Neutrophic factors from three different families have distinct effects on the proliferation and neuronal differentation of embryonic dorsal root ganglion cells
by Katherine Maida Nielsen

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
Montana State University

Abstract:
There is increasing evidence that the development of a neuron depends on the action of several neurotrophic factors at different developmental stages. This study was designed to further elucidate the roles that three such factors play in the development of the dorsal root ganglia (DRG). A key stage in the development of the DRG is sensory neurogenesis which peaks in the chick at embryonic day 4.5. Previous work has shown that at this stage, two-thirds of the cells are neurons and 30% of the cells are mitotically active neural and glial progenitor cells. The factors which regulate the behavior of these mitotically active progenitor cells are unidentified; this study thus identified factors which were likely candidates and studied their effects on this cell population in vitro. We used a 6-hour cell culture assay to determine the effects of ciliary neurotrophic factor (CNTF), neurotrophin-3 (NT-3), and pituitary adenylate cyclase-activating peptide (PACAP) on cellular proliferation and neuronal differentiation. Virtually no cell death occurs during this six-hour period, thus ruling out significant apoptotic and survival effects. Since there is evidence of interactions between neurotrophic factors and the expression of CNTF, PACAP, and NT-3 and/or their receptors overlaps temporally and spatially, the combined effects of all three factors were examined. Our results indicate that each of these neurotrophic factors has a distinct effect on the proliferation and/or neuronal differentiation of the cells of the DRG at the peak of neurogenesis. In addition, we have found that NT-3 and CNTF completely block PACAP's effect on neuronal differentiation. Each of these neurotrophic factors utilizes different intracellular signal transduction pathways, but these pathways share some common substrates. Our data may indicate distinct behavioral effects resulting from cross talk between these pathways.

NEUROTROPHIC FACTORS FROM THREE DIFFERENT FAMILIES HAVE DISTINCT EFFECTS ON THE PROLIFERATION AND NEURONAL DIFFERENTIATION OF EMBRYONIC DORSAL ROOT GANGLION CELLS

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APPROVAL

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Katherine Maida Nielsen

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date November 26, 1999

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ABSTRACT

There is increasing evidence that the development of a neuron depends on the action of several neurotrophic factors at different developmental stages. This study was designed to further elucidate the roles that three such factors play in the development of the dorsal root ganglia (DRG). A key stage in the development of the DRG is sensory neurogenesis which peaks in the chick at embryonic day 4.5. Previous work has shown that at this stage, two-thirds of the cells are neurons and 30% of the cells are mitotically active neural and glial progenitor cells. The factors which regulate the behavior of these mitotically active progenitor cells are unidentified; this study thus identified factors which were likely candidates and studied their effects on this cell population *in vitro*. We used a 6-hour cell culture assay to determine the effects of ciliary neurotrophic factor (CNTF), neurotrophin-3 (NT-3), and pituitary adenylate cyclase-activating peptide (PACAP) on cellular proliferation and neuronal differentiation. Virtually no cell death occurs during this six-hour period, thus ruling out significant apoptotic and survival effects. Since there is evidence of interactions between neurotrophic factors and the expression of CNTF, PACAP, and NT-3 and/or their receptors overlaps temporally and spatially, the combined effects of all three factors were examined. Our results indicate that each of these neurotrophic factors has a distinct effect on the proliferation and/or neuronal differentiation of the cells of the DRG at the peak of neurogenesis. In addition, we have found that NT-3 and CNTF completely block PACAP's effect on neuronal differentiation. Each of these neurotrophic factors utilizes different intracellular signal transduction pathways, but these pathways share some common substrates. Our data may indicate distinct behavioral effects resulting from cross talk between these pathways.

INTRODUCTION

The neurons of the dorsal root ganglia (DRG) are an extremely heterogeneous cell population. These neurons can differ in their sensory modality, morphology, target innervation site, neurotransmitter content, and growth factor dependencies (Scott, 1992). Yet, these cells share a common background: they are derived from the neural crest (Weston, 1963). Specifically, these cells leave the neural tube, migrate ventral laterally, and then aggregate to form the nascent DRG (Weston, 1963; Teillet et al., 1987; Lallier and Bronner-Fraser, 1988). A period of intense cell proliferation and differentiation follows, resulting in the production of several thousand neurons. In sum, this series of events includes migration, aggregation, proliferation, and differentiation. What are the cues involved in these different pathways?

There is increasing evidence that neurotrophic factors play a key role in these developmental processes (Korsching, 1993) and that the development of a neuron depends on the action of several neurotrophic factors at different developmental stages (Davies, 1994). Neurotrophic factors were first documented as target-derived factors which regulate the growth and survival of responsive neurons. For example, the family of neurotrophins, whose members include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), -4/5, and -6, protect postmitotic neurons from programmed cell death (Bothwell, 1995). Several recent studies also demonstrate potential roles for neurotrophins in key early events in sensory neurogenesis which occur prior to target innervation (Davies, 1994; Henion et al., 1995; Farinas et al., 1996; Elshamy and Ernfors, 1996; Sieber-Blum and Zhang, 1999). Indeed, research now indicates that the cooperative and sequential action of different neurotrophic factors, including the above-

described neurotrophins, cytokines such as ciliary neurotrophic factor and leukemia inhibitory factor, and growth factors such as fibroblast growth factor and insulin-like growth factor, affects progenitor cell proliferation, cell fate determination, neuronal survival, and differentiation.

Previous work has indicated that growth factors influence neuronal development in the DRG. The neurotrophin family has repeatedly been shown to promote DRG neuronal survival (Ernsberger and Rohrer, 1988; Davies et al., 1986; Crowley et al., 1994; Buchman and Davies, 1993) and neurotrophins have also enhanced DRG neuronal survival *in vitro* (Memberg and Hall, 1995). The cytokine leukemia inhibitory factor (LIF) can promote sensory neuron differentiation (Murphy et al., 1991; Memberg and Hall, 1995).

At the peak of sensory neurogenesis in the DRG, 30% of the cells are mitotically active neural and glial progenitors (Lefcort, personal communication; Farinas et al., 1998; v. Holst et al., 1997) and about two-thirds are neurons (Carr and Simpson, 1978). In order to gain a better understanding of the factors involved in sensory neurogenesis and differentiation at this developmental stage, three neurotrophic factors, neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF) and pituitary adenylate-cyclase activating peptide (PACAP), were chosen for study. These proteins and/or their receptors are expressed in embryonic DRG and have been shown to affect survival, proliferation, and/or differentiation in other regions of the nervous system. Our goal was to determine these factors' effects on the proliferation and neuronal differentiation of progenitor cells at the peak of sensory neurogenesis in the DRG.

NT-3 is a member of the neurotrophin family and its primary high-affinity receptor is the trkC receptor, a tyrosine kinase receptor. Expression of the trkC receptor is first observed on a small subset of migrating neural crest cells. By chick E4/E4.5, the peak of

neurogenesis, 63% of the cells express trkC (Lefcort et al., 1996) and 20% of the dividing cells in the interior region of the DRG are trkC+ (Rifkin et al., submitted). Moreover, when a blocking antibody to trkC was injected *in ovo*, the number of neurons in the ventrolateral region of the DRG decreased by 77% by E7.5/8 (at this stage, the trkC-expressing cells reside primarily in the ventrolateral region). A 36% decrease in the number of small-diameter, dorsomedially located neurons was also observed, indicating that these cells (the vast majority of which express trkA during the period of postmitotic, naturally occurring cell death) require NT-3 either directly or indirectly during at least one stage of their development. Overall, a 47% decrease in neuronal numbers in the DRG was noted (Lefcort et al., 1996) and similar results were found after injection of a blocking antibody to NT-3 *in ovo* (Gaese et al., 1994). In addition, when embryos were injected with the blocking antibody to trkC, there was a 27% decrease in the number of neurons at E4.5/5 (Lefcort et al., 1996). This stage of development is just before the period of postmitotic, target-mediated neuronal cell death; these results thus demonstrate that a functional trkC receptor is required for early differentiation of cells within the DRG. Several studies have shown that NT-3 is produced in developing limb buds (Henderson et al., 1993), spinal cord (Pinco et al., 1993; Elkabes et al., 1994) and DRG (Schecterson and Bothwell, 1992; Pinco et al., 1993; Elkabes et al., 1994; Zhang et al., 1994); cells in the DRG could presumably be exposed to NT-3 from one or more of these sources. Combined, these observations clearly point to a role for NT-3 in the nascent DRG, possibly influencing the survival, proliferation, and/or differentiation of dividing progenitor cells. There is *in vitro* evidence for such a role: NT-3 has been shown to promote proliferation of neural crest cells (Pinco et al., 1993; Kalcheim et al., 1992; Chalazonitis et al., 1994) and dorsal root ganglia neuronal precursor cells (Memberg and Hall, 1995),

promote the survival of sympathetic neuroblasts before their dependence on NGF (Birren et al., 1993; DiCicco-Bloom et al., 1993) and to promote neuronal differentiation (Wright et al., 1992; Pinco et al., 1993).

Ciliary neurotrophic factor (CNTF) is a neuropoietic cytokine and was originally identified as a survival factor for chick ciliary neurons (Adler et al., 1979; Lin et al., 1989; Stockli et al., 1989). CNTF's actions are mediated by a three-component receptor complex which consists of a CNTF-specific binding component known as CNTF Receptor α (CNTFRα), and two signal-transducing subunits, LIFR (Leukemia Inhibitory Factor Receptor)-β and gp130, which it shares with its cytokine relatives (Stahl and Yancopoulos, 1994; Ip et al., 1992). These three components, unassociated on the cell surface, form a complex in response to CNTF. CNTF first binds to its α receptor component, then LIFR-β and gp130 are recruited. LIFR-β and gp130 do not bind to CNTF in the absence of CNTFRα (Ip et al., 1992). CNTFRα is linked to the cell surface via a glycosylphosphatidylinositol linkage and plays no role in signaling; its sole function appears to be conferring binding specificity (Stahl and Yancopoulos, 1994). There is a high degree of conservation in CNTFRα between species; for example, the sequence of rat CNTFRα is 94% identical to its human counterpart (Ip et al., 1993).

In the developing chick DRG, CNTFRα is first seen in a few cells at stage 19 (E3) and its expression then increases during development with maximal expression seen at stages 27-30 (E6-E7) and continued high expression to at least stage 38 (E12) (v. Holst et al., 1997). In one-day cultures of dissociated cells from E5 DRG, all cells with neuronal morphology expressed CNTFRα and were positive for a neuronal marker. The nonneuronal cells did not express CNTFRα. As with trkC, CNTFRα is expressed on dividing cells at the peak of sensory neurogenesis (v. Holst et al., 1997), indicating a

potential role for CNTF in proliferation, differentiation, and/or survival. CNTFRα is expressed throughout the adult rat nervous system (Ip et al., 1993). CNTFRα expression is detected in rat DRG at E11 (a stage at which the DRG is comprised predominantly of neuronal precursors, Murphy et al., 1991), E15 (a stage at which the DRG contains postmitotic neurons), and in the adult (Ip et al., 1993).

CNTF has been shown to exert a variety of effects on different neuronal populations. Barbin et al. (1984) found that CNTF did not support the survival of chick E8 DRG neurons, but did support the survival of E10 DRG neurons. CNTF supports the survival of embryonic hippocampal neurons and promotes neurite outgrowth (Ip et al., 1991); promotes the survival of chick E6 spinal motoneurons (Arakawa et al., 1990); and induces differentiation of chick sympathetic neurons (Ernsberger et al., 1989). It inhibits the proliferation of sympathetic neurons (Ernsberger et al., 1989) and MAH cells (an immortalized sympathoadrenal progenitor cell line, Ip et al., 1992).

While CNTF has been shown to affect many neuronal populations, there is evidence that endogenous CNTF does not play a critical role in neuronal development. Mice homozygous for null mutations in the *CNTF* gene appear remarkably normal: they are viable and initially thrive, and only in adulthood do they exhibit a very mild loss of motor neurons (Masu et al., 1993; DeChiara et al., 1995). In addition, a study of the Japanese population found that approximately 2.5% of the population are homozygous for mutations that inactivate the *CNTF* gene and that these individuals have not yet been shown to exhibit any associated neurologic abnormalities (Takahashi et al., 1994). Consistent with these findings, it has been found that CNTF is normally expressed only at very low levels in the embryo (Stockli et al., 1991; Ip et al., 1993). However, in contrast to mice lacking CNTF, mice lacking CNTFRα die shortly after birth and exhibit profound deficits in all

motor neuron populations examined (DeChiara et al., 1995). It has thus been postulated that CNTFRα is utilized by a ligand other than CNTF and that this factor is critical for normal development and postnatal viability.

Pituitary adenylate cyclase-activating peptide (PACAP) was originally isolated from the hypothalamus and was shown to activate adenylate cyclase in rat pituitary cells (Miyata et al., 1990; 1989). PACAP belongs to the glucagon/secretin/vasoactive intestinal peptide family (Arimura, 1992; Miyata et al., 1989) and exists in two active forms, PACAP38 and PACAP27. PACAP38 is equivalent to PACAP27 plus an 11-amino acid C-terminal extension. PACAP's biological effects are mediated by multiple isoforms of PACAP receptors (Journot et al., 1994; Spengler et al., 1993). The sequence of PACAP has been remarkably conserved during evolution; for example, PACAP38 is absolutely conserved at the amino acid level in rats, sheep, and humans (Arimura, 1992), as is PACAP27 in species ranging from fish to humans (Waschek et al., 1998). Such sequence conservation suggests that PACAP regulates important biological functions.

PACAP and its receptors are widely expressed in the brain during development and in adulthood, for example in the cerebral cortex, hippocampus, hypothalamus, and cerebellum (Skoglosa et al., 1997; D'Agata et al., 1996; Spengler et al., 1993; Arimura et al., 1991; Kivioelto et al., 1992). In both E16 and adult rats, 20% of the neurons in the DRG are positive for PACAP mRNA (Lioudyno et al., 1998; Mulder et al., 1994; Zhang et al., 1995). There are numerous PACAP-positive cells in human DRG (Dun et al., 1996). Both PACAP and its type I receptor are widely expressed in E10.5 mouse neural tube (Waschek et al., 1998) and the gene for this receptor has been detected in both the DRG and the central nervous system of E14.5 mice.

Vasoactive intestinal peptide (VIP), which exhibits 68% sequence identity with PACAP27, has been shown to stimulate mitosis, promote neurite outgrowth, and enhance the survival of sympathetic neuron precursor cells in culture (Pincus et al., 1990). VIP, PACAP27, and PACAP38 all promoted neurite extension in PC12 cells (Colbert et al., 1994). PACAP38 promotes the survival of DRG post-mitotic neurons from E20 or P1 rats and enhances neurite outgrowth after one week in culture (Lioudyno et al., 1998). PACAP38 has also been shown to promote the survival and neuronal differentiation of cultured granule cells from the cerebellum (Gonzalez et al., 1997).

While the effects of these factors on some areas of the nervous system have been studied, very little research has been done on the cells of the DRG, particularly at the peak of sensory neurogenesis. However, the expression of these proteins and/or their receptors hints intriguingly of their involvement in this important developmental stage. This study therefore focused on CNTF and PACAP's effects on the proliferation and neuronal differentiation of the cells of the chick DRG at E4.5. The effects of an antagonist to PACAP were also examined. Research on the effects of NT-3 alone was conducted by Sharon Hapner in the same lab. Steps were taken to ensure that we were assaying proliferation and/or neuronal differentiation, and not survival (see "Results"). Our results demonstrate distinct roles for each of these neurotrophic factors during the development of the DRG.

In addition, given the temporal and spatial overlap in the expression of these three growth factors and/or their receptors, the possibility of synergistic or antagonistic actions was tested. Each of these growth factors utilizes different receptors linked to divergent downstream signaling pathways: NT-3 binds to a receptor tyrosine kinase, thereby initiating the Ras/MAPK pathway; CNTF primarily activates a JAK/STAT pathway; and,

PACAP stimulates both an increase in cAMP and the PLC/PKC pathway. The combined effects of these proteins were examined, and our results provide strong evidence for interactive relationships between these factors and their respective signaling pathways.

EXPERIMENTAL OBJECTIVES AND RATIONALE

The goal of this study was to determine whether CNTF, PACAP, and NT-3 influence cellular proliferation and neuronal differentiation at the peak of sensory neurogenesis. These three proteins were chosen because previous work indicated their likely involvement in this important developmental stage of the peripheral nervous system. That is, these proteins and/or their receptors are expressed in the embryonic DRG and have been shown to affect survival, proliferation, and/or differentiation in other areas of the nervous system.

We used a 6-hour cell culture assay to determine the effects of CNTF and PACAP on the differentiation and proliferation of cells cultured from E4.5 DRG, the peak of neurogenesis. Virtually no cell death occurs during this six-hour period (Hapner, personal communication), thus ruling out significant apoptotic and survival effects. Since there is evidence of interactions between neurotrophic factors and the expression of CNTF, PACAP, and NT-3 and/or their receptors overlaps temporally and spatially, the combined effects of all three factors were examined.

MATERIALS AND METHODS

Embryos

Fertilized White Leghorn chicken embryos were obtained from Truslow Farms (Chestertown, MD) and incubated at 37°C in a rocking incubator (Kuhl, Flemington, NJ). Embryos were staged according to Hamburger and Hamilton (1951).

DRG Cell Cultures

Dorsal root ganglia (DRG) from E4.5 (Stage 25) chick embryos were dissected into Hanks' Balanced Salts, Calcium and Magnesium Free (Sigma, St. Louis, MO). The DRG from several embryos were combined in each DRG cell culture: depending on the number of treatments in the experiment, between four and eight embryos were dissected, with an average yield of 30 DRG per embryo. To obtain a single cell suspension, the DRG were incubated in 0.25% trypsin - 1 mM EDTA (Gibco, Grand Island, NY) for 5 minutes at 37°C and then triturated with pulled glass pipettes. The culture media consisted of Nutrient Mixture F-12 (Sigma) supplemented with Hybrimax Antibiotic/Antimycotic (1:100, Sigma), 0.4 mg/ml BSA (Bovine Albumin A-7638, Sigma), T_3 (10 ng/ml, Sigma), T_4 (25 ng/ml, Sigma), transferrin (250 µg/ml, Gibco), and selenium (100 ng/ml, Gibco). Cells were plated in 8-well Nunc glass chamber slides that were coated with poly-d-lysine (10 µg/ml in F-12, Sigma) for 30 minutes at room temperature, rinsed twice with F-12, and coated with mouse laminin (20 µg/ml in F-12, Gibco) overnight at 37°C. Approximately equal number of cells (roughly 10,000) were plated in each well.

Immediately after plating, if proliferation was being assayed, bromodeoxyuridine (BrdU, Sigma) was added at 10 µg/ml to each well. The growth factor in question was

added at the stated concentration to each well with each experimental condition repeated in quadruplicate. The cells were then cultured for 6 hours at 37°C, 7% CO_2.

The growth factors used were NT-3 (Genentech, South San Francisco, CA), CNTF (R & D Systems; Minneapolis, MN; kind gift of Dr. C. Paden, Montana State University), and PACAP38 (Peninsula Laboratories, Inc.; San Carlos, CA; kind gift of Dr. R. Zigmond, Case Western Reserve University). The antagonist to PACAP (PACAP 6-38) was also from Peninsula Laboratories, Inc. and kindly provided by Dr. R. Zigmond.

Immunocytochemistry

Proliferation Assay

The culture media was removed, cells were rinsed twice with F-12, and then fixed with methanol/5% acetic acid at -20°C for 10 minutes. The slides were then washed four times with F-12 and two times with TBS (10mM Tris, 150mM NaCl, pH 7.4), five minutes per wash. They were then treated with 2N HCl for 15 minutes at room temperature, followed by four 10-minute washes with TBS. The slides were then placed in blocking buffer (10% normal goat serum, 1% glycine, 0.4% Triton X-100 in 30mM TRIS, 150mM NaCl) for one hour at room temperature, followed by overnight incubation in primary antibody at 4°C.

Differentiation Assay

FBS (Hyclone, Logan, UT) was added to each well at a 1:5 concentration. Cells were then fixed for 30 minutes at room temperature by adding 8% paraformaldehyde in a buffer (0.1 M PO_4, 0.15 N NaCl, pH 7.4) at a 1:1 concentration. By adding FBS, followed by the addition of paraformaldehyde solution directly to the wells without first

removing the culture media, the majority of neurons is maintained during the fixing procedure (Hapner, personal communication). The slides were washed for two minutes with TBS, then placed in blocking buffer (see "Proliferation Assay") for twenty minutes at room temperature, followed by overnight incubation in primary antibody at 4°C.

Differentiation and Proliferation Assay

When both differentiation and proliferation needed to be assayed in the same experiment, cells were fixed as in the differentiation assay and then went through the steps for BrdU staining as described in the proliferation assay, beginning with the F-12 and TBS washes.

Antibodies

For BrdU staining, an anti-BrdU monoclonal antibody (Novacastra, Vector Laboratories, Burlingame, CA) was used at 1:300 in blocking buffer. To identify neurons, either an antibody to the 180-kDa neurofilament subunit (1:500 in blocking buffer, kind gift of Dr. B. Granger, Montana State University) or Tuj-1, an antibody to a neural β-tubulin isoform (1:700 in blocking buffer, kind gift of Dr. Robert Oakley, George Washington University), was used.

After an overnight incubation in primary antibodies, the slides were rinsed four times with blocking buffer, five minutes each time. They were then placed in secondary antibodies for one hour at room temperature. Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and Rhodamine (TRITC)-conjugated AffiniPure Goat Anti-Mouse (Jackson ImmunoResearch Laboratories, Inc.) were both used at 1:300 in blocking buffer. To

visualize chromatin, DAPI (4'6 diamidino-2-phenylindole dihydrochloride; Molecular Probes, Eugene, OR) was added to this incubation at 1:1000. After the incubation, the slides were washed with TBS (four times, six minutes each time). The slides were then mounted in Prolong Antifade (Molecular Probes).

Quantitation and Statistical Analyses

All slides were examined on a Nikon FXA microscope. For each well, cells were first identified by their chromatin (DAPI) staining, which gives no indication of whether a cell is neuronal or non-neuronal. A small number of dividing cells can be identified by their chromatin staining, but the vast majority of them cannot be so identified. Every cell within the field of view, identified by its chromatin staining, was counted. The number of neurons (defined as neurofilament+ or Tuj-1+ and bearing at least one process one cell diameter in length, except for the studies at 5 and 25 ng/ml CNTF where all positively stained cells were counted) and/or BrdU+ cells was then determined by switching filters and noting the immunopositive cells. A total of 500 cells per well were counted. Each experiment included a control treatment which did not receive any growth factors.

Within each experiment, each treatment was tested in four wells and each well within an experiment was considered an independent measure. Thus, within each experiment, the effects of the various treatments were compared with the use of ANOVAs and Student-Newman-Keuls Method. All the results reported as significant when compiled (see below) were significant within each experiment.

Each experiment was repeated at least once and several treatments were tested many times. Data from the experiments was compiled as follows: first, the four control wells within an experiment were averaged; second, the percent change of each treatment

well (four treatment wells per experiment) relative to the control average was determined; and, finally, t-tests or ANOVAs, depending on the comparison in question, were run on these normalized percentage values (with n equal to the total number of wells, see "Results"). As stated above, the statistical significance of the combined data coincides completely with the statistical signficance found within each experiment (with four wells per treatment). The only exception to this trend occurs with the experiments which assayed the effects of CNTF on neuronal differentiation by using an antibody to neurofilament: in all five experiments, there were fewer neurons in the CNTF treatment relative to the control, and in two of the five experiments, the difference was statistically significant (as measured by an ANOVA and Student-Newman-Keuls Method). When the data was combined, the difference was not statistically significant (see "Results").

RESULTS

This study examined the effects of CNTF, PACAP, and NT-3 on proliferation and neuronal differentiation of cells cultured from DRG at the peak of sensory neurogenesis (E4.5 in the chick). The thymidine analog BrdU was added to the cultures as an assay for proliferation; the use of antibodies to neurofilament and a neural tubulin along with cell morphology were used to examine neuronal differentiation. To ensure that survival effects were not complicating our results, we used a very short culture period of 6 hours. No TUNEL labeling (Apotag, Oncor, Gaithersburg, MD) has been seen during this 6-hour culture period (Hapner, personal communication), thus ruling out significant apoptotic and potential survival effects. During differentiation assays, steps were taken to ensure that the majority of neurons were retained during the fixing procedure (see "Differentiation Assay, Immunocytochemistry" in Materials and Methods).

NT-3 and CNTF's effects on proliferation were strikingly similar: the addition of each one at 10 ng/ml resulted in a 24 $\pm$2% increase in the number of BrdU+ cells, relative to the control (n=16 for NT-3 data, n=28 for CNTF data, $p \leq 0.0001$ for both NT-3 versus control and CNTF versus control, two separate t-tests, Figure 1). No additive or synergistic effects of CNTF and NT-3 on proliferation were noted (n=8, $p \leq 0.002$, Figure 1), suggesting that these two neurotrophic factors are acting on the same cell population.

At 10 ng/ml, CNTF also induced a decrease in the number of neurons: a 25 $\pm$5% decrease in the number of Tuj-1+ cells (n=8, $p \leq 0.005$) and an 17 $\pm$3% decrease in the number of neurofilament+ (NF+) cells (n=12, $p \leq 0.1$, Figure 2). A possible explanation for the discrepancy in significance between Tuj-1+ and NF+ cells is that Tuj-1 is a very

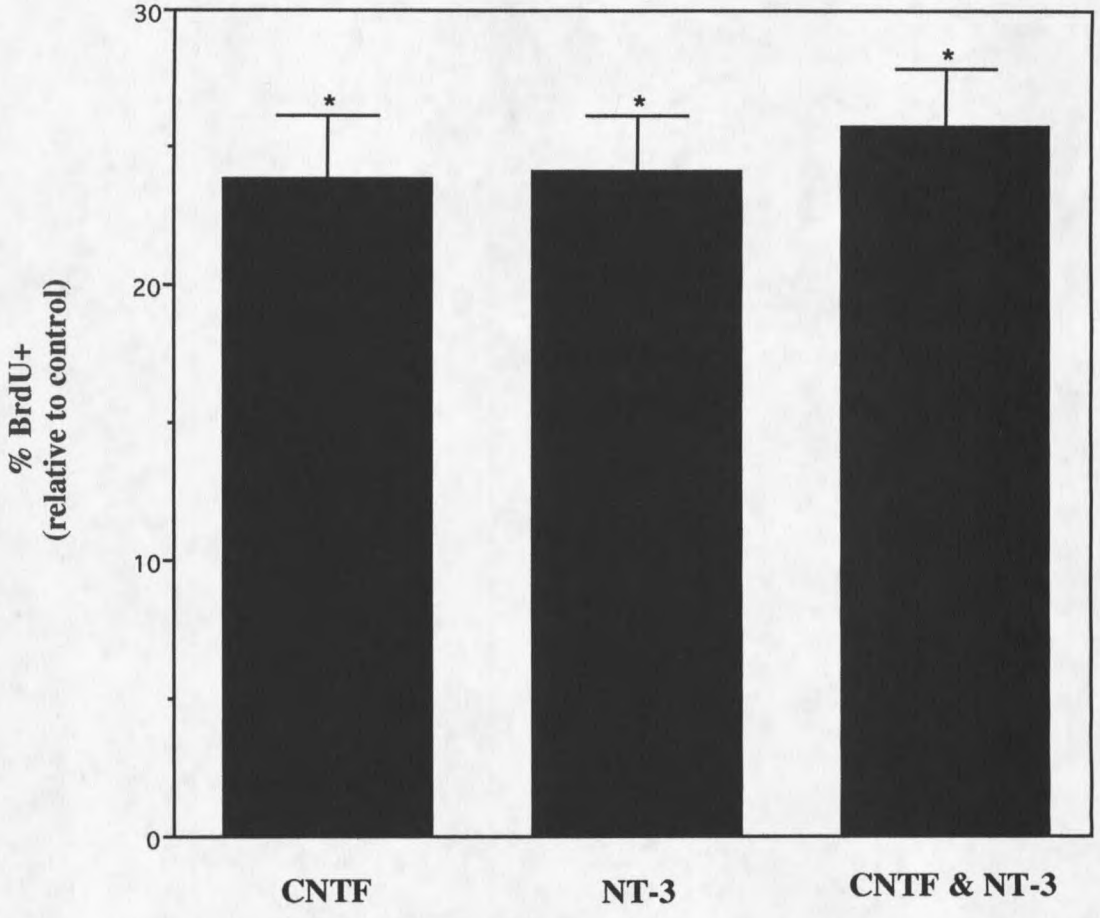

Figure 1. CNTF and NT-3 promote proliferation of embryonic DRG cells, but their effects are not additive. DRG from chick E4.5 were dissociated and then cultured for 6h. CNTF (10 ng/ml) and/or NT-3 (10 ng/ml) and BrdU were added at the time of plating. Within each experiment, each treatment was tested in four wells. Each well was considered an independent measure. Values represent the mean difference in the percent of BrdU+ cells for each treatment relative to the control over the same period, plus SEM from 8 or more separate wells. Three separate t-tests, each conducted relative to its control, are combined in this graph; $p \leq 0.002$.

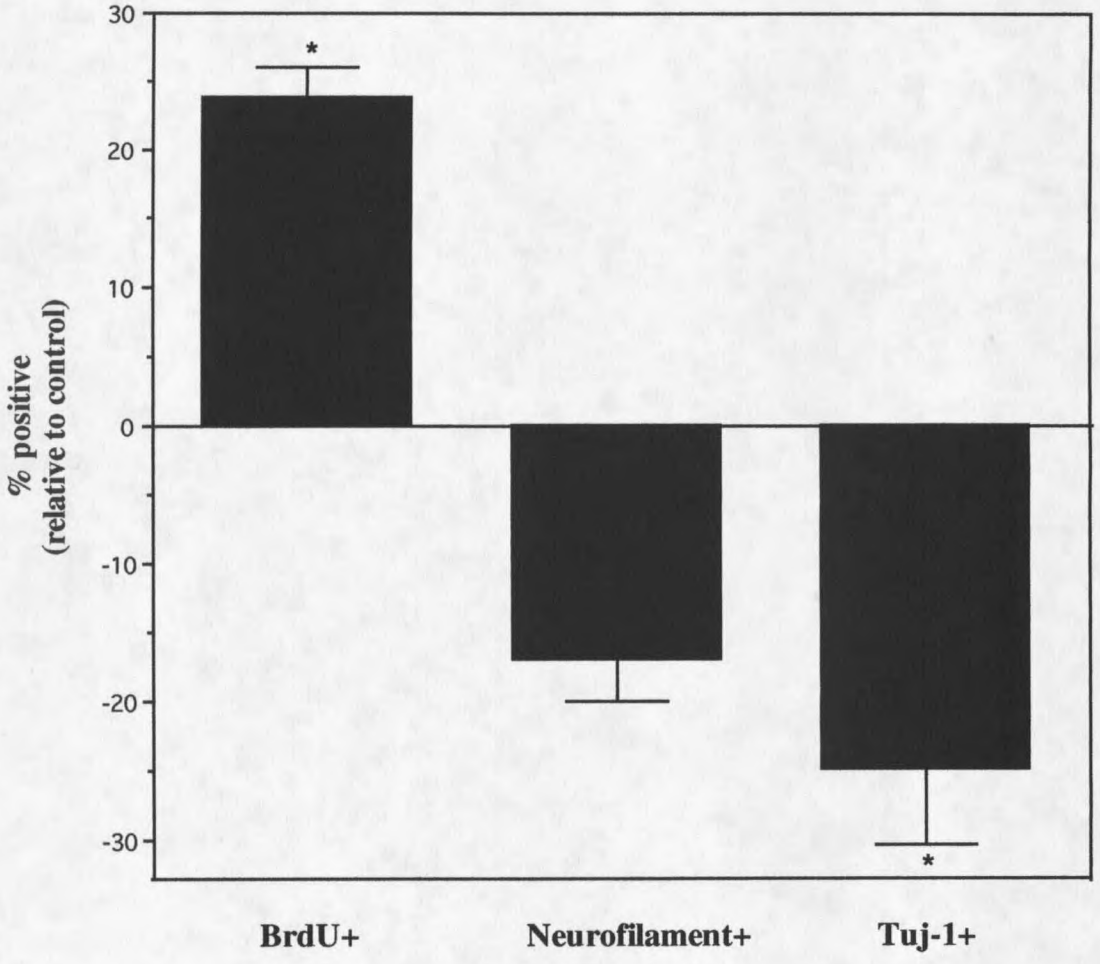

Figure 2. CNTF's effects on proliferation and neuronal differentiation of embryonic DRG cells. DRG from chick E4.5 were dissociated and then cultured for 6 h. CNTF (10 ng/ml) and BrdU (for proliferation assays) were added at the time of plating. Within each experiment, each treatment was tested in four wells with each well being considered an independent measure. Values represent the mean difference in the percent of positive cells for each treatment relative to the control over the same period, plus SEM from 8 or more separate wells. Three separate t-tests, each conducted relative to its control, are combined in this graph; $p \leq 0.002$.

cleanly staining antibody making it easy to determine positively-stained cells, whereas NF antibody staining results in cells immunopositive to varying degrees. At 25 ng/ml, CNTF did not have a significant effect on proliferation (n=12) or neuronal differentiation (n=8, Figure 3). In addition, at 5 ng/ml, CNTF did not significantly decrease the number of neurons (n=8, Figure 3). NT-3 at 10 ng/ml did not induce neuronal differentiation (n=12, Figure 4B). Interestingly, preliminary data indicate that NT-3 can override CNTF's inhibition of neuronal differentiation: when NT-3 and CNTF were combined, the number of neurons was similar to control values (n=4, Figure 4B). Research on NT-3's effects at other concentrations, such as the generation of dose response curves, was conducted by Sharon Hapner in the same lab (unpublished data).

In contrast to CNTF and NT-3, PACAP significantly increased the number of NF+ cells over control conditions. The peak response, a 73 $\pm$7% increase in the number of NF+ cells relative to the control, was seen at 50 nM PACAP (n=15, $p \leq 0.0001$, Figure 5). At all other concentrations tested between 1 nM and 150 nM PACAP, there was a 36% to 54% increase in the number of NF+ cells relative to the control (n=8 to 20, $p \leq 0.0001$, Figure 5). These results were corroborated by Tuj-1 staining: similar trends at concentrations ranging from 1 nM to 100 nM PACAP were seen (n=4, Figure 5A). Taken together, these results suggest that PACAP was not simply upregulating neurofilament, but was promoting neuronal differentiation. Morphological observations also support this idea: when compared to the control cultures, there were more cells with neurites in the 1 nM to 150 nM PACAP cultures (see cells treated with 50 nM PACAP in Figure 7). Only cells with at least one process one cell diameter in length were counted as neurons; most cells counted as neurons had two processes that were several times the cell diameter. PACAP was incapable of promoting neuronal differentiation at 0.5 nM (n=8, Figure 5B).

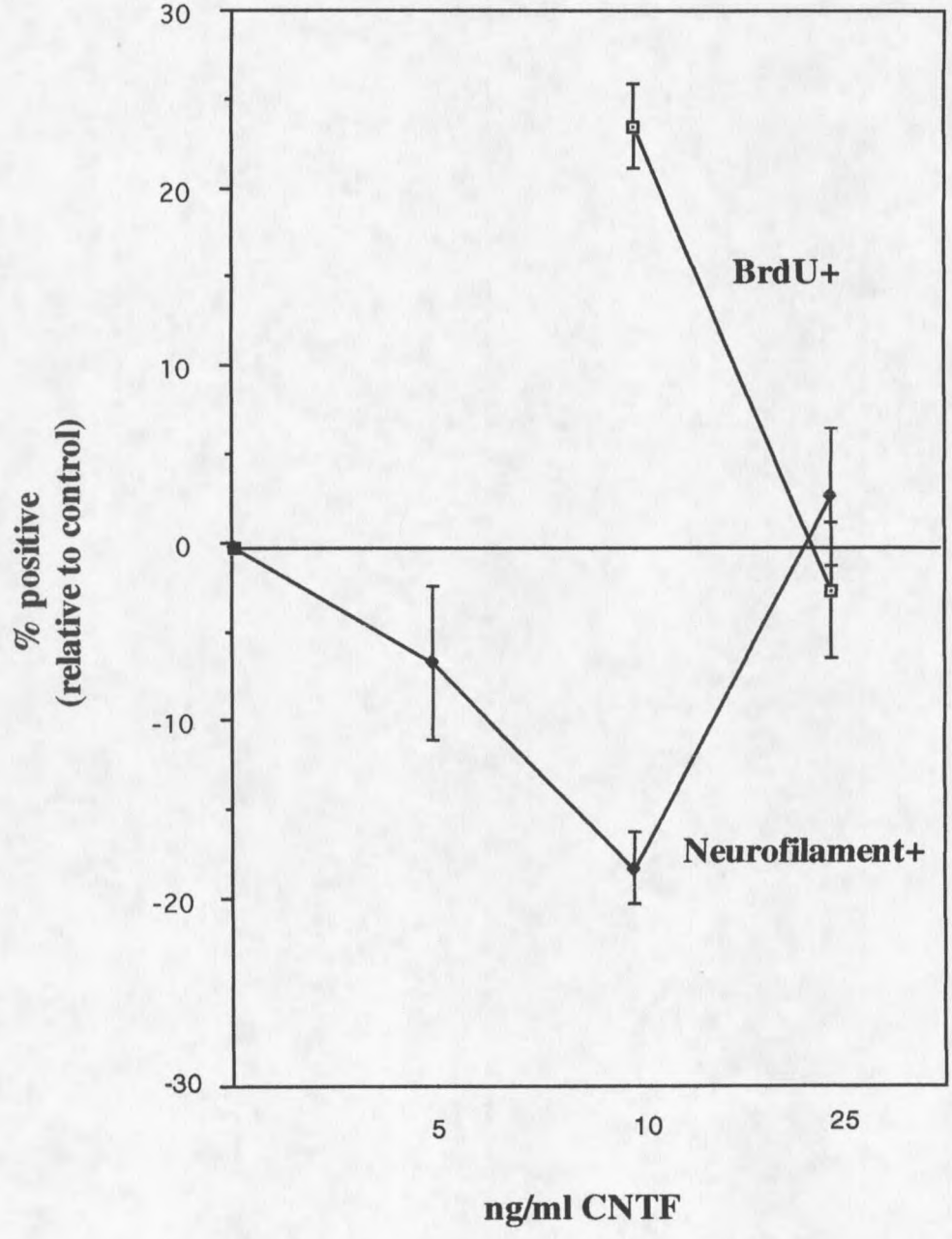

Figure 3. CNTF's effects at different concentrations on proliferation and neuronal differentiation of embryonic DRG cells. DRG from chick E4.5 were dissociated and then cultured for 6 h. CNTF and BrdU (for proliferation assays) were added at the time of plating. Within each experiment, each treatment was tested in four wells. Each well was considered an independent measure. Values represent the mean difference in the percent of positive cells for each treatment relative to the control over the same period, plus SEM from 8 or more separate wells.

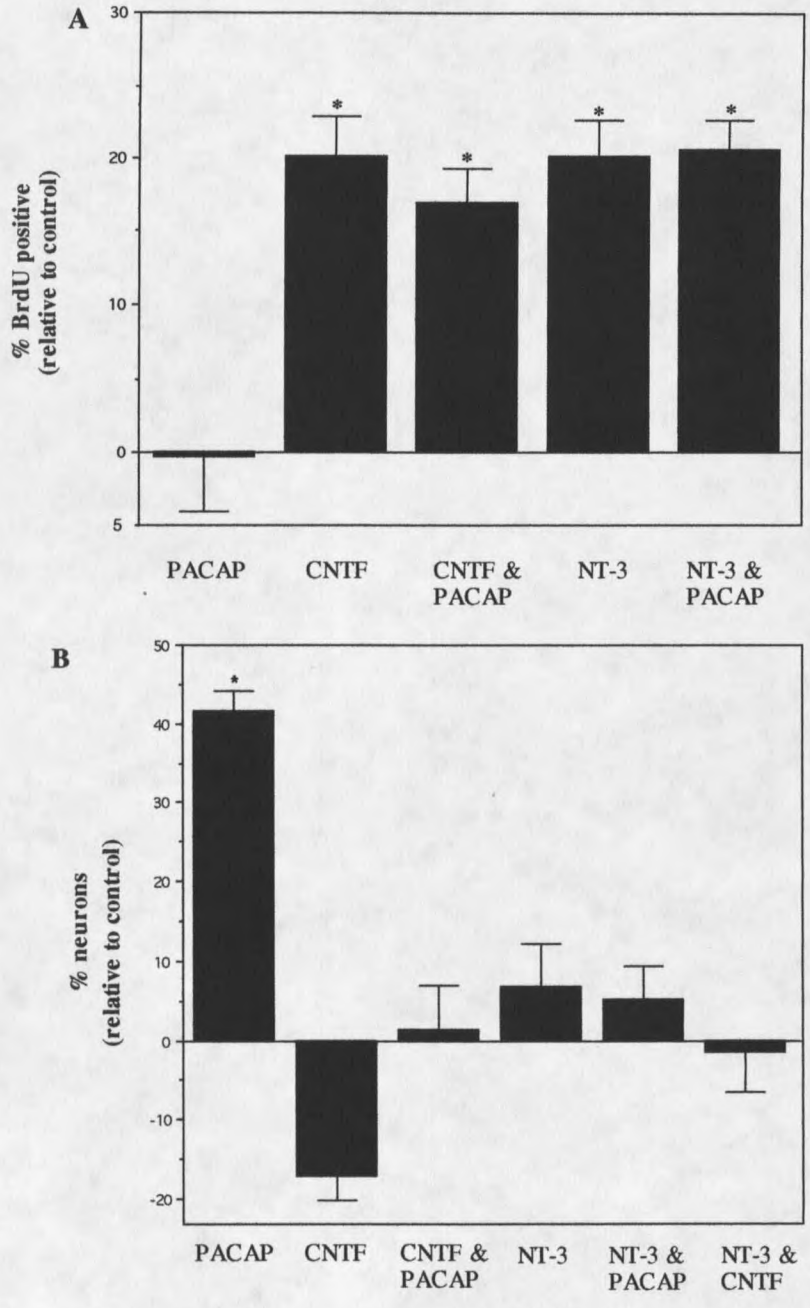

Figure 4. PACAP, CNTF, and NT-3's individual and combined effects on proliferation or neuronal differentiation of embryonic DRG cells. DRG from chick E4.5 were dissociated and then cultured for 6 h. PACAP (10 nM), CNTF (10 ng/ml), NT-3 (10 ng/ml) and/or BrdU (for proliferation assays) were added at the time of plating. Within each experiment, each treatment was tested in four wells with each well being considered an independent measure. (A) Effects on proliferation. Values represent the mean difference in the percent of positive cells for each treatment relative to the control over the same period, plus SEM from 8 or more separate wells. Five separate t-tests, each conducted relative to its control, are combined in this graph; $p \leq 0.002$. (B) Effects on differentiation. Cells were counted as neurons if they were immunopositive to an antibody to neurofilament and had at least one process one cell diameter in length. Values represent the mean difference in the percent of positive cells for each treatment relative to the control over the same period, plus SEM from 8 or more separate wells, except for NT-3 and CNTF where the SEM is from 4 separate wells. ANOVA and Student-Newman-Keuls Method; $p < 0.0001$.

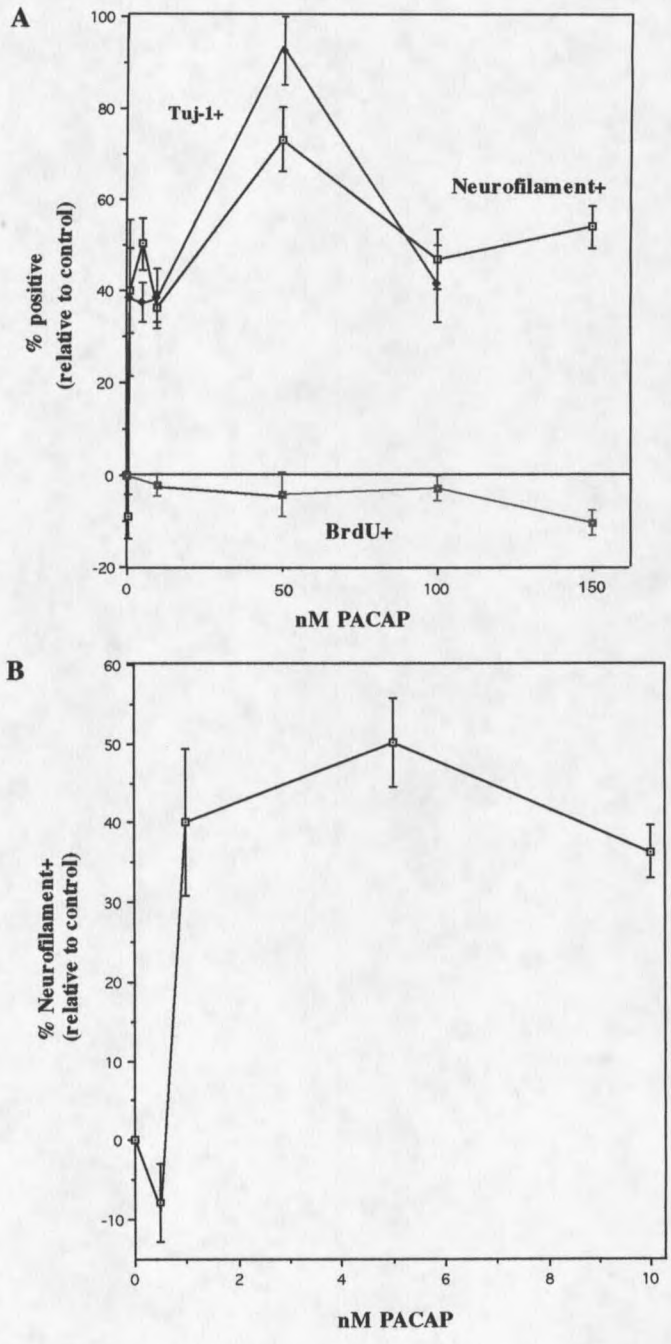

Figure 5. PACAP's effects at different concentrations on proliferation and neuronal differentiation of embryonic DRG cells. DRG from chick E4.5 were dissociated and then cultured for 6 h. PACAP and BrdU (for proliferation assays) were added at the time of plating. Within each experiment, each treatment was tested in four wells. Each well was considered an independent measure. Values represent the mean difference in the percent of positive cells for each treatment relative to the control over the same period, plus SEM from 8 or more separate wells (except for the Tuj-1 data and the 50 nM PACAP/BrdU+ data which are from 4 separate wells). (A) PACAP's effects at 0 to 150 nM. (B) Detailed view of PACAP's effects at 0 to 10 nM.

At concentrations of 10 (n=32), 100 (n=20), and 150 (n=12) nM PACAP, no significant difference in the number of BrdU+ cells, relative to the control, was observed (Figure 5A). Preliminary data indicates that this trend continues at 50 nM PACAP, the concentration at which the greatest increase in neuronal number was noted. In one experiment, there was no significant difference in the number of BrdU+ cells at 50 nM PACAP versus the control (n=4, Figure 5A).

While PACAP does not inhibit the proliferative effects of CNTF and NT-3 (n=8 to 11, Figure 4A), CNTF and NT-3 effectively block the neuronal differentiation promoted by PACAP (n=8 to 12, Figure 4B). At 10 nM PACAP, the number of neurofilament-positive cells with processes is 36 $\pm$3% greater than in control conditions. When cells are cultured with PACAP and either CNTF or NT-3, this effect completely drops out: the result is a 1% or 5% increase in the number of neurons respectively, neither of which is significantly different from the control results. This antagonistic effect suggests that these factors are affecting the same cell population.

An antagonist to PACAP38 (called PACAP6-38) has been shown to specifically block its effects (Lazarovici et al., 1998; Gonzalez et al., 1997). At 1 µm concentration, this antagonist unquestionably blocked PACAP38's effects. Specifically, when 50 nM PACAP (the concentration at which we observed the largest increase in NF+ cells) and 1 µm antagonist were combined, a 50 $\pm$ 2.5% decrease in the number of NF+ cells relative to the control was seen (n=8, $p \leq 0.0001$, Figure 6).

When only the antagonist to PACAP38 was added to the cell cultures, a striking decrease in the number of NF+ cells was observed. The number of NF+ cells was nearly 58 $\pm$6% lower than in the control treatments (n=8, $p \leq 0.0001$, Figure 6). Our results provide no evidence that this antagonist is acting as a toxin. To test this possibility, we

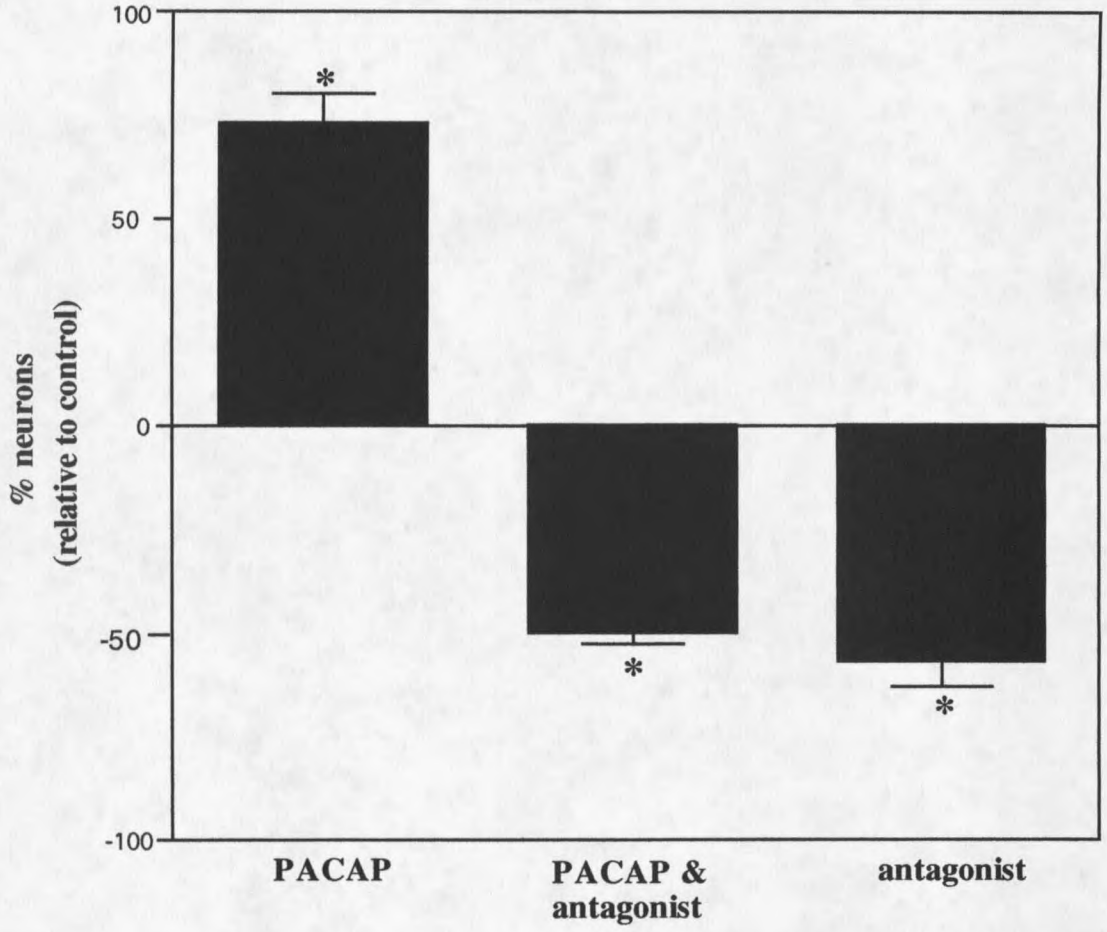

Figure 6. PACAP and its antagonist's effects on neuronal differentiation of embryonic DRG cells. DRG from chick E4.5 were dissociated and then cultured for 6 h. PACAP (50 nM) and/or its antagonist (1 μm) were added at the time of plating. Within each experiment, each treatment was tested in four wells. Each well was considered an independent measure. Values represent the mean difference in the percent of positive cells for each treatment relative to the control over the same period, plus SEM from 8 or more separate wells. ANOVA and Student-Newman-Keuls Method; $p < 0.0001$.

investigated whether there was a significant difference in the number of cells in the following three treatments: 1 μm antagonist, 1 μm antagonist and 50 nM PACAP, and the control. A "snapshot" of the number of cells was taken by counting the total number of cells in the same five fields of view (from five distinct regions) of a well. Each treatment was tested in four wells; an ANOVA test showed no significant difference in cell numbers for each treatment (Table 1). In addition, at 1 μm antagonist, there is no significant difference in the number of BrdU+ cells as compared to (1) the control and (2) 1 μm antagonist and 50 nM PACAP treatments (Table 1). Finally, the morphology and chromatin (DAPI staining) of the cells in the 1 μm antagonist treatment indicate that they are healthy cells (Figure 7). Thus, these results argue that PACAP exerts an endogenous effect on neuronal differentiation in this cell population *in vitro*.

TABLE 1. Effects of PACAP antagonist on cell number and BrdU+ cells. Results are mean ± SEM (n=4).

Treatment	**Number of Cells**	**BrdU+ Cells**
Control (no PACAP and no antagonist)	578.75 ± 22.9	104.8 ± 3.94
1 μm antagonist	538.0 ± 42.7	104.5 ± 3.93
1 μm antagonist and 50 nM PACAP	596.75 ± 33.6	103.5 ± 5.81

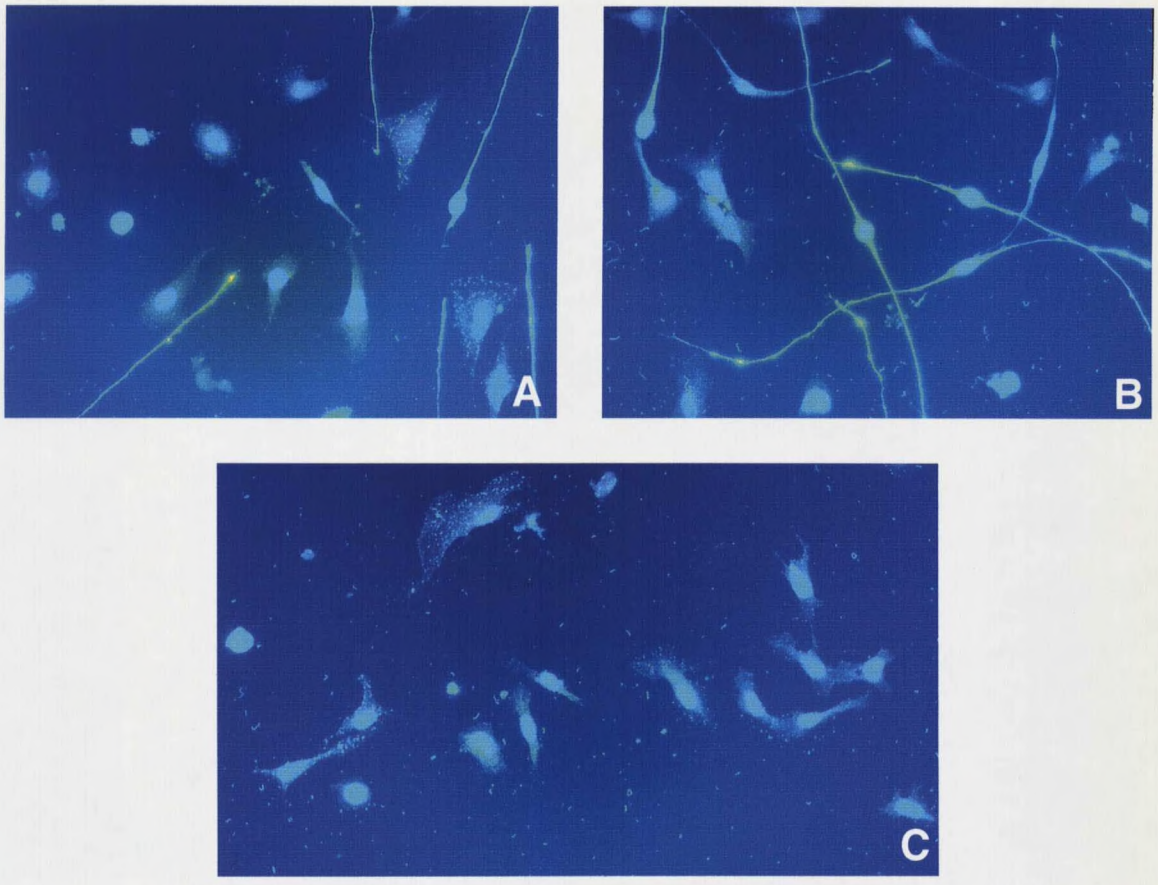

Figure 7. PACAP and its antagonist's effects on cell morphology. DRG from chick E4.5 were dissociated and then cultured for 6h as described in Methods. Cells were cultured either under control conditions - no neurotrophic factors added (A); 50 nM PACAP (B); or 1μm PACAP antagonist (C). Images are representative of the results of these three treatments. Cells are double labeled with an antibody to neurofilament and the chromatin stain, DAPI. Note the increase in neurons in (B) and the morphology and chromatin staining in (C).

DISCUSSION

This study was designed to further elucidate the roles that growth factors play in the development of the DRG. Specifically, a key stage in DRG development is sensory neurogenesis which peaks in the chick at E4.5 (Lallier and Bronner-Fraser, 1988). Previous work has shown that at this stage, two-thirds of the cells are neurons (Carr and Simpson, 1978) and 30% of the cells are mitotically active neural and glial progenitors (Lefcort, personal communication; Farinas et al., 1998; v. Holst et al., 1997). The factors which regulate the behavior of these mitotically active progenitor cells are unidentified; this study thus identified factors which were likely candidates in this developmental stage and then studied their effects on this cell population *in vitro*. Our results indicate that these neurotrophic factors, NT-3, CNTF, and PACAP, have distinct effects on the proliferation and/or neuronal differentiation of the cells of the DRG at the peak of neurogenesis. In addition, we have found that NT-3 and CNTF completely block PACAP's effect on neuronal differentiation. Each of these neurotrophic factors utilizes different intracellular signal transduction pathways, but these pathways share some common substrates. Our data may indicate distinct behavioral effects resulting from cross talk between these pathways.

TrkC (the primary NT-3 receptor) and the CNTF receptor are expressed on DRG cells at the peak of neurogenesis (Rifkin et al., submitted; Lefcort et al., 1996; v. Holst et al., 1997) pointing to potential roles for these receptors and their ligands in cell survival, proliferation, and/or differentiation. Indeed, we found that both NT-3 and CNTF promoted proliferation of this cell population. NT-3's effect on proliferation is not surprising in light of previous work. NT-3 can cause the proliferation of neural crest cells *in vitro* (Pinco et

al., 1993; Kalcheim et al., 1992; Chalazonitis et al., 1994) and our lab has recently shown that ectopic overexpression and activation of trkC in neural crest cells *in vitro* can promote their proliferation (Hapner et al., 1998). In addition, NT-3 has previously been shown to promote a 17% increase in proliferation in 48 hour, rat E12.5 DRG cell cultures (Memberg and Hall, 1995) and blocking trkC prior to the peak of neurogenesis reduces DRG neuronal cell number (Lefcort et al., 1996).

In contrast, to my knowledge, CNTF has not previously been shown to act as a mitogen in the nervous system. CNTF inhibits the proliferation of chick sympathetic ganglia (E7) neurons (Ernsberger et al., 1989) and does not affect the proliferation of cortical precursor cells (Bonni et al., 1997). LIF, which belongs to the same family as CNTF and utilizes two of the same receptor components, did not affect the proliferation of rat E12.5 DRG cells (Memberg and Hall, 1995). CNTF has been shown to support the survival of embryonic motoneurons (Arakawa et al., 1990), embryonic hippocampal neurons (Ip et al., 1991), and ciliary, sympathetic, and dorsal root ganglia neurons (Verdi and Anderson, 1994; Barbin et al., 1984).

CNTF's effects on neuronal differentiation are varied. CNTF promotes differentiation of embryonic sympathetic ganglia neurons (Ernsberger et al., 1989) and neural crest cells (Murphy et al., 1994) in culture. In contrast, CNTF has been shown to cause sympathetic neurons to retract their dendrites *in vitro* (Guo et al., 1999). Others have found that CNTF promotes glial differentiation of cortical precursor cells (Bonni et al., 1997; Marmur et al., 1998). Our data indicate that CNTF inhibits neuronal differentiation of DRG cells.

Of particular interest is that two embryonic cell populations, both derived from the neural crest, respond to the same growth factor in opposite ways. That is, we found that

CNTF promoted cellular proliferation and inhibited neuronal differentiation of the DRG. In contrast, CNTF inhibits proliferation of sympathetic ganglia and promotes their neuronal differentiation (Ernsberger et al., 1989). Thus, these two populations, which share a common heritage, are somehow programmed to respond in different ways to the same protein.

The expression patterns of trkC and the CNTF receptor clearly overlap at E4.5/5; both are expressed over much of the DRG. 63% of the cells at this stage are trkC+ (Lefcort et al., 1996) and 44% of the cells express the CNTF receptor (v. Holst et al., 1997). We therefore tested the combined effects of these neurotrophic factors. No additive effect was seen, suggesting that NT-3 and CNTF both influence the same group of cells. Interestingly, Chalazonitis et al. (1998) found that NT-3 and CNTF exerted an additive effect on the development of neurons from enteric neural crest-derived cells and that their respective receptors were rarely co-expressed. Both of these cells and those of the DRG are derived from the neural crest, but it may be that, at this later stage of development, they exhibit different patterns of receptor expression. Differences such as these are one way for different cell populations to utilize the same ligands to different ends. By double labeling chick E4.5 DRG with antibodies to trkC and CNTFRα, we could definitively tell whether these receptors are co-expressed and the extent of co-expression.

Previous work has shown that PACAP promoted neuronal differentiation in several regions of the nervous system. Specifically, PACAP has been shown to promote neurite outgrowth in sympathetic ganglia neurons (Pincus et al., 1990), chromaffin cells (Wolf and Krieglstein, 1995), cortical precursor cells (Lu et al., 1998), cerebellar neuroblasts (Gonzalez et al., 1997), and PC12 cells (Colbert et al., 1994). PACAP also induced the outgrowth of embryonic and neonatal rat DRG neurons when cells were

cultured for one week in its presence (Lioudyno et al., 1998). Similarly, we found that PACAP significantly stimulated neurite outgrowth and promoted neuronal differentiation within a 6-hour culture.

Others have found varied effects of PACAP on cellular proliferation. For example, PACAP inhibits proliferation of hindbrain neuroepithelial cells (Waschek et al., 1998) and cerebral cortical precursor cells (Lu et al., 1998); yet, it stimulates proliferation of sympathetic ganglia cells (Pincus et al., 1990; Lu et al., 1998). Our results indicate that PACAP does not affect proliferation in embryonic DRG cell cultures.

The two best characterized PACAP receptors are PACAP type I and II: PACAP type I receptor binds PACAP27 and PACAP38 more potently than VIP; while the type II receptor binds the three with approximately equal nanomolar affinities (Arimura, 1992). Both receptors are seven-transmembrane domain, G-protein-coupled receptors and both are positively coupled to cAMP, whereas only the type I receptor activates phospholipase C (PLC) pathways (Spengler et al., 1993). PACAP6-38 (hereafter referred to as PACAP antagonist) is an antagonist to PACAP38 and has previously been shown to inhibit PACAP38's effect via the type I receptor (Lioudyno et al., 1998). The PACAP antagonist inhibited PACAP38-induced neurite outgrowth in PC12 cells (Lazarovici et al., 1998) and it significantly attenuated PACAP38's survival effect on cerebellar neuroblasts (Gonzalez et al., 1997). Similarly, our results show a marked decrease in the number of neurons in cultures treated with the PACAP antagonist in conjunction with PACAP38. Therefore, the noted effect of an increase in neuronal differentiation are likely occurring via activation of the type I receptor.

Other studies further support the idea that PACAP38's effects on differentiation occur via the type I receptor and one particular intracellular signal transduction pathway.

Deutsch and Sun (1992) found that in PC12 cells, treatment with PACAP27 or PACAP38 led to equal activation of adenylate cyclase and elevation of cAMP, but PACAP38 was 200-fold more potent than PACAP27 in promoting the phospholipase C pathway and that PACAP38 was a much stronger stimulant of neurite outgrowth than PACAP27. Lazarovici et al. (1998) found that PACAP38-induced neurite outgrowth and that activation of extracellular signal-regulated kinase (Erk) kinase activity in PC12 cells is primarily protein kinase C (PKC) and MEK (threonine/tyrosine dual-specificity extracellular signal-regulated kinase; MAP/Erk kinase) dependent. Thus, our evidence suggests that the neuritogenesis promoted by PACAP38 in DRG cells primarily utilizes this same pathway (Figure 8). To test this, we could incubate cell cultures with inhibitors to distinct substrates within each of the two pathways, such as PKA and PKC, and then treat them with PACAP38. Doing so would enable us to decipher by which pathway(s) PACAP38 was promoting neuritogenesis.

Intriguingly, we also observed a significant decrease in neuronal number in cell cultures treated with only the PACAP antagonist and we saw no evidence that the antagonist was acting as a toxin (see "Results"). Such results clearly indicate an endogenous role for PACAP38 in the neuronal development of the DRG. Obvious steps for further study include the use of mice homozygous for null mutations in the *PACAP38* gene and injecting the PACAP antagonist *in ovo*.

There is a growing body of evidence that indicates that collaborative actions between growth factors are important in cell fate decisions and that cross talk occurs between signaling pathways (Ip et al., 1994; Janknecht and Hunter, 1999). In contrast, there is very little evidence of antagonistic actions between growth factors. We did, however, find such an effect: CNTF and NT-3 blocked PACAP's promotion of neuronal

differentiation. The simplest interpretation of these results is that each of these factors is affecting precursor cells which express receptors for each of these neurotrophic factors. As precursor cells, they can either divide or differentiate and their fate is determined by an integration of the signals they receive. Given our results, the scenario would be the following: if PACAP and CNTF or NT-3 bind to a precursor cell, the fate promoted by CNTF or NT-3, proliferation, is favored for some reason.

Our data, however, do not fully support the above-described model. Specifically, the noted increase in proliferation caused by both NT-3 and CNTF is higher than expected given the number of mitotically-active cells expressing these receptors. That is, twenty percent of the dividing cells of the interior region of the DRG are trkC+ and none of the mitotically active cells in the outer areas of the DRG are trkC+ (Rifkin et al., submitted). In other words, a small percentage of the 30% mitotically-active cells at this stage in the development of the DRG are trkC+. Furthermore, after a 3 hour [^{3}H]thymidine pulse at E5, only 5% of the [^{3}H]thymidine+ cells expressed the CNTF receptor (v. Holst et al., 1997). Thus, the 24% increase in proliferation induced by both NT-3 and CNTF was higher than expected if these factors are acting directly on precursor cells. One possible explanation is that these neurotrophic factors are upregulated by these cells in culture.

Another interpretation is that these neurotrophic factors are acting on post-mitotic cells which are in turn affecting the mitotically-active cells in this cell population. Previous research indicates that the axonal signal β-neuregulin regulates precursor cell survival and promotes mitosis and survival of perinatal Schwann cells (Jessen and Mirsky, 1999). Perhaps NT-3 is inducing post-mitotic cells to signal glial precursor cells to divide via β-neuregulin. In support of this interpretation, cells in these cultures, though not plated at such a density that they must touch each other, are often seen in contact with each other,

usually a neurite to a cell body. And, neurites have been observed in contact with dividing cells.

Given that PACAP promoted neuronal differentiation, but did not affect proliferation, the simplest interpretation is that PACAP is inducing recently post-mitotic cells to become neurons. In contrast, CNTF induced proliferation and decreased neuronal numbers. Thus, perhaps CNTF is acting directly on precursor cells, inducing them to proliferate instead of differentiate. However, given the distinct effects of CNTF and NT-3 and that we did not see an additive effect of CNTF and NT-3, a clear argument in favor of an indirect or direct effect of CNTF cannot be made. Double staining with antibodies to the CNTF receptor and trkC would tell us the extent of co-expression of these two receptors. We also observed antagonistic effects between NT-3 or CNTF and PACAP, which supports the idea that these factors are affecting the same group of cells. The most direct way to understand which cell population(s) these factors are acting on would be to isolate both the mitotically-active and post-mitotic cells and then study the effects of these neurotrophic factors on these two subpopulations in a manner similar to that done in this study.

A possible argument in favor of these factors acting directly on precursor cells is that the noted results occured within a very short culture period. That is, one may find it hard to imagine the following events occurring in six hours: a growth factor binds to a post-mitotic cell, its signal is transduced, the cell in response upregulates and secretes a protein, this protein binds to a precursor cell, its signal is transduced, and finally the cell responds by dividing or differentiating. This scenario becomes even more complex when one considers the observed antagonistic effects. Yet, it is possible for this series of events

to occur in such a short-time period. And, as stated above, cells in these cultures are often seen in contact with each other, potentially simplifying the scenario described above.

Whether these neurotrophic factors are acting on precursor cells or post-mitotic cells or some combination, an examination of the signal transduction pathways activated by each of these growth factors provides a possible model for the integration of these signals. Indeed, such signal integration is becoming a common part of detailing the steps involved in cell fate decisions. For example, Janknecht and Hunter (1999) note that p300, a nuclear transcriptional coactivator, facilitates synergistic cross talk between two different signaling pathways, those of LIF and BMP2 (bone morphogenetic protein 2). Reviewing the work of others on signal transduction pathways, we find that each of the three neurotrophic factors used in this study primarily activate different pathways, but that there is some overlap in these pathways. Specifically, NT-3 binds to trkC, a receptor tyrosine kinase, which leads to the phosphorylation of GRB2/SHC and a pathway which includes Sos, Ras, Raf, MEK, and MAPK/Erk (Figure 8). CNTF is known to stimulate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Bonni et al., 1997; v. Holst et al., 1997; Stahl and Yancopoulos, 1994; Boulton et al., 1994; Ip, 1998). Of more interest to us, CNTF has also been shown to activate Erks, Raf, and SHC/GRB2, implying a shared pathway with receptor tyrosine kinases (Figure 8, Boulton et al., 1994). Indeed, in cortical precursor cells, activation of the JAK-STAT pathway by CNTF promoted gliogenesis; when such activation was combined with inhibition of the Ras-Erk signaling pathway, CNTF's promotion of the JAK-STAT pathway increased (Bonni et al., 1997). The authors thus propose that CNTF receptor activation of the Ras-Erk signaling pathway opposes the JAK-STAT pathway in promoting gliogenesis and suggest the possibility that the Ras-Erk signaling pathway is important for proliferation or neuronal

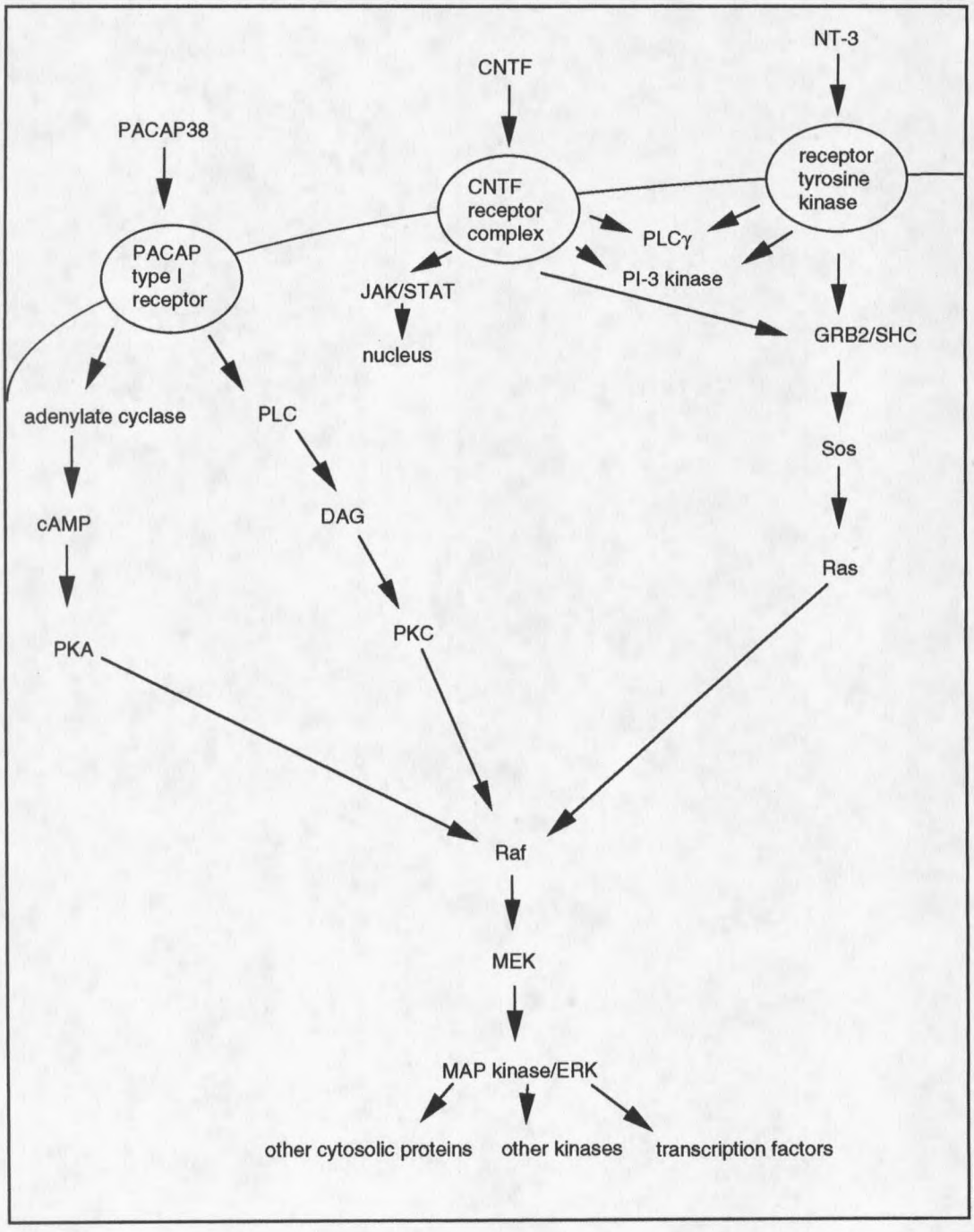

Figure 8. Signal transduction pathways of PACAP, CNTF, and tyrosine kinase receptors.

differentiation of cortical precursor cells. Interestingly, we found CNTF to promote proliferation in our cell population and it is the Ras-Erk pathway which can also be activated by NT-3 and PACAP. It could be that in this cell population, CNTF promotes proliferation via the Ras-Erk pathway and promotes gliogenesis (possibly observed as a decrease in neuronal number) via the JAK-STAT pathway. Our preliminary data indicates that when NT-3 and CNTF are combined, NT-3 blocks CNTF's inhibition of neuronal differentiation; a possible explanation is that the Ras-Erk pathway is now favored over the JAK-STAT pathway. To test this, one could separately block both the Ras-Erk or JAK-STAT pathways, treat the cells with CNTF, and note any changes in either of the noted effects (the increase in proliferation and decrease in neuronal differentiation).

While these three growth factors utilize different receptors and signal transduction pathways, all three activate pathways which converge downstream on the same substrates (Figure 8). In particular, given our current understanding of these pathways, Raf is the most likely point of cross talk as both PACAP pathways and the Ras-Erk pathways of CNTF and NT-3 converge on it. It is also possible that CNTF and NT-3 are interacting via phospholipase Cγ (PLCγ) and/or phosphatidylinositol-3 kinase (PI-3 kinase) as both of their respective receptors have been shown to associate with these signaling molecules. There is not, however, a common substrate with PACAP's signaling pathways and these molecules. It is also possible that the receptors for PACAP, CNTF, and NT-3 are interacting with each other, but there is nothing in the literature indicating such interactions.

Given our understandings of these signal transduction pathways, one explanation for our results is that through downstream convergence the cellular fate of proliferation versus neuronal differentiation is somehow determined. One possibility is that CNTF and NT-3 may more quickly activate their respective pathways with the result being that

proliferation is favored over neuronal differentiation. Intriguingly, it appears that in a different cell population, again one that is derived from the neural crest, the opposite occurs: PACAP38 has been shown to almost completely abolish bFGF, NGF, and IGF-II's induction of proliferation in neonatal chromaffin cells (Frodin et al., 1995). How is it that in one cell population one factor "wins out" and in another population the other factor does? This contrast serves to highlight the complexities involved in cell fate determination and our current simplistic understandings of these complexities. Future studies examining the above-described potential cross talk points of these pathways would likely improve our understandings of how such cell fate decisions are made.

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