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
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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.

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

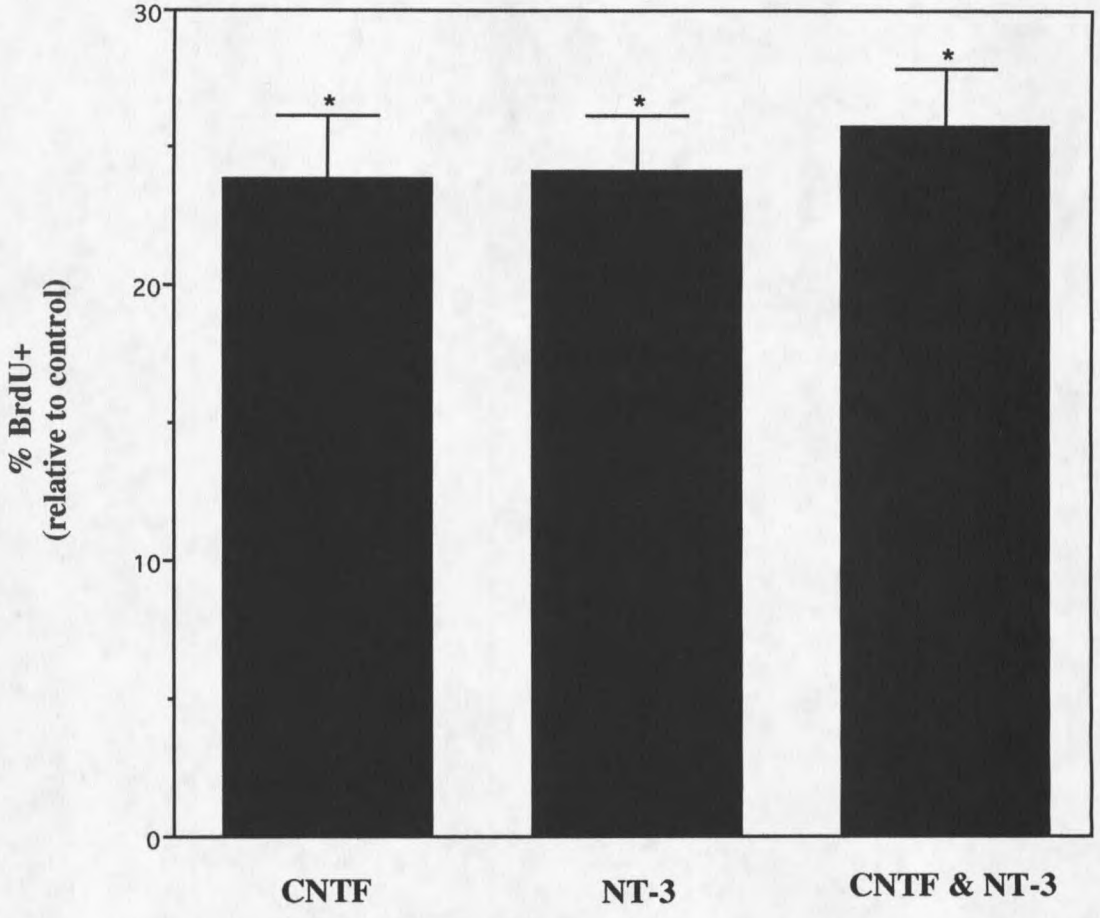

Figure 1. CNTF and NT-3 promote proliferation of embryonic DRG cells, but their effects are not additive. DRG from chick E4.5 were dissociated and then cultured for 6h. CNTF (10 ng/ml) and/or NT-3 (10 ng/ml) and BrdU were added at the time of plating. Within each experiment, each treatment was tested 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; $p \leq 0.002$.

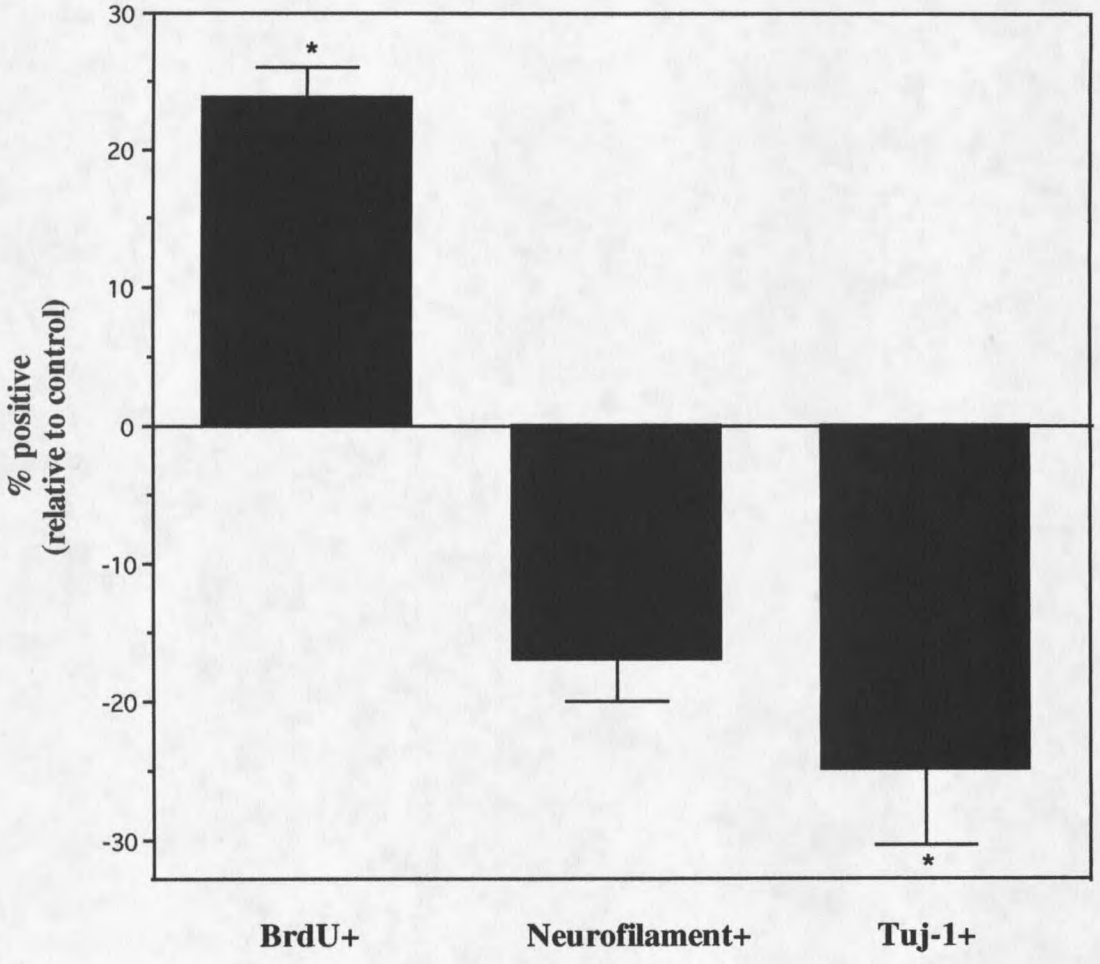

Figure 2. CNTF's effects on proliferation and neuronal differentiation of embryonic DRG cells. DRG from chick E4.5 were dissociated and then cultured for 6 h. CNTF (10 ng/ml) and BrdU (for proliferation assays) were added at the time of plating. Within each experiment, each treatment was tested in four 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).