated in the avian, rat and murine nervous systems, both *in vivo* and *in vitro*.

Direct evidence for a change in neurotransmitter phenotype in individual cells was obtained in an elegant series of microculture experiments (Furshpan *et al.*, 1976, 1986 a,b, Landis, 1976, Potter *et al.*, 1986, Matsumoto *et al.*, 1987). Sympathetic neurons grown on a small island of heart myocytes or fibroblasts will form functional synapses (Furshpan *et al.*, 1976). The neurotransmitter released at these synapses can be assayed using various agonists and antagonists. Using this assay system it was possible to demonstrate transitions of neurotransmitter phenotype from noradrenergic to dual function and dual function to cholinergic. Thus, in culture at least, single identified post mitotic neurons can, under the influence of external agents over a period of time, change their neurotransmitter status.

Similarly, in cultures of avian sympathetic neurons, cholinergic function can also be induced in the catecholaminergic subpopulation in culture by manipulation of the culture conditions. When neurons are grown on collagen with either fetal calf or horse serum, all neurons express catecholaminergic traits (Iacovetti *et al.*, 1987) and little or no ChAT activity is expressed; however, when embryonic eye extract is added to cultures the ChAT activity is increased. Similarly (Acheson and Rutishauser, 1988), density and depolarization can influence neurotransmitter expression in chick sympathetic cultures. Furthermore, the density dependent increase in ChAT can be duplicated by treatment with neuronal membranes, indicating that an external environmental signal modulates neurotransmitter expression.

Neuropeptide expression can also be modulated in culture. For example, Substance P levels can be modulated by a wide variety of agents including activity, non-neuronal cells and cell contact (Adler and Black, 1985; Kessler, 1984 a,b; Kessler, 1985 a,b; Kessler, *et al.*, 1986; Wong and Kessler, 1987). Since Substance P is not normally present in detectable amounts in sympathetic *neurons* by immunocytochemistry, this *de novo* expression of Substance P, therefore,

represents the ability of postmitotic neurons to modulate their peptidergic phenotype. Somatostatin and VIP levels can also be modulated by activity and denervation (Kessler *et al.*, 1984 a, Sachs *et al.*, Neuroscience abstract, 1990).

Many neurons of the embryonic rat cerebral cortex that do not normally express TH can be made to do so when placed in the appropriate culture environment or when transplanted to the adult cortex (Park, Joh and Ebner, 1986, Iacovetti *et al.*, 1987). The induction of TH is stimulated by the coculture of cortical neurons with primary cultures of skeletal, smooth and cardiac muscle (Iacovetti *et al.*, 1989).

During normal development, too, there appears evidence for plasticity. For example, transient TH immunoreactivity is seen during murine embryonic development in several organs including the kidney, dorsal mesentry and pancreas (Teitelman *et al.*, 1981a, Teitelman and Lee, 1987). At embryonic day 11.5 in the developing rat gut, cells which express TH and DBH-IR and catecholamine fluorescence can be detected (Cochard *et al.*, 1979, Teitelman *et al.*, 1979). No TH IR cells or catecholamine fluorescence is evident at a later date. Several lines of evidence suggest that the loss of catecholaminergic properties from the developing gut is not due to the disappearance of the cells, but rather due to a change in their properties (Jonakait *et al.*, 1979, 1985, Gershon *et al.*, 1984, Rothman *et al.*, 1978, 1980).

Transient TH IR cells are also seen in several other developing systems. At embryonic days 10.5 and 12.5, TH IR cells are seen in both sensory and parasympathetic ganglia in the rat. With subsequent embryonic development, neither TH IR or catecholamine uptake can be demonstrated in the sensory ganglia (Jonakait *et al.*, 1984, 1985, Katz *et al.*, 1983, Katz and Black, 1986) or can be demonstrated in only a very small proportion of the cells, as in the sphenopalatine ganglia (Leblanc and Landis, 1988 b) and particular dorsal root ganglion (Price and Mudge, 1983).

The most convincing example of neurotransmitter plasticity in normal development *in vivo* is seen in the developing innervation of the rat eccrine sweat glands. In the adult, fibers are immunoreactive with antibodies to acetylcholinesterase (AChE) and cholineacetyl transferase (ChAT), tissue homogenates contain high levels of ChAT activity (Leblanc and Landis 1986, Landis and Keefe, 1983) and functional assays demonstrate that transmission is cholinergic (Stevens and Landis, 1987). Thus, the innervation is morphologically and functionally cholinergic. The neurotransmitter properties initially expressed by the

developing sweat gland, however, are strikingly different (Landis and Keefe 1983, Leblanc and Landis, 1986, Stevens and Landis, 1987, Landis *et al.*, 1988). At day four, when sympathetic fibers first become associated with developing sweat glands, they exhibit intense catecholamine histofluorescence and immunoreactivity for DBH (Dopa-ß-hydroxylase) and TH (Tyrosine hydroxylase) properties characteristic of the noradrenergic phenotype. In contrast, no cholinergic properties can be detected. Evidence that this change in neurotransmitter properties takes place in a single population of fibers comes from ultrastructural studies (Landis and Keefe 1983), the persistence of certain catecholaminergic traits (Landis *et al.*, 1988, Landis and Keefe, 1983), and the failure of cholinergic innervation to develop in animals treated with adrenergic neurotoxins during their first week of postnatal development (Yodlowski *et al.*, 1984, Leblanc and Landis, 1986).

Studies in the developing avian peripheral nervous system have also demonstrated striking evidence for plasticity. Recently, Coulombe and Bronner-Fraser (1986) showed that when postmigratory postmitotic cholinergic ciliary neurons were harvested and injected into the trunk neural crest pathway, many translocated to positions appropriate for cells derived from the trunk level of the neural crest. Following migration to the sympathetic ganglia or adrenal primordia, these cholinergic neurons began to express a catecholaminergic phenotype. Equally important, retrogradely labelled cells that settled in other locations did not contain catecholamine histofluorescence. Using chick-quail chimeras, Le Douarin and her colleagues (see Le Douarin, 1986) have demonstrated that back transplantation of sensory precursors from the dorsal root ganglion to the neural crest will give rise to cells which will migrate to the sympathetic ganglia and express catecholaminergic traits. Since cells in the the dorsal root ganglion do not express catecholaminergic properties, it is reasonable to assume that that this property rose *de novo* in either committed or uncommitted precursors.

In the adult rat, Macmahon and Gibson (1987) have shown that crossinnervation results in a change in the peptide phenotype. Sensory fibers innervating the skin contain substance P, while the sensory afferents from muscle do not. When skin and muscle hind limb nerves are crossinnervated, then, ten to twelve weeks later, the former skin nerve (now innervating muscle) has substantially reduced Substance P immunoreactivity. Conversely, the muscle nerve now innervating skin shows increased Substance P immunoreactivity. These experiments, done in adult rats, show that some postmitotic neurons of the sensory system are plastic with respect to the peptides that they synthesize and, further,

that at least in this case, the target, in some manner, specified the phenotype.

Thus, a number of examples of plasticity of either the neurotransmitter synthesizing enzymes, peptides, or classical neurotransmitter phenotype exist and plasticity in this context is not a unique or isolated event. It is, in fact, relatively common and has been observed in postmitotic neurons in a wide variety of species, both vertebrate and invertebrate. These changes have been described both *in vivo* and *in vitro* both in the CNS and in the PNS. These examples are inconsistent with the development of the nervous system following a strictly hemopoietic pattern. Such plasticity is far more consistent with an additional layer of environmental regulation determining the expression of peptide and classical transmitters in the nervous system.

Factors can modulate neurotransmitter phenotype.

A second prediction that can be made is that it should be possible to identify factors which will modulate transmitters in postmitotic neurons. In the simplest form of the hypothesis it should be possible to identify factors which regulate a single neurotransmitter. In more complicated versions of the hypothesis factors which modulate subsets of transmitters or inhibit the expression of a neurotransmitter should exist. Evidence for factors modulating transmitters over the long term or inducing *de novo* expression of a neurotransmitter has come mainly from cell culture experiments.

Factors modulating neurotransmitter levels in sympathetic neuron cultures

When sympathetic neurons are cultured with non-neuronal cells or conditioned medium from a wide variety of cells, they will switch from a adrenergic to cholinergic phenotype (see review, Landis, 1989). Using this as an assay system, several different factors were isolated and partially purified. Some of these factors are poorly defined: for example, chick embryo extract, human placental serum, and rat serum increase acetylcholine synthesis and/or ChAT activity (Higgins *et al.*, 1981; Iacovitti *et al.*, 1981, 1982; Wolinsky *et al.*, 1985). Other factors have been partially characterized. A soluble 50 kDa factor has been obtained from brain by heparin affinity chromatography (Kessler *et al.*, 1983). A membrane-associated neurotransmitter-stimulating factor (MANS) has been solubilized and partially purified from rat spinal cord; this activity is associated with a 29 kDa band (Wong and Kessler, 1987). Another factor of approximately the same apparent molecular

weight as MANS but possessing somewhat different properties has been partially purified from spinal cord membranes (Adler *et al.*, 1989). The cholinergic differentiation factor (CDF) present in heart cell-conditioned medium (HCM) has been purified to homogeneity. It is a basic 45 kDa glycoprotein, with at least six glycosylation sites (Fukada, 1985). The deglycosylated protein has a molecular weight of 22 kDa and retains biological activity. A very similar molecule, present in skeletal muscle cell-conditioned medium (Weber *et al.*, 1985), increases cholinergic function in spinal cord and nodose sensory neurons as well as sympathetic neurons (Geiss and Weber, 1984; Mathieu *et al.*, 1984). Most recently, ciliary neurotrophic factor (CNTF), which was identified as a trophic factor for ciliary neurons (Adler *et al.*, 1979; Varon *et al.*, 1979; Barbin *et al.*, 1984; Manthorpe *et al.*, 1986) has been shown to switch cultured rat sympathetic neurons from a noradrenergic to a cholinergic phenotype (Saadat *et al.*, 1989).

More recently, several factors have been partially characterized which can modulate the expression of neuropeptides in sympathetic neuron cultures. Two additional factors have been isolated and partially purified from heart cell-conditioned medium which can modulate VIP and Somatostatin levels respectively (Nawa and Patterson, 1990, Nawa and Sah, 1990). MANS originally isolated as a cholinergic differentiation factor has been shown to modulate the expression of several neuropeptides in culture (Lee *et al.*, 1990). It elevates the levels of Somatostatin and vasoactive intestinal peptide while it has no effects on the levels of neuropeptide Y or Leu- enkephalin (Lee *et al.*, 1990). CNTF has also been shown to modulate the expression of vasoactive intestinal peptide in embryonic chick sympathetic neuron cultures (Ernsberger *et al.*, 1989).

Factors which modulate the transmitter levels of other neurons.

Several factors have been shown to modulate the levels of ChAT in motor neuron cultures. A 22 Kd protein has been isolated from skeletal muscle (Mcmanaman *et al.*, 1988) which increases ChAT levels two- to three fold. It has been shown to have similar effects *in vivo* (Mcmanaman *et al.*, 1990). It is an acidic protein with a pI of 4.8, is not glycosylated and is biochemically distinct from CNTF, CDF/LIF, and the heparin-binding growth factors (Mcmanaman *et al.*, 1989). A selective increase in ChAT levels is also observed if spinal cord neurons are grown in muscle cell-conditioned medium or in extracts of neonatal muscle (*Kaufman*, Barry and Barrett 1985). The ChAT-inducing activity is associated with a 40 Kd protein.

A muscle-derived factor (MDF) has been shown to induce TH-IR and TH mRNA in cultures of embryonic rat cerebral cortex cells (Iacovitti *et al.*, 1989). Basic FGF, EGF, Insulin and Insulin-like growth factors have been to shown to increase ChAT and dopaminergic levels in hippocampal and septal cultures (Knusel *et al.*, 1989).

Similarly, factors which modulate the levels of NPY and Leu-enkephalin in chromaffin cells have been identified in culture and *in vivo*. Glucocorticoids elevate the levels of enkephalin severalfold over cultures grown without steroid agonists (Naranjo *et al.*, 1986, La Gamma and Adler, 1987, Stachowiak *et al.*, 1988, Henion *et al.*, unpublished observations). Similarly, insulin treatment in the animal (which reflexively stimulates the splanchnic nerve) will cause a six- to seven fold *increase* in Leu-enkephalin mRNA (Fischer-Colbrie *et al.*, 1988). Paradoxically, bilateral transection of the splanchnic nerve also results in an increase in the number of L-enk immunoreactive cells as assayed by immunocytochemistry (Schultzber *et al.*, 1978, Lewis *et al.*, 1987, Henion and Landis, 1990). Reserpine treatment will increase NPY levels in animals with intact splanchnic innervation, suggesting that this effect is associated with impulse activity (De Quidt and Emson, 1986, Schalling *et al.*, 1988).

A factor which can modulate the somatostatin levels in embryonic ciliary neuron cultures of the chick has also been partially purified from choroid cultures (Coulombe *et al.*, 1991). This factor, named Somatostatin stimulating activity (SSA), can induce more than 90% of cultured ciliary neurons to express somatostatin. The molecular weight of this factor is between 30 to 40 Kd, and it appears distinct from the other peptidergic factors described.

A number of smaller molecules, hormones, peptides, and transmitters have been found to induce the expression of particular phenotypic properties in developing neurons. Estrogen, for example, can differentially regulate the expression of cholecystokinin (CCK) and Substance P in a sexually dimorphic pathway in the amygdala (Simerly *et al.*, 1989). Serotonin can increase the number of neurons expressing tryptophan hydroxylase in cultures of embryonic hypothalamus (De Vitry *et al.*, 1986). Angiotensin II increases CA production in primary cultures of rat hypothalamus and brainstem neurons (Maclean, Raizada and Sumners, 1990). VIP stimulates proEnk A mRNA levels in cultured chromaffin cells (Wan and Livett, 1989), and several neuronal markers in spinal cord cultures (Foster, Eiden and Brenneman, 1989).

Factors can affect neurons of different lineages.

The third prediction of our hypothesis was that factors should be able to modulate neurons of different lineages with certain caveats. If the neurons innervate the same target as can occur in the periphery, the factors should either affect only one population or should modulate the levels of neurotransmitters differently in the two populations. Data for this is not as convincing as it is for the presence of factors. One reason for this is that few of the factors have been purified to homogeneity, and few, if any, have been cloned. Perhaps the best example is that of heart cell-conditioned medium. It has been recently demonstrated that HCM will cause cholinergic induction in septal cholinergic neurons (unpublished results), in sensory nodose cultures (Geis and Weber, 1984), as well as in dorsal root ganglion cells (Nawa and Patterson, 1990), and that the same or closely related factor will cause induction in motor neurons (Kato and Patterson, unpublished results) and hippocampal neurons (Hefti and Patterson, unpublished results).

Similarly, MANS has been shown to cause cholinergic induction in spinal cord cultures as well as in sympathetic neuron cultures (Lombard-Golly *et al.*, 1990, Wong and Kessler, 1987, Adler *et al.*, 1989).

CNTF was originally identified as a survival factor for chick embryonic ciliary neurons (Barbin *et al.*, 1984, Manthorpe *et al.*, 1986). Subsequent experiments with purified CNTF have shown that it has cholinergic-inducing properties on cultures of rat sympathetic neurons (Saadat *et al.*, 1989) and VIP-inducing effects on embryonic chick ciliary neurons (Ernsberger *et al.*, 1989). Perhaps, somewhat surprisingly, while it has survival effects in ciliary neuron cultures, CNTF does not specifically increase the CHAT levels in ciliary neuron cultures (Eckenstein *et al.*, 1990), indicating that for this population of neurons it is not a cholinergic factor.

Thus, clearly none of the factors that have been tested on different populations is specific to cells of any particular lineage, rather, in keeping with our hypothesis, both central and peripheral nervous system neurons in culture respond to these factors, indicating that the receptors for these factors are fairly widespread and that a large variety of neurons will respond to these factors.

While several key components of the hypothesis appear to be true, several questions remain to be clarified before we can accept the validity of this hypothesis. It is not clear if we have identified one factor, an adrenergic to cholinergic conversion factor, or a class of these factors. Nor is it clear if any other class of

factors exists. Clarifying this will enable us to predict if many such factors can be expected, or a single unique moiety will specify a particular phenotype. It is also not clear how general is the response of neurons of different lineages to differentiation factors.

One very important problem is that, while as predicted a large number of factors have been identified which can modulate neurotransmitter phenotype in culture, none of these factors has been shown to play a role in normal development. Is it possible that the effects that have been identified are not physiologically relevant? This is an important question, as some of these factors have been purified from cultured cells. For example, CDF has been purified from heart cell-conditioned medium. Others have been purified from what *a priori* seems an inappropriate location. For example, CNTF has been isolated from sciatic nerve which contains numerous noradrenergic sympathetic fibers. Both CNTF and CDF from HCM have functions in addition to their cholinergic-inducing activity (see Ch. 1 and references therein). Thus, it would be important to demonstrate either a role for these factors in phenotypic conversion in the course of normal development, or the presence of these factors in a system in which a phenotypic conversion occurs.

In my thesis I have addressed certain specific questions raised by this hypothesis.

a) As an extension of the hypothesis that these factors may be capable of modulating the levels of several different neurotransmitters, I have assessed the neurotransmitter modulating properties of CNTF to define the subset of transmitters and peptides that CNTF modulates. I will present evidence that CNTF can alter levels of several different peptides in sympathetic neuron cultures, but not in dorsal root ganglion cultures.

b) I have approached the question of how many cholinergic factors there are by comparing the immunological, biochemical and biological properties of these factors. I will show that CNTF and CDF are distinct molecules, that CNTF and MANS are immunologically and biologically similar, and that CDF from skeletal muscle is not a cholinergic differentiation molecule when added to sympathetic neuron cultures.

c) I have then undertaken to assess if any of these factors is present in a biological system where normal development is characterized by a noradrenergic to cholinergic switch. I have identified a differentiation factor(s) present in sweat gland extracts which mediates a similar phenotypic change in culture and is likely to be the biologically relevant factor(s) in the development of the sweat gland innervation.

d) I have compared the immunological, biochemical and biological properties of the sweat gland-derived cholinergic differentiation activity with four other cholinergic factors: MANS, CDF from skeletal muscle, CNTF, and CDF. I will present data on its similarities to and differences from CNTF and LIF.

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Chapter 1.

The Cholinergic Neuronal Differntiation Factor from heart cell conditioned medium is different from the cholinergic factors in sciatic nerve and spinal cord.

ABSTRACT

Environmental cues play an important role in determining the transmitter phenotype of developing sympathetic neurons. Several factors have been described which can induce cholinergic function in cultured sympathetic neurons. We have compared biological and immunological properties of three of them: cholinergic differentiation factor (CDF), membrane-associated neurotransmitter-stimulating factor (MANS) and ciliary neurotrophic factor (CNTF) to determine whether they are different. As previously reported, all three increased acetylcholine synthesis in cultured sympathetic neurons. In addition, MANS as well as CNTF and CDF decreased catecholamine synthesis. CNTF and MANS, but not CDF, promoted the survival of embryonic chick ciliary neurons. Affinity-purified antibodies raised against a synthetic peptide corresponding to the N-terminal sequence of CDF immunoprecipitated CDF, but not MANS or CNTF. These results indicate that although CDF, MANS and CNTF have similar effects on transmitter synthesis by cultured sympathetic neurons, CDF lacks the ciliary neurotrophic activity of MANS and CNTF. Further, CDF possesses an N-terminal epitope which is absent from both MANS and CNTF. Thus, CDF is distinct from MANS and CNTF, and at least two factors exist which can alter the transmitter phenotype of sympathetic neurons *in vitro*.

INTRODUCTION

The neurotransmitter phenotype of neural crest-derived neurons is remarkably plastic and is sensitive to developmental signals present in the environment. In culture, noradrenergic sympathetic neurons dissociated from superior cervical ganglia (SCG) of newborn rats can be induced to become cholinergic. Sympathetic neurons express noradrenergic properties early in embryonic development (Eranko, 1972; Cochard *et al.*, 1979; Teitelman *et al.*, 1979) and shortly after plating, cultured sympathetic neurons exhibit noradrenergic but not cholinergic properties (Mains and Patterson, 1973; Johnson *et al.*, 1976; 1980; Patterson and Chun, 1977b; Landis, 1980). When grown in the presence of certain nonneuronal cells such as heart myocytes and fibroblasts or in medium conditioned by heart or skeletal muscle cells, the neurons undergo a transition from noradrenergic to cholinergic function; catecholaminergic properties including tyrosine hydroxylase activity and catecholamine synthesis are reduced and choline acetyltransferase (ChAT) and acetylcholine synthesis are induced (Patterson and Chun, 1977a; Weber, 1981; Swerts *et al.*, 1983; Wolinsky *et al.*, 1983; Potter *et al.*, 1986; Raynaud *et al.*, 1987). The transmitter plasticity and role of environmental cues revealed in these cell culture studies reflect the normal developmental events *in situ*. For example, a similar noradrenergic to cholinergic transition has been documented during the development of the cholinergic sympathetic neurons that innervate sweat glands (Landis and Keefe, 1983; Leblanc and Landis, 1986) and this transition is dependent upon target-derived cues (Schotzinger and Landis, 1988).

The cholinergic differentiation factor (CDF) present in heart cell conditioned medium (HCM) has been purified to homogeneity. It is a basic 45 kDa glycoprotein, with at least six glycosylation sites (Fukada, 1985). The deglycosylated protein has a molecular weight of 22 kDa and retains biological activity. A very similar molecule, present in skeletal muscle cell conditioned medium (Weber *et al.*, 1985), increases cholinergic function in spinal cord and nodose sensory neurons as well as sympathetic neurons (Geiss and Weber, 1984; Mathieu *et al.*, 1984).

A number of other sources contain factors that can induce cholinergic function in cultured sympathetic neurons. Some of these factors are poorly defined: for example, chick embryo extract, human placental serum and rat serum increase acetylcholine synthesis and/or ChAT activity (Higgins *et al.*, 1981; Iacovitti *et al.*, 1981; 1982; Wolinsky *et al.*, 1985). Other factors have been partially characterized. A soluble 50kDa factor has been obtained from brain by heparin

affinity chromatography (Kessler *et al.*, 1983). A membrane-associated neurotransmitter-stimulating factor (MANS) has been solubilized and partially purified from rat spinal cord; this activity is associated with a 29 kDa band (Wong and Kessler, 1987). Another factor of approximately the same apparent molecular weight as MANS but possessing somewhat different properties has been partially purified from spinal cord membranes (Adler *et al.*, 1989). Most recently, ciliary neurotrophic factor (CNTF) which was identified as a trophic factor for ciliary neurons (Adler *et al.*, 1979; Varon *et al.*, 1979; Barbin *et al.*, 1984; Manthorpe *et al.*, 1986) has been shown to switch cultured rat sympathetic neurons from a noradrenergic to a cholinergic phenotype (Saadat *et al.*, 1989).

The relationship between these cholinergic factors is unclear. It was possible that the same molecule had been isolated independently from a number of sources since MANS, CNTF and the deglycosylated form of CDF are of approximately the same molecular weight. Defining the relationship of these factors will be important in addressing the issue of how the factors(s) act during normal development. We have compared the biological properties of these three factors and used an antiserum generated against a peptide corresponding to the N-terminal amino acid sequence of CDF to look for determinants shared between CDF, MANS and CNTF. Our findings indicate that CDF is different from MANS and CNTF in its spectrum of activity and that it is immunologically distinct from them.

RESULTS

Effects on acetylcholine and catecholamine synthesis

To examine the effects of CDF, CNTF and MANS, equal protein concentrations (10 ug/ml) were added to dissociated sympathetic neurons from neonatal rats cultured in the absence of nonneuronal cells. Transmitter synthesis was assayed on the fourteenth day of culture (Table 1). The protein concentration used had been previously determined for each of the factor preparations to cause a significant cholinergic induction but not to yield the maximal cholinergic induction. The DEAE fraction of CDF caused a 29-fold increase in acetylcholine synthesis and a 32% decrease in the synthesis of catecholamines when compared to control cultures, results similiar to those reported by Fukada (1985). CNTF treatment resulted in a 13-fold increase in acetylcholine synthesis and a 74% decrease in catecholamine synthesis findings consistent with a previous report (Saadat *et al.*, 1989). Similarly, MANS caused an eight-fold induction and a 53% reduction in the synthesis of acetylcholine and catecholamines, respectively. The induction of cholinergic function confirms a previous report (Wong *et al.*, 1987), but the reduction in catecholaminergic properties has not been described before. The alterations in transmitter synthesis by these factors were not accompanied by changes in morphology or in neuron survival (Table 1). Thus, CDF, CNTF and MANS had similar effects on transmitter status of cultured sympathetic neurons.

Ciliary neuron trophic activity

CNTF was initially identified because it promotes the survival of E8 chick ciliary neurons in culture (Adler *et al.*, 1979; Varon *et al.*, 1979; Barbin *et al.*, 1984; Manthorpe *et al.*, 1986). Do CDF and MANS share this trophic property? Aliquots of the same preparations that induce cholinergic function in rat sympathetic neurons were serially diluted from an initial concentration of 5ug/ml and were assayed for their ability to enhance the survival of chick ciliary neurons. Both CNTF and MANS had ciliary neurotrophic activity (Fig1). Control cultures showed no surviving neurons after 24 hours, but wells to which CNTF and MANS had been added contained numerous neurons with extensive neurites (Fig. 1). In contrast, neither DEAE nor Sephadex fractions of CDF had detectable ciliary neurotrophic activity at any dose tested (Fig. 1). Since the failure of survival could have been due to the presence of a toxic component in the fractions, aliquots of CNTF were combined with the DEAE fraction of CDF. The number of surviving ciliary neurons and the extent of neurite extension was similiar to that in cultures which had only CNTF added to them

(data not shown). Thus, the CDF preparation did not contain detectable amounts of material either toxic to, nor trophic for, ciliary neurons.

Immunoprecipitation of biological activities

The above data suggest that CDF is different from MANS and CNTF. It was possible, however, that there were actually two factors present in the partially purified fractions of MANS and CNTF, one which was responsible for inducing cholinergic function, identical to the cholinergic factor present in CDF fractions, and a second, the ciliary trophic activity, that is absent from CDF fractions. To address this question, we used affinity-purified rabbit antibodies generated against a synthetic peptide corresponding to the N-terminal sequence of CDF (Fukada, 1985). In one series of experiments, the antibodies were used to immunoprecipitate the three factor preparations and the resulting supernatants were then assayed for their ability to induce ChAT activity in cultured rat sympathetic neurons. Parallel aliquots were incubated without the antibody or with the antibody plus 10μM of the synthetic peptide used as the antigen. The antibodies completely precipitated the cholinergic-inducing activity present in the DEAE fraction of CDF, and the immunoprecipitation was blocked by preincubation with the N-terminal peptide (Fig 2). In contrast, the antibody had no effect on the ability of CNTF or MANS to induce cholinergic function.

These results indicate that the cholinergic factor/s in CNTF and MANS does not share the N-terminal epitope with CDF. In a second series of experiments, the supernatants obtained after immunoprecipitation were tested for their ability to promote ciliary neuron survival. The DEAE fraction of CDF had no survival activity, and the trophic activities of both MANS and CNTF were not affected by the antibodies (Fig. 3). Thus, neither the cholinergic inducing nor the ciliary neurotrophic activities in MANS and CNTF share the N-terminal epitope of CDF.

Immunoprecipitation of labeled fractions

It is possible that the failure to immunoprecipitate activity from the CNTF and MANS fractions was due to the relatively crude nature of the preparations. To examine this possibility, the partially purified CNTF and MANS fractions were run on SDS PAGE gels and the regions which had been reported to have cholinergic activity were eluted. These proteins, as well as the DEAE and Sephadex fractions of CDF, were radioactively labeled by the Bolton-Hunter method. The labeled fractions were then subjected to immunoprecipitation with the same antibodies as described previously. The amount of radioactivity in the immunoprecipitates was determined,

and an aliquot of each was analyzed by SDS PAGE. Precipitation of radioactive protein was only detected in the CDF preparations and this was blocked by preincubation with the peptide antigen (Fig. 4). As expected (Fukada, 1985), SDS-PAGE analysis revealed that a protein of 45 kDa was specifically precipitated from both the Sephadex and DEAE CDF fractions (Fig. 5). In contrast, no labelled proteins were specifically immunoprecipitated from the MANS and CNTF fractions. The counts immunoprecipitated represent about 7% of the total protein present in the Sephadex fraction of CDF and about 1.5% of the protein in the DEAE fraction. These fractions were, therefore, at least as heterogeneous as the CNTF and MANS fractions (Wong *et al.*, 1987; Manthorpe *et al.*, 1986) from which no specific protein bands could be precipitated.

DISCUSSION

A number of factors have been described that induce cholinergic function in cultured rat sympathetic neurons. Among the best characterized of these are CDF, MANS and CNTF (Fukada, 1985; Wong and Kessler, 1987; Saadat *et al.*, 1989). CDF, MANS and CNTF not only induce cholinergic function in cholinergic sympathetic neurons but they also share a number of other properties, including similar molecular weights and similar effects on the neuropeptide expression by sympathetic neurons. While CDF produced by cultured heart cells has a molecular weight of 45 kDa, this form is glycosylated. Treatment of CDF with endoglycosidases yields multiple bands ranging from 45 to 22kDa and both the deglycosylated and glycosylated forms are biologically active (Fukada, 1985). The molecular weight of CNTF isolated from rat sciatic nerve is 22.5 to 24kDa (Manthorpe *et al.*, 1986; Saadat *et al.*, 1989) while that of MANS is 29kDa (Wong and Kessler, 1987). Therefore, it was possible that MANS was a partially glycosylated form of CDF while CNTF was the completely deglycosylated CDF protein. In addition, CDF, like MANS, increases neuronal Substance P (Wong and Kessler, 1987; Nawa and Sah, 1990; Nawa and Patterson, 1990) and both CDF and CNTF increase VIP expression (Ernsberger *et al.*, 1989; Nawa and Sah, 1990; Nawa and Patterson, 1990). The effects of MANS on VIP and CNTF on Substance P have not been reported. Given these similarities, it was important to determine whether these three cholinergic inducing factors obtained from different sources were identical.

These factors were tested in two biological assays: transmitter synthesis by neonatal rat sympathetic neurons and survival of E8 chick ciliary neurons. In addition, affinity-purified antibodies raised against the N-terminal region of CDF were used to determine whether MANS and CNTF share this site. All three factors cause an increase in acetylcholine synthesis and ChAT activity as previously reported (Fukada, 1985; Wong and Kessler, 1987; Saadat *et al.*, 1989). In addition, all three factors decrease catecholamine synthesis; these observations confirm earlier studies of the effects of CDF and CNTF and extend our understanding of the actions of MANS. CDF can be distinguished from MANS and CNTF, however. Both MANS and CNTF promote survival of E8 chick ciliary neurons while CDF does not. Furthermore, the affinity-purified antibodies immunoprecipitate both the cholinergic-inducing activity and a 45kDa protein from CDF. In contrast, the antibodies have no effect on the cholinergic-inducing activity or the ciliary neuron trophic activity present *in* MANS and CNTF, nor do they specifically immunoprecipitate a protein band from

these preparations. The assays used in the present studies do not permit us to distinguish between CNTF and MANS.

The results of the assay of ciliary neuron survival indicate that while all three factors are competent to induce cholinergic function, only CNTF and MANS have trophic activity for ciliary neurons. Our finding that MANS promotes the survival of ciliary neurons raises the possibility that it is a member of the family of ciliary neuron trophic molecules. It should be noted, however, that the MANS preparation still contains many proteins. CDF, on the other hand, had no trophic activity at any dose tested. Since both DEAE and Sephadex fractions of CDF were tested, and the addition of both CNTF and CDF to the same culture resulted in survival of the ciliary neurons, it is unlikely that the failure of survival was due to a toxic component. It is of interest that while CDF from HCM does not support ciliary neuron survival, extracts of bovine heart do contain ciliary neurotrophic activity which is similar to CNTF (Bonyhady *et al.*, 1980; Watters and Hendry, 1987).

Further evidence for the distinct nature of CDF comes from immunoprecipitation studies using affinity-purified antibodies generated against the N-terminal domain of CDF. These antibodies immunoprecipitate all cholinergic-inducing activity from both the DEAE and the Sephadex fractions. In contrast, neither the cholinergic-inducing activity nor a protein band could be precipitated from MANS or CNTF purified by SDS PAGE electrophoresis. Since the antibody was generated against a peptide domain of CDF and can precipitate the glycosylated form of CDF, it seems unlikely that the failure to immunoprecipitate the biological activity from CNTF and MANS was due to differential glycosylation. The results, indicate therefore, that CDF possesses an N-terminal domain that is not expressed by either MANS or CNTF.

CDF and CNTF also appear to differ in their charge. CNTF which has been purified from chick embryo ocular tissues and rat sciatic nerve has an isoelectric point of approximately 5.0 (Barbin *et al.*, 1984; Manthorpe *et al.*, 1986; Saadat *et al.*, 1989). In contrast, glycosylated CDF is a basic protein (Fukada, 1985). Since glycosylation would add negatively charged residues to the protein, one would predict that a deglycosylated form of CDF would be far more basic than pI 5.0. Thus, the difference in charge between the two proteins can not easily be explained by differential glycosylation. Other, secondary modifications are not ruled out, however.

The present studies indicate that CDF is distinct from MANS and CNTF but we can not at present rule out the possibility that they share some sequence homology. These factors not only act on sympathetic neurons, but they also induce cholinergic

function in other neuronal populations. CDF or a closely related molecule increases ChAT in spinal cord neurons and nodose sensory neurons (Geiss and Weber, 1984; Matheiu *et al.*, 1984) and CNTF increases ChAT in retinal neurons (Hofmann, 1988). In addition, other factors, whose relationship to the proteins examined in the present study remain to be defined, induce cholinergic function in ciliary (Nishi and Berg, 1981) and spinal cord neurons (McManaman *et al.* , 1989), populations that respond to CDF and CNTF. Thus, it seems very likely that there are multiple factors that have the same effect, the induction of cholinergic function, on a variety of responsive neurons.

Why are there several different molecules that cause such specific changes in phenotypic expression by sympathetic neurons, and induce cholinergic function in other neuronal populations as well? One possibility is that the functional roles of the protein are segregated in space and/or time. An analogy may be found in the developmental roles of brain-derived neurotrophic factor (BDNF) and NGF. BDNF is present in the central nervous system (Barde *et al.*, 1982; Leibrock *et al.*, 1989) while NGF is present in peripheral target tissues as well as the central nervous system (Korsching and Thoenen, 1983; Shelton and Reichardt, 1984; Large *et al* ., 1986; Auburger *et al.*, 1987; Davies *et al.*, 1987). Both are able to support the survival of sensory neurons (Levi-Montalcini and Angeletti, 1963; Johnson *et al..*, 1980; Hamburger *et al.*, 1981; Davies *et al.*, 1986; Hofer and Barde, 1988) but each has trophic activity for other distinct neuronal populations (for example, Levi-Montalcini and Booker, 1960a,b; Johnson *et al* , 1986). Most recently, BDNF and NGF have been shown to exhibit significant sequence homology (Leibrock *et al* ., 1989). Moreover, since CDF, CNTF and MANS influence not only classical transmitters but also neuropeptide expression (Wong and Kessler, 1986; Ernsberger *et al.*, 1989; Nawa and Sah, 1990; Nawa and Patterson, 1990), they may be members of a large group of phenotype specifying factors that act on neurons in a combinatorial fashion to produce the exceedingly large number of different combinations of neurotransmitters and neuropeptides found in the nervous system. In the hematopoetic system, for example, differentiation choices are affected by a group of proteins, the hematopoetic regulators or lymphokines; four colony stimulating factors (CSF), granulocyte-macrophage (GM-CSF), granulocyte (G-CSF), macrophage (M-CSF) and multipotential (multi-CSF), can stimulate multipotential stem cell precursors to differentiate into granulocytes and/or macrophages (reviewed by Clark and Kamen, 1987 and Metcalf, 1989). Thus, multiple hematopoietic and neurotransmitter phenotypic regulators can have

distinct, but overlapping or partially redundant, biological effects. Having defined the presence of several factors which alter the transmitter phenotype of cultured sympathetic neurons, it will be important to determine which of these proteins play roles in normal development. The sympathetic innervation of the sweat gland, which undergoes a noradrenergic to cholinergic transition in transmitter properties during normal development (Landis and Keefe, 1983; Leblanc and Landis, 1986; Landis *et al.*, 1988), provides an excellent system in which to examine this question.

Table 1.

	No of Cells	CA/Dish	Ach/Dish	Ach/CA	Fold Induction
Medium	1046 ± 52	8.61	0.28	0.032 ± .012	1
CDF	1171 ± 286	5.86	8.33	1.42 ± .297	44
CNTF	1013 ± 118	2.23	3.72	1.67 ± .08	52
MANS	979 ± 33	4.04	2.27	0.56 ± .116	17

Effects of cholinergic factors on neurotransmitter function.
10 ug/ml of CDF (DEAE fraction), CNTF (DEAE fraction) or MANS (Sephadex fraction) was added to cultured sympathetic neurons. After 14 days in culture, neurons were counted and neurotransmitter properties were determined by measuring the incorporation of tritiated precursors into catecholamines and acetylcholine. This assay measures the net synthesis and accumulation of acetylcholine and catecholamines by living neurons (Mains and Patterson, 1973). Samples were run in triplicate, Data is expressed as mean picomoles of Ach or Catecholamines per dish.

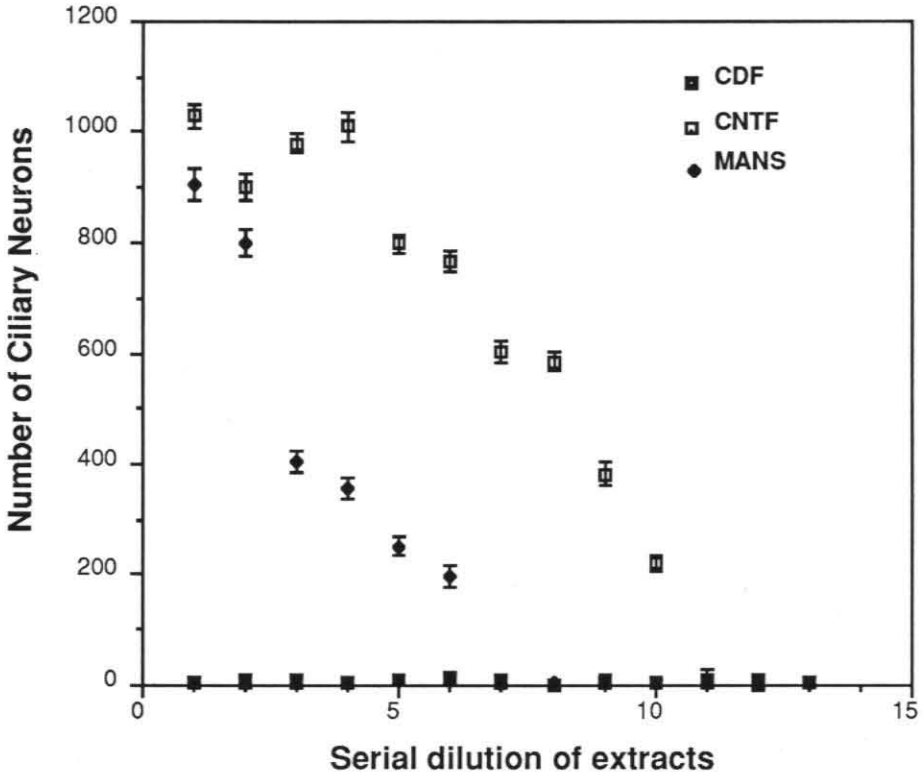
Figure 1.

Effect of CDF, CNTF and MANS on survival of E8 chick ciliary neurons.

(a) Ciliary neurons were grown in serial dilutions of 10ug/ml of CDF (DEAE fraction), CNTF (DEAE fraction) or MANS (Sephadex fraction). The number of phase bright cells was counted after 24 hours in culture. Samples were run in triplicate.

(b) No surviving neurons are evident after 24 hours in culture in the presence of CDF alone. X240. (c) Neurons with long neurites are present after 24 hours in the presence of CDF and CNTF. X240.

Figure 1.


1200
1000
800
600
400
200
0
Number of Ciliary Neurons
0
5
10
15
Serial dilution of extracts
CDF
CNTF
MANS

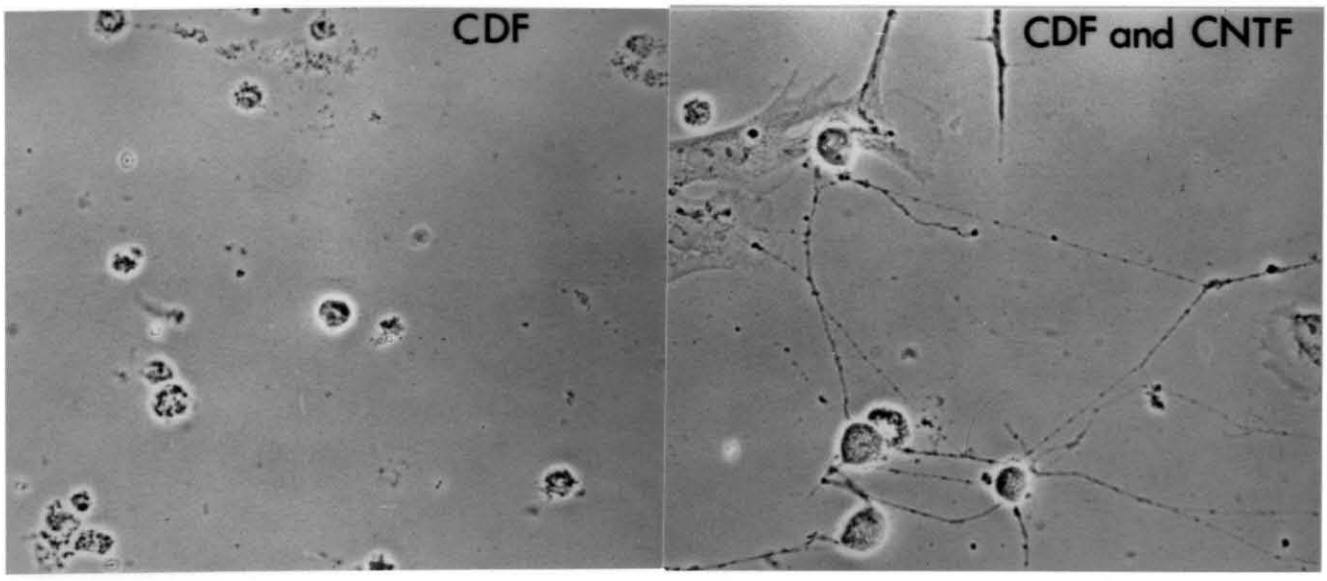

CDF
CDF and CNTF

Figure 2.

Immunoprecipitation of cholinergic-inducing activity.

CDF (DEAE fraction), CNTF (DEAE fraction) or MANS (Sephadex fraction) were incubated with Protein A sepharose (A), affinity-purified antibodies to the N-terminal sequence of CDF (B) or affinity purified antibodies preincubated with the peptide antigen (C). After immunoprecipitation, supernatants were added to sympathetic neuron cultures. After 10 days, the cultures were assayed for ChAT activity by the method of Fonnum. The results are expressed as the fold induction of ChAT in comparison to the activity observed in neurons grown in medium without extracts. All samples were run in duplicate.

Figure 2.

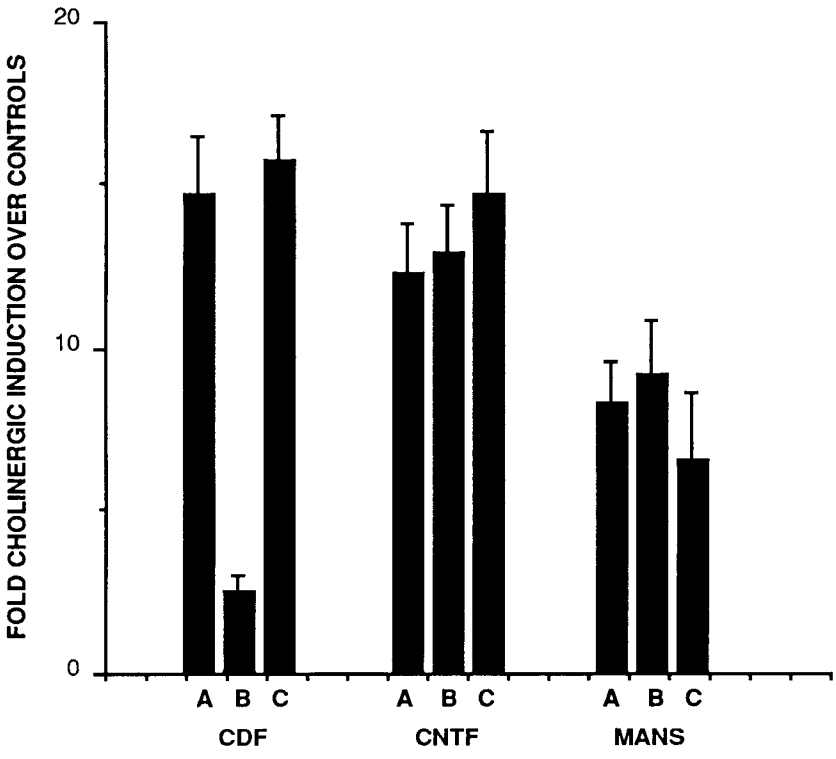

FOLD CHOLINERGIC INDUCTION OVER CONTROLS
20
10
0
A B C
CDF
A B C
CNTF
A B C
MANS

Figure 3.

Immunoprecipitation of ciliary neurotrophic activity.

CDF (DEAE fraction), CNTF (DEAE fraction) or MANS (Sephadex fraction), were incubated with affinity-purified antibodies to the N-terminal sequence of CDF (B) or affinity-purified antibodies preincubated with the peptide antigen (C). After immunoprecipitation, the supernatants were added to ciliary neuron cultures. The number of phase bright cells was counted after 24 hours. Wells were run in duplicate.

Figure 3.

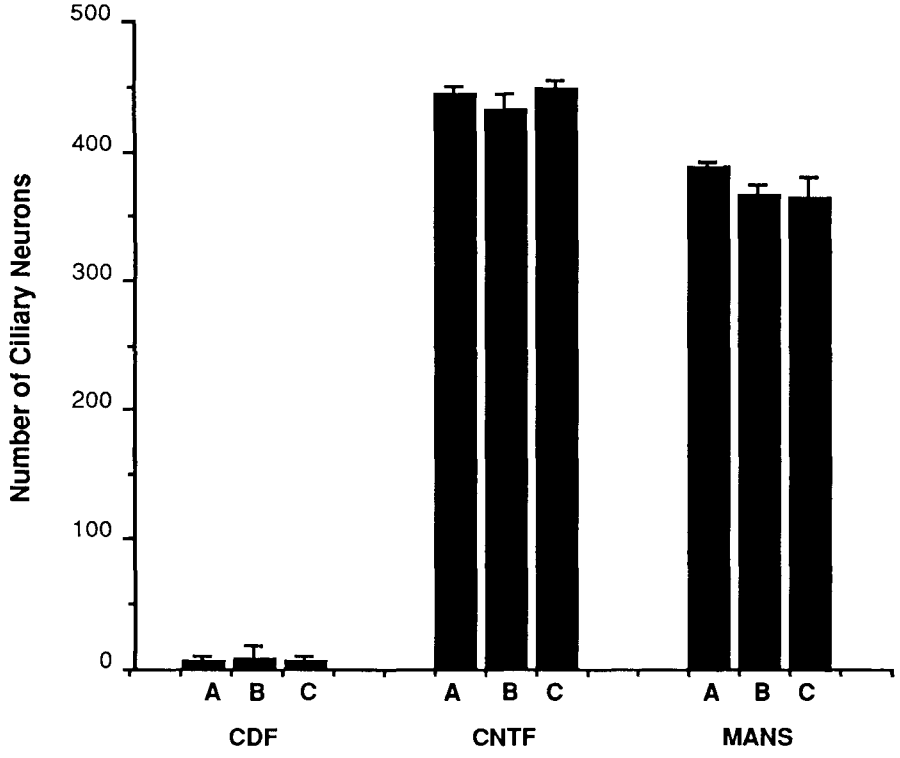

Number of Ciliary Neurons
500
400
300
200
100
0
A B C
CDF
A B C
CNTF
A B C
MANS

Figure 4.

Immunoprecipitation of ^{125}I labeled fractions of CDF, CNTF and MANS.

CDF^{a} (DEAE fraction), CDF^{b}(Sephadex fraction), CNTF (extract purified with SDS PAGE) and MANS (extract purified with SDS PAGE) were labeled with Bolton-Hunter reagent. Approximately 10^{6} cpm of each labeled extract was incubated with Protein A sepharose (A), affinity-purified antibodies to the N-terminal sequence of CDF (B) or affinity-purified antibodies preincubated with the peptide antigen (C). The counts immunoprecipitated are expressed as cpm. Samples were run in duplicate.

Figure 4.

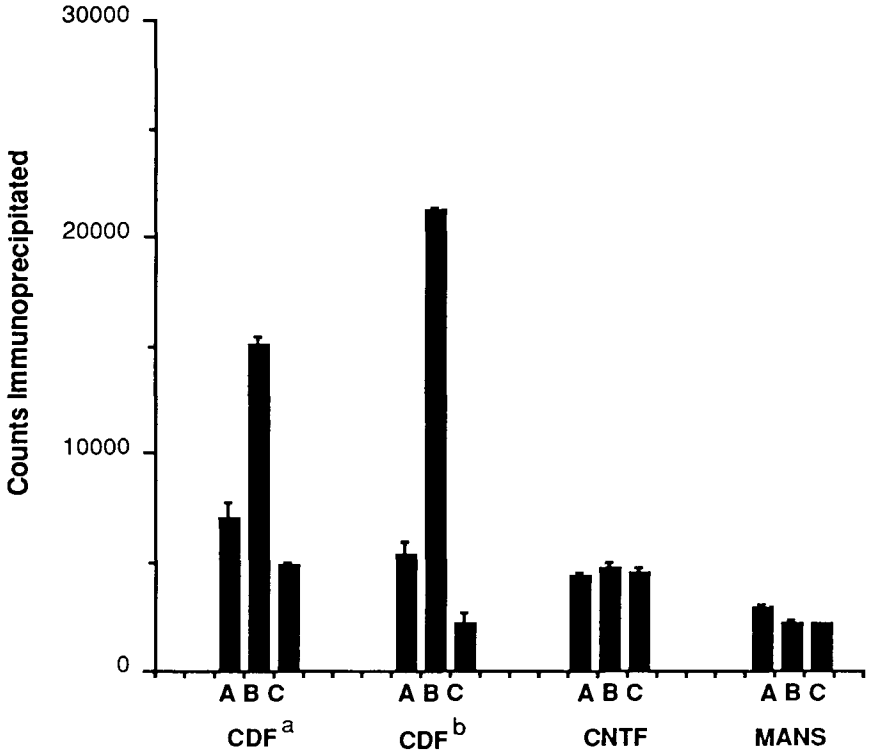
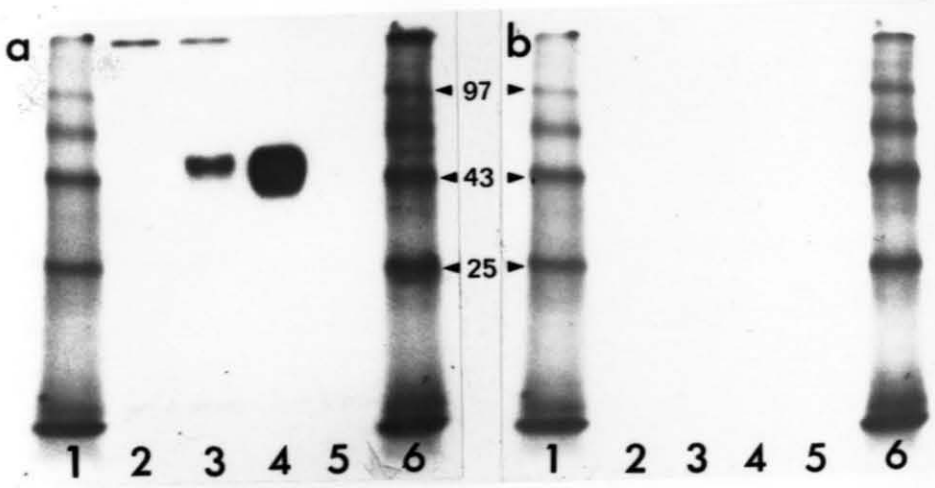

Counts Immunoprecipitated
30000
20000
10000
0
A B C
CDF a
A B C
CDF b
A B C
CNTF
A B C
MANS

Figure 5.

Autoradiographic localization of immunoprecipitated proteins.

Following immunoprecipitation by affinity-purified antibodies to the N-terminal sequence of CDF, labeled proteins were extracted by boiling in SDS sample buffer and subjected to SDS PAGE electrophoresis in a 10% gel. The labeled proteins were localized on X-ray films developed after a seven-day exposure. Panel a shows CDF fractions labeled with Bolton Hunter reagent. The affinity-purified antibodies immunoprecipitate a 45 kDa band from the DEAE fraction (lane 3) and the Sephadex fraction (lane 4) of CDF. Preincubation of the antibodies with antigen prevents immunoprecipitation of a specific band (lane 2, DEAE fraction; lane 4, Sephadex fraction). Panel b shows CNTF (SDS PAGE extract) in lanes 2 and 4 and MANS (SDS PAGE extract) in lanes 3 and 5. In lanes 4 and 5, the antibodies were preincubated with antigen prior to immunoprecipitation. ^{14}C standards were run in lanes 1 and 6; a-chymotrypsin (25,700), ovalbumin (43,000) and phosphorylase b (97,400) are indicated.


a
b
97
43
25
1 2 3 4 5 6
1 2 3 4 5 6

MATERIALS AND METHODS

Cell culture reagents were obtained from Gibco (Grand Island, NY.). 24 and 96-well culture plates were purchased from Corning (Corning, NY.). Affi-gel 10 was obtained from Bio-rad (Richmond, CA.). The Centricon filters were purchased from Amicon (Danvers, MA). ^{125}I Bolton-Hunter reagent and ^{3}H methyl choline were obtained from Amersham (Arlington Heights, Ill.). ^{3}H acetyl -CoA and ^{3}H tyrosine were purchased from New England Nuclear (Wilmington, DE.). Dispase was purchased from Boeringer-Mannheim (Indianapolis, IN.) and collagenase from Worthington Biochemicals (Freehold, NJ.). The Purified Protein Derivative (PPD) conjugation kit was obtained from Cambridge Research Biochemicals (Atlantic Beach, NY.). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Nerve Growth Factor (NGF) was prepared from male mouse submaxillary glands as described by Bocchini and Angeletti (1969), and rat tail collagen was prepared by acid extraction from one or two rat tails under sterile conditions.

Antibody generation

Antisera were generated against an N-terminal peptide sequence derived from purified CDF. An 11 amino acid synthetic peptide corrresponding to the N-terminal region (Fukada, 1986; Yamamori *et al.*, in preparation) was prepared. Approximately 2 mg of this peptide was conjugated to PPD using the aldehyde conjugation method (Lachmann *et al.*, 1986). The conjugate was injected into rabbits previously primed with Bacillus Calmette Guerin (BCG) and boosted at two week intervals until a titer of 1:30,000 was obtained. Serum from several bleeds was pooled and purified on a peptide affinity column prepared by conjugating the N-terminal peptide to a Biorad Affigel 10 column. The bound antibody was eluted from the column using 1M glycine, pH 2.5. The eluate was neutralized with Tris buffer and then dialysed and concentrated using a Centricon microconcentrator with a 30 kDa cutoff. Affinity-purified antibodies were used for all experiments.

Cholinergic Factors

CNTF was purified from rat sciatic nerve according to the method of Manthorpe *et al.* (1986) with the omission of the sucrose density gradient step. The eluate from the DEAE column was used in most experiments. An additional purification step was added for studies involving immunoprecipitation of labeled proteins. The CNTF preparation was subjected to SDS PAGE and the region between 17 and 25 kD was eluted using either an electroeluter or 0.1% Triton X-100. This relatively broad molecular weight range was chosen in view of the different

molecular weights that have been reported for CNTF (Manthorpe *et al.*, 1986; Saadat *et al.*, 1989)

MANS was partially purified according to the protocol of Wong and Kessler (1987). Adult rat spinal cord was homogenized and the cell nuclei and debris removed by centrifugation. Membranes were recovered by high-speed centrifugation and the protein was extracted using 4M NaCl. The extracted protein was dialysed against phosphate buffer and then separated into two fractions using a Centricon microconcentrator with a 30 kDa cutoff. For some experiments, the fraction containing MANS was subjected to SDS PAGE and the material between 25 and 30 kDa was eluted and used.

CDF from heart cell CM was purified according to the protocol of Fukada (1985). The DEAE fraction was used for most experiments; in some cases, however, the Sephadex fraction was used.

Cell culture

Cultures of rat sympathetic neurons were prepared as described by Hawrot and Patterson (1979). Neurons were dissociated enzymatically with dispase (5mg/ml) and collagenase (1mg/ml) and plated in 35 mm collagen-coated dishes. About 2000-3000 neurons were plated per dish. The neurons were grown in Leibovitz's L15-CO_2 medium with NGF (100 ng/ml), 100 units of penicillin, 100 µg of streptomycin and 10 uM cytosine arabinocide and 5% rat serum and the medium changed every second day. The cholinergic factors were diluted in growth medium, sterilized by passage through a 0.2 micron filter and added to the neurons from the third day of culture on. Neurons were harvested for assay between the ninth or fourteenth days of culture.

Ciliary ganglia were dissected from E8 (embryonic day eight) chicks, dissociated and plated in DMEM with 10% fetal calf serum as described by Varon *et al.* (1979). Approximately 1000-2000 neurons were plated directly into medium in 96-well cell culture plates that had been coated sequentially with polyornithine and laminin. The cultures were incubated for 24 hours at 37° C and then fixed with 2% glutaraldehyde as described by Barbin *et al.* (1984). The number of surviving neurons was determined by counting the phase bright cells.

Iodination

Iodination was carried out by the Bolton-Hunter method (1973). 1 to 5 µg of protein in 10 µl of 0.1M borate buffer pH 8.5 was added to the dried iodinated ester and the reaction was allowed to proceed for 15 minutes at 0°C and then for 15 minutes at room temperature. 100 µl of 0.2 M glycine was added to stop the reaction.

The labeled protein was separated from the unreacted label by dialysis using a Centricon microconcentrator with a 10 kDa cutoff. Labeling efficiency was between 40-60%. Labeled fractions were stored at 4°C after adding 0.2mg/ml of BSA to prevent loss due to adsorbtion.

Immunoprecipitation

For the immunoprecipitation experiments in which the biological activity of the factors was tested, aliquots of CNTF, MANS and CDF sufficient for a cell culture assay were added to buffer (PBS pH 7.3 with 2% BSA, 0.2% Triton X-100, and 0.02% PEG 6000). Affinity-purified antibody was added to each vial to a final concentration of 10 uM. After an overnight incubation, the antigen-antibody complex was absorbed to 10 ul of protein A Sepharose for a two more hours at room temperature. The bound complexes were separated by centrifugation and the supernatant was diluted into L15-CO_2 medium and used for cell culture assays. Two controls were performed to insure that the loss of activity consequent to absorption was due to a specific effect of the antibody. Aliquots of CDF were incubated without the antibody and treated as described above and other aliquots were treated with antibody that had been previously adsorbed with 50uM of the synthetic peptide originally used as antigen. A similar procedure was used for immunoprecipitation experiments with labeled fractions. After precipitation with protein A Sepharose, the radioactivity in the pellet was measured with a gamma scintillation counter. To determine the molecular weight of the protein bands immunoprecipitated by the affinity-purified antibody, the protein A Sepharose pellet was boiled in sample buffer for 5 minutes and the extracted material was subjected to SDS-PAGE. Labeled bands were visualized by autoradiography.

Assays

The neurotransmitter status of the neurons was determined in two ways. In some experiments, the incorporation of labeled precursors, ^{3}H-tyrosine into catecholamines and ^{3}H choline into acetylcholine, was assayed by high-voltage paper electrophoresis as described by Mains and Patterson (1973). In other experiments, the induction of cholinergic function was determined by assaying choline acetyltransferase (ChAT) activity in homogenates, essentially according to the method of Fonnum (1969). To increase the sensitivity of the assay, an incubation period of an hour was used. All of the activity was inhibitable by 500μM napthylvinyl pyridine, a specific inhibitor of CHAT activity. Protein concentration was assayed by the method of Lowry, with BSA as a standard.

Appendix to Chapter 1.

MANS preparations contain a CNTF like molecule.

Introduction

Previous experiments (see Chapter 1) comparing the biological activities of CDF/LIF, CNTF and MANS and immunoprecipitation experiments utilizing an antibody against the N-terminal epitope of CDF/LIF had suggested that CDF/LIF was distinct from CNTF and MANS. Subsequent to these experiments, CNTF and CDF/LIF were cloned (Lin *et al.*, 1989, Stockli *et al.*, 1989, Yamamori *et al.*, 1989). Examination of the sequences of CDF/LIF and CNTF confirmed our findings that CNTF was distinct from CDF/LIF and that the two proteins shared no sequence homology or even short stretches of identity.

The relationship between CNTF and MANS, however, was not clear. Both CNTF and MANS had ChAT-inducing activity as well as the ability to reduce levels of catecholamines. Further, MANS preparations, like CNTF, had ciliary neurotrophic activity. The similarity in their spectrum of activities led us to postulate that MANS and CNTF may be related (Rao *et al.*, 1990). Since the fractions compared, however, were only partially purified it was not possible to distinguish between the possibilities that CNTF was a contaminant in the MANS preparation, or that MANS and CNTF, while similar in their ability to induce cholinergic properties, were actually distinct molecules. The cloning of CNTF (Lin *et al.*, 1989, Stockli *et al.*, 1989) and CDF/LIF (Yamamori *et al.*, 1989) also led to the availability of CNTF in large quantities and a polyclonal antibody to rCNTF was generated by Dr. Donna Marrissey (Regeneron Pharmaceuticals). We obtained antibodies to rCNTF (recombinant CNTF) and tested their ability to recognize a CNTF-like molecule in spinal cord membrane preparations.

Results

Antibodies to rCNTF crossreact with a 24-kd band in crude MANS preparations

A polyclonal antibody generated to rCNTF was obtained, (The kind gift of Dr. Donna Marissey--Regeneron Pharmaceuticals) and used to probe Western blots of crude MANS preparations. Figure 1 shows that this antibody recognizes a 24-kd band in sciatic nerve preparations and also recognizes a similar band in spinal cord preparations. Both preparations of membrane extract from spinal cord, as well as preparations of soluble protein from sciatic nerve extract, contain CNTF-like immunoreactivity.

Antibodies to rCNTF immunoprecipitate the cholinergic and ciliary neurotrophic activity in MANS extracts

Our western blotting result suggested that a CNTF/CNTF like protein was present in crude MANS preparation. To distinguish between the possibility of whether two cholinergic molecules are present in the extracts or only one CNTF-like molecule is present, we performed immunoprecipitation experiments. Figure 2a shows that a 1:4 dilution of the CNTF antisera is adequate to completely immunoprecipitate all the cholinergic-inducing activity in sciatic nerve preparations. This dilution of the polyclonal antibody does not alter the cholinergic-inducing ability of LIF (Fig 2b), a molecule which does not share any sequence homology with CNTF (Yamamori *et al.*, 1989, Lin *et al.*, 1989, Stockli *et al.*, 1989, Rao *et al.*, 1990). This dilution of the antibody was then used to precipitate activity from spinal cord preparations. Figure 3 shows that this dilution of the antibody can completely immunoprecipitate activity from both soluble and membrane protein fractions of spinal cord preparations.

Discussion

To define further the relationship between MANS and CNTF, we have assayed the ability of an antibody generated against rCNTF to detect related molecules in spinal cord extracts by immunoprecipitation and Western blots. The antibody raised against rCNTF recognizes a 24-kD band the reported molecular weight of CNTF (Manthorpe *et al.*, 1986, Lin *et al.*, 1989, Stockli *et al.*, 1989) in spinal cord membrane preparations. Further, our immunoprecipitation experiments show that all the cholinergic and ciliary neurotrophic activity in spinal cord extracts can be completely precipitated by the antibody to rCNTF. Thus, our data would indicate that spinal cord membrane extracts contain cholinergic-inducing activity and that all this activity is due to CNTF or a CNTF-like molecule.

What then of MANS? Are there two different cholinergic factors present in spinal cord membrane extracts or is MANS a CNTF-like molecule?

Given the similarity in the biological (Wong and Kessler, 1987, Rao *et al.*, 1990, Sendtner *et al.*, 1989) and biochemical properties (Wong and Kessler, 1987, Adler *et al.*, 1989, Manthorpe *et al.*, 1986, Lin *et al.*, 1989, Stockli *et al.*, 1989) of CNTF and MANS and our data that antibodies to CNTF can immunoprecipitate virtually all the cholinergic-inducing activity in spinal cord membrane preparations and that that CNTF like immunoreactivity can be detected on Western blots, it is likely that MANS and CNTF are closely related molecules. Further, since message for CNTF can be detected by Northern blot hybridization in spinal cord tissue, it would appear that the two factors may be closely related or even identical. MANS, however, is a membrane associated protein (Wong and Kessler 1987, Adler *et al.*, 1989) unlike CNTF which is cytoplasmic (Lin *et al.*, 1989, Stockli *et al.*, 1989) and its reported molecular wt is 29 kD, while that of CNTF is 22-24 kD (Manthorpe *et al.*, 1986, Lin *et al.*, 1989, Stockli *et al.*, 1989). These observations, however, are not inconsistent with our results, as the association of MANS with the membrane is weak and can be easily dissociated by incubating the membrane pellet in 100 mM salt (Adler *et al.*, 1989), which is less than the osmolarity of serum; thus, it is possible that some fraction of the CNTF/MANS present in the cytoplasmic fraction associates with the membrane fraction during the extraction process and is subsequently released on incubation with a 100 mM or higher salt solution. Consistent with this hypothesis is our finding that spinal cord supernatants comprising the soluble protein also contain cholinergic-inducing activity and that Western blots of this preparation also contain a 24-kD molecule recognized by an antibody generated against rCNTF. Recent reports on a cell surface

bound form of CNTF (John Rudge., Neuroscience abstract, 1990) also support this possibility. The small difference in molecular weights is also easily attributed to a difference in methodology. The molecular weight of MANS was estimated by gel permeation of non-denatured protein, while that of CNTF by SDS-PAGE. Estimate of the molecular weight of CNTF by gel permeation also gives a somewhat higher value (Manthorpe, personal communication).

An alternative hypothesis that would be consistent with the data is the possibility that MANS is a completely distinct molecule which is, in fact, membrane-bound (or even associated with the extracellular matrix) and interacts synergistically with CNTF to cause cholinergic induction, but by itself is incapable of cholinergic induction. According to this hypothesis, CNTF/CNTF-like molecule would now be present (albeit in small quantities), as would MANS in spinal cord extracts. Immunoprecipitation of CNTF with the polyclonal antibody would result in a complete loss of cholinergic induction with spinal cord extracts, even though the antibody did not actually immunoprecipitate MANS. Indirect support for this hypothesis comes from the work of Laura *et al.* (1990 a, b), who have demonstrated that two factors are required for the differentiation of O2A progenitor cells (Laura *et al.*, 1990 a). One of these is a soluble factor and is most likely CNTF (Hughes *et al.*, 1988, Laura *et al.*, 1990 b), and the other is a molecule associated with the extracellular matrix and probably secreted by the mesothelial cells, which can be dissociated from the matrix by high salt solutions (1 to 2 M NaCl). This molecule, would therefore, appear very similar to MANS. No data on purification of this extracellular matrix component is available at present. It is difficult to prove or disprove this hypothesis as no experiments to test the ability of CNTF or purified MANS to induce cholinergic induction in defined medium or in single cell cultures of sympathetic neurons have been performed as have been done for heart cell conditioned medium (Furshpan *et al.*, 1976, 1986 a,b). However, given that CNTF protein as well as message is present in spinal cord extracts and giventhe fact that purified CNTF (Sadat *et al.*, 1989, Rao *et al.*, 1990) and rCNTF (see Chapter 2) can cause cholinergic induction in defined medium, it does not seem necessary to postulate the existence of a cofactor for the cholinergic differentiation function of CNTF. Conclusive proof, however, that MANS is not a distinct factor will require amino acid sequencing of MANS.

Figure 1.

CNTF-like immunoreactivity is detectable in spinal cord extracts in Western blot assays.

Equal protein concentration (100 μg) of (a) sciatic nerve, (b) spinal cord supernatant and (c) spinal cord membrane extracts were electrophorised and transferred to nitrocellulose. The blots were probed with a polyclonal antiserum raised against recombinant rat CNTF. As expected, the antiserum recognizes a 24-kD band present in sciatic nerve extracts (a). It also recognizes a 24-Kd band in both spinal cord soluble and membrane extracts (lane b and c, respectively).

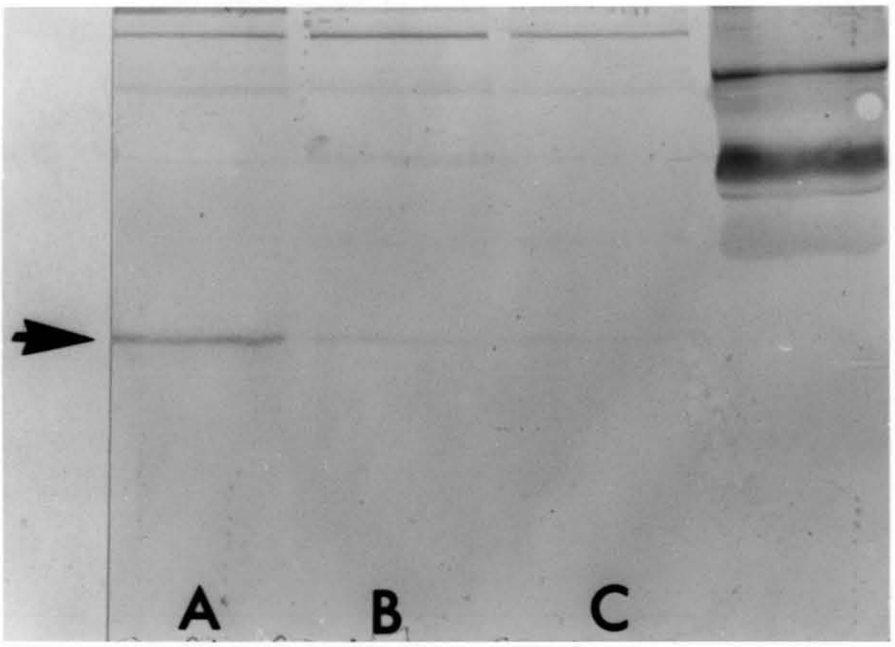

A
B
C

Figure 2.

Specificity of the polyclonal antiserum.

In a. Sciatic nerve extract (100 µg/ml) was incubated with different dilutions of the polyclonal antiserum against CNTF. After immunoprecipitation samples were added to sympathetic neuron cultures. Seven days after the addition of the extracts, the cultures were assayed for ChAT activity by the method of Fonnum. The results are expressed as fold induction of ChAT ± s.e.m. compared to the activity observed in neurons grown in medium without extracts. All samples were run in duplicate (n= 3 experiments).

In b. Sciatic nerve extract (100 µg/ml) and recombinant LIF (25 ng/ml) were incubated with (+) or without (-) a 1:4 dilution (25 µl/100 µl) of the polyclonal antisera to rCNTF. After immunoprecipitation, the samples were added to sympathetic neuron cultures. Seven days after addition of the extracts, samples were assayed for ChAT activity by the method of Fonnum. The results are expressed as the fold induction of ChAT activity ± s.e.m. observed in neurons grown in medium without extract. All samples were run in triplicate.

Figure 2a

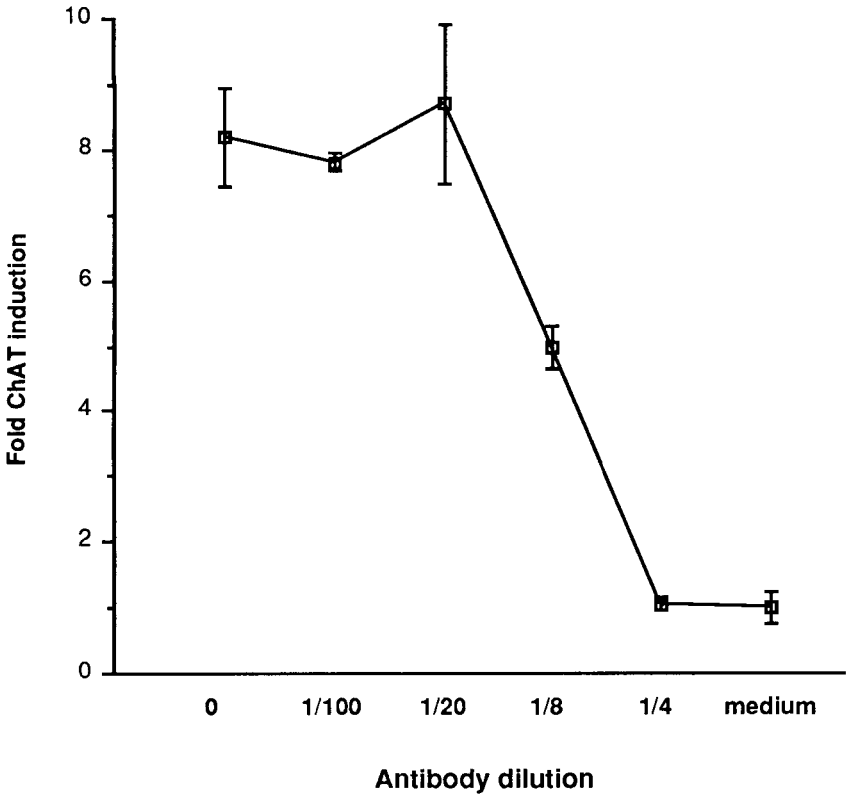


Figure 2b

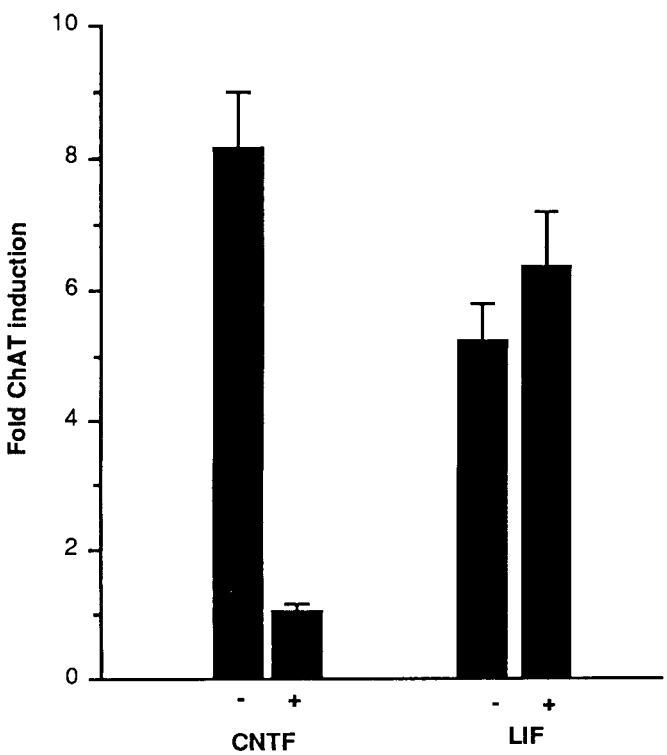
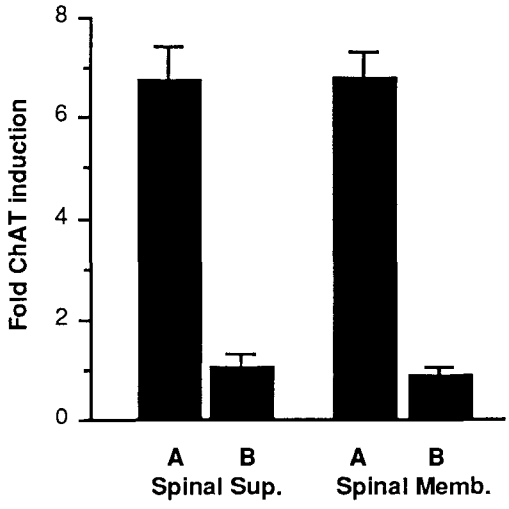


Figure 3.

Polyclonal antisera to CNTF immunoprecipitates the cholinergic-inducing activity in spinal cord extracts.

Spinal cord soluble protein (100 μg/ml) and spinal cord membrane extract (100 μg/ml) were incubated without (a) or with (b) a 1:4 dilution (25 μl/100 μl) of the polyclonal antisera to rCNTF. After immunoprecipitation the samples were added to sympathetic neuron cultures. Seven days after addition of the extracts, samples were assayed for ChAT activity by the method of Fonnum. The results are expressed as the fold induction of ChAT activity ± s.e.m. observed in neurons grown in medium without extract. All samples were run in triplicate.


Fold ChAT induction
8
6
4
2
0
A
B
Spinal Sup.
A
B
Spinal Memb.

Materials and Methods

All materials and methods are as described in Chapter 1 except as described below. The antisera to CNTF was obtained from Dr. Donna Marissey - Regeneron Pharmaceuticals.

Western blotting with antisera to CNTF

Aliquots of extracts (60 ug/lane) were run on a 15% SDS PAGE minigel (Biorad) and the proteins were blotted onto nitrocellulose (overnight 30mA). The nitrocellulose blots were blocked in blocking buffer (5% defatted milk in Tris buffered saline, pH 7.2) and then incubated for two hours with a polyclonal antibody against CNTF (1:1000 dilution) (the kind gift of Dr. Donna Marissey, Regeneron Pharmaceuticals). The blots were then sequentially incubated with a biotinylated secondary antibody (One hour), and avidin conjugated to alkaline phosphatase (one half hour). The bound enzyme was detected with NBT (Nitroblue tetrazolium) and BCIP(Bromo chloro indoyl phosphate) in 10 mM bicarbonate buffer pH 9.5. After optimal color development, the reaction was stopped by rinsing in distilled water.

Immunoprecipitation of cholinergic-inducing activity.

For the immunoprecipitation experiments in which the biological activity of the factors was tested, aliquots of sciatic nerve extract, spinal cord membrane preparations or spinal cord soluble extract sufficient for a cell culture assay were added to buffer (PBS pH 7.3 with 2% BSA, 0.2% Triton X-100, and 0.02% PEG 6000). Affinity-purified antibody was added to each vial to a final dilution of 1:4. After an overnight incubation, the antigen-antibody complex was absorbed to 10 µl of protein A Sepharose for a further two hours at room temperature. The bound complexes were separated by centrifugation and the supernatant was diluted into L15-CO_2 medium and used for cell culture assays. Two controls were performed to insure that the loss of activity consequent to absorption was due to a specific effect of the antibody. Aliquots of LIF were incubated with the antibody and treated as described above, and the two results were compared with aliquots incubated without the antibody but otherwise treated as described above.

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Chapter 2.

The effect of ciliary neurotrophic factor on neuropeptide expression by sympathetic and dorsal root ganglion neurons.

Abstract

Ciliary neurotrophic factor (CNTF) and sciatic nerve extracts have been shown to cause ChAT induction in cultured sympathetic neurons (Saadat *et al.*, 1989, Rao *et al.*, 1990). To determine if CNTF alters levels of other neurotransmitter candidates we have examined the effects of crude sciatic nerve extracts on the expression of several peptides in cultures of rat sympathetic neurons. Vasoactive intestinal peptide (VIP), Substance P, and somatostatin levels were increased several fold, while Neuropeptide Y (NPY) levels were significantly reduced in comparison to levels in control cultures. No change in levels of Leu-enkephalin (L-enk) were detected. The effects of crude sciatic nerve extract on expression of peptides were abolished by immunoprecipitating CNTF-like molecules from the extract with a polyclonal antibody raised against recombinant CNTF (rCNTF). rCNTF had the same effect on neuropeptide levels as crude sciatic nerve extracts. These observations suggest that the peptide modulating factor present in sciatic nerve extracts is CNTF.

The changes in peptide levels observed with rCNTF treatment occurred in a dose-dependent fashion with maximal induction seen at a concentration of 5-25 ng/ml. Peptide levels were altered three days after exposure to CNTF and levels continued to increase up to 14 days in culture. Treatment of sympathetic neuron cultures with 30 mM KCl, which has been shown to block the cholinergic differentiation effect of heart cell conditioned medium (Walicke *et al.*, 1977) did not block the effects of CNTF on ChAT induction and peptide expression. Similar treatment with 30 mM K^+ blocked the effect of cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) on ChAT induction. Thus, the cholinergic induction in sympathetic neurons, by CNTF and CDF/LIF, is differentially modulated by depolarization. This suggests that the mechanism of cholinergic and peptide induction in sympathetic cultures by CNTF differs from CDF/LIF.

CNTF also differed from CDF/LIF in its effects on ChAT and peptide levels on cultures of DRG neurons. No change is detected in ChAT, VIP, Substance P, and somatostatin levels in response to 25 ng/ml of CNTF. These observations provide additional evidence of the overlapping but distinct spectrum of action of CNTF and CDF/LIF.

Introduction

Over the past several years it has become increasingly evident that the transmitters and neuropeptides produced by a particular neuron are not specified by lineage alone (see Introduction). Rather, the transmitter profile represents a dynamic balance between cell lineage and environmental signals. The role of the environment in inducing the *de novo* expression and/or modulating existing levels of classical neurotransmitter and peptides has been examined both *in vivo* and *in vitro*. The target that the neuron innervates, the level of preganglionic activity and the ganglionic environment (including cell density and presence of non-neuronal cells), have all been suggested as sources of extrinsic signals that regulate the expression of transmitters in neurons *in vivo*.

Evidence for target specification of neurotransmitter phenotype comes from cell culture, crossinnervation and tissue transplant experiments. Co-culture experiments in which pineal cells or salivary glands were grown with sympathetic neurons from the superior cervical ganglion (SCG) have shown that Substance P and somatostatin levels are elevated (Kessler *et al.*, 1984). Subsequent studies have demonstrated that the elevation in levels of somatostatin and Substance P by target tissue is mediated by a soluble factor (Kessler, 1984). MacMahon and Gibson (1985) have shown that sensory neurons can alter their expression of Substance P depending on whether they innervate skin or muscle. When sensory fibers innervating the muscle and exhibiting low levels of Substance P immunoreactivity are made to innervate skin, Substance P immunoreactivity in the fibers increases ; conversely, Substance P immunoreactivity in skin sensory fibers decreases when they innervate muscle spindles. These data suggest that levels of Substance P *in vivo* are regulated by the target. Tissue transplantation experiments (Schotzinger and Landis, 1989, 1990) demonstrate that the normal noradrenergic to cholinergic and peptidergic switch that characterizes sweat gland innervation (Landis and Keefe, 1983 Leblanc and Landis, 1986, Landis *et al.*, 1988) is specified by the target. When sympathetic fibers that normally innervate noradrenergic targets are made to innervate sweat glands (Schotzinger and Landis, 1989, 1990), they express VIP immunoreactivity and increased choline acetyl transferase (ChAT) activity. Noradrenergic markers such as tyrosine hydroxylase and catecholamine histofluorescence are decreased. Conversely, when fibers innervating the sweat gland innervate a noradrenergic target like parotid gland, they fail to express VIP and choline esterase immunoreactivity and maintain their noradrenergic markers

(Schotzinger and Landis, 1990). The actual mechanism by which the sweat gland influences the neurotransmitter expression is not known. A factor(s) has been solubilized and partially characterized, however, which can mediate a similar noradrenergic to cholinergic switch in culture (Rao and Landis 1990).

Preganglionic activity can also influence neurotransmitter and neuropeptide levels. Denervation, for example, results in expression of Substance P in sympathetic neurons, and impulse activity and depolarization in culture reduce Substance P and somatostatin levels (Kessler 1983 a). Levels of VIP are also two-fold higher in the decentralized SCG as compared to sham operated animals (Sachs et. al., Neuroscience abstract, 1990). In contrast to the decrease in Substance P and somatostatin levels, impulse activity and depolarization augmented noradrenergic characteristics (Thoenen, 1974, Walicke *et al.*, 1977), indicating that the effects on Substance P and somatostatin are specific. Denervation and preganglionic stimulation also have specific effects on increasing L-enk and NPY expression in the adrenal gland (Schultzber *et al.*, 1978, Lewis *et al.*, 1987, De Quidt and Emson, 1986, Schalling *et al.*, 1988, La Gamma *et al.*, 1984). Experiments with cultured adrenal chromaffin cells also demonstrate that cells grown under depolarizing conditions will up-regulate the levels of enkephalins (Henion, P.D., personal communication) in rat chromaffin cell cultures. Thus, preganglionic activity and depolarization can modulate the expression of several different peptides.

Considerable evidence suggests that cell density and non-neuronal cells can also modulate the expression of catecholaminergic, cholinergic and peptidergic properties in sympathetic neuron cultures (Patterson and Chun, 1974, Bunge *et al.*, 1984, Kessler 1983, 1984, 1985). For example, increasing cell density in culture increases levels of ChAT and Substance P (Kessler, 1985, Adler and Black, 1985, Acheson and Rutishauser, 1988) without increasing the expression of noradrenergic traits or somatostatin (Kessler, 1985). Substance P levels can also be increased by co-culture of dissociated sympathetic neurons with ganglionic nonneuronal cells and nonneuronal cell conditioned medium (Kessler, 1984, Kessler, 1985). Nonneuronal cell membranes/membrane extract can also increase ChAT activity (Kessler *et al.*, 1986, Wong and Kessler, 1987). Similar increases in somatostatin, but not Substance P, levels have been described when avian sensory neurons are grown with non-neuronal cells or non-neuronal conditioned medium (Mudge 1981).

Both soluble and membrane-associated factors have been identified which could mediate the effects of the target and/or non-neuronal cells on transmitter and peptide expression. A membrane-associated neurotransmitter-stimulating substance

(MANS) (Wong and Kessler, 1987, Adler *et al.*, 1989) was identified as a cholinergic-inducing factor for sympathetic neurons in culture. MANS preparations also increase the levels of Substance P and somatostatin (Wong and Kessler, 1987, Lee *et al.*, 1990). It has been suggested that MANS is the factor responsible for the density-mediated change in ChAT activity and peptide levels. CDF/LIF, another cholinergic factor (Fukada, 1985, Yamamori *et al.*, 1989), differentially regulates several peptides (Nawa and Patterson 1990 a, b). The evidence suggests that this factor is secreted by fibroblasts (Patterson and Chun, 1977, Fukada, 1980, Hozumi *et al.*, 1984). CNTF purified and cloned from sciatic nerve (Manthorpe *et al.*, 1986, Stockli *et al.*, 1990, Lin *et al.*, 1990) is made by Schwann cells and cultured type 1 astrocytes (Saadat *et al.*, 1989), Rudge *et al.*, Neuroscience abstract, 1990). CNTF increases ChAT activity and reduces TH levels in sympathetic neuron cultures (Saadat *et al.*, 1989, Rao *et al.*, 1990). In addition, CNTF induces expression of VIP in embryonic chick sympathetic neuron cultures (Ernsberger *et al.*, 1989). A cholinergic and peptide differentiation activity has also been solubilized from sweat gland tissue (Rao and Landis, 1990). The sweat gland differentiation activity increases VIP levels and decreases NPY levels in sympathetic neuron cultures.

The interaction between cholinergic differentiation factors and other environmental influences on neurotransmitter and peptide levels in cultured sympathetic neurons is complex and incompletely defined. The effects of heart cell conditioned medium on induction of ChAT and reduction of catecholamines can be modulated by the activity of the neurons (Walicke *et al.*, 1977, Walicke and Patterson, 1981 a, b). Depolarization can almost completely block the adrenergic to cholinergic switch (Walicke *et al.*, 1977). The presence of killed ganglionic non-neuronal cells or skin fibroblasts can block the induction of somatostatin by fibroblast-conditioned medium in sympathetic neuron cultures (Spiegel *et al.*, 1990). The factor present in the fibroblast-conditioned medium was not identified but is likely to be CDF/LIF (Patterson and Chun, 1977, Fukada, 1980, Hozumi *et al.*, 1984). The inhibitory factor(s) associated with killed fibroblasts is not MANS (Spiegel *et al.*, 1990), but appears to be due to some as yet uncharacterized factor.

Identifying the molecular signals that mediate the effects of poorly defined conditioned medium and tissue extracts on peptide levels will be critical for further analysis. We have begun examining the effects of CNTF on peptide expression and its interaction with other environmental influences to identify a possible role for CNTF in peptide regulation. In this chapter, we have examined the effects of CNTF on peptide expression by sympathetic and sensory neurons in culture. In initial

experiments the effects of crude sciatic nerve extracts, a rich source of CNTF, were determined. Subsequent experiments were undertaken with recombinant CNTF. We demonstrate that sciatic nerve extracts, in addition to regulating the levels of ChAT and TH (Saadat *et al.*, 1989, Rao *et al.*,1990) also contain a molecule(s) that can modulate the levels of several different peptides. Using polyclonal antibodies to rCNTF, we show that the induction of peptides by crude sciatic nerve extracts is due to CNTF. We characterize the interaction of CNTF and depolarization on ChAT and peptide induction and compare it with the effects of depolarization on the effects of CDF/LIF.

Results

Sciatic nerve extracts have peptidergic activity

We examined the effects of crude sciatic nerve extracts on the expression of VIP, somatostatin, Substance P, NPY and L-enk. Sympathetic neuron cultures treated with sciatic nerve extracts were assayed for peptide levels by radioimmunoassay. The levels of VIP, somatostatin, and Substance P were elevated in comparison to control cultures grown without extracts (Table 1). Levels of NPY were reduced and L-enk levels remained unchanged. Since sciatic nerve extracts do not have any effects on cell number in sympathetic cultures over a period of nine days (Saadat *et al.*, 1989, Rao *et al.*, 1990), sciatic nerve extracts affect differentially the levels of several different peptides in cultured sympathetic neurons.

The peptidergic activity present in sciatic nerve extracts is due to CNTF

A likely source of the peptidergic activity in the sciatic nerve preparations is CNTF since it has been shown to cause VIP induction in embryonic chick sympathetic neurons (Ernsberger *et al.*, 1989). To ascertain whether CNTF and/or any other peptidergic factor(s) were present in sciatic nerve extracts, we used a polyclonal antiserum generated against rCNTF to immunoprecipitate CNTF-like molecules from the extract. Sciatic nerve extracts incubated with and without antiserum were added to sympathetic neuron cultures and the ability of sciatic nerve extracts which had been depleted of CNTF to alter peptides levels was assayed. Figure 1 shows that no significant induction of VIP, somatostatin or Substance P is seen. Likewise, no changes in NPY levels were detected in cultures treated with extracts preincubated with a 1: 4 dilution of a polyclonal antibody to CNTF. This suggests that the factor(s) in sciatic nerve extracts responsible for altering the levels of four different peptides tested is immunologically related to CNTF.

To confirm that CNTF has a similar effect on peptide levels as crude sciatic nerve extract, we added rCNTF to sympathetic neuron cultures and assayed peptide levels. rCNTF (Table 2) and sciatic nerve extract (Table 1) have similar effects on peptide levels. VIP, somatostatin and Substance P levels were raised as compared to those in control cultures grown without rCNTF. NPY levels were reduced and L-enk levels were unchanged. This suggests that CNTF is the molecule responsible for the effects of sciatic nerve extracts on the levels of the peptides tested and no other peptide regulating factor (for the peptides tested) is present.

Dose response of peptide induction

We have determined the concentration of CNTF required to cause detectable changes in peptide levels. Figure 2 shows that the addition of as little as 200 pg/ml of CNTF caused a significant change in NPY and Substance P levels. The amount of rCNTF required to alter NPY, VIP and Substance P levels is similar to the concentration of rCNTF required for detectable effects on embryonic day-eight ciliary neuron survival (Stockli *et al.*, 1989, Lin *et al.*, 1989). Higher levels (1-5 ng/ml) were required to see a detectable change in somatostatin levels. The maximal induction of all peptides is seen between 5-25 ng/ml of CNTF. This concentration of CNTF, required for maximal induction of peptides is five-fold higher than that required for ciliary neuron survival (Stockli *et al.*, 1989, Lin *et al.*, 1989) and is similar to levels of CNTF required for ChAT induction in sympathetic neuron cultures (Saadat *et al.*, 1989, our unpublished results).

Time course of peptide induction

The time course of peptide induction in sympathetic neuron cultures in response to CNTF was examined (Fig. 3) to determine if it parallels the reported time course of altered ChAT activity (Saadat *et al.*, 1989). Detectable increases in peptide levels (as determined by radioimmunoassay) are first seen as early as three days after exposure to 2 ng/ml of CNTF, and levels continue to increase up to day 14, the last time point examined. The increase in peptide levels paralleled the time course of ChAT induction in response to CNTF (Saadat *et al.*, 1989, unpublished observations), and suggests that the peptide response does not occur secondary to the ChAT differentiation. Alteration in peptide levels in response to CDF/LIF is, in general, slower with induction first detected seven to ten days after exposure to CDF/LIF (Nawa and Patterson, 1990, Nawa and Patterson, Cold Spring Harbor Symposium, 1991).

The effect of depolarization on neurotransmitter modulation by CNTF

The effects of CDF/LIF and CNTF on ChAT activity, catecholamine levels and peptide expression are very similar (Saadat *et al.*, 1989, Rao *et al.*, 1990, Nawa and Patterson, 1990). To further examine the similarities and differences in ChAT induction by CNTF and CDF/LIF, we have assayed the effect of CNTF and CDF/LIF on neurons grown in 30 mM KCl. Depolarization with 30 mM KCl will block the ChAT induction of heart cell conditioned medium (Walicke *et al.*, 1977, Raynaud and

Weber, 1987) and recombinant LIF (Fig. 4). The ChAT induction by CNTF is not blocked by high potassium culture conditions. Levels of ChAT activity in cultures grown with CNTF and 30 mM KCl are higher than in cultures grown in CNTF alone. Thus, CNTF and CDF/LIF differ in their ability to induce cholinergic function under depolarizing conditions.

We have also examined the effect of depolarization and CNTF on peptide levels. As can be seen in Fig. 5, depolarization caused an elevation of NPY and VIP levels. No changes are seen in somatostatin and Substance P levels. The cell number is not altered by depolarization, although cells appear larger in diameter when compared to controls (unpublished observations). Depolarization did not block the effects of CNTF on peptide levels. The levels of VIP, somatostatin and Substance P are unchanged or higher in cultures grown with the addition of CNTF and 30 mM KCl when compared to control cultures grown with CNTF alone (Fig. 5). The change in NPY levels in cultures grown with CNTF and 30 mM KCl may represent the additive effect of depolarization and CNTF. Thus, depolarization with 30 mM KCl did not alter the response of sympathetic neurons to CNTF.

Effect of CNTF on DRG neuron cultures

CNTF receptors are present on rat dorsal root ganglion (DRG) neurons (Squinto *et al.*, 1991). CNTF has also been shown to be a survival factor for DRG neurons (Eckenstein *et al.*, 1990). It was possible that in addition to its effects on survival, CNTF would also alter peptide levels. To determine whether DRG neurons responded to CNTF by altering transmitter and peptide levels, we assayed the effect of CNTF on neonatal rat DRG cultures. As can be seen in figure 6, DRG neurons in culture expressed high levels of Substance P but contain little VIP, somatostatin, NPY or ChAT. L-enk was undetectable in DRG cultures (less than 1 pg/well). This is similar to the peptide expression reported in cultured DRG neurons (Nawa and Patterson, Cold Spring Harbor Symposium, 1991). Addition of CNTF caused no significant changes in either ChAT activity or in the levels of the peptides examined.

Discussion

We have presented evidence for the effects of CNTF and crude sciatic nerve extracts on expression of neuropeptides by sympathetic neurons in culture. Our data comparing the effects of depolarization on ChAT and peptide induction by CNTF and CDF/LIF, as well as the effects of CNTF and CDF/LIF on sensory neurons, has provided additional evidence of their distinct but overlapping effects.

Extracts of sciatic nerve modulate the expression of several different peptides in sympathetic neuron cultures. VIP, somatostatin and Substance P are elevated and NPY levels are reduced, while no changes are seen in the levels of L-enk. Since the levels of induction are high and there is no significant difference in cell survival (Saadat *et al.*, 1989, Rao *et al.*, 1990), the varied effects on neuropeptide levels are not due to differential cell survival. An antiserum generated against rCNTF can completely immunoprecipitate the ChAT (see Chapter 1 appendix) and peptide-inducing activities present in sciatic nerve extracts. Further, Western blot analysis of sciatic nerve extracts probed with the same antibody reveal a single 24-kD band (see Chapter 1 appendix) the reported molecular weight of CNTF (Manthorpe *et al.*, 1986, Stockli *et al.*, 1989). It is, therefore, reasonable to assume that the activity in sciatic nerve extracts is due to a single molecule that is immunologically related or identical to CNTF. This conclusion is supported by the fact that rCNTF, like sciatic nerve extracts, differentially regulates the levels of the identical peptides in a dose-dependent fashion. VIP, somatostatin and Substance P are elevated, while the levels of NPY are reciprocally down-regulated with no detectable change in L-enk levels.

The alteration of peptide expression in response to rCNTF occurs at low concentrations. The half maximal dose for peptide induction is between 1-5 ng and maximal levels of VIP, Substance P and somatostatin are seen between 5-25 ng/ml of CNTF. The concentration of CNTF required to cause maximal changes in peptide levels is similar to that reported for changes in ChAT and catecholamine levels in sympathetic neuron cultures (Saadat *et al.*, 1989).

The peptides altered by CNTF and CDF/LIF (Nawa and Patterson, 1990), a second cholinergic-inducing molecule, in sympathetic neuron cultures are identical. Thus, at least two different factors, CNTF and CDF/LIF, can alter peptide levels in sympathetic neuron cultures. Other, less characterized, peptide-inducing *molecules* have also been described (Wong and Kessler, 1987, Lee *et al.*, 1990, Rao and Landis 1990, Nawa and Sah, 1990, Nawa and Patterson, 1990) which alter peptide levels

when added to sympathetic neuron cultures. MANS (Wong and Kessler, 1986, Adler *et al.*, 1989, Lee *et al.*, 1990) and sweat gland cholinergic differentiation factor (Rao and Landis, 1990) have similar effects on VIP, NPY, Substance P and somatostatin (Wong and Kessler, 1986, Lee *et al.*, 1990, Rao and Landis, 1990, and unpublished results), and it is possible that these peptide-inducing molecules represent CNTF isolated from different sources.

The time course of induction of these peptides is similar to that reported for changes in ChAT activity and catecholamine levels in sympathetic neuron cultures (Saadat *et al.*, 1989). An increase in ChAT activity is seen as early as two days in culture (Saadat *et al.*, 1989, our unpublished results). Significant induction of Substance P, VIP and somatostatin is seen as early as three days after the exposure to CNTF, suggesting that the change in peptide levels is not secondary to the noradrenergic to cholinergic switch. The time course of induction of both peptides and ChAT activity differs from that of CDF/LIF (Nawa and Patterson 1990). SCG neurons respond to CDF/LIF with an increase in ChAT levels first detectable four to five days after exposure to CDF/LIF, followed two to three days later with a change in VIP and Substance P levels (Nawa and Patterson 1990, Nawa and Patterson, Cold Spring Harbor Symposium, 1991). It is, therefore, possible that the short-term regulation of VIP, Substance P and somatostatin that has been described in sympathetic neuron cultures and ganglion explants (Kessler, 1983 a, Sachs *et al.*, Neuroscience abstract, 1990) is mediated by CNTF.

The failure of CNTF to alter ChAT and peptide levels in DRG cultures was somewhat surprising, as recent reports have indicated that receptors for CNTF are present on neonatal and adult rat DRG neurons (Squinto *et al.*, 1991). Binding of CNTF to its receptor leads to a specific induction of c-jun, an early intermediate gene, in neonatal DRG neurons, indicating that the receptors present are active. CNTF is also a survival factor for embryonic chick DRG neurons (Eckenstein *et al.*, 1990). A failure to respond to CNTF with altered ChAT activity and peptide levels may suggest that different kinds of CNTF receptors mediate the effects of CNTF on cell survival and alteration of peptide levels and ChAT activity. An alternative explanation is that DRG neurons are developmentally incapable of regulating neurotransmitter and peptide levels. Since CDF/LIF in DRG cultures causes a 10-fold induction of ChAT and also differentially regulates peptide levels (Nawa and Patterson, Cold Spring Harbor Symposium, 1991), the inability of the DRG neurons to increase ChAT activity or alter peptide levels in response to CNTF is not due to a developmental restriction on plasticity.

CDF/LIF has also been shown to regulate the levels of peptides, including Substance P, somatostatin, Neuropeptide Y, and VIP (Nawa and Patterson, 1990, Nawa and Patterson, Cold Spring harbor Symposium, 1990). Given this remarkable similarity in the modulation of neurotransmitter levels of CNTF and CDF/LIF, it is tempting to speculate that these factors are acting by a common cascade of intracellular regulatory events to alter a developmental program in these neurons. Since LIF and CNTF share no sequence homology, it is unlikely that they bind to a common receptor. Further, LIF does not have any ciliary neurotrophic (Rao *et al.*, 1990) activity, indicating that LIF does not bind to at least one CNTF receptor. Thus, it is possible that their distinct receptors impinge on a common second messenger pathway. To explore the hypothesis that these two molecules were acting via a common mechanism, we compared the effects of depolarization on ChAT induction by CDF/LIF and CNTF. Depolarization has no effect on the induction of ChAT or peptides by CNTF, while it completely antagonizes the effects of LIF on ChAT induction. This failure of depolarization to antagonize the effects of CNTF suggests that CNTF acts differently from LIF in regulating the cholinergic status of cultured sympathetic neurons. Determining the hierarchy of interactions between factors *in vitro* will provide evidence for the mechanism of their actions, and will be the focus of subsequent studies.

	VIP	Substance P	Somatostatin	NPY	L-enk
Medium	42 ± 24	85 ± 15	55 ± 29	1868 ± 74	< 4.0
Sciatic N. ext	214 ± 77	2255 ± 255	709 ± 242	730 ± 196	< 4.0

Table 1.

Sciatic nerve extracts can modulate neuropeptide levels in sympathetic neuron cultures.

Sciatic nerve extract (50 µg/ml) was added to sympathetic neuron cultures. Seven days after the addition of the extracts, samples were homogenized and assayed for peptide levels by radioimmunoassay. The results are expressed as picograms of peptide/well ± s.d. All peptides were assayed in triplicate in sister wells from a single culture (n=3 experiments).

	VIP	Substance P	Somatostatin	NPY	L-enk
Medium	176 ± 21	291 ± 20	41 ± 15	880 ± 30	< 4.0
CNTF	800 ± 80	2750 ± 450	290 ± 20	495 ± 15	< 4.0

Table 2.

CNTF modulates neuropeptide levels in sympathetic neuron cultures.

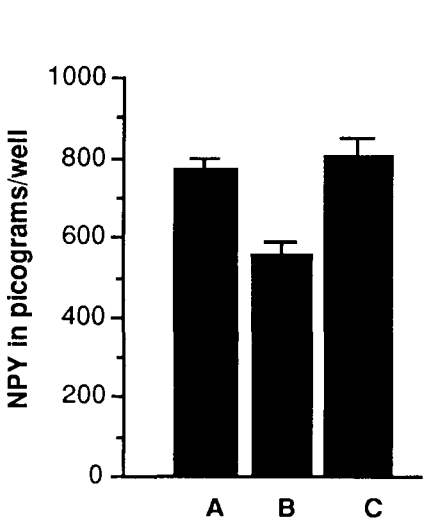
CNTF (2 ng/ml) was added to sympathetic neuron cultures. Seven days after the addition of the recombinant protein samples were homogenized and assayed for peptide levels by radioimmunoassay. The results are expressed as picograms of peptide/well ± s.e.m. All peptides were assayed in duplicate from samples pooled from three different experiments (n=3 experiments).

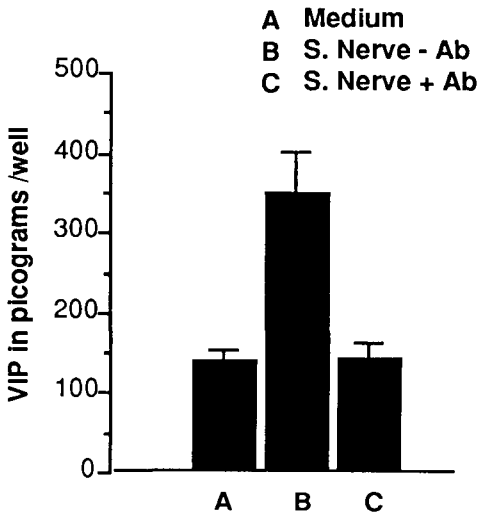
Figure 1.

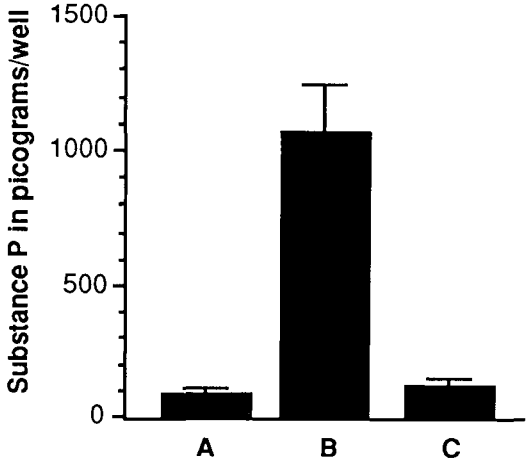
The peptide-regulating activity present in sciatic nerve extracts is immunoprecipitated with antibodies to rCNTF.

Sciatic nerve extract (DEAE fraction) was incubated with either protein A sepharose or polyclonal antibodies to CNTF. After immunoprecipitation, the supernatants were added to sympathetic neuron cultures. Seven days after the addition of extract, cultures grown with (a) L15CO_2, (b) sciatic nerve extract incubated with protein A sepharose, and (c) sciatic nerve extracts incubated with polyclonal antibody and protein A sepharose were assayed for peptide levels by radioimmunoassay. The results are expressed as picograms/well $\pm$ s.e.m. All samples were run in duplicate (n=3 experiments).

Figure 1.


NPY in picograms/well
1000
800
600
400
200
0
A
B
C


A Medium
B S. Nerve - Ab
C S. Nerve + Ab
VIP in picograms /well
500
400
300
200
100
0
A
B
C


Substance P in picograms/well
1500
1000
500
0
A
B
C

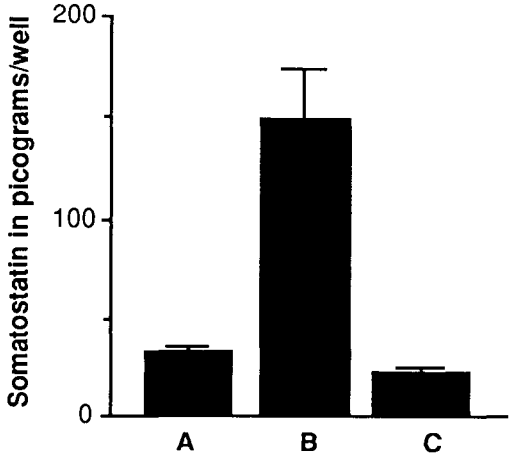
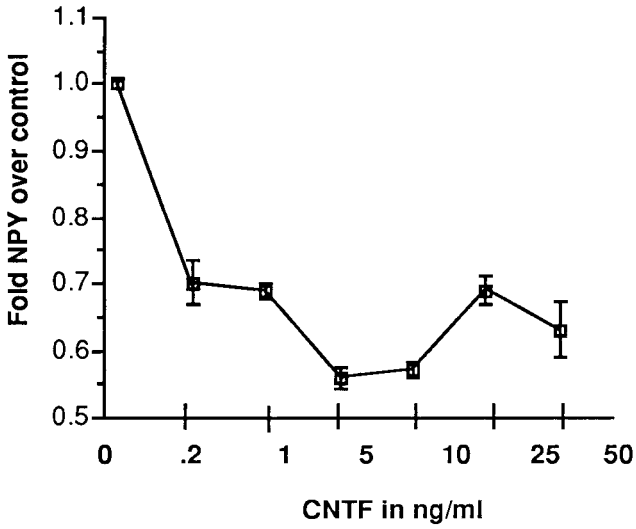
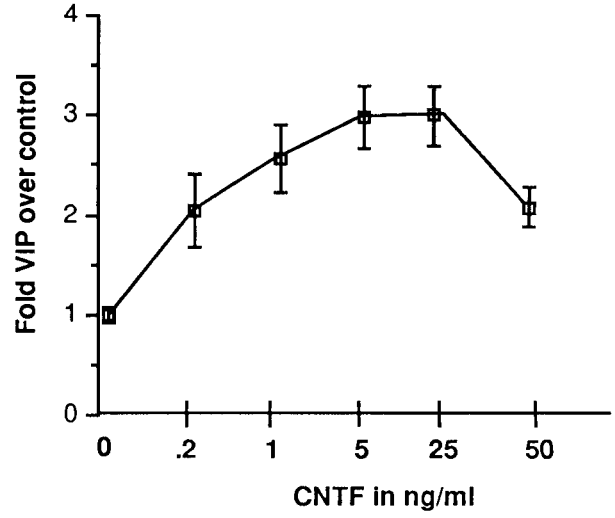

Somatostatin in picograms/well
200
100
0
A
B
C

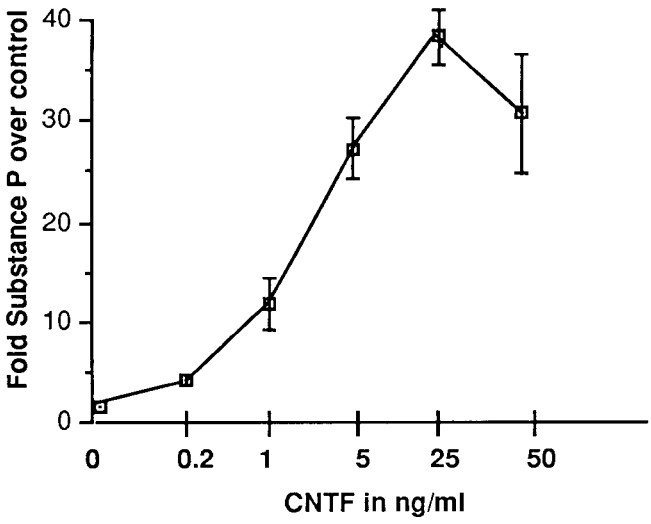
Figure 2.

Effect of increasing concentrations of CNTF on neuropeptide levels. Increasing concentrations of CNTF were added to sympathetic neuron cultures. Five days after the addition of CNTF, neurons were homogenized and aliquots were assayed for peptide levels by radioimmunoassay. Samples were run in triplicate. The results are expressed as fold induction, as compared to control cultures grown without the addition of CNTF $\pm$ s.e.m. All samples were run in duplicate (n=3 experiments).

Figure 2 a.


Fold NPY over control
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
.2
1
5
10
25
50
CNTF in ng/ml


Fold VIP over control
4
3
2
1
0
0
.2
1
5
25
50
CNTF in ng/ml


Fold Substance P over control
40
30
20
10
0
0
0.2
1
5
25
50
CNTF in ng/ml

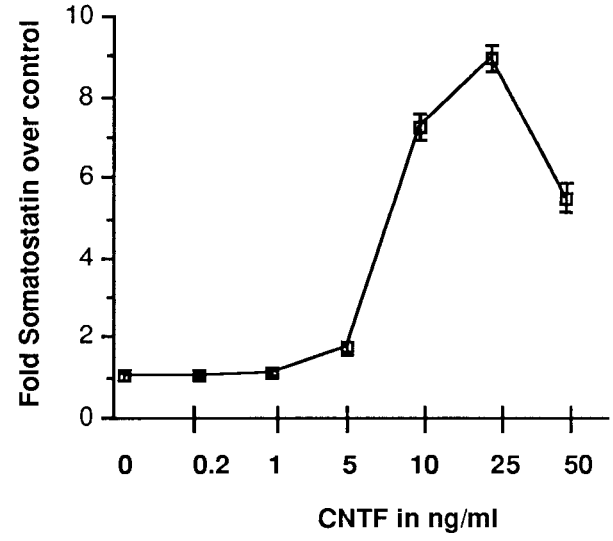
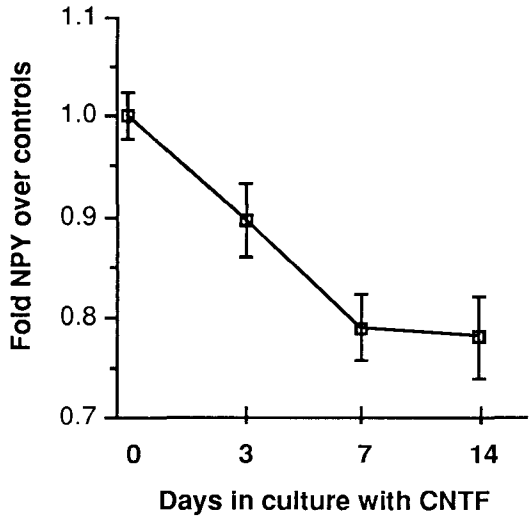

Fold Somatostatin over control
10
8
6
4
2
0
0
0.2
1
5
10
25
50
CNTF in ng/ml

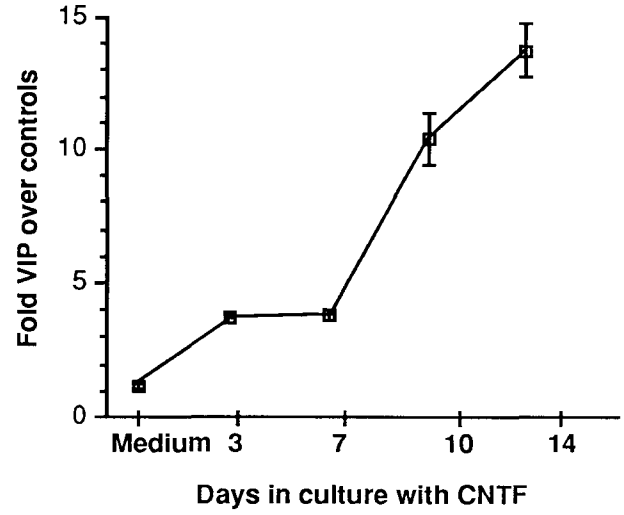
Figure 3.

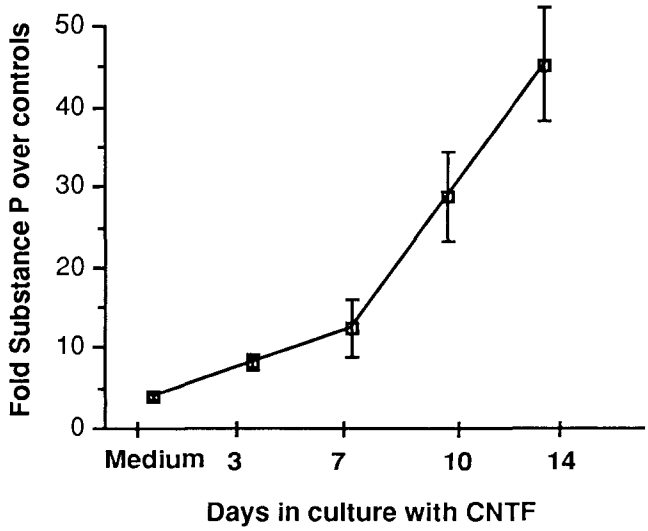
Time course of response to CNTF.

2 ng/ml of rCNTF was added to sympathetic neuron cultures. Samples were homogenized at appropriate intervals after the addition of CNTF and assayed for peptide levels by radioimmunoassay. The results are expressed as fold induction, as compared to control cultures grown without the addition of CNTF $\pm$ s.e.m. All samples were run in duplicate (n=3 experiments).

Figure 3.


Fold NPY over controls
1.1
1.0
0.9
0.8
0.7
0
3
7
14
Days in culture with CNTF


Fold VIP over controls
15
10
5
0
Medium
3
7
10
14
Days in culture with CNTF


Fold Substance P over controls
50
40
30
20
10
0
Medium
3
7
10
14
Days in culture with CNTF

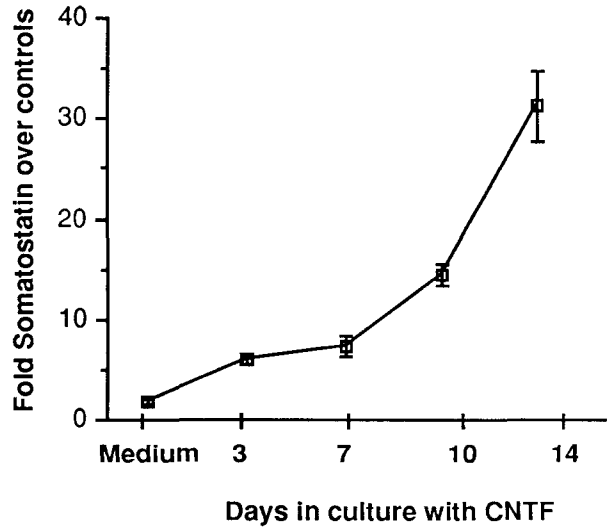

Fold Somatostatin over controls
40
30
20
10
0
Medium
3
7
10
14
Days in culture with CNTF

Figure 4.

Effect of 30 mM KCl on the ChAT-inducing effect of CDF/LIF and CNTF. Sympathetic neurons were grown with (a) L15CO_2, (b) CDF/LIF (5 ng/ml) or (c) CNTF with (+) or without (-) the addition of 30 mM KCl. Seven days after the addition of CDF/LIF and CNTF, samples were homogenized and assayed for ChAT activity by the method of Fonnum. All samples were run in duplicate (n=3 experiments). Results are expressed as fold induction, as compared to controls grown with L15CO_2 medium.± s.e.m.

Figure 4.

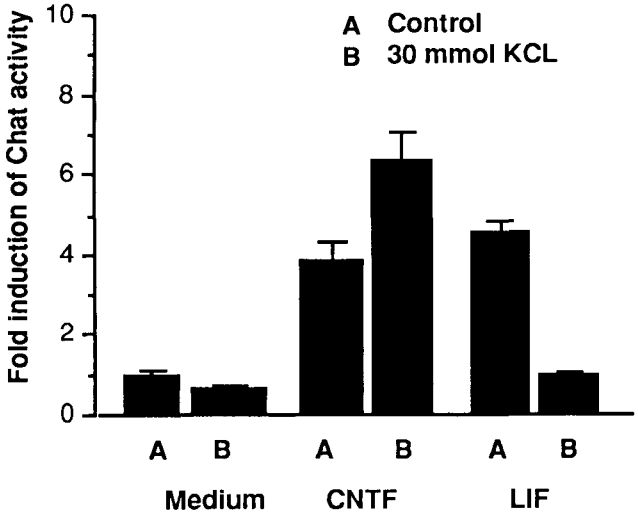

Fold induction of Chat activity
10
8
6
4
2
0
A Control
B 30 mmol KCL
A B A B A B
Medium CNTF LIF

Figure 5.

Effect of 30 mM KCl and CNTF on neuropeptide levels.

Sympathetic neurons were grown with (a)L15CO_2, (b) L15CO_2 and 30 mM KCl, (c) CNTF (d) CNTF and 30 mM KCl. Seven days after the addition of CDF/LIF, CNTF and/or 30 mM KCl samples were homogenized and assayed for peptide levels by radioimmunoassay. All samples were run in duplicate (n=3 experiments). Results are expressed as picograms/well $\pm$ s.e.m.

Figure 5.

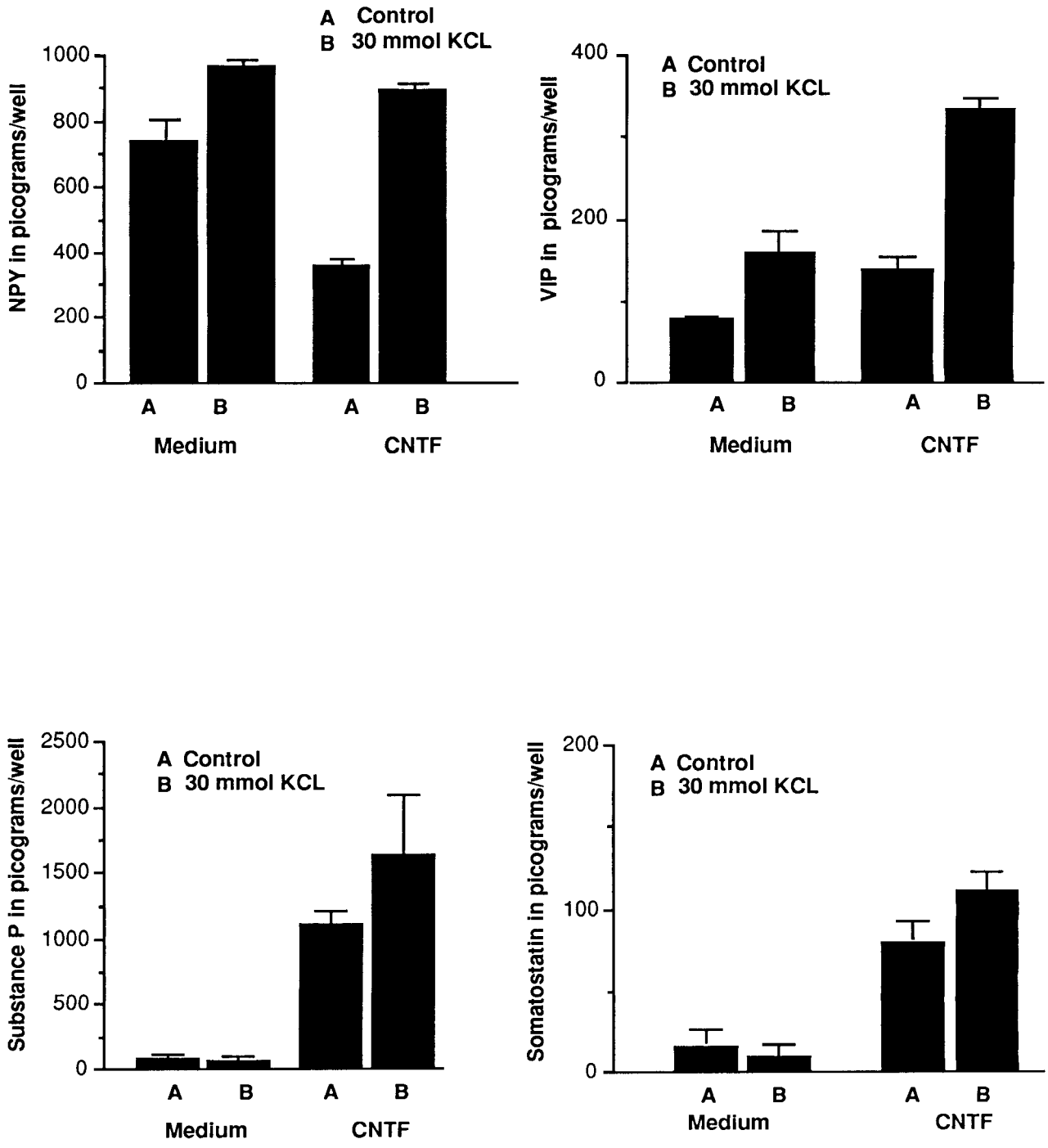
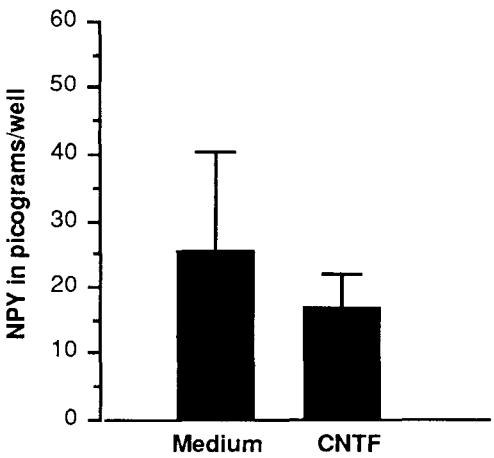

A Control
B 30 mmol KCL
NPY in picograms/well
1000
800
600
400
200
0
A B A B
Medium CNTF
A Control
B 30 mmol KCL
VIP in picograms/well
400
200
0
A B A B
Medium CNTF
A Control
B 30 mmol KCL
Substance P in picograms/well
2500
2000
1500
1000
500
0
A B A B
Medium CNTF
A Control
B 30 mmol KCL
Somatostatin in picograms/well
200
100
0
A B A B
Medium CNTF

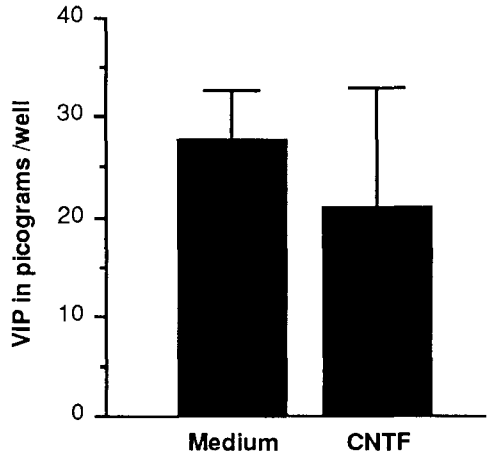
Figure 6.

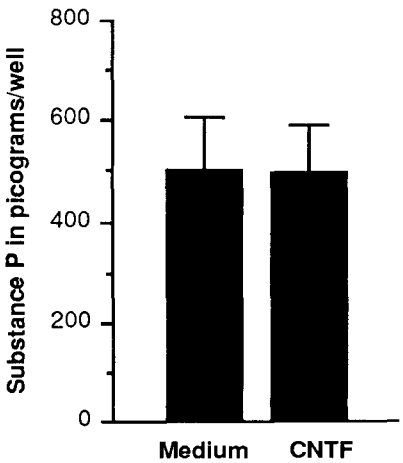
The effect of CNTF on neuropeptide levels in dorsal root ganglion cultures.

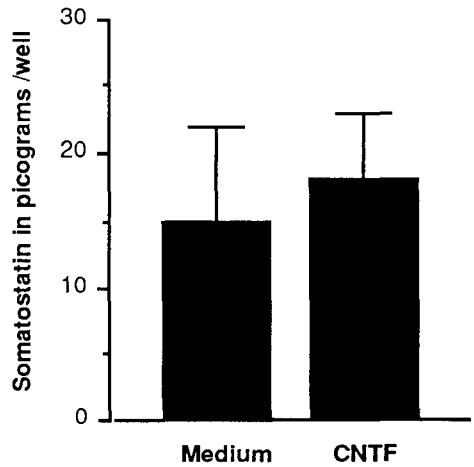
CNTF (25 ng/ml) was added to DRG cultures. Seven days after the addition of CNTF samples were homogenized and assayed for peptide levels by radioimmunoassay. The results are expressed as picograms of peptide/well $\pm$ s.e.m.

Figure 6


NPY in picograms/well
60
50
40
30
20
10
0
Medium
CNTF


VIP in picograms /well
40
30
20
10
0
Medium
CNTF


Substance P in picograms/well
800
600
400
200
0
Medium
CNTF


Somatostatin in picograms /well
30
20
10
0
Medium
CNTF

MATERIALS AND METHODS

Materials

Cell culture reagents were obtained from Gibco (Grand Island, NY) and culture plates from Corning (Corning, NY). The Centricon filters were purchased from Amicon (Danvers, MA). ^{3}H acetyl -CoA was purchased from New England Nuclear (Wilmington, DE). Dispase was obtained from Boeringer-Mannheim (Indianapolis, IN) and collagenase from Worthington Biochemicals (Freehold, NJ). VIP, Substance P and somatostatin RIA kits were obtained from Incstar (Stillwater, MN) and NPY RIA reagents from Amersham. Nerve Growth Factor (NGF) (the kind gift of Dr. K. Neet, Dept. of Biochemistry, CWRU) was prepared from male mouse submaxillary glands as described by Bocchini and Angeletti (1969). Pierce protein assay kit was obtained from Pierce (Rockford, Il). Other chemicals were purchased from Sigma (St. Louis, MO).

Tissue Extracts

To prepare sciatic nerve extracts, nerves were dissected from adult rats or frozen dissected sciatic nerves were obtained from (Pelfreez). Tissue from twenty animals was generally processed at one time. The tissue was homogenized for 5 seconds in 10 volumes of 10 mM phosphate buffer pH 7.0 with a Polytron. The extract was then centrifuged at 100,000g for one hour. The supernatant was collected, filtered through a 0.2 mm filter and concentrated using a Centricon filter with a 10 kD cutoff. The protein concentration was determined with a Pierce protein assay kit.

DEAE Chromatography

Aliquots of the soluble extract were diluted five-fold with 10 mM phosphate buffer, pH 7.0, and applied to a 0.9 cm/10 cm DEAE column (Whatman/Bioprobe) at a flow rate of 10ml /hr. The column was washed with an equal volume of phosphate buffer. The wash and the flowthrough were pooled and concentrated using a Centricon filter with a 10 kD cutoff. The bound protein was eluted with 10ml of 0.25M NaCl and concentrated in a similar manner. This extract, termed sciatic nerve extract (Manthorpe *et al.*, 1986), was used in all experiments.

Immunoprecipitation

For the immunoprecipitation experiments in which the biological activity of the factors was tested, aliquots of sciatic nerve extracts (DEAE fraction) sufficient for a cell culture assay were added to buffer (PBS pH 7.3 with 2% BSA, and 0.02% PEG 6000). A polyclonal antiserum generated against rCNTF (a kind gift of Dr. Donna Morissey, Regeneron Pharmaceuticals) was added to each vial to a final dilution of 1:4 (see Chapter 1 appendix). After an overnight incubation, the antigen-antibody complex was absorbed to 10 µl of protein A Sepharose for a further two hours at room temperature. The bound complexes were separated by centrifugation and the supernatant was diluted into L15 CO_2 medium and used for cell culture assays. To insure that the loss of activity consequent to absorption was due to a specific effect of the antibody, aliquots of the cholinergic factors were incubated without the antibody and treated as described above

Cell culture

Cultures of rat sympathetic neurons and dorsal root ganglion neurons were prepared as described by Hawrot and Patterson (1979). Neurons from the superior cervical ganglia or the dorsal root ganglia (thoracic and lumbar levels) of newborn rats were dissociated enzymatically with dispase (5 mg/ml) and collagenase (1 mg/ml) and plated in 96-well plates coated sequentially with polylysine (0.1 mg/ml) and laminin (10 µg/15 ml). About 2000 to 3000 neurons were plated per well except where indicated. The neurons were grown in Leibovitz's L15CO_2 medium with NGF (100 ng/ml), 100 units of penicillin, 100 mg of streptomycin, 10 mM cytosine arabinocide and 5% rat serum, and the medium changed every third or fourth day. The tissue extracts were diluted in growth medium, sterilized by passage through a 0.2 µm filter, and added to the neurons from the second day of culture on. Neurons were harvested for assay between the ninth and fourteenth days of culture.

Assays

Choline acetyl transferase activity

The induction of cholinergic function was determined by assaying choline acetyltransferase activity in homogenates, essentially according to the method of Fonnum (1969). To increase the sensitivity of the assay, an incubation period of an hour was used. All of the activity was inhibitable by 500 mM napthylvinyl pyridine,

a specific inhibitor of CHAT activity. Protein concentration was assayed by the method of Lowry using bovine serum albumin as a standard.

Peptide assays

Neuropeptide levels were determined by radioimmunoassay. Cultures were rinsed once with phosphate-buffered saline (PBS), and then homogenized in 100 ml of 2M acetic acid. After boiling for five minutes, samples were centrifuged for one minute in an Eppendorf microfuge. The supernatants were dried under vacuum and stored at -70^{o}C for subsequent assays. VIP, Substance P and somatostatin were assayed using general protocols as specified in the RIA kits. The primary antibodies have been previously shown to display minimal crossreactivity with other peptides (see Refs in RIA kit). Since the antibody to NPY shows only 64% cross reactivity with rat NPY, standards were run with rat NPY (Peninsula Laboratories) and sample values read of that standard curve.

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Chapter 3.

Characterization of a target-derived neuronal cholinergic differentiation factor.

Chapter 3

ABSTRACT

The sympathetic innervation of rat sweat glands undergoes a target-induced switch from a noradrenergic to cholinergic and peptidergic phenotype during development. Treatment of cultured sympathetic neurons with sweat gland extracts mimics many of the changes seen *in vivo*. Extracts induce choline acetyltransferase activity and vasoactive intestinal peptide expression in the neurons in a dose-dependent fashion, while reducing catecholaminergic properties and neuropeptide Y. The cholinergic differentiation activity appears in developing glands at postnatal day five and is maintained in adult glands. It is a heat-labile, trypsin-sensitive, acidic protein which does not bind to heparin agarose. The sweat gland activity is a likely candidate for mediating the target-directed change in sympathetic neurotransmitter function observed *in vivo*.

INTRODUCTION

Most sympathetic neurons are noradrenergic; however, a minority population, including those that innervate sweat glands, are cholinergic. The sympathetic neurons which innervate sweat glands are further distinguished in that they contain vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) immunoreactivity (Lundberg *et al*, 1979; Landis and Fredieu, 1986; Lindh *et al.*, 1989) while many noradrenergic neurons contain neuropeptide Y (NPY; Lundberg *et al.*, 1982; 1983; Jarvi *et al.*, 1986). Although the mature sweat gland innervation is functionally cholinergic, the developing innervation is noradrenergic (Landis and Keefe, 1983; Leblanc and Landis, 1986; Stevens and Landis, 1987; Landis *et al.*, 1988). When sympathetic axons first innervate the developing sweat glands, they possess intense catecholamine histofluorescence and immunoreactivity for the catecholamine synthetic enzymes, tyrosine hydroxylase and dopamine β hydroxylase. As the gland innervation matures, catecholamine histofluorescence disappears, tyrosine and dopamine β hydroxylase immunoreactivities decrease and cholinergic and peptidergic properties appear. For example, acetylcholinesterase is detectable at postnatal day 7, VIP immunoreactivity at day 10, choline acetyltransferase activity at day 11 and cholinergic transmission at day 14. Thus, the cholinergic sympathetic innervation of sweat glands undergoes a striking change in neurotransmitter properties during postnatal development.

Several lines of evidence indicate that the change from noradrenergic to cholinergic function in the developing sweat gland innervation is mediated by interactions with the target tissue. First, when the innervation of developing sweat glands is delayed by seven to ten days, there is a corresponding delay in the disappearance of catecholamine histofluorescence and the appearance of cholinergic properties (Stevens and Landis, 1988). Second, if the superior cervical ganglion, which contains noradrenergic sympathetic neurons, is transplanted to the anterior chamber of the eye with footpad tissue containing sweat glands, the neurons innervate the glands, reduce their expression of catecholamine histofluorescence and NPY and develop immunoreactivity for choline acetyltransferase and VIP (Stevens and Landis, 1990). Finally, cross-innervation experiments provide direct evidence for a target role. When footpad skin is transplanted in place of hairy skin in the thoracic region of early postnatal rats, the transplant is innervated by sympathetic neurons whose normal targets are piloerectors and blood vessels. The sympathetic

fibers which innervate hairy skin are noradrenergic and do not normally contain choline acetyltransferase activity, acetylcholinesterase staining or VIP immunoreactivity (Schotzinger and Landis, 1990a). Several weeks after innervating the transplanted sweat glands, however, the fibers show a marked reduction in catecholamine fluorescence and express properties characteristic of the innervation of their novel target; they exhibit choline acetyltransferase activity, acetylcholinesterase staining and VIP immunoreactivity (Schotzinger and Landis, 1988; and unpublished observations). Conversely, if parotid gland, a target of noradrenergic sympathetic neurons, is transplanted to the footpad in place of the sweat glands, it is innervated predominantly by fibers which normally innervate sweat glands and become cholinergic; in this case, the fibers innervating the transplanted parotid fail to acquire cholinergic properties and continue to express catecholaminergic properties typical of the sympathetic innervation of the parotid glands (Schotzinger and Landis, 1990b). Thus, the normal expression of cholinergic properties in the sweat gland innervation depends on the presence of this particular target and sweat glands are able to induce cholinergic and certain peptidergic properties in sympathetic neurons which would not normally express them. Since sympathetic axons never contact sweat gland cells or the basal lamina that surrounds them directly (Landis and Keefe, 1983; Uno and Montagna, 1975; Quick *et al.*, 1984), it seems likely that the target effect is mediated by a soluble factor.

To identify the target factor responsible for the adrenergic to cholinergic switch observed in the developing sweat gland innervation and to explore the possible *in vivo* role of the candidate cholinergic factors identified in cell culture in this decision, we assayed the effects of sweat gland extracts on the transmitter properties of cultured sympathetic neurons. We found that sweat glands contain a soluble factor(s) with the appropriate spectrum of activities; it reduces the expression of catecholamines and tyrosine hydroxylase and induces the expression of choline acetyltransferase and VIP. This activity is present when the phenotype of the sweat gland innervation is changing. Our initial characterization of the sweat gland-derived choline acetyltransferase-inducing activity permits a comparison with the cholinergic factors previously identified in cell culture.

RESULTS

Sweat gland extracts induce choline acetyltransferase activity in sympathetic neurons

To examine the effects of soluble factors present in sweat glands on neurotransmitter status, extracts from the footpads of adult rats were added to sympathetic neuron cultures at a concentration of 250μg extract protein/ml. Neurons in sister wells were grown either in medium without added tissue extract or in medium containing an equal protein concentration of liver, hairy skin or parotid gland extracts prepared in the same manner as the sweat gland extracts. The addition of sweat gland extract caused a 15-fold induction in choline acetyltransferase activity compared to neurons grown in medium alone or with extracts of liver, hairy skin or parotid gland (Fig. 1a).

One possible source of the cholinergic-inducing activity in the sweat gland extracts is CNTF possibly present in the peripheral nerve plexus of the footpad tissue. Comparison of the cholinergic-inducing activity in sciatic nerve extracts and sweat gland extracts (Fig. 1b), however, indicated that the level of induction per mg of extract protein was similar despite the fact that the peripheral nerve plexus constitutes only a small proportion of the gland tissue. This observation, and the finding that extracts of hairy skin did not cause choline acetyltransferase induction in cultured sympathetic neurons even though the hairy skin contains a plexus of sympathetic and sensory nerve fibers and endings comparable to that in sweat gland-containing skin, make it unlikely that the ability of sweat gland extracts to induce cholinergic function is due to CNTF potentially derived from Schwann cells (Stockli *et al.*, 1989). Further evidence to support this conclusion was obtained in immunoblot experiments using antisera generated against recombinant CNTF (see below).

The induction of choline acetyltransferase activity by the sweat gland extract was due to a direct effect on sympathetic neurons. Since the neurons were grown in the continuous presence of 10μM cytosine arabinoside, nonneuronal cells were virtually absent; thus, it is unlikely that the sweat gland extracts exerted their influence indirectly *via* nonneuronal cells. In addition, when sympathetic neuron cultures were maintained in serum-free medium which yielded cultures free of nonneuronal cells, cholinergic induction was seen following treatment with sweat gland extracts (Table 1). This observation also makes it very unlikely that the sweat

gland extract potentiated the cholinergic-inducing effects of the rat serum present in normal growth medium (Wolinsky *et al.*, 1985; Wolinsky and Patterson, 1985).

Sweat gland extracts were tested for their ability to support the survival of cultured sympathetic neurons. Table 2 shows that neurons plated in medium lacking NGF but containing sweat gland extracts at a dose of 1mg/ml did not survive more than three days in culture. Further, there was no significant difference in neuron number in cultures grown with or without sweat gland extract, even at extract doses as high as 1mg/ml in the presence of 50ng/ml of NGF. Since the levels of choline acetyltransferase activity and acetylcholine synthesis are initally very low in dissociated sympathetic neuron cultures (Johnson *et al.*, 1976; 1980; Patterson and Chun, 1977b), and there was no significant change in cell number with a 50- to 100-fold induction of choline acetyltransferase activity, it is extremely unlikely that the induction of choline acetyltransferase observed in the presence of sweat gland extract is due to the selective survival of pre-existing cholinergic neurons.

Choline acetyltransferase induction is dose-dependent

Serial dilutions of sweat gland extracts were added to cultured sympathetic neurons and the wells were assayed for choline acetyltransferase activity seven days later. Induction was seen with doses as low as 10μg/ml and increased with the addition of increasing amounts of extract to the maximum dose tested (Fig. 2a). When extract concentrations much higher than 1mg/ml were used, some toxicity was evident in the cultures; neuron number was reduced and cell bodies were smaller in size. Similiar effects of high doses of other cholinergic-inducing factors have been described (Fukada, 1985; Saadat *et al.*, 1989).

The time course of induction was also determined. Elevated choline acetyltransferase activity was detected as early as the second day in culture and continued to increase through day 14, the last time point assayed (Fig 2b). This time course of cholinergic induction in sympathetic neuron cultures is similar to that reported for heart and muscle cell conditioned medium factors, presumably CDF/LIF (Patterson and Chun, 1977b; Raynaud *et al.*, 1987), and for CNTF (Saadat *et al.*, 1989). In contrast, increased choline acetyltransferase activity is seen significantly sooner following treatment of sympathetic neurons with MANS (Adler *et al.*, 1989) or treatment of spinal cord cultures with a soluble factor isolated from skeletal muscle (MacManaman *et al.*, 1988).

Sweat gland extracts cause a reduction in the expression of noradrenergic properties

Not only does choline acetyltransferase activity appear during the normal development of the sweat gland innervation, but there is also a concomitant reduction in tyrosine hydroxylase immunoreactivity and catecholamine histofluorescence (Landis and Keefe, 1983; Landis *et al.*, 1988). If the sweat gland extracts contained a factor(s) which played a role in altering neurotransmitter phenotype, one would predict that it would decrease the expression of noradrenergic properties in cultured sympathetic neurons. To assay the effect of sweat gland extracts on tyrosine hydroxylase levels, equal protein aliquots of neurons grown with and without extract were subjected to SDS PAGE electrophoresis, blotted onto nitrocellulose and probed with a monoclonal antibody to tyrosine hydroxylase (Rohrer *et al.*, 1986; the kind gift of Dr A. Acheson, University of Edmonton). A single band was evident at 62-kD, the expected molecular weight (Lamoroux *et al.*, 1979). Visual inspection of the immunoblots suggested that the amount of immunoreactivity was significantly reduced in cultures grown with sweat gland extracts (Fig. 3). When the color intensity was read with a laser densitometer, cultures grown with 100µg/ml sweat gland extract exhibited a 2.5-fold reduction in the level of tyrosine hydroxylase detected. In contrast, levels of immunoreactivity for a cell surface adhesion molecule, L1 (Rathjen and Schachner, 1984), revealed with a polyclonal antiserum (the kind gift of Dr. U. Rutishauser, CWRU) were not reduced when assayed in a similiar manner (data not shown).

To determine whether the change in the level of tyrosine hydroxylase was associated with a corresponding change in the level of catecholamines, the catecholamine content was determined in cultures of sympathetic neurons grown with and without sweat gland extract. The total catecholamine content of wells incubated with sweat gland extracts was reduced compared to that of control cultures (Table 3). An inverse correlation was observed between choline acetyltransferase activity and catecholamine content; as the induction of choline acetyltransferase increased, the catecholamine content decreased. This relationship has been observed previously in studies with heart and skeletal muscle cell conditioned medium (Patterson and Chun, 1977a; Raynaud *et al.*, 1987).

Sweat gland extracts alter neuropeptide expression

Changes in neuropeptide expression are observed in the developing sweat gland innervation. VIP immunoreactivity is initially absent but becomes detectable

by postnatal day 10; the immunoreactivity increases in extent and intensity with subsequent development. Since the sympathetic innervation of footpads transplanted to the thorax acquires VIP immunoreactivity, sweat glands are able to induce VIP expression in addition to choline acetyltransferase activity (Schotzinger and Landis, 1988; unpublished observations). We therefore assayed cultures of sympathetic neurons treated with sweat gland extract by radioimmunoassay to determine whether extracts increased VIP levels. Sympathetic neurons grown in control medium contain relatively little VIP immunoreactivity. Sweat gland extracts significantly increase VIP (Fig. 4a); a dose of 100μg/ml causes an induction of 80 pg/well of VIP, a more than four-fold increase over the levels present in control cultures. The induction of VIP expression increased with increasing concentrations of sweat gland extracts (Fig. 4a).

The effect of sweat gland extract on NPY content was examined because previous studies have shown that while many noradrenergic sympathetic neurons contain NPY immunoreactivity, cholinergic sympathetic neurons, including those that innervate sweat glands, do not (Landis *et al.*, 1988; Lindh *et al.*, 1989). The content of NPY-like immunoreactivity was high in control cultures as observed in previous studies (Marek and Mains; 1989; Nawa and Sah, 1990). Growth in the presence of sweat gland extract led to a reduction in NPY content (Fig. 4b). This reduction is in marked contrast to the elevation of VIP content and indicated that sweat gland extracts regulate the levels of the two peptides differentially. The decreased expression of NPY in sympathetic neurons grown with sweat gland extract is consistent with results of a previous study of target effect on peptide expression *in vivo*; following transplantation of the superior cervical ganglion from newborn rats to the anterior chamber of the eye, NPY-IR was absent when the ganglion was cotransplanted with sweat glands, but present when the ganglion was cotransplanted with the pineal gland (Stevens and Landis, 1990).

Age dependence of choline acetyltransferase-inducing activity in extracts of sweat glands

The change in neurotransmitter properties in the developing sweat gland innervation occurs postnatally and is essentially complete by postnatal day 21. To determine the earliest age at which detectable cholinergic inducing activity was present in developing glands, extracts were prepared from footpads of animals ranging in age from two to 21 days and were assayed for their ability to induce

choline acetyltransferase activity (Fig. 5). Increased levels of choline acetyltransferase were detected in cultures treated with extracts from postnatal day 5 glands and choline acetyltransferase inducing activity was present at all subsequent ages. Less than a two-fold difference was evident in the specific cholinergic inducing activity present in footpads of postnatal day 5 and adult animals when the inducing activity was expressed as the amount of choline acetyltransferase activity detected per mg of extract protein. The amount of protein extracted from twenty footpads, however, varied almost fifteen-fold from the youngest to the oldest animals. Thus, the absolute amount of choline acetyltransferase-inducing activity increased approximately thirty-fold during development. These results indicate that cholinergic differentiation activity is present in developing glands when the properties of the innervation change. In addition, extracts from sweat glands of postnatal day nine, 14 and 21 rats were found to increase the expression of VIP and decrease levels of tyrosine hydroxylase as well as increasing choline acetyltransferase (data not shown).

Initial characterization of the factor(s) responsible for choline acetyltransferase induction

Preliminary characterization of the choline acetyltransferase-inducing activity is summarized in Table 4. It was heat and trypsin labile, and retained by a Centricon filter with a 10 kD cutoff indicating that the activity is a protein. The activity was only partially retained by a Centricon filter with a 30 kD cutoff suggesting that a low molecular weight protein is responsible for the induction of choline acetyltransferase. The cholinergic-inducing activity was relatively stable; little activity was lost with storage at -20^{o}C and on repeated freeze-thawing. Since none of the activity bound to a heparin agarose column, the sweat gland cholinergic factor does not appear to be a heparin-binding protein like the 50 kD soluble cholinergic factor from brain (Kessler *et al.*, 1986). Almost all choline acetyltransferase-inducing activity and 35% of the protein was recovered in the 0.25M eluate from a DEAE column indicating that the differentiation activity is an acidic protein(s) and that this can be used as an initial purification step. The 0.25M DEAE eluate not only induced choline acetyltransferase activity but also increased levels of VIP and reduced levels of tyrosine hydroxylase (data not shown). Thus, the several effects of the sweat gland extract on neurotransmitter properties of cultured sympathetic neurons are not readily separated.

DISCUSSION

Consideration of the developmental history of the sweat gland innervation (Landis and Keefe, 1983; Leblanc and Landis, 1986; Landis *et al.*, 1988) and of the results of cross-innervation experiments (Schotzinger and Landis, 1988; 1990b) enabled us to predict some properties of a target-derived signal expected to play a role in the noradrenergic to cholinergic and peptidergic switch that these neurons undergo. In order to identify the molecule(s) responsible, we prepared low salt extracts of sweat gland tissue and tested the ability of these extracts to modify the neurotransmitter phenotype of cultured sympathetic neurons. Extracts of sweat glands but not of liver, hairy skin or parotid gland increase levels of choline acetyltransferase activity and of VIP-like immunoreactivity in a dose-dependent fashion. As the levels of choline acetyltransferase activity increase in the cultured neurons, there is a concomitant decrease in the catecholamine content and tyrosine hydroxylase. Thus, extracts of soluble protein from sweat glands cause many of the phenotypic changes in cultured sympathetic neurons that characterize the developing sweat gland innervation *in vivo* and that are induced by the glands in cross-innervation experiments.

The ability to alter neurotransmitter properties is present in sweat gland extracts of animals between 5 and 21 days postnatal when the fibers innervating the sweat glands are changing from noradrenergic to cholinergic (Landis and Keefe, 1983; Leblanc and Landis, 1986; Landis *et al.*, 1988). Extracts from postnatal day 5 animals increase choline acetyltransferase activity and when extracts of glands from animals between 9 and 21 days of age are tested, they alter all three transmitter properties examined; they increase choline acetyltransferase and VIP-like immunoreactivity and reduce tyrosine hydroxylase. Further, since elevated levels of choline acetyltransferase activity are detectable after two days of treatment in culture, the extract is able to induce changes with a time course consistent with *in vivo* studies. Establishing a more precise temporal correlation is difficult since the changes in the terminal plexus in the sweat glands *in situ* presumably reflect retrograde transport of a target-derived signal, altered expression of transmitter synthetic enzymes and peptides, and anterograde transport of these molecules to the terminals.

Sweat glands of adult, as well as developing animals, contain activity. The functional significance, however, of the continued expression is unclear. Studies of cholinergic induction in cultured sympathetic neurons by heart and skeletal muscle

cell conditioned medium have shown that neurons once induced to acquire cholinergic function maintain it for a period, even if the inducing factor is removed from the culture medium (Patterson and Chun, 1977b; Vidal *et al.*, 1987). In contrast, the suppression of noradrenergic properties is reversible shortly after exposure to conditioned medium, although with continued exposure, catecholaminergic suppression is maintained for a period as well. Whether or not cholinergic sympathetic neurons *in vivo* require continued exposure to their target tissue for maintenance of the cholinergic phenotype is not known. Since interactions with sweat glands also influence neuropeptide expression, it is possible that while cholinergic function once induced is stable, peptidergic properties remain modifiable. According to this scenario, the continued expression of a particular peptide phenotype would require the continued presence of an inducing factor. This is, indeed, true for the peptidergic induction observed in cultured sympathetic neurons with CDF/LIF (Nawa *et al.*, 1990); for example, withdrawal of CDF/LIF results in the return of Substance P content to control levels. The finding in cross-innervation experiments involving adult sensory nerves that peptide phenotype can be altered is also consistent with this possibility (McMahon and Gibson, 1987). When a muscle nerve is cross-anastomosed to a cutaneous nerve and induced to innervate targets in the skin, the regenerated muscle nerves appear to acquire immunoreactivity for Substance P and, conversely, when the cutaneous nerve was cross-anastomosed to a muscle nerve, Substance P immunoreactivity is decreased in the cutaneous nerve. Although neuropeptide expression by sympathetic neurons can be qualitatively influenced by target interactions during development *in vivo* (Stevens and Landis, 1990; Schotzinger and Landis, 1990b), it remains to be determined whether similar plasticity is exhibited by mature sympathetic neurons *in vivo*.

Although comparison of the levels of biological activity observed *in vitro* with those present *in vivo* is difficult, it is of interest to estimate whether the glands contain enough cholinergic-inducing activity to mediate the switch. Retrograde tracing studies suggest that at least 200 neurons innervate the six palmar pads (Siegel and Landis, unpublished observations). Our extraction procedure yields about 10mg of soluble protein per gram of footpad tissue from 21-day old animals, or approximately 80µg/single pad. Since choline acetyltransferase activity is induced at concentrations as low as 10µg/ml in cultures containing several thousand neurons, it appears that they do. The concentration of cholinergic-inducing activity present in sweat gland extracts is greater than that in spinal cord extracts (Wong

and Kessler, 1987; Adler *et al.*, 1989) and at least as high as that in sciatic nerve extracts (Sendtner *et al.*, 1989; Rao *et al.*, 1990).

The extracts tested in these studies are relatively crude. It will be important to determine whether the activity present in sweat glands that increases choline acetyltransferase activity and VIP-like immunoreactivity and decreases tyrosine hydroxylase and catecholamine levels is represented by a single molecule or several molecules, each of which influences one aspect of the transmitter phenotype. Since CDF/LIF (Fukada, 1985; Yamamori *et al.*, 1989; Nawa and Patterson, 1990; Nawa *et al.*, 1990) and CNTF (Sendtner *et al.*, 1989; Ernsberger *et al.*, 1989) cause the induction of cholinergic function, the reduction of catecholaminergic function and increase VIP expression in sympathetic neuron cultures, it is clear that a single molecule can effect changes in all these properties. Two observations from the present studies are consistent with the notion that one molecule in the extracts is responsible. Extracts prepared from animals of different ages influence the several properties assayed and, more importantly, the several effects were not resolved into distinct activities in the preliminary characterization that we have performed. Thus, a single molecule seems likely; however, the possibility that several factors are involved cannot be formally excluded. The cholinergic-inducing activity, whose properties we have examined, was obtained by extracting soluble proteins from gland tissue.

It is of interest to compare the properties of the activity in sweat gland extract with the several factors which been previously described to induce cholinergic function in cultured sympathetic neurons. Since the cholinergic-inducing activity present in the sweat gland extracts is easily extracted in low salt solutions and no detectable activity is associated with membranes (unpublished observations), it is not likely to be related to the membrane-associated factors that induce choline acetyltransferase (Wong and Kessler, 1987; Adler *et al.*, 1989) and reduce levels of tyrosine hydroxylase (Rao *et al.*, 1990, Lee *et al.*, 1990) in cultured sympathetic neurons. In addition, there is a difference in the time course of induction of choline acetyltransferase activity; cultures treated with sweat gland extracts exhibit a small increase in activity after two days, while cultures treated with a membrane-associated cholinergic-inducing activity show high levels of activity in the same time period (Adler *et al.*, 1989). Two soluble factors, CDF/LIF and CNTF, are similar in their effects on sympathetic neurons; they increase choline acetyltransferase and VIP expression and reduce tyrosine hydroxylase and

catecholamine content (Fukada, 1985; Yamamori *et al.*, 1989; Sendtner *et al.*, 1989; Ernsberger *et al.*, 1989; Nawa and Patterson, 1990). In addition, like sweat gland extract, both CDF/LIF (Nawa and Patterson, 1990) and extracts of sciatic nerve containing CNTF (unpublished observations) decrease NPY expression. Further purification will however be required to determine the relationship of the sweat gland cholinergic-inducing activity to CDF/LIF and CNTF.

In summary, we have shown that a cholinergic-sympathetic target tissue, sweat glands, contains cholinergic-differentiating activity which mimics in culture the effects of the target on sympathetic neurons *in vivo*. The activity was obtained by extracting soluble proteins from gland tissue. Secreted proteins will be represented in such an extract but so will cytoplasmic. As the purification and characterization of this activity proceeds, it will be important to establish that it is normally secreted by the target tissue and to examine its role in mediating the target-induced changes in transmitter phenotype observed *in vivo*. Our preliminary purification and analysis indicate that the cholinergic-inducing activity present in the sweat gland extracts represents an excellent candidate for mediating the target-induced phenotypic changes in the the cholinergic sympathetic neurons that innervate sweat glands. Subsequent efforts will be directed at further purifying this activity and comparing its physiological and biochemical properties with those of CNTF and CDF/LIF.

Table 1.

	Choline Acetyltransferase Activity	
	Medium	**S.G. extract**
Defined medium	**8.02 ± 1.64**	**109.93 ± 5.57**
L15CO2 + serum	**18.05 ± 5.57**	**126.06 ± 5.58**

The cholinergic-inducing effect of sweat gland extracts is independent of serum.

Sympathetic neurons were cultured in L15-CO_2, either lacking serum or containing 5% rat serum with 300μg/ml of extract protein. Cells were harvested seven days after the addition of extract and aliquots were tested for choline acetyltransferase activity by the method of Fonnum. Samples were run in triplicate. Data are expressed as picomoles of activity/min/well ± sem.

Table 2

	Cell Number			
	- NGF	- NGF + ext	+ NGF	+NGF + ext
DAY 2	5027 ± 35	4734 ± 164	5027 ± 231	5324 ± 186
DAY 5	10 ± 12	12 ± 13	4624 ± 112	4867 ± 28

Lack of trophic effect of sweat gland extracts.

Sympathetic neurons were cultured in L15CO_2 with NGF (50ng/ml) for two days. On the second day, the medium was replaced with medium containing a) no NGF, b) no NGF but with 1mg/ml sweat gland extract, c) NGF (50ng/ml) or d) NGF (50ng/ml) and 1mg/ml sweat gland extract. Cells were counted after an additional three days of culture. Samples were run in triplicate. Data are expressed as the number of cells surviving/well ± sem.

Table 3.

		CA in picomoles/well	% Reduction
Control medium		**14.85 ± 2.55**	**1**
Sweat gland Extract	**A (11)**	**10.65 ± 0.9**	**28.3**
	B (26)	**7.95 ± 0.15**	**46.5**
	C (47)	**6.0 ± 0.6**	**60**

Sweat gland extracts cause a reduction in the detectable levels of catecholamines.

Sympathetic neurons were grown with sweat gland extracts (100µg/ml, 250µg/ml and 1mg/ml). Seven days after the addition of extract, the cultures were harvested and assayed for catecholamine content by HPLC . Samples were run in triplicate. Data are expressed as mean picomoles of catecholamines per dish ± sem. The figures in brackets are the mean fold induction of choline acetyltransferase assayed in sister wells by the method of Fonum.

Table 4.

	% activity retained
Thermal stability	
-20/-70 storage	**95**
Freeze thawing	**90**
Boiling (100 C°for 5 min)	**0**
Protease treatment	
Trypsin	**0**
Trypsin+ inhibitor	**97**
Heparin agarose chromatography	
Flow through	**95**
Eluate	**0**
Centricon Retention	
10kDa cutoff	**95**
30kDa cutoff	**50**
DEAE chromatography	
Flow through	**2**
0.25M eluate	**90**

Physiochemical characterization.

Aliquots of sweat gland extracts (100µg/ml) were incubated as described below. To examine the effects of protease treatment, aliquots were incubated for 1 hr with trypsin (1mg/ml) or with trypsin and trypsin inhibitor (3mg/ml). For Centricon separation samples were spun in a SS34 rotor until the retentate volume was 25µl. The retentate was diluted to 1ml and spun again. After three such spins the flow through was collected and concentrated. Choline acetyl transferase activity was determined in cultures seven days after the addition of treated extracts. 100% represents activity evident in cultures exposed to untreated extract. 0% represents activity in cultures grown without the addition of extract.

Figure 1.

Sweat gland extracts induce choline acetyltransferase activity.

Soluble protein extracted from sweat glands, hairy skin, parotid gland, liver or sciatic nerve of adult rats was added to cultures of dissociated sympathetic neurons. Seven days after the addition of extracts, neurons were homogenized and aliquots were assayed for levels of choline acetytransferase (ChAT) activity by the method of Fonnum. Samples were run in triplicate. In a, 250μg of protein extracted from the indicated tissues was added. The data are expressed as the fold induction of ChAT activity compared to that present in control cultures grown without added extract. In b, 250μg of protein extracted from sciatic nerve or sweat gland was added. The data are expressed as the fold induction of specific activity/mg of extract protein added.

Fig 1a

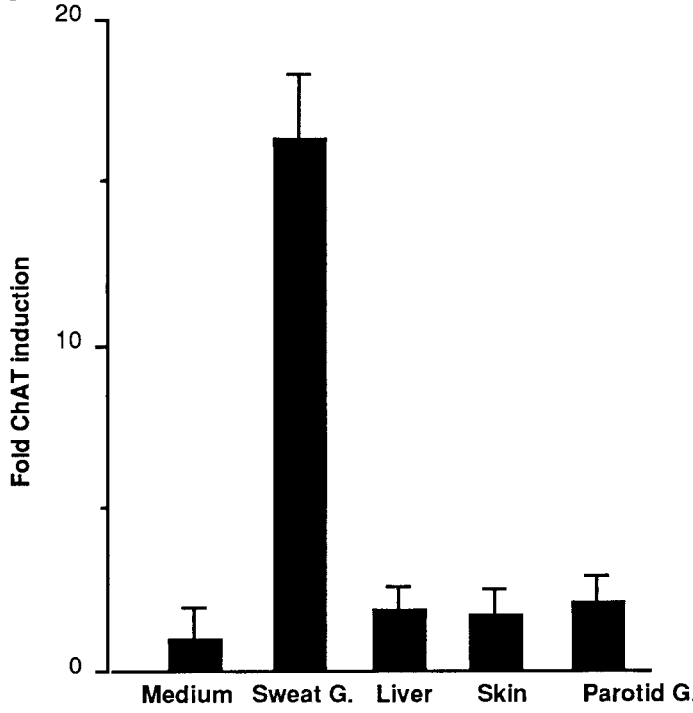

20
10
0
Fold ChAT induction
Medium
Sweat G.
Liver
Skin
Parotid G.

Fig 1b

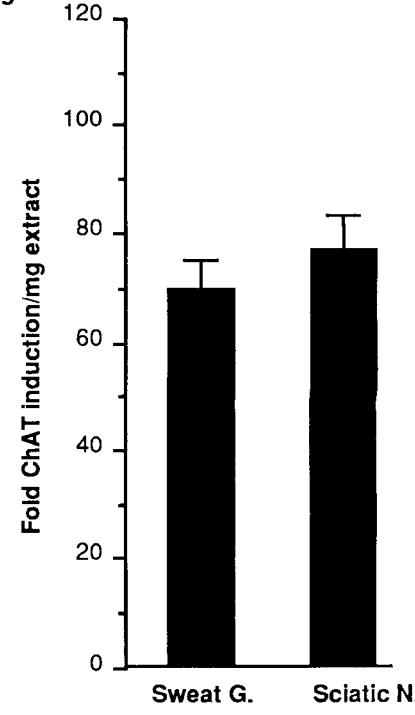

120
100
80
60
40
20
0
Fold ChAT induction/mg extract
Sweat G.
Sciatic N.

Figure 2a.

Increasing concentrations of sweat gland extracts cause increased induction of choline acetyltransferase activity.

Increasing concentrations of soluble protein extracted from sweat glands of adult rats were added to sympathetic neuron cultures. Seven days after the addition of extract, neurons were homogenized and aliquots were assayed for choline acetyltransferase activity by the method of Fonnum. Samples were run in triplicate. Data are expressed as picomoles of activity/minute/well $\pm$ s.d.

Figure 2b.

Time course of induction of choline acetyltransferase activity.

100μg/ml of soluble protein extracted from adult sweat glands was added to sympathetic neuron cultures. Duplicate samples were homogenized at appropriate intervals after the addition of extract and assayed for choline acetyltransferase activity. Data are expressed as picomoles of activity/min /well $\pm$ s.d.

Figure 2a

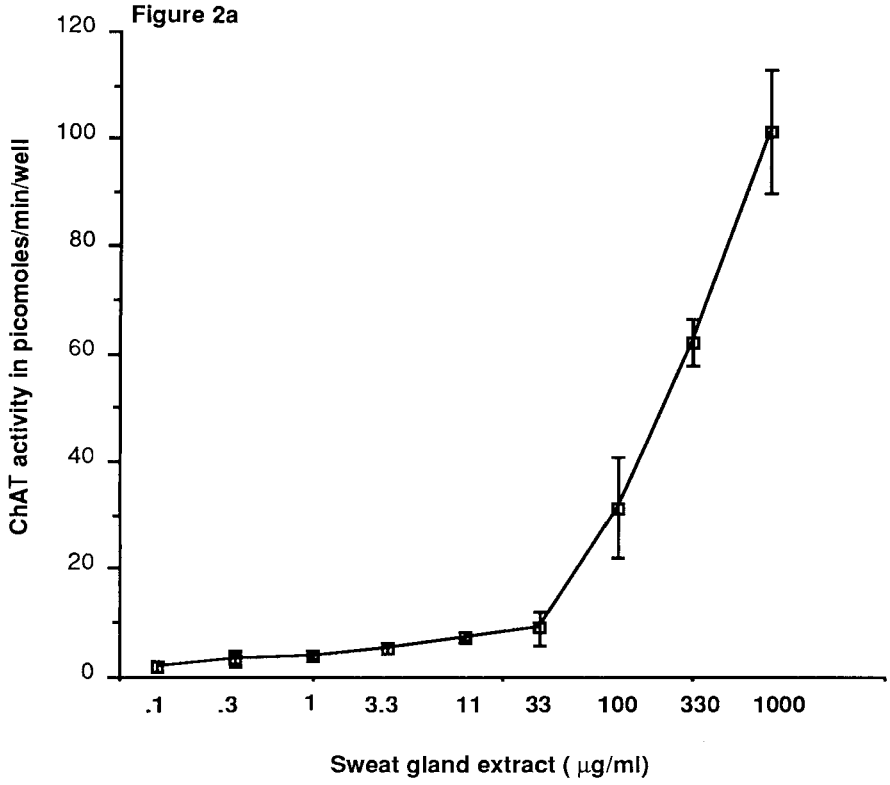


Figure 2b

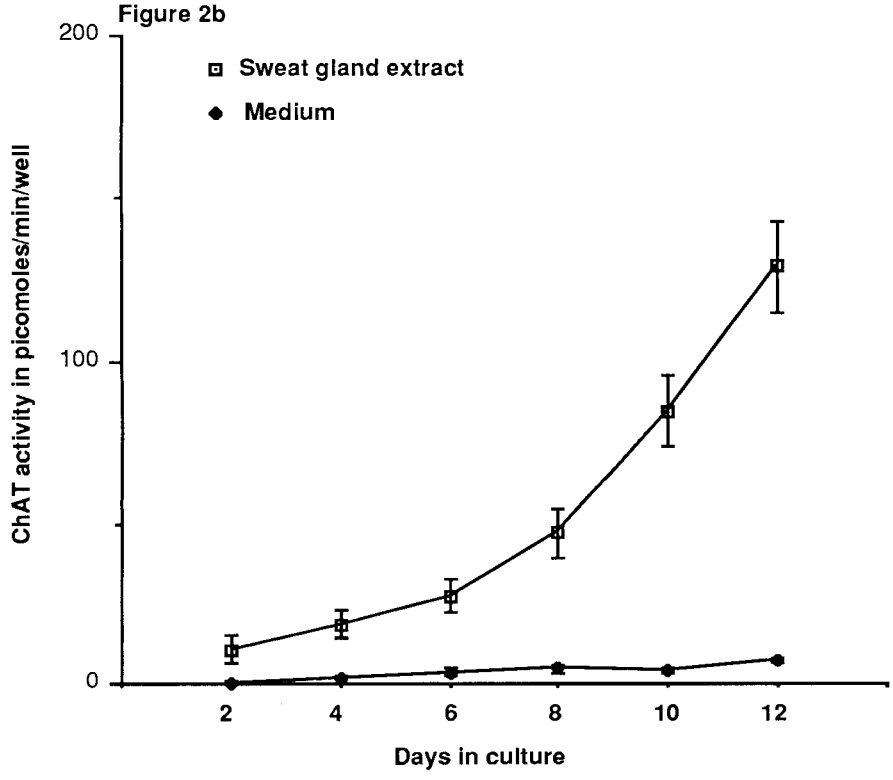


Figure 3.

Sweat gland extracts reduce tyrosine hydroxylase.

Sympathetic neurons were grown in medium without sweat gland extract (a) or with 100μg/ml of sweat gland extract (b). Samples were pooled from several wells and homogenized in sample buffer, electrophoresed and blotted onto nitrocellulose. The blots were probed with a monoclonal antibody to tyrosine hydroxylase (inset). The laser densitometer scan (absorbance of 600 nm) of the staining intensity of the bands from control and treated cultures is shown.

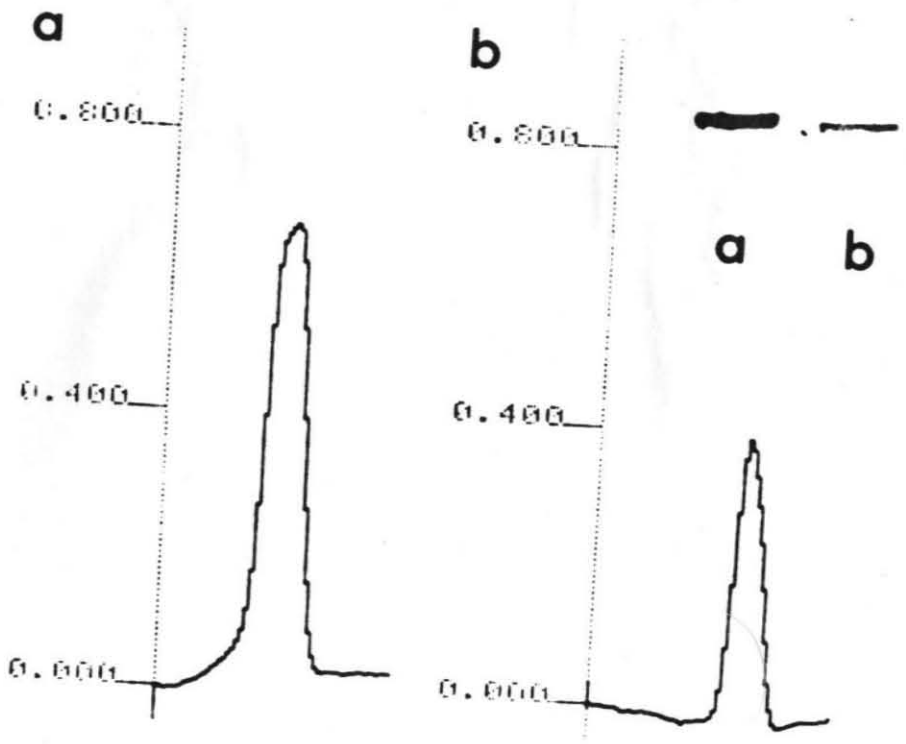

a
0.800
0.400
0.000
b
0.800
a
b
0.400
0.000

Figure 4a.

Sweat gland extracts modulate the expression of VIP.

Serial dilutions of the soluble protein extracted from adult rat sweat glands were added to sympathetic neuron cultures. Cultures were harvested on the eighth day after the addition of extract. Sister wells were assayed for either vasoactive intestinal peptide levels using radioimmunoassay or for choline acetyltransferase activity. All samples were run in duplicate. The data are expressed as picograms of VIP ± s.d./well or as picomoles of choline acetyltransferase activity ±s.d./well.

Figure 4b.

Sweat gland extracts reduce the levels of NPY and elevate the levels of VIP.

Sweat gland extracts (100μg/ml) were added to sympathetic neuron cultures. Cultures were harvested on the eighth day after the addition of extract. Sister wells were assayed for VIP or for NPY by radioimmunoassay. All samples were run in triplicate. Data are expressed as picograms of VIP or NPY ± s.d./well.

Figure 4a

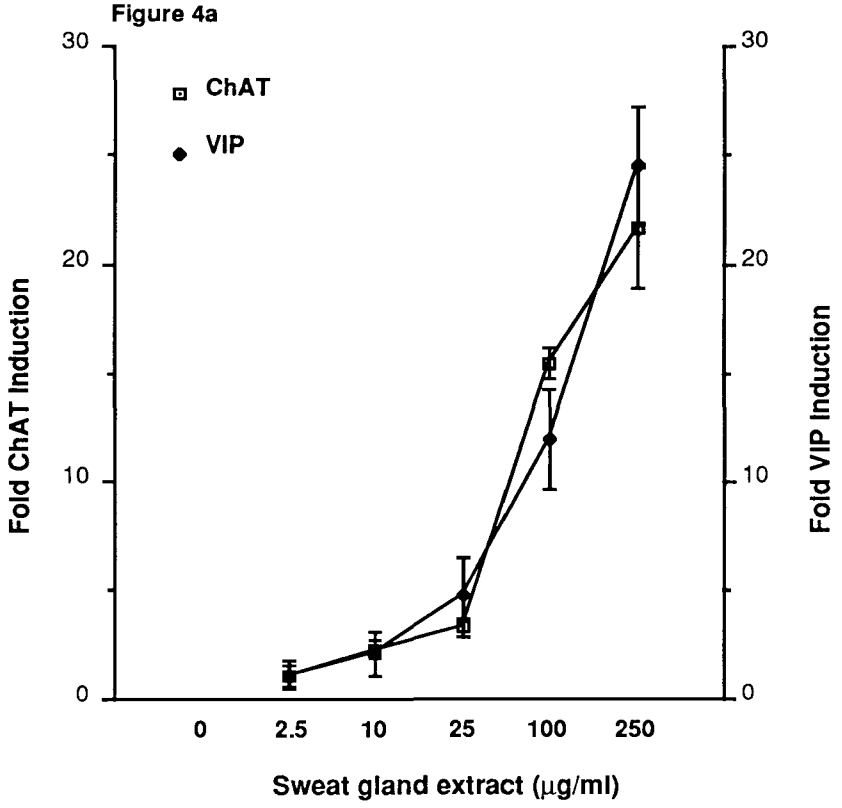


Figure 4b

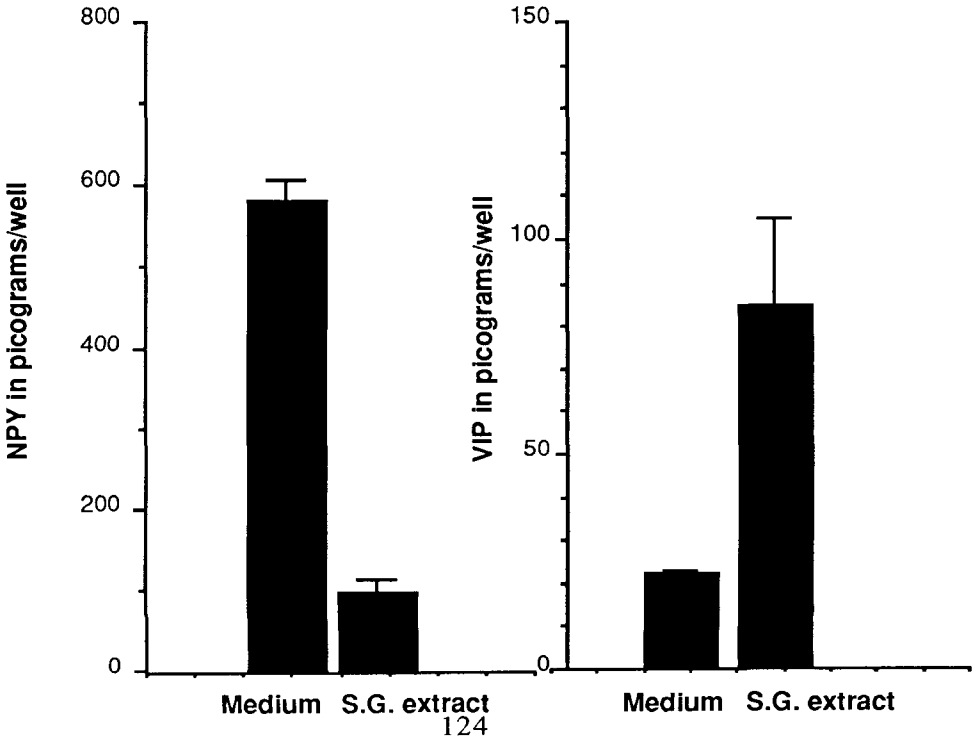


Figure 5.

Appearance of cholinergic differentiation activity in sweat gland extracts.

Sweat gland extracts were prepared from animals at the indicated ages. Approximately equal protein concentrations (100 μg/ml) were added to sympathetic neuron cultures. Seven days after the addition of extract, neurons were harvested and aliquots assayed for choline acetyltransferase activity. At least three different preparations at each age were tested. Data are expressed as fold induction of choline acetyltransferase/mg extract protein ± s.d.

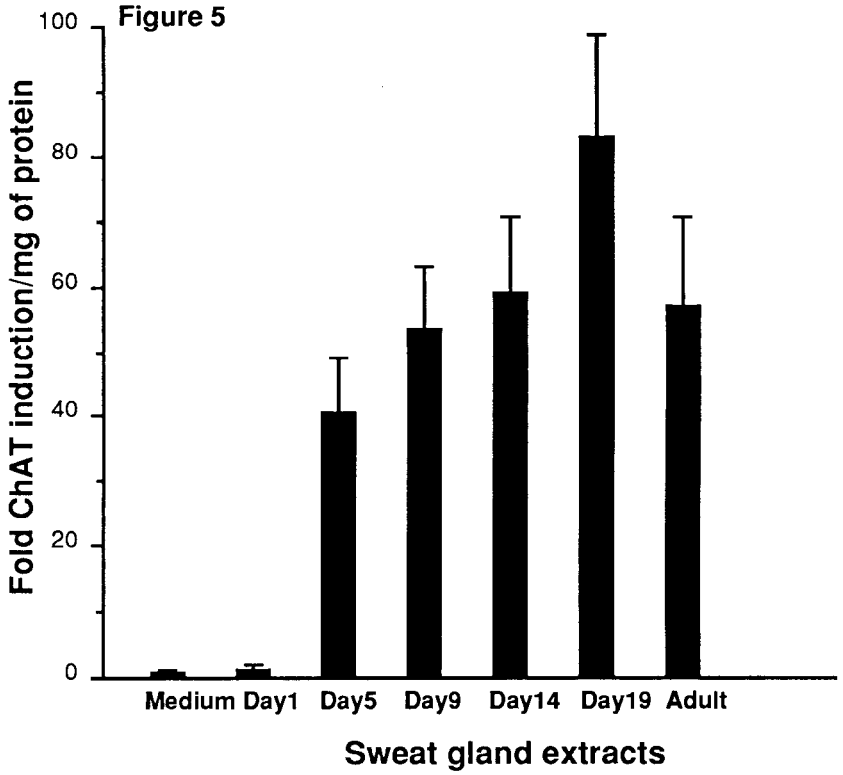


Figure 5
Fold ChAT induction/mg of protein
100
80
60
40
20
0
Medium
Day1
Day5
Day9
Day14
Day19
Adult
Sweat gland extracts

MATERIALS AND METHODS

Materials

Cell culture reagents were obtained from Gibco (Grand Island, NY) and culture plates from Corning (Corning, NY). The Centricon filters were purchased from Amicon (Danvers, MA). ^3H acetyl-CoA and Bolton Hunter reagent were purchased from New England Nuclear (Wilmington, DE). Dispase was obtained from Boeringer-Mannheim (Indianapolis, IN) and collagenase from Worthington Biochemicals (Freehold, NJ). VIP RIA kits were obtained from Incstar (Stillwater, MN) and NPY RIA reagents from Amersham. Nerve Growth Factor (NGF; the kind gift of Dr. K. Neet, Dept. of Biochemistry, CWRU) was prepared from male mouse submaxillary glands as described by Bocchini and Angeletti (1969). Pierce protein assay kit was obtained from Pierce (Rockford, Il), ITS Premix from Collaborative Research (Bedford, MA) and reagents for SDS/PAGE from BioRad (Richmond, CA). Avidin-conjugated alkaline phosphatase was obtained from Cappel (Westchester, PA) and goat anti-mouse and anti-rabbit secondary antibodies from Jackson Immunologicals (Westgrove, PA). Other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture

Cultures of rat sympathetic neurons were prepared as described by Hawrot and Patterson (1979). Neurons from the superior cerivcal ganglia of newborn rats were dissociated enzymatically with dispase (5mg/ml) and collagenase (1mg/ml) and plated in 96 well plates coated sequentially with polylysine (0.1mg/ml) and laminin (10 μg/15ml). About 2000-3000 neurons were plated per well except where indicated. The neurons were grown in Leibovitz's L15-CO_2 medium with NGF (100ng/ml), 100 units of penicillin, 100μg of streptomycin, 10μM cytosine arabinocide and 5% rat serum and the medium changed every third or fourth day. In some experiments, cells were grown without rat serum in L15-CO_2 supplemented with transferrin, selenium, bovine serum albumin, insulin and fatty acids.

The tissue extracts were diluted in growth medium, sterilized by passage through a 0.2 μm filter and added to the neurons from the third day of culture on. Neurons were harvested for assay between the ninth and fourteenth days of culture.

Tissue Extracts

To prepare sweat gland extracts, footpads were dissected from rats of various postnatal ages and weighed. Tissue from twenty animals was generally processed at

one time. The weight of footpads from twenty rats varied from 0.5gms to 5gms depending upon the age of the animals. The tissue was homogenized for 5 secs in 10 volumes of 10 mmol phosphate buffer pH 7.0 with a Polytron. The extract was then centrifuged at 100,000g for one hour. The supernatant was collected, filtered through a 0.2μm filter and concentrated using a Centricon filter with a 10kD cutoff. The protein concentration was determined with a Pierce protein assay kit. Extracts of liver, sciatic nerves and parotid gland were prepared in a similiar manner. To prepare hairy skin extracts, the skin over the thoracic region was shaved and dissected free from the underlying panniculosis carnosus muscle and weighed. The skin was then cut into smaller pieces before being homogenized and processed as described above.

Assays

The induction of cholinergic function was determined by assaying choline acetyltransferase activity in homogenates essentially according to the method of Fonnum (1969). To increase the sensitivity of the assay, an incubation period of an hour was used. All of the activity was inhibitable by 500μM napthylvinyl pyridine, a specific inhibitor of CHAT activity. Protein concentration was assayed by the method of Lowry using bovine serum albumin as a standard.

Catecholamine content was assayed by high performance liquid chromatography (Rittenhouse *et al.*, 1988) on a 5μm pore reverse phase C-18 column (Altex Ultrasphere-IP; Beckman, Berkeley, CA) using a coulometric detector (5100A; ESA, Bedford, MA). Three electrodes were set in series at +0.36, +0.03 and -0.38V relative to a reference electrode. Standards at known dilutions (5 picomoles) were run at the same time to estimate the concentration. The total catecholamine content of a well was obtained by summing the levels of norepinephrine, dopamine and DOPAC, a metabolite, present in each extract. Neither epinephrine nor DOPA was detected.

The amount of tyrosine hydroxylase present in the cultured neurons was determined by semiquantitative analysis of immunoblots. Cell cultures were homogenized in sample buffer (50 mM Tris pH 6.8 with 2% SDS, 10% glycerol and .004% bromophenol blue and 5% ßmercaptoetanol), aliquots of the extract were run on a 10% SDS-PAGE gel and the proteins were blotted onto nitrocellulose. The nitrocellulose blots were blocked in blocking buffer (5% defatted milk in Tris buffered saline pH 7.2) and then incubated with a monoclonal antibody against TH (the kind gift of Dr. Ann Acheson, University of Alberta, Edmonton) overnight. The

blots were then sequentially incubated with a biotinylated secondary antibody and avidin conjugated to alkaline phosphatase. The reaction product was developed with NBT (Nitroblue tetrazolium) and BCIP(5-Bromo-4-chloro 3-indoyl phosphate) in 10mm bicarbonate buffer, pH 9.5. After optimal color development, the reaction was stopped by rinsing in distilled water. The blots were allowed to dry and the color intensity was read on a scanning laser densitometer (Shimadzu). Comparisons were made between samples run in parallel lanes and treated identically.

Neuropeptide levels were determined by radioimmunoassay. Cultures were rinsed once with phosphate buffered saline (PBS) and then homogenized in 100μl of 2M acetic acid. After boiling for five minutes, samples were centrifuged for one minute in an Eppendorf microfuge. The supernatants were dried under vaccum and stored at -70^{o}C for subsequent assays. VIP was assayed using a kit obtained from INCSTAR with primary antibodies previously demonstrated to show minimal crossreactivity with other peptides. To assay NPY by radioimmunoassay, antibodies, standards and labeled tracer were obtained from Amersham and peptide content was determined by the delayed tracer method. Since the antibody shows only 64% cross reactivity with rat NPY, standards were also run with rat NPY (Peninsula Laboratories), and sample values read off the standard curve.

Appendix to Chapter 3.

Decreased content of cholinergic differentiation activity in footpad extracts of mutant mice that lack sweat glands.

Introduction

Sweat glands in the rat and the mouse are found on the palmar, plantar pads and in the plicae digitalis (Ring and Randall, 1947, Sofaer, 1969). Sweat gland rudiments are first evident by postnatal day 2 and have acquired their adult morphology by postnatal day 21. Unlike most other sympathetic targets, adult rat sweat glands are innervated by cholinergic sympathetic neurons (Hayashi and Nagawa, 1963, Sjoqvist, 1963 a, b). The development of rat sweat gland innervation has been described in detail (Landis and Keefe, 1983, Landis 1983, Leblanc and Landis 1986, Landis *et al.*, 1988). The initial innervation of the sweat glands is noradrenergic and fibers express catecholaminergic markers (Landis and Keefe, 1983), but soon after innervating the target the fibers switch from a noradrenergic to a cholinergic phenotype (Landis and Keefe, 1983, Leblanc and Landis 1986, Landis *et al.*, 1988). Transplant experiments (Schotzinger and Landis 1988, 1990) show that the noradrenergic to cholinergic switch is target mediated. Studies of the innervation of the mouse sweat gland suggest that its developmental history is similar to that documented for the rat, and it undergoes a similar noradrenergic to cholinergic switch (Landis, S. C., unpublished results).

Sweat gland extracts from footpad rich tissue in developing and adult rats can cause induction of ChAT activity in cultures of sympathetic neurons (Rao and Landis 1990). In addition to increasing ChAT levels, extracts can also increase the levels of VIP and decrease levels of TH and NPY. Thus, sweat gland extracts induce many of the changes *in vitro* that are seen during development of the sweat gland innervation *in vivo*. In view of the similar effects on the neurotransmitter phenotype and the appropriate temporal expression of the differentiation activity, we have postulated that the molecule(s) present in the extract plays a role in the adrenergic to cholinergic switch that occurs in the developing sweat gland innervation.

Our data (Rao and Landis, 1990) suggest that some cells in the footpads make this factor. Since extracts are made by grinding entire pads, it is difficult to determine which cells in the footpads are making the molecule(s). Dissecting individual sweat glands free of contamination is not feasible and no well-characterized sweat gland cell line, which could serve as a source for the differentiation activity, is presently available.

An alternative approach to identifying which tissues in footpads provide the differentiation activity in the extracts is the use of mutants defective in sweat gland development. Three such mutants have been identified : crinkled, downless and tabby

(Ta) (see Sofaer, 1969). Tabby is the best characterized of the three. It is a X-linked mutant first identified by Falconer (1952) which, in males and homozygous females, produces a syndrome of ectodermal dysplasia. The syndrome includes anhidrosis due to the absence of sweat glands (Grunber, 1971, Blecher *et al.*, 1982). Several other glands are also affected, including the respiratory mucous glands (Grunber, 1971). In addition, other epidermal derivatives, including dermal ridges, hair follicles, and tooth germs, are absent or abnormal (Dun, 1957, Kindred, 1967, Sofaer, 1969 a). The defects are due, at least in part, to an abnormal developmental and functional role of EGF (Blecher *et al.*, 1982, 1990 a, b); when Tabby males are treated with EGF, sweat glands with an appropriate morphology develop and sweat in response to agonist treatment (Blecher *et al.*, 1990 a).

We have taken advantage of this mutant to determine whether differentiation factor(s) present in footpad extracts are produced by the sweat gland by comparing the morphology and cholinergic differentiation ability of Tabby footpads with control mice.

Results

An initial examination of the adult sweat pads in tabby mutant *mice was* performed to determine the morphology of the tabby footpad. Semi-thin sections of control and tabby pads were stained with toluidine blue (Fig. 1) and examined for gross histological abnormalities. The epidermis, connective tissue, blood vessels and nerve bundles coursing through the tissue appear to be normal when compared to corresponding sections from control mice footpads. However, no glandular tissue is apparent in the sections in tabby footpad sections. Thus, in the plantar pads, the defect in tabby mutants appears to be limited to the absence of sweat gland ducts and secretory cells. These results are similar to previous observations on the deficit in sweat gland development in Tabby mice (Grunber, 1971, Blecher *et al.*, 1982).

To analyze the innervation of the aglandular pads, fixed footpad sections were stained for CGRP and VIP, markers for sensory and cholinergic sympathetic innervation, respectively (Yodlowski *et al*, 1984, Ishida-Yamamoto *et al.*, 1988). The sensory innervation of the epidermis as assessed by CGRP immunoreactivity appears normal or even increased (fig. 2) as compared to the CGRP immunoreactivity in sections from control mice. No VIP positive fibers which characterize the sympathetic cholinergic innervation of sweat glands are seen in sections of tabby footpads, indicating that the sympathetic innervation of the sweat glands is not present in the adult tabby mice. In contrast, VIP positive fibers are seen around the sweat gland ducts in sections of footpad from control mice.

We prepared extracts of footpad tissue from adult control mice and Tabby mice and assayed them for cholinergic-inducing activity by the method of Fonnum (1969). Figure 3 shows that extracts of control mice footpads caused cholinergic induction in rat sympathetic neuron cultures. In contrast, cultures treated with extracts prepared from the aglandular pads of Tabby mice showed significantly less cholinergic-inducing activity. Equal protein concentrations of Tabby footpad extract resulted in 70% less cholinergic induction as compared to controls.

Discussion

We have compared the morphology and the cholinergic-inducing ability of tissue extracts made from footpads from Tabby and control mice. Semi-thin sections of tabby footpads show that sweat gland secretory tubules, ducts and the associated sympathetic innervation are absent. Other tissue in the footpad of Tabby mice is normal; specifically, the sensory innervation of the epidermis in tabby footpads is present and appears qualitatively identical to that in control pads.

The lack of sweat glands in the footpads of tabby mice was associated with a 70 to 80 percent of cholinergic-inducing activity in footpad extracts as assayed on sympathetic neuron cultures. Since the gross morphology and immunocytochemical examination suggests that the major difference in the control and tabby footpads is the absence of sweat glands and their assciated innervation, it appears likely that the differentiation activity is being made by sweat glands cells or by Schwann cells associated with the innervating fibers. Schwann cells in the sciatic nerve in the rat are known to make CNTF (Stockli *et al.*, 1989, Lin *et al.*, 1989., see also Chapter 2) and presumably Schwann cells along the sympathetic and sensory fibers in the footpad will also make CNTF. CNTF is a cholinergic differentiation factor for cultured sympathetic neurons (Saadat *et al.*, 1989, Rao *et al.*, 1990). Thus, footpad extracts could contain CNTF made by Schwann cells and the reduction in differentiation activity in tabby extracts is explained by the absence of sympathetic innervation. Two observations are inconsistent with this hypothesis. First, extracts prepared from hairy skin and parotid gland containing a large number of nerve endings do not have cholinergic-inducing properties in similar experiments (see Chapter 3), and second, tabby pads containing a large number of sensory endings show little cholinergic-inducing activity. Thus, it appears unlikely that the large reduction in cholinergic-inducing activity in Tabby pad extracts can be attributed to a small number (relative to the sensory innervation) of sympathetic fibers. We do note, however, that tabby pads still contain a small amount of cholinergic-inducing activity. We cannot identify the source of this activity. It is possible that the remaining activity in the pads represents CNTF activity from Schwann cells. The alternative possibility is that the differentiation activity in sweat gland extracts is due to sweat gland cells themselves. Although we cannot at present conclusively prove that this is true, it appears more likely when taken together with our data on the failure of hairy skin extracts to cause cholinergic induction (Chapter 3) and that the sensory innervation of the tabby footpads is normal. We would, therefore, suggest that the cholinergic differentiation activity is made by the sweat gland cells.

Figure 1.

Morphology of control and tabby mice footpads.

Toluidine stained semi-thin sections were examined for overall sweat gland morphology. Panel a shows footpads from control mice with a number of sweat gland ducts. Panel b shows a similar section of footpad from tabby mice with no sweat gland tissue present.

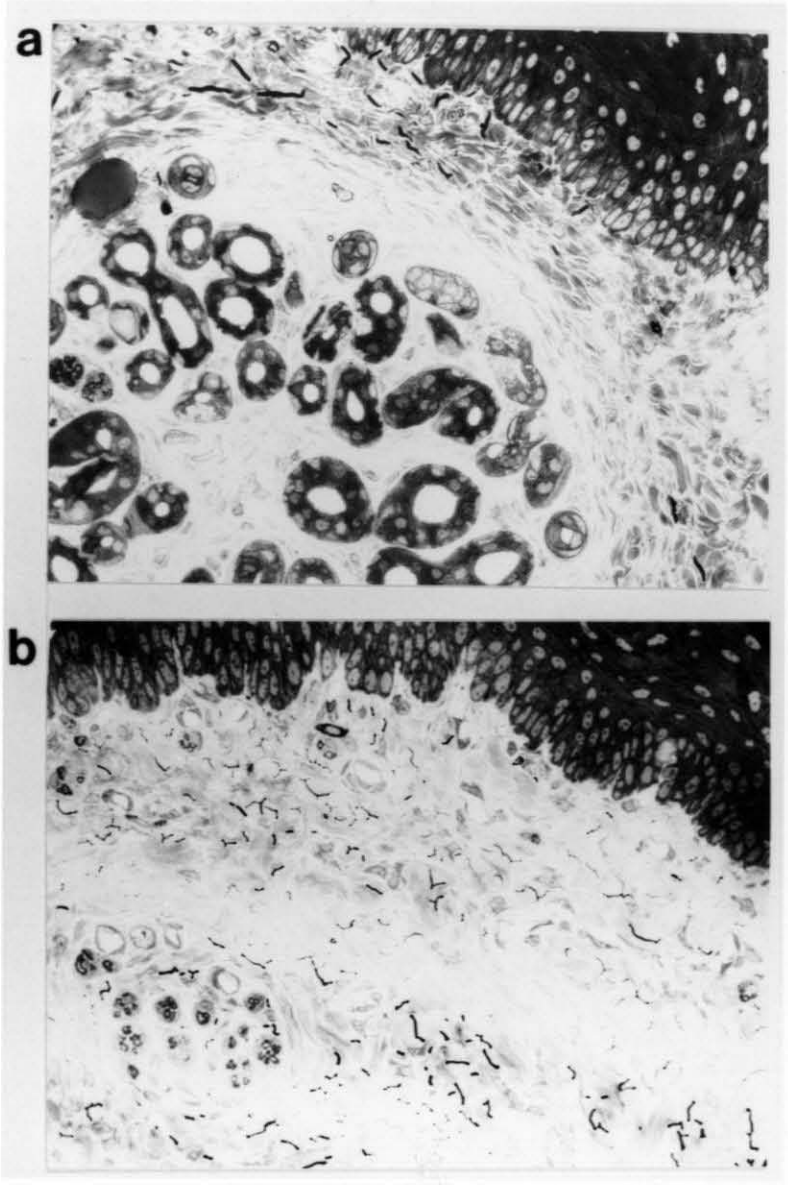

a
b

Figure 2.

Sympathetic cholinergics are absent in Tabby footpads.

Footpad Cryostat sections of adult control and Tabby footpads were stained for CGRP and VIP. CGRP and VIP positive fibers can be seen in sections from control mice (Panel a and b, respectively). Similar CGRP immunoreactivity is seen in tabby mice footpad sections (Panel c) but no VIP positive fibers (Panel d) can be detected.

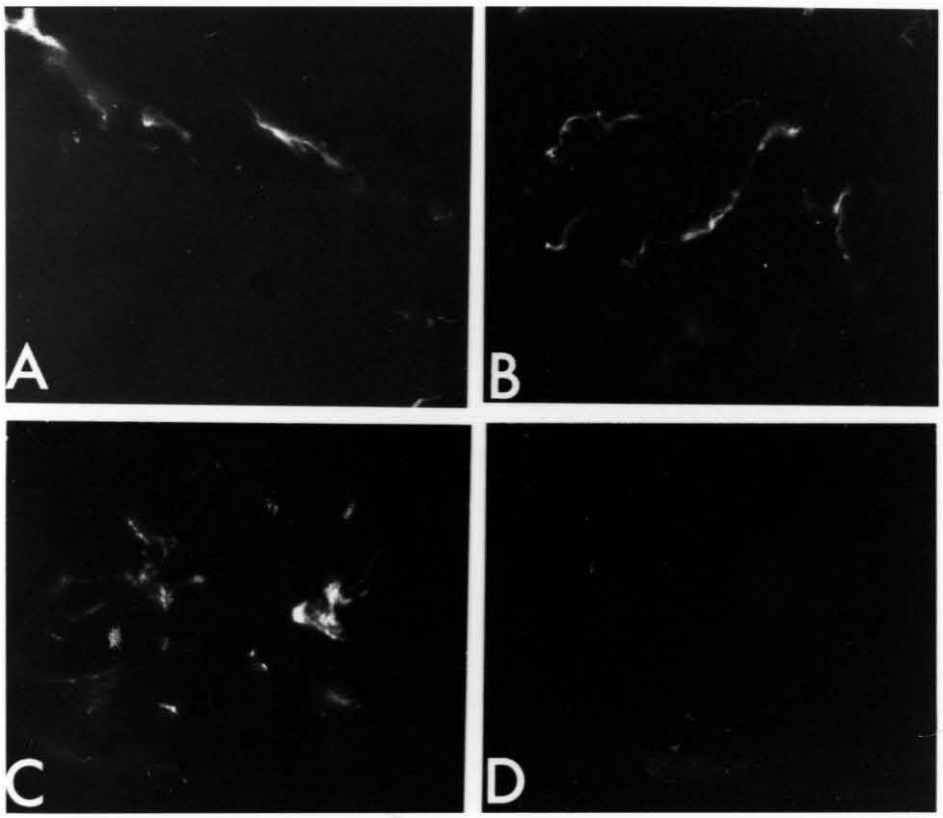
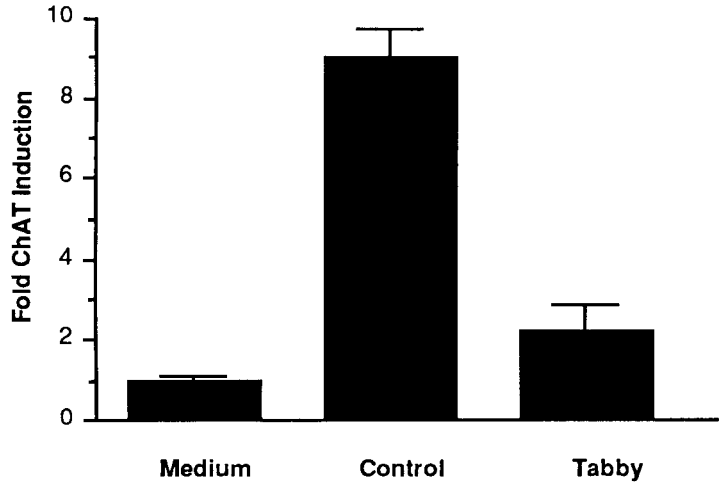

A
B
C
D

Figure 3.

Sweat gland extracts from mice alter ChAT activity in rat sympathetic neuron cultures.

Footpad extracts from control and Tabby mice (25 μg/ml) were added to sympathetic neuron cultures. Seven days after the addition of the extracts samples were homogenized and assayed for ChAT activity. The results are expressed as fold induction of ChAT activity ± s.e.m as compared to control cultures grown in medium without the addition of extract. All samples were assayed in duplicate (n=3 experiments).


Fold ChAT Induction
10
8
6
4
2
0
Medium
Control
Tabby

Materials and Methods

All materials are as described in Chapter 3. Polybed 812 was obtained from Polysciences (Warrington, PA). The CGRP antiserum was obtained from Amersham (Arlington Heights, Il). The VIP antiserum was generated in guinea pig against porcine VIP (Paul Henion and Mahendra Rao, CWRU). Rhodamine-conjugated goat anti-guinea pig was obtained from Antibodies Incorporated (Davis, CA) and the flourescein-conjugated goat anti-rabbit was from Tago (Burlingame, CA). Mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Tissue Histology

Control and tabby mice were killed with ether and perfused through the heart with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for ten minutes. The footpads were dissected and further fixed by immersion in the fixative overnight. Tissue was then rinsed in 0.12 M phosphate buffer and placed in 1% osmium in phosphate buffer for one hour. After washing, tissue was stained en bloc using uranyl acetate overnight at 4°C overnight. The tissue was then dehydrated in ethanol and embedded. Semi-thin sections were stained with toludine blue.

Immunocytochemistry

Mice were killed with ether and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for ten minutes. The footpads were further fixed by immersion in the paraformaldehyde solution for one hour. After rinsing with phosphate buffered saline (PBS), the tissue was equilibrated with 30% sucrose in PBS. Fifteen µm cryostat sections were thaw mounted onto gelatin coated slides and were processed for indirect immunoflourescence according to the method of Coons (1958). After sequential incubation with the primary antiserum (CGRP 1:800, VIP 1:300) overnight and the secondary antiserum for 2hrs the sections were rinsed and mounted in a 1:1 mixture of PBS and glycerol. The specificity of the antiserum has been previously determined (Schotzinger and Landis, 1990).

Footpad extracts

Extracts were prepared as described in Chapter 3. In brief, footpads were collected from five to six adult animals. The tissue was homogenized and the soluble protein collected and concentrated.

Cell culture

Sympathetic neuron cultures from neonatal animals were prepared as described (Chapter 3). Two days after plating footpad extracts to be assayed were added to the cells. Seven days after the addition of the extracts, neurons were harvested and assayed for ChAT activity by the method of Fonnum (1969). Unless otherwise stated, 25 µg/ml of the footpad extracts was added to cultures.

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Chapter 4.

Comparison of the sweat gland differentiation activity with other cholinergic differentiation factors.

Abstract

Treatment of cultured sympathetic neurons with sweat gland extracts mimics many of the changes in neurotransmitter properties seen *in vivo* during development of the sweat gland innervation (Rao and Landis, 1990). Further purification of the differentiation activity from extracts of adult sweat gland indicates that the activity is associated with a soluble protein(s) having an approximate molecular weight of 22-26 kD and a pI of 5.0. Comparison of its immunological and biochemical properties with three different cholinergic differentiation molecules, CDF (cholinergic differentiating factor), CDF/LIF (cholinergic differentiation factor/Leukemia inhibitory factor), and CNTF (ciliary neurotrophic factor) suggests that it is related to CNTF. CDF differs from the sweat gland activity in that it does not cause cholinergic induction in sympathetic neuron cultures. The major sweat gland cholinergic activity is biochemically distinct from LIF and, in contrast to CDF/LIF, its effects on cholinergic induction are not antagonized by depolarization with 30 mM KCl. Further, antibodies generated against the N-terminal sequence of CDF/LIF do not immunoprecipitate the cholinergic-inducing activity from sweat gland extracts. Comparison of partially purified sweat gland cholinergic-activity with CNTF indicates that these two factors are biochemically very similar. In addition, purified sweat gland activity like CNTF supports the survival of E8 chick ciliary neurons. Finally, antibodies to CNTF can partially precipitate the differentiation activity from extracts of sweat glands. The sweat gland cholinergic inducing activity may not be identical to CNTF, however, as western blots, northern blots and *in situ* hybridization experiments fail to detect CNTF or CNTF message in sweat gland tissue. These observations raise the possibility that the major sweat gland activity is a molecule closely related to CNTF.

Introduction

The sympathetic innervation of the adult rat sweat glands is cholinergic (Landis and Keefe, 1983; Leblanc and Landis, 1986, Stevens and Landis, 1987). The fibers which initially innervate the sweat gland, however, are noradrenergic. During development the neurotransmitter-related properties of these fibers undergo a developmental switch from a noradrenergic to a cholinergic phenotype (Landis and Keefe, 1983, Leblanc and Landis, 1986). As discussed in Chapter 3, several lines of evidence suggest that this switch in neurotransmitter properties is target mediated. Sympathetic neurons which normally innervate sweat glands and become cholinergic fail to acquire cholinergic properties when they innervate the parotid gland, a target whose innervation is normally noradrenergic (Schotzinger and Landis, 1990). Further, normally noradrenergic sympathetic neurons will become cholinergic when they innervate transplanted sweat glands (Schotzinger and Landis, 1988). Although the evidence for a role of the target in directing this transmitter switch is compelling, little is known about the actual molecules involved.

To identify the component in sweat gland tissue that is responsible for instructing the sympathetic neurons to alter their neurotransmitter phenotype, we have assayed extracts from sweat glands for differentiating activity. As previously reported (Rao and Landis, 1990), we have isolated and partially characterized a factor(s) present in crude extracts of sweat gland tissue which *in vitro* can mediate many of the changes in the neurotransmitter phenotype seen during the development of sweat gland innervation. Extracts from sweat gland tissue, but not from parotid gland or hairy skin, induce choline acetyltransferase activity and increase vasoactive intestinal peptide expression in cultures of sympathetic neurons. The induction of cholinergic properties is accompanied by a concomitant reduction in catecholaminergic properties and in levels of neuropeptide Y. The cholinergic differentiation activity that can be extracted from sweat gland tissue appears to be present in developing glands as early as postnatal day five and increases over a period of two weeks to reach adult levels. Thus, it is present at the appropriate time in development to be the factor responsible for the target-mediated adrenergic to cholinergic switch. In view of its appropriate spatial and temporal expression, we have postulated that this extracted activity represents the developmentally relevant molecule.

Several proteins have been identified which cause a similar noradrenergic to cholinergic switch when added to cultures of sympathetic neurons and, *therefore,* represent potential candidates for the differentiation signal produced by sweat glands.

Some of these factors are poorly defined; for example, chick embryo extract, human placental serum and rat serum increase acetylcholine synthesis and/or ChAT activity (Higgins et al., 1981; Iacovitti et al., 1981, 1982; Wolinsky et al., 1985). Other cholinergic molecules like CDF from skeletal muscle, CDF/LIF, CNTF and MANS (membrane-associated neurotransmitter-stimulating factor) from spinal cord membranes have been more completely characterized. CDF prepared from skeletal muscle extracts has been purified and characterized (Mcmanaman et al., 1989). It is biochemically distinct from CDF/LIF and CNTF and has been shown to increase levels of ChAT in spinal cord motor neurons both *in vitro* and *in vivo* (Mcmanaman et al., 1990). Although the ability of CDF to induce cholinergic properties in cultures of sympathetic neurons has not been tested, it is a potential candidate since two of the factors that influence cholinergic function in sympathetic neurons, CDF/LIF and CNTF, have a similar effect on spinal cord cultures (Sendtner et al 1990, Yoshihiro et al., 1990, Geis and Weber, 1984). The cholinergic differentiation factor (CDF), purified from heart cell conditioned medium (Patterson and Chun, 1977, Fukada, 1985), has been shown to be identical to leukemia inhibitory factor (LIF; Yamamori et al., 1989). A very similar, if not identical, molecule is present in skeletal muscle cell conditioned medium (Weber et al., 1985) and increases cholinergic function in spinal cord and nodose sensory neurons as well as sympathetic neurons (Geiss and Weber, 1984, Mathieu et al., 1984). Ciliary neurotrophic factor (CNTF), originally identified as a survival factor for ciliary neurons (Adler et al., 1979; Barbin et al., 1984; Manthorpe et al., 1986) and recently cloned (Lin et al., 1989; Stockli et al., 1989), induces cholinergic and reduces catecholaminergic function in cultured sympathetic neurons (Saadat et al., 1989). MANS has been solubilized and partially purified from rat spinal cord. The latter activity is associated with a 29-kD protein (Wong and Kessler, 1987; Adler et al., 1989). We have previously demonstrated (see Chapter 2) that the membrane-associated cholinergic-inducing activity is most likely to be similar/related to CNTF. MANS shares with CNTF the ability to support the survival of chick ciliary neurons (Rao et al., 1990) and antibodies to CNTF can immunoprecipitate the cholinergic-inducing activity and detect a 24-kD band on Western blots (see Chapter 2).

While the list of well-characterized cholinergic molecules is not extensive, several molecules including CDF, CDF/LIF, and CNTF have the appropriate biological properties to play a role in the adrenergic to cholinergic switch that characterizes sweat gland innervation. It is not clear, however, if any of these proteins is related to or identical with the cholinergic-inducing activity present in sweat gland extracts. Nor is it clear that the cholinergic-inducing ability of these factors represents their primary,

or even a relevant function, in normal development. Several of these factors have been shown in cell culture systems to have additional functions. For example, CDF/LIF inhibits proliferation and induces macrophage differentiation in the M1 myeloid cell line (Hilton et al., 1988) and maintains the developmental potential of embryonic stem cells (Smith et al., 1988; Williams et al., 1988) while CNTF has trophic activity for ciliary neurons (Barbin et al, 1984, Manthorpe et al., 1986) and motor neurons (Yoshihiro et al., 1990, Sendtner et al., 1990). In addition, it induces astrocytic properties in 02A progenitor cells (Hughes et al., 1988).

To determine if any of the previously identified factors are present in sweat gland extracts, we have further purified the cholinergic-inducing activity from sweat gland tissue and compared its biochemical and immunological properties with three of the better characterized cholinergic factors, CDF/LIF, CNTF and CDF (Fukada et al., 1985, Stockli et al., 1990, Mcmanaman et al., 1990). Our results suggest that the activity present in sweat gland extracts is distinct from CDF from skeletal muscle and CDF/LIF, but shares many properties with those of recombinant CNTF. Western blots, *in situ* hybridization and Northern blot analysis suggest, however, that the sweat gland cholinergic-inducing activity is not identical to CNTF.

Results

Characterization of the sweat gland cholinergic differentiation activity.

Anionic exchange chromatography

As an initial step in purification, we prepared sweat gland extracts from adult tissue and chromatographed it on a anionic exchange column (Mono Q). Figure 1a shows that all the cholinergic activity bound to the column and eluted as a single peak with 0.25 mM NaCl. This is similar to the behavior of CNTF and CDF (Manthorpe et al., 1986, Mcmanaman et al., 1988, 1989), but different from that reported for CDF/LIF (Fukada, 1985), a basic protein.

Isoelectric focussing

The Mono Q peak fraction was chromatographed on a MONO P column and 0.5 ml fractions were collected and assayed for cholinergic activity. Figure 1b shows that the ChAT activity eluted between pH 4.8-5.2 with a peak of activity at pH 5.0, indicating that the pI of the active protein was in this range. This is similar to the value reported for CNTF purified from sciatic nerve extracts (Manthorpe et al., 1986).

Size fractionation

To determine the molecular weight of the cholinergic-inducing activity, peak fractions from the anionic exchange column were chromatographed on a sizing column. Fractions were tested for cholinergic-inducing activity on sympathetic neuron cultures. Figure 2 shows that the cholinergic inducing activity eluted in a peak between the 16 kD and 32 kD protein markers. Comparison of its size with the molecular weights reported for the other cholinergic factors indicates that the size of the sweat gland differentiating activity is similar to that reported for MANS (Wong and Kessler, 1987), CNTF (Manthorpe et al., 1986) and deglycosylated LIF (Fukada et al., 1985).

To determine more accurately the molecular weight of the sweat gland differentiating activity, fractions of the sweat gland extract purified on an anionic exchange column were run on a SDS-PAGE gel. Slices of the gel between the appropriate molecular weight standards were cut and the proteins eluted from them using an electroeluter. Aliquots of the extracted proteins were tested for activity. Figure 3a shows that the activity has a molecular weight between 22-26 kD.

The 22-26 kD fraction which had cholinergic-inducing activity was also tested for its ability to modulate levels of NPY and VIP. Figure 3b shows that the same SDS-PAGE eluted fraction induces VIP and reduces NPY levels. These observations indicate that a single protein or several closely related proteins with similar molecular weights

and pI's may mediate the several effects on the neurotransmitter phenotype that one observes after addition of sweat gland extracts to sympathetic neurons.

Comparison of sweat gland cholinergic differentiating activity with skeletal CDF.

A skeletal muscle polypeptide has been isolated from skeletal muscle from young rats (Mcmanaman et al., 1988) which can stimulate the development of ChAT activity in embryonic day 14 rat spinal cord cultures. This factor has a molecular weight of 22-Kd and a pI of 4.8 biochemical properties similar to that of sweat gland factor (Mcmanaman et al., 1988). The time course of expression of CDF activity in skeletal muscle extracts and that of sweat gland cholinergic-inducing activity in sweat gland extracts are also similar (Rao et al., 1990, Mcmanaman et al., 1989). To ascertain whether the sweat gland differentiating activity and CDF are similar, we obtained partially purified CDF (Hap7 fraction, a kind gift of Dr. Mcmanaman), and tested the ability of this preparation to induce cholinergic activity in sympathetic neuron cultures. Figure 4 shows that this fraction does not induce cholinergic function in cultures of sympathetic neurons. The number of neurons in cultures with and without extracts were similar, indicating that the failure to observe an effect was not due to toxicity of the preparations. In contrast, the same fraction of CDF caused a maximal ChAT induction of 150% in spinal cord cultures (Dr. Mcmanaman, personal communication).

Comparison of sweat gland cholinergic factor with LIF.

The major cholinergic differentiation activity in sweat glands is biochemically distinct from LIF

Additional purification of the sweat gland activity indicated that the biochemical properties of the soluble cholinergic-inducing activity were distinct from glycosylated LIF. The sweat gland differentiating activity is an acidic protein retained on an anionic column and has a molecular weight between 22-26 kD. It was possible, however, that the cholinergic-inducing activity in the extracts represented a deglycosylated or partially glycosylated form of LIF that interacted with another protein present in sweat gland extracts and, therefore, behaved differently during our purification procedures. To examine this possibility, we added recombinant deglycosylated LIF to sweat gland homogenates and after an overnight incubation determined whether LIF was retained on a DEAE column. Figure 5a shows that when recombinant deglycosylated LIF is incubated

with crude sweat gland extracts and then chromatographed on an anion exchange column it still does not bind to the column.

Depolarization antagonizes the cholinergic induction by LIF but not by the sweat gland differentiating activity

The action of heart cell conditioned medium but not CNTF can be antagonized by the addition of 30 mM KCl to cell cultures (Walicke et al., 1977, Chapter 2). Figure 5b shows that this is true for recombinant LIF also. The action of sweat gland extracts on cholinergic induction, however, is not antagonized by depolarization with 30 mM KCl (Fig. 5b).

Failure to immunoprecipitate biological activity with antibodies to CDF/LIF

Antisera generated against a synthetic peptide whose sequence corresponds to the N-terminal peptide sequence of CDF/LIF immunoprecipitate the cholinergic-inducing activity from a partially purified fraction (the DEAE flow through) from heart cell conditioned medium (Fukada, Neuroscience abstract, 1988, Yamamori et al., 1989, Rao et al., 1990). When the DEAE eluate fraction of the sweat gland extract was treated with affinity-purified antibodies to CDF/LIF, there was no detectable decrease in the ability of the extract to induce choline acetyltransferase activity (Fig 6a). Since the DEAE fraction is relatively crude, it was possible that inhibitory proteins or proteases were present in the sweat gland extract which were responsible for the inability to immunoprecipitate activity. In parallel experiments, however, the same antibodies added to the DEAE fraction of sweat gland extracts were able to precipitate iodinated recombinant CDF/LIF (the kind gift of T. Yamamori, California Institute of Technology) as the appropriate 20-kD band (Figure 6b). Thus, the principal cholinergic-inducing activity present in the sweat gland extracts is not likely to be CDF/LIF.

Comparison of sweat gland cholinergic factor with CNTF.

Sweat gland extracts have ciliary neurotrophic activity

CNTF was originally isolated on the basis of its ability to support the survival of E8 chick ciliary neurons in culture (Manthorpe et al., 1986, Barbin et al., 1984) and was later shown to induce cholinergic properties in sympathetic neuron cultures (Saadat et al., 1989). Since the molecular weight and pI of the differentiating activity in sweat

gland extracts was very similar to that reported for CNTF (Manthorpe et al., 1986, Ernsberger et al., 1989, Sendtner et al., 1990, Lin et al., 1990) and crude sweat gland extracts had ciliary neurotrophic activity (data not shown), we assayed the ability of SDS-PAGE purified extracts of sweat gland extract to support the survival of ciliary neurons. Figure 7 shows that purified sweat gland extracts supported the survival of E8 chick ciliary neurons.

Antibodies raised against recombinant CNTF immunoprecipitate cholinergic-inducing activity from sciatic nerve extracts and from sweat gland extract

Immunoprecipitation of peptide and cholinergic-inducing activity from sciatic nerve extracts is complete with a 1:4 dilution of a polyclonal antiserum generated against rCNTF (see Chapter 2). In similar immunoprecipitation experiments, a 1:4 dilution of the antibody failed to precipitate activity from LIF solutions and precipitated approximately 80% of the cholinergic-inducing activity in sweat gland extracts. Thus, the major cholinergic-inducing activity present in sweat gland extracts displays some cross-reactivity with CNTF.

Data from our partial purification suggested that the 22-26 kD fraction could be responsible for the spectrum of neurotransmitter modulating activities that are present in sweat gland extracts. To assess if antibodies to rCNTF also altered the ability of sweat gland extracts to modulate NPY and VIP levels, we assayed the effect of sweat gland extract incubated with and without antisera on peptide levels. Figure 8b shows that the peptidergic-inducing activities present in sweat gland extracts are only partially immunoprecipitated by this antibody. Fifty percent of the VIP-inducing and little, if any, of the NPY-reducing ability of sweat gland extracts is precipitable. These observations provide additional evidence that the molecule(s) responsible for the differentiating activity present in sweat gland extracts, while immunologically related to CNTF, may not be identical to it.

Antisera against recombinant rat CNTF fails to detect CNTF in sweat gland extracts

Our immunoprecipitation and biochemical data suggested that the sweat gland differentiating activity was either identical or closely related to CNTF. To examine these possibilities further, equal amounts of cholinergic-inducing activity from sciatic nerve extracts and sweat gland extracts were loaded on an SDS-PAGE gel, electrophorised and probed with a polyclonal antiserum generated against recombinant rat CNTF. The antiserum recognized recombinant CNTF (Figure 9a) and a 24-kD band present in the sciatic nerve extracts (Figure 9b). Binding was completely blocked by preincubating the

antibody with 10 mM recombinant CNTF. No specific band was evident in the lanes containing either hairy skin extracts, which do not have cholinergic-inducing activity, (Rao and Landis, 1990), or sweat gland extracts even though the lanes containing the sweat gland and sciatic nerve extracts contained the same amount of cholinergic-inducing activity for cultured sympathetic neurons. Loading ten-fold more cholinergic-inducing activity and overstaining the blots failed to reveal any specific binding in lanes containing sweat gland extracts (data not shown).

Northerns and in situ *hybridization analysis with an oligonucleotide probe against rat CNT*

To determine if message for CNTF could be detected in sweat glands we prepared RNA from sweat gland from adult rats and probed the Northern blots for message. Figure 10 shows that a band of 1.2 kb, the expected size of CNTF message, can be detected in sciatic nerves and a faint band can be detected in the lane containing RNA prepared from dorsal root ganglia which may also contain CNTF (see Chapter 2). In contrast, no specific binding can be detected in lanes containing RNA from liver or sweat glands

The same probe was also used in the *in situ* hybridization studies to probe sections of sciatic nerve and sweat gland as a more sensitive assay for its presence that may be making CNTF/CNTF-like molecule. Figure 11 shows that a subpopulation of Schwann cells in the sciatic nerve exhibit a specific hybridization signal with the antisense probe as compared to the sense control. No specific signal, however, can be detected in sweat gland tissue either over the glands or over the Schwann cells present in the nerve fibers in the tissue.

Discussion

We have previously shown that sweat gland extracts contain a cholinergic-differentiating activity as assayed on cultures of sympathetic neurons (Rao and Landis, 1990). Further purification of this differentiating activity revealed it to be either a single, or several closely related molecules, with a molecular weight(s) between 22-24 kD and a pI of 5.0. Since the activity has not been purified to homogeneity yet, we cannot at present distinguish between one or several molecules with very similar properties in sweat gland extracts. Given that several molecules (CDF, CNTF, MANS) have been identified which have a similar spectrum of activities, we would consider a single molecule more likely. It is of interest to compare the properties of the differentiation activity in sweat gland extract with these previously characterized molecules. We have compared the the sweat gland differentiation activity with three different cholinergic factors: CDF/LIF, CNTF and CDF from skeletal muscle. We did not consider MANS (Wong and Kessler, 1987; Adler et al., 1989) to be a likely candidate because the sweat gland differentiating activity is easily extracted in low salt solutions and no detectable activity is associated with membranes (unpublished observations). Further, our immunoprecipitation and Western blot experiments with an antisera to rCNTF (see Chapter 1) suggested that MANS is closely related or identical to CNTF.

CDF , a cholinergic factor for motor neurons with a pI of 4.8 and a molecular weight of 22 kd (Mcmanaman et al., 1988, 1989, 1990) is biochemically very similar to the activity partially purified from sweat gland extracts. Further, expression of CDF in skeletal muscle extracts is temporally similar to that of differentiation activity in sweat gland extracts (Mcmanaman et al., 1989, Rao and Landis, 1990). Although CDF had never been shown to cause cholinergic induction in sympathetic neurons, it represented a potential candidate for the sweat gland differentiation factor(s) as other factors which cause cholinergic induction in sympathetic neuron cultures, increase levels of ChAT in spinal motor neuron cultures (Sendtner et al., 1990, Yoshihiro et al., 1990, Geis and Weber, 1984, Kato and Patterson, unpublished results). We, therefore, tested the ability of purified fractions of CDF to induce cholinergic function in sympathetic neuron cultures under conditions identical to those in which sweat gland extract, CDF/LIF and CNTF cause 10-to-20 fold induction. CDF, however, had no effect on either ChAT or TH levels (unpublished results) in sympathetic neuron cultures. Thus, CDF differs from the other cholinergic factors and cannot be responsible for mediating the adrenergic to cholinergic switch in sweat gland innervation.

Two soluble factors, CDF/LIF and CNTF, are similar in their effects on sympathetic neurons; they increase choline acetyltransferase and VIP expression and reduce tyrosine hydroxylase and catecholamine content (Fukada, 1985; Yamamori et al., 1989; Sendtner et al., 1989; Ernsberger et al., 1989; Nawa and Patterson, 1990). In addition, like sweat gland extract, both CDF/LIF (Nawa and Patterson, Cold Spring Harbor Symposium, 1991) and extracts of sciatic nerve containing CNTF (see Chapter 2) decrease NPY expression. Thus, they are potential candidates for a sweat gland differentiation activity.

CDF/LIF is an attractive candidate; it has a consensus signal sequence, it is glycosylated and it is secreted by heart cells (Patterson and Chun, 1977 a; Yamamori et al., 1989). Comparison of the biochemical characteristics of CDF/LIF with the sweat gland factor(s), however, indicate that this protein does not appear to be related to LIF. LIF is a basic protein of a molecular weight of 45 kD which does not bind to a DEAE column and has a pI of >7 (Fukada et al., 1985). It is also unlikely that the protein in sweat gland extracts is the deglycosylated or partially deglycosylated form of LIF; glycosylation would tend to add negative residues to the protein and, therefore, the deglycosylated form of LIF would be even more basic. Indeed, an estimate of the charge on deglycosylated LIF shows 10 excess positive charges in rat (12 in human) with an estimated pI of 8.5. It is also unlikely that the protein present in the extracts is LIF bound to some other protein which causes it to behave differently on anionic exchange columns. Incubation of rLIF with sweat gland extracts does not alter the behavior of LIF on an anionic exchange column. Finally, affinity-purified antibodies raised against the N-terminal region of CDF/LIF can immunoprecipitate the cholinergic-inducing activity from the DEAE or Sephadex fractions of heart cell conditioned medium (Fukada, Neuroscience abstract, 1988, Yamamori et al., 1989, Rao et al., 1990) but these antibodies do not immunoprecipitate the cholinergic-inducing activity from sweat gland extracts. Thus, it is unlikely that the major cholinergic-inducing activity present in the sweat gland extracts is identical to or related to LIF.

It is possible, however, that two molecules are present in the extracts, the major component being a molecule distinct from LIF and a minor LIF-like component. Since antibodies to rCNTF which immunoprecipitate virtually all the activity from crude homogenates of sciatic nerve immunoprecipitate about 80% of the cholinergic activity from sweat glands, and even less of the peptide modulating activity, it is possible that the non-precipitable differentiation activity which is yet to be characterized represents CDF/LIF. This notion is consistent with the recent PCR results suggesting a specific expression of LIF in sweat gland extracts (Yamamori, T., Caltech,.personal

communication). Clearly, however, there are several alternative explanations for our failure to immunoprecipitate all the cholinergic-inducing activity. For example, the immunoprecipitation experiments were done with a polyclonal antiserum using crude homogenates. Further, no additional peaks of activity corresponding to LIF were detected during purification. Further experimentation will be required to determine the biological and biochemical characteristics of minor differentiation component if, indeed, one is present.

Even if the minor cholinergic-inducing component in the sweat gland extracts is not CDF/LIF, we cannot also at present rule out the possibility that LIF is present in the sweat gland tissue and is either lost or destroyed during the extraction process. While we cannot rule out the possibility that LIF is present in sweat glands in amounts that we cannot detect, we still have to determine which of the two cholinergic-inducing molecules present in the same tissue is the biologically relevant molecule.

The biochemical and immunological properties of the major sweat gland cholinergic-inducing activity show remarkable similarity with those reported for CNTF. CNTF is also a acidic protein (Manthorpe et al., 1986) with a pI of 5.0 (Saadat et al., 1989, Ernsberger et al., 1989, Stockli et al., 1989, Lin et al., 1989) and a molecular weight of 24 kD (Manthorpe et al., 1986). CNTF, like sweat gland extracts, is capable of mediating an adrenergic to cholinergic switch in sympathetic neurons (Saadat et al., 1989), as well as inducing VIP both in chick (Ernsberger et al., 1989) and rat sympathetic neuron cultures and reducing NPY levels (Chapter 2). Since sweat gland extracts also have ciliary neurotrophic activity and this trophic activity copurifies with the cholinergic-inducing activity, the biological properties of the sweat gland factor(s) very similar to that of CNTF. Thus, it would seem that the major sweat gland activity is at the least immunologically and biochemically similar and probably even identical to CNTF.

Several pieces of evidence are inconsistent with the notion that the sweat gland differentiating activity is identical to CNTF. Immunoblotting experiments with a polyclonal antiserum generated against, and recognizing, rat CNTF failed to reveal any CNTF-like immunoreactivity in sweat gland extracts. Further, Northern blot analysis (Sendtner, Neuroscience abstract, 1989, see results) and *in situ* hybridization experiments fail to reveal detectable message for CNTF. In contrast, message is easily detectable both in Northern blot and *in situ* hybridization analysis of sciatic nerve. Preliminary evidence that sweat gland protein does not support survival of a CNTF-dependent cell line (Dr. S. Squinto personal communication) is also consistent with the view that the factor(s) in sweat glands is not identical to CNTF.

One possible explanation for a failure to detect CNTF by Northerns, *in situ* hybridization and Western blots is that, as discussed above, two molecules are present in the extract. One is CNTF and the other is an as yet unidentified component, possibly LIF. This would then explain the failure to detect CNTF on Western blots or CNTF message in sweat glands, and also our finding that antibodies to CNTF can only partially immunoprecipitate the activity. An alternative explanation, also consistent with the results, is that the molecule present in the extracts is a novel factor immunologically related to CNTF. A third possibility is that a large amount of the immunologically identifiable CNTF in sciatic nerve extracts is biologically inactive and, hence, loading equal amounts of activity on Western blots does not necessarily imply equal amounts of detectable protein. According to this scenario, the sweat gland activity is identical to CNTF but the amount of protein and message present is much less than that in sciatic nerve and, hence, we fail to detect it in sweat gland preparations. Our data cannot distinguish between these possibilities. Further purification of the sweat gland protein(s), amino acid sequencing and *in vivo* perturbation experiments will help distinguish between these possibilities.

If the factor in sweat gland extracts is indeed CNTF or a related molecule, a question that arises is how does CNTF synthesized by the sweat glands reach the fibers innervating the sweat gland? CNTF does not appear to be a secreted protein, it has no signal peptide (see review Verner and Schatz, 1988) and expression of CNTF in a cell line results in high levels of intracellular CNTF which is not secreted into the medium. It is possible, however, that novel secretory mechanisms may be present in the appropriate cell type to ensure the release of CNTF. For example, FGF and IL6 both lack signal peptides and are secreted by alternative mechanisms (Rifkin and Moscatelli, 1989, Kostura et al., 1989). Alternatively, modified or related forms of CNTF may exist. We have shown (see Chapter 2 Appendix) that MANS, a membrane-associated protein, can be precipitated by antibodies generated against recombinant CNTF and, thus, a membrane-bound form of CNTF or a CNTF-like molecule is potentially present in spinal cord preparations. Recent evidence from astrocyte cultures (Rudge et al., Neuroscience abstract, 1990) demonstrates the existence of a membrane-bound form of CNTF. While some evidence for a membrane-bound form of CNTF exists there is little evidence for a secreted form of CNTF. Although an extracellular soluble CNTF-like molecule has been reported (Hughes et al., 1988, Laura et al., 1990), it is not clear if the CNTF-like molecule is actually released into the media (Laura et al., 1990). Clarification of these issues will require additional experiments.

In summary, there are two possible explanations for our results on the comparison of the sweat gland differentiation activity with CDF, CDF/LIF and CNTF. First, two factors are present in the extracts, a major CNTF/CNTF-like component and a minor, as yet uncharacterized component, which may be LIF. Alternatively, a novel cholinergic activity is present in sweat gland extracts which, when compared to several known cholinergic molecules, is very similar but not identical to CNTF. Further purification, amino-acid sequencing and *in vivo* perturbation experiments will distinguish between these possibilities.

Figure 1 a.

The major cholinergic-inducing activity in sweat gland extracts is an acidic protein.

Soluble protein extracted from sweat glands of adult rats was chromatographed on a Mono Q anionic exchange column and 0.5 ml fractions were collected, concentrated and added to sympathetic neuron cultures. Seven days after the addition of the extracts, neurons were homogenized and aliquots were assayed for ChAT activity by the method of Fonnum. All samples were run in duplicate. The data are expressed as the fold induction of ChAT activity compared to that present in control cultures grown without added extract.

Figure 1b.

The major cholinergic-inducing activity in sweat gland extracts has a pI of 5.0.

The peak fraction of activity from the Mono Q column was chromatographed on a Mono P chromatofocussing column. The fractions collected were concentrated and added to sympathetic neuron cultures. Seven days after the addition of the extracts, neurons were homogenized and aliquots were assayed for ChAT activity by the method of Fonnum. All samples were run in duplicate. The data are expressed as the fold induction of ChAT activity compared to that present in control cultures grown without added extract.

Figure 1a.

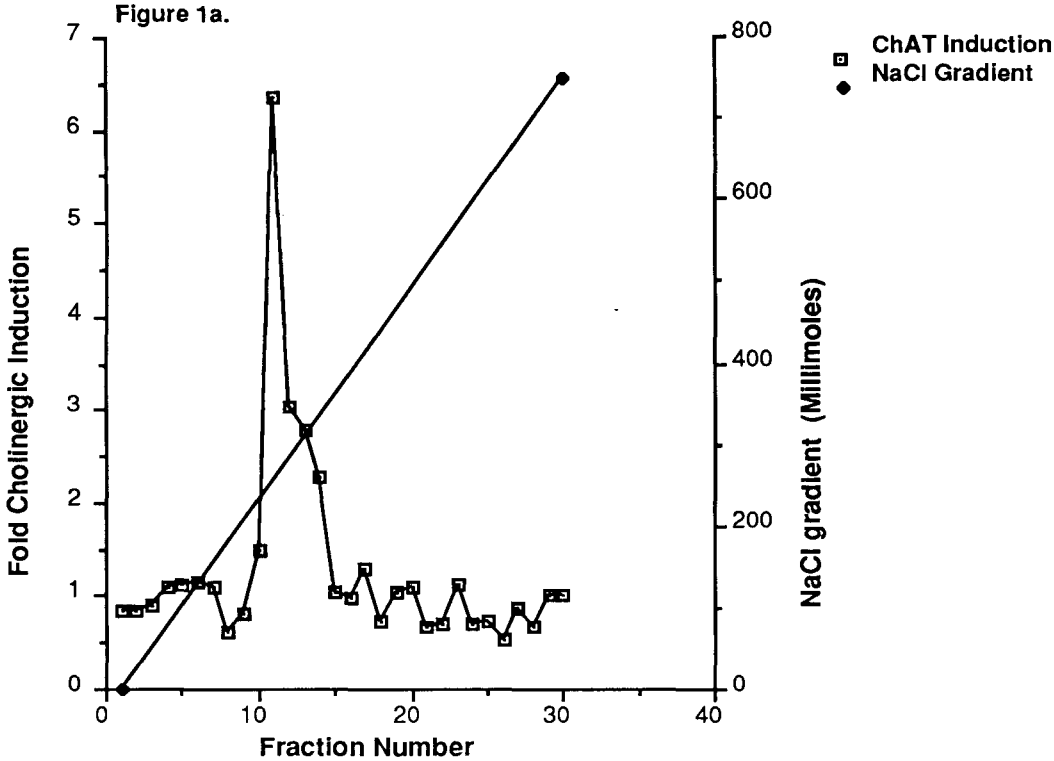


Figure 1b

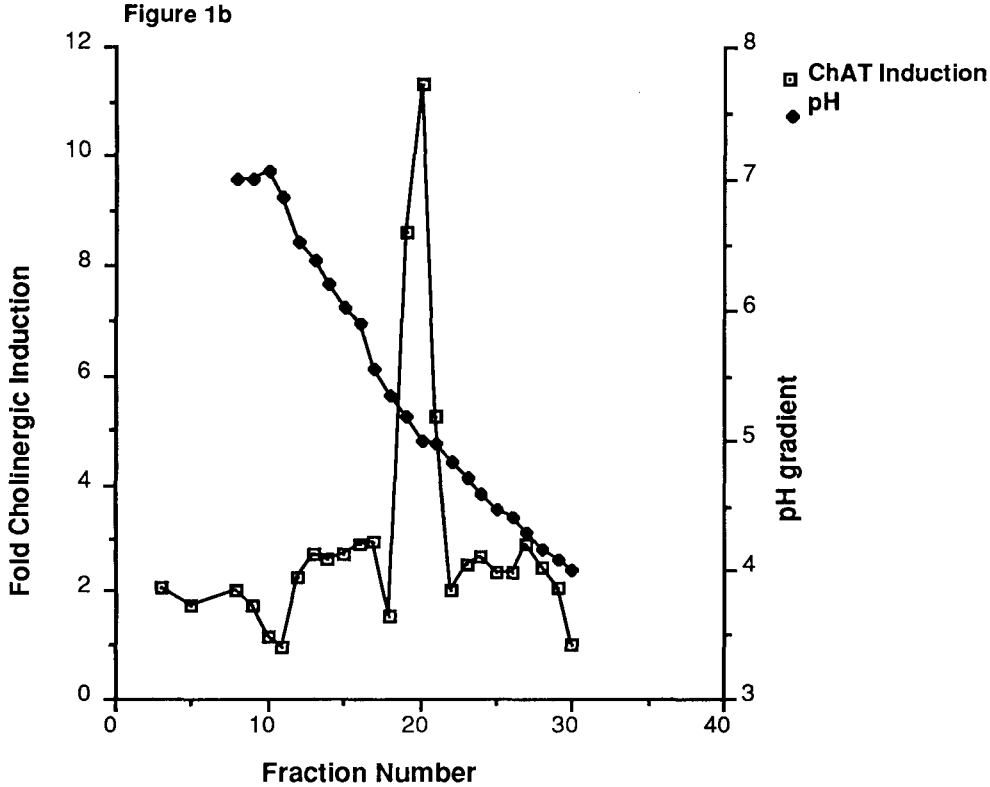


Figure 2.

The major cholinergic-inducing activity in sweat gland extracts is a low molecular weight protein.

The peak fraction of activity from the Mono P column was chromatographed on a Sepharose 12 sizing column. The fractions collected were concentrated and added to sympathetic neuron cultures. Seven days after the addition of the extracts, neurons were homogenized and aliquots were assayed for ChAT activity by the method of Fonnum. The data are expressed as the fold induction of ChAT activity compared to that present in control cultures grown without added extract.

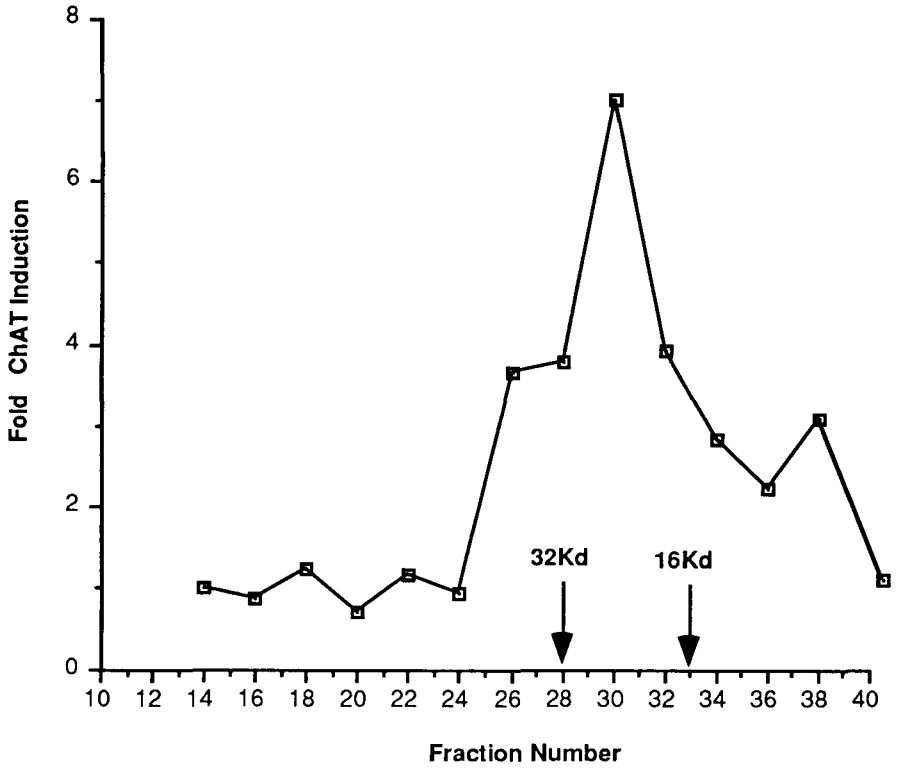

Fold ChAT Induction
0
2
4
6
8
10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Fraction Number
32Kd
16Kd

Figure 3.

Sweat gland SDS gel extracts induce ChAT activity.

In a, SDS gel fractions between 22-26 kd and 26-32 kd were eluted and added to cultures of dissociated sympathetic neurons. Seven days after the addition of extracts, neurons were homogenized and aliquots were assayed for levels of ChAT activity by the method of Fonnum. Samples were run in triplicate. The data are expressed as the fold induction of ChAT activity compared to that present in control cultures grown without added extract $\pm$ s.e.m.

In b., Sister cultures treated with SDS gel extracts as described above were assayed for NPY (neuropeptide Y) and VIP (Vasoactive Intestinal Peptide) levels by radio immunoassay. Samples were run in duplicate. The data are expressed as fold induction of peptide as compared to control cultures treated with SDS gel extract from native gels $\pm$ s.e.m.

Figure 3.A

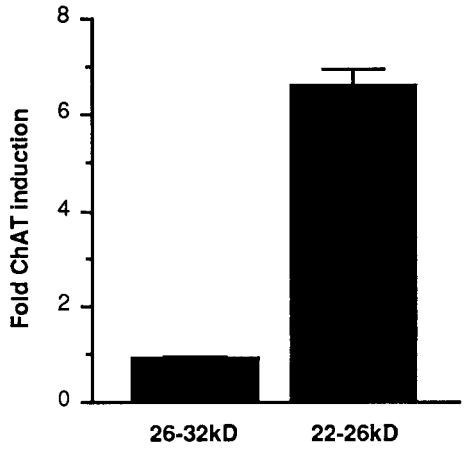
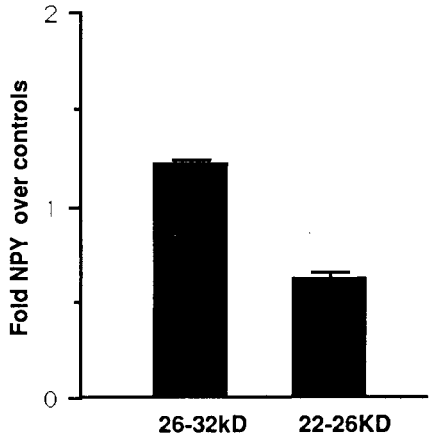


Figure 3.B



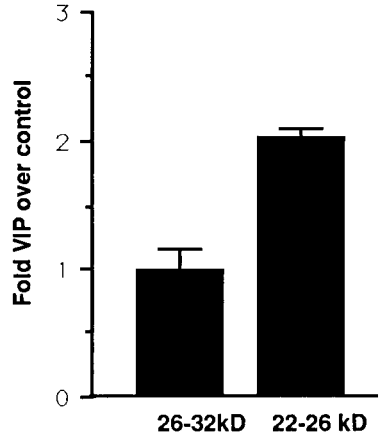


Figure 4.

Skeletal Cholinergic differentiation factor does not induce cholinergic properties in sympathetic neuron cultures.

Aliquots of the Hap 7 fraction of the skeletal cholinergic differentiation factor (0 µl, 1 µl, 5 µl, and 15 µl) were added to sympathetic neuron cultures. Seven days after the addition of the extracts, samples were assayed for ChAT activity by the method of Fonnum. Results are expressed as cpm/hr/well ± s.e.m. The cell number, seven days after addition of the Hap 7 fraction, was 1952 ± 162, 2077 ± 15, 1993 ± 28, 2033 ± 26, respectively. All samples were run in duplicate (n=three experiments).

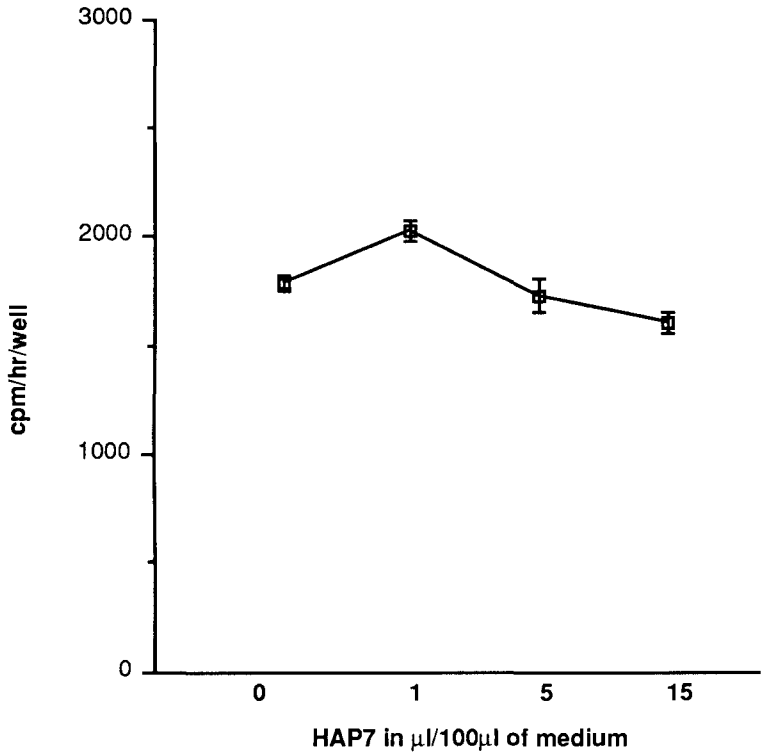

3000
2000
1000
0
cpm/hr/well
0
1
5
15
HAP7 in µl/100µl of medium

Figure 5a.

Sweat gland cholinergic-inducing activity is biochemically distinct from CDF/LIF.

Sweat gland extracts (25 μg/ml) incubated a) with LIF (25 ng/ml) or b) without rLIF was chromatographed on a DEAE column. The flow through was collected, concentrated and added to sympathetic neuron cultures. Seven days after the addition of the extracts, samples were assayed for ChAT activity. Results are expressed as fold ChAT induction ± s.e.m. over controls grown in the presence of L15CO_2 medium. All samples were run in duplicate (n=2 experiments).

Figure 5b.

Cholinergic induction by sweat gland extracts is not antagonized by depolarization.

Aliquots of a) sweat gland extract (25μg/ml), b) sweat gland extract (25μg/ml), and 30 mM KCl, c) rLIF (25 ng/ml), d) rLIF (25 ng/ml) and 30 mM KCl were added to sympathetic neuron cultures. Seven days after the addition of the extracts, samples were assayed for ChAT activity by the method of Fonnum. Results are expressed as fold ChAT induction over control cultures grown without the addition of extracts ± s.e.m. All samples were run in duplicate (n=three experiments).

Figure 5A

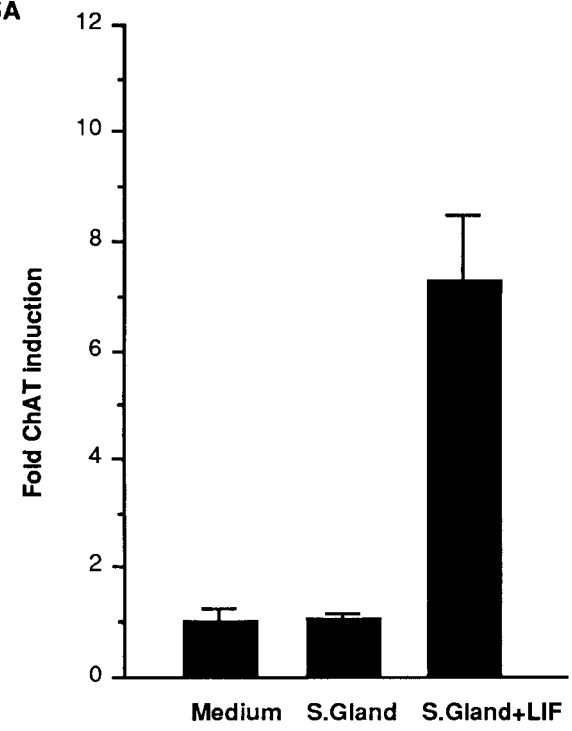


Figure 5 B

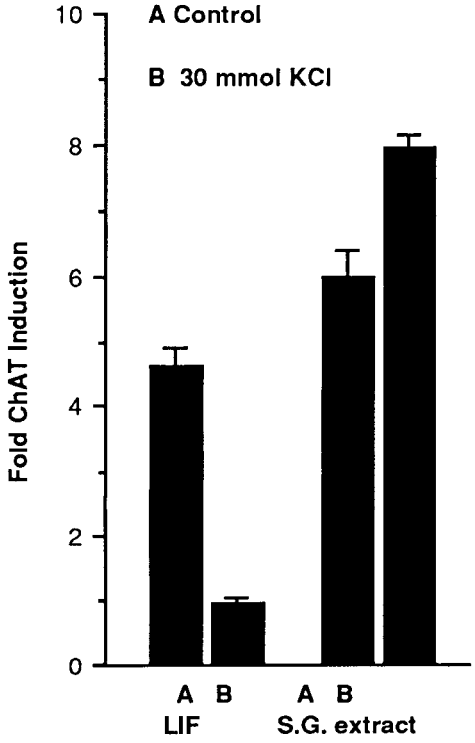


Figure 6.

The major cholinergic-inducing activity present in sweat gland extracts is not immunoprecipitated with antibodies to CDF/LIF.

a. Sweat gland extracts (DEAE fraction) were incubated with (a) protein A sepharose, (b) affinity-purified antibodies to the N-terminal sequence of CDF or (c) affinity-purified antibodies preincubated with the peptide antigen. After immunoprecipitation, supernatants were added to sympathetic neuron cultures. Ten days after the addition of extract, the cultures were assayed for choline acetyl transferase activity by the method of Fonnum. The results are expressed as the fold induction of ChAT compared to the activity observed in neurons grown in medium without extract. All samples were run in duplicate.

b. ^{125}I labeled recombinant CDF/LIF (20,000 cpm) was incubated with affinity-purified antibodies to the N-terminal sequence of CDF (1 and 3) or affinity-purified antibodies preincubated with the peptide antigen (2 and 4) in buffer (1 and 2) or with 100 µg of soluble protein extracted from adult rat sweat gland (3 and 4). Following immunoprecipitation by affinity-purified antibodies to the N-terminal sequence of CDF, the labeled proteins were extracted by boiling in SDS sample buffer and subjected to SDS-PAGE electrophoresis in a 10% gel. The labeled proteins were localized on X-ray films developed after a seven-day exposure. The arrowhead indicates a specific 20-kD band in lanes 1 and 3.

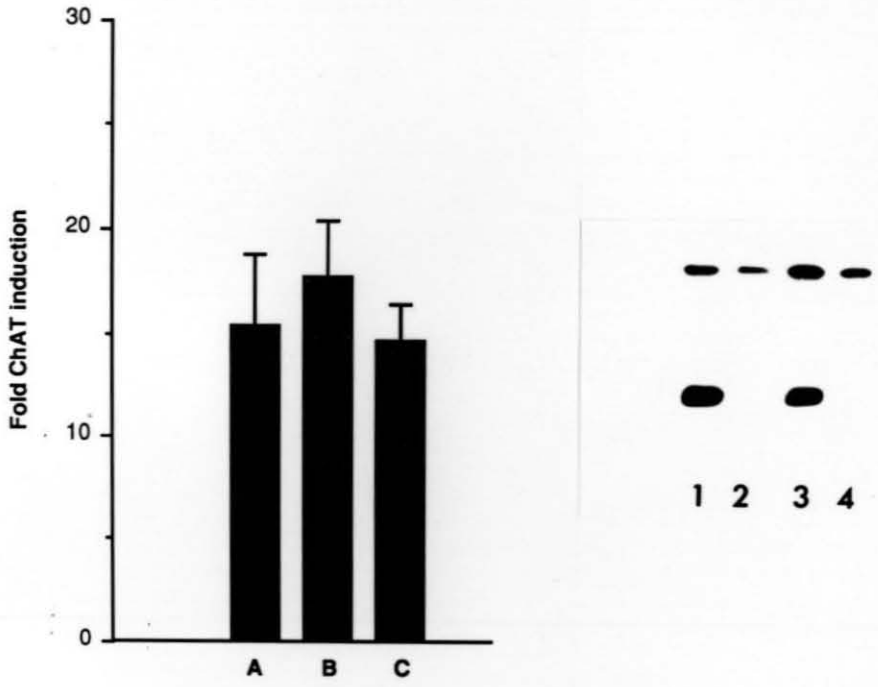

Fold ChAT induction
30
20
10
0
A
B
C
1 2 3 4

Figure 7.

The SDS fraction which has cholinergic-inducing activity also has ciliary neurotrophic activity.

The SDS gel eluate from the a) 22-26 kD fraction or the b) 26-32 kD fraction (100 ng/ml) was added to freshly dissociated chick ciliary neuron cultures. Twenty four hours later, the cultures were fixed and the number of phase bright cells with neurites was counted. Results are expressed as the number of surviving cells /well ± s.e.m. All samples were run in triplicate.

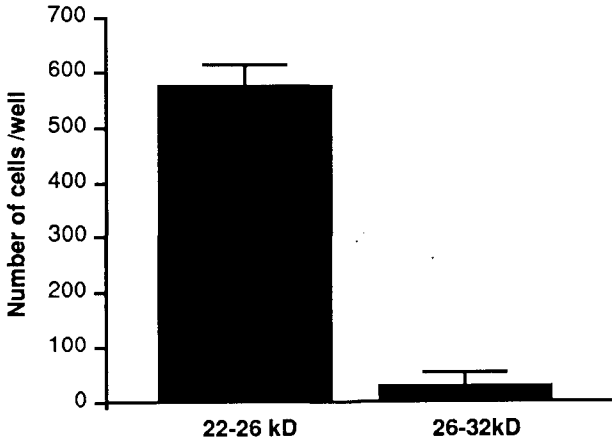
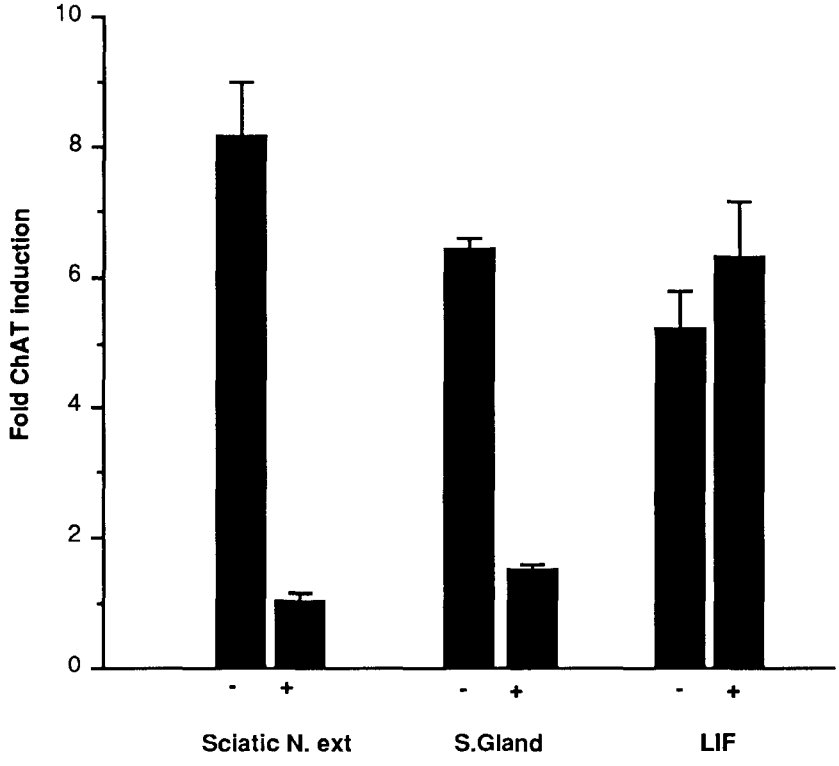
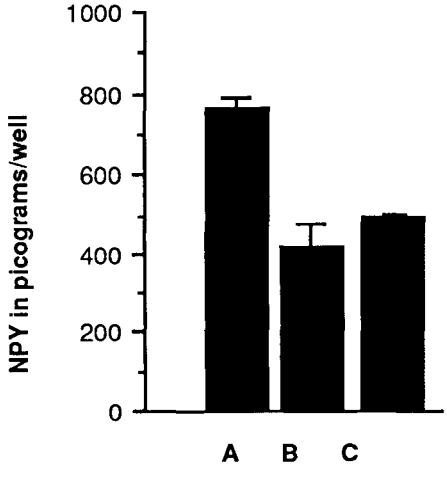

700
600
500
400
300
200
100
0
Number of cells /well
22-26 kD
26-32kD

Figure 8.

Antibodies to CNTF can partially immunoprecipitate cholinergic differentiation activity from sweat gland extracts.

In a, aliquots of sciatic nerve extract (a), sweat gland extract (b), or LIF (c) were incubated with (+) or without a polyclonal antibody generated against rCNTF. After overnight incubation, proteins specifically recognized by the CNTF antibody were adsorbed with Protein A sepharose. The supernatants were then added to sympathetic neuron cultures. Eight days after the addition of the supernatants, the samples were assayed for ChAT induction by the method of Fonnum. The results are expressed as fold ChAT induction $\pm$ s.e.m. as compared to control sympathetic neuron cultures grown in medium without the addition of extracts. All samples were run in triplicate In b., neurons were grown either (a) in medium without the addition of extract or with sweat gland extract (50 μg/ml of crude homogenate) incubated without (b) or with (c) an antiserum to rCNTF. Cultures were then assayed for NPY and VIP induction by radioimmunoassay. Results are expressed as picograms of peptide per well $\pm$ s.e.m. All samples were run in triplicate.


Fold ChAT induction
10
8
6
4
2
0
- +
- +
- +
Sciatic N. ext
S.Gland
LIF


NPY in picograms/well
1000
800
600
400
200
0
A B C

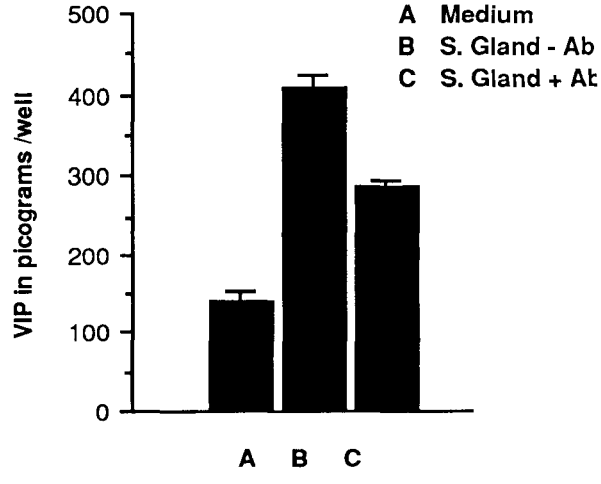

VIP in picograms /well
500
400
300
200
100
0
A B C
A Medium
B S. Gland - Ab
C S. Gland + Ab

Figure 9.

CNTF is not detectable in sweat gland extracts in Western blot assays. In panel a, 10 ng of recombinant CNTF was blotted onto nitrocellulose. In panels b and c, 60 μg of soluble protein (DEAE fractions) from sciatic nerve extract (lane 1), from hairy skin extract of adult rat (lane 2) or from sweat gland extract of adult (lane 3) or 21-day (lane 4) animals (panels b and c) were blotted onto nitrocellulose. In panels a and b, the blots were probed with a polyclonal antiserum raised against recombinant rat CNTF, while in panel c the blot was probed with antiserum preincubated with 10 μg recombinant CNTF. Panel a documents that the antiserum recognizes CNTF (arrowhead). As expected, the antiserum recognizes a 24-kD band present in sciatic nerve extracts (lane 1 b, c), but no specific bands were evident in hairy skin extracts (lane 2) or in sweat gland extracts from 21-day (lane 3) or adult (lane 4) animals. Arrowheads in b and c indicate 92, 30 and 22.5 kD standards.

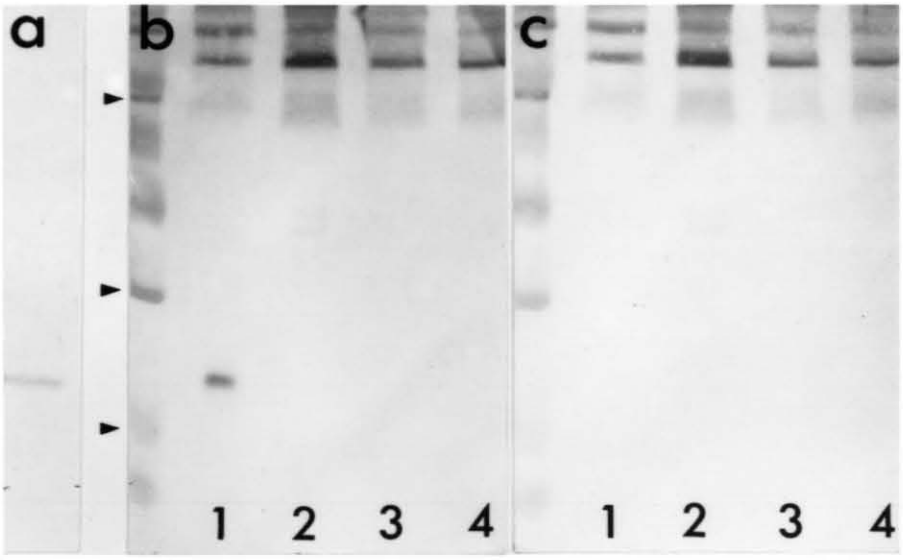

a
b
c
1
2
3
4
1
2
3
4

Figure 10.

CNTF message is not detectable in sweat gland extracts.

30μg of total RNA from (a) adult sciatic nerves, (b) sweat glands, (c) liver and (d) dorsal root ganglia was electrophorised and transferred onto nylon membrane. The membrane was then probed with an oligonucleotide probe to rat CNTF. Arrow shows a positive 1.3 kb band in lane (a), containing sciatic nerve RNA, and a fainter band in the same position in lane (d), containing dorsal root ganglia. No specific signal is detected in lanes b and c, containing sweat gland and liver RNA, respectively.

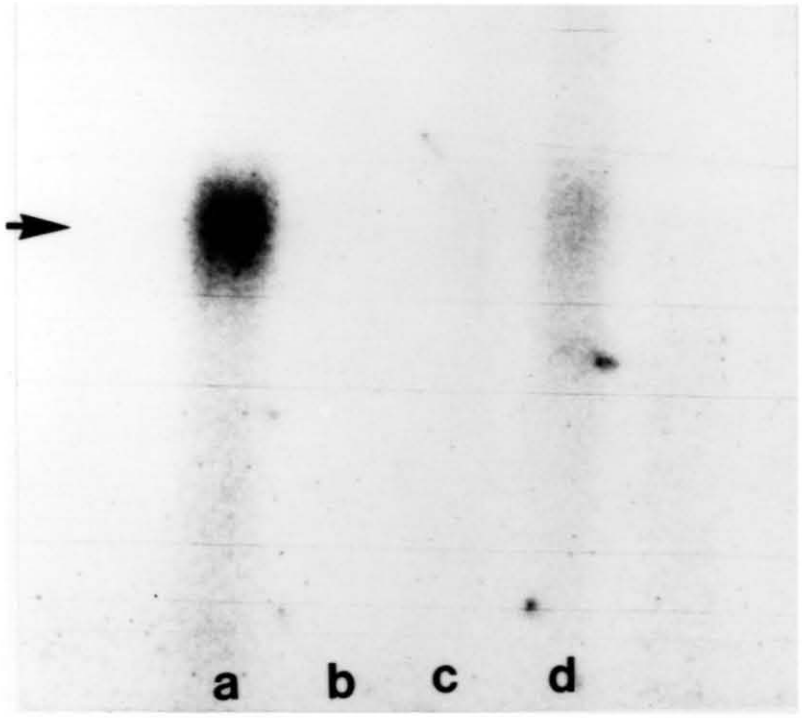
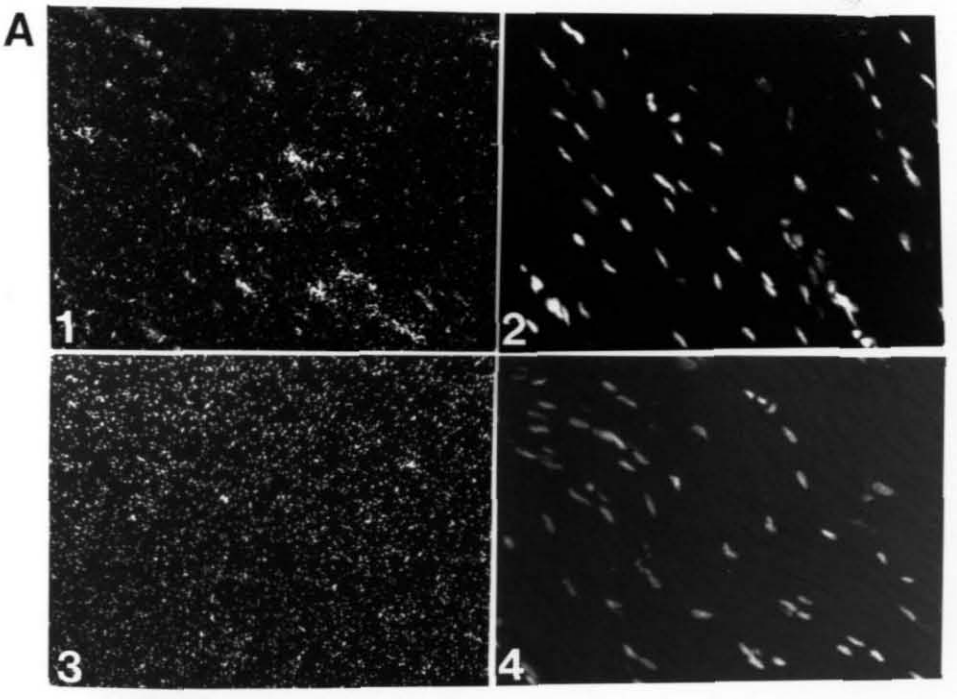

a
b
c
d

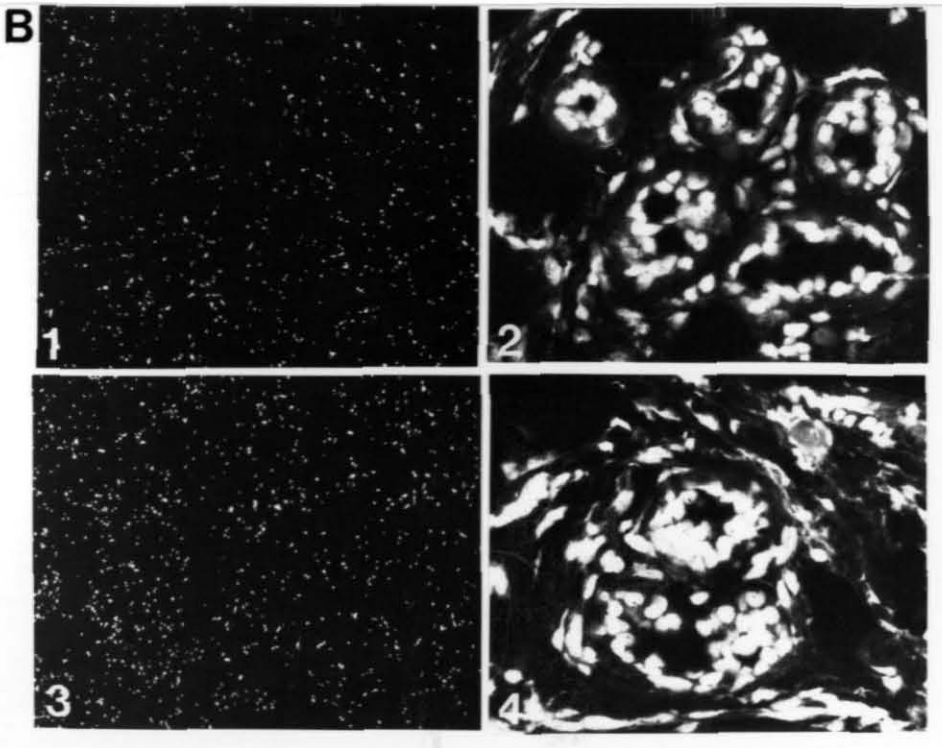
Figure 11.

CNTF message is not detectable in sweat gland extracts by *in situ* Hybridization.

Sections of sciatic nerve and sweat gland were probed with a oligonucleotide probe to rat CNTF. Panel A shows sciatic nerve sections hybridized with a antisense (1 and 2) and sense (3 and 4) oligonucleotide probe made against rat CNTF. Specific hybridization to Schwann cells is seen with the antisense probe.

In Panel B, sweat gland sections hybridized with the sense (3 and 4) and antisense probe (1 and 2) do not exhibit specific binding to the sweat glands tissue or any other cells in the glaborous skin.


A
1
2
3
4


B
1
2
3
4

Materials and Methods

Cell culture reagents were obtained from Gibco (Grand Island, NY). 24 and 96-well culture plates were purchased from Corning (Corning, NY). Affi-gel 10 was obtained from BioRad (Richmond, CA). The Centricon and centriprep filters were purchased from Amicon (Danvers, MA). ^{125}I Bolton-Hunter reagent and ^{3}H methyl choline were obtained from Amersham (Arlington Heights, Il). ^{3}H acetyl -CoA and ^{3}H tyrosine were purchased from New England Nuclear (Wilmington, DE). Dispase was purchased from Boheringer-Mannheim (Indianapolis, IN) and collagenase from Worthington Biochemicals (Freehold, NJ). Nerve Growth Factor (NGF) was prepared from male mouse submaxillary glands as described by Bocchini and Angeletti (1969). Avidin-conjugated alkaline phosphatase was obtained from Cappel (Westchester, PA) and goat anti-mouse and anti-rabbit secondary antibodies from Jackson Immunologicals (Westgrove, PA). T4 polynucleotide kinase and gamma ^{32}P, and {a ^{35}S}dATP were obtained from Amersham (Arlington Heights, Ill.) Other chemicals were purchased from Sigma (St. Louis, MO).

Cholinergic Factors

rCNTF was a kind gift of Regeneron pharmaceuticals. LIF was a kind gift of Dr. Yamamori. The cholinergic differentiation factor from skeletal muscle was obtained from Dr. Mcmanaman. The HAP7 fraction was used for all experiments (Mcmanaman et al 1989). Sweat gland extracts were prepared as described previously (see Chapter 3). Unless otherwise stated, the DEAE fraction of adult sweat gland extracts was used for all experiments

Cell culture

Cultures of rat sympathetic neurons were prepared as described by Hawrot and Patterson (1979). Neurons were dissociated enzymatically with dispase (5 mg/ml) and collagenase (1 mg/ml) and plated in 96-well polylysine-laminin coated dishes. About 1000-to-2000 neurons were plated per dish. The neurons were grown in Leibovitz's L15-CO_2 medium with NGF (100 ng/ml), 100 units of penicillin, 100 µg of streptomycin and 10 µM cytosine arabinocide and 5% rat serum and the medium changed every second day. The cholinergic factors were diluted in growth medium, sterilized by passage through a 0.2 micron filter and added to the neurons from the third day of culture on. Neurons were harvested for assay between the ninth and fourteenth days of culture.

Ciliary ganglia were dissected from E8 (embryonic day eight) chicks, dissociated and plated in DMEM with 10% fetal calf serum as described by Varon et al. (1979). Approximately 1000-to-2000 neurons were plated directly into medium in 96 well cell culture plates that had been coated sequentially with polylysine and laminin. The cultures were incubated for twenty four hours at 37° C and then fixed with 2% glutaraldehyde as described by Barbin et al. (1984). The number of surviving neurons was determined by counting the phase bright cells.

Iodination

Iodination was carried out by the Bolton-Hunter method (1973). 1-5 µg of protein in 10 µl of 0.1M borate buffer pH 8.5 was added to the dried iodinated ester and the reaction was allowed to proceed for 15 minutes at 0°C, and then for 15 minutes at room temperature. 100 µl of 0.2 M glycine was added to stop the reaction. The labeled protein was separated from the unreacted label by dialysis using a Centricon microconcentrator with a 10 kDa cutoff. Labeling efficiency was between 40-60%. Labeled fractions were stored at 4°C after adding 0.2 mg/ml of BSA to prevent loss due to adsorbtion.

Immunoprecipitation

For the immunoprecipitation experiments in which biological activity of the factors was subsequently tested, aliquots of CNTF, LIF and sweat gland extracts (DEAE fraction) sufficient for a cell culture assay were added to buffer (PBS pH 7.3 with 2% BSA, 0.2% Triton X-100, and 0.02% PEG 6000). Affinity-purified antibody against LIF (Rao et al., 1990) or a polyclonal antibody generated against rCNTF (a kind gift of Dr. Donna Marrissey, Regeneron Pharmaceuticals) was added to each vial to a final concentration of 10 µM or a final dilution of 1:4, respectively. After an overnight incubation, the antigen-antibody complex was absorbed to 10 µl of protein A Sepharose for a further two hours at room temperature. The bound complexes were separated by centrifugation and the supernatant was diluted into L15CO_2 medium and used for cell culture assays. Two controls were performed to insure that the loss of activity consequent to absorption was due to a specific effect of the antibody. Aliquots of the cholinergic factors were incubated without the antibody and treated as described above and for the LIF antibody experiments other aliquots were treated with antibody that had been previously adsorbed with 50 µM of the synthetic peptide originally used as antigen.

Immunoprecipitation of labeled fractions

A similar procedure was used for immunoprecipitation experiments with labelled fractions. After precipitation with protein A Sepharose, the radioactivity in the pellet was measured with a gamma scintillation counter. To determine the molecular weight of the protein bands immunoprecipitated by the affinity-purified antibody, the protein A Sepharose pellet was boiled in sample buffer for 5 minutes and the extracted material was subjected to SDS-PAGE. Labeled bands were visualized by autoradiography.

CNTF Western Blotting

Aliquots of extracts (60 µg/lane) were run on a 15% SDS-PAGE minigel (BioRad) and the proteins were blotted onto nitrocellulose (overnight 30mA). The nitrocellulose blots were blocked in blocking buffer (5% defatted milk in Tris buffered saline pH 7.2) and then incubated for two hours with a polyoclonal antibody against CNTF (1:1000 dilution) or with the antibody preincubated with 10 µM rCNTF. The blots were then sequentially incubated with a biotinylated secondary antibody (one hour) and avidin conjugated to alkaline phosphatase (30 min). The bound enzyme was detected with NBT (Nitroblue tetrazolium) and BCIP (Bromo chloro indoyl phosphate) in 10 mM bicarbonate buffer pH 9.5. After optimal color development, the reaction was stopped by rinsing in distilled water.

Northern Blots

Total RNA was prepared from liver, sweat gland and sciatic nerve using the single step guadinium-isothyocyanate method (Chomczynski and Sacchi 1987). 30µg of total RNA was loaded per lane and transferred to a genescreen nylon membrane. Blots were probed with a 45 base pair oligonucleotide probe against rat CNTF sequence (from 99-144). The probe was labelled at the 5' end using T4 polynucleotide kinase and gamma ^{32}P. Blots were sequentially washed and then examined by autoradiography.

In situ *hybridization*

The probe used in the Northern blot experiments was also used for *in situ* hybridization. It was labeled at the 3' end using terminal transferase and {a ^{35}S}dATP. The *in situ* hybridization experiments were performed as described by Siegel (1989). Briefly, fresh frozen sections of tissue were fixed in 4% formaldehyde for five minutes, rinsed three times in PBS, incubated in acetic anhydride/triethanolamine for 10 minutes, rinsed two times briefly in 2X SSC, and finally dehydrated through a series of graded alcohols. The slides were air-dried and processed for hybridization. After a one

hour prehybridization incubation, hybridization was performed at room temperature for 15 hours in humidified containers. After washing, the sections were dipped in Kodak NTB-3 emulsion and exposed for six weeks. Sections were developed, fixed and counterstained with ethidium bromide.

Fonnum assay

The induction of cholinergic function was determined by assaying choline acetyltransferase (ChAT) activity in homogenates, essentially according to the method of Fonnum (1969). To increase the sensitivity of the assay, an incubation period of an hour was used. All of the activity was inhibitable by 500 μMol napthylvinyl pyridine, a specific inhibitor of CHAT activity. Protein concentration was assayed by the method of Lowry, with BSA as a standard.

Peptide Assays

Neuropeptide levels were determined by radioimmunoassay. Cultures were rinsed once with phosphate buffered saline (PBS) and then homogenized in 100 ml of 2M acetic acid. After boiling for five minutes, samples were centrifuged for one minute in an Eppendorf microfuge. The supernatants were dried under vacuum and stored at -70^{o}C for subsequent assays. VIP was assayed using a kit obtained from INCSTAR (Stillwater, MN) with primary antibodies previously demonstrated to show minimal crossreactivity with other peptides. To assay NPY by radioimmunoassay, antibodies, standards and labelled tracer were obtained from Amersham (Arlington Hts, Il) and peptide content was determined by the delayed tracer method. Since the antibody shows only 64% cross reactivity with rat NPY, standards were also run with rat NPY (Peninsula Laboratories) and sample values read off the standard curve.

Column chromatography

Sweat gland homogenates (soluble fractions) were pooled and concentrated using a centriprep microconcentrator (Amicon). The final buffer concentration of the sample was 10mM PO_4 pH 7.0. 10 mg of protein was chromatographed on a MonoQ column (Pharmacia- Sweden) using a Whatman HPLC. 0.5 ml fractions from a 0-1M NaCl gradient were collected and assayed for biological activity.

Peak activity from different runs on the Mono Q column were pooled and and made to pH 7.0 Samples were injected on a Mono P HR 5/5 column (Pharmacia- Sweden) equilibrated with 0.025 M bis-tris pH 7.1. Fractions were eluted with polybuffer made to pH 4.0 with a saturated solution of iminodiacetic acid. Unbound protein and 0.25 ml

fractions between a 4.0 to 7.0 pH gradient were collected and the pH in the eluted sample was determined. Samples were stored at -70 degrees celsius until used. No attempt was made to remove the polybuffer as separate experiments had shown that it did not harm the neuronal cultures.

Peak fractions from the Mono P run (pH 4.9-5.2) were pooled and chromatographed on a Sepharose 12 column (Pharmacia) equilibrated in 100mM NaCl and 10 mmPO buffer. 0.5 ml fractions were collected and assayed for biological activity. The retention time of the peak activity was compared to protein standards (Sigma) chromatographed under identical conditions.

Elution of proteins from SDS-PAGE

The active peak from the Mono P column run was electrophorised on a 15% preparative polyacrylamide gel using a Biorad gel apparatus. The appropriate regions of the gel were sliced and loaded onto a Biorad electroeluter. Proteins were electroeluted (10 mA/tube) for a period of four hours in elution buffer (50 mM Ammonium bicarbonate, 0.1% SDS). Samples were concentrated and the SDS was precipitated by adding KCl to a final concentration of 100 mM as described by Suzuki and Terada (1988). 1% BSA was added to the samples which were then dialyzed using a centricon (10 Kd cutoff) to remove the excess KCl. Samples were stored at -70^{o} C until used.

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