Cytokine Control of Neuronal Phenotype

Thesis by

Ming-Ji Fann

In Partial Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy

California Institute of Technology
Pasadena, California
1994
(Submitted May 12, 1994)

Acknowledgments

This was a great experience for me to study at Caltech. I gained so much understanding in both science and life from so many people that I have no way to say "Thanks" to everyone for helping me.

First, I would like to thank Paul Patterson for his advice and support during my research. Sometimes I wonder how he obtained so many cytokines from so many different sources. I will always remember his enthusiasm for science and persistent search for the truth.

I would also like to thank several other faculty members for guiding me to the field of neuroscience. The Introductory Neurobiology course given by David Van Essen and Mark Tanouye directed my mind towards neurobiology. Ion Channel courses, both lecture and laboratory, given by Henry Lester were fascinating. What I learned from Paul's Developmental Neurobiology course was very useful for my research. I am grateful to my thesis committee members, David Anderson, Norman Davidson, Paul Sternberg, and Kai Zinn, for giving me valuable suggestions at critical times. The range of their knowledge seems to have no bounds.

The members of the Patterson group were always so warm to me that I did not experience too much homesickness. Nagesh Mahanthappa taught me neuronal culture and helped me overcome the difficulties of the first year. Doreen McDowell prepared the cultured medium and maintained the laboratory. Bill Lease supplied me with critically needed equipment on several occasions. Tetsuo Yamamori introduced molecular biology techniques to me. Whenever I got into trouble, both personal and professional, I could always go to Zaven Kaprielian for the best solution. Michael Hadjiargyrou and Lisa Banner were good companions when I needed persons to consult. Thanks especially to Susan Ou, who cheered me up all the time. She was so generous to me that whenever I needed the computer, her office became mine.

Thanks also to Kangsheng Wang, who made living in the States easier; to Derek Stemple, who gave me good advice; to Maribelle Denslow, who taught me first-handedly what life should be.

Finally, I would like to thank Li-Ann Lai. She adjusts her own dreams to fit mine without any complaint. I am a lucky man to have her.

Abstract

Diffusible proteins regulate neural development at a variety of stages. Using a novel neuronal culture assay, I have identified several cytokines that regulate the expression of neurotransmitters and neuropeptides in sympathetic neurons. These cytokines fall into two families. The first group is termed the neuropoietic cytokines, while including CDF/LIF, CNTF, OSM and GPA, induces expression of the same set of neuropeptide mRNAs in cultured sympathetic neurons. These four factors not only exhibit similar biological activities; they also share a predicted secondary structure and bind to a signal-transducing receptor subunit in common with IL-6 and IL-11. The latter two cytokines display a weaker activity in this assay. In addition, I find that several members of the TGF-β superfamily, activin A, BMP-2, and BMP-6, have a selective overlap with the neuropoietic family in the spectrum of neuropeptides that these cytokines induce in sympathetic neurons. Different patterns of neuropeptides induced by the TGF-β family members, however, demonstrate that the activities of these cytokines are distinct from those of the neuropoietic family. Another 30 cytokines are without detectable effect in this neuronal assay.

Activin A induces a set of neurotransmitters and neuropeptides that is somewhat similar to the phenotype of sympathetic neurons innervating sweat glands in rat footpads. *In situ* hybridization and RNase protection were carried out to test whether activins were involved in the phenotypic transition when sympathetic neurons contact sweat glands. I find that activin mRNA is present in both cholinergic and noradrenergic targets. Moreover, homogenates of footpads do not contain activin-like activity in the neuronal assay *in vitro*. Taken together, these data do not support activins as the best candidates for the sweat gland factor.

Several novel factors that regulate neuropeptide expression exist in heart cell conditioned medium. I attempted to purify these factors in collaboration with Dr. Jane Talvenheimo. Our results suggest that these factors are sensitive to the storage conditions used. Several modifications of purification strategy are discussed.

Table of Contents

List of Illustrations

CHAPTER 1. Introduction

The development of the nervous system involves several stages, from initial proliferation of progenitor cells, to differentiation and migration of these cells, through the outgrowth of neurites and formation of synapses, to the rearrangement of connections. The goal of this sequence is to build an appropriate information network by which neurons can communicate with their targets to control body functions. The task of building this network is tremendously complex, given the enormous number of neurons in vertebrates, and their morphologically and molecularly extreme diversity. Mechanisms for each step are, however, beginning to be elucidated. For example, the variety of cell types is controlled, in part, by cell cycle-dependent intrinsic changes (McConnell, 1992), contact with extracellular matrix and cell surface molecules (Bronner-Fraser, 1992; Stemple and Anderson, 1993) and diffusible proteins (Jessell and Melton, 1992).

In addition to the physical meeting of appropriate partners, functional communication between neurons and their targets requires that the presynaptic expression of neurotransmitters and neuropeptides is matched with the proper receptors for decoding these signals postsynaptically. There are approximately a dozen classical transmitters and over 75 neuropeptides currently known, and new candidates are continuously being discovered in the nervous system. A recent example is agmatine, which binds to α_2-adrenergic and imidazolin receptors and stimulates release of catecholamines from adrenal chromaffin cells (Li et al., 1994). Not only is there a very large repertoire of transmitters and neuropeptides, but individual neurons produce reproducible combinations of these molecules (Gibbins and Morris, 1987; Lundberg and Hokfelt, 1986), which further complicates the developmental task. How neurons "choose" a particular set of transmitters and neuropeptides is therefore an intriguing problem. Recent studies have highlighted how neuronal phenotypic decisions can be based on neuronal cell lineage as well as on environmental cues and instructive differentiation signals (Patterson and Landis, 1992).

Phenotype based on lineage

Because of the ease in identifying specific stem cells and following their progeny during development, insects have provided valuable insights into the role of cell lineage in determining transmitter phenotype. In the locust, Taghert and Goodman (1984) reported that only a subpopulation of the progeny of neuroblast 7-3 uses serotonin as a transmitter. Neuroblast 7-3 divides three times *in situ*, generating six neurons. However, only the progeny of the first two cell divisions express serotonin, suggesting that birth order is involved in establishing this phenotype. The expression of GABA is also confined to particular progeny in the thoracic ganglion of the tobacco hornworm (Witten and Truman, 1991a). These two examples illustrate that a particular transmitter may be restricted to subsets of neurons in discrete lineages. Further evidence that cell lineage is involved in phenotypic choice was demonstrated by ablating of neuroblasts that give rise to progeny expressing specific transmitters. Work by Sulston and Horvitz (1977) in the nematode, Stuart et al. (1987) in the leech, Targhert and Goodman (1984) in the locust, and Witten and Truman (1991b) in the moth showed that, if the appropriate neuronal stem cells are removed, the nervous system does not compensate for the loss, and the transmitter phenotype (dopamine, GABA or serotonin) disappears from that region of the central nervous system (CNS).

Studies using retroviruses as cell lineage tracers have shed light on the role of lineage in determining neuronal identity in the vertebrate CNS. In the cerebral cortex there are two main populations of neurons, the pyramidal and the nonpyramidal cells, which respectively adopt glutamate and GABA as their transmitters (Fagg and Foster, 1983). Clonally related neurons, marked by retrovirus injection into rat telencephalic ventricles at any time point between embryonic day 14 and day 19, display remarkable homogeneity, expressing either of the two types of morphology and its corresponding transmitter (Mione et al., 1994). This restriction of morphology and transmitter is specific to those traits, as clonal relatives express different calcium binding proteins. Since clonally-related neurons in the cerebral cortex display widespread migration and are presumably exposed to different environments (Walsh and Cepko, 1992), this result suggests that commitment of cortical neurons to a particular phenotype takes place in the ventricular zone, where progenitor cells are still actively dividing, and that lineage plays a role in determining this aspect of phenotype in cortical neurons.

Influence of environmental signals

Although lineage tracing experiments reveal that neuronal properties can be specified at the progenitor cell stage, a role for cellular interactions in specifying phenotype is not ruled out. In the *Drosophila* ommatidium, differentiation choices are invariant under normal conditions. Nonetheless, cell-cell interactions are required to generate the final identical pattern of photoreceptor cells (Cagan and Zipursky, 1992). Analysis of the vertebrate peripheral nervous system (PNS) further illustrates that environmental cues, especially interactions between neurons and their targets, modulate neuronal phenotypic choices (Patterson, 1978; Patterson and Landis, 1992; Patterson and Nawa, 1993).

Evidence supporting that environmental cues influence neuronal phenotype

The avian parasympathetic ciliary ganglion contains two distinct populations of neurons: choroid neurons, which innervate vasculature smooth muscle in the eye's choroid layer, and ciliary neurons, which innervate striated muscle of the ciliary body and iris (Marwitt et al., 1971). Both populations of neurons utilize acetylcholine (ACh) as a transmitter (Martin and Pilar, 1963; Meriney and Pilar, 1987). However, choroid neurons, but not ciliary neurons, express somatostatin (SOM) (Epistein et al., 1988). Since both neurons are derived from the neural crest (Narayanan and Narayanan, 1978), develop presumably in the same ganglionic environment, and receive the same preganglionic input from the Edinger-Westphal nucleus, the expression of SOM in the choroid neurons suggests that the target tissues are involved in generating the difference in neuropeptide expression. The expression of ACh may also be specified by the target, because the developmental increase in the ACh-synthetic enzyme (choline acetyltransferase; ChAT) activity coincides with innervation of the eye (Coulombe and Bronner-Fraser, 1990). Different phenotypic traits expressed in neurons with soma in the same location but with axons innervating different targets also occur in sympathetic ganglia (Elfvin et al., 1993; Gibbins, 1990). For instance, neurons in the superior cervical ganglion sending axons to blood vessels usually contain NPY, but those with axons innervating salivary glands and fat cells do not, even though

both populations of neurons use noradrenaline as a transmitter (Cannon et al., 1986). Thus target tissues may induce some neuronal traits *de novo.*

One of the most intensively studied examples is the sympathetic innervation of sweat glands in the rat; these neurons switch phenotypes from noradrenergic to cholinergic when their axons reach the target, demonstrating postmitotic plasticity (Landis, 1990). Before sympathetic axons first contact the developing sweat gland in neonatal rats, they exhibit catecholamine histofluorescence and express the noradrenaline synthetic enzymes, tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH). As the gland innervation matures, catecholamine histoflurescence diminishes and expression of TH and DBH decreases gradually (Landis and Keefe, 1983). At the same time, these axons begin to express ACh and novel neuropeptides. ChAT is first detectable at postnatal day 11 (P11) (Leblanc and Landis, 1986) and vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) immunoreactivities can be demonstrated at P10 and P21, respectively (Landis et al., 1988). The induction of these neuropeptides is specific, because there is no detectable expression of neuropeptide Y (NPY), somatostatin (SOM), substance (SP), or leu- and met-enkephalin (ENK) in either developing or mature sweat gland axons (Landis et al., 1988). The close association in the appearance of ChAT and VIP raises the possibility that these two genes are regulated coordinately, while expression of CGRP may be regulated by a different mechanism (Patterson and Landis, 1992). Several studies indicate that this transition occurs in a single population of axons. First, the axons innervating sweat glands retain TH and DBH immunoreactivities and the catecholamine uptake system in adulthood, although at much reduced levels. Thus, coexistence of cholinergic and noradrenergic traits in single axons can be detected during the phenotypic transition (Schotzinger and Landis, 1988). Second, the expression of ChAT and VIP is not observed in the adult glands if neonatal rats are treated with 6-hydroxydopamine to destroy catecholaminergic neurons. This indicates that cholinergic and VIP-positive neurons are derived from initially catecholaminergic neurons (Yodlowski et al., 1984).

Transplantation as a tool to define neuronal plasticity and environmental influences

The role of the sweat gland in regulating the transmitter and neuropeptide phenotype of innervating sympathetic axons has been further explored with transplantation experiments. When the superior cervical ganglion (SCG), which contains noradrenergic neurons and does not innervate sweat glands normally, is transplanted to the anterior chamber of the eye together with developing sweat glands, the neurons initially exhibit catecholamine histofluorescence that declines with further development. After four weeks, ChAT and VIP immunoreactivities become evident in these neurons (Stevens and Landis, 1990). These results suggest that, as in the neurons that innervate sweat glands *in situ*, noradrenergic traits are suppressed and cholinergic function is induced in SCG neurons upon innervating the glands in the eye. Moreover, cross-innervation experiments provide direct evidence for the role of the target. When footpad tissue containing sweat glands is transplanted in place of hairy skin that normally receives noradrenergic sympathetic innervation, sympathetic axons innervating sweat glands express cholinergic traits four weeks later (Schotzinger and Landis, 1988). Conversely, if sweat glands are replaced with the parotid gland, a target of noradrenergic sympathetic neurons, the innervation of the parotid in the footpad does not display a cholinergic phenotype. Instead, these sympathetic neurons maintain the noradrenergic phenotype, which they would have lost during normal development (Schotzinger and Landis, 1990). Thus, the expression of cholinergic properties in the sweat gland innervation depends on the presence of this particular target, and sweat glands are able to alter the phenotype in sympathetic neurons that do not normally contact the glands (Landis, 1990). This phenomenon that targets have effects on neuronal phenotype is not restricted to vertebrates. Similar transplantation studies also demonstrate that the morphological and physiological properties of Retzius neurons in the leech are determined by their peripheral targets (French and Kristan, 1992).

Phenotypic plasticity and target influences are not restricted to the embryo or to the young animal. When skin sensory fibers are made to innervate muscle in adult rats, these axons lose substance P (SP) immunoreactivity. Conversely, SP immunoreactivity appears if muscle sensory axons are made to innervate skin (McMahon and Gibson, 1987). When adult SCG neurons are placed in culture in the presence of heart cell

conditioned medium, these neurons change phenotype from noradrenergic to cholinergic (Patterson, 1978). Thus, neurons can respond to environmental cues in the adult animal.

Further evidence supporting the role of environmental influences is provided by results from other transplantation experiments. For example, when postmitotic cholinergic ciliary neurons are labeled and injected into the chick embryo in the region of active neural crest migration, some of these cells migrate to adrenal medulla and sympathetic ganglia primodia and express catecholamine histofluorescence (Coulombe and Bronner-Fraser, 1986). In the rat embryo, neocotical neurons transiently express TH. If early embryonic neocotical neurons are transplanted into adult neocortex, they continually express TH (Park et al., 1986), suggesting inhibitory factors may be present in the perinatal but not adult neocortex that suppress TH expression. Implantation and integration of multipotent neural cell lines into different regions of the brain in neonatal rodents has recently been demonstrated. Most strikingly, the grafted cells differentiate into neurons or glia, and exhibit appropriate morphologies and intercellular connections, according to the developmental stage and site of injection (Renfranz et al., 1991; Snyder et al., 1992). This region-specific differentiation of multipotent cells suggests that commitment and differentiation of progenitor cells in the CNS is controlled in part by local cell-cell interactions.

Molecules that specify transmitter and neuropeptide phenotype

Several factors have been characterized in terms of their ability to modulate neuronal phenotype. These include small molecules, such as hormones, peptides and transmitters, as well as proteins, such as cytokines and growth factors (Patterson, 1993; Patterson and Landis, 1992; Patterson and Nawa, 1993).

The effects of small molecules

Changes in expression of transmitters and neuropeptides have been correlated with natural fluctuations of hormones during metamorphosis and the estrous cycle. In the hawkmoth, *Manduca sexta*, abdominal lateral neurosecretory cells produce both a cardioacceleratory peptide and a low level

of the tanning hormone, bursicon. During metamorphosis, neuronal gene expression changes so that only bursicon is produced by these neurons (Tublitz and Sylwester, 1990). This alteration is initiated by both prepupal peaks of 20-hydroxyecdysone and a decline of the juvenile hormone (Weeks and Levine, 1990). In female rats, the expression of galanin (GAL) in the luteinizing hormone-releasing hormone (LHRH)-containing neurons of the medial preoptic area is regulated according to the estrous cycle. These neurons express higher levels of GAL during proestrous than during estrous, while expression of co-localized LHRH does not show cyclic changes (Merchenthaler et al., 1991). Ovariectomy results in a significant decrease in the number of cells expressing GAL, and estradiol treatment of such animals restores the number of GAL-containing cells to normal levels. In male rats, estradiol injection does not enhance expression of GAL in LHRH-containing neurons. Apparently, neonatal hormonal imprinting is involved in the process (Merchenthaler et al., 1993). A similar example is the differential regulation of cholecystokinin and SP in the amygdala by estrogen (Simerly, 1990).

Evoked activity through release of transmitters and neuropeptides from presynaptic neurons can also influence postsynaptic neuronal phenotype. For example, olfactory afferent innervation is important in the maintenance of the dopamine phenotype of olfactory bulb neurons. Deprivation of odorant stimulation decreases TH expression without cell loss or change of expression of GABA or aromatic L-amino acid decarboxylase that coexist with TH in olfactory bulb neurons (Baker, 1990). This *in vivo* manipulation can be replicated *in vitro*. TH expression is enhanced when olfactory bulb neurons are co-cultured with olfactory epithelial neurons. This effect can be mimicked by administrating CGRP, which is a peptide produced and released by olfactory epithelial neurons (Denis-Donini, 1989).

In tissue culture, constant depolarization by elevated extracellular potassium concentration increases the expression of TH, ENK, NPY, SOM, VIP in various neurons (Goodman, 1990; Morris et al., 1988; Tolon et al., 1994; Zigmond, et al., 1989). In denervated sympathetic ganglia, however, depolarization blocks the induction of SP expression (Roach et al., 1987). Direct application of transmitters or neuropeptides can also affect neuronal phenotype. Adding noradrenaline to cultured rat pineal cells suppresses the

expression of serotonin (Araki and Tokunaga, 1990). Noradrenaline has a positive feedback effect on its synthesis by upregulating expression of TH and DBH in cultured quail neural crest cells (Zhang and Sieber-Blum, 1992). CGRP, VIP, SP, SOM and substance K also regulate TH activity in catecholaminergic neurons (Denis-Donini, 1989; Friedman et al., 1988; Kessler et al., 1983; Zurn et al., 1993). In adrenal chromaffin cells, factors differentially regulate expression of ENK and the adrenaline-synthesizing enzyme, phenylethanolamine N-methyltransferase (PNMT). The expression of PNMT, but not ENK, is induced by glucocoticoids. Conversely, VIP, through an increase of intracellular cAMP, induces ENK but not PNMT (Wan et al., 1991).

A peptide of 11 amino acids has been purified and sequenced from hippocampal extracts by virtue of its ability to stimulate ACh synthesis in medial septal nuclei in explant culture. This peptide is designated as hippocampal cholinergic neurostimulating peptide (HCNP). Chemically-synthesized HCNP induces ACh synthesis of cultured medial septal nuclei in a dose-dependent manner, but has no effect on cultures of corpus striatum or anterior spinal cord (Ojika et al., 1992). Since explants were used in the experiment, the effect of HCNP could be due to an indirect effect via glia or to differential survival effect on cholinergic neurons. The gene for HCNP has not been cloned yet, and its tissue distribution has not been published.

The effects of cytokines and growth factors

Given the evidence that target tissues can control the transmitter phenotype of the neurons that innervate them, it is reasonable to assume that there are target-derived factors that act as trans-synaptic, retrogradely transported, phenotype-specifying agents. Soluble factors are more likely in some cases, because neurons do not physically contact certain target tissues, such as sweat gland cells (Landis and Keefe, 1983; Quick et al., 1984; Uno and Montagna, 1975). Cell culture of neurons has been used to characterize and identify several factors that regulate neuronal gene expression. These factors include members of the neurotrophin, neuropoietic, and TGF-β families.

Neurotrophins. Originally identified as a survival factor for sensory and sympathetic neurons (Levi-Montalcini and Hamburger, 1951), nerve growth factor (NGF) was the first member of the neurotrophin family that now

contains at least four proteins (Altin and Bradshaw, 1993; Thoenen, 1991). In addition to acting as a survival factor, NGF can enhance neuronal differentiation and trigger mitosis in chromaffin cells (Claude et al., 1988; Frodin and Gammeltoft, 1994; Stemple et al., 1988). Neurotrophins can also act as instructive factors to regulate expression of transmitters and neuropeptides. For example, NGF selectively induces the expression of particular neuropeptides in sensory neurons (Lindsay and Harmar, 1989). Similarly, brain-derived neurotrophic factor induces the expression of SOM and NPY by directly acting on cultured cortical neurons without affecting neuronal survival (Nawa et al., 1993).

Neuropoietic cytokines. In addition to the neurotrophins, several other families of proteins have both neuronal survival and differentiation activities. The cholinergic neuronal differentiation factor (CDF) is a protein initially purified from heart cell conditioned medium by virtue of its ability to change cultured sympathetic neurons from a noradrenergic to a cholinergic phenotype without affecting survival or growth (Fukada, 1985; Patterson and Chun, 1977). When the cDNA for CDF was cloned and sequenced, it turned out to be identical to leukemia inhibitory factor (LIF) (Yamamori et al., 1989). CDF/LIF is a pleiotrophic cytokine, regulating cell proliferation and differentiation in a variety of tissues (Metcalf, 1992). CDF/LIF has recently been shown to also act as a survival factor for embryonic sensory and motor neurons (Martinou et al., 1992; Murphy et al., 1991). Conversely, ciliary neurotrophic factor (CNTF), which was characterized as a survival factor for ciliary neurons (Alder et al., 1979), was shown to switch cultured sympathetic neurons from a noradrenergic to a cholinergic phenotype (Saadat et al., 1989). In addition to inducing ChAT and decreasing TH expression, both CDF/LIF and CNTF show very similar activities on neuropeptide expression, inducing SP, SOM, and VIP in cultured sympathetic neurons (Nawa et al., 1991; Nawa et al., 1990; Rao et al., 1992d), and acting as survival factors for motor neurons (Arakawa et al., 1990; Martinou et al., 1992; Sendtner et al., 1990). In addition, CDF/LIF and CNTF sustain survival and differentiation of glial cells. The oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells in the optic nerve and cerebral cortex can develop into oligodendrocytes or type-2 astrocytes under appropriate conditions (Raff, 1989). CNTF triggers conversion of

progenitor cells to type-2 astrocytes (Hughes et al., 1988), although this effect is transient and other factors are required to drive the process to completion. Both CDF/LIF and CNTF also promote survival and maturation of cultured oligodendrocytes, and induce myelin basic protein expression (Barres et al., 1993; Louis et al., 1993). CNTF also mimics the effects of CDF/LIF on embryonic stem cells and hepatocytes (Conover et al., 1993; Schooltink et al., 1992). Despite there being many biological activities in common, the primary sequences of CDF/LIF and CNTF contain very limited homology (Lin, et al., 1989; Stockli et al., 1989; Yamamori et al., 1989). Nonetheless, Bazan (1991) predicted that CDF/LIF and CNTF, together with IL-6, oncostatin M (OSM), and granulocyte colony-stimulating factor (G-CSF), share similar secondary structures (Bazan, 1991). The cloning of receptors for this family showed that CDF/LIF, CNTF, IL-6, OSM, and IL-11 utilize the same protein, gp130, as a signal transduction subunit in the receptor complex (Gearing et al., 1992; Ip et al., 1992; Kishimoto et al., 1994; Yin et al., 1993). In addition, OSM and CDF/LIF share the same receptor complex, as OSM competes with CDF/LIF for receptor binding with high affinity (Gearing and Bruce, 1991; Gearing et al., 1992). Signal transduction pathways involving tyrosine phosphorylation have been characterized for these factors (Lutticken et al., 1994; Stahl et al., 1994), and stimulation of immediate early gene expression has been identified (Ip et al., 1992; Kishimoto et al., 1994; Lord et al., 1991; Yamamori, 1991). Not surprisingly, CDF/LIF, OSM, IL-6, and IL-11 have similar bioactivities in various tissues (Kishimoto et al., 1992). Prior to the present work, however, the effects of OSM, IL-6, and IL-11 on neuronal phenotype had not been explored in detail. Preliminary result from Rao et al. (1992c) showed that OSM induces VIP expression in neuroblastoma cells.

Three other neuronal differentiation factors are related to CNTF. Growth promoting activity (GPA) was purified from chick eye and has the same ciliary neurotrophic activity as mammalian CNTF (Eckenstein et al., 1990). Upon cloning, GPA was found to be approximately 50% identical to rat CNTF (Leung et al., 1992). GPA is expressed in chick eye during the period of naturally occurring cell death for ciliary neurons that innervate eye muscles, and is secreted from transfected cells (Leung et al., 1992). CNTF, on the other hand, is not expressed in the embryo, and is not secreted. These differences between CNTF and GPA raise the possibility that GPA may not be the chick

equivalent of CNTF. Recently, GPA has been demonstrated to have neuronal differentiation activity and induce VIP expression in chick sympathetic neurons (Heller et al., 1993). Two other proteins related to CNTF are the membrane-associated neurotransmitter-stimulating (MANS) factor (Wong and Kessler, 1987) and an activity in sweat gland extracts, sweat gland factor (SGF) (Rao and Landis, 1990). SGF and MANS have not been sequenced as yet. CNTF, MANS and SGF show similar activities on neuronal phenotype in cultured sympathetic neurons (Rao et al., 1992a; Rao et al., 1992b). Anti-CNTF antisera immunoprecipitate all MANS activity but only part of SGF activity (Rao et al., 1992a; Rao et al., 1992b). This raises the possibility that MANS may be a membrane-bound form of CNTF, and that there is a distinct, CNTF-like neuronal differentiation factor in sweat glands (Rao et al., 1992b; Rohrer, 1992). The latter notion is supported by biochemical analysis, which indicates that SGF is not CDF/LIF or CNTF (Rao,et al., 1992b; Rohrer, 1992). Moreover, the innervation of sweat glands is phenotypically normal in CDF/LIF- and CNTF- deficient mutant mice (Masu et al., 1993; Rao et al., 1994). Interpretation of negative results with these knockout mice is difficult, however, given the overlapping activities of multiple factors. A likely candidate for the SGF is the mammalian version of GPA.

Since CDF/LIF, CNTF, GPA, MANS, SGF, IL-6 and IL-11 have overlapping activities in a variety of tissues, share a predicted secondary structure, and utilize the same receptor subunit as a signal transducer, these proteins are grouped together as a family, even though their primary sequences are not conserved. The term neuropoietic cytokines is used, because they regulate cell differentiation and gene expression both in the nervous and hematopoietic systems (Anderson, 1989; Bazan, 1991; Patterson and Nawa, 1993).

TGF-β superfamily. The factor likely to be responsible for SOM expression in choroid neurons of the chick ciliary ganglion has been identified as activin A (Coulombe et al., 1993), a member of the TGF-β superfamily (Kingsley, 1994; Massague, 1990). Activin A message and protein are present in cultured ciliary choroid cells, but not in cultured skeletal muscle cells. Follistatin, an endogenous activin inhibitor, can block SOM-inducing activity in the choroid cell conditioned medium. Furthermore, recombinant activin A can induce

expression of SOM in cultured ciliary neurons. Since several members of the TGF-β family are localized primarily in the nervous system (Basler et al., 1993; Lee, 1991) or have neurotrophic effects (Lin et al., 1993), this family deserves further analysis in neuronal development.

Other factors. Other known growth factors have effects on neuronal phenotype. For example, insulin and insulin-like growth factors induce TH in cultured embryonic quail dorsal root ganglia (Xue et al., 1988). Both acidic FGF and basic FGF can induce a cholinergic phenotype in cultured chick sympathetic neurons (Zurn, 1992). Neither of these phenomena has been further analyzed to date. A number of additional neuronal differentiation factors have been partially purified. Nawa and Patterson (1990) showed that two factors biochemically distinct from CDF/LIF exist in heart cell conditioned medium; one induces VIP and the other induces SOM in sympathetic neurons (Nawa and Patterson, 1990). A factor termed muscle-derived differentiation factor (MDF) can increase expression of TH in several cultured CNS neurons without enhancing neuronal survival (Iacovitti, 1991; Iacovitti et al., 1992). There is a critical period for neurons to respond to MDF. Another muscle-derived factor, ChAT development factor (CDF, note: not the same as CDF/LIF), increases the level of ChAT activity in cultured spinal cord neurons (McManaman et al., 1988). This protein can selectively rescue motor neurons during the period of naturally occurring cell death (McManaman et al., 1990), but not other neurons which undergo cell death during the same period. CDF is a 22 kDa protein; peptide sequence analysis indicates that it is a novel protein (McManaman and Oppenheim, 1993)

The goal of the present work

The fact that the cytokine CDF/LIF can act as a growth and differentiation factor in the nervous system raises the issue as to whether other cytokines and growth factors may have effects on neuronal cells. Indeed, several interleukins are now known to have neurotrophic activity (Kamegai et al., 1990; Loughlin and Fallon, 1993; Mehler et al., 1993). As in the case of neurotrophins and neuropoietic cytokines, these interleukins may also have neuronal differentiation activities. To screen for more than 60 known

cytokines and growth factors on the neuronal expression of transmitters and neuropeptides, a convenient assay was needed. Conventional methods, such as radioimmunoassays and metabolic assays, are laborious and time-consuming. Moreover, such methods screen one phenotypic trait at a time and require many thousands of cells for each data point. To resolve this problem, I will describe in Chapter 2 a method based on the reverse transcription-polymerase chain reaction (RT-PCR) technique to measure the expression of 13 genes commonly found in the PNS. The specificity of primers will be demonstrated and the conditions of PCR will be defined. The effects of CDF/LIF and CNTF on cultured sympathetic neurons are then assessed by this assay in order to compare with the results from previous assay methods.

The effects of 33 cytokines are analyzed with the RT-PCR assay in Chapter 3. Evidence is presented to show that CDF/LIF, CNTF, GPA, OSM, IL-6 and IL-11 have similar activities on sympathetic neurons. In addition, activin A is shown to induce a particular set of neuronal traits that is different from that of the neuropoietic cytokines. This result supports our hypothesis that there are factors released by target tissues that generate a wide variety of neuronal phenotypes. That most cytokines are without detectable activity in this assay illustrates the specificity of these effects.

To further pursue the effects of TGF-β family members on neuronal phenotype, TGF-β1, TGF-β3, BMP-2, and BMP-6 were tested and the results are described in Chapter 4. Since activins have cholinergic-inducing activity, the possibility that these factors are involved in the phenotypic switch that occurs in the sweat gland innervation is analyzed by *in situ*, RNase protection and tissue homogenate experiments. The results are presented in Chapter 4 as well.

I also collaborated with Dr. Jane Talvenheimo in an attempt to purify novel factors from heart cell conditioned medium. These results are presented in Chapter 5.

Finally, I participated in the cloning of CDF/LIF. The Appendix includes a paper describing this effort.

References

Alder, R., Landa, K. B., Manthorpe, M., and Varon, S. (1979). Cholinergic neuronotrophic factors: Intraocular distribution of trophic activity for ciliary neurons. Science, **204**, 1434.

Altin, J. G. and Bradshaw, R. A. (1993). Nerve growth factor and related substances: Structure and mechanism of action. In S. E. Laughlin & J. H. Fallon (Eds.), Neurotrophic factors (pp. 129-180). New York: Academic Press.

Anderson, D. J. (1989). The neural crest cell lineage problem: Neuropoiesis? Neuron, **3**, 1-12.

Arakawa, Y., Sendtner, M., and Thoenen, H. (1990). Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: Comparison with other neurotrophic factors and cytokines. J. Neurosci., **10**, 3507-3515.

Araki, M. and Tokunaga, F. (1990). Norepinepherin suppresses both photoreceptor and neuron-like properties expressed by cultured rat pineal glands. Cell Diff. Dev., **31**, 129-135.

Baker, H. (1990). Unilateral neonatal olfactory deprivation alters tyrosine hydroxylase expression but not aromatic amino acid decarboxylase or GABA immunoreactivity. Neurosci., **36**, 761-771.

Barres, B. A., Schmid, R., Sendtner, M., and Raff, M. C. (1993). Multiple extracellular signals are required for long-term oligodendrocyte survival. Development, **118**, 283-295.

Basler, K., Edlund, T., Jessel, T. M., and Yamada, T. (1993). Control of cell pattern in the neural tube: Regulation of cell differentiation by dorsalin-1, a novel TGF beta family member. Cell, **73**, 687-702.

Bazan, J. F. (1991). Neuropoietic cytokines in the hematopoietic fold. Neuron, **7**, 197-208.

Bronner-Fraser, M. (1992). Cell lineage segregation in the vertebrate neural crest. In M. Shankland & E. R. Macagno (Eds.), Determinants of neuronal identity. (pp. 359-389). New York: Academic Press.

Cagan, R. L. and Zipursky, S. L. (1992). Cell choice and patterning in the *Drosophila* retina. In M. Shankland & E. R. Marcagno (Eds.), Determinants of neuronal identity. (pp. 189-224). New York: Academic Press.

Cannon, B., Nedergaard, J., Lundberg, J. M., Hokfelt, T., Terenius, L., and Goldstein, M. (1986). Neuropeptide tyrosine (NPY) is co-stored with noradrenaline in vascular but not in parenchymal sympathetic nerves of brown adipose tissue. Exp. Cell Res., **164**, 546-550.

Claude, P., Parada, I. M., Gordon, K. A., D'Amore, P. A., and Wagner, J. A. (1988). Acidic fibroblast growth factor stimulates adrenal chromaffin cells to proliferate and to extend neurites, but is not a long-term survival factor. Neuron, **1**, 783-790.

Conover, H. C., Ip, N. Y., Poueymirou, W. T., Bates, B., Goldfarb, M. P., DeChiara, T. M., and Yancopoulos, G. D. (1993). Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. Development, **119**, 559-565.

Coulombe, J. N. and Bronner-Fraser, M. E. (1986). Cholinergic neurons acquire adrenergic neurotransmitters when transplanted into an embryo. Nature, **324**, 569-572.

Coulombe, J. N. and Bronner-Fraser, M. E. (1990). Development of cholinergic traits in the quail ciliary ganglion: Expression of choline acetyltransferase-like immunoreactivity. Neurosci., **37**, 259-270.

Coulombe, J. N., Schwall, R., Parent, A. S., Eckenstein, F. P., and Nishi, R. (1993). Induction of somatostatin immunoreactivity in cultured ciliary ganglion neurons by activin in choroid cell-conditioned medium. Neuron, **10**, 899-906.

Denis-Donini, S. (1989). Expression of dopaminergic phenotype in the mouse olfactory bulb induced by the calcitonin gene-related peptide. Nature, **339**, 701-703.

Eckenstein, F. P., Esch, F., Holbert, T., Blacher, R. W., and Nishi, R. (1990). Purification and characterization of a trophic factor for embryonic peripheral neurons: Comparison with fibroblast growth factors. Neuron, **4**, 623-631.

Elfvin, L.-G., Lindh, B., and Hokfelt, T. (1993). The chemical neuroanatomy of sympathetic ganglia. Annu. Rev. Neurosci., **16**, 471-507.

Epistein, M., Davis, J., Gelman, L., Lamb, J., and Dahl, J. (1988). Cholinergic neurons of the chick ciliary ganglion contain somatostatin. Neurosci., **25**, 1053-1060.

Fagg, G. E. and Foster, A. C. (1983). Amino acid neurotransmitters and their pathways in the mammalian central nervous system. Neurosci., **9**, 701-719.

French, K. A. and Kristan, W. B. (1992). Target influences of the development of leech neurons. Trends Neurosci., **15**, 169-174.

Friedman, W. J., Dreyfus, C. F., McEwen, B., and Black, I. B. (1988). Presynaptic transmitters and depolarizing influences regulate development of the substantia nigra in culture. J. Neurosci., **8**, 3616-3623.

Frodin, M. and Gammeltoft, S. (1994). Insulin-like growth factors act synergistically with basic fibroblast growth factor and nerve growth factor to promote chromaffin cell proliferation. Proc. Natl. Acad. Sci. USA, **91**, 1771-1775.

Fukada, K. (1985). Purification and partial characterization of a cholinergic neuronal differentiation factor. Proc. Natl. Acad. Sci. USA, **82**, 8795-8799.

Gearing, D. P. and Bruce, A. G. (1991). Oncostatin M binds the high-affinity leukemia inhibitory factor receptor. New Biol., **4**, 61.

Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., Ziegler, S. F., and Cosman, D. (1992). The IL-6 signal transducer, gp130: An oncostatin M receptor and affinity converter for the LIF receptor. Science, **255**, 1434-1437.

Gibbins, I. L. (1990). Peripheral autonomic nervous system. In G. Paxinos (Eds.), The human nervous system. (pp. 93-123). New York: Academic Press.

Gibbins, I. L. and Morris, J. L. (1987). Coexistence of neuropeptides in sympathetic, cranial autonomic and sensory neurons innervating the iris of the guinea pig. J. Auton. Nerv. Syst., **29**, 137-150.

Goodman, R. H. (1990). Regulation of neuropeptide gene expression. Annu. Rev. Neurosci., **13**, 111-127.

Heller, S., Huber, J., Finn, T. P., Nishi, R., and Rohrer, H. (1993). GPA and CNTF produce similar effects in sympathetic neurons but differ in receptor binding. NeuroReport, **5**, 357-360.

Hughes, S. M., Lillien, L. E., Raff, M. C., Rohrer, H., and Sendtner, M. (1988). Ciliary neurotrophic factor induces type-2 astrocyte differentiation in culture. Nature, **335**, 70-73.

Iacovitti, L. (1991). Effects of a novel differentiation factor on the development of catecholamine traits in noncatecholamine neurons from various regions of the rat brains: Studies in tissue culture. J. Neurosci., **11**, 2403-2409.

Iacovitti, L., Evinger, M. J., and Stull, N. D. (1992). Muscle-derived differentiation factor increases expression of the tyrosine hydroxylase gene and enzyme activity in cultured dopamine neurons from the rat midbrain. Mol. Brain Res., **16**, 215-222.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N., and Yancopoulos, G. D. (1992). CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. Cell, **69**, 1121-1132.

Jessell, T. M. and Melton, D. A. (1992). Diffusible factors in vertebrate embryonic induction. Cell, **68**, 257-270.

Kamegai, M., Niijima, K., Kunishita, T., Nishizawa, M., Ogawa, M., Araki, M., Ueki, A., Konishi, Y., and Tabira, T. (1990). Interleukin-3 as a trophic factor for central cholinergic neurons *in vitro* and *in vivo*. Neuron, **2**, 429-436.

Kessler, J. A., Adler, J. E., and Black, I. B. (1983). Substance P and somatostatin regulate sympathetic noradrenergic function. Science, **221**, 1059-1061.

Kingsley, D. M. (1994). The TGF-β superfamily: New members, new receptors, and new genetic tests of function in different organisms. Genes & Dev., **8**, 133-146.

Kishimoto, T., Akira, S., and Taga, T. (1992). Interleukin-6 and its receptor: A paradigm for cytokines. Science, **258**, 593-597.

Kishimoto, T., Taga, T., and Akira, S. (1994). Cytokine signal transduction. Cell, **76**, 253-262.

Landis, S. C. (1990). Target regulation of neurotransmitter phenotype. Trends Neurosci., **13**, 344-350.

Landis, S. C. and Keefe, D. (1983). Evidence for neurotransmitter plasticity *in vivo*: Developmental change in properities of cholinergic sympathetic neurons. Dev. Bio., **98**, 349-372.

Landis, S. C., Siegel, R. E., and Schwab, M. (1988). Evidence for neurotransmitter plasticity *in vivo*, II. Immunocytochemical studies of rat sweat gland innervation during development. Dev. Biol., **126**, 129-140.

Leblanc, G. and Landis, S. C. (1986). Development of choline acetyltransferase (CAT) in the sympathetic innervation of rat sweat glands. J. Neurosci., **6**, 260-265.

Lee, S. J. (1991). Expression of growth/differentiation factor 1 in the nervous system: Conservation of a bicistronic structure. Proc. Natl. Acad. Sci. USA, **88**, 4250-4254.

Leung, D. W., Parent, A. S., Cachianes, G., Esch, F., Coulombe, J. N., Nikolics, K., Eckenstein, F. P., and Nishi, R. (1992). Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion neurons. Neuron, **8**, 1045-1053.

Levi-Montalcini, R. and Hamburger, V. (1951). Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J. Exp. Zool., **116**, 321-363.

Li, G., Regunathan, S., Barrow, C. J., Eshraghi, J., Cooper, R., and Reis, D. J. (1994). Agmatine: An endogenous clonidine-displacing substance in the brain. Science, **263**, 966-969.

Lin, L.-F., Doherty, D. H., Lile, J. D., Bektrsh, S., and Collins, F. (1993). GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science, **260**, 1130-1132.

Lin, L.-F. H., Mismer, D., Lile, J. D., Armes, L. G., Bulter, E. T. I., Vannice, J. L., and Collins, F. (1989). Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). Science, **246**, 1023-1025.

Lindsay, R. M. and Harmar, A. J. (1989). Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature, **337**, 362-364.

Lord, K. A., Abdollahi, A., Thomas, S. M., Demarco, M., Brugge, J. S., Hoffman-Liebermann, B., and Leibermann, D. A. (1991). Leukemia inhibitory factor and interleukin-6 trigger the same immediate early response, including tyrosine phosphorylation, upon induction of myeloid leukemia differentiation. Mol. Cell. Bio., **11**, 4371-4379.

Loughlin, S. E. and Fallon, J. H. (Ed.). (1993). Neurotrophic factor. New York: Academic Press, Inc.

Louis, J.-C., Magal, E., Takayama, S., and Varon, S. (1993). CNTF protection of oligodendrocytes against natural and tumor necrosis factor-induced death. Science, **259**, 689-692.

Lundberg, J. M. and Hokfelt, T. (1986). Multiple coexistence of peptides and classical neurotransmitters in peripheral autonomic and sensory neurons - functional and pharmacological implications. In T. Hokfelt, K. Fuxe, & B. Pernow (Eds.), Progress in Brain Research. Amerstdam: Elsevier.

Lutticken, C., Wegenka, U. M., Yuan, J., Buschmann, J., Schindler, C., Ziemiecki, A., Harpur, A. G., Wilks, A. F., Yasukawa, K., Taga, T., Kishimoto, T., Barbieri, G., Pellegrini, S., Sendtner, M., Heinrich, P. C., and Horn, F. (1994). Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. Science, **263**, 89-92.

Martin, A. R. and Pilar, G. (1963). Transmission through the ciliary ganglion of the chick. J. Physiol., **168**, 464-475.

Martinou, J.-C., Martinou, I., and Kato, A. C. (1992). Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons in vitro. Neuron, **8**, 737-744.

Marwitt, R., Pilar, G., and Weakly, J. (1971). Charaterization of two ganglion cell populations in avian ciliary ganglia. Brain Res., **25**, 317-334.

Massague, J. (1990). The transforming growth factor-β family. Annu. Rev. Cell Biol., **6**, 597-641.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G., and Thoenen, H. (1993). Disruption of the CNTF gene results in motor neuron degeneration. Nature, **365**, 27-32.

McConnell, S. K. (1992). The determination of neuronal identity in the mammalian cerebral cortex. In M. Shankland & E. R. Macagno (Eds.), Determinants of neuronal identity. (pp. 391-432). New York: Academic Press.

McMahon, S. B. and Gibson, S. (1987). Peptide expression is altered when afferent nerves innervate inappropriate tissue. Neurosci. Lett., **73**, 9-15.

McManaman, J., Crawford, F., Stewart, S. S., and Appel, S. (1988). Purification of a skeletal muscle polypeptide which stimulates choline acetyltransferase activity in cultured spinal cord neurons. J. Biol. Chem., **263**, 5890-5897.

McManaman, J., Oppenheim, R. W., Prevette, D., and Marchetti, D. (1990). Rescue of motoneurons from cell death by a purified skeletal muscle polypeptide: Effects of the ChAT development factor, CDF. Neuron, **4**, 891-898.

McManaman, J. L. and Oppenheim, R. W. (1993). Skeletal muscle-derived neurotrophic factors and motoneuron development. In S. E. Loughlin & J. H. Fallon (Eds.), Neurotrophic factors (pp. 475-487). New York: Academic Press.

Mehler, M. F., Rozental, R., Dougherty, M., Spray, D. C., and Kessler, J. A. (1993). Cytokine regulation of neuronal differentiation of hippocampal progenitor cells. Science, **362**, 62-65.

Merchenthaler, I., Lennard, D. E., Lopez, F. J., and Negro-Vilar, A. (1993). Neonatal imprinting predetermines the sexually dimorphic, estrogen-dependent expression of galanin in luteinizing hormone-releasing hormone neurons. Proc. Natl. Acad. Sci. USA, **90**, 10479-10483.

Merchenthaler, I., Lopez, F. J., Lennard, D. E., and Negro-Villar, A. (1991). Sexual differences in the distribution of neurons coexpressing galanin and luteinizing hormone in the rat brain. Endocrinol., **129**, 1977-1986.

Meriney, S. D. and Pilar, G. (1987). Cholinergic innervation of the smooth muscle cells in the choroid coat of the chick eye and its development. J. Neurosci., **7**, 3827-3839.

Metcalf, D. (Ed.). (1992). Polyfunctional cytokines: IL-6 and LIF. New York: John Wiley & Sons Ltd.

Mione, M. C., Danevic, C., Boardman, P., Harris, B., and Parnavelas, J. G. (1994). Lineage analysis reveals neurotransmitter (GABA or glutamate) but not calcium-binding protein homogeneity in clonally relates cortical neurons. J. Neurosci., **14**, 107-123.

Morris, B. J., Feasey, K. J., Bruggencate, G. J., Herz, A., and Hollt, V. (1988). Electrical stimulation *in vivo* increase the expression of proenkephalin mRNA and decrease the expression of prodynorphin mRNA in rat hippocampal granule cells. Proc. Natl. Acad. Sci. USA, **85**, 3226-3230.

Murphy, M., Reid, K., Hilton, D. J., and Bartlett, P. F. (1991). Generation of sensory neurons is stimulated by leukemia inhibitory factor. Proc. Natl. Acad. Sci. USA, **88**, 3498-3501.

Narayanan, C. and Narayanan, Y. (1978). On the origin of the ciliary ganglion in birds. J. Embryol. Exp. Morphol., **47**, 137-148.

Nawa, H., Bessho, Y., Carnahan, J., Nakanishi, S., and Mizuno, K. (1993). Regulation of neuropeptide expression in cultured cerebral cirtical neurons by BDNF. J. Neurochem., **60**, 772-775.

Nawa, H., Nakanishi, S., and Patterson, P. H. (1991). Recombinant cholinergic differentiation factor (leukemia inhibitory factor) regulates sympathetic neuron phenotype by alternations in the size and amounts of neuropeptide mRNAs. J. Neurochem., **56**, 2147-2150.

Nawa, H. and Patterson, P. H. (1990). Separation and partial characterization of neuropeptide-inducing factors in heart cell conditioned medium. Neuron, **4**, 269-277.

Nawa, H., Yamamori, T., Le, T., and Patterson, P. H. (1990). Generation of neuronal diversity: Analogies and homologies with hematopoiesis. Cold Spring Harbor Symp. Quant. Biol, **55**, 247-253.

Ojika, K., Kojima, S., Ueki, Y., Fukushima, N., Hayashi, K., and Yamamoto, M. (1992). Purification and structural analysis of hippocampal cholinergic neurostimulating peptide. Brain Res., **572**, 164-171.

Park, J. K., Joh, T. H., and Ebner, F. F. (1986). Tyrosine hydrxylase is expressed by neocortical neurons after transplantation. Proc. Natl. Acad. Sci. USA, **83**, 7495-7498.

Patterson, P. H. (1978). Environmental determination of autonomic neurotransmitter functions. Annu. Rev. Neurosci., **1**, 1-17.

Patterson, P. H. (1993). Instructive neuronal differentiation factors. In S. E. Laughlin & J. H. Fallon (Eds.), Neurotrophic factors (pp. 527-558). New York: Academic Press.

Patterson, P. H. and Chun, L. L. Y. (1977). The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. I. Effects of conditioned medium. Dev. Biol., **56**, 263-280.

Patterson, P. H. and Landis, S. C. (1992). Phenotype specifying factors and the control of neuronal differentiation decisions. In I. E. Hendry & C. E. Hill (Eds.), Development, Regeneration and Plasticity of the Autonomic System (pp. 231-265). Chur, Switzerland: Harwood Academic Publishers.

Patterson, P. H. and Nawa, H. (1993). Neuronal differentiation factor/cytokines and synaptic plasticity. Cell, **72**, 123-137.

Quick, D. C., Kennedy, W. R., and Yoon, K. S. (1984). Ultrastructure of the secretory epithelium, nerve fibers and capillaries on the mouse sweat gland. Anat. Rec., **208**, 491-499.

Raff, M. (1989). Glial cell diversification in the rat optic nerve. Science, **243**, 1450-1455.

Rao, M. S. and Landis, S. C. (1990). Characterization of a target-derived neuronal cholinergic differentiation factor. Neuron, **5**, 899-910.

Rao, M. S., Patterson, P. H., and Landis, S. C. (1992a). Membrane-associated neurotransmitter-stimulating (MANS) factor is very similar to ciliary neurotrophic factor (CNTF). Dev. Biol., **153**, 411-416.

Rao, M. S., Patterson, P. H., and Landis, S. C. (1992b). Multiple cholinergic differentiation factors are present in footpad extracts: Comparison with known cholinergic factors. Development, **116**, 731-744.

Rao, M. S., Sun, Y., Escary, J. L., Perreau, J., Tresser, S., Patterson, P. H., Zigmond, R. E., Brulet, P., and Landis, S. C. (1994). Leukemia inhibitory factor mediates an injury response but not a target-directed developmental transmitter switch in sympathetic neurons. Neuron, **11**, 1175-1185.

Rao, M. S., Symes, A., Malik, N., Shoyab, M., Fink, J. S., and Landis, S. C. (1992c). Oncostatin M regulates VIP expression in a human neuroblastoma cell line. NeuroReport, **3**, 865-868.

Rao, M. S., Tyrrell, S., Landis, S. C., and Patterson, P. H. (1992d). Effects of ciliary neurotrophic factor (CNTF) and depolarization on neuropeptide expression in cultured sympathetic neurons. Dev. Biol., **150**, 281-293.

Renfranz, P. J., Cunningham, M. G., and McKay, R. D. G. (1991). Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. Cell, **66**, 713-729.

Roach, A., Adler, J. E., and Black, I. B. (1987). Depolarizing influences regulate preprotachykinin mRNA in sympathetic neurons. Proc. Natl. Acad. Sci. USA, **84**, 5078-5081.

Rohrer, H. (1992). Cholinergic neuronal differentiation factors: Evidence for the presence of both CNTF-like and non-CNTF-like factors in developing rat footpad. Development, **114**, 689-698.

Saadat, S., Sendtner, M., and Rohrer, H. (1989). Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. J. Cell Bio., **108**, 1807-1816.

Schooltink, H., Stoyan, T., Roeb, E., Heinrich, P. C., and Rose-John, S. (1992). Ciliary neurotrophic factor induces acute-phase protein expression in hepatocytes. FEBS Let., **314**, 280-284.

*Schotzinger, R. J. and Landis, S. C. (1988). Cholinergic phenotype developed by noradrenergic sympathetic neurons after innervation of a novel cholinergic target in vivo. Nature, **335**, 637-639.

Schotzinger, R. J. and Landis, S. C. (1990). Acquisition of cholinergic and peptidergic properties by sympathetic innervation of rat sweat glands requires interaction with normal target. Neuron, **5**, 91-100.

Sendtner, M., Kreutzberg, G. W., and Thoenen, H. (1990). Ciliary neurotrophic factor prevents the degeneration of motor neurons after axonomy. Nature, **345**, 440-441.

Simerly, R. B. (1990). Hormonal control of neuropeptide gene expression in sexually dimorphic olfactory pathways. Trends Neurosci., **13**, 104-110.

Snyder, E. Y., Deitcher, D. L., Walsh, C., Arnold-Aldea, S., Hartweig, E. A., and Cepko, C. L. (1992). Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. Cell, **68**, 33-51.

Stahl, N., Boulton, T. G., Farruggella, T., Ip, N. Y., Davis, S., Wittuhn, B., Quelle, F. W., Silvennoinen, O., Barbieri, G., Pellegrini, S., Ihle, J. N., and Yancopoulos, G. D. (1994). Association and activation of Jak-Tyk kinase by CNTF-LIF-OSM-IL-6 receptor components. Science, **263**, 92-95.

Stemple, D. L. and Anderson, D. J. (1993). Lineage diversification of the neural crest: *in vitro* investigations. Dev. Biol., **159**, 12-23.

Stemple, D. L., Mahanthappa, N. K., and Anderson, D. J. (1988). Basic FGF induces neuronal differentiation, cell division, and NGF dependence in chromaffin cells: A sequence of events in sympathetic development. Neuron, **1**, 517-525.

Stevens, L. M. and Landis, S. C. (1990). Target influence on transmitter choice by sympathetic neurons developing in the anterior chamber of the eye. Dev. Biol., **137**, 109-124.

Stockli, K. A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., Gotz, R., Lindholm, D., and Thoenen, H. (1989). Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. Nature, **342**, 920-923.

Stuart, D. K., Blair, S. S., and Weisblat, D. A. (1987). Cell lineage, cell death, and developmental origin of identified serotonin- and dopamine-containing neurons in the leech. J. Neurosci., **7**, 1107-1122.

Sulston, J. E. and Horvitz, H. R. (1977). Postembryonic cell lineages of the nematode, *Caenorhabditis elegans*. Dev. Biol., **56**, 110-156.

Taghert, P. H. and Goodman, C. S. (1984). Cell determination and differentiation of identified serotonin-immunoreactive neurons in the grasshopper embryo. J. Neurosci., **4**, 989-1000.

Thoenen, H. (1991). The changing scene of neurotrophic factors. Trends Neurosci., **14**, 165-170.

Tolon, R. M., Franco, F. S., Frailes, M. T. d. l., Lorenzo, M. J., and Cacicedo, L. (1994). Effect of potassium-induced depolarization on somatostatin gene expression in cultured fetal rat cerebrocortical cells. J. Neurosci., **14**, 1053-1059.

Tublitz, N. J. and Sylwester, A. W. (1990). Postembryonic alteration of transmitter phenotype in individually identified peptidergic neurons. J. Neurosci., **10**, 161-168.

Uno, H. and Montagna, W. (1975). Catecholamine-containing nerve terminals of the ecrine sweat glands of macaques. Cell Tissue Res., **158**, 1-13.

Walsh, C. and Cepko, C. L. (1992). Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. Science, **255**, 434-440.

Wan, D. C. C., Marley, P. D., and Livett, B. G. (1991). Regulation of opioid and PNMT gene expression in adrenal chromaffin cells. J. Auton. Nerv. Syst., **33**, 130-131.

Weeks, J. C. and Levine, R. B. (1990). Postembryonic neuronal plasticity and its hormonal control during insect metamorphosis. Annu. Rev. Neurosci., **13**, 183-194.

Witten, J. L. and Truman, J. W. (1991a). The regulation of transmitter expression in postembryonic lineages in the moth *Manduca sexta*. I. Transmitter identification and developmental acquistion of expression. J. Neurosci., **11**, 1980-1989.

Witten, J. L. and Truman, J. W. (1991b). The regulation of transmitter expression in postembryonic lineages in the moth *Manduca sexta*. II. Role of cell lineage and birth order. J. Neurosci., **7**, 1990-1997.

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

Xue, Z. G., LeDouarin, N. M., and Smith, J. (1988). Insulin and insulin-like growth factor-1 can trigger the differentiation of catecholaminergic precusors in cultures of dorsal root ganglia. Cell Diff. Dev., **25**, 1-10.

Yamamori, T. (1991). CDF/LIF selectively increase c-fos and jun-B transcripts in sympathetic neurons. Neuroreport, **2**, 173-176.

Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M.-J., and Patterson, P. H. (1989). The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. Science, **246**, 1412-1416.

Yin, T., Taga, T., Tsang, M. L.-S., Kishimoto, T., and Yang, Y. C. (1993). Involvement of interleukin-6 signal transducer gp130 in interleukin-11-mediated signal transduction. J. Immunol., **151**, 2555-2561.

Yodlowski, M., Fredieu, J. R., and Landis, S. C. (1984). Neonatal 6-hydroxydopamine treatment eliminates cholinergic sympathetic innervation and induces sensory sprouting in rat sweat glands. J. Neurosci., **4**, 1535-1548.

Zhang, J.-M. and Sieber-Blum, M. (1992). Characterization of the norepinepherine uptake system and the role of norepinepherine in the expression of the adrenergic phenotype by quail neural crest cells in the clonal culture. Brain Res., **570**, 251-258.

Zigmond, R. E., Schwazschild, M. A., and Rittenhouse, A. R. (1989). Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitter via phosphorylation. Annu. Rev. Neurosci., **12**, 415-461.

Zurn, A. (1992). Fibroblast growth factor differentially modulates the neurotransmitter phenotype of cultured sympathetic neurons. J. Neurosci., **12**, 4195-4201.

Zurn, A. D., Fauquet, M., Shaw, P., and Kocher, J. (1993). The neuropeptide VIP regulates the expression of the tyrosine hydroxylase gene in cultured avian sympathetic neurons. Mol. Brain Res., **20**, 125-129.

CHAPTER 2. A novel approach to screen for cytokine effects on neuronal gene expression

Journal of Neurochemistry
Raven Press, Ltd., New York

A Novel Approach to Screen for Cytokine Effects on Neuronal Gene Expression

Ming-Ji Fann and Paul H. Patterson

Division of Biology, California Institute of Technology, Pasadena, California, U.S.A.

Abstract: We describe an assay based on reverse transcription–polymerase chain reaction to detect the expression of mRNAs for a variety of transmitter synthetic enzymes and neuropeptides present at low levels in primary neuronal cultures. The assay is specific for mRNA-derived templates and is not affected by the presence of genomic DNA. Using this method, we demonstrate that cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) and ciliary neurotrophic factor (CNTF) induce mRNAs for choline acetyltransferase, somatostatin, substance P, vasoactive intestinal polypeptide, cholecystokinin, and enkephalin. The induction of cholecystokinin and enkephalin by CDF/LIF and CNTF had not been shown previously. These data illustrate that the assay can reproduce findings obtained with other methods, as well as provide the sensitivity necessary to produce new results. These results also extend the overlap of CDF/LIF and CNTF in controlling gene expression in cultured sympathetic neurons, supporting the idea that these cytokines may share receptor subunits and signal transduction pathways. **Key Words:** Cholinergic differentiation factor—Leukemia inhibitory factor—Ciliary neurotrophic factor—Cholecystokinin—Enkephalin—Choline acetyltransferase—Somatostatin—Substance P—Vasoactive intestinal peptide.
J. Neurochem. **61,** 1349–1355 (1993).

Neuronal communication is mediated by approximately 12 known classical transmitters and over 75 neuropeptides. As individual neurons are known to express several transmitters and as many as eight different neuropeptides, the number of potential combinations is enormous. The choice of which transmitters and neuropeptides to produce is determined by the cell's lineage history and environmental influences (Nawa et al., 1990; Patterson and Landis, 1992; Patterson and Nawa, 1993). One of the most extensive analyses of environmental influences on neuronal phenotype involves sympathetic neurons, both in culture and in their innervation of various target tissues in vivo (Black et al., 1987; Hendry and Hill, 1992). In the latter work, Landis and colleagues (reviewed in Landis, 1990) have shown that the phenotypic choice made by sympathetic neurons is controlled, in part, by the particular target tissue they innervate. Moreover, this choice is altered as the neurons contact certain targets, such as the sweat glands, during normal development.

Neuronal cultures have been used to identify candidate factors that may mediate this control of neuronal gene expression. Cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) was characterized and cloned on the basis of its ability to convert cultured rat sympathetic neurons from a noradrenergic to a cholinergic phenotype (Yamamori et al., 1989). In addition, CDF/LIF induces the expression of substance P (SP), somatostatin (SOM), and vasoactive intestinal polypeptide (VIP) by increasing transcription of these genes (Nawa et al., 1990, 1991). Saadat et al. (1989) found that ciliary neurotrophic factor (CNTF) can mediate the same noradrenergic-to-cholinergic switch of phenotype in these neurons. CNTF also alters expression of the same neuropeptides as CDF/LIF (Rao et al., 1992*a*). Other, as yet unidentified, proteins can influence neuronal phenotype. For example, two proteins in the conditioned medium from cultured heart cells selectively induce the expression of VIP and SOM (Nawa and Patterson, 1990). Moreover, a protein(s) in sweat gland extracts has CDF/LIF-CNTF-like effects on these neurons (Rao et al., 1992*b*; Rohrer, 1992). Identification of these factors will enable a further understanding of how neuronal phenotype is determined.

Primary neurons have been used in such assays be-

Received December 4, 1992; revised manuscript received February 10, 1993; accepted February 16, 1993.

Address correspondence and reprint requests to Dr. P. H. Patterson at Division of Biology, California Institute of Technology, Pasadena, CA 91125, U.S.A.

Abbreviations used: CCK, cholecystokinin; CDF/LIF, cholinergic differentiation factor/leukemia inhibitory factor; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; CNTF, ciliary neurotrophic factor; DYN, dynorphin; ENK, enkephalin; GAD65, 65-kDa glutamic acid decarboxylase; GAD67, 67-kDa glutamic acid decarboxylase; NPY, neuropeptide Y; RT-PCR, reverse transcription–polymerase chain reaction; SOM, somatostatin; SP, substance P; TPH, tryptophan hydroxylase; TH, tyrosine hydroxylase; VIP, vasoactive intestinal polypeptide.

cause they presumably reflect the response of normal neurons more accurately than do neuronal cell lines. This approach does, however, result in limiting amounts of material for the assays. One way to circumvent this problem is to increase the sensitivity of the assays for particular transmitters and neuropeptides. In this report, we present a new approach based on reverse transcription–polymerase chain reaction (RT-PCR) that enables us to follow simultaneously the expression of mRNAs for 13 transmitters/neuropeptides in small numbers of neurons. Using this assay, we demonstrate that CDF/LIF and CNTF induce expression of mRNAs for choline acetyltransferase (ChAT), SP, VIP, cholecystokinin (CCK), and enkephalin (ENK) in cultured sympathetic neurons. The induction of the latter two neuropeptides by CDF/LIF and CNTF has not been shown previously. As small numbers of neurons and minimal amounts of factors are required in the assay, and numerous genes can be analyzed at the same time, this PCR-based method may facilitate identification of new neuronal differentiation factors.

MATERIALS AND METHODS

Animals and reagents

Neonatal Sprague–Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA, U.S.A.). Most of the reagents for tissue culture, Superscript reverse transcriptase (RTase), and 1-kb DNA marker were purchased from GIBCO/BRL (Grand Island, NY, U.S.A.). Transferrin was obtained from Calbiochem (La Jolla, CA, U.S.A.). Nerve growth factor, glycogen, dATP, dCTP, dGTP, and dTTP were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). RNase inhibitor (RNasin) and *Taq* DNA polymerase were purchased from Promega (Madison, WI, U.S.A.). Oligo dT was obtained from Pharmacia (Piscataway, NJ, U.S.A.). Primers were synthesized in the Biopolymer Synthesis and Analysis Resource Center at California Institute of Technology, Pasadena, CA, U.S.A. All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.). Human recombinant CDF/LIF and rat recombinant CNTF were kindly provided by Dr. Babru Samal and colleagues at Amgen, Inc. (Thousand Oaks, CA, U.S.A.).

Neuronal culture

Neurons were prepared and cultured as described previously (Hawrot and Patterson, 1979; Wolinsky et al., 1985) with the modification noted below. Briefly, superior cervical ganglia were dissected from neonatal rats, enzymatically dissociated with 0.1% trypsin, and cultured in L15-CO_2 medium supplemented with 100 μg/ml of transferrin, 100 ng/ml of nerve growth factor, 5 μg/ml of insulin, 16 μg/ml of putrescine, 30 n*M* sodium selenite, and 4 μg/ml of aphidicolin. Aphidicolin was used here because it is a relatively nontoxic antimitotic agent used to eliminate nonneuronal cells (Wallace and Johnson, 1989). For ease and reproducibility, neurons were seeded in 96-well plates (Falcon, Oxnard, CA, U.S.A.) at a density of one ganglion per well. Half of the medium was changed every 36 h in an effort to ensure the activity of the cytokines. After 7 days, each well contained approximately 3,000 cells (Wolinsky et al., 1985) and more than 95% of surviving cells were neurons. Cytokines were added from the second day of culture, and duplicate wells were prepared for each condition.

Preparation of RNA and cDNA

Adult rat spinal cord RNA was prepared by disruption of the tissue in 4 *M* guanidinium thiocyanate using a syringe, and extraction with acidic phenol and chloroform (Chomczynski and Sacchi, 1987). Total RNA from cultured neurons was prepared in the same manner, but smaller amounts of material were used (Belyavsky et al., 1989). Briefly, 160 μl of lysis buffer (4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7.0, 100 m*M* 2-mercaptoethanol, and 0.5% sodium lauroyl sarcosinate) was added to each well. The lysate was transferred to 1.7-ml Eppendorf tubes and vortexed vigorously to shear DNA. Ten microliters of 1 *M* sodium acetate, pH 4.0, 200 μl of water-saturated phenol, and 40 μl of chloroform were added sequentially, with vigorous vortexing after each addition. The samples were kept at 4°C for 20 min and centrifuged at 12,000 *g* for 15 min. The water phase was transferred to a new tube containing 10 μl of 3 mg/ml of glycogen. RNA was precipitated by addition of 200 μl of isopropanol and stored at −20°C overnight.

Spinal cord cDNA was prepared as follows: 10 μg of total RNA in 6 μl was denatured in 16 μl of 13 m*M* methylmercury hydroxide in water for 10 min, and 5 μl of 75 m*M* 2-mercaptoethanol in water for 5 min. Reverse transcription was performed in a total volume of 50 μl containing 50 m*M* Tris-HCl, pH 8.3, 75 m*M* KCl, 3 m*M* $MgCl_2$, 0.5 m*M* dNTP, 0.5 μg of oligo dT, 40 U of RNasin, and 200 U of RTase for 1 h at 37°C. To produce cDNA from cultured neurons, total RNA was centrifuged at 12,000 *g* for 15 min, washed once with 70% ethanol, and dried in a Speedvac (Savant, Farmingdale, NY, U.S.A.). The RNA was dissolved directly in 10 μl of 13 m*M* methylmercury hydroxide for 10 min, and an additional 2 μl of 75 m*M* 2-mercaptoethanol was added for 5 min. Reverse transcription was performed in a final volume of 20 μl, with the buffer described above, and 100 U of RTase and 20 U of RNasin for 1 h at 37°C.

PCR

Most of the oligonucleotides were selected as primers according to the criteria described by Lowe et al. (1990). For those genes without available genomic DNA sequences, several distinct oligonucleotides were synthesized and tested in PCR pilot experiments using spinal cord cDNA as template. Primers that could discriminate cDNA from genomic DNA were selected for further study. The sequences of each pair of primers are given in Table 1. Amplification of cDNA was performed in a thermal cycler (MJ Research, Watertown, MA, U.S.A.) with each tube containing a final volume of 20 μl, consisting of 1 μl of cDNA, 1× PCR buffer, 0.5 U of *Taq* DNA polymerase, 0.25 m*M* dNTP, and one set of primers (200 n*M*). The following conditions were used for PCR: 94°C for 45 s, annealing temperature (temperatures for each cDNA listed in Table 1) for 75 s, and 72°C for 30 s. The numbers of cycles used to amplify each cDNA were chosen to allow the PCR to proceed in a linear range, but with sufficient yield to be visualized by ethidium bromide staining; and the cycling profile for each gene is listed in Table 1. The appropriate cycling profiles were determined empirically. Eight microliters of each PCR sample was ana-

lyzed on a 2% agarose gel, and the products were visualized with ethidium bromide staining and UV illumination.

RESULTS

Selection of primers

An RT-PCR assay was designed to facilitate the measurement of mRNAs for neuropeptides and neurotransmitter synthetic enzymes using small numbers of cultured neurons. Fourteen sets of oligonucleotide primers were made to detect mRNAs for β-actin, the neurotransmitter synthetic enzymes ChAT, 65-kDa glutamic acid decarboxylase (GAD65), 67-kDa glutamic acid decarboxylase (GAD67), tyrosine hydroxylase (TH), and tryptophan hydroxylase (TPH), and the neuropeptides calcitonin gene-related peptide (CGRP), CCK, dynorphin (DYN), ENK, neuropeptide Y (NPY), SOM, SP, and VIP. For those genes expected to yield more than one transcript due to alternative splicing, including DYN, ENK, TPH, and SP (Krause et al., 1987; Darmon et al., 1988; Garrett et al., 1989), the selected primers recognize regions common to each transcript and generate a single PCR product for each of these genes; the exception is SP, which may have four different PCR products. There are two different genes encoding CGRP, and the primers were designed to identify one of them, CGRPα (Amara et al., 1982, 1985). The sequences of primers, annealing temperatures, amplification cycles, and the predicted sizes of the PCR products are given in Table 1.

Specificity of the primers

We first tested whether these primers specifically identify the mRNAs for the intended neuromodulators. Total RNA was prepared from adult rat spinal cord and reverse-transcribed into cDNA. All cDNAs except actin were amplified for 35 cycles; actin was amplified for 20 cycles. Each set of primers (except those for SP) generated a single PCR product whose size was predicted from the known gene sequences (Fig. 1, "+" lanes). Reverse transcription was essential for generation of these expected products, as they did not appear in the samples that were not treated with reverse transcriptase (Fig. 1, "−" lanes). The primers for GAD65, SOM, and TH presumably also recognized genomic DNA and produced PCR products whose sizes were larger than expected in the samples not treated with reverse transcriptase (Fig. 1, GAD65, SOM, TH, "−" lanes). This did not interfere with the assay, however, because these bands disappeared when there is competition with the proper templates present in the cDNA. The SP primers produced three products whose sizes (375, 331, and 276 bp) corresponded to the mRNAs for β-, γ-, and δ-SP, respectively (Krause et al., 1987; Harmar et al., 1990). The assay also yielded the expected relative levels of these mRNAs; β-SP and γ-SP were more abundant than δ-SP, whereas α-SP comprised less than 1% of the total SP mRNAs (Carter and Krause, 1990; Harmar et al., 1990) and was undetectable in our assay. We used two sets of primers to detect GAD67 and GAD65, and judging from the intensity of the products, there was more GAD67 than GAD65 mRNA in the spinal cord.

These results demonstrate that the chosen primers yield a specific assay for these neuromodulators. We sequenced three PCR products (β-actin, because there are many actin homologues, and CCK and ENK, because our findings for these two genes are novel, as described below). All the clones that were sequenced (at least three clones for each PCR product) contained the sequences expected for these genes. This was expected, because the annealing temperature used in the PCR for each set of primers was close to or above the predicted melting temperature in an effort to increase the specificity of the PCR. In addition, we have demonstrated that these PCR products only appear when RT is present, that there is only one PCR product for each gene analyzed, and that the size of each PCR product is identical to that predicted from the known sequences. Although the PCR can generate artifacts when unknown sequences are cloned from ambiguous primers, this type of artifact rarely occurs with primers that are identical to the known sequences, especially when the PCR is carried out under high-stringency conditions, as in these experiments. Therefore, the PCR products obtained here should represent products of the genes indicated. The data also show that the adult rat spinal cord expresses a variety of neuromodulator mRNAs, albeit at different levels, some of which (DYN, GAD65) had not been readily identified in the spinal cord by other methods.

CDF/LIF induces CCK and ENK in cultured sympathetic neurons

To test further the applicability of the assay, we asked whether it can reproduce the known effects of CDF/LIF on cultured sympathetic neurons. It had been shown that CDF/LIF induces the expression of SP, SOM, VIP, and ChAT (Nawa et al., 1990, 1991). Sympathetic neurons were cultured in 96-well plates for 7 days in serum-free medium to avoid the inductive effects of serum (Wolinsky and Patterson, 1985). Different concentrations of recombinant human CDF/LIF were added between days 2 and 7. Duplicate samples were used for each concentration to compensate for an occasional failure of amplification (about 5%). At the end of 7 days of culture, RNA was extracted from each well and reverse transcribed to cDNA. The cDNA derived from a single well was used as template for 14 PCRs for each gene, in 14 separate tubes. β-Actin expression was used to monitor the amount of RNA in each sample. The expression pattern of these neuromodulators, with and without CDF/LIF, was similar to that previously reported (Nawa et al., 1990). CDF/LIF induced expression of

TABLE 1. *Sequences of the primers, predicted sizes of PCR fragments, and amplification cycles used in the PCR*

Neuromodulator	Sequence	Predicted size (bp)	Annealing temp.	Amplification cycles	Reference
β-Actin	TCATGAAGTGTGACGTTGACATCCGT CCTAGAAGCATTTGCGGTGCACGATG	285	58	18	Nudel et al., 1983
CGRP	TTCTGAAGTTCTCCCCTTTCC GCATATAGTTCTGCACCAGTGC	165	58	35	Amara et al., 1982
CCK	GACTCCGCATCCGAAGAT CTACGATGGGTATTCGTA	366	50	35	Deschenes et al., 1985
ChAT	GCTTACTACAGGCTTTAC GACAAACCGGTTGCTCAT	338	50	30	Hahn et al., 1992
DYN	ATCAACCCCCTGATTTGCTCC GCCTGTTTTCTCAAGTCCTCC	368	60	33	Douglass et al., 1989
ENK	ATCAACTTCCTGGCATGC GCTCGTGCTGTCTTCATC	429	50	28	Rosen et al., 1984
GAD65	GCTCTGGCTTTTGGTCCTTCG TTTTGGTCACAGGTGCAGGCG	220	58	40	Erlander et al., 1991
GAD67	CTGCCAATACCAATATGTTCAC TTGTCAGTTCCAAAGCCAAGCG	297	58	35	Wyborski et al., 1990
NPY	GCTAGGTAACAAACGAATGGGG CACATGGAAGGGTCTTCAAGC	288	58	22	Larhammar et al., 1987
SOM	CCAGACTCCGTCAGTTTCTGC AGTTCTTGCAGCCAGCTTTGC	238	58	28	Montminy et al., 1984
SP	ATGAAAATCCTCGTGGCG GTAGTTCTGCATTGCGCT	$\alpha = 321, \beta = 375$ $\gamma = 331, \delta = 276$	58	27	Carter and Krause, 1990
TPH	GATGCAAAGGAGAAGATG TAAGCAGGTGACAGAAAT	410	50	31	Darmon et al., 1988
TH	CGGGCTATGTAAACAGAATGGG GATGGAGACTTTGGGAAAGGC	418	58	24	Grima et al., 1985
VIP	AGTGTGCTGTTCTCACAGTCG GCTGGTGAAAACTCCATCAGC	216	58	28	Nishizawa et al., 1985

SP, VIP, ChAT, and SOM in a dose-dependent manner (Fig. 2). In addition, CDF/LIF induced expression of CCK and ENK. These data clearly illustrate that by this RT-PCR assay we can reproduce previous results, as well as provide the sensitivity to uncover new findings.

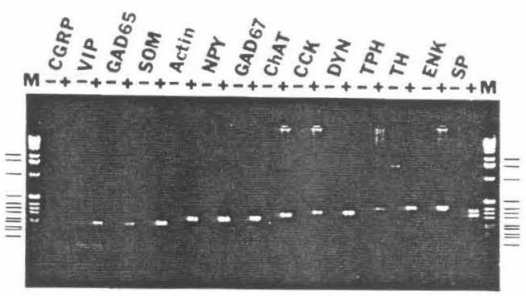


FIG. 1. Primers amplify cDNA-derived, but not genomic, DNA templates. Total RNA was prepared from adult rat spinal cord and either converted to cDNA with reverse transcriptase ("+" lanes) or not treated with reverse transcriptase ("−" lanes). All samples were amplified for 35 cycles in the PCR, except actin, which was amplified for 20 cycles. The samples were loaded in ascending order of expected sizes, based on the known gene sequences, as listed in Table 1. A 1-kb DNA marker was used as DNA molecular weight standard ("M" lanes) and the measured sizes are, from bottom to top, 75, 134, 154, 201, 220, 298, 344, 396, 517/506, 1,018, 1,635, 2,036 bp.

The concentrations of CDF/LIF needed to induce various mRNAs to maximal levels varied. The signals for SP, VIP, and ENK reached plateau at 0.4 ng/ml of CDF/LIF, whereas more than 1.6 ng/ml was needed to induce the expression of ChAT, SOM, and CCK fully. It may be that different genes are induced by different transcription factors, which are turned on at different concentrations of CDF/LIF (e.g., Lord et al., 1991; Yamamori, 1991; Ip et al., 1992).

The assay was further used to determine the effect of CDF/LIF on other neuromodulators found previously to be expressed by sympathetic neurons (Sah and Matsumoto, 1987; Nawa and Sah, 1990; Vandenbergh et al., 1991). In the absence of CDF/LIF, these neurons detectably expressed mRNAs for the transmitter/synthetic enzymes TH, TPH, and GAD67, as well as the neuropeptides DYN, NPY, and CGRP (Fig. 2). GAD65 was undetectable, even after 40 cycles of amplification (data not shown). Addition of CDF/LIF did not increase expression of these genes. The apparent increase in expression of GAD67 by CDF/LIF (Fig. 2) was not reproducible. We were unable to determine whether CDF/LIF decreased the expression of these genes (TH, NPY, CGRP, TPH, DYN) because the β-actin control also decreased at high concentrations of CDF/LIF. Because this type of deleterious effect had not been found with purified heart cell CDF/LIF (Fukada, 1985), the apparent toxicity may be due to *Esche-*

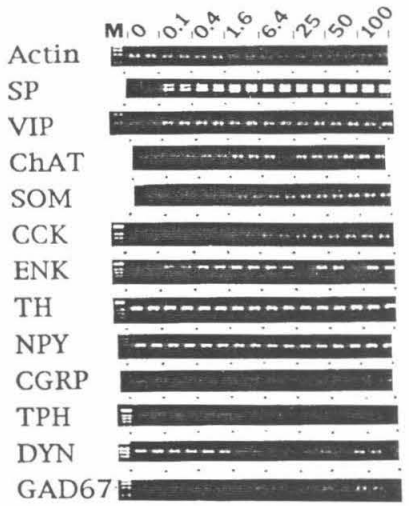


FIG. 2. CDF/LIF induces expression of SP, VIP, ChAT, SOM, CCK, and ENK, but not TH, NPY, CGRP, TPH, DYN, or GAD67 in cultured sympathetic neurons. Different concentrations of CDF/LIF (nanograms per milliliter) were added to cultured sympathetic neurons for 6 days and the RT-PCR assay was used to monitor a variety of mRNAs. Duplicate samples were run at each concentration. Actin was used as control to monitor the amount of mRNA in the sample. Some lanes yield no products due to failure of amplification. The 1-kb DNA marker ("M" lane) was used as in Fig. 1.

richia coli-derived products in the recombinant protein preparation.

CNTF induces the same neuromodulators as CDF/LIF

Another cytokine that has been shown to induce the expression of several neuromodulators is CNTF (Saadat et al., 1989; Rao et al., 1992*a*). CNTF is predicted to have a structure similar to that of CDF/LIF (Bazan, 1991; Rose and Bruce, 1991). In addition, CNTF shares receptor subunits and signal transduction pathways with CDF/LIF (Ip et al., 1992). When added to the sympathetic neuronal cultures and analyzed by RT-PCR assay, CNTF induced the same pattern of neuromodulators as CDF/LIF (Fig. 3). The induction of SP, VIP, ChAT, SOM, CCK, and ENK mRNAs was clear. As with CDF/LIF, a higher concentration of CNTF was required to induce SOM, ChAT, and CCK. The induction of SP, VIP, ChAT, and SOM by CNTF had been shown previously by other methods (Saadat et al., 1989; Rao et al., 1992*a*). CNTF had no detectable inductive effects on TH, NPY, CGRP, TPH, DYN, or GAD67 (data not shown). Although higher concentrations of CNTF appeared to be required for induction of neuromodulators in Fig. 3, such concentration differences between the effects of CNTF and CDF/LIF were not reproducible. The decrease of β-actin with increasing CNTF may indicate, however, that toxicity is associated with the recombinant protein preparation.

DISCUSSION

We describe a method, based on RT-PCR, for detecting mRNAs for a variety of transmitters and neuropeptides present at low levels in primary neuronal cultures. Specific sets of primers were designed for 14 different genes. When tested in RT-PCR using adult spinal cord RNA, these primers amplify cDNA even in the presence of genomic DNA, and single PCR products are generated that have the sizes predicted from the known sequences of the genes (except for SP, which yielded three products of expected sizes). This demonstrates that genomic DNA does not interfere with the assay even though it may contaminate the samples.

Sympathetic neurons have been used to study neuronal phenotypic choice and to identify neuronal differentiation factors (Patterson, 1978, 1992). Using the RT-PCR method, we demonstrate that cultured sympathetic neurons have the capacity to express CGRP, CCK, ChAT, DYN, ENK, GAD67, NPY, SOM, SP, TPH, TH, and VIP. The expression of this variety of genes makes sympathetic neurons a good choice for a responder cell to test the effects of candidate neuronal differentiation factors. When CDF/LIF and CNTF are applied to sympathetic neurons, they induce expression of mRNAs for CCK, ChAT, ENK, SOM, SP, and VIP in a very similar manner. The finding that CCK and ENK are induced by CDF/LIF and CNTF had not been reported previously, and suggests that these cytokines may be responsible for the induction of CCK and ENK by heart cell conditioned medium (Nawa and Sah, 1990).

Rao et al. (1992*a*) did not observe induction of leu-enkephalin by CNTF using a radioimmunoassay for

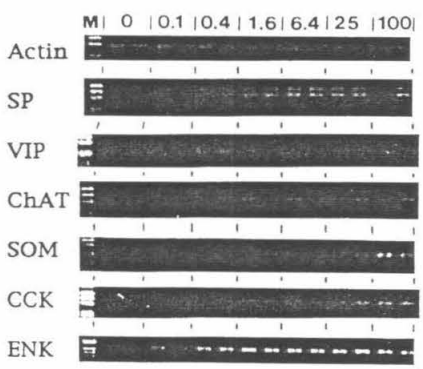


FIG. 3. CNTF induces the same set of neuromodulators as does CDF/LIF in cultured sympathetic neurons. Various concentrations of CNTF (nanograms per milliliter) were added to cultured sympathetic neurons for 6 days and the RT-PCR assay was used to monitor the expression of neuromodulators as in Fig. 2.

the neuropeptide. This discrepancy with our results may be due to a difference in sensitivity of the assays or to induction of ENK mRNA but not ENK peptide. Such a differential regulation of mRNA and protein has been shown previously. For example, nonneuronal cells induce SOM mRNA but not SOM peptide in cultured sympathetic neurons (Spiegel et al., 1990). In addition, ENK mRNA is induced in the adrenal medulla when it is placed in culture (Zhu et al., 1992). It may be interesting to determine whether CDF/LIF or CNTF is responsible for this induction.

The concentration of CDF/LIF and CNTF needed for full induction of SP, ENK, and VIP was different from that required for full induction of ChAT, SOM, and CCK. This suggests that these genes are regulated by different signal transduction pathways. It has been shown that CDF/LIF and CNTF induce the expression of several early genes, including c-*jun*, *junB*, *junD*, c-*fos*, and *tis11* (Lord et al., 1991; Yamamori, 1991; Ip et al., 1992). The concentration dependence of the induction of these early genes has not been reported, however.

The present results further characterize the effects of CDF/LIF and CNTF on cultured sympathetic neurons. These data are consistent with indications that these cytokines can share receptor subunits and signal transduction pathways (Ip et al., 1992), as predicted by Bazan (1991). The emerging picture of a family of neuropoietic cytokines that have redundant effects on the nervous system is consistent with similar findings on the control of hematopoietic phenotypes (Nawa et al., 1990; Patterson and Nawa, 1993).

Acknowledgment: We thank Doreen McDowell for help with tissue culture materials, Derek Stemple for advice on design of primers, and Lisa Banner, Herman Govan, and Zaven Kaprielian for constructive comments on the manuscript. This project was supported by grants from Amgen and NINDS (Javits Neuroscience Investigator Award) to P.H.P., and by a Fellowship from the Ministry of Education, Taiwan, R.O.C., to M.-J.F.

REFERENCES

Amara S. G., Jonas V., Rosenfeld M. G., Ong E. S., and Evans R. M. (1982) Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* **298,** 240–244.

Amara S. G., Arriza J. L., Leff S. E., Swanson L. W., Evans R. M., and Rosenfeld M. G. (1985) Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. *Science* **229,** 1094–1097.

Bazan J. F. (1991) Neuropoietic cytokines in the hematopoietic fold. *Neuron* **7,** 197–208.

Belyavsky A., Vinorgradova T., and Rajewsky K. (1989) PCR-based cDNA library construction: general cDNA libraries at the level of a few cells. *Nucleic Acids Res.* **17,** 2919–2932.

Black I. B., Adler J. E., Dreyfus C. F., Friedman W. F., LaGamma E. F., and Roach A. H. (1987) Biochemistry of information storage in the nervous system. *Science* **236,** 1263–1268.

Carter M. S. and Krause J. E. (1990) Structure, expression, and some regulatory mechanism of the rat preprotachykinin gene encoding substance P, neurokinin A, neuropeptide K, and neuropeptide γ. *J. Neurosci.* **10,** 2203–2214.

Chomczynski P. and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.

Darmon M. C., Guibert B., Leviel V., Ehret M., Maitre M., and Mallet J. (1988) Sequence of two mRNAs encoding active rat tryptophan hydroxylase. *J. Neurochem.* **51,** 312–316.

Deschenes R. J., Haun R. S., Funckes C. L., and Dixon J. E. (1985) A gene encoding rat cholecystokinin: isolation, nucleotide sequence, and promoter activity. *J. Biol. Chem.* **260,** 1280–1286.

Douglass J., McMurray C. T., Garrett J. E., Adelman J. P., and Calavetta L. (1989) Characterization of the rat prodynorphin gene. *Mol. Endocrinol.* **3,** 2070–2078.

Erlander M. G., Tillakaratne N. J. K., Feldblum S., Patel N., and Tobin A. J. (1991) Two genes encode distinct glutamate decarboxylases. *Neuron* **7,** 91–100.

Fukada K. (1985) Purification and partial characterization of a cholinergic neuronal differentiation factor. *Proc. Natl. Acad. Sci. USA* **82,** 8795–8799.

Garrett J. E., Collard M. W., and Douglass J. O. (1989) Translational control of germ cell-expressed mRNA imposed by alternative splicing: opioid peptide gene expression in rat tissue. *Mol. Cell. Biol.* **9,** 4381–4389.

Grima B., Lamouroux A., Blanot F., Biguet N. F., and Mallet J. (1985) Complete coding sequence of rat tyrosine hydroxylase mRNA. *Proc. Natl. Acad. Sci. USA* **82,** 617–621.

Hahn M., Hahn S. L., Stone D. M., and Joh T. H. (1992) Cloning of the rat gene encoding choline acetyltransferase, a cholinergic neuron-specific marker. *Proc. Natl. Acad. Sci. USA* **89,** 4387–4391.

Harmar A. J., Hyde V., and Chapman K. (1990) Identification and cDNA sequence of delta-preprotachykinin, a fourth splicing variant of the rat substance P precursor. *FEBS Lett.* **275,** 22–24.

Hawrot E. and Patterson P. H. (1979) Long-term culture of dissociated sympathetic neurons. *Methods Enzymol.* **58,** 574–584.

Hendry I. A. and Hill C. E., eds (1992) *The Autonomic Nervous System, Vol. 2: Development, Regeneration and Plasticity of the Autonomic Nervous System.* Harwood Academic Publishers, Chur, Switzerland.

Ip N. Y., Nye S. H., Boulton T. G., Davis S., Taga T., Li Y., Birren S. J., Yasukawa K., Kishimoto T., Anderson D. J., Stahl N., and Yancopoulos G. D. (1992) CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* **69,** 1121–1132.

Krause J. E., Chirgwin J. M., Carter M. S., Xu Z. S., and Hershey A. D. (1987) Three rat preprotachykinin mRNA encode the neuropeptides substance P and neurokinin A. *Proc. Natl. Acad. Sci. USA* **84,** 881–885.

Landis S. C. (1990) Target regulation of neurotransmitter phenotype. *Trends Neurosci.* **13,** 344–350.

Larhammar D., Ericsson A., and Persson H. (1987) Structure and expression of the rat neuropeptide Y gene. *Proc. Natl. Acad. Sci. USA* **84,** 2068–2072.

Lord K. A., Abdollahi A., Thomas S. M., Demarco M., Brugge J. S., Hoffman-Liebermann B., and Liebermann D. A. (1991) Leukemia inhibitory factor and interleukin-6 trigger the same immediate early response, including tyrosine phosphorylation, upon induction of myeloid leukemia differentiation. *Mol. Cell. Biol.* **11,** 4371–4379.

Lowe T., Sharefkin J., Yang S. Q., and Dieffenbach C. W. (1990) A computer program for selection of oligonucleotide primers for polymerase chain reaction. *Nucleic Acids Res.* **18,** 1757–1761.

Montminy M. R., Goodman R. H., Horovitch S. J., and Habener J. F. (1984) Primary structure of the gene encoding rat preprosomatostatin. *Proc. Natl. Acad. Sci. USA* **81,** 3337–3340.

Nawa H. and Patterson P. H. (1990) Separation and partial characterization of neuropeptide-inducing factors in heart cell conditioned medium. *Neuron* **4,** 269–277.

Nawa H. and Sah D. W. Y. (1990) Different biological activities in conditioned media control the expression of a variety of neuropeptides in cultured sympathetic neurons. *Neuron* **4**, 279–287.

Nawa H., Yamamori T., Le T., and Patterson P. H. (1990) Generation of neuronal diversity: analogies and homologies with hematopoiesis. *Cold Spring Harb. Symp. Quant. Biol.* 55, 247–253.

Nawa H., Nakanishi S., and Patterson P. H. (1991) Recombinant cholinergic differentiation factor (leukemia inhibitory factor) regulates sympathetic neuron phenotype by alterations in the size and amounts of neuropeptide mRNAs. *J. Neurochem.* **56**, 2147–2150.

Nishizawa M., Hayakawa Y., Yanaihara N., and Okamoto H. (1985) Nucleotide sequence divergence and functional constraint in VIP precursor mRNA evolution between human and rat. *FEBS Lett.* **183**, 55–59.

Nudel U., Zakut R., Shani M., Neuman S., Levy Z., and Yaffe D. (1983) The nucleotide sequence of the rat cytoplasmic beta-actin gene. *Nucleic Acids Res.* **11**, 1759–1771.

Patterson P. H. (1978) Environmental determination of autonomic neurotransmitter functions. *Annu. Rev. Neurosci.* **1**, 1–17.

Patterson P. H. (1992) The emerging neuropoietic cytokine family: first CDF/LIF, CNTF and IL-6; next ONC, MGF and GCSF? *Curr. Opin. Neurobiol.* **2**, 94–97.

Patterson P. H. and Landis S. C. (1992) Phenotype specifying factors and the control of neuronal differentiation decisions, in *Development, Regeneration and Plasticity of the Autonomic Nervous System* (Hendry I. A. and Hill C. E., eds), pp. 231–265. Harwood Academic Publishers, Chur, Switzerland.

Patterson P. H. and Nawa H. (1993) Neuronal differentiation factors/cytokines and sympathetic plasticity. *Cell* **72**, 123–137.

Rao M. S., Tyrrell S., Landis S. C., and Patterson P. H. (1992*a*) Effects of ciliary neurotrophic factor (CNTF) and depolarization on neuropeptide expression in cultured sympathetic neurons. *Dev. Biol.* **150**, 281–293.

Rao M. S., Patterson P. H., and Landis S. C. (1992*b*) Multiple cholinergic differentiation factors are present in footpad extracts: comparison with known cholinergic factors. *Development* **116**, 731–744.

Rohrer H. (1992) Cholinergic neuronal differentiation factors: evidence for the presence of both CNTF-like and non-CNTF-like factors in developing rat footpad. *Development* **114**, 689–698.

Rose T. M. and Bruce A. G. (1991) Oncostatin M is a member of a cytokine family which includes leukemia inhibitory factor, granulocyte colony-stimulating factor and interleukin-6. *Proc. Natl. Acad. Sci. USA* **88**, 8641–8645.

Rosen H., Douglass J., and Herbert E. (1984) Isolation and characterization of the rat proenkephalin gene. *J. Biol. Chem.* **259**, 14309–14313.

Saadat S., Sendtner M., and Rohrer H. (1989) Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. *J. Cell Biol.* **108**, 1807–1816.

Sah D. W. Y. and Matsumoto S. G. (1987) Evidence for serotonin synthesis, uptake and release in dissociated rat sympathetic neurons in culture. *J. Neurosci.* **7**, 391–399.

Spiegel K., Wong V., and Kessler J. A. (1990) Translational regulation of somatostatin in cultured sympathetic neurons. *Neuron* **4**, 303–311.

Vandenbergh D. J., Mori N., and Anderson D. J. (1991) Co-expression of multiple neurotransmitter enzyme genes in normal and immortalized sympathoadrenal progenitor cells. *Dev. Biol.* **148**, 10–22.

Wallace T. L. and Johnson E. M. (1989) Cytosine arabinoside kills postmitotic neurons: evidence that deoxycytidine may have a role in neuronal survival that is independent of DNA synthesis. *J. Neurosci.* **9**, 115–124.

Wolinsky E. J. and Patterson P. H. (1985) Rat serum contains a developmentally regulated cholinergic inducing activity. *J. Neurosci.* **5**, 1509–1512.

Wolinsky E. J., Landis S. C., and Patterson P. H. (1985) Expression of noradrenergic and cholinergic traits by sympathetic neurons cultured without serum. *J. Neurosci.* **5**, 1497–1508.

Wyborski R. J., Bond R. W., and Gottlieb D. I. (1990) Characterization of a cDNA coding for rat glutamic acid decarboxylase. *Mol. Brain Res.* **8**, 193–198.

Yamamori T. (1991) CDF/LIF selectively increase c-*fos* and *jun*-B transcripts in sympathetic neurons. *Neuroreport* **2**, 173–176.

Yamamori T., Fukada K., Aebersold R., Korsching S., Fann M.-J., and Patterson P. H. (1989) The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science* **246**, 1412–1416.

Zhu Y.-S., Branch A. D., Robertson H. D., Huang T. H., Franklin S. O., and Inturrisi C. E. (1992) Time course of enkephalin mRNA and peptides in cultured rat adrenal medulla. *Mol. Brain Res.* **12**, 173–180.

CHAPTER 3. Neuropoietic cytokines and activin A differentially regulate the phenotype of cultured sympathetic neurons

Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 43–47, January 1994
Neurobiology

Neuropoietic cytokines and activin A differentially regulate the phenotype of cultured sympathetic neurons

MING-JI FANN AND PAUL H. PATTERSON

Biology Division, California Institute of Technology, Pasadena, CA 91125

Communicated by Norman Davidson, August 31, 1993 (received for review July 2, 1993)

ABSTRACT **A number of cytokines sharing limited sequence homology have been grouped as a family because of partially overlapping biological activities, receptor subunit promiscuity, and the prediction of a shared secondary structure. Since several of these cytokines regulate gene expression and cell number in the nervous and hematopoietic systems, this specific group is termed the neuropoietic cytokine family. Using a reverse transcription–polymerase chain reaction-based assay system for monitoring the expression of multiple phenotypic markers in cultured sympathetic neurons, we present further evidence that, in addition to cholinergic differentiation factor/leukemia inhibitory factor and ciliary neurotrophic factor, oncostatin M, growth promoting activity, interleukin 6, and interleukin 11 belong in this family. In addition, one member of the transforming growth factor β superfamily, activin A, shares a selective overlap with the neuropoietic family in the spectrum of neuropeptides that it induces in sympathetic neurons. The particular neuropeptides induced by activin A, however, demonstrate that the activity of this cytokine is distinct from that of the neuropoietic family. Twenty-six other cytokines and growth factors were without detectable activity in this assay.**

Diffusible proteins can regulate neural development at a number of stages, from the initial proliferation and differentiation of progenitor cells, through the outgrowth of processes and formation of synapses, to the rearrangement of connections in postnatal life (1). Two of the families of proteins involved in these events include the neurotrophins and the neuropoietic cytokines. The prototype of the former group is nerve growth factor (NGF), and subsequent family members were cloned by utilizing the extensive homology between NGF and the second neurotrophin identified, brain-derived neurotrophic factor. In contrast, the neuropoietic family is emerging not through strong homologies among cytokine sequences, but rather by shared biological activities and predicted secondary structures (2, 3). Subsequent studies on the receptors for the neuropoietic cytokines have revealed not only structural homology among the receptors but the sharing of a common receptor transducing subunit and similar signal-transducing pathways (4–8).

Inaugural members of the neuropoietic family include a protein we termed the cholinergic differentiation factor (CDF; refs. 9–11), also widely known as leukemia inhibitory factor (LIF; ref. 12), and ciliary neurotrophic factor (CNTF; refs. 13–15). These two proteins display identical profiles of activity in the regulation of neuropeptide expression in cultured sympathetic neurons (16, 17) and in the support of motor neuron survival (18, 19). In addition, Bazan (2) predicted that CDF/LIF and CNTF would share a similar secondary structure with interleukin (IL)-6, oncostatin M (OSM), and granulocyte colony-stimulating factor (G-CSF). Similar predictions were made by Rose and Bruce (20). While it was known that CDF/LIF and IL-6 elicited many of the same responses in nonneural cells (21), the linkage with OSM was not previously suspected. Further evidence for a close relationship between these proteins is the finding that OSM can displace CDF/LIF from its binding sites on M1 cells (22). Moreover, OSM was recently found to induce the same neuropeptide in a neuroblastoma line as CDF/LIF (23). Another candidate for membership in this family is IL-11, a cytokine that phosphorylates the same gp130 receptor subunit as do CDF/LIF, CNTF, and IL-6 (5, 24). Recently, growth-promoting activity (GPA), a protein that shares trophic activity for ciliary neurons with CNTF, was cloned from chicken and found to have a limited sequence homology with mammalian CNTF (25). Finally, extracts of rat foot pads (which contain sweat glands, the target of cholinergic sympathetic neurons) were found to contain a protein, termed the sweat gland factor (SGF), that exhibits both CDF/LIF and CNTF activities and is similar to, but distinct from, CNTF (26, 27). SGF has not as yet been sequenced. All of these proteins are therefore candidates for membership in the neuropoietic cytokine family.

Other cytokines may be involved in the development of the nervous system. IL-3 acts as a trophic factor for central cholinergic neurons (28), and IL-5, -7, -9, and -11 regulate differentiation of murine hippocampal progenitor cells (29). IL-1 and tumor necrosis factor α (TNFα), two inflammatory cytokines released after injury, can induce NGF and CDF/LIF, which in turn have effects on neuronal survival and gene expression (30–32). Activin A, a member of the transforming growth factor β (TGFβ) superfamily, stimulates expression of the neuropeptide somatostatin in cultured ciliary ganglion neurons (33). Accordingly, these cytokines are candidate neuronal differentiation factors. Nonetheless, there has been no systematic analysis of the effects of these cytokines on neuronal phenotype.

We have designed a reverse transcription (RT)-PCR assay to analyze the effects of soluble factors on the expression of neuropeptides and neurotransmitter synthesizing enzymes in cultured sympathetic neurons. In this assay, CDF/LIF and CNTF induce an identical set of neuropeptide and neurotransmitter enzyme mRNAs (34). We here present the results of testing the effects of 33 different recombinant cytokines and growth factors on neuronal gene expression. These

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CCK, cholecystokinin; CDF/LIF, cholinergic differentiation factor/leukemia inhibitory factor; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; CNTF, ciliary neurotrophic factor; DYN, dynorphin; ENK, enkephalin; FGF, fibroblast growth factor; GAD65, 65-kDa glutamic acid decarboxylase; GAD67, 67-kDa glutamic acid decarboxylase; G-CSF, granulocyte colony-stimulating factor; GPA, growth-promoting activity; GROα, growth-related cytokine α; IL, interleukin; M-CSF, macrophage colony-stimulating factor; MIP1α, macrophage inflammatory protein 1α; NGF, nerve growth factor; NPY, neuropeptide Y; OSM, oncostatin M; rh-, recombinant human; RT, reverse transcription; SGF, sweat gland factor; SOM, somatostatin; SP, substance P; TGFα and -β, transforming growth factor α and β; TH, tyrosine hydroxylase; TNFα, tumor necrosis factor α; TPH, tryptophan hydroxylase; VIP, vasoactive intestinal polypeptide.

results support the hypothesis that GPA and OSM belong in the neuropoietic family. In addition, weak activities of IL-6 and IL-11 can also be detected in this assay. The pattern of neuronal gene expression induced by activin A, however, sets it apart from this family.

MATERIALS AND METHODS

Animals and Reagents. Neonatal Sprague–Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA). Most of the tissue culture reagents, Superscript reverse transcriptase II, and the 1-kb DNA marker were purchased from GIBCO/BRL. NGF, glycogen, dATP, dCTP, dGTP, dTTP, acidic fibroblast growth factor (FGF) and basic FGF were purchased from Boehringer Mannheim. RNase inhibitor (RNasin) and *Taq* DNA polymerase were purchased from Promega. Oligo(dT) was obtained from Pharmacia. Oligonucleotide primers were synthesized in the Biopolymer Synthesis and Analysis Resource Center at Caltech. The recombinant proteins murine IL-1, murine TNF-α, human activin A, human growth-related cytokine α (GROα), and rat CNTF were gifts from Genentech. Chick GPA was kindly provided by T. Finn and Rae Nishi of Oregon Health Sciences University (Portland). Human recombinant activin A was also obtained from Jim Smith at the National Institute for Medical Research (London). Human macrophage inflammatory protein 1α (MIP1α) was provided by Mary Freshney and Gerry Graham at the Beatson Institute for Cancer Research (Glasgow, U.K.). The recombinant proteins murine IL-3, -4, -6, -7, and -9, human IL-8, and macrophage colony stimulating factor (M-CSF) were purchased from R & D Systems. The recombinant protein human IL-10 was supplied from Genzyme. The recombinant protein human IL-11 was a gift from Yu-Chung Yang at Indiana University. The recombinant protein murine IL-12 was provided by Stanley Wolf from Genetics Institute (Cambridge, MA). The recombinant protein human OSM was provided by David Gearing at Immunex. Other cytokines and growth factors used in this study were recombinant human (rh) proteins provided by James Miller and colleagues at Amgen. All other reagents were purchased from Sigma.

Neuronal Culture. Dissociated sympathetic neurons were prepared and cultured in serum-free medium as described previously (34–36). For reproducibility, neurons were seeded in 96-well plates (Falcon), at a density of one ganglion per well. Cultures were maintained for 7 days, and half of the medium was changed every 36 hr in an effort to ensure that cytokines maintained activity. The antimitotic agent aphidicolin was added to the cultures at the concentration of 4 μg/ml, effectively eliminating nonneuronal cells. After 7 days, each well contained approximately 3000 neurons, and more than 95% of the surviving cells were neurons, as judged by phase-contrast microscopy (36). Cytokines and growth factors were added from the second day of culture, and duplicate wells were prepared for each condition. All results reported here were reproduced in at least two separate neuronal platings.

Preparation of RNA and cDNA. Total RNA preparation and cDNA synthesis from cultured neurons were as described previously (34). Briefly, 160 μl of lysis buffer was added to each well. The lysate was transferred to a 1.7-ml Eppendorf tube and swirled vigorously to shear DNA, and then one round of acidic phenol/chloroform extraction was performed to deplete DNA and proteins. RNA was coprecipitated with 30 μg of glycogen in a 50% isopropyl alcohol solution and stored at −20°C overnight.

To produce cDNA from the cultured neurons, the total RNA was centrifuged, dried, and directly dissolved in 10 μl of 13 mM methylmercury hydroxide for 10 min, and an additional 2 μl of 75 mM 2-mercaptoethanol was added for 5 min. Oligo(dT)-primed RT was done in a final volume of 20 μl, containing the RT buffer (34), 100 units of reverse transcriptase, and 20 units of RNasin, for 1 hr at 37°C.

PCR. The sequences of the primers and conditions used in the PCR for each neuronal gene analyzed were described in a previous report (34). Amplification of cDNA was performed in a thermal cycler (MJ Research, Watertown, MA). Each tube contained a final volume of 20 μl, consisting of 1 μl of cDNA, 1× PCR buffer (Promega), 0.5 unit of *Taq* DNA polymerase, 0.25 mM dNTP, and one set of primers (200 nM). After the reaction, 8 μl of each PCR sample was analyzed on a 2% agarose gel, and the products were visualized after ethidium bromide staining and UV illumination. The appropriate amplification cycle schedule for each neuropeptide gene was determined empirically, to allow a minimal, yet detectable, signal for control samples (neurons grown without cytokines or growth factors). Cytokines were deemed to have regulatory effects on specific genes if the intensities of the PCR products were about 3-fold different from those of negative controls, as judged by eye.

RESULTS

Screening of 33 Cytokines and Growth Factors for Effects on Neuronal Gene Expression. In the first screen for effects of cytokines and growth factors on neuronal phenotype, two concentrations (10 ng/ml and 100 ng/ml) of each recombinant protein were applied to neonatal rat sympathetic neurons in culture for 6 days. The expression pattern of different neuromodulators in the presence of cytokines and growth factors was elucidated by the RT-PCR assay and the results are summarized in Table 1. Twenty-six factors showed no effect on the intensity of the PCR products for any of the six genes analyzed, in at least two independent experiments; these factors were not analyzed further. In addition to reporting our findings with CDF/LIF and CNTF, we report here in detail for the first time the effects of GPA, OSM, IL-6, IL-11, and activin A on neuronal gene expression. Some of the inductive effects of CDF/LIF, CNTF, and OSM have been demonstrated previously (16, 17, 23, 34, 37).

GPA Induces the Same Set of Neuropeptides and Neurotransmitter Synthetic Enzymes as CNTF. GPA was purified from chick and has the same ciliary neurotrophic activity as mammalian CNTF. Moreover, the amino acid sequence of GPA shows a 50% identity to the sequences for human, rat, and rabbit CNTF (25). In contrast to CNTF, however, GPA has been shown to be secreted from cells in a transient transfection experiment (25). We tested whether GPA has the same effects as CNTF on cultured sympathetic neurons, and the results are illustrated in Fig. 1. The β-actin band was used to monitor the amount of mRNA present in each sample, and duplicate samples were prepared for each condition. GPA induces mRNAs for the neuropeptides substance P (SP), enkephalin (ENK), vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), and somatostatin (SOM), as well as the enzyme for synthesizing acetylcholine, choline acetyltransferase (ChAT), in a dose-dependent manner. This pattern of neuropeptide induction is very similar to that of CNTF and CDF/LIF. We also analyzed expression of the mRNAs for the enzymes synthesizing γ-aminobutyric acid (glutamic acid decarboxylase; GAD65 and GAD67 for the 65- and 67-kDa forms), the enzyme for synthesizing catecholamines (tyrosine hydroxylase; TH), the enzyme for synthesizing serotonin (tryptophan hydroxylase; TPH), and the neuropeptides calcitonin gene-related peptide (CGRP), dynorphin (DYN), and neuropeptide Y (NPY). As with CNTF, GPA has no detectable inductive effect on these genes at the mRNA level (data not shown). This identity in the expression patterns of 13 neuropeptide and neurotransmitter mRNAs

Table 1. Regulation of gene expression in sympathetic neurons

Cytokines	CCK	ChAT	ENK	SOM	SP	VIP	CGRP, DYN, NPY	GAD, TH, TPH
Neuropoietic								
CDF/LIF, CNTF, GPA	++	+	++	++	+++	+	–	–
OSM	+	+	+	+	++	+	–	–
IL-6, IL-11	–	–	–	–	+	–	–	–
G-CSF	–	–	–	–	–	–	–	–
Other								
Activin A	+	+	–	++	–	–	++	–
IL-1α, -2, -3, -4, -5, -7, -8, -9, -10, -12, IFN-γ, GM-CSF, M-CSF, EPO, TGFα, TGFβ, EGF, IGF-1, TNFα, SCF, MIP1α, GROα, PDGF, acidic FGF, basic FGF	–	–	–	–	–	–	ND	ND

A + indicates that there is induction effect; a – indicates that there is no induction effect; ND, not determined. Cytokines not previously defined: IFN-γ, interferon γ; EPO, erythropoietin; TGFα and -β, transforming growth factor α and β; EGF, epidermal growth factor; IGF-1, insulin-like growth factor 1; SCF, stem cell factor; PDGF, platelet-derived growth factor. Neuropeptides and enzymes in column heads are defined in the text.

strengthens the idea that GPA and CNTF share many biological activities.

OSM, IL-6, and IL-11 Have Activity in the Sympathetic Neuron Assay. OSM and CDF/LIF have the same biological activities in several assay systems, including triggering differentiation of M1 leukemia cells and the induction of the acute phase response in hepatocytes (38, 39). Recently, Gearing and colleague (22) provided evidence that OSM binds to the CDF/LIF receptor with high affinity. To test the effects of OSM on sympathetic neurons, various concentrations of human OSM were applied and the expression of neuronal genes was analyzed. For comparison, CDF/LIF at 1.6 ng/ml was included in sister cultures. The data in Fig. 2 illustrate that OSM induces mRNAs for SP, ENK, ChAT, and CCK, but only at concentrations (>25 ng/ml) higher than required for similar inductive effects by CDF/LIF. OSM induces a low level of mRNA for SOM, even at 400 ng/ml. It is possible that this quantitative difference is due to the species difference between OSM (human) and the target neurons (rat). Reduced responsiveness of rat cells to OSM was also demonstrated in an acute phase protein assay (39). Like GPA, OSM does not induce the expression of the mRNAs for GAD65, GAD67, TH, TPH, CGRP, DYN, and NPY (data not shown).

As outlined in the introduction, there are results raising the possibility that several other cytokines may belong to the neuropoietic family. These include G-CSF, IL-6, and IL-11. These proteins were tested in the sympathetic neuron assay, along with CDF/LIF for comparison, and the results are illustrated in Fig. 3. G-CSF has no detectable activity on the expression of any of the genes analyzed. IL-6 and IL-11, in contrast, induce preprotachykinin mRNA (which produces the SP neuropeptide). This effect is weak and is observed only at concentrations of >100 ng/ml. Among the several genes influenced by CDF/LIF and CNTF, SP is the one that displays the most striking response (>100-fold increase; ref. 16). Therefore, rather than displaying more specificity than CDF/LIF and CNTF, it could be that IL-6 and IL-11 are such weak inducers that only an increase in SP can be observed.

Activin A Induces a Different Set of Neuronal Genes. Activin A is a member of the TGFβ superfamily and has effects on erythroid differentiation, embryonic mesoderm induction and axis formation, and sexual development (40). Although this protein does not belong in the neuropoietic cytokine family on the basis of structure, it does have effects on neuronal gene expression. Since it was recently demonstrated that activin A can induce SOM in ciliary ganglion neurons (33), it was of interest to determine whether activin A has similar effects on sympathetic neurons. In fact, activin A elicits an induction pattern of mRNAs for neuropeptides and neurotransmitter synthetic enzymes that is distinct from that of CNTF and CDF/LIF. As illustrated in Fig. 4, CDF/LIF induces mRNAs for SP, ENK, and VIP but does not alter the expression of CGRP and DYN mRNAs. Activin A, in contrast, does not alter the expression of SP, ENK, and VIP mRNAs, even at the concentration of 400 ng/ml, but it does have potent effects on CGRP and DYN mRNAs (Fig. 4).

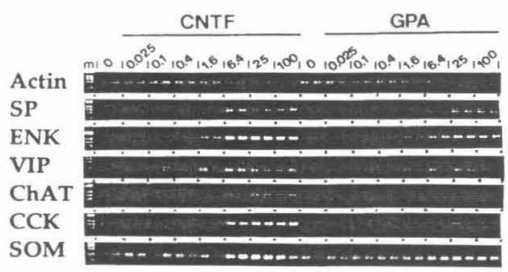


FIG. 1. Rat CNTF and chicken GPA induce mRNAs for SP, ENK, VIP, ChAT, CCK, and SOM with a similar dose dependency. Various concentrations (ng/ml) of rat CNTF and chicken GPA were added to cultured sympathetic neurons from day 2 to day 7. Duplicate samples were run for each concentration. The relative abundance of each mRNA was analyzed by RT-PCR. The expression of β-actin was used to monitor the amount of RNA in each sample. The DNA molecular weight standard (m lane) is a 1-kb DNA marker.

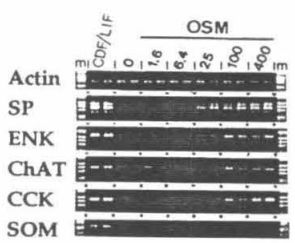


FIG. 2. OSM induces mRNAs for SP, ENK, ChAT, CCK, and SOM, but only at high concentrations. Various concentrations (ng/ml) of rhOSM, or rhCDF/LIF at 1.6 ng/ml, were added to cultured sympathetic neurons from day 2 to day 7 and RT-PCR was used to monitor mRNA expression of neuropeptides and transmitter biosynthetic enzymes. Duplicate samples were prepared for each condition. Induction of mRNAs for SP and ENK occurs at 25 ng/ml. At 100 ng/ml, rhOSM begins to induce mRNAs for ChAT and CCK. Induction of SOM was just visible at 400 ng/ml.

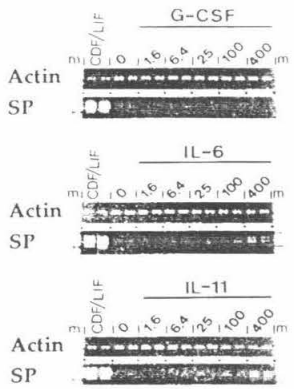


FIG. 3. High concentrations of IL-6 and IL-11 induce expression of mRNA for SP, while G-CSF has no effect on the neuronal genes tested. Various concentrations (ng/ml) of rhIL-11, rhG-CSF, recombinant murine IL-6, or rhCDF/LIF at 1.6 ng/ml were added to neuronal cultures and the effects were analyzed by RT-PCR. Duplicate samples were prepared for each condition. IL-6 and IL-11 begin to show induction of SP at 100 ng/ml, but they do not induce other neuromodulators.

Moreover, while CDF/LIF down-regulates the expression of mRNA for TH and NPY, activin A induces higher levels of NPY mRNA, while TH mRNA levels appear to be unaltered. Activin A and CDF/LIF do have some similarities in action; both induce ChAT, SOM, and CCK mRNAs, and both have no effect on GAD67, GAD65, and TPH mRNAs.

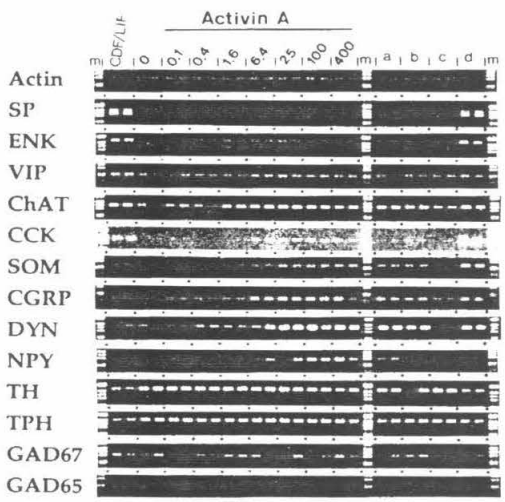


FIG. 4. Recombinant human activin A induces a novel set of neuronal genes; this induction is not blocked by recombinant human inhibin A and is additive to the effects of CDF/LIF. rhCDF/LIF at 1.6 ng/ml or activin A at various concentrations (ng/ml) was applied to neurons and the effects were analyzed by RT-PCR. For the blocking experiment, activin A at 25 ng/ml and inhibin A at 200 ng/ml were added to the cultures and the results are shown in lane b. The effects of activin A alone at 25 ng/ml are shown in lane a, and inhibin A alone at 200 ng/ml in lane c. To observe additive effects, CDF/LIF at 1.6 ng/ml and activin A at 25 ng/ml were added together (lane d). Duplicate samples were prepared for each condition. Although inhibin A inhibits NPY induction by activin A in this set of samples, this result was not observed consistently, and no other inhibitory effects were seen.

Two proteins, inhibin and follistatin, inhibit the action of activin A. We tested one of them, inhibin A, at the concentration (200 ng/ml) that was shown previously to inhibit induction of erythroid differentiation by activin A at 25 ng/ml (33). In the sympathetic neuron assay inhibin has no effect by itself on the neuronal genes tested (Fig. 4, lane c), nor does it block the effects of activin A when these two proteins are added together (Fig. 4, lane b). This lack of effect suggests that these neurons have the type of activin A receptor that does not recognize inhibin A. When activin A and CDF/LIF are added together in the assay, each at a concentration sufficient to generate maximal inductive effects in this assay, additive effects are observed (Fig. 4, lane d). This implies that activin A and CDF/LIF use different mechanisms to regulate neuronal gene expression.

DISCUSSION

The utility of an RT-PCR-based assay for screening factors that may affect neuronal gene expression is borne out by the present results. Thirty-three cytokines and growth factors were tested for their effects on the expression of a variety of neuropeptides and transmitter-synthesizing enzymes that are known to be present in the peripheral nervous system. Active factors can be quickly identified, and dose–response experiments allow a qualitative assessment of which genes respond to each factor. As illustrated by the results presented here, assays of a smaller subset of neuronal genes could have led to conclusions entirely different from those we derive from the larger data set. Although this method is qualitative, comparison of dose–response results obtained with known cytokines allows an accurate analysis of the effects of novel factors.

As summarized in Table 1, most of the factors tested did not alter the expression of phenotypic markers in cultured sympathetic neurons. We cannot rule out, however, the possibility that some of these cytokines might have been active had we been able to screen the recombinant rat proteins. Further studies using neurons and cytokines derived from the same species are necessary to clarify this uncertainty. Indeed, Mehler and colleagues (29) have provided evidence that IL-5, -7, -9, and -11 are survival and/or differentiation factors for murine hippocampal progenitor neurons. Another example is FGF. Both acidic FGF and basic FGF induce the cholinergic phenotype in cultured chick sympathetic neurons (41), but we were not able to observe any change of expression in rat sympathetic neurons in several trials. Other differences in responsiveness to factors have been observed between rat and chicken sympathetic neurons (e.g., ref. 42).

CNTF was identified and cloned from human, rat, and rabbit by its ability to support the survival of ciliary neurons *in vitro*, and later it was also found to maintain the survival of motor neurons. It does not contain a signal peptide for secretion, however, and it is not detected in the medium of transfected cells (14, 15). Moreover, CNTF is not found in tissue fluids or extracellular matrix preparations, except after axonal injury (43). A protein with CNTF-like activity (SGF) is found in the soluble fraction of sweat gland homogenates, raising the possibility that there exists a secretable CNTF homologue (26, 27). This idea is supported by the finding that the chick expresses a secretable protein that displays 50% amino acid sequence identity with CNTF (25). Our laboratory has recently cloned a partial cDNA for a different CNTF sequence homologue from chicken, however, and this molecule appears to be the chicken version of mammalian CNTF (44). Therefore, chicken GPA is also likely to be a family member. The present finding that GPA possesses an activity on sympathetic neurons identical to that of CNTF and CDF/LIF further solidifies the position of GPA in this family.

The present results also provide further support for OSM as a neuropoietic cytokine. Although OSM shows only a modest sequence homology with CDF/LIF, it can displace the latter from its receptor on M1 cells, and if this is also the case for the sympathetic neurons, the mechanism for how these two cytokines can regulate the same set of neuronal genes is straightforward. We provide evidence that IL-6 and IL-11 weakly induce expression of SP, and not the other genes tested. Since it appears that IL-6, IL-11, CNTF, CDF/LIF, and OSM use the same receptor subunit as a signal transducer, gp130, the relatively weak effects observed here for IL-6 and IL-11 may indicate very low levels of the ligand-binding subunits for the latter two cytokines in sympathetic neurons.

The observation that activin A induces a distinctive subset of neuronal genes is important for two reasons. First, it demonstrates that the various neuropeptide genes assayed can, in fact, be regulated independently of each other. That is, the observations that CCK, ENK, SP, VIP, and ChAT are all induced together by CDF/LIF, CNTF, GPA, and OSM is not simply due to a mandatory coordinate regulation of these neuropeptide genes. The activin A results thus serve to further link the neuropoietic group together. The lack of induction of SP by activin A is particularly striking, given the extraordinarily strong induction of this neuropeptide by all of the neuropoietic cytokines. The second point of interest in the activin A findings is that the particular set of neuropeptides induced by this factor closely resembles the neuropeptide phenotype of the cholinergic sympathetic neurons that innervate the sweat gland in the rat foot pad. The presence of CGRP induction and the absence of SP induction are important because cholinergic sympathetic neurons express CGRP, but not SP, in rats (45). On this basis, activin A is a legitimate candidate, along with SGF, for the target-derived neuronal differentiation factor that induces sympathetic neurons innervating sweat glands to become cholinergic. It will therefore be of interest to determine if activin A is expressed in the sweat glands during normal development.

We thank Doreen McDowell for help with tissue culture materials and Drs. James Miller, Rae Nishi, Jim Smith, David Shelton, David Gearing, Yu-Chung Yang, Stanley Wolf, Mary Freshney, and Gerry Graham for providing cytokines. We are grateful to Dr. Rae Nishi for providing information concerning GPA and activin A activities before publication. We thank Drs. Lisa Banner, Herman Govan, Zaven Kaprielian, and Manhendra Rao and two reviewers for constructive comments on the manuscript. This project is supported by grants from Amgen and the National Institute of Neurological Disorders and Stroke (Javits Neuroscience Investigator Award) to P.H.P., as well as a Fellowship from the Ministry of Education, Taiwan, Republic of China, to M.-J.F.

1. Jessell, T. M. & Melton, D. A. (1992) *Cell* **68,** 257–270.
2. Bazan, J. F. (1991) *Neuron* **7,** 197–208.
3. Patterson, P. H. (1992) *Curr. Opinion Neurobiol.* **2,** 94–97.
4. Gearing, D. P., Thut, C. J., VandenBos, T., Gimpel, S. D., Delaney, P. B., King, J., Price, V., Cosman, D. & Beckmann, M. P. (1991) *EMBO J.* **10,** 2839–2848.
5. Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N. & Yancopoulos, G. D. (1992) *Cell* **69,** 1121–1132.
6. Lord, K. A., Abdollahi, A., Thomas, S. M., Demarco, M., Brugge, J. S., Hoffman-Liebermann, B. & Leibermann, D. A. (1991) *Mol. Cell. Biol.* **11,** 4371–4379.
7. Davis, S., Aldrich, T. H., Valenzuela, D. M., Wong, V., Furth, M. E., Squinto, S. P. & Yancopoulos, G. D. (1991) *Science* **253,** 59–63.
8. Kishimoto, T., Akira, S. & Taga, T. (1992) *Science* **258,** 593–597.
9. Patterson, P. H. & Chun, L. L. Y. (1977) *Dev. Biol.* **56,** 263–280.
10. Fukada, K. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8795–8799.
11. Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M.-J. & Patterson, P. H. (1989) *Science* **246,** 1412–1416.
12. Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kelso, A. & Metcalf, D. (1987) *EMBO J.* **6,** 3995–4002.
13. Alder, R., Landa, K. B., Manthorpe, M. & Varon, S. (1979) *Science* **204,** 1434–1436.
14. Lin, L.-F. H., Mismer, D., Lile, J. D., Armes, L. G., Bulter, E. T. I., Vannice, J. L. & Collins, F. (1989) *Science* **246,** 1023–1025.
15. Stockli, K. A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., Gotz, R., Lindholm, D. & Thoenen, H. (1989) *Nature (London)* **342,** 920–923.
16. Nawa, H., Yamamori, T., Le, T. & Patterson, P. H. (1990) *Cold Spring Harbor Symp. Quant. Biol.* **55,** 247–253.
17. Rao, M. S., Tyrrell, S., Landis, S. C. & Patterson, P. H. (1992) *Dev. Biol.* **150,** 281–293.
18. Martinou, J.-C., Martinou, I. & Kato, A. C. (1992) *Neuron* **8,** 737–744.
19. Oppenheim, R. W., Prevette, D., Qin-Wei, Y., Collins, F. & MacDonald, J. (1991) *Science* **251,** 1616–1618.
20. Rose, T. M. & Bruce, A. G. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8641–8645.
21. Metcalf, D., ed. (1992) *Polyfunctional Cytokines: IL-6 and LIF* (Wiley, Chichester, U.K.).
22. Gearing, D. P. & Bruce, A. G. (1991) *New Biol.* **4,** 61–65.
23. Rao, M. S., Symes, A., Malik, N., Shoyab, M., Fink, J. S. & Landis, S. C. (1992) *NeuroReport* **3,** 865–868.
24. Yang, Y.-C. & Yin, T. (1992) *BioFactors* **4,** 15–21.
25. Leung, D. W., Parent, A. S., Cachianes, G., Esch, F., Coulombe, J. N., Nikolics, K., Eckenstein, F. P. & Nishi, R. (1992) *Neuron* **8,** 1045–1053.
26. Rao, M. S., Patterson, P. H. & Landis, S. C. (1992) *Development* **116,** 731–744.
27. Rohrer, H. (1992) *Development* **114,** 689–698.
28. Kamegai, M., Niijima, K., Kunishita, T., Nishizawa, M., Ogawa, M., Araki, M., Ueki, A., Konishi, Y. & Tabira, T. (1990) *Neuron* **2,** 429–436.
29. Mehler, M. F., Rozental, R., Dougherty, M., Spray, D. C. & Kessler, A. J. (1993) *Science* **362,** 62–65.
30. Wetzler, M., Talpaz, M., Lowe, D. G., Baiocchi, G., Gutterman, J. U. & Kurzrock, R. (1991) *Exp. Hematol.* **19,** 347–351.
31. Gadient, R. A., Cron, K. C. & Otten, U. (1990) *Neurosci. Lett.* **117,** 335–340.
32. Shadiack, A. M., Hart, R. P., Carlson, C. D. & Jonakait, G. M. (1993) *J. Neurosci.* **13,** 2601–2609.
33. Coulombe, J. N., Schwall, R., Parent, A. S., Eckenstein, F. P. & Nishi, R. (1993) *Neuron* **10,** 899–906.
34. Fann, M.-J. & Patterson, P. H. (1993) *J. Neurochem.* **61,** 1349–1355.
35. Hawrot, E. & Patterson, P. H. (1979) *Methods Enzymol.* **58,** 574–583.
36. Wolinsky, E. J., Landis, S. C. & Patterson, P. H. (1985) *J. Neurosci.* **5,** 1497–1508.
37. Saadat, S., Sendtner, M. & Rohrer, H. (1989) *J. Cell Biol.* **108,** 1807–1816.
38. Bruce, A. G., Hoggatt, I. H. & Rose, T. M. (1992) *J. Immunol.* **149,** 1271–1275.
39. Richards, C. D., Brown, T. J., Shoyab, M., Baumann, H. & Gauldie, J. (1992) *J. Immunol.* **148,** 1731–1736.
40. Massagué, J. (1990) *Annu. Rev. Cell Biol.* **6,** 597–641.
41. Zurn, A. (1992) *J. Neurosci.* **12,** 4195–4201.
42. Smith, J., Vyas, S. & Garcia-Arraras, J. E. (1993) *J. Neurosci. Res.* **34,** 346–356.
43. Sendtner, M., Stockli, K. A. & Thoenen, H. (1992) *J. Cell Biol.* **118,** 139–148.
44. Govan, H. L. & Patterson, P. H. (1993) *Soc. Neurosci. Abstr.* **19,** 252.
45. Landis, S. C. (1990) *Trends Neurosci.* **13,** 344–350.

CHAPTER 4. Regulation of neuronal phenotype by members of the TGF-β superfamily.

Introduction

Members of the neuropoietic cytokine family modulate neuronal gene expression (Fann and Patterson, 1994). These cytokines induce a particular set of neurotransmitter synthetic enzymes and neuropeptides in cultured sympathetic neurons, including cholecystokinin (CCK), enkephalin (ENK), somatostatin (SOM), substance P (SP), vasoactive intestinal polypeptide (VIP) and choline acetyltransferase (ChAT). In addition to these cytokines, several lines of evidence indicate that members of transforming growth factor β (TGF-β) superfamily also regulate neuronal gene expression. For example, activin A induces SOM in cultured ciliary neurons and induces ChAT and several neuropeptides in cultured sympathetic neurons (Coulombe et al., 1993; Fann and Patterson, 1994). Bone morphogenetic proteins (BMP) induce the expression of the neural cell adhesion molecules N-CAM and L1 in neuroblastoma cells and stimulate PC12 cell neuronal differentiation (Paralkar et al., 1992; Perides et al., 1994). Although its function is unknown, growth/differentiation factor 1 is expressed mainly in the nervous system (Lee, 1991). Dorsalin, a protein expressed preferentially in the dorsal portion of the developing neural tube, promotes the migration of neural crest cells and inhibits the formation of motor neurons (Basler et al., 1993). Glial-derived neurotrophic growth factor enhances the survival and differentiation of dopaminergic neurons (Lin et al., 1993). Thus, it is of interest to test the effects of TGF-β cytokines on neuronal phenotype.

The expression of transmitters and neuropeptides in neurons is also regulated by presynaptic stimulation (Patterson and Nawa, 1993). Membrane depolarization of neurons that mimics neuronal activity causes rapid alterations in protein phosphorylation (Nairn et al., 1985), and induces the expression of ENK, neuropeptide Y (NPY), SOM, tyrosine hydroxylase (TH), and VIP in various neurons (Goodman 1990; Morris et al., 1988; Tolon et al., 1994; Zigmond et al., 1989). Moreover, an interaction between neuropoietic cytokines and membrane depolarization on transmitter and neuropeptide expression has been demonstrated. Depolarization blocks most of the noradrenergic-to-cholinergic switch induced by cholinergic neuronal differentiation factor/leukemia inhibitory factor (CDF/LIF) in heart cell conditioned medium (Walicke et al., 1977). The cholinergic activity of ciliary

neurotrophic factor (CNTF) is reported not to be affected by depolarization, however (Rao et al., 1992b). The actions of these two factors on the expression of neuropeptides are also differentially regulated by depolarization (Rao et al., 1992b). These results indicate that it will also be of interest to investigate the influence of depolarization on gene regulation by members of the TGF-β superfamily.

One of the ways in which cytokines and growth factors can regulate neuronal phenotype is as target-derived factors. One of the most intensively studied examples of target influence is the innervation of sweat glands by sympathetic neurons (Landis, 1990). Upon innervating these glands, sympathetic neurons increase expression of ChAT, calcitonin gene-related peptide (CGRP), and VIP, while decreasing expression of TH. The induction of these traits is specific, because no other neuropeptides are detectable in these neurons (Landis et al., 1988). A putative factor(s) involved in this phenotypic transition has been partially characterized and has similar activities on sympathetic neurons to those of CDF/LIF and CNTF (Rao et al., 1992a; Rohrer, 1992). However, null mutant mice that are CDF/LIF- or CNTF-deficient display sweat gland innervation of a normal phenotype (Masu et al., 1993; Rao et al., 1994). These results suggest that CDF/LIF and CNTF may not be the factor responsible for the phenotypic transition and raise the possibility that other cholinergic-inducing factors may be involved in this process.

As a first step in defining the role of the TGF-β superfamily in the sympathetic nervous system, we focus on effects of these factors on neuronal phenotype *in vitro*. The 24 members in the TGF-β superfamily are distributed into four groups based on sequence homology: TGF, activin, dpp, and 60A subfamilies (Kingsley, 1994). We analyze representative members of each subfamily for this effect on neuronal gene expression using the reverse transcription-polymerase chain reaction (RT-PCR) method (Fann and Patterson, 1993). We find that, in addition to the activin subfamily (Fann and Patterson, 1994), members of the dpp and 60A subfamilies also induce a particular set of transmitters and neuropeptides in cultured sympathetic neurons. The TGF subfamily is, however, without detectable effect in the assay. The effect of depolarization on gene regulation by these factors is also analyzed. Our results demonstrate that depolarization differentially alters the induction patterns of neuropeptide genes by activin A, BMP-2, and BMP-6.

Since activin A acts as a cholinergic factor in cultured sympathetic neurons, inducing ChAT and CGRP expression, it was of interest to investigate whether this cytokine could be the sweat gland factor. We have used *in situ* hybridization, RNase protection, and analysis of neuronal gene-inducing activities in tissue extracts to test whether members of the activin subfamily are present in developing sweat glands. Our results show that activin B is present in target tissues of both cholinergic and noradrenergic sympathetic neurons. The cholinergic activity in sweat gland extracts do not, however, resemble activins. These results suggest that the activins are not responsible for the phenotypic switch during sweat gland innervation.

Materials and Methods

Animals and reagents

Neonatal Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA). Most of the tissue culture reagents, Superscript reverse transcriptase II (RTase) and the 1-kb DNA marker were purchased from Gibco/BRL (Grand Island, NY). Nerve growth factor, glycogen, dATP, dCTP, dGTP, and dTTP were purchased from Boehringer Mannheim (Indianapolis, IN). RNase inhibitor (RNasin) and *Taq* DNA polymerase were purchased from Promega (Madison, WI). Oligo dT was obtained from Pharmacia (Piscataway, NJ). Oligonucleotide primers were synthesized in the Biopolymer Synthesis and Analysis Resource Center at Caltech. Recombinant human activin A was a gift from Genentech (S. San Francisco, CA). Recombinant proteins human BMP-2 and BMP-6 were provided by Dr. John M. Wozney (Genetics Institute, Cambridge, MA). Recombinant proteins human TGF-β1 and TGF-β3 were purchased from R&D Systems (Minneapolis, MN). All other reagents were purchased from Sigma (St. Louis, MO).

Neuronal culture

Dissociated sympathetic neurons were prepared and cultured in serum-free medium as described previously (Fann and Patterson, 1993; Hawrot and Patterson, 1979; Wolinsky et al., 1985). Neurons were seeded in 96-well plates (Falcon; Oxnard, CA) at a density of one ganglion per well. Cultures were maintained for 7 days, and half of the medium was changed every 36 hours in an effort to ensure that cytokines maintained activity. The antimitotic agent, aphidicolin, was added to the cultures at a concentration of 4 μg/ml to effectively eliminate non-neuronal cells. After 7 days, each well contained approximately 3000 neurons and about 95% of the surviving cells were neurons, as judged by phase microscopy (Wolinsky et al., 1985). Cytokines, with or without 40 mM KCl, were added from the second day of culture, and duplicate wells were prepared for each condition. All results were reproduced in at least two separate neuronal platings.

Preparation of RNA and cDNA

Total tissue RNA was prepared by disruption of the tissue in 4 M guanidinium thiocyanate using a polytron and extraction with acidic phenol and chloroform (Chomczynski and Sacchi, 1987). The kit from 5-Prime-3 -Prime Inc. (Boulder, CO) was used to select poly-A RNA from total RNA. Total RNA from cultured neurons was prepared in the same manner, but smaller amounts of material were used (Belyavsky et al., 1989). Briefly, 160 µl of lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 100 mM 2-mercaptoethanol, 0.5% sodium lauroyl sarcosinate) was added to each well. The lysate was transferred to 1.7 ml eppendorf tube and vortexed vigorously to shear DNA. Ten µl of 1 M sodium acetate, pH 4.0, 200 µl of water-saturated phenol and 40 µl of chloroform were added sequentially, with vigorous vortexing after each addition. The samples were kept at 4°C for 20 min and centrifuged at 12,000 g for 15 min. The water phase was transferred to a new tube containing 10 µl of 3 mg/ml glycogen. RNA was precipitated by addition of 200 µl isopropanol and stored at -20°C overnight.

To produce cDNA from the cultured neurons, the total RNA was centrifuged, dried and directly dissolved in 10 µl of 13 mM methylmercury hydroxide for 10 min, and an additional 2 µl of 75 mM 2-mercaptoethanol was added for 5 min. Oligo-dT primed reverse transcription was done in a final volume of 20 µl, containing the reverse transcription buffer, 100 U reverse transcriptase and 20 U RNasin, for 1 hour at 37°C.

Polymerase chain reaction

The sequences of the primers and conditions used in the PCR for each neuronal gene analyzed were described previously (Fann and Patterson, 1993). Amplification of cDNA was performed in a thermal cycler (MJ Research, Watertown, MA). Each tube contained a final volume of 20 µl, consisting of 1 µl of cDNA, 1X PCR buffer, 0.5 U *Taq* DNA polymerase, 0.25 mM dNTP, and one set of primers (200 nM). After the reaction, 8 µl of each PCR sample were analyzed on a 2% agarose gel, and the products were visualized after ethidium bromide staining and UV illumination. The appropriate amplification cycle schedule for each neuropeptide gene was determined empirically, to allow a minimal, yet detectable, signal for control samples (neurons grown without cytokines). Cytokines were deemed to have

regulatory effects on specific genes if the intensity of the PCR products were about three-fold different from those of negative controls, as judged by eye.

RNase protection assay

Plasmids containing rat inhibin α (from nucleotide 260 to nucleotide 1398), β_A (from nucleotide 149 to nucleotide 1459), and β_B (from nucleotide 1 to nucleotide 1562) (Feng et al., 1989; Woodruff et al., 1987) were cloned by PCR using embryonic day 12 placenta cDNA as template. The PCR products were ligated into TA Cloning kit purchased from Invitrogen (San Diego, CA) and sequenced to confirm their identity. GAPDH was used as internal control and obtained from Dr. Lisa Banner (Banner and Patterson, 1994). The assay was performed according to the protocol of Patterson and Fann (1992). Briefly, plasmids were linearized by restriction enzymes and ^{32}P-labeled RNA sense probes generated by *in vitro* transcription were hybridized to various poly-A RNAs. The sizes of probes for α, β_A, β_B, and GAPDH are 466, 428, 338, and 206 nucleotides, respectively. These probes yielded protected fragments of 386 nucleotides for α, 348 nucleotides for β_A, 267 nucleotides for β_B, and 136 nucleutides for GAPDH. Radioactivity was quantitated by scanning the protected fragments on a Phosphoimager 400S (Molecular Dynamic, Sunnyvale, CA) and computing with ImageQuant software. The intensities of the protected fragments corresponding to α, β_A, and β_B, were expressed as ratios to that of GADPH, and the values expressed in arbitrary units.

***In situ* hybridization**

Antisense and sense, digoxigenin-labeled RNA probes for inhibin α (from nucleotide 260 to nucleotide 1398), β_A (from nucleotide 149 to nucleotide 1459), and β_B (from nucleotide 1 to nucleotide 1562) were generated using the Ambion (Austin, Texas) Megascript *in vitro* transcription kit, after digesting plasmids with restriction enzymes. *In situ* hybridization was performed according to the protocol of Birren et al. (1993). Briefly, 10 μm sections of fixed tissue were hybridized overnight with 1 μg/ml of either sense or antisense probes. The hybridization temperture for α and β_A probes was 60°C, and 64°C for β_B probes. After RNase digestion and washing with 0.2 X SSC, 0.3% CHAPS solution at the hybridization temperatures, sections were incubated overnight with an alkaline phosphatase-conjugated anti-

digoxygenin antibody (Boehringer Mannheim). Nitro Blue Tetrazolium and 5-bromo-4-cholro-3-indoyl phosphate were used as substrates to visualize the enzyme activity.

Tissue homogenate preparation

To prepare tissue homogenates, footpads, skin from the thoracic region, and submaxillary glands were dissected from P7 and P21 rats. Each tissue was homogenized for 20 s, three times, in 10 vol of 10 mM phosphate buffer (pH 7.0) with a polytron. The extracts were centrifuged at 100,000 x G for 1 hour. The supernatants were collected and concentrated using Centricon filters with 10 kDa molecular weight cutoff. The protein concentration was determined with a Pierce (Rockford, IL) protein assay kit.

Results

BMP-2 and BMP-6 induce a particular set of transmitters and neuropeptides.

The TGF-β superfamily includes 24 members and is divided into 4 subfamilies, according to sequence homologies (Kingsley, 1994). We selected representative members from each subfamily and examined the effects on the expression of transmitter synthetic enzymes and neuropeptides in cultured sympathetic neurons by the RT-PCR method (Fann and Patterson, 1993). The TGF subfamily is represented by TGF-β1 and TGF-β3, the activin subfamily by activin A, the dpp subfamily by BMP-2, and the 60A subfamily by BMP-6. We have reported the effects of activin A on neuronal gene expression previously (Fann and Patterson, 1994). Activin A induces mRNAs for ChAT, CCK, CGRP, dynorphin (DYN), NPY, and SOM, but not ENK, SP, VIP, TH, tryptophan hydroxylase (TPH) and 67 kDa glutamic acid decarboxylase (GAD67).

When two concentrations (40 ng/ml and 400 ng/ml) of TGF-β1 or TGF-β3 were applied to neonatal rat sympathetic neurons in culture for 6 days, these cytokines show no effect on the intensity of PCR products for any of the 11 genes analyzed (Fig. 1). The β-actin band is used to monitor the amount of mRNA present in each sample, and duplicate samples are prepared for each condition. In contrast, both BMP-2 and BMP-6 elicit induction patterns of mRNA for neuropeptides and transmitter synthetic enzymes that are distinct from those of the neuropoietic cytokines and activin A. As illustrated in Fig. 2, BMP-2 and BMP-6 have no effect on the expression of mRNAs for ChAT, CGRP, GAD 67, ENK, SP, and TH. Both do induce DYN, NPY, SOM, VIP, and TPH in a dose-dependent manner. Although BMP-2 and BMP-6 show similar patterns of induction, there are two noticeable differences between the effects of these two factors. First, BMP-2 is more potent than BMP-6. Second, BMP-2 induces CCK expression but BMP-6 does not. The latter difference may, however, be due to the lower potency of BMP-6, since CCK induction by BMP-2 is weak. As the members in each subfamily are highly conserved, we predict that the effects of TGFs, activin A, and BMPs analyzed here are representative of most or all family members.

Depolarization modulates the induction patterns of activin A and BMPs.

Depolarization by elevation of extracellular potassium concentration (K^+) can alter neuronal gene expression. When 40 mM K^+ is added into cultured sympathetic neurons to mimic presynaptic stimulation, expression of mRNAs for CGRP and NPY is slightly increased while that for ChAT was decreased (Fig. 3). Depolarization also alters the induction patterns of neuropeptides by members of the TGF-β superfamily (Table I). Depolarization inhibits expression of CGRP induced by activin A, although high K^+ alone slightly increases CGRP. Conversely, expression of VIP is increased when activin A and high K^+ are added together. A reverse pattern was observed when BMPs and high K^+ were applied together. The induction of VIP expression by BMP-2 and BMP-6 is inhibited by depolarization while the expression of CGRP is increased in the combination of BMP-2 and high K^+. This dramatic difference between the effect of depolarization on CGRP and VIP induction by activin A versus BMP-2 and BMP-6 is surprising, as receptors for all these cytokines are highly conserved and all contain serine-threonine kinase domains (Xie et al., 1994). This inhibition by depolarization is specific, as high K^+ inhibits the induction of ChAT by activin A but has no effect on ChAT when added together with BMPs. Moreover, depolarization has no effect on other genes induced by either activin A or BMPs (CCK, DYN, NPY, SOM, TPH). These results are summarized in Table I.

Activins are unlikely to be the factors involved in the switch of neuronal phenotype during sweat gland innervation.

One of the most extensively studied systems of target-derived influence on neuronal development is the sympathetic innervation of sweat glands (Landis, 1990). Contact with these glands induces the innervating neurons to change their phenotype from noradrenergic to cholinergic. Contact with other targets does not induce this switch in gene expression (Schotzinger and Landis, 1988; 1990). In considering potential candidates for the sweat gland cholinergic factor, only neuropoietic cytokines and activins elicit ChAT expression in cultured sympathetic neurons (Fann and Patterson, 1994). To test whether activins are involved *in vivo*, we asked two questions. First, are the mRNAs for activins localized in footpads containing sweat glands, but not in skin or submaxillary glands, which are noradrenergic targets? The distribution of activins in these tissues was analyzed by *in situ* hybridization

and *RNase* protection. Second, do tissue homogenates for footpads contain activin-like activity when assayed on cultured sympathetic neurons? Homogenates were assayed by the RT-PCR method.

The activin subfamily are dimers containing one or two of the family members: α, β_A, or β_B. Hetrodimers of α and either one of the β subunits are termed inhibins. Homodimers or heterodimers of β subunits are termed activins. Digoxigenin-labeled RNA probes were used to detect these three genes in footpads by *in situ* hybridization. There is no staining for the α and β_A genes in P7 and P14 rat footpads (Figs. 4 and 5). There is, however, a positive signal using the β_B antisense probe. The β_B mRNA is localized in sweat glands but not muscle, epidermal, endothelial cells (Fig. 6). Thus, activin B mRNA is selectively expressed at the right time and place to be involved in the changes of neuronal phenotype mediated by the sweat glands.

We next tested whether activins are present in tissues which are innervated by noradrenergic sympathetic axons: hairy skins and submaxillary glands. The mRNAs for the subunits were analyzed using the RNase protection method; a sample gel is shown in Fig. 7. Protected fragments were quantified by the Phosphoimager and expressed as ratios to the control gene, glyceraldehyde phosphate dehydrogenase (GAPDH) (Figs. 8). There is expression of the β_B gene in footpads at all stages analyzed, with a peak at P7. There is expression of α and β_A in footpads, also peaking at P7. Since the specific activities of the α and β_A probes are 3- and 2-fold higher, respectively, than that of the β_B probe, the relative expression of α and β_A is much lower than β_B. This likely explains our failure to detect α and β_A mRNAs in footpad by *in situ* hybridization. The expression of the α and β_B genes is more abundant in submaxillary glands than in footpads (Fig. 8). Since the amounts of α and β_B genes are similar in submaxillary glands, these glands may produce more inhibins than activins. All three genes are expressed at low levels in skin during development, and there is a large increase of β_B gene in the adult (Fig. 8). Thus, these are striking differences in the expression of the activins and inhibins during development and between tissues. The results do not, however, support the notion that activin B is selectively expressed in targets of cholinergic sympathetic neurons.

To further analyze the possibility that activins participate in regulating the innervation of sweat glands, tissue homogenates were prepared from

footpads, hairy skin of the thoracic region, and submaxillary glands from P21 rats. High speed supernatants from these homogenates were applied to neuronal cultures to detect neuropeptide-inducing activities by the RT-PCR assay. Extracts from submaxillary glands are toxic to neurons and were not analyzed further. Both footpad and skin homogenates at the concentration of 400 µg/ml induce SP, VIP, and ENK mRNAs (Fig. 9). Extracts from footpads but not skin induce the expression of ChAT, a result similar to that of Rao and Landis (1990). There is, however, no induction of CGRP and DYN, two neuropeptides strongly induced by activin A. Although activin A, but not activin B, was used as the positive control in this assay, there are no known functional differences between these two factors (Schwall et al., 1989). We obtained similar results from P7 tissue homogenates, though with lower neuropeptide-inducing activity (data not shown). Thus, the high speed supernatants of footpad extracts do not contain detectable activin activity in the sympathetic neuronal assay, although activin B mRNA is expressed in sweat glands.

Discussion

The TGF-β superfamily presently contains 24 members. Although some of them are primarily located in the nervous system, the functions of these proteins in the nervous system are not yet clear. To detect the effects of this superfamily on cultured sympathetic neurons, we selected TGF-β1, TGF-β3, BMP-2, BMP-6, and activin A as representatives of the subfamilies and analyzed their effects on neuronal phenotype by the RT-PCR method. Effects of activin A on the expression of neurotransmitters and neuropeptides have been reported previously (Fann and Patterson, 1994). This cytokine induces mRNAs for ChAT, CCK, CGRP, DYN, NPY, and SOM, but not the other genes analyzed. Although activins and BMPs have about 60% similarity in primary amino acid sequences, they display different inductive patterns of transmitters and neuropeptides. Both BMP-2 and BMP-6 increase the mRNA expression of DYN, NPY, SOM, VIP, and TPH, with BMP-2 being more potent than BMP-6. BMP-2 also weakly induces the expression of CCK mRNA. Although activin A and BMPs induce some of the same genes (CCK, DYN, NPY, and SOM), there are striking differences: activin A induces ChAT and CGRP while BMPs do not, and BMPs induce TPH and VIP while activin A does not. These various effects are specific in another way; TGF-β1 and TGF-β3 have no effects in the assay.

The induction of these neuropeptides by BMPs is not abnormal, as Gibbins (1992) reported that VIP, NPY, and DYN co-localized in 15% of guinea-pig lumbar sympathetic ganglia. The induction of TPH is also not completely unexpected as several groups reported that sympathetic neurons transiently express serotonin (Garcia-Arraras and Martinez, 1990; Happola et al., 1986; Soinila et al., 1988). The potential involvement of BMPs in these inductions *in vivo* merits further study. The result that BMPs and activin A display different induction patterns of neurotransmitters and neuropeptides is in dramatic contrast to the picture with the neuropoietic cytokines. While members of the latter family have very limited sequence homology, they induce very similar patterns of neuropeptide induction in cultured sympathetic neurons (Fann and Patterson, 1994). These results suggest that, unlike the neuropoietic cytokines that channel signals through the same

receptor subunit (Kishimoto et al., 1994), the activin and BMP receptors have diverged to mediate different signal cascades.

Several lines of evidence, both *in vivo* and *in vitro*, have shown that presynaptic activity can regulate the neuronal gene expression (Castren et al., 1992; Goodman, 1990). Recent progress suggests that calcium influx through voltage-sensitive calcium channels (VSCC) is involved (Franklin and Johnson, 1992; Ghosh et al., 1994). Depolarization inhibits the sympathetic neuron phenotypic switch induced by CDF/LIF (Walicke et al., 1977). This inhibition is Ca^{+2}-dependent and may utilize VSCC (Walicke and Patterson, 1981). To test whether depolarization modulates the effects of BMP-2, BMP-6 and activin A, we applied these cytokines to sympathetic neuronal cultures in the presence of high K^+. Our results demonstrate that depolarization differentially regulates the effects of BMPs and activin A (Table I). High K^+ strongly decreases the induction of CGRP and ChAT by activin A, but has a synergistic effect with activin A on the expression of VIP. Very different effects of depolarization are observed with BMP-2 and BMP-6. Depolarization decreases the induction of VIP expression by BMP-2 and BMP-6, but has synergistic effects with BMP-2 on the expression of CGRP. The fact that depolarization does not change the expression of other genes illustrates the specificity of these effects. These results demonstrate the complexity of neuronal gene regulation: the same gene is differentially regulated by homologous proteins and presynaptic stimulation.

Coulombe et al. (1993) have shown that activin A is the likely factor in the eye to induce the expression of SOM in choroid neurons. Since activin A also induces the expression of ChAT and CGRP in sympathetic neurons, we asked whether this cytokine could be involved in the phenotypic switch mediated by the sweat gland. We performed *in situ* hybridization and demonstrated that the message for activin B is present in sweat glands but not in other cells of the footpad. The other two genes in activin subfamily could not be detected by the *in situ* technique. To determine whether activin B mRNA is also expressed in tissues innervated by noradrenergic sympathetic axons, the RNase protection assay was utilized. The β_B mRNA is present in footpads at all time points analyzed, with a peak at P7. The other two genes, α and β_A, are expressed at lower levels in footpads. This result is consistent to our *in situ* hybridization result. The mRNA for the β_B gene is, however, also

found in skin and submaxillary glands. The expression of the β_B gene in submaxillary glands is higher than in footpads at all corresponding time points. Since submaxillary glands also express higher amounts of the α gene, it is likely that submaxillary glands form more inhibin B protein than activin B protein. Skin also contains similar level of expression of the β_B gene to that of footpads at early stages of development. There is, however, a dramatic increase of β_B mRNA in adult skin. Whether this increase correlates with any developmental change in skin requires further analysis. Together, these results indicate that the expression of the β_B gene is not only in footpads but also in targets of noradrenergic sympathetic neurons.

Further evidence that activins are not the factors involved in the phenotypic switch of the sweat gland innervation comes from analysis of footpad homogenates. When these homogenates are added to cultured sympathetic neurons, an induction of mRNAs for SP, ENK, VIP, and ChAT is detected. This induction pattern is similar to that of the neuropoietic cytokines, but is different from that of activins. There is no increase of the expression of CGRP and DYN, which are two neuropeptides strongly induced by activin A. Since the detection sensitivity of RT-PCR assay for activin A is about 5 ng/ml (Fann and Patterson, 1994), it means that less than 5 ng/ml of activins are present in 400 μg/ml of footpad extract. The fact that less than 1/80000 of total proteins in footpads may be an activin makes these cytokines less likely candidates for the sweat gland factor. Since the induction pattern of footpad extract is similar to that of the neuropoietic cytokines, these are more likely candidates for the sweat gland factor.

Figure 1. TGF-β1 and TGF-β3 have no effect on neuronal gene expression.

Two concentrations (40 ng/ml and 400 ng/ml) of TGF-β1 or TGF-β3 were added into cultured sympathetic neurons for six days. The expression of mRNAs for 11 transmitter synthetic enzymes and neuropeptides was analyzed by RT-PCR. Duplicate samples were prepared for each condition. The β-actin PCR product was used to monitor the amount of mRNA present in each sample. There is no difference of expression of these 11 genes with cytokine addition.

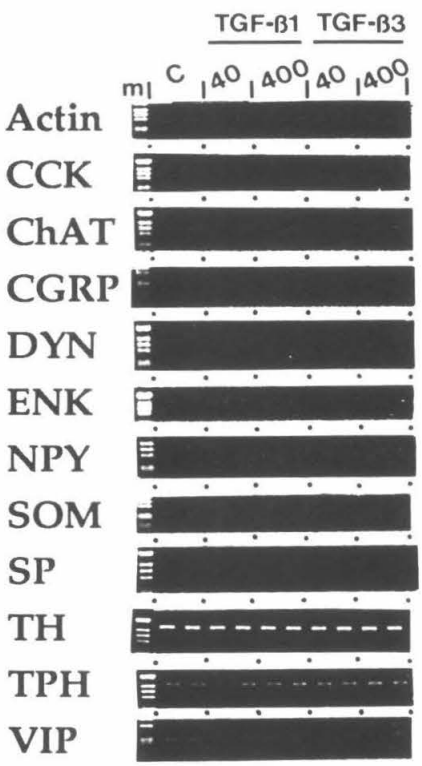

TGF-β1
TGF-β3
ml
C
40
400
40
400
Actin
CCK
ChAT
CGRP
DYN
ENK
NPY
SOM
SP
TH
TPH
VIP

Figure 2. BMP-2 and BMP-6 selectively induce the expression of mRNAs for several neuropeptides and TPH.

Neonatal rat sympathetic neurons were cultured with different concentrations (ng/ml) of BMP-2 and BMP-6 for six days. Duplicate samples were prepared for each condition. Total RNA was extracted from cells after the end of the incubation and the expression of mRNA for 12 transmitter synthetic enzymes and neuropeptides was analyzed by PCR. The intensity of β-actin PCR product was used to monitor the amount of mRNA present in each sample. BMP-2 and BMP-6 strongly increase the expression of DYN. Both cytokines also enhance the expression of NPY, SOM, TPH and VIP. CCK is weakly induced by higher concentrations of BMP-2. ChAT, CGRP, ENK, GAD67, SP, and TH were not affected by BMP-2 and BMP-6.

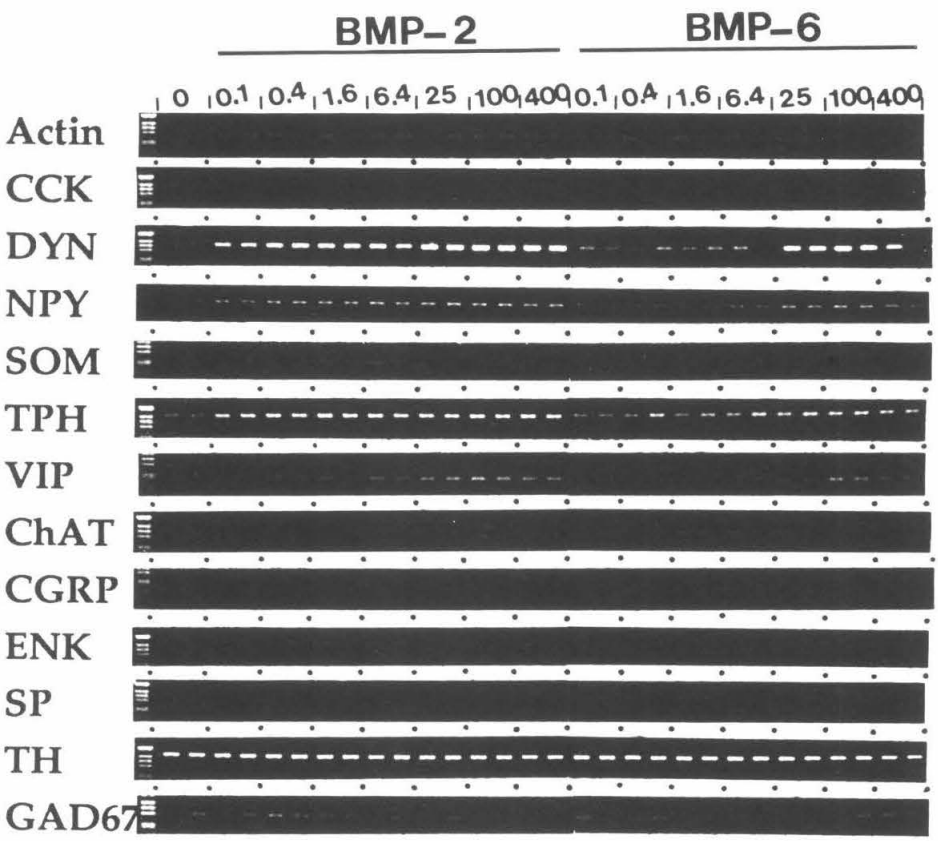

BMP-2
BMP-6
0
0.1
0.4
1.6
6.4
25
100
400
0.1
0.4
1.6
6.4
25
100
400
Actin
CCK
DYN
NPY
SOM
TPH
VIP
ChAT
CGRP
ENK
SP
TH
GAD67

Figure 3. The effect of depolarization on neuropeptide induction by activin A, BMP-2, and BMP-6.

Neonatal rat sympathetic neurons were cultured in the presence of cytokines, with or without 40 mM KCl (K^+) for six days. The concentrations of cytokines were 50 ng/ml for activin A, and 100 ng/ml for BMP-2 and BMP-6. Duplicate samples were prepared for each condition. Depolarization of cells alone (C, +) slightly increases the expression of CGRP and NPY, while decreasing the expression of ChAT. The induction of ChAT and CGRP by activin A is inhibited by depolarization. Expression of VIP, however, is enhanced when activin A and K^+ are added together. The induction of VIP by BMP-2 and BMP-6, in contrast, is inhibited by depolarization. Expression of CGRP is enhanced when BMP-2 and K^+ are added together. Although depolarization enhances CCK expression induced by activin A in this panel, this effect was not observed in other experiments. We cannot consistently demonstrate that depolarization inhibits SOM expression induced by BMP-6 either. Depolarization has no effect on other genes analyzed.

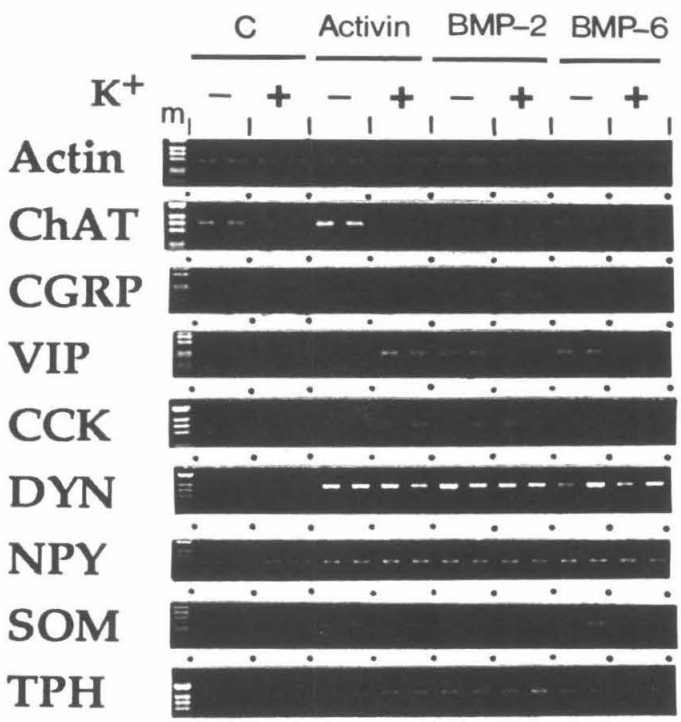

C
Activin
BMP-2
BMP-6
K+
– + – + – + – +
m
Actin
ChAT
CGRP
VIP
CCK
DYN
NPY
SOM
TPH

Table I. Summary of depolarization effect on neuropeptide induction by activin A, BMP-2 and BMP-6.

	activin A		BMP-2		BMP-6	
K^+	—	+	—	+	—	+
CGRP	↑	–	–	↑	–	–
VIP	–	↑	↑	–	↑	–
ChAT	↑	–	–	–	–	–
CCK	↑	↑	↑	↑	–	–
DYN	↑	↑	↑	↑	↑	↑
NPY	↑	↑	↑	↑	↑	↑
SOM	↑	↑	↑	↑	↑	↑
TPH	–	–	↑	↑	↑	↑

↑: induced; –: uninduced

Figure 4. The message for the inhibin α subunit is not detectable in sweat glands by *in situ* hybridization.

Ten μm sections of P14 footpads were hybridized with sense or antisense inhibin α probes labeled with digoxigenin. After washing and RNase digestion, sections were reacted with an alkaline phosphatase-conjugated anti-digoxigenin antibody and were developed with enzyme substrates. There is no difference on the staining between sections labeled with sense or antisense probes. Scale bar is 40 μm.

P14 α sense

P14 α antisense

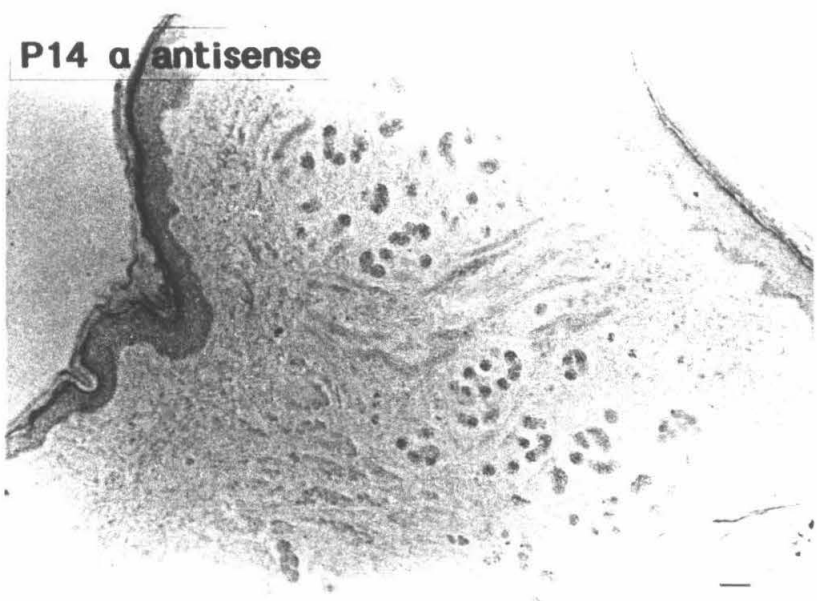


Figure 5. The message for the activin β_A subunit is not detectable in sweat glands by *in situ* hybridization.

Sections of P14 footpads were processed in the same way as in Fig. 4. There is no difference on the staining between sections labeled with sense or antisense probes. Scale bar is 40 μm.

P14 β_A sense

P14 β_A antisense

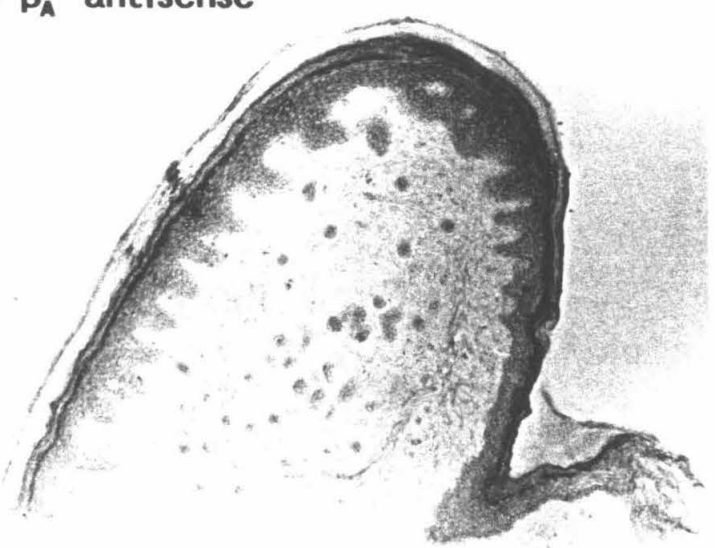


Figure 6. The message for the activin β_B subunit is present in sweat glands by *in situ* hybridization.

Sections of footpads were processed in the same way as in Fig. 4. Specific hybridization to sweat glands is seen with the antisense probe. Scale bar is 20 μm.

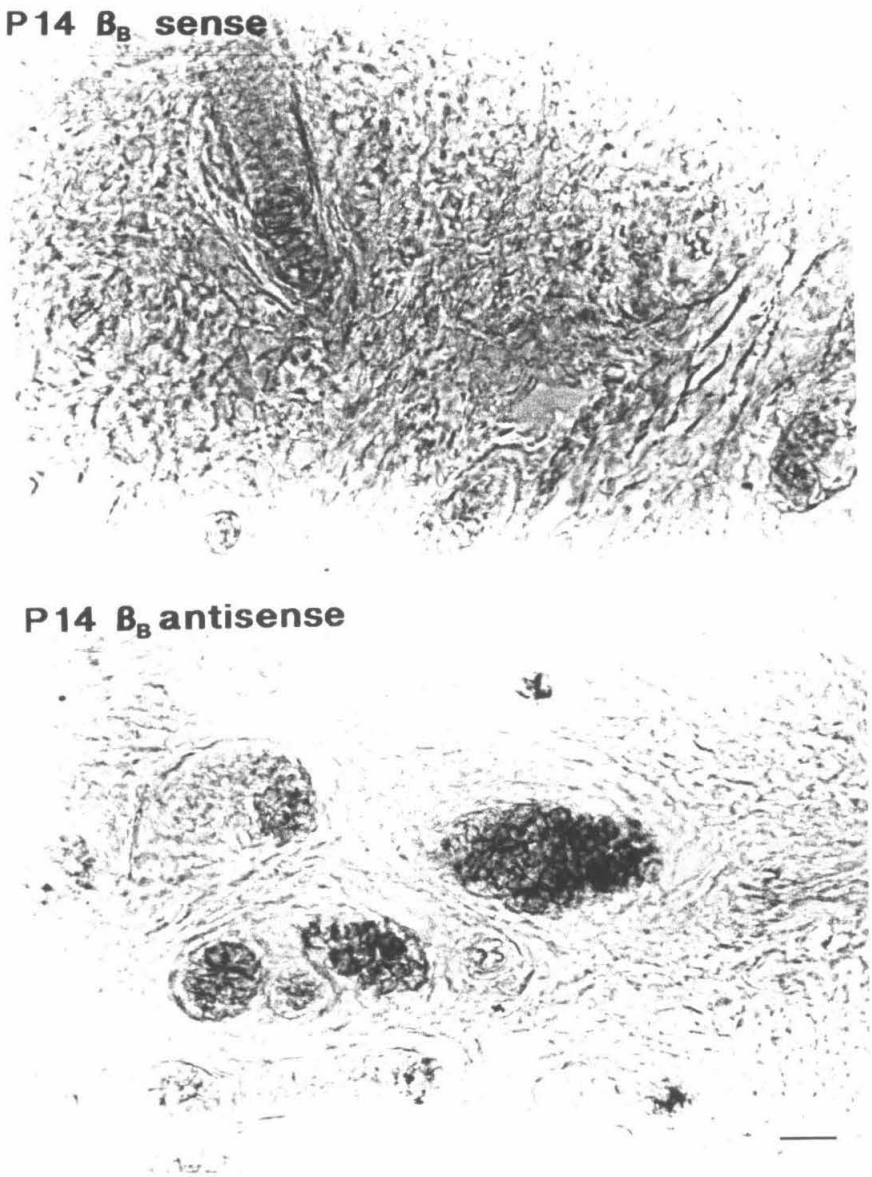

P14 β_B sense
P14 β_B antisense

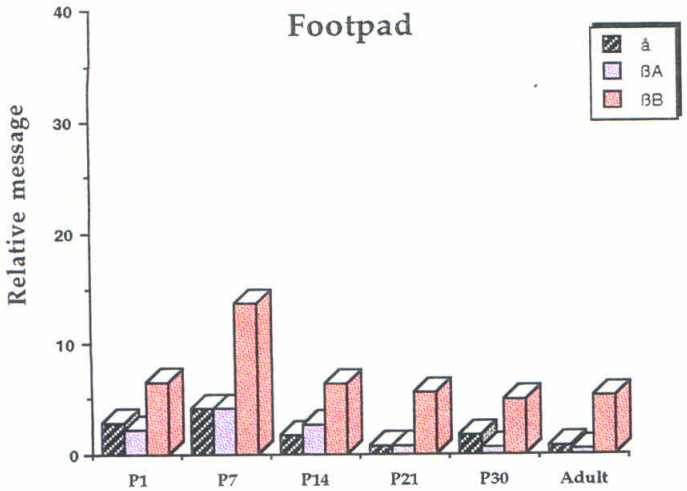
Figure 7. RNase protection analysis of mRNAs for α, β_A, and β_B activins.

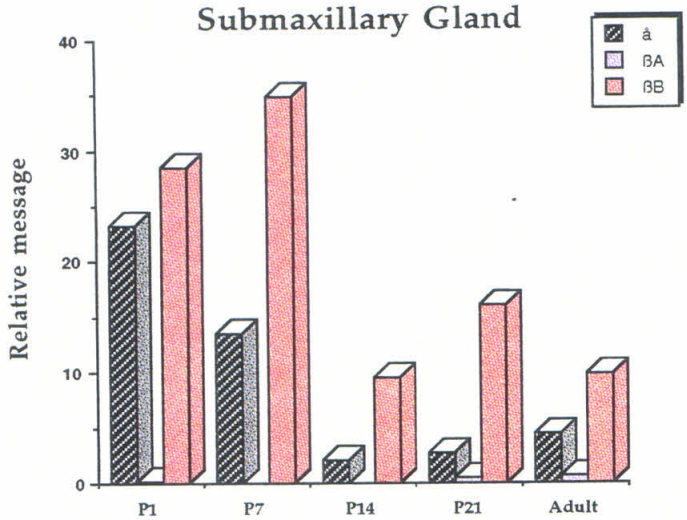
Footpads (FP), submaxillary glands (S. G.), and skin were dissected from rats at various developmental stages. RNA was extracted and expression of the α, β_A, and β_B genes were assayed by the RNase protection method, as described in the Methods section. Arrows on the right side of the panel delineate the sizes of undigested probes. The size of the undigested GAPDH probe is not shown. Arrows on the left side of the panel delineate the predicted sizes of the protected probes. tRNA (tRNA) was used as a negative control in the assay.

probe
P1 FP
P7 FP
P14 FP
P21 FP
P30 FP
adult FP
P1 S.G.
P7 S.G.
P14 S.G.
P21 S.G.
adult S.G.
P1 skin
P7 skin
P14 skin
P21 skin
P30 skin
adult skin
tRNA
α ←
β_A ←
β_B ←
← α'
← β_A'
← β_B'
← GAPDH'

Figure 8. Quantitative analysis of mRNA levels for α, β_A, and β_B genes in footpads, submaxillary glands and hairy skin at different developmental stages.

Each protected fragment for α, β_A, and β_B genes shown in Fig. 7 was quantified using the Phosphoimager and expressed as a ratio to the control gene, GAPDH.


Footpad
Relative message
40
30
20
10
0
å
βA
βB
P1
P7
P14
P21
P30
Adult


Submaxillary Gland
Relative message
40
30
20
10
0
å
βA
βB
P1
P7
P14
P21
Adult

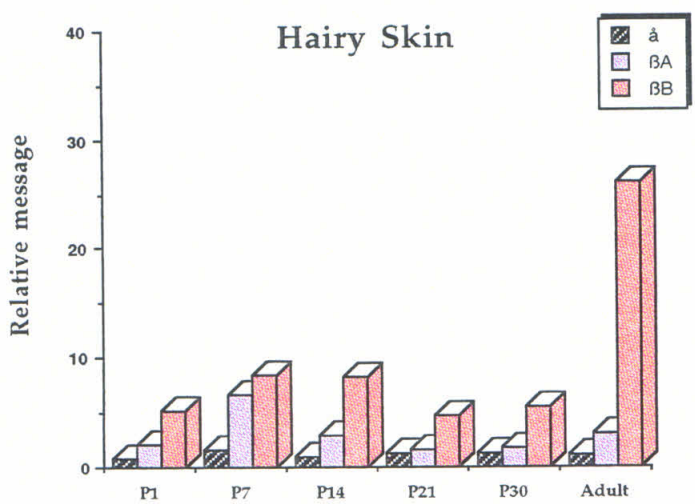
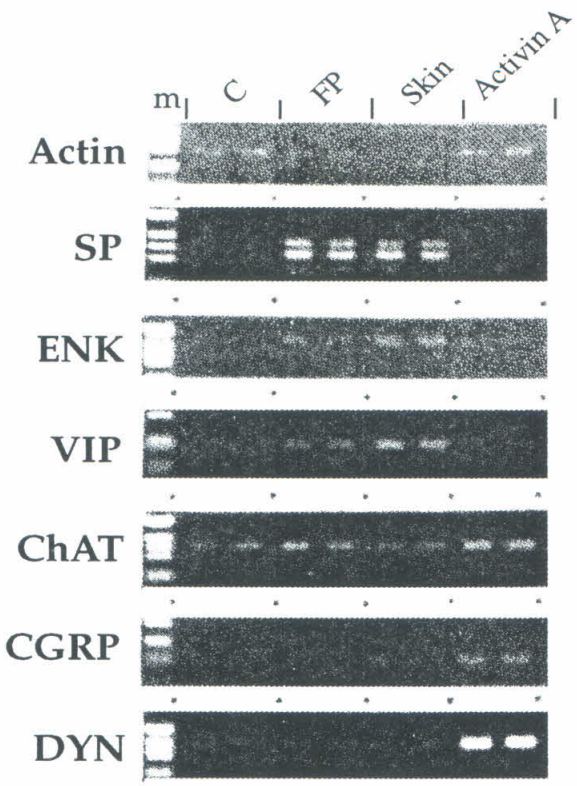

Hairy Skin
Relative message
40
30
20
10
0
å
βA
βB
P1
P7
P14
P21
P30
Adult

Figure 9. Tissue homogenates of footpads and skin do not contain activin-like, neuropeptide-inducing activities.

High speed supernatants of footpad and skin homogenates from P21 rats were added into cultured sympathetic neurons for 6 days. Activin A (100 ng/ml) was also added for comparison. The expression of mRNAs for six transmitter synthetic enzymes and neuropeptides was analyzed by RT-PCR. The selected genes are either induced by neuropoietic cytokines (SP, ENK, VIP, and ChAT) or activin A (ChAT, CGRP, and DYN). Duplicate samples were prepared for each condition. Both homogenantes have SP-, ENK-, and VIP-inducing activities. Footpad extract has also ChAT-inducing activity. The lack of induction of CGRP and DYN by the homogenates does not support the presence of activins in these tissues. Similar results were obtained from P7 rats, though with lower levels of activity.


m
C
FP
Skin
Activin A
Actin
SP
ENK
VIP
ChAT
CGRP
DYN

References

Banner, L. R. and Patterson, P. H. (1994). Major changes in the expression of the mRNA for CDF/LIF and its receptor following injury to adult peripheral nerves and ganglia. Proc. Natl. Acad. Sci. USA, in press.

Basler, K., Edlund, T., Jessel, T. M., and Yamada, T. (1993). Control of cell pattern in the neural tube: Regulation of cell differentiation by dorsalin-1, a novel TGF beta family member. Cell, **73**, 687-702.

Belyavsky, A., Vinorgradova, T., and Rajewsky, K. (1989). PCR-based cDNA library construction: General cDNA libraries at the level of a few cells. Nucleic Acids Research, **17**, 2919-2932.

Birren, S. J., Lo, L.-C., and Anderson, D. J. (1993). Sympathetic neuroblasts undergo a developmental switch in trophic dependence. Development, **119**, 597-610.

Castren, E., Zafra, F., Thoenen, H., and Lindholm, D. (1992). Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. Proc. Natl. Acad. Sci. USA, **89**, 9444-9448.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-choloform extraction. Anal. Biochem., **162**, 156-159.

Coulombe, J. N., Schwall, R., Parent, A. S., Eckenstein, F. P., and Nishi, R. (1993). Induction of somatostatin immunoreactivity in cultured ciliary ganglion neurons by activin in choroid cell-conditioned medium. Neuron, **10**, 899-906.

Fann, M.-J. and Patterson, P. H. (1993). A novel approach to screen for cytokine effects on neuronal gene expression. J. Neurochem., **61**, 1349-1355.

Fann, M.-J. and Patterson, P. H. (1994). Neuropoietic cytokines and activin A differentially regulate the phenotype of cultured sympathetic neurons. Proc. Natl. Acad. Sci. USA, **91**, 43-47.

Feng, Z.-M., Li, Y.-P., and Chen, C.-L. C. (1989). Analysis of the 5'-flanking regions of rat inhibin alpha- and beta-B-subunit genes suggests two different regulatory mechanisms. Mol. Endocrinol., **3**, 1914-1925.

Franklin, J. L. and Johnson, E. M. (1992). Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium. Trends Neurosci., **15**, 501-508.

Garcia-Arraras, J. E. and Martinez, R. (1990). Developmental expression of serotonin-like immunoreactivity in the sympathoadrenal system of the chicken. Cell Tissue Res., **262**, 363-372.

Ghosh, A., Carnahan, J., and Greenberg, M. E. (1994). Requirement for BDNF in activity-dependent survival of cortical neurons. Science, **263**, 1618-1623.

Gibbins, I. L. (1992). Vasoconstrictor, vasodilator and pilomotor pathways in sympathetic ganglia of guinea-pigs. Neurosci., **47**, 657-672.

Goodman, R. H. (1990). Regulation of neuropeptide gene expression. Annu. Rev. Neurosci., **13**, 111-127.

Happola, O., Paivarinta, H., Soinila, S., and Stenbusch, H. (1986). Pre- and post-natal development of 5-hydroxytryptamine-immunoreactive cells in the superior cervical ganglion of the rats. J. Auton. Nerv. Syst., **15**, 21-31.

Hawrot, E. and Patterson, P. H. (1979). Long-term culture of dissociated sympathetic neurons. Methods Enzymol., **58**, 574-583.

Kingsley, D. M. (1994). The TGF-β superfamily: New members, new receptors, and new genetic tests of function in different organisms. Genes & Dev., **8**, 133-146.

Kishimoto, T., Taga, T., and Akira, S. (1994). Cytokine signal transduction. Cell, **76**, 253-262.

Landis, S. C. (1990). Target regulation of neurotransmitter phenotype. Trends Neurosci., **13**, 344-350.

Landis, S. C., Siegel, R. E., and Schwab, M. (1988). Evidence for neurotransmitter platicity *in vivo*, II. Immunocytochemical studies of rat sweat gland innervation during development. Dev. Biol., **126**, 129-140.

Lee, S. J. (1991). Expression of growth/differentiation factor 1 in the nervous system: Conservation of a bicistronic structure. Proc. Natl. Acad. Sci. USA, **88**, 4250-4254.

Lin, L.-F., Doherty, D. H., Lile, J. D., Bektrsh, S., and Collins, F. (1993). GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science, **260**, 1130-1132.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G., and Thoenen, H. (1993). Disruption of the CNTF gene results in motor neuron degeneration. Nature, **365**, 27-32.

Morris, B. J., Feasey, K. J., Bruggencate. G. J., Herz, A., and Hollt, V. (1988). Electrical stimulation *in vivo* increase the expression of proenkephalin

mRNA and decrease the expression of prodynorphin mRNA in rat hippocampal granule cells. Proc. Natl. Acad. Sci. USA, **85**, 3226-3230.

Nairn, A. C., Hemmings, H. C., and Greengard, P. (1985). Protein kinase in the brain. Annu. Rev. Biochem., **54**, 931-976.

Paralkar, V. M., Weeks, B. S., Yu, Y. M., Kleinman, H. K., and Reddi, A. H. (1992). Recombinant human bone morphogenetic protein 2B stimulates PC12 cell differentiation: Potentiation and binding to type IV collagen. J. Cell Biol., **119**, 1721-1728.

Patterson, P. H. and Fann, M.-J. (1992). Further studies of the distribution of CDF/LIF mRNA. Ciba Symp., **167**, 125-140.

Patterson, P. H. and Nawa, H. (1993). Neuronal differentiation factor/cytokines and synaptic plasticity. Cell, **72**, 123-137.

Perides, G., Safran, R. M., Downing, L. A., and Charness, M. E. (1994). Regulation of neural cell adhesion molecule and L1 by the transforming growth factor-β superfamily. J. Biol. Chem., **269**, 765-770.

Rao, M. S. and Landis, S. C. (1990). Characterization of a target-derived neuronal cholinergic differentiation factor. Neuron, **5**, 899-910.

Rao, M. S., Patterson, P. H., and Landis, S. C. (1992a). Multiple cholinergic differentiation factors are present in footpad extracts: Comparison with known cholinergic factors. Development, **116**, 731-744.

Rao, M. S., Sun, Y., Escary, J. L., Perreau, J., Tresser, S., Patterson, P. H., Zigmond, R. E., Brulet, P., and Landis, S. C. (1994). Leukemia inhibitory factor mediates an injury response but not a target-directed developmental transmitter switch in sympathetic neurons. Neuron, **11**, 1175-1185.

Rao, M. S., Tyrrell, S., Landis, S. C., and Patterson, P. H. (1992b). Effects of ciliary neurotrophic factor (CNTF) and depolarization on neuropeptide expression in cultured sympathetic neurons. Dev. Biol., **150**, 281-293.

Rohrer, H. (1992). Cholinergic neuronal differentiation factors: Evidence for the presence of both CNTF-like and non-CNTF-like factors in developing rat footpad. Development, **114**, 689-698.

Sah, D. W. Y. and Matsumoto, S. G. (1987). Evidence for serotonin synthesis, uptake and release in dissociated rat sympathetic neurons in culture. J. Neurosci., **7**, 391-399.

Schotzinger, R. J. and Landis, S. C. (1988). Cholinergic phenotype developed by noradrenegic sympathetic neurons after innervation of a novel cholinergic target in vivo. Nature, **335**, 637-639.

Schotzinger, R. J. and Landis, S. C. (1990). Acquisition of cholinergic and peptidergic properties by sympathetic innervation of rat sweat glands requires interaction with normal target. Neuron, **5**, 91-100.

Schwall, R., Schmelzer, C. H., Matsuyama, E., and Mason, A. J. (1989). Multiple actions of recombinant activin-A *in vivo*. Endocrinol., **125**, 1420-1423.

Soinila, S., Ahonen, M., Joh, T. H., and Steinbusch, H. W. M. (1988). 5-Hydroxytryptamine and catecholamines in developing sympathetic cells of the rats. J. Auton. Nerv. Syst., **22**, 193-202.

Tolon, R. M., Franco, F. S., de los Frailes, M. T., Lorenzo, M. J., and Cacicedo, L. (1994). Effect of potassium-induced depolarization on somatostatin gene expression in cultured fetal rat cerebrocortical cells. J. Neurosci., **14**, 1053-1059.

Walicke, P. A., Campenot, R., and Patterson, P. H. (1977). Determination of transmitter function by neuronal activity. Proc. Natl. Acad. Sci. USA, **74**, 5767-5771.

Walicke, P. A. and Patterson, P. H. (1981). On the role of Ca^{2+} in the transmitter choice made by cultured sympathetic neurons. J. Neurosci., **1**, 343-350.

Wolinsky, E. J., Landis, S. C., and Patterson, P. H. (1985). Expression of Noradrenergic and cholinergic traits by sympathetic neurons cultured without serum. J. Neuronsci., **5**, 1497-1508.

Woodruff, T. K., Meunier, H., Jones, P. B., Hsueh, A. J., and Mayo, K. E. (1987). Rat inhibin: Molecular cloning of alpha- and beta-subunit complementary deoxyribonucleic acids and expression in the ovary. Mol. Endocrinol., **1**, 561-568.

Xie, T., Finelli, A. L., and Padgett, R. W. (1994). The *Drosophila* saxophone gene: A serine-threonine kinase receptor of the TGF-β superfamily. Science, **263**, 1756-1759.

Zigmond, R. E., Schwazschild, M. A., and Rittenhouse, A. R. (1989). Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitter via phosphorylation. Annu. Rev. Neurosci., **12**, 415-461.

CHAPTER 5. Purification steps for neuropeptide-inducing factors in rat heart cell conditioned medium

with Jane Talvenheimo and Lin Cai

Introduction

We have demonstrated that members of the neuropoietic family and the TGF-β superfamily can regulate the expression of neuronal genes in cultured sympathetic neurons (see Chapters 3 and 4). Although factors from these families induce different sets of transmitters and neuropeptides, these inductive patterns cannot account for diverse combinations of transmitters and neuropeptides observed in sympathetic neurons *in vivo*. Other novel factors that have different effects on neuronal gene expression may also be required. A number of proteins have been partially characterized as to their transmitter-inducing activities (see Chapter 1). Previous studies in this laboratory showed that conditioned media (CM) from several cell types contain neuropeptide-inducing activities (Nawa and Sah, 1990). Heart cell CM contains CDF/LIF (Fukada, 1985) and two other partially characterized neuropeptide-inducing proteins (Nawa and Patterson, 1990). One factor is a 45 kDa acidic protein with SOM-inducing activity; the other is an about 80 kDa basic protein with VIP-inducing activity. We have further characterized these two proteins through ion exchange, gel filtration and carbohydrate-binding columns and HPLC. We follow neuropeptide-inducing activities of these fractions using cultured sympathetic neurons and the RT-PCR method of detecting neuropeptide mRNAs (Fann and Patterson, 1993). We find that both proteins are labile and exist in low amounts in heart cell CM. The purification of these two proteins is further hampered by fractions with CDF/LIF. A modified version of this purification scheme will be required to successfully identify these two proteins.

Materials and Methods

Most of the experimental procedures used are described in Chapter 4 with the following exception.

Cell culture

Heart cell CM was prepared in serum-free medium developed by Fukada (1980). Neonatal rat sympathetic neurons were prepared and cultured in serum-free medium as described previously (see Chapter 4). PBS-dialyzed column fractions were mixed with the same volume of neuronal medium and sterilized through 0.2 μm filters before being added into neuronal culture.

Biochemical separation of active components

The fractionation of proteins was accomplished by Jane Talvenheimo and Lin Cai at Amgen, Inc. (Thousand Oaks, CA). Most of the separation steps were done at 4°C. The proteins in heart cell CM were precipitated by 80% (W/V) saturation of ammonium sulfate, and the pellet was dialyzed against 10 volumes of the buffer containing 5 mM Tris-HCl (pH 7.1) (Fukada, 1985). The dissolved precipitate was loaded on a 2 cm x 25 cm, 50 ml bed volume Q-Sepharose column (Pharmacia) preequilibrated with the same buffer. The column was washed with 5 mM Tris-HCl at the same volume of applied sample, which were collected as the flow-through fraction. Then the column was eluted with a linear gradient of 0 to 1 M NaCl in 5 mM Tris-HCl buffer at a flow rate of 5 ml/min. Every 30 ml were collected into one fraction. A total of 24 fractions were collected.

Sephadex G-100 and G-75 (superfine grade) columns were used for gel filtration analysis. Samples were applied to a 2 cm x 100 cm, 300 ml bed volume Sephadex columns equilibrated in PBS, pH 7.3. The columns were eluted with the same buffer at the flow rate of 1 ml/min. Each fraction contained 6 ml and 50 fractions were collected. The columns were calibrated with aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen (25 kDa).

Lentil lectin and Concanavalin A columns were purchased from Pharmacia. The Q-Sepharose flow-through fractions in PBS (pH 7.3) were concentrated to 100 ml and supplemented with 1 mM $MgCl_2$ and $MnCl_2$ prior

to application to the lentil lectin column. Samples were mixed with 75 ml of lentil lectin agarose, the material transferred to a column (2.6 cm x 25 cm) for washing with 150 ml of a buffer containing 40 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM $MnCl_2$, pH 7.2, and eluted in one fraction with 200 ml 0.3 M α-methylmannoside in the same buffer. The lentil lectin column flow-through fraction, which contains about 98% of the starting protein, was concentrated to 100 ml prior to application to a 50 ml Concanavalin A (ConA) agarose column (2.6 cm x 25 cm). The column was washed with 100 ml of a buffer containing 40 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM $MnCl_2$, pH 7.2. The protein bound to the ConA column was eluted into a fraction with 100 ml of the same buffer containing 0.3 M α-methylmannoside. The lentil lectin eluate, the ConA eluate and the ConA flow-through fraction were extensively dialyzed against 20-fold volume of PBS, and then concentrated to 10 ml before being added to the neuronal culture.

Fractions eluted from lectin columns were further concentrated to 400 µl, dialyzed against water, and acidified to a final concentration of 80% buffer A (0.1% TFA) plus 20% buffer B (0.1% TFA, 90% CH_3CN) prior to application to a C4 reverse phase HPLC column (4.6 x 250 mm; Waters). The material was eluted with a linear gradient of 20% to 80% buffer B at a flow rate of 1 ml/min. One ml was collected into each fraction so that the fraction number corresponded to the elution time. Absorbance was monitored at 214 nm. Fractions were dried and then re-dissolved in 2 ml PBS before being added to the neuronal culture.

Results

Several neuropeptide-inducing activities exist in Q-Sepharose fractions.

Nawa and Patterson (1990) reported that heart cell CM contains at least three different proteins with neuropeptide-inducing activities and these proteins can be separated by anion exchange columns. We therefore adopted a Q-Sepharose anion exchange column as a first purification step. Five liters of serum-free heart cell CM were concentrated by ammonia sulfate precipitation, dialyzed against 5 mM Tris-HCl buffer at neural pH, applied to a Q-Sepharose column in the same buffer, and eluted with a zero to 1 M NaCl gradient. Fig. 1 shows the protein elution profile of Q-Sepharose chromatography. Most of the protein was eluted as a single peak. These fractions (containing negatively charged proteins) as well as flow-through fractions (containing positively charged proteins) were analyzed for their transmitter- and neuropeptide-inducing activities on cultured sympathetic neurons by the RT-PCR method. Fractions 4 and 6-10 have ChAT-, SOM-, SP-, and VIP-inducing activity (Fig. 2). There is, however, almost no detectable VIP signal in either the original heart cell CM or the column flow-through (Fig. 2). This result was unexpected, since the CM and the column flow-through should contain CDF/LIF, which induces VIP. Because signals for other neuropeptides are also weak in the original CM and flow-through fractions, it is possible that the active factors were either too dilute to be detected, or perhaps subject to inhibition by other components before purification. To address these concerns, the Q-Sepharose flow-through fractions were concentrated and assayed again (Fig. 3). An aliquot was also applied to a Sephadex G-100 column to assess the molecular weight (MW) of the VIP-inducing activity. When concentrated, the Q-Sepharose flow-through fractions display activity similar to that of CDF/LIF. Weak VIP and a significant SP signal are found in the 50-70 kDa fractions from the gel filtration column. Thus, our results from this anion exchange column are similar to those of Nawa and Patterson (1990) who used a different anion exchange column, and the MW of these activities is similar to that previously reported.

Isolation of SOM-inducing activity from the acidic protein fractions.

Since there was a SOM-inducing activity from the Q-Sepharose fractions (Fig. 2), we first worked on isolation of this activity from the acidic proteins. Nawa and Patterson (1990) found a SOM-inducing activity in heart cell CM that is distinct from CDF/LIF with an apparent MW of 45 kDa by gel filtration. We find a similar activity in the Q-Sepharose fractions. Fractions 4 and 8 were each applied to a Sephadex G-100 column, and bioassay results are shown in Fig. 4. Although Q-Sepharose fraction 4 continues to give a strong SOM signal in this assay, Q-Sepharose fraction 8 no longer exhibits activity. There is, however, no activity in any of Sephadex G-100 fractions derived from either Q-Sepharose fraction. There are at least three explanations for the negative results: (1) the SOM-inducing activity is labile, (2) the gel filtration fractions were too dilute, or (3) multiple components required for activity were separated by the gel filtration step. To test the possibility that samples were too dilute, we concentrated aliquots of the gel filtration fractions and assayed these fractions again (Fig. 5). The Sephadex G-100 fractions derived from Q-Sepharose fraction 4 still showed no activity, even though fraction 4 itself continued to induce SOM. This indicated that SOM-inducing activity in Q-Sepharose fraction 4 might contain multiple components that were separated by the gel filtration step. In contrast, a SOM signal is detected in the concentrated fraction with 73-100 kDa MW range derived from Q-Sepharose 8 and in concentrated Q-Sepharose fraction 8. Taken together, these results suggest that the SOM-inducing activity detected in the assay shown in Fig. 2 is labile or consists of multiple components. In other Q-Sepharose preparations using CM that had been stored in freezers for long periods, SOM-inducing activity was difficult to detect. Thus, the purification of SOM-inducing activity was not pursued further.

Isolation of VIP-inducing activity from the basic protein fractions.

In order to separate CDF/LIF from non-CDF/LIF VIP-inducing activities in the Q-Sepharose flow-through fraction, we used lentil lectin chromatography to eliminate most of CDF/LIF, a scheme used by other investigators to purify CDF/LIF to homogeneity (Hilton et al., 1988). The Q-Sepharose flow-through fractions were mixed with 75 ml of lentil lectin agarose, the material transferred to a column for washing, and eluted with α-methylmannoside. We expected CDF/LIF to be present in the glycoprotein

fraction eluted from this column. The lentil lectin column flow-through fraction, which contains about 98% of the starting protein, was applied to a 50 ml Concanavalin A (ConA) agarose column to remove any residual CDF/LIF (ConA and lentil lectin have similar carbohydrate binding specificity, ConA having a higher affinity). This scheme produced four samples for assay: (1) Q-Sepharose flow-through (100% of starting protein), (2) Lentil lectin eluate (2% of starting protein), (3) ConA flow-through (96% of starting protein), and (4) ConA eluate (2% of starting protein). The assay results for these samples are shown in Fig. 6. SP and ChAT are strongly induced by the lentil lectin eluate, consistent with the presence of CDF/LIF in this fraction. The ConA eluate also induces SP and ChAT, but less strongly than the lentil lectin eluate. VIP is induced more strongly in the ConA eluate than in the lentil lectin eluate, suggesting that the ConA eluate may contain, in addition to CDF/LIF, a novel VIP-inducing activity. We were unable to obtain conclusive results for the ConA flow-though fraction in this assay because it was toxic to the neurons.

To further separate CDF/LIF from other VIP-inducing activities, we subjected the ConA eluate to reverse phase HPLC. To test where CDF/LIF is eluted from HPLC, the lentil lectin eluate was applied to a Superdex 75 column. The fractions in the MW range of 20 to 90 kDa were pooled and run on a C4 HPLC column in 80% buffer A (0.1% TFA) plus 20% buffer B (0.1% TFA, 90% CH_3CN), and eluted with a linear gradient of 20% to 80% buffer B (Fig. 7). The fractions containing CDF/LIF were identified by three assays: (1) Immunoblots using an anti-CDF/LIF antiserum (Yamamori et al., 1989) reveals a broad band, possibly a doublet, at 40 kDa in fraction 37 (Fig. 8), which elutes at 68% buffer B; (2) N-terminal sequencing of fraction 37 confirms it as CDF/LIF; (3) The bioassay detected CDF/LIF-like activity in fractions 36 and 38 (Fig. 9, H36 and H38). We conclude that the lentil lectin eluate from the Q-Sepharose flow-though contains CDF/LIF and that this may be the only activity detectable after reverse phase HPLC.

We next subjected the ConA eluate to HPLC using similar conditions (Fig. 10). Immunoblot analysis does not detect a CDF/LIF band in the ConA eluate and the ConA flow-through fraction (Fig. 8, ConA E and ConA FT), nor in its HPLC fractions (data not shown). The bioassay results (Fig. 11) revealed that the early-eluting HPLC fractions (4, 5, and 21-26) contain moderate ChAT-inducting activity distributed over several protein peaks. Moderate

VIP and *ENK* signals are obtained with fractions 25-27, overlapping the ChAT-inducing activity. The lack of SP-inducing activity in these fractions is notable. Fractions 31 and 32 show CDF/LIF-like activities, inducing SP expression. Fractions 39-43 exhibit moderate VIP and CCK induction without SP induction. Since we had not observed a combination of VIP- and CCK-inducing activities previously, fractions 39-43 were combined together as Pool 3 and assayed again. In the repeat assay this sample had no activity, suggesting that this sample was not stable. We also combined fractions 25-27 as Pool 1 and fractions 31-32 as Pool 2 and examined them by SDS-PAGE and silver-staining (Fig. 12). Pool 1 contains major bands at 78, 41, and 37 kDa, as well as other minor bands. While the 37 and 41 kDa bands are in the size range of CDF/LIF, the lack of SP induction by this pool indicates that if these bands are the active agents, these are novel proteins. Pool 2 (containing the CDF/LIF-like activity) displays one major band at 78 kDa and several minor bands. There is no band detectable at the MW range expected for CDF/LIF. No protein bands are detectable in Pool 3.

VIP-inducing activity was lost during the larger scale of purification.

To produce a larger quantity of material for purification, we utilized 20 L heart cell CM and a sample provided by Dr. K. Fukada. The latter material represents 200 L of heart cell CM that had been passed through a DEAE column to remove the acidic protein fraction, then through a CDF/LIF affinity column using CDF/LIF antisera to remove CDF/LIF (Fukada et al., 1991). This sample should provide a good source for non-CDF/LIF activities.

Twenty liters of CM were treated as described in the flow chart shown in Fig. 13. Samples were concentrated, buffer-exchanged, and passed over a Q-Sepharose column at neural pH to separate acidic and basic proteins. The Q-Sepharose flow-through fractions containing basic proteins were applied to a lentil lectin column, and the lentil lectin column flow-through was applied to a ConA column. This procedure yielded three samples: the lentil lectin eluate (proteins specifically absorbed by lentil lectin), the ConA eluate (proteins that passed through the lentil lectin column but bound to the ConA column), and the ConA flow-through (protein that did not bind to either lectin column). The ConA flow-through fraction contains about 95% of the protein from the original Q-Sepharose flow-through. The sample from K. Fukada was

first dialyzed extensively against PBS, then applied to lentil lectin and ConA columns. Bioassay results for fractions from these preparations are shown in Fig. 14. Most of the neuropeptide-inducing activity appears in the lentil lectin eluate and the ConA eluate, the two samples enriched in glycoproteins. Surprisingly, only a weak VIP-inducing activity is observed in fractions derived from K. Fukada's sample. Moderate VIP-inducing activity is present in lentil lectin eluate and ConA eluate derived from Q-Sepharose flow-through. The CCK-inducing activity eluted from lentil lectin column appears to have been lost upon ConA chromatography. A major difference between the results from this large scale lentil lectin-ConA preparation and those from the first analytical lentil lectin-ConA procedure is the absence of a neuropeptide-inducing activity that lacks SP induction. That is, unlike the first lentil lectin-ConA experiment, the second preparation does not have a clear, non-CDF/LIF activity.

Because the lentil lectin eluate and ConA eluate derived from these two sources of starting material display similar activity profiles, we combined samples from these two sources. The pooled lentil lectin eluate and pooled ConA eluate were each subjected to reverse phase HPLC purification under identical conditions. The bioassay results for two sets of HPLC fractions are shown in Figs. 15 and 16. HPLC fractions derived from the pooled lentil lectin eluate show a broad peak of CDF/LIF-like activity centered on fractions 30-35 (Fig. 15). Surprisingly, HPLC fractions derived from the ConA eluate also show a broad peak of CDF/LIF-like activity centered on fractions 30-35 (Fig. 16). To determine whether the ConA eluate contains any non-CDF/LIF activity, we re-tested the activity of both ConA eluate and HPLC fractions 30-35 in the presence of a CDF/LIF neutralizing antiserum. This antiserum blocks the activity in the ConA eluate and in the HPLC fractions derived from it (Fig. 17). After treatment with the antiserum, the ConA eluate shows only very weak induction of VIP and ChAT, slightly above the background level induced in the negative control. The early-HPLC fractions from the lentil lectin eluate, fractions 22-24, had weak VIP, SP, and ENK-inducing activity (Figs. 15). Thus, unlike the first analytical lentil lectin-ConA preparation, the large scale, second preparation contains primary CDF/LIF as the neuropeptide-inducing protein. There is, however, some residual VIP-inducing activity in the ConA flow-through fraction.

Discussion

Our primary goal was to identify non-CDF/LIF, neuropeptide-inducing factors. Nawa and Patterson (1990) reported that heart cell CM contains two non-CDF/LIF factors that induce expression of VIP or SOM in cultured sympathetic neurons. The SOM-inducing activity adsorbed to an anion exchange column when applied in low salt at neutral pH, and has an apparent MW of 45 kDa. The VIP-inducing activity, by contrast, is a basic protein with an apparent MW of 80 kDa.

We were able to confirm that the SOM-inducing activity is an acidic protein(s) that adsorbed to a Q-Sepharose column. We lost this activity when we attempted further purification through gel filtration, however. Moreover, later preparations of heart cell CM which were stored in freezers longer than 6 months contain very low level of SOM-inducing activity in such Q-Sepharose fractions. Thus, it is reasonable to assume that this activity is labile.

We also attempted to identify the VIP-inducing activity that co-exists with CDF/LIF in the Q-Sepharose flow-through fraction. CDF/LIF was effectively eliminated by the lentil lectin affinity column in our initial, analytical experiment. Three distinct activities were demonstrated after the ConA column and reverse phase HPLC. One of the activities (Pool 2, HPLC fractions 31 and 32, Fig. 11) may be residual CDF/LIF, as judged from its pattern of neuropeptide induction. Another activity (Pool 1, HPLC fractions 25-27, Fig. 11) is more hydrophilic than CDF/LIF and induces ChAT, ENK, VIP, but not SP expression. The other activity (Pool 3, HPLC fractions 39-43, Fig. 11) increases expression of CCK and VIP without inducing SP, and has more hydrophobic properties. A large scale preparation that combined 20 l of heart cell CM and a sample equivalent to 200 l of heart cell CM provided by Dr. Fukada was purified using the same scheme and failed to reproduce similar results. Instead, most of the VIP-inducing activity was attributed to CDF/LIF. We did observe VIP-inducing activity in early HPLC fractions derived from lentil lectin eluate. Those fractions contain < 5% of the total CM protein. Since the second preparation of heart cell CM had been stored in freezers for more than two years or processed through several columns (Dr. Fukada's sample), a likely reason for the lack of reproducibility of the initial data is a loss of this novel VIP-inducing activity. The use of fresh heart cell

CM with minimal storage time in freezers seems important for isolation of both the SOM- and VIP-inducing factors.

The purification of VIP-inducing activity is also hampered by the contamination of CDF/LIF in the Q-Sepharose flow-through fraction. Since CDF/LIF also induces VIP expression, elimination of CDF/LIF in the early steps of purification is necessary to detect the novel VIP-inducing activity. This may be feasible through repeated lentil lectin chromatography or through a CDF/LIF affinity column. Although we failed to eliminate CDF/LIF using these steps in our second preparation, it may be that the column was overloaded. An alternative approach would be to obtain heart cell CM from CDF/LIF-deficient mice (Escary et al., 1993; Stewart et al., 1992).

The RT-PCR assay employed for this purification purpose could also be modified as well. The length of time required for the bioassay is nine days. Most of this time is for incubating the neurons (six days). It may be worth investigating various cell lines to reach a faster bioassay response. In addition, this assay is qualitative and does not allow calculation of specific activities for each fraction. Whether the assay should be upgraded to a quantitative PCR is worth considering.

An alternative approach to identify these neuropeptide-inducing factors is by expression cloning. We attempted to find novel factors from a neonatal brain expression library using cultured sympathetic neurons and the RT-PCR assay without success. The major technical obstacle was a background problem. We had many false-positive clones, because variations of culture condition for Cos cells (or any other protein-generating cells *in vitro*) can have effects on the cultured neurons. Maintaining very uniform neuronal cultures is also very difficult. Neuronal cell lines could be useful in this respect. Another question in the expression cloning approach is the size of each pool used in the first screen. This is related to the sensitivity of the assay and the yield of the expression plasmid. The sensitivity of the assay seems dependent on the proteins of interest. We can consistently detect CNTF and CDF/LIF at concentration of >1 ng/ml (Fann and Patterson, 1993). For activins and BMP, 5 ng/ml are required. This assay is not nearly as sensitivie as those used for expression cloning of interleukins. Thus, the size of each pool cannot be too large. Our preliminary results indicate that 300 colonies

per pool may be the upper limit, which means that many pools are needed to screen the library.

In summary, we have provided further evidence for the existence of novel neuropeptide-inducing factors. Although we were unable to purifiy these proteins to homogeneity, the lessons learned from it may benefit other researchers for pursuit of these factors.

Figure 1. Anion exchange chromatography of heart cell conditioned medium.

1.8 liters of concentrated heart cell CM were applied to a column of Q-Sepharose equilibrated at 4°C in a buffer containing 5 mM Tris-HCl at pH 7.1. The column was eluted with 1.7 liters of equilibrated buffer that were collected as two fractions (FT1 and FT2), followed by a 720 ml linear gradient of zero to 1 M sodium chloride that were collected in 24 fractions. Sodium chloride concentration was measured by a conductivity monitor and the protein concentration of each fraction was determined using the Bio-Rad protein assay kit.

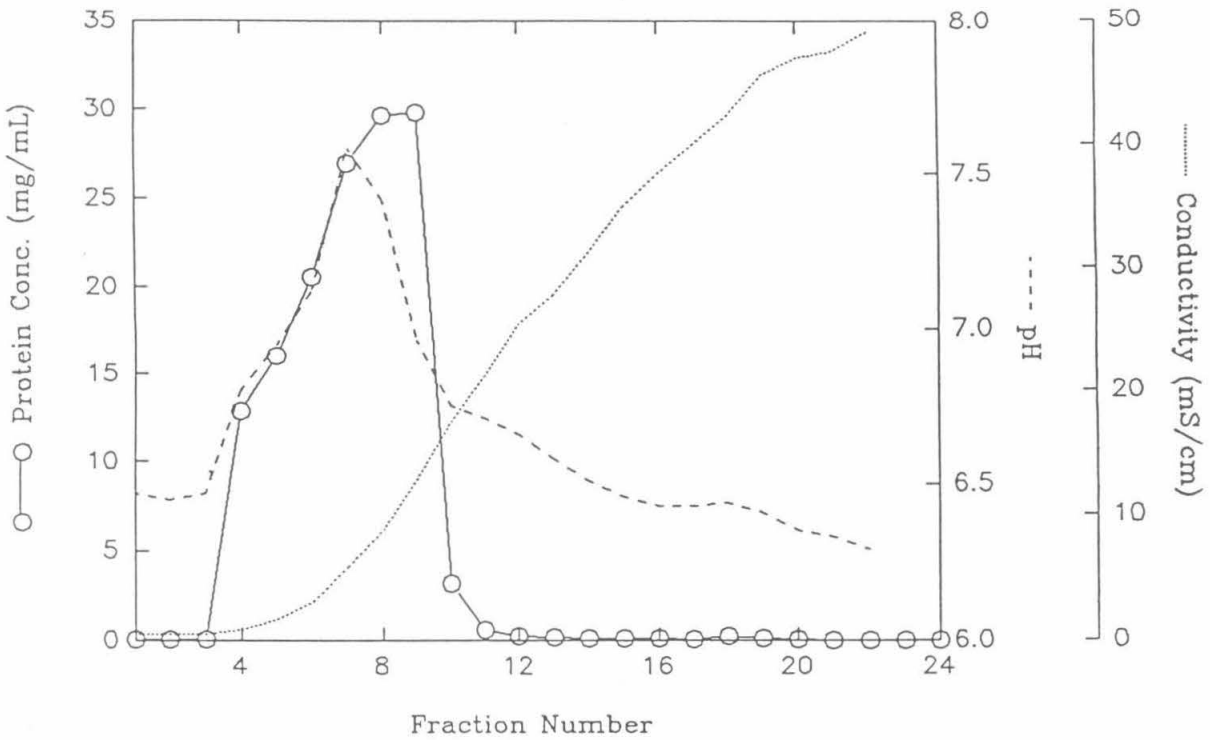

Protein Conc. (mg/mL)
35
30
25
20
15
10
5
0
8.0
7.5
7.0
6.5
6.0
pH
50
40
30
20
10
0
Conductivity (mS/cm)
4
8
12
16
20
24
Fraction Number

Figure 2. The profile of neuropeptide-inducing activities in Q-Sepharose fractions.

An aliquot (1 ml) of each Q-Sepharose fractions (shown in Fig. 1) was dialyzed against PBS (pH 7.3), mixed with the same volume of serum-free neuronal cultured medium and filtered. Sympathetic neurons were growing in this 50% column fraction medium for six days. One ml of heart cell CM (HC CM), $(NH_4)_2SO_4$ precipitate after dialysis (P), and heart cell serum-free medium (HC M, which nerve contacts heart cells) were also mixed with 1 ml neuronal cultured medium and added to the neurons. Another control (C) was neurons cultured in neuronal medium only. The expression of mRNAs for transmitter synthetic enzymes and neuropeptides was analyzed by the RT-PCR assay. Duplicate samples were prepared for each condition, and the β-actin PCR product was used to monitor the amount of mRNA present in each sample.

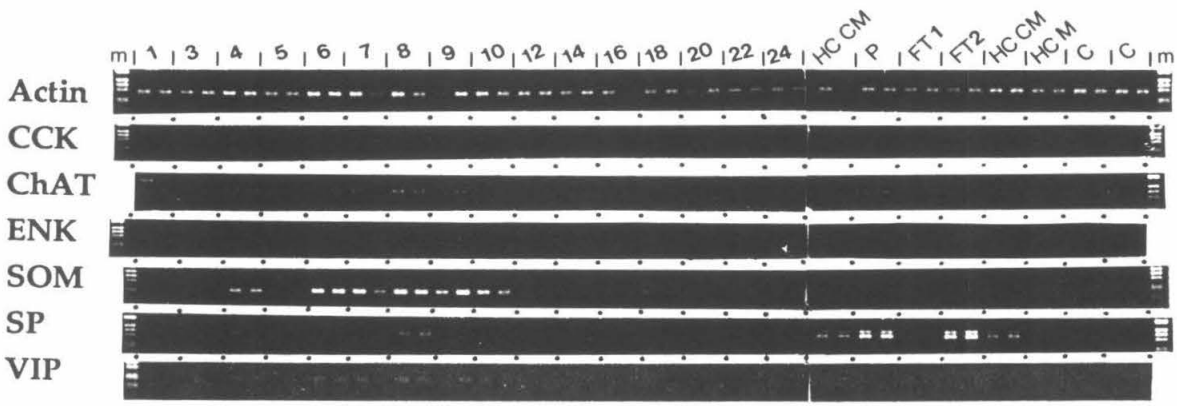

m
1
3
4
5
6
7
8
9
10
12
14
16
18
20
22
24
HC CM
P
FT1
FT2
HC CM
HC M
C
C
m
Actin
CCK
ChAT
ENK
SOM
SP
VIP

Figure 3. Q-Sepharose flow-through fractions contain VIP-inducing activity.

Q-Sepharose fractions FT1 and FT2 were concentrated five-fold before being added to neuronal culture. An aliquot of FT2 was also applied to a Sephadex G-100 column to measure the apparent MW of VIP-inducing activity. The MW ranges were estimated based on calibration of the column with MW standards. Neuronal culture and the RT-PCR were processed as in Fig. 2.

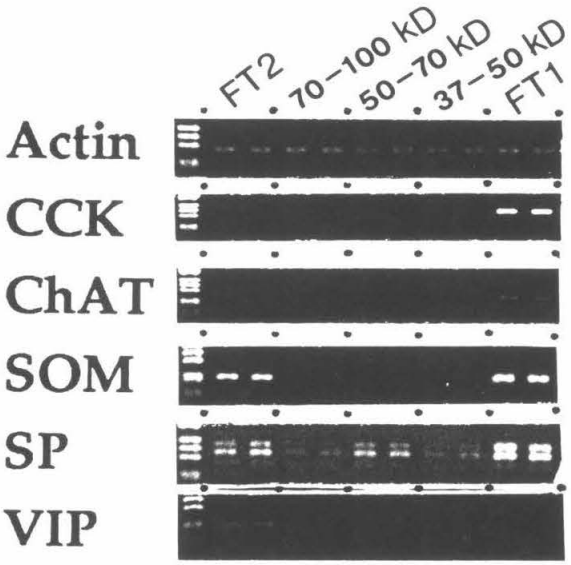

FT2
70–100 kD
50–70 kD
37–50 kD
FT1
Actin
CCK
ChAT
SOM
SP
VIP

Figure 4. The neuropeptide-inducing profile of Sephadex G-100 fractions derived from Q-Sepharose fractions 4 and 8.

148 mg of protein from Q-Sepharose fraction 4 (F4) and 8 (F8) were applied to a column of Sephadex G-100 column to further purify SOM-inducing activity. The MW ranges (kDa) were estimated based on calibration of the column with MW standards.

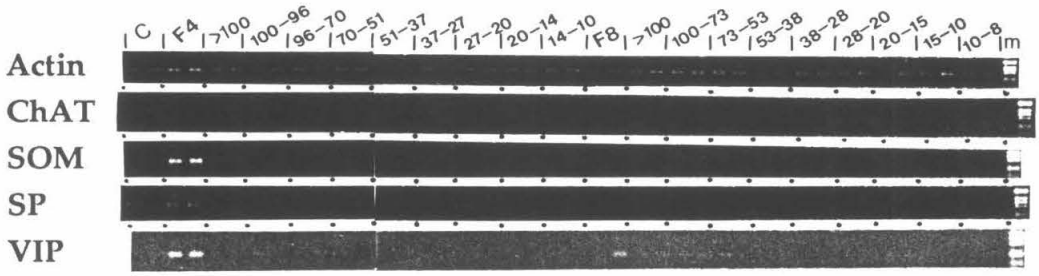

C
F4
>100
100–96
96–70
70–51
51–37
37–27
27–20
20–14
14–10
F8
>100
100–73
73–53
53–38
38–28
28–20
20–15
15–10
10–8
m
Actin
ChAT
SOM
SP
VIP

Figure 5. The profile of neuropeptide-inducing activity in concentrated Sephadex G-100 fractions.

Fractions from a Sephadex G-100 column were concentrated five-fold before being subjected to the neuronal assay. Q-Sepharose fractions 4 and 8 (F4 and F8) were concentrated two-fold. The fractions derived from F8 with MW 73-100 kDa and higher contained SOM-inducing activity.

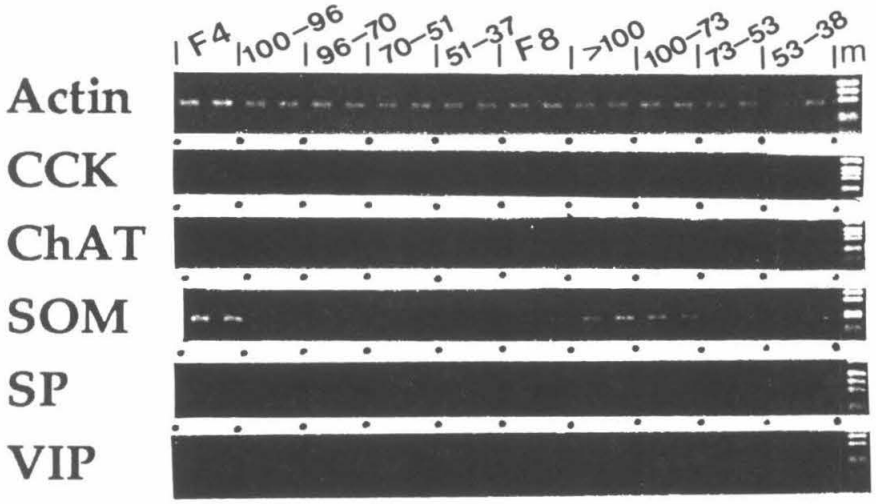

F4
100–96
96–70
70–51
51–37
F8
>100
100–73
73–53
53–38
m
Actin
CCK
ChAT
SOM
SP
VIP

Figure 6. Bioassay of fractions from sequential lentil lectin and ConA columns.

The Q-Sepharose flow-through (Q. FT) fraction was applied to a lentil lectin column. The absorbed protein was eluted in a single fraction (LL E) with 0.3 M α-methylmannoside. The lentil lectin column flow-through fraction was applied to a ConA agarose column. The absorbed protein was eluted in a single fraction (ConA E) with 0.3 M α-methylmannoside. The ConA column flow-through was also collected in a single fraction (ConA FT). These samples were extensively dialyzed against PBS before being assayed on the neuronas. Neurons were not healthy in ConA FT.

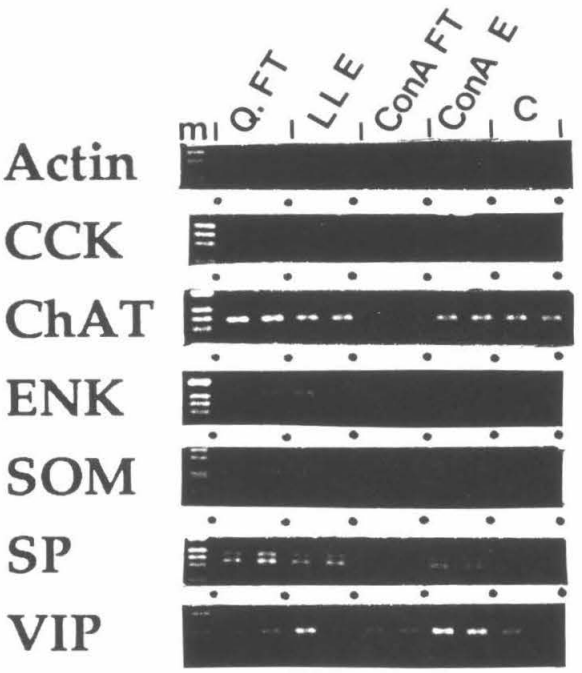

m
Q. FT
L L E
ConA FT
ConA E
C
Actin
CCK
ChAT
ENK
SOM
SP
VIP

Figure 7. Reverse phase HPLC fractionation of the lentil lectin eluate.

Eluted material from the lentil lectin column was concentrated, dialyzed against PBS and loaded on a Superdex 75 column. The fractions in the MW range of 20 to 90 kDa were pooled, concentrated, dialyzed against water, and acidified to a final concentration of 0.1% tetrafluoroacetic acid (TFA). Reverse phase HPLC was performed on a C4 column in 80% buffer A (0.1% TFA) plus 20% buffer B (0.1% TFA, 90% CH_3CN) and eluted with a linear gradient of 20% to 80% buffer B at 1 ml/min. One ml was collected into each fraction so that the fraction number corresponds to the elution time. Absorbance was monitored at 214 nm. The solid line is protein concentration profile. The dotted line is generated by the detector artificially to separate overlapping peaks, which does not relate to the fractions collected. The elution time (min after injection) of each peak is marked on the top of the peak.

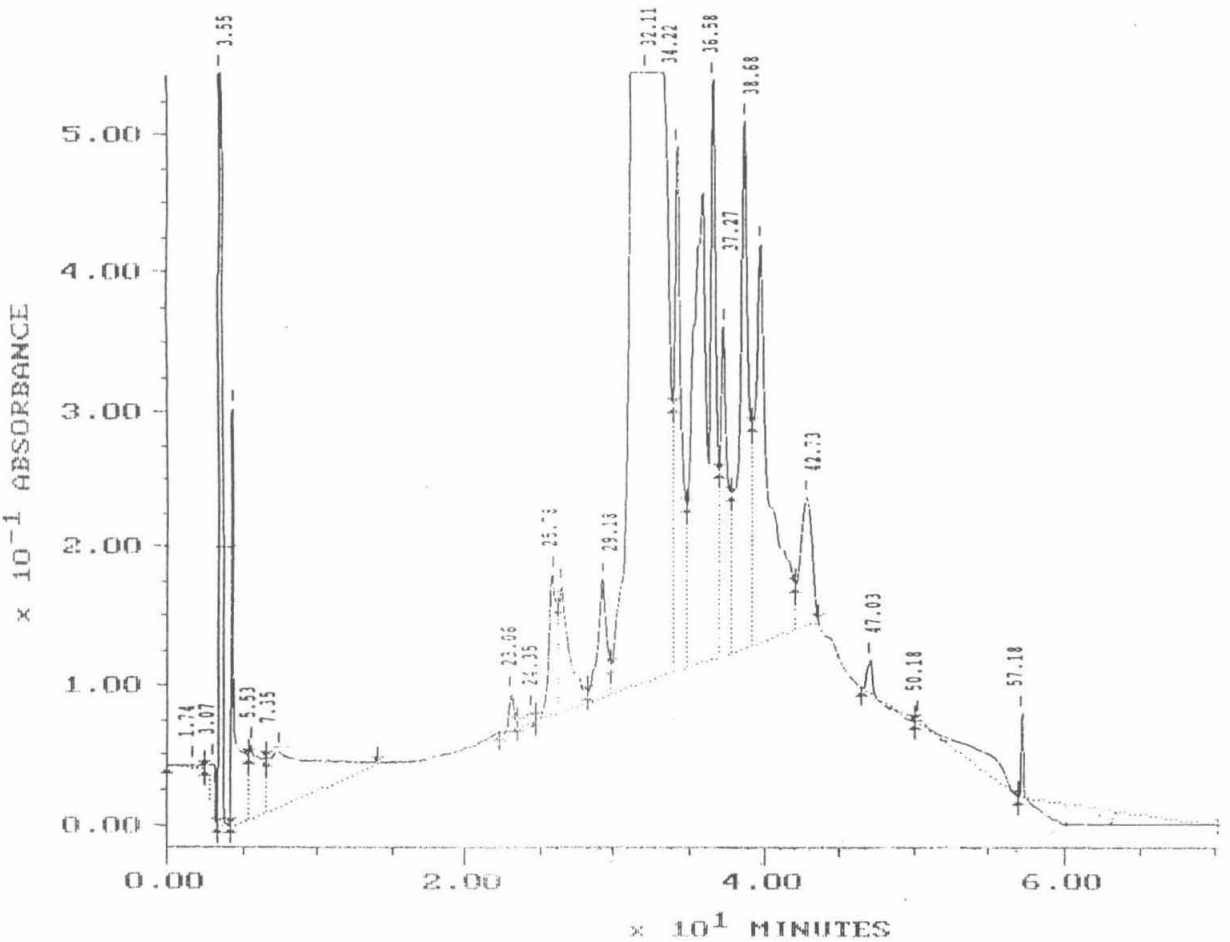

1.74
3.07
3.55
5.53
7.35
22.66
24.35
25.73
29.33
32.11
34.22
36.58
37.27
38.68
42.73
47.03
50.18
57.18
5.00
4.00
3.00
2.00
1.00
0.00
x 10⁻¹ ABSORBANCE
0.00
2.00
4.00
6.00
x 10¹ MINUTES

Figure 8. Immunoblot of lentil lectin, its HPLC, and ConA fractions using an anti-CDF/LIF antiserum.

Proteins in the ConA eluate (ConA E), the ConA flow-though (ConA FT), the lentil lectin eluate (LL E) and the HPLC fractions derived from lentil lectin eluate were separated by 10-20% gradient SDS-PAGE. After blotting, the nitrocellulose strips were incubated with 1: 100 anti-CDF/LIF N-terminal peptide antiserum and developed with alkaline phosphatase substrates. Only the bands at 43 and 37 kDa can be reversed by incubating the antiserum with recombinant CDF/LIF (data not shown). Other bands are therefore cross-reactive proteins. Incubation of the strips with preimmune serum did not reveal any bands (data not shown).

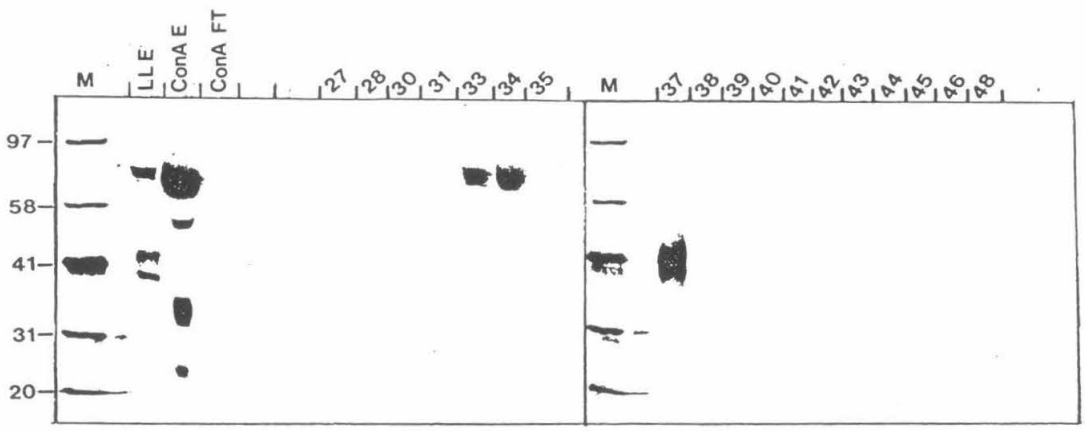

M
LL E
ConA E
ConA FT
27
28
30
31
33
34
35
M
37
38
39
40
41
42
43
44
45
46
48
97
58
41
31
20

Figure 9. Bioactivity analysis of Superdex 75 fractions and reverse phase HPLC fractions derived from lentil lectin eluate.

The lentil lectin eluate was subjected to the Superdex 75 column chromatography. Fractions 21-23 derived from this column were further separated by reverse phase HPLC (see Fig. 7). Superdex fractions (S) (MW about 20-90 kDa) and HPLC fractions (H) were also subjected to the neuronal assay. Only 1/100 of HPLC fraction 37 (H37) was applied to the neuronal assay, because most of this fraction was used for peptide sequencing. Superdex fractions 19, 20 and 24 display a typical CDF/LIF neuropeptide inductive pattern. HPLC fractions 36, 37, and 38 contained CDF/LIF-like activity.

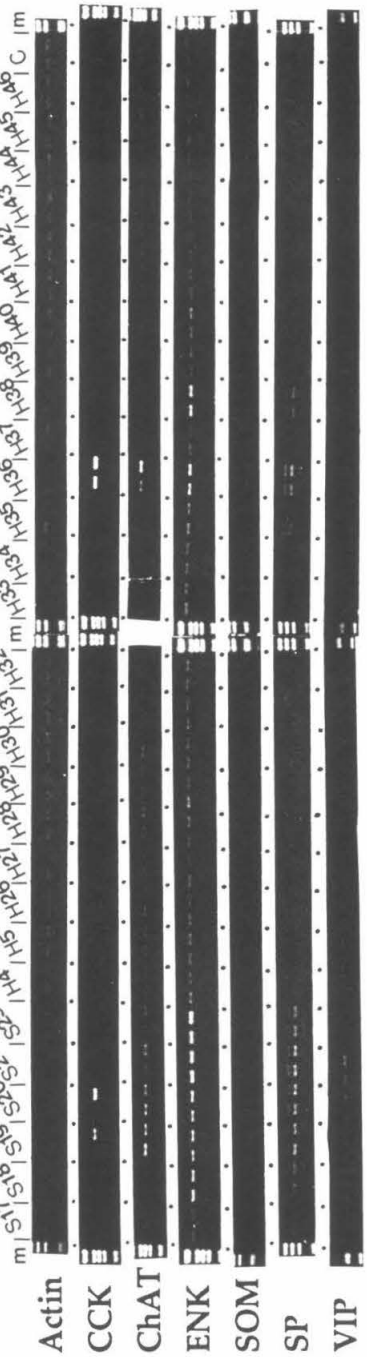

Actin
CCK
ChAT
ENK
SOM
SP
VIP

Figure 10. Reverse phase HPLC chromatography of the ConA eluate.

The ConA eluate was concentrated, dialyzed against water, and acidified to a final concentration of 0.1% TFA. Reverse phase HPLC was performed on a C4 column in 70% buffer A (0.1% TFA), and 30% buffer B (0.1% TFA, 90% CH_3CN) and eluted with a linear gradient of 30% to 80% buffer B at 1 ml/min. One ml was collected into each fraction so that fraction number is corresponding to the elution time. Absorbance was monitored at 214 nm. The solid line is protein concentration profile. The dotted line is generated by the detector artificially to separate overlapping peaks, which does not relate to the fractions collected. The elution time of each peak is marked on the top of the peak.

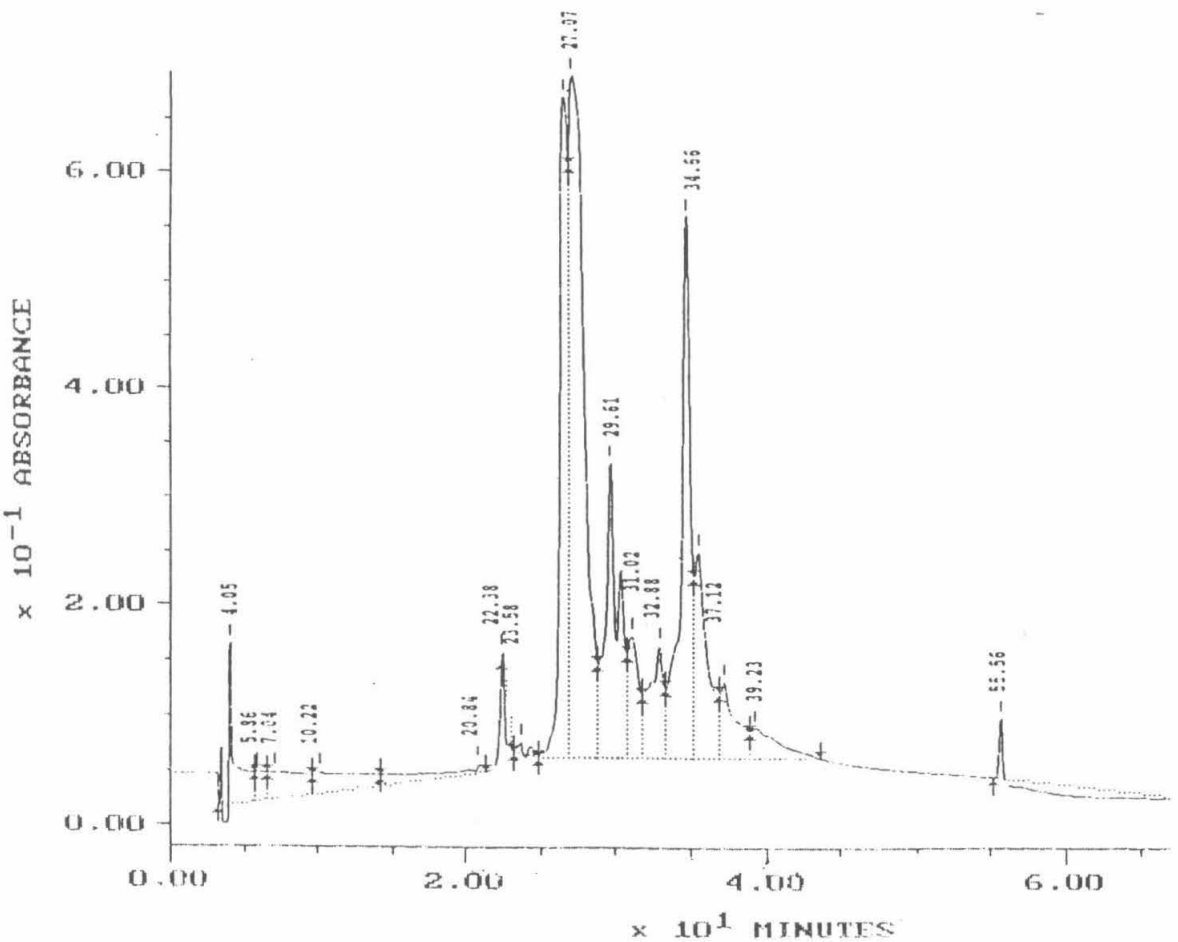

6.00
4.00
2.00
0.00
x 10⁻¹ ABSORBANCE
0.00
2.00
4.00
6.00
x 10¹ MINUTES
4.05
5.86
7.04
10.22
20.84
22.38
23.58
27.07
29.61
31.02
32.88
34.56
37.12
39.23
55.56

Figure 11. Bioactivity analysis of ConA eluate and its reverse phase HPLC fractions.

The ConA eluate was subjected to reverse phase HPLC (see Fig. 10). Fractions were evaporated to dryness, resuspended in PBS, and assayed on cultured sympathetic neurons. Fractions 25-27 contained ChAT-, ENK- and VIP-inducing activity, but not SP-inducing activity. CDF/LIF-like activity was present in fractions 31 and 32. A broad CCK- and VIP-inducing activity is present in fractions 39-43.


ConA E
4
5
21
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
56
Actin
CCK
ChAT
ENK
SOM
SP
VIP

Figure 12. SDS-PAGE analysis of HPLC fractions derived from the ConA eluate.

HPLC fractions from the ConA eluate were combined into three pools according to their neuropeptide-inducing activity. An aliquot of each pool was analyzed by 10% SDS-PAGE and silver staining. The sizes (kDa) of the MW standards are shown on the left side of the panel. Pool 1 (I) contains several major and minor bands. Pool 2 (II) contains one major and several faint bands. Pool 3 (III) has no detectable bands at this level of sensitivity.

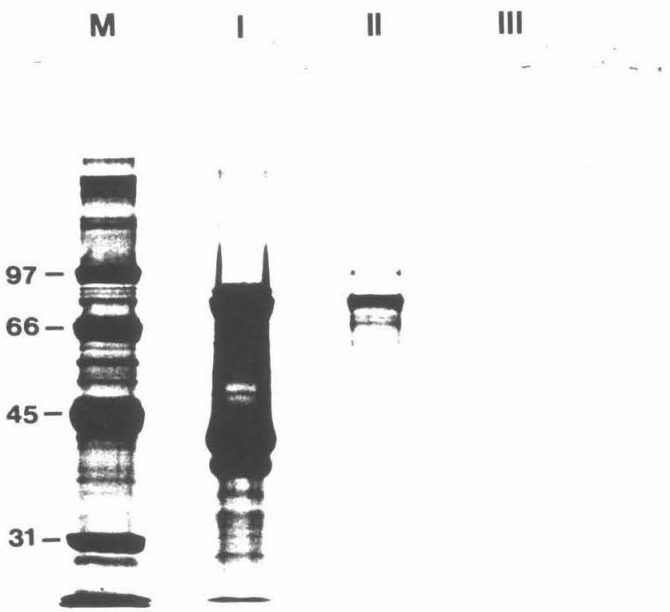

M
I
II
III
97
66
45
31

Figure 13. Flow chart of purification steps.

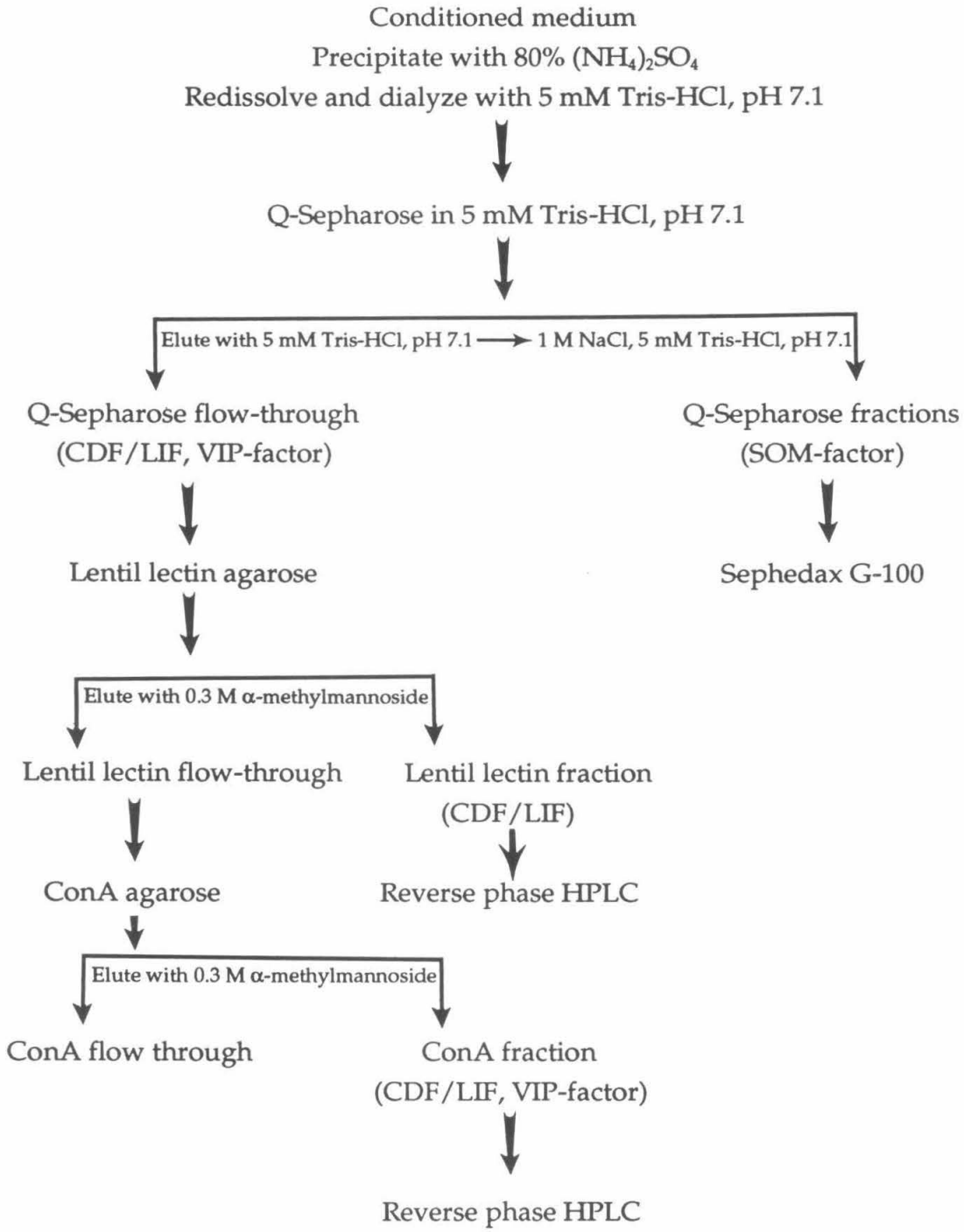

Conditioned medium
Precipitate with 80% $(NH_4)_2SO_4$
Redissolve and dialyze with 5 mM Tris-HCl, pH 7.1
Q-Sepharose in 5 mM Tris-HCl, pH 7.1
Elute with 5 mM Tris-HCl, pH 7.1 → 1 M NaCl, 5 mM Tris-HCl, pH 7.1
Q-Sepharose flow-through
(CDF/LIF, VIP-factor)
Q-Sepharose fractions
(SOM-factor)
Lentil lectin agarose
Sephedax G-100
Elute with 0.3 M α-methylmannoside
Lentil lectin flow-through
Lentil lectin fraction
(CDF/LIF)
ConA agarose
Reverse phase HPLC
Elute with 0.3 M α-methylmannoside
ConA flow through
ConA fraction
(CDF/LIF, VIP-factor)
Reverse phase HPLC

Figure 14. Bioassay of fractions from sequential lentil lectin and ConA columns derived from 20 liters of heart cell CM and from Dr. Fukada's sample.

The purification procedure was as described for Fig. 6. The concentration of LL E2 was 1/20 of LL E1. Cells were not healthy in ConA FT from Dr. FuKada's sample. Both sets of samples show similar activity patterns.

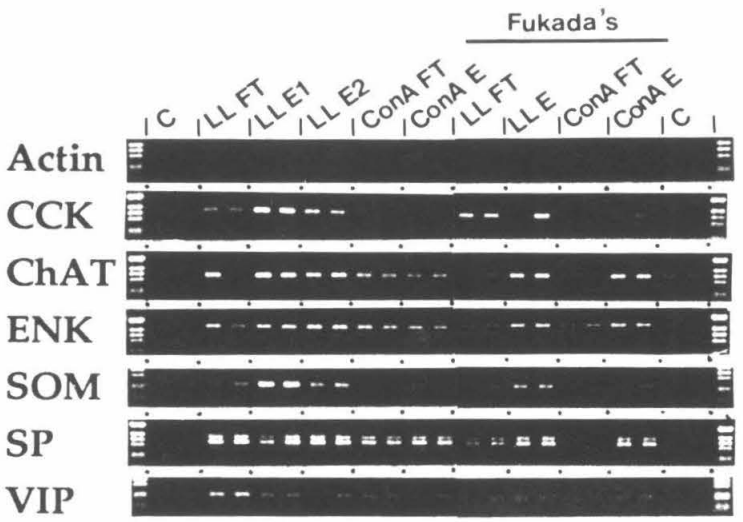

Fukada's
C
LL FT
LL E1
LL E2
ConA FT
ConA E
LL FT
LL E
ConA FT
ConA E
C
Actin
CCK
ChAT
ENK
SOM
SP
VIP

Figure 15. Bioactivity analysis of reverse phase HPLC fractions derived from the second preparation of lentil lectin eluate.

The lentil lectin eluate was separated by reverse phase HPLC without passing through a gel filtration column. HPLC was performed as described for Fig. 10. Fractions 30-37 contains CDF/LIF-like activity. Fractions 22-24 have weak ENK-, SP-, and VIP-inducing activity.

m C 19 20 21 22 23 24 25 26 27 28 29 30 31 32 m 33 34 35 36 37 38 39 40 41 42 43 44 45 E C m
Actin
CCK
ChAT
ENK
SOM
SP
VIP

Figure 16. Bioactivity analysis of reverse phase HPLC fractions derived from the second preparation of ConA eluate.

HPLC was performed as described for Fig. 10. Fractions 30-35 contain CDF/LIF-like activity. Weak inducing activity for ENK, SP, and VIP is detected in fractions 22-24.

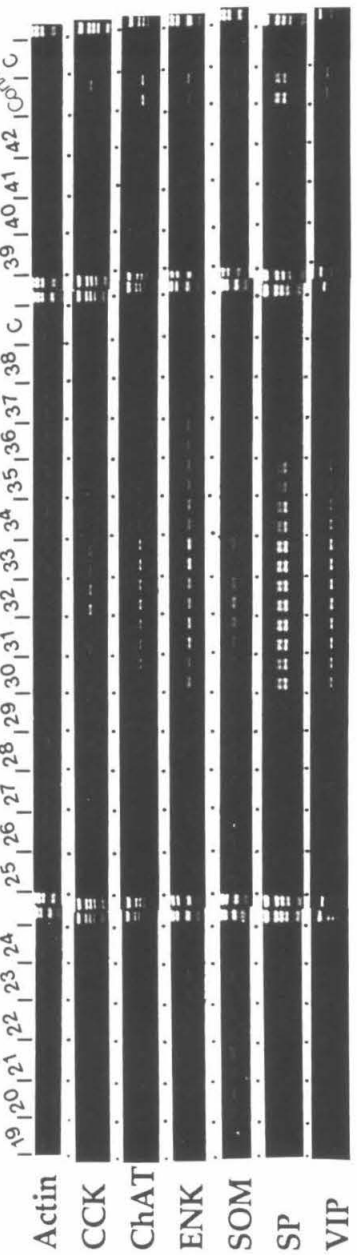
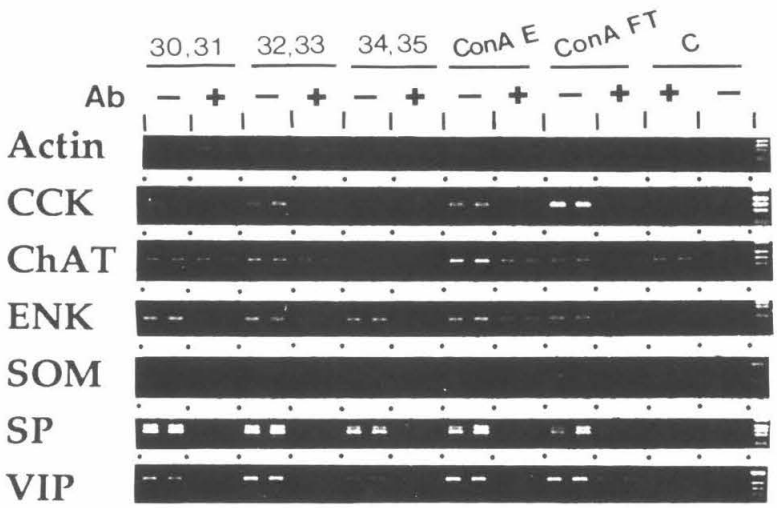

Actin
CCK
ChAT
ENK
SOM
SP
VIP
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 C 39 40 41 42 CDNA C

Figure 17. Blockade of the activity in the ConA eluate, ConA flow-through, and HPLC fractions derived from the ConA eluate by an anti-CDF/LIF antiserum.

Sympathetic neurons were cultured in the presence of fractions, with or without 100 μg/ml anti-CDF/LIF neutralizing antiserum (Ab), for six days. Adjacent HPLC fractions were pooled and analyzed together. The anti-CDF/LIF antiserum blocks all activity in the ConA eluate (ConA E) and its HPLC fractions. Most of the activity in the ConA flow-through (ConA FT) is also blocked by the antiserum. Residual VIP-inducing activity is still present in the antibody-treated sample, however.


30,31
32,33
34,35
ConA E
ConA FT
C
Ab
— + — + — + — + — + + —
Actin
CCK
ChAT
ENK
SOM
SP
VIP

References

Escary, J.-L., Perreau, J., Dumenil, D., Ezine, S., and Brulet, P. (1993). Leukemia inhibitory factor is necessary for maintenance of haematopoietic stem cell and thymocyte stimulation. Nature, **363**, 361-364.

Fann, M.-J. and Patterson, P. H. (1993). A novel approach to sreen for cytokine effects on neuronal gene expression. J. Neurochem., **61**, 1349-1355.

Fukada, K. (1980). Hormonal Control of Neurotransmitter Choice in Sympathetic Neurone Cultures. Nature, **287**, 553-555.

Fukada, K. (1985). Purification and partial characterization of a cholinergic neuronal differentiation factor. Proc. Natl. Acad. Sci. USA, **82**, 8795-8799.

Fukada, K., Rushbrook, J. I., and Towle, M. F. (1991). Immunoaffinity purification and dose-response of cholinergic neuronal differentiation factor. Dev. Brain Res., **62**, 203-214.

Hilton, D. J., Nicola, N. A., Gough, N. M., and Metcalf, D. (1988). Resolution and purification of three distinct factors produced by krebs ascites cells which have differentiation-inducing activity on murine myeloid leukemic cell lines. J. Biol. Chem., **263**, 9238-9243.

Nawa, H. and Patterson, P. H. (1990). Separation and partial characterization of neuropeptide-inducing factors in heart cell conditioned medium. Neuron, **4**, 269-277.

Nawa, H. and Sah, D. W. Y. (1990). Different biological activities in conditioned media control the expression of a variety of neuropeptides in cultured sympathetic neurons. Neuron, **4**, 279-287.

Stewart, C. L., Kasper, P., Brunet, L., Bhatt, H., Gadi, I., Kontgen, F., and Abbondanzo, S. (1992). Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. Nature, **359**, 76-79.

Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M.-J., and Patterson, P. H. (1989). The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. Science, **246**, 1412-1416.

APPENDIX . The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor.

The Cholinergic Neuronal Differentiation Factor from Heart Cells Is Identical to Leukemia Inhibitory Factor

TETSUO YAMAMORI, KEIKO FUKADA, RUEDI AEBERSOLD, SIGRUN KORSCHING, MING-JI FANN, PAUL H. PATTERSON*

A protein secreted by cultured rat heart cells can direct the choice of neurotransmitter phenotype made by cultured rat sympathetic neurons. Structural analysis and biological assays demonstrated that this protein is identical to a protein that regulates the growth and differentiation of embryonic stem cells and myeloid cells, and that stimulates bone remodeling and acute-phase protein synthesis in hepatocytes. This protein has been termed D factor, DIA, DIF, DRF, HSFIII, and LIF. Thus, this cytokine, like IL-6 and TGFβ, regulates growth and differentiation in the embryo and in the adult in many tissues, now including the nervous system.

A GROUP OF PROTEINS, OFTEN CALLED CYTOKINES, REGULATE growth and differentiation in a wide variety of tissues, both in the embryo and in the adult organism. Some of these proteins, such as interleukin-6 (IL-6), were first recognized for their effects on myeloid cells. The generation of the diverse array of myeloid cells is under the control of cytokines and proteins termed

The authors are or were with the Biology Division, California Institute of Technology, Pasadena, CA 91125. Present addresses: R. Aebersold, Biomedical Research Center, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5; K. Fukada, Department of Anatomy and Cell Biology, SUNY Health Science Center, Brooklyn, NY 11203; S. Korsching, Max-Planck-Institut für Entwicklungsbiologie, Abteilung Physikallische Biologie, D-7400 Tubingen 1, Federal Republic of Germany.

*To whom correspondence should be addressed.

hematopoietic regulators. The genes for many of these proteins and their receptors have been cloned, and considerable information is available on their biological activities in vitro, as well as some data on their actions in vivo in mouse and man (*1*). In the nervous system, it is also clear that phenotypic decisions can be controlled by factors in the local environment, both in vitro (*2, 3*) and in situ (*4–6*). It has been difficult to characterize these neuronal differentiation factors because the protein concentrations are very low, and the assays are lengthy and utilize nondividing, primary neurons. Nonetheless, a 45-kD glycoprotein that can control neuronal phenotypic decisions has been purified from rat heart cell conditioned medium (*7, 8*). This protein, usually referred to as the cholinergic neuronal differentiation factor, acts on postmitotic, rat sympathetic neurons to specifically induce the expression of acetylcholine (ACh) synthesis and cholinergic function, while suppressing catecholamine (CA) synthesis and noradrenergic function (*7–10*). The protein can also specifically alter neuropeptide gene expression in these neurons (*11*), and it very likely affects the development of other types of peripheral and central neurons as well (*12*). It is termed a differentiation factor because it controls phenotypic choices in these neurons without affecting their survival or growth (*9*).

Using highly sensitive protein sequencing methods, we have obtained amino acid sequence data for seven peptides from the cholinergic factor. An affinity purified antiserum made against a synthetic peptide corresponding to the NH_2-terminal 11 amino acids is able to specifically precipitate the differentiation activity, demonstrating that the sequenced protein is, in fact, the active factor. The amino acid sequences of the seven peptides are nearly identical to the corresponding peptides of the hematopoietic factor that can inhibit the proliferation and induce macrophage differentiation of the leukemic myeloid M1 cell line [leukemia inhibitory factor, LIF (*13*), which is also known as D factor and human macrophage differentiation inducing factor, DIF (*14*)], maintain the developmental potential of embryo carcinoma cells and embryonic stem cells [differentiation inhibitory activity, DIA, and differentiation retarding factor, DRF (*15*)], and support the proliferation of the leukemic DA-1a cell line (HILDA) (*16*), although the latter identification is in doubt (*17*). We used our amino acid sequence data and the available complementary DNA (cDNA) sequence information to clone a cDNA for the rat protein. Comparison of the sequences of the rat cholinergic factor with mouse and human LIF confirms their identity. In biological assays, recombinant murine LIF (recLIF) duplicates the cholinergic differentiation activity.

Protein sequencing of the cholinergic factor and production of antisera. In order to sequence the NH_2-terminal of the cholinergic factor, we collected 40 liters of serum-free conditioned medium from secondary, rat heart cell cultures, and purified the protein (*7*). An estimated 0.5 to 0.8 μg of the 45-kD protein was electroblotted onto an activated glass fiber filter, and sequenced on a Caltech gas phase sequenator at an initial signal of 18 pmol (*18*). Three separate samples yielded an NH_2-terminal, 14–amino acid sequence of EPLPITPVSATXAI (*19*). To minimize the possibility that the 45-kD band containing the cholinergic activity was contaminated by another protein, we isolated the band by SDS–polyacrylamide gel electrophoresis (PAGE); the material in the band was eluted, chemically deglycosylated with anhydrous hydrogen fluoride, purified further by SDS-PAGE, and sequenced (*20*). The chemically deglycosylated protein was approximately the same size, 20 kD, as that obtained after enzymatic deglycosylation with endoglycosidases (*7*). The sequence obtained for the 20-kD band was EPLPITPV, identical to that of the intact protein.

In order to be certain that the sequenced protein was, in fact, the cholinergic factor, we obtained antisera to an 11–amino acid peptide corresponding to the NH_2-terminal sequence (*21*). These antisera (and affinity purified antibodies) could precipitate both the 45-kD protein (Fig. 1) and the cholinergic activity (Table 1) (*22*). Prior incubation of the antisera with the synthetic peptide prevented this precipitation, indicating that the protein of interest had been sequenced. The antisera to the NH_2-terminus recognized the native protein, suggesting that this portion of the protein is exposed on its surface. The precipitates in Fig. 1 also reveal other, weaker bands near the 45-kD protein, and addition of the synthetic peptide abolished the precipitation of all of these bands. Since the 45-kD protein has several glycosyl chains (*7*), the additional bands in the precipitated fractions may be glycosylation variants of this protein or the result of partial proteolytic degradation.

The extremely high degree of degeneracy in the codons for the NH_2-terminal sequence made cloning by oligonucleotide screening difficult; we therefore purified a large amount of the cholinergic factor from 35 liters of conditioned medium (*23*) and determined the sequences of a number of internal peptides (*24*). The following sequences were obtained: LGQGEPFPNNVDKL; SQLAQLPVV; LGASL; LVE; EAFE; LIAT; and EPLPITPV. The fact that one of the peptides corresponded to the same NH_2-terminal sequence already obtained showed that the tryptic peptides came from the same protein. A search of the computer database revealed a close match of each of these peptides with a corresponding peptide in the

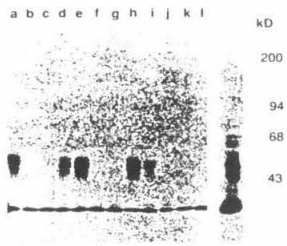


Fig. 1. Precipitation of the 45-kD protein by antisera to a synthetic peptide corresponding to the NH_2-terminal sequence of the protein. Rabbit antisera to the peptide coupled to the carrier proteins KLH (lanes a to d), ovalbumin (lanes e to h), or not coupled to any carrier (lanes i and j). The partially purified factor (Sephadex fraction) was labeled with ^{125}I, and the labeled proteins are shown in the lane next to the molecular sizes. The ^{125}I-labeled proteins were incubated (*21*) with antisera that had been incubated with the following blockers: free peptide (lanes b, f, and j), peptide conjugated to the appropriate carrier proteins (lanes c and g), KLH (lane d), ovalbumin (lane h), or was incubated with antiserum to ovalbumin (lane l) or without any antiserum (lane k). The immune complexes were adsorbed by protein A-Sepharose, washed, eluted, and analyzed by SDS-PAGE. A similar experiment, with affinity-purified antibodies, yielded similar results.

Table 1. Precipitation of cholinergic activity by antibodies to peptide. Immunoprecipitation experiments were performed as in Fig. 1 (*21*), with antibodies purified by peptide affinity-column chromatography. When antibodies were incubated with free peptide, the sample was extensively dialyzed after the incubation to remove free peptide. The immune complexes were centrifuged and proteins were eluted from the pellets by acid treatment. Antibodies could also be eluted by this treatment. However, addition of excess peptide to the eluate before neutralization to block rebinding of the eluted antibodies to the eluted factors resulted in no change in cholingeric activity. Both the eluates from the immune pellets and the immune supernatants (Sup) were added to sympathetic neuronal cultures from days 2 to 16, at 2-day intervals. The ratio of transmitters produced (acetylcholine to catecholamine; ACh/CA) (*7*) is a quantitative measure of the two transmitter systems and reflects, in part, the levels of choline acetyltransferase and tyrosine hydroxylase, respectively (*9*).

Addition to Sephadex fraction	ACh/CA	
	Sup	Eluate
Antibodies to peptide-KLH	0.20	2.40
Antibodies to peptide-KLH plus free peptide	2.13	0.05
Antibodies to KLH	2.64	0.24
No antibodies added	4.46	

murine and human LIF-DIF-D-factor-DIA-DRF protein (*13*), which we refer to as LIF.

Cloning the cDNA for the cholinergic factor and its identity with LIF. To determine the correct sequence for rat cholinergic factor or LIF, we used the polymerase chain reaction (PCR), with

```
rlif ATGAAGGTCTTGGCCCGCAGGGATTGTGCCCCTACTGCTC---ATTCTGCAC 48
mlif ATGAAGGTCTTGGCCCGCAGGGATTGTCCCCT       GCTGG
RLIF                                LeuLeuLeu---IleLeuHis -7
MLIF MetLysValLeuAlaAlaGlyIleValPro          LeuVal
HLIF                        Val              ---Val

                          ++1
rlif TGGAAACACGGGGCAGGGAGCCCCCTTCCCATCACCCCTGTAAATGCCACC 99
mlif                         T
RLIF TrpLysHisGlyAlaGlySerProLeuProIleThrProValAsnAlaThr 11
MLIF
HLIF

rlif TGCCCCATACGCCACCCGTGTCACGGCAACCTCATGAACCAGATCAAGAGT 150
mlif  T              A  C                                A
RLIF CysAlaIleArgHisProCysHisGlyAsnLeuMetAsnGlnIleLysSer 28
MLIF                                                    Asn
HLIF                            Asn                   ArgSer

rlif CAACTGGCTCAACTCAACGGCAGTGCCAATGCCCTCTTTATTTCCTATTAC 201
mlif       A  G     T    C       T    C
RLIF GlnLeuAlaGlnLeuAsnGlySerAlaAsnAlaLeuPheIleSerTyrTyr 45
MLIF
HLIF                                              Leu

rlif ACAGCTCAAGCGGAACCATTTCCCAACAACGTGGATAAGCTATGTGCGCCA 252
mlif           A  G  G                 A                T
RLIF ThrAlaGlnGlyGluProPheProAsnAsnValAspLysLeuCysAlaPro 62
MLIF                                   Glu
HLIF                                Leu             Gly

rlif AACATGACGGATTTCCCACCTTTCCATGCCAATGGGACAGAGAAGACCAAG 303
mlif         A  C     T         G  C
RLIF AsnMetThrAspPheProProPheHisAlaAsnGlyThrGluLysThrLys 79
MLIF                    Ser      Gly
HLIF    Val                                       Ala

rlif TTGGTCGAGCTGTATCGGATGGTCGCGTACCTGGGAGCCTCCCTGACCAAC 354
mlif      G                    A     A C                T
RLIF LeuValGluLeuTyrArgMetValAlaTyrLeuGlyAlaSerLeuThrAsn 96
MLIF                                   Ser
HLIF                      Ile   Val          Thr      Gly

rlif ATCACCTGGGATCAGAAAAACCTCAACCCCACTGCCGTGAGCCTCCAGATC 405
mlif       C    C     GGT   G                          G
RLIF IleThrTrpAspGlnLysAsnLeuAsnProThrAlaValSerLeuGlnIle 113
MLIF       Arg          Val                              Val
HLIF       Arg          Ile          Ser   Leu       HisSer

rlif AAACTCAATGCGACTACAGACGTCATGAGGGGGCTCCTTAGCAACGTGCTT 456
mlif  G        T    T                 C    C     T
RLIF LysLeuAsnAlaThrThrAspValMetArgGlyLeuLeuSerAsnValLeu 130
MLIF               Ile
HLIF               Ala   IleLeu

rlif TGCCGTCTGTGCAACAAGTACCATGTGGGCCATGTGGATGTGCCCTGTGTC 507
mlif                      G         C              ACC
RLIF CysArgLeuCysAsnLysTyrHisValGlyHisValAspValProCysVal 147
MLIF                      Arg                      Pro
HLIF             Ser                            ThrTyrGly

rlif CCCGACAACTCTAGCAAAGAAGCCTTCCAAAGGAAGAAGTTGGGCTGCCAG 558
mlif       C     GA                    A          T
RLIF ProAspAsnSerSerLysGluAlaPheGlnArgLysLysLeuGlyCysGln 164
MLIF       His   Asp
HLIF       Thr   Gly   AspVal      Lys

rlif CTCCTGGGGACATACAAGCAAGTCATAAGTGTGTTGGCCCAGGCCTTCTAG 609
mlif  T                GTCATAAGTGTGGTGGTCCAGGCCTTCTAG
RLIF LeuLeuGlyThrTyrLysGln                          180
MLIF                     ValIleSerValValValGlnAlaPheTER
HLIF          Lys        Ile   Ala   LeuAla        TER
```

1414

oligonucleotides corresponding to the signal peptide and COOH-terminal sequences of murine and human LIF (*25*). The rat, murine, and human amino acid sequences are aligned in Fig. 2, and the corresponding peptides that were sequenced are underlined. Although the COOH-terminus of the rat sequence is not yet complete, there is a strong homology with both the murine (92 percent identity) and the human (82 percent identity) LIF amino acid sequences. It is striking that there are highly nonconservative differences between all three species at residues 103, 113, 146, 150, and 152, and a cysteine residue (at 146) is present only in the rat sequence. The differences between the deduced rat amino acid sequence (Fig. 2) and sequences derived from the tryptic peptides (given above) are found primarily in the NH_2-terminal ends of the peptides and at putative *N*-glycosylation sites. These are sites known to present difficulties for accurate amino acid sequencing.

In order to confirm that the cloned product of the murine LIF (*13*) does indeed have cholinergic neuronal differentiation activity, we obtained an authentic sample of this material (*26*). Purified murine recLIF, expressed in *Escherichia coli* (*27*), was added to cultures of neonatal rat sympathetic neurons, and its effects on the choice of transmitter phenotype were assayed; LIF was able to induce ACh synthesis as well as suppress CA synthesis at low concentrations (Table 2). These are the activities observed for the cholinergic differentiation factor purified from heart cell conditioned medium (*7*).

We have presented several lines of evidence to demonstrate that the proteins previously called LIF and the cholinergic neuronal differentiation factor are the same molecule. The purified neuronal factor and recLIF have the same biological activity when tested with cultured neurons. The slight differences between the amino acid sequences obtained from the tryptic peptides and the deduced sequence for rat LIF can be attributed to limitations of protein sequencing methods. In addition, Gearing *et al.* (*13*) detected only one gene on Southern blots of murine genomic DNA digested with nine restriction endonucleases and probed with a LIF cDNA at both high and low stringencies.

The various names that have been used for this protein may deserve reconsideration. For example, the previous term used with neurons, cholinergic differentiation factor, is inappropriate for two reasons. First, recent results with pure factor and recLIF on cultured sympathetic neurons show that the protein specifically induces the expression of several neuropeptides in addition to the biosynthetic enzyme for acetylcholine (*11*). Second, several reports on the effects of the protein on myeloid cell lines and embryonic stem cells have led to the introduction of at least five different names. Furthermore, the protein has been implicated in bone remodeling and in the induction of acute phase plasma protein synthesis in liver cells (*14, 17, 28*).

Although the role of the cholinergic factor in the normal development of the nervous system is not yet known, the fact that

Fig. 2. The nucleotide sequence of the coding region of rat LIF (*rlif*) and its deduced amino acid sequence (RLIF) are compared to those of mouse (*mlif*, MLIF) and human (HLIF), which have been described earlier (*13*). The suggested NH_2-terminal residue is designated as +1 with an arrow. MLIF has an additional amino acid in the signal sequence compared to the rat and human sequences; this difference is indicated by the dashed lines. Serine is suggested to be the NH_2-terminal residue because our NH_2-terminal amino acid sequencing data yielded one residue before proline, and Gly-Ser is a likely signal cleavage site. The regions of the deduced protein that correspond to the peptides obtained in our amino acid sequencing experiments are underlined. The first and last 30 nucleotides correspond to the primers used for the PCR reactions and were taken from the mouse or human sequences.

Table 2. Cholinergic differentiation activity of LIF. Sympathetic neurons were grown for 2 weeks in the presence of 100 percent volume equivalents of heart cell conditioned medium, recombinant (rec) murine LIF, or no additions other than the usual medium constituents. The concentration of cholinergic factor protein in 100 percent heart cell conditioned medium is estimated to be between 10 and 100 ng/ml. Transmitter synthesis from radioactive precursors was assayed as described (7); the data are expressed as means ± SEM, with $n = 4$. The ACh/CA values are the means of the ratios from individual cultures. ACh, acetylcholine; CA, catecholamine.

Material tested	Activity per neuron (fmol/hour)		
	ACh	CA	ACh/CA
Control	0.04 ± 0.01	2.70 ± 0.41	0.02 ± 0.01
recLIF (1 ng/ml)	0.20 ± 0.04	1.10 ± 0.23	0.29 ± 0.13
recLIF (10 ng/ml)	1.30 ± 0.02	0.50 ± 0.08	2.70 ± 0.45
Heart cell conditioned medium	3.20 ± 1.25	0.52 ± 0.16	6.60 ± 0.35

sympathetic neurons [and possibly sensory and spinal cord neurons as well (*12*)] show a striking and specific response to it suggests that they have receptors for the protein as well as a compatible second messenger system. It is important to ascertain where these receptors are localized during neuronal development. Although there is good evidence for the localized production of a phenotype-specifying factor by a target tissue in situ (*6*), all types of neurons that innervate a given target do not respond similarly to that environment. Thus, the known diversity of neuronal phenotypes could be due to different combinations of instructive cues present at various locations and to differential responsiveness to a given mixture of cues at any one site. The availability of the appropriate genes, proteins, and antisera should lead to clarification of the similarities and differences among the various factors that have been reported to influence neuronal phenotype. This includes not only the cholinergic factors (*29*), but other neuronal differentiation activities as well (*30*), because the cholinergic factor itself can turn on (and off) the expression of several different neurotransmitters and neuropeptides (*11*).

If the same molecule influences the differentiation of myeloid, neural, and other cells, how then is the specificity of its control on each of these different systems maintained? Side effects, if any, could be averted if the distribution of the factor is highly localized, or if the various systems are responsive during different developmental periods. There are other examples of proteins that are potentially important for the development of both the immune and nervous systems. Interleukin-1 (IL-1) and possibly IL-3, and receptors for IL-1, -2, and -4 are found in the brain and in neural cell lines (*31, 32*). IL-1 stimulates glial proliferation, somatostatin, and nerve growth factor (NGF) production, and it has transmitter-like effects on neurons (*33*). IL-2 stimulates oligodendroglial proliferation and maturation as well as corticotropin release from pituitary cells (*32*). Gamma-interferon can enhance astrocyte maturation and indirectly increase the cholinergic differentiation of cultured spinal cord neurons, and rat interferon enhances the expression of acetylcholine receptors in cultured rat myotubes (*34*). B cell stimulatory factor 2 (or IL-6) is detected in glial cell lines and can induce neurite extension and voltage-dependent sodium channels in PC12 cells (*35*). Conditioned medium from activated lymphocytes can maintain sympathetic neurons in culture, and brain macrophages can release NGF in vitro (*36*). NGF can promote hemopoietic colony growth and differentiation, and injection of NGF into neonatal rats increases the number of mast cells (*37*). In addition, subsets of thymocytes and lymphocytes express NGF receptors (*37*). In fact, there are many parallels in the questions and phenomenology of lineage decisions in the neural crest and hematopoietic systems (*38*). Our data add a new dimension to this overlap; very different lineage choices may be controlled by the same molecules.

Note added in proof: We have determined the 3′ sequence of the rat cDNA, and the final 30 bases are GTCATTAGTGCGGTGGTCCAGGCCTTCTAG.

REFERENCES AND NOTES

1. S. C. Clark and R. Kamen, *Science* **236**, 1229 (1987); J. E. Groopman, *Cell* **50**, 5, (1987); C. M. Heyworth, I. L. O. Ponting, T. M. Dexter, *J. Cell Sci.* **91**, 239 (1988); D. Metcalf, *Proc. R. Soc. London Ser. B* **230**, 389 (1987); *ibid.*, *Nature* **339**, 27 (1989).
2. P. H. Patterson, *Annu. Rev. Neurosci.* **1**, 1 (1978).
3. M. C. Raff, *Science* **243**, 1450 (1989).
4. A. Tomlinson, *Development* **104**, 183 (1988).
5. N. M. LeDouarin and J. Smith, *Annu. Rev. Cell Biol.* **4**, 375 (1988).
6. R. J. Shotzinger and S. C. Landis, *Nature* **335**, 637 (1988).
7. K. Fukada, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8795 (1985).
8. A very similar protein (to the cholinergic factor) has been partially characterized from skeletal muscle conditioned medium [M. J. Weber, *J. Biol. Chem.* **256**, 3447 (1981); M. J. Weber, B. Raynaud, C. Delteil, *J. Neurochem.* **45**, 1541 (1985)].
9. P. H. Patterson and L. L. Y. Chun, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3607 (1974); P. H. Patterson, L. F. Reichardt, L. L. Y. Chun, *Cold Spring Harbor Symp. Quant. Biol.* **40**, 389 (1975); P. H. Patterson and L. L. Y. Chun, *Dev. Biol.* **56**, 263 (1977).
10. D. D. Potter, S. C. Landis, S. G. Matsumoto, E. J. Furshpan, *J. Neurosci.* **6**, 1080 (1986).
11. H. Nawa and P. H. Patterson, *Neuron*, in press.
12. M.-C. Giess and M. J. Weber, *J. Neurosci.* **4**, 1442 (1984); C. Mathieu, A. Moisand, M. J. Weber, *Neuroscience* **13**, 1373 (1984).
13. D. P. Gearing *et al.*, *EMBO J.* **6**, 3995 (1987); N. M. Gough *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2623 (1988); D. P. Gearing *et al.*, *Nucleic Acids Res.* **16**, 9857 (1988).
14. T. Abe *et al.*, *J. Biol. Chem.* **264**, 8941 (1989); D. G. Lowe *et al.*, *DNA* **8**, 351 (1989).
15. P. Koopman and R. G. H. Cotton, *Exp. Cell Res.* **154**, 233 (1984); A. G. Smith and M. L. Hooper, *Dev. Biol.* **121**, 1 (1987); R. L. Williams *et al.*, *Nature* **336**, 684 (1988); A. G. Smith *et al.*, *ibid.*, p. 688.
16. J.-F. Moreau *et al.*, *Nature* **336**, 690 (1988).
17. N. M. Gough and R. L. Williams, *Cold Spring Harbor Rev.*, in press.
18. A small fraction of the factor preparation was labeled with ^{125}I and added to the unlabeled protein as a tracer. For the final step in the purification, the sample was run on a 12.5 percent SDS-polyacrylamide gel (0.7 mm thick) and electroblotted onto an activated glass fiber filter. Whatman GF/F glass fiber filters were covalently derivatized with trimethoxysilylpropyl-*N*,*N*,*N*-trimethylammonium chloride after etching the glass with trifluoracetic acid as described (R. H. Aebersold, D. B. Teplow, L. E. Hood, S. B. H. Kent, *J. Biol. Chem.* **261**, 4229 (1986)). Electroblotting was carried out in 25 m*M* tris, 192 m*M* glycine, 0.5 m*M* dithiothreitol (DDT), *p*H 8.3 at 50 V for 2 hours at 10°C. The filter was air dried at room temperature and the proteins were visualized by autoradiography. Alternatively, the blot was stained with the fluorescent dye, 3,3′-dipentyloxacarbocyanine iodide, and bands were visualized by illumination at 254 nm. The region of the filter at 45 kD was cut out with a razor blade and inserted into the cartridge of a sequenator. Phenylthiohydantoin (PTH) derivatives from each cleavage were then analyzed [M. W. Hunkapiller and L. E. Hood [*Methods Enzymol.* **91**, 486 (1983)] on an IBM cyano column, except that 5 to 7 percent (v/v) tetrahydrofuran was added to buffer A and the *p*H was adjusted to 5.1.
19. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met;, N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and X, undetermined.
20. For chemical deglycosylation, the gel-eluted protein was extensively dried; redistilled HF (100 μl) was added and the reaction was allowed to proceed on ice for 30 minutes. The HF was evaporated under a stream of nitrogen, residual HF was neutralized with 100 m*M* $(NH_4)HCO_3$, and the sample was dried at reduced pressure. The protein was dissolved in SDS-PAGE sample buffer for the final electrophoresis.
21. The peptide was synthesized and coupled to the carrier proteins keyhole limpet hemocyanin (KLH) and ovalbumin by standard methods. The free peptide and the peptide conjugates were diluted to 1 mg/ml in PBS (phosphate-buffered saline), *p*H 7.5, and mixed with an equal volume of complete Freund's adjuvant. Each peptide (1 mg) was injected into each of three New Zealand White rabbits (6 to 10 weeks old), subcutaneously. After 1 month, the animals were injected with 0.5 mg of antigen in incomplete Freund's adjuvant, and bled 10 days after each injection. Antibody titers were determined by a solid phase radioimmunoassay, with ^{125}I-labeled protein A for detection of bound antibody. The antibodies to the peptides were purified on a peptide affinity column. Long-chain alkylamine-controlled pore glass (Sigma) (1 g) was activated with 2 mg of maleimidobenzoyl *N*-hydroxysuccinimide ester in dimethylformamide and coupled with 3 mg of reduced peptide in 100 m*M* sodium phosphate (*p*H 7.0) at 30°C for 1 hour. Unreacted groups were blocked by excess DTT, and the beads washed and equilibrated with PBS. The antiserum or its protein A-Sepharose purified immunoglobulins were incubated in batches with the column material for 3 to 15 hours and washed twice with 20 ml of PBS, *p*H 7, then with 9 ml of PBS containing 0.5 percent Tween and 1*M* NaCl, followed by 100 ml of PBS. Specifically bound antibodies were eluted with 18 ml of 0.1*M* glycine-HCl, *p*H 2.5. The eluate was promptly neutralized with 1*M* tris-HCl, *p*H 8, and concentrated by Centricon 30 (Amicon). The antibodies were dialyzed against PBS, frozen, and stored. The ratio of antibodies to the peptide to

antibodies to the carrier was >10:1, on the basis of binding curves with the peptide and the carrier proteins as antigens.
22. Partially purified cholinergic factor was iodinated with the Bolton-Hunter reagent (7) and used as a tracer. The Sephadex fraction, containing 2×10^5 cpm of tracer, was first incubated with preimmune serum (overnight at 4°C) in the presence of protease inhibitors [1 m*M* phenylmethylsulfonyl fluoride (PMSF), and Trasylol, leupeptin, and turkey egg white trypsin inhibitor at 2 μg/ml each]. The complexes that bound to preimmune serum were absorbed by protein A-Sepharose in PBS, 0.02 percent azide, 0.01 percent polyethylene glycol (PEG) for 2 hours, and were removed by centrifugation. The supernatant was then incubated overnight at 4°C with antiserum to the peptides (which had been incubated overnight at 4°C with or without blockers) in the presence of protease inhibitors and reprecipitated. The protein A-Sepharose bearing immune complexes were washed once with PBS, 0.02 percent azide, 0.01 percent PEG, four times with high salt buffer (0.1*M* tris-HCl, *p*H 7.8, 0.5*M* NaCl, 0.02 percent azide, and 0.01 percent PEG), and then with PBS, azide, and PEG. Immune complexes were eluted by 0.1*M* glycine-HCl, *p*H 2.5, 0.01 percent PEG and neutralized promptly with 1*M* tris-HCl, *p*H 8. These results were reported in an abstract [K. Fukada, *Soc. Neurosci. Abstr.* **12**, 106.5 (1986)].
23. The cholinergic factor was purified as described (7), except that the precipitation with 60 percent ammonium sulfate was omitted. The purification was monitored by precipitation of the iodinated factor (which had been added to the preparation) with the antiserum to the factor. This procedure works well only for the CM-cellulose and Sephadex fractions.
24. The preparation and sequencing of internal peptides followed the protocol of Aebersold *et al.* [R. H. Aebersold, J. Leavitt, R. A. Saavedra, L. E. Hood, S. B. H. Kent, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6970 (1987)]. Partially purified cholinergic factor (2 to 3 μg) was separated from contaminating protein by SDS-PAGE. The proteins were transferred to nitrocellulose, and the band corresponding to the cholinergic factor was digested with trypsin on the nitrocellulose. The resulting peptides were released into the supernatant and separated by high-performance liquid chromatography (HPLC). The most prominent peptides were then sequenced.
25. Four oligomers were used to obtain the PCR fragment of rat LIF: (i) a 21-nucleotide (nt) oligomer (ATGAAGGTCTTGGCCGCAGGG) starting from the initiation codon of murine LIF; (ii) 20 nt (CTAGAAGGCCTGGACCACACA) starting from the antisense sequence of the stop condon of the murine and human factor; (iii) 30 nt (ATGAAGGTCTTGGCCGCAGGGATTGTGCCC) corresponding to the 5′ end of the signal peptide of murine LIF; (iv) 30 nt (CTAGAAGGCCTGGACCACACACTTATGAC) corresponding to the COOH-terminus of the protein. Complementary DNA was synthesized from polyadenylated cultured heart cell RNA with a MuLV (murine leukemia virus) reverse transcriptase kit (BRL) and oligomer (ii) as the primer. During the first 30 cycles of the PCR reaction [94°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes; R. K. Saiki *et al.*, *Science* **239**, 487 (1988)] the oligomers of (i) and (ii) were the primers. The first PCR product of approximately 600 bp was purified and used as the template of the second PCR reaction with oligomers of (iii) and (iv). The second PCR product contained a major band of the expected size (609 bp) in agarose gel electrophoresis. The fragment was subcloned to the vector of PGEM-3zf(−) (Promega), and analyzed with the Sequenase (U.S. Biochemical) system. Both strands were sequenced not only with the T7 and SP6 primers, but also with oligomers (iii) and (iv) and three internal sequences (TTTTTCTGATCCCAGGTGAT, TTCCCCTTGAGCTGTGTAATAGGAA, GAGAAGACCAAGTTGGTCGAGCTGTA).
26. The authentic sample was obtained from D. Metcalf (see *13*).
27. D. P. Gearing *et al.*, *Biotechnology*, in press.
28. H. Baumann and G. G. Wong, *J. Immunol.* **143**, 1163 (1989); D. Metcalf and D. P. Gearing, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5948 (1989).
29. N. Brookes, D. R. Burt, A. M. Goldberg, G. G. Bierkamper, *Brain Res.* **186**, 474 (1980); E. W. Godfrey, B. K. Schrier, P. G. Nelson, *Dev. Biol.* **77**, 403 (1980); E. Hawrot, *ibid.* **74**, 136 (1980); R. Nishi and D. K. Berg, *J. Neurosci.* **1**, 505 (1981); M. Manthorpe, W. Luvten, F. M. Longo, S. Varon, *Brain Res.* **267**, 57 (1983); G. Barbin, M. Manthorpe, S. Varon, *J. Neurochem.* **43**, 1468 (1984); C. E. Henderson, M. Huchet, J. P. Changeux, *Dev. Biol.* **104**, 336 (1984); J. E. Adler and I. B. Black, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4296 (1985); E. J. Wolinsky and P. H. Patterson, *J. Neurosci.* **5**, 1509 (1985); L. M. Kaufman, S. R. Barry, J. N. Barrett, *ibid.*, p. 160; R. G. Smith, K. Vaca, J. McManaman, S. H. Appel, *ibid.* **6**, 439 (1986); V. Wong and J. A. Kessler, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8726 (1987); A. Acheson and U. Rutishauser, *J. Cell Biol.* **106**, 479 (1988); I. A. Hendry, C. E. Hill, D. Belford, D. J. Watters, *Brain Res.* **475**, 160 (1988); J. L. McManaman, F. G. Crawford, S. S. Stewart, S. H. Appel, *J. Biol. Chem.* **263**, 5890 (1988); J-C. Martinou, F. Bierer, A. L. V. Thai, M. J. Weber, *Dev. Brain Res.* **47**, 251 (1989); S. Saadat, M. Sendtner, H. Rohrer, *J. Cell Biol.* **108**, 1807 (1989).
30. J. A. Kessler, J. E. Adler, W. O. Bell, I. B. Black, *Neuroscience* **9**, 309 (1983); J. A. Kessler, *ibid.* **15**, 827 (1985); D. W. Y. Sah and S. G. Matsumoto, *J. Neurosci.* **7**, 391 (1987); J. P. Davis, M. L. Epstein, *Dev. Biol.* **124**, 512 (1987).
31. K. S. Frei, S. Bodmer, C. Schwerdel, A. Fontana, *J. Immunol.* **135**, 4044 (1985); *ibid.* **137**, 3521 (1986); W. L. Farrar, M. Vinocour, J. M. Hill, *Blood* **73**, 137 (1989); W. L. Farrar, P. L. Kilian, M. R. Ruff, J. M. Hill, C. B. Pert, *J. Immunol.* **139**, 459 (1987); J. W. Lowenthal *et al.*, *J. Immunol.* **140**, 456 (1988); C. D. Breder, C. A. Dinarello, C. B. Saper, *Science* **240**, 321 (1988).
32. E. N. Benveniste and J. E. Merrill, *Nature* **321**, 610 (1986); L. R. Smith, S. L. Brown, J. E. Blalock, *J. Neuroimmunol.* **21**, 249 (1989).
33. A. Fontana, R. Dubs, R. Merchant, S. Balsinger, P. J. Grob, *J. Neuroimmunol.* **2**, 71 (1982); A. Fontana, U. Otz, A. L. deWeck, P. J. Grob, *ibid.*, p. 81; D. Lindholm, R. Heumann, M. Meyer, H. Thoenen, *Nature* **330**, 658 (1987); D. Giulian, K. Vaca, B. Johnson, *J. Neurosci. Res.* **21**, 487 (1988); D. Giulian, D. G. Young, J. Woodward, D. C. Brown, L. B. Lachman, *J. Neurosci.* **8**, 709 (1988); J. E. Merrill and K. Matsushima, *J. Biol. Reg. Homeost. Agents* **2**, 77 (1988); D. E. Scarborough, S. L. Lee, C. A. Dinarello, S. Reichlin, *Endocrinology* **124**, 549 (1989); T. Hori *et al.*, *Brain Res. Bull.* **20**, 75 (1988).
34. L. Erkman, L. Wuarin, D. Cadelli, A. C. Kato, *Dev. Biol.* **132**, 375 (1989); P. Andre *et al.*, *J. Neurosci. Res.* **19**, 297 (1988).
35. K. Yasukawa *et al.*, *EMBO J.* **6**, 2939 (1987); T. Satoh *et al.*, *Mol. Cell. Biol.* **8**, 3546 (1988).
36. Y. Gozes, M. A. Moskowitz, T. B. Strom, I. Gozes, *Dev. Brain Res.* **6**, 97 (1983); M. Millat, R. Houlgarte, P. Brachet, A. Prochiantz, *Dev. Biol.* **133**, 309 (1989).
37. L. Aloe and R. Levi-Montalcini, *Brain Res.* **133**, 358 (1977); A. Cattaneo, in *Molecular Aspects of Neurobiology*, R. Levi-Monatalcini, Ed. (Springer-Verlag, Berlin, 1986), pp. 31–36; H. Matsuda, M. D. Coughlin, J. Bienenstock, J. A. Denburg, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6508 (1988); B. Morgan, L. W. Thorpe, D. Marchetti, J. R. Perez-Polo, *J. Neurosci. Res.* **23**, 41 (1989); I. Stamenkovic, E. A. Clark, B. Seed, *EMBO J.* **8**, 1403 (1989). There is also the still unresolved issue of the role of "neuroleukin" in neural development [M. Chaput *et al.*, *Nature* **332**, 454 (1988); P. Faik, J. I. H. Walker, A. A. M. Redmill, M. J. Morgan, *ibid.*, p. 455; M. E. Gurney, *ibid.*, p. 456].
38. D. J. Anderson, *Neuron* **3**, 1 (1989).
39. We thank D. Metcalf and colleagues at the Walter and Eliza Hall Institute for Medical Research in Melbourne for the recLIF; M. Tanouye and M. Ramaswami for help with the PCR DNA amplification; T. Hunkapiller and D. Jackson for help with the computer searches; D. Anderson, H. Nawa, and M. Rao for advice, D. McDowell for help with the preparation of tissue culture materials; J. Carnahan for NGF, J. Frost, M. Ryder, and R. Pittman for conditioned medium, S. Horvath and colleagues for oligonucleotide and peptide synthesis, B. Jentoft-Nilsen and M. Aranda for help with nucleic acid sequencing, and S. Ou for help with antibodies. Supported by NINCDS (Javits Neuroscience Investigator Award), a McKnight Foundation Neuroscience Research Project Award, a Lucille P. Markey Charitable Trust Developmental Biology grant, the Gustavus and Louise Pfeiffer Research Foundation, and the Joseph Drown Foundation (P.H.P.); by the Muscular Dystrophy Association and Dysautonomia Foundation (K.F.); by the American Heart Association and the Otto Hahn Medaille (S.K.); by the Alzheimer's Disease and Related Disorders Association (T.Y.); a Swiss National Foundation Fellowship (R.A.); and the Ministry of Education, Taiwan, R.O.C., and the Helen G. and Arthur McCallum Foundation (M.-J.F.).

15 September 1989; accepted 14 November 1989