Neurotrophins and the Neuroendocrine Brain: Different Neurotrophins Sustain Anatomically and Functionally Segregated Subsets of Hypothalamic Dopaminergic Neurons

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Hypothalamic neurons control a variety of important hormonal and behavioral functions. Little is known, however, about the neurotrophic factors that these neurons may require for survival and/or maintenance of their differentiated functions. We conducted experiments to examine this issue, utilizing a combination of immunohistochemical, *in situ* hybridization and cell culture approaches. We found that the low affinity receptor for nerve growth factor (p75 NGFR) is present in small subsets of hypothalamic peptidergic neurons identified as such by their content of galanin, luteinizing hormone-releasing hormone (LHRH) and vasointestinal peptide (VIP). More prominently, however, examination of hypothalamic dopaminergic (DA) neurons for the presence of p75 NGFR-like immunoreactivity revealed that the receptor was present on tyrosine hydroxylase (TH)-positive neurons of the zona incerta and periventricular region, but not on neuroendocrine DA neurons of the tuberoinfundibular region. *In situ* hybridization experiments using a p75 NGFR cRNA confirmed this distribution. Regardless of the presence or absence of p75 NGFR, neither DA group expresses *trk*A mRNA, indicating that these two major hypothalamic subsets of DA neurons are NGF-insensitive. A substantial fraction of TH mRNA-positive cells in the zona incerta expresses *trk*B mRNA, which encodes the receptor for brain derived neurotrophic factor (BDNF); in turn BDNF supports the *in vitro* survival of hypothalamic TH neurons bearing p75-NGFR, suggesting that BDNF is trophic for DA neurons of the zona incerta. In contrast, tuberoinfundibular DA neurons do not express *trk*B mRNA, but some have *trk*C mRNA, which encodes the receptor for neurotrophin-3 (NT-3). The *in vitro* survival of TH neurons devoid of p75-NGFR is supported by NT-3, implying that NT-3 may be trophic for a subset of tuberoinfundibular DA neurons. These results suggest that, in spite of expressing an identical neurotransmitter phenotype, anatomically and functionally segregated DA neurons of the neuroendocrine brain are sustained by different neurotrophic factors.

[Key words: neurotrophins, neurotrophin receptors, hypothalamic neurons, dopaminergic neurons, neuronal survival, neuroendocrine brain]

It is well established that the CNS produces a variety of soluble polypeptides which support the growth, cellular differentiation, and survival of neurons. Prominent among these proteins is the neurotrophic family of growth factors which include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (Levi-Montalcini, 1987; Leibrock et al., 1989; Maisonpierre et al., 1990b; Ernfors et al., 1990; Hohn et al., 1990; Rosenthal et al., 1990; Berkemeier et al., 1991; Hallböök et al., 1991; Ip et al., 1992). Different members of the neurotrophin family have been shown to promote the survival of specific neuronal subpopulations in brain. Thus, while the trophic effects of NGF are limited to cholinergic neurons of the basal forebrain (Hefti, 1986), BDNF supports the survival and differentiation of both cholinergic neurons of the basal forebrain (Alderson et al., 1990; Knüsel et al., 1991; Lamballe et al., 1994) and dopaminergic (DA) neurons of the substantia nigra/ventral mesencephalon (Hyman et al., 1991; Knüsel et al., 1991). In contrast to BDNF and NGF, NT-3 does not affect the survival and/or differentiation of cholinergic neurons of the basal forebrain (Knüsel et al., 1991). NT-3 does, however, promote the survival of mesencephalic DA neurons (Hyman et al., 1994) and exert trophic effects on developing hippocampal cells (Collazo et al., 1992).

All neurotrophins bind with low affinity to a membrane spanning receptor molecule known as the low-affinity NGF receptor (p75-NGFR) (reviewed in Meakin and Shooter, 1992). Although p75-NGFR may contribute to the initiation of NGF biological actions (Hempstead et al., 1991, reviewed in Bothwell, 1991, and Meakin and Shooter, 1992) it is now well established that the trophic effects of all neurotrophins, including NGF (Jing et al., 1992), require binding to recognition molecules of the *trk* tyrosine protein kinase family of receptors (Barbacid et al., 1991; Meakin and Shooter, 1992). This family includes *trk*A, which preferentially binds NGF (Kaplan et al., 1991; Klein et al., 1991); *trk*B, which recognizes BDNF and NT-4/5 (Squinto et al., 1991; Soppet et al., 1991; Klein et al., 1992; Ip et al., 1993); and *trk*C, the receptor preferred by NT-3 (Lamballe et al., 1991; Tsoulfas et al., 1993).
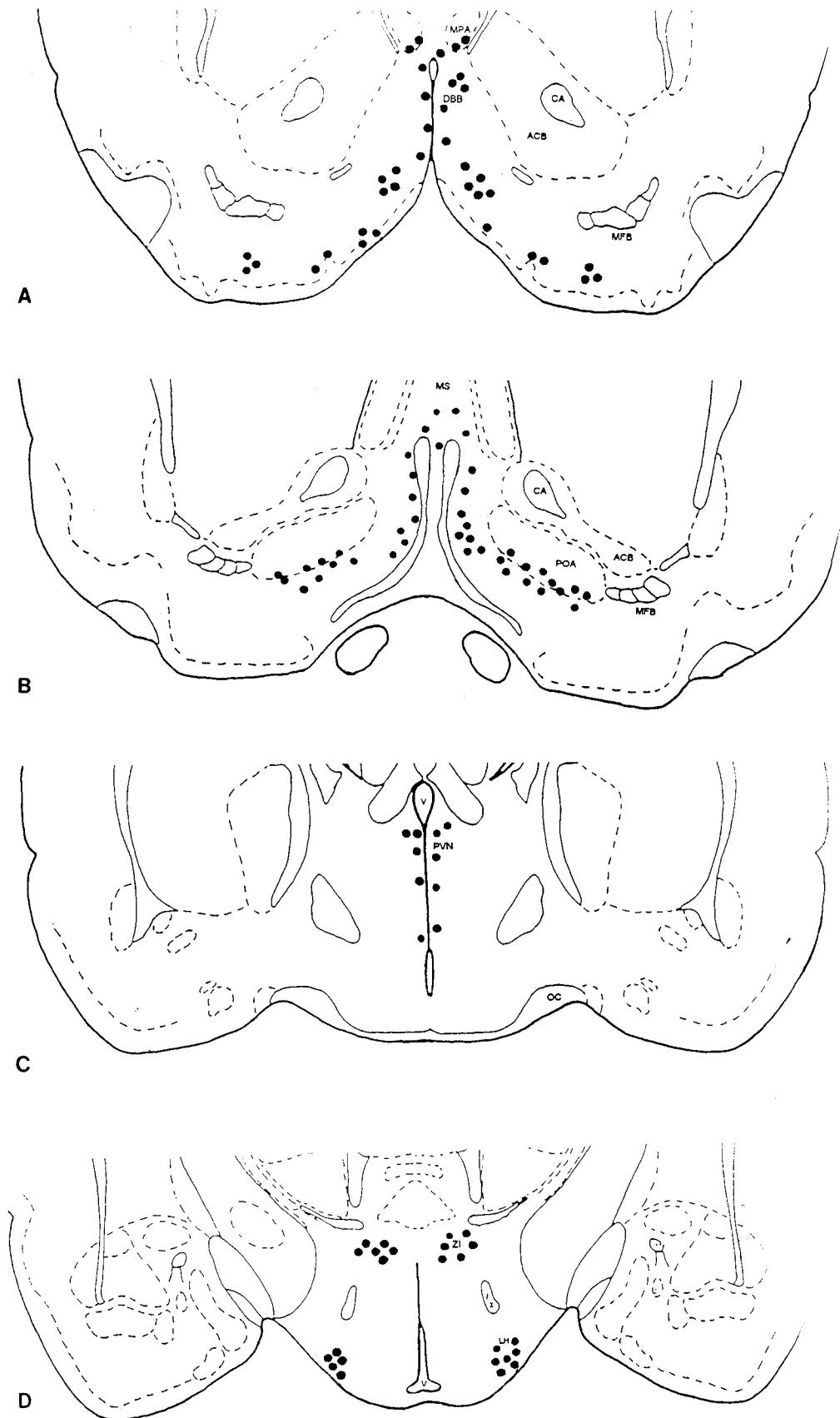
Figure 1. Drawing depicting the rostrocaudal (*A–D*) distribution of p75-NGFR-positive neurons localized by immunohistochemistry in the immature rat forebrain and hypothalamus. The number of dots represents the relative density of positive cells within the respective regions. *MPA*, Medial preoptic area; *POA*, preoptic area; *DBB*, diagonal band of Broca; *PVN*, paraventricular nucleus; *LH*, lateral hypothalamus; *ZI*, zona incerta; *CA*, commissura anterior; *OC*, optic chiasm; *FX*, fornix; *V*, third ventricle; *ACB*, nucleus accumbens; *MFB*, median forebrain bundle; *MS*, medial septum.
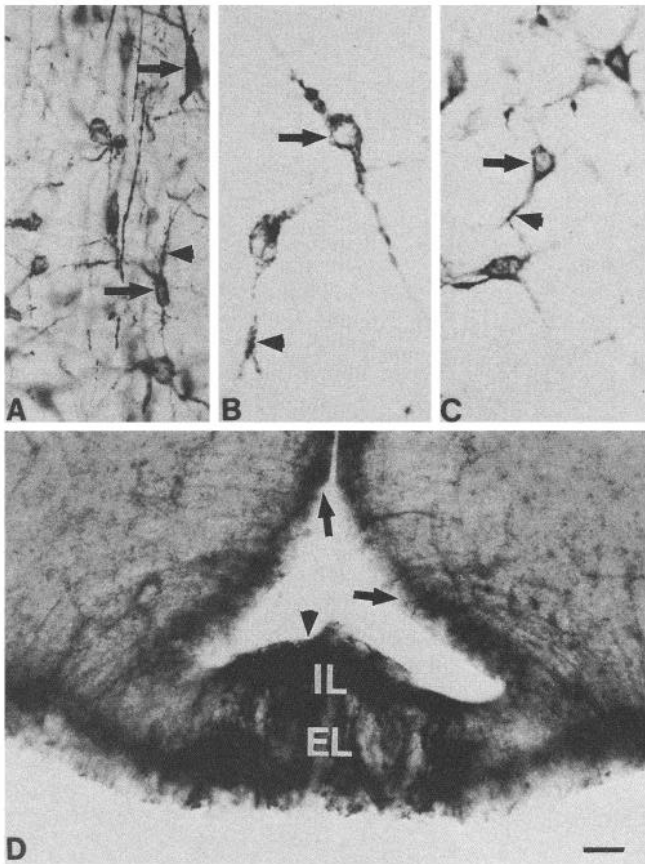
Figure 2. Localization of the p75-NGFR immunoreactive material in the immature rat forebrain and hypothalamus. *A–C*, Note the immunoreactivity on the soma (*arrows*) and processes (*arrowheads*) of neurons in the diagonal band of Broca (*A*), zona incerta (*B*), and lateral hypothalamic area (*C*). *D*, Depicts the intense immunoreactivity on the ependymal (tanycyte) cells lining the wall (*arrows*) and the floor (*arrowheads*) of the third ventricle, and on their processes within the internal and external layers of the median eminence. *IL*, Internal layer; *EL*, external layer. Scale bars: *A*, 15 μm; *B* and *C*, 7 μm; *D*, 30 μm.

To date, a great deal of attention has been devoted to the presence of neurotrophins and their receptors in different brain areas and to the trophic effects that neurotrophins may exert on cholinergic neurons of the basal forebrain and mesencephalic DA neurons. Little is known, however, about the neuronal populations that may be responsive to neurotrophins in the hypothalamus—a region of the CNS critically important not only for the control of the endocrine system, but also for the manifestation of a variety of behavioral functions. Distribution studies have shown that while the developing postnatal hypothalamus expresses BDNF mRNA (Maisonpierre et al., 1990a; Friedman et al., 1991), it contains only very low levels of NGF mRNA (Korsching et al., 1985; Gonzalez et al., 1990) and no NT-3 mRNA (Ernfors et al., 1990). Within the hypothalamus, BDNF expression is more prominent in the ventromedial nucleus of the medial basal hypothalamus and the preoptic area in the rostral hypothalamus (Friedman et al., 1991).

Neurotrophic receptors are also found in the neuroendocrine brain. For instance, the hypothalamus expresses p75-NGFR mRNA (Ojeda et al., 1990) and displays active p75-NGFR protein, as assessed by cross-linking assays (Ojeda et al., 1990). At the cellular level, the most predominant sites of expression of p75-NGFR have been localized immunohistochemically to the suprachiasmatic nucleus and tanycytes of the third ventricle (Yan and Johnson, 1988; Koh et al., 1989). Less information exists concerning the hypothalamic expression of *trk* genes. Although a general inspection revealed no *trk*A mRNA-expressing cells in this region (Holtzman et al., 1992), *trk*B mRNA was detected throughout the medial hypothalamic region (Valenzuela et al., 1993) and in the median eminence (Klein et al., 1990). Recent *in situ* hybridization studies revealed the presence of *trk*C mRNA in the dorsal and ventromedial nuclei of the medial hypothalamus and the median eminence (Lamballe et al., 1994).

The present study was undertaken to identify some of the neuronal populations responsive to neurotrophins in the developing hypothalamus. To this end, we first identified the neurotransmitter phenotypes of those hypothalamic neurons displaying immunoreactive p75-NGFR, then defined the type of *trk* receptor expressed by the most prominent subset of p75-NGFR-containing neurons and, lastly, determined the ability of different neurotrophins to promote the survival of these neurons in culture. The results suggest that the two major groups of hypothalamic dopaminergic neurons—those of the zona incerta and those of the tuberoinfundibular region—differ in their sensitivity to neurotrophins. While both the p75-NGFR and *trk*B genes are expressed in DA neurons of the zona incerta, no detectable levels of either receptor mRNA were seen in tuberoinfundibular DA neurons; however, some of these neurons contain *trk*C mRNA. As cultured hypothalamic DA neurons expressing p75-NGFR were found to require BDNF for survival, whereas DA neurons devoid of p75-NGFR are sustained by NT-3, the results suggest that BDNF is mostly trophic for DA neurons of the zona incerta whereas NT-3 mainly supports the survival of tuberoinfundibular DA neurons.

A partial report of these findings has appeared (Dees et al., 1993).

Materials and Methods

Animals

Localization of neurotrophin receptors in hypothalamic neurons by either immunohistochemistry or hybridization histochemistry was performed in immature 28–29 d old female Sprague–Dawley rats. Assessment of neuronal survival in response to neurotrophins was carried out in primary neuronal cultures prepared from 18 d old rat fetuses.

Antibodies

p75-NGFR was identified with monoclonal antibody IgG 192 (kindly provided by Dr. Eugene Johnson, Washington University School of Medicine, St. Louis, MO). This antibody, developed by Chandler et al. (1984), has been extensively used for the immunohistochemical detection of p75-NGFR by several investigators (Taniuchi et al., 1988; Yan and Johnson, 1988), including ourselves (Dissen et al., 1991). Neurons were identified with an antiserum to neuron specific enolase (NSE, Polysciences Inc., Warrington, PA), the $\gamma\gamma$-isoenzyme of enolase (2-phospho-D-glycerate hydrolase) which is expressed in neurons and neuroendocrine cells (Marangos and Schmechel, 1987).

Double-label immunohistochemistry was performed using anti-NGFR and the following polyclonal antisera: anti-choline acetyltransferase (CAT, kindly provided by Dr. Louis B. Hersh, Southwestern Medical Center, Dallas, TX), anti-galanin (GAL, kindly provided by Dr. Jim Koenig, Immunobiology Research Institute, Allendale, NJ), anti-interleukin Iα (IL-1α, Genzyme, Boston, MA), anti-tyrosine hydroxylase (TH, Eugene Tech, Allendale, NJ), and anti-neuropeptide Y (NPY, Dr. J. K. McDonald, Emory University School of Medicine, Atlanta, GA). Luteinizing hormone releasing hormone (LHRH)-containing neurons were identified using monoclonal antibody 4H3 (kindly provided by Dr. Henryk Urbanski, Oregon Regional Primate Research Center, Beaverton, OR).
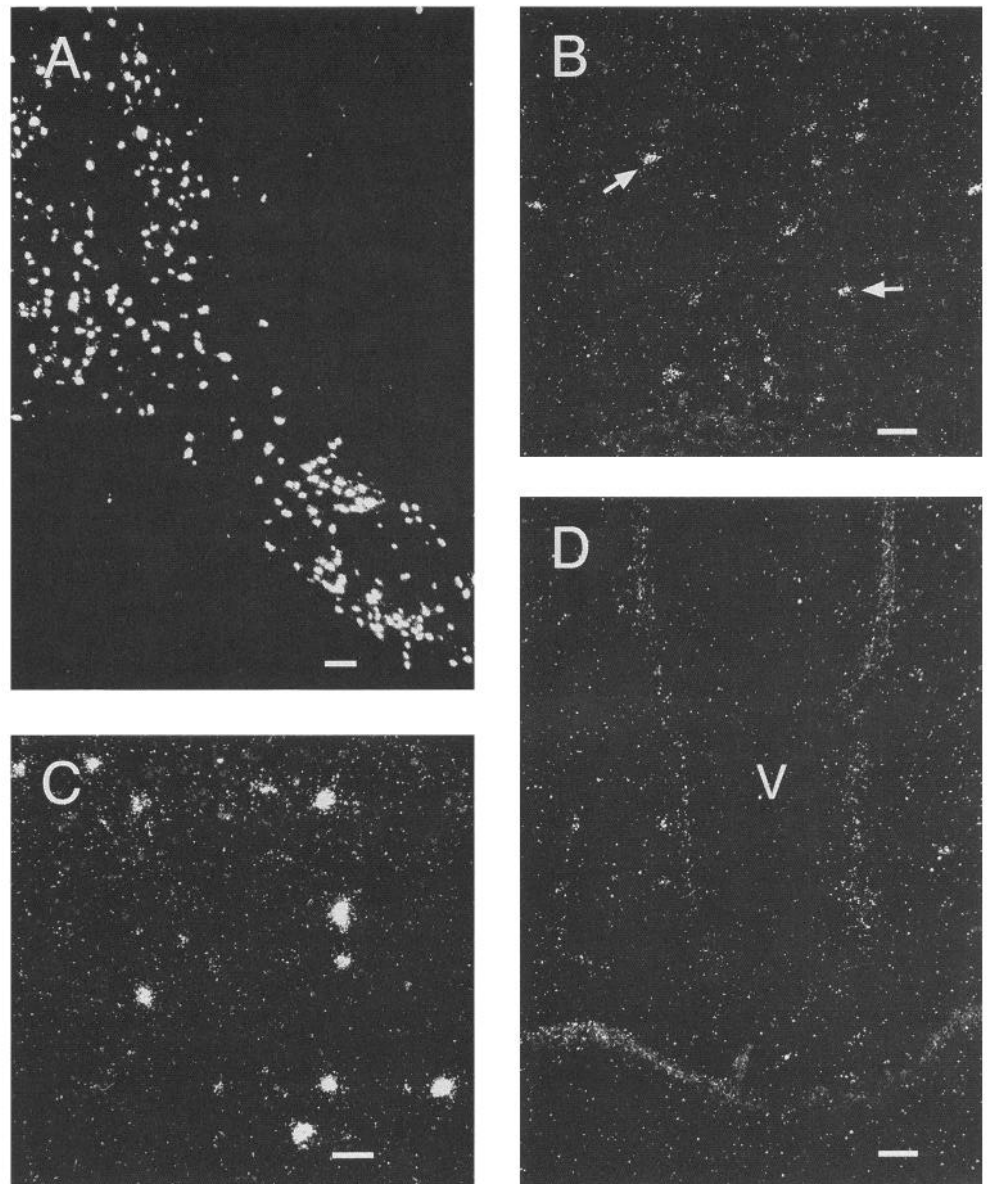
Figure 3. Localization of p75-NGFR mRNA in the forebrain and hypothalamus of immature rats by hybridization histochemistry. *A,* The diagonal band of Broca; *B,* zona incerta; *C,* lateroventral hypothalamus; *D,* absence of detectable p75-NGFR mRNA in the arcuate nucleus-median eminence region of the hypothalamus. Scale bars: *A,* 40 μm; *B* and *D,* 20 μm; *C,* 10 μm.

Immunohistochemistry

NGFR. Animals were transcardially perfused with isotonic saline and then perfusion-fixed with Zamboni's solution. The brains were removed and postfixed in the same fixative for 24 hr, then kept in 0.05 M Tris buffered saline (TBS) pH 7.6 at 4°C until sectioning.

Immunocytochemical staining was performed on 40 μm Vibratome sections which were floated in 0.05 M TBS, pH 7.6. After three 10 min washes in TBS, the sections were floated for 2 hr in TBS containing 1% H_2O_2 to inhibit endogenous peroxidases. Sections were rinsed in TBS, and then washed twice for 10 min each in TBS A (TBS plus 0.5% bovine serum albumin and 0.2% Triton X-100) and twice for 10 min each with TBS B (TBS plus 1.5% normal goat serum). Brain sections from the diagonal band of Broca, rostrally, to the arcuate nucleus/median eminence region, caudally, were incubated with a 7.5 μg/ml dilution of the anti-p75-NGFR serum for 24 hr at 4°C. Tissues were rinsed as above in TBS A and TBS B, then incubated for 1 hr at room temperature with preabsorbed biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:250. After washes in the two buffers, the sections were incubated in the avidin–biotin complex (ABC) for 75 min at room temperature. The sections were rinsed and exposed to TBS containing 0.02% 3,3'-diaminobenzidine HCl (DAB) with 0.003% H_2O_2 and 2.5% nickel for 10 min, generating a black reaction product due to the nickel intensification of the dark brown, DAB stain. The reaction was stopped by washing in TBS; then the sections were mounted on gelatin-coated slides. Finally, they were dehydrated in graded alcohols, cleared in Histoclear (VWR Scientific, Philadelphia, PA), coverslipped, and viewed with a Leitz microscope.

Double-label immunohistochemistry. These experiments were performed to determine the neuronal phenotypes expressing p75-NGFR. The tissues were first overlaid with either (1) anti-CAT (1:1000), (2) anti-GAL (1:250), (3) anti-IL-Iα (1:400), (4) anti-NPY (1:500), (5) anti-LHRH (1:1000), or (6) anti-TH (1:500). The localization of each of these substances was visualized using the procedure described above except that the second antibody was biotinylated goat anti-rabbit-IgG (1:250) and the tissues were exposed to DAB/H_2O_2 only (without nickel) yielding a dark brown final reaction product. After the DAB stain, the tissues were washed in TBS, then the above described procedure for immunohistochemical localization of NGFR was followed except the DAB/nickel intensification staining procedure was replaced with the previously described benzidine dihydrochloride procedure (Lokos and Basbaum, 1986), which yields a punctate dark blue reaction product (Junier et al., 1991).

In the case of hypothalamic cultures, TH positive neurons were identified using the TH antiserum at a 1:1500 dilution and p75-NGFR using
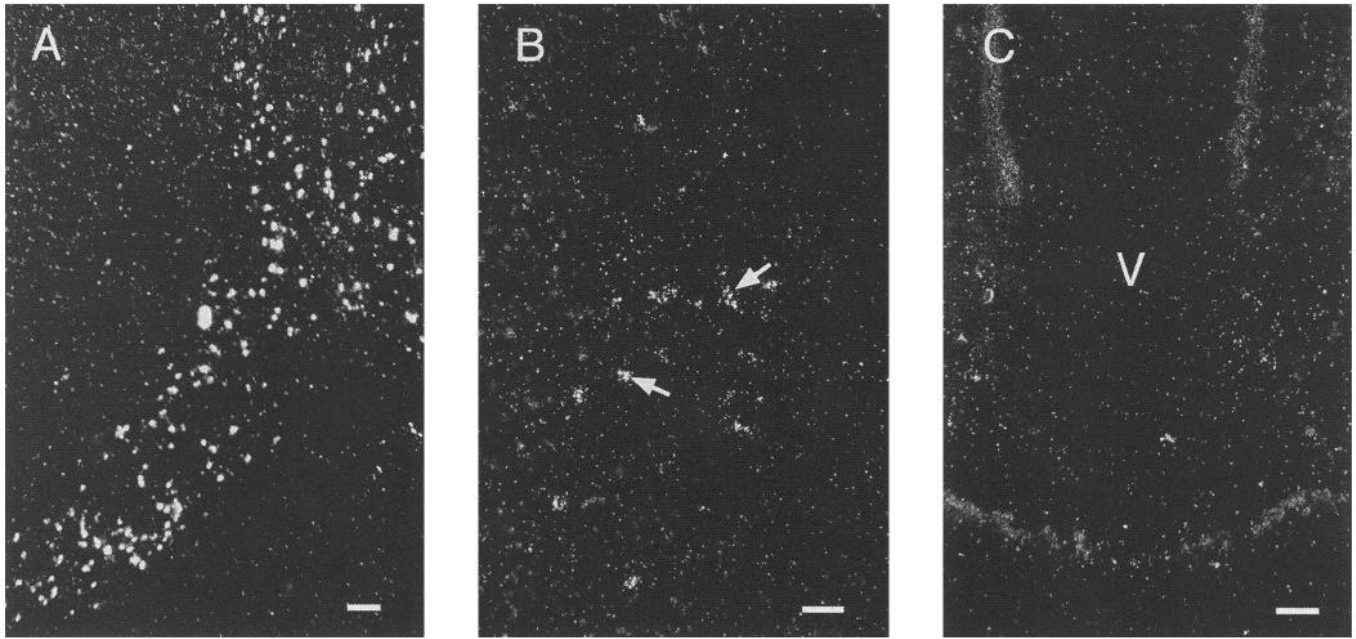
Figure 4. Localization of *trk*A mRNA in the forebrain and hypothalamus of immature rats by hybridization histochemistry. *A,* Diagonal band of Broca; *B,* lateroventral hypothalamus; *C,* absence of detectable *trk*A mRNA in the arcuate nucleus-median eminence region of the hypothalamus. Scale bars: *A,* 40 μm; *B* and *C,* 20 μm.

antibody IgG 192 at 2 μg/ml. The NSE antiserum employed for the broad identification of neurons was used at a 1:500 dilution. The immunohistochemical reactions were developed as described above.

Hybridization histochemistry

The hybridization protocol used, which is based on that reported by Simmons et al. (1989), has been described elsewhere (Dissen et al., 1991; Junier et al., 1991). The brains were fixed by transcardiac perfusion with 4% paraformaldehyde in borate buffer pH 9.5, postfixed at 4°C for 18 hr in the same fixative containing 10% sucrose, blocked, and stored at −85°C in air-tight containers before further processing. Twenty micrometer sections were prepared with a sliding microtome, mounted on Superfrost Plus (Fisher Scientific, Kent, WA) precleaned slides, dried overnight, and stored at −85°C before hybridization. Following treatment with proteinase K and acetic anhydride (Simmons et al., 1989), the sections were overlaid with 70 μl of hybridization solution (50% formamide, 0.2 M NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA, 2× Denhardt's solution) containing 1 × 10⁷ cpm/ml labeled probe (p75-NGFR, *trk*A or TH cRNA, see below). Hybridization was for 18–20 hr at 55–60°C; thereafter, the slides were washed four times in 4× sodium chloride-sodium citrate (SSC) prior to RNase A digestion (20 μg/ml for 30 min at 37°C), rinsed at room temperature in decreasing concentrations of SSC (2, 1, and 0.5×) containing 1 mM dithiothreitol (DTT) to a final stringency of 0.1× SSC at 65°C for 30 min. After dehydration in graded alcohols also containing 1 mM DTT and 0.1× SSC, the slides were exposed to Hyperfilm x-ray film for 3 d, defatted, and dipped in NTB-2 emulsion. The reaction was developed 3 weeks later, the sections were counterstained with 0.1% thioneine and analyzed under both bright- and dark-field illumination.

For experiments involving double label *in situ* hybridization, a combination of the above protocol and a modification of the nonisotopic *in situ* hybridization protocol described by Springer et al. (1990) was used. Digoxygenin UTP-labeled *trk*B and *trk*C cRNAs were prepared by *in vitro* transcription using 1 μl of a 2 mM digoxigenin-UTP solution; 500 ng of cDNA template (see below); 2.5 mM of each ATP, CTP, and GTP; and 15–20 U of either SP6 (*trk*B) or T3 (*trk*C) RNA polymerase. Following 1 hr of incubation at either 40°C (*trk*B) or 37°C (*trk*C), the reaction mixtures were treated with DNase, stabilized with DTT and salt, and the volume was adjusted to 100 μl with 20 mM DTT. The cRNA transcripts were then precipitated with ethanol, dried and resolubilized in 100 μl diethyl pyrocarbonate (DEPC)-treated water. Five microliters of the mixture were then run on a formaldehyde-

agarose gel to assess the integrity of the cRNAs and the yield of the reaction. For hybridization, 25–50 μl of either digoxygenin-labeled cRNA were added to 500 μl of hybridization buffer containing 1 × 10⁷ cpm/ml of ³⁵S-UTP TH cRNA. Following hybridization and post-hybridization washes, which were carried out as described above, the slides were not dehydrated after the last 0.1 SSC wash, but instead were incubated overnight in 2× SSC containing 0.05% Triton X-100 and 2% normal goat serum at room temperature. The next day the slides were incubated with anti-digoxygenin-alkaline phosphatase conjugate (1:1000) for 5 hr at room temperature. The slides were then rinsed and incubated overnight at room temperature in a chromogen solution containing 0.338 mg/ml 4-nitroblue tetrazolium chloride (NTB) and 0.175 mg/ml 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and 3 mg/ml of levamisole. The staining reaction was then stopped by placing the slides in 10 mM Tris-HCl with 1 mM EDTA, pH 8. After extensive washes in potassium phosphate-sodium chloride buffer pH 7.4, the sections were quickly dehydrated in ethanol containing DTT and 2× SSC, dried under vacuum, examined for digoxygenin-labeled *trk*B or *trk*C cRNA hybridization, and processed for autoradiography to detect ³⁵S-labeled TH mRNA hybrids. To avoid chemoautoradiographic signals, the slides were dipped in Ilford K5 emulsion (without defatting) instead of the NTB-2 emulsion used for regular isotopic hybridization. After 2 weeks of exposure the slides were developed, quickly dehydrated, dried under vacuum for 30 min, and coverslipped for microscopic examination.

Nucleic acid probes

p75-NGFR mRNA was detected with a 271 nucleotide (nt) cRNA transcribed from a rat cDNA complementary to nt 430–700 in p75-NGFR mRNA (Buck et al., 1988) subcloned into the pGEM-3Z vector. Transcription of the antisense strand was performed using SP6 polymerase. *trk*A mRNA was detected with a *trk*A cRNA complementary to nt 273–747 in *trk*A mRNA (Meakin et al., 1992). Transcription of the antisense strand from the cDNA template subcloned into pGEM-3Z was also directed by SP6 polymerase. TH mRNA was detected with a 280 nt TH cRNA complementary to nt 1241–1521 in TH mRNA (Grima et al., 1985); the probe was transcribed with SP6 polymerase from a cDNA template subcloned into the pGEM-3Z vector. *trk*B mRNA was detected with a 241 nt antisense RNA complementary to nt 2344–2585 in the intracellular-encoding domain of *trk*B mRNA (Middlemas et al., 1991); the cDNA template was subcloned into the pGEM-3Z vector. *trk*C was identified with a 409 nt cRNA probe complementary to nt 2266–2675

Figure 5. Double-label immunohistochemistry illustrating specific neuronal phenotypes expressing p75-NGFR. *A, Arrows* depict three CAT-positive neurons (*brown*) in the diagonal band of Broca that also express p75-NGFR (*punctate blue*). *Arrowheads* depict other neurons that are CAT-negative, but NGFR-positive. *B, Arrow* depicts a GAL-positive neuron (*brown*) in the paraventricular nucleus of the hypothalamus that also expresses the NGFR (*punctate blue*). The *arrowhead* depicts a GAL-positive neuron that is NGFR-negative. *C* demonstrates two immunopositive IL-Iα-immunopositive neurons in the lateral preoptic area that are NGFR-negative (*arrows*) and one neuron that is IL-Iα-negative but p75-NGFR-positive (*arrowhead*). *D* and *E* illustrate NPY-positive neurons in the cortex and arcuate nucleus of the hypothalamus, respectively. Note that only NPY neurons in the cortex express NGFR, as illustrated in *D* by the punctate reaction on the soma. *F* and *G* demonstrate LHRH-positive neurons in the medial preoptic area. Note that one LHRH neuron is NGFR-positive (*F*) as depicted by the punctate blue reaction, whereas the other LHRH neuron is NGFR-negative (*G*). Scale bar: *A*, 10 μm; *B–E*, 7 μm.
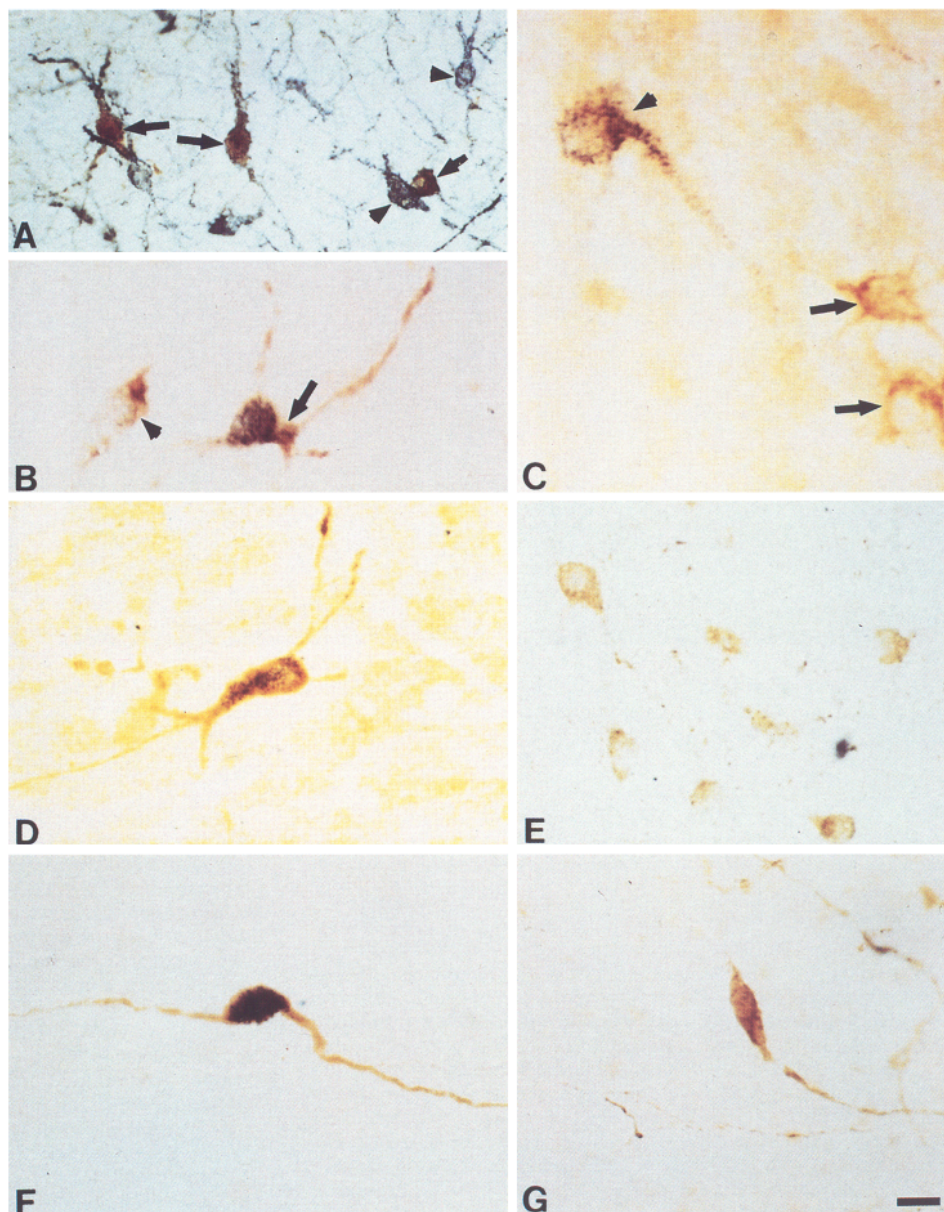
in the intracellular-encoding domain of *trk*C mRNA (Valenzuela et al., 1993); the antisense RNA probe was transcribed from a cDNA template subcloned into the pBluescript SK-vector. For each probe, hybridization controls consisted of tissue sections hybridized in the same assay with sense RNAs transcribed from the same cDNA templates used to synthesized the antisense probes.

Hypothalamic cultures

Hypothalami from 18 d old rat fetuses were mechanically dissociated using a Stomacher 80 blender (Tekmar Company, Cincinnati, OH) (1 min at 90% power) and seeded on eight-well Lab-Tek plastic culture chamber slides (Nunc, Inc., Napperville, IL) coated with polyornithine-laminin. For the coating, each chamber was treated overnight with polyornithine diluted in 15 mM borate buffer, pH 8.4, at 100 μg/ml (Edgar et al., 1984). Following extensive washing to remove unpolymerized polyornithine, the chambers were treated with laminin at 2 μg/ml in PBS for 2 hr at room temperature (Manthorpe et al., 1983). After drying, the cells were seeded at a density of 50,000 cells per well in DMEM-F12 medium containing 7.5% fetal calf serum (Hyman et al., 1991). After an overnight culture, the medium was replaced by serum-free medium supplemented with L-glutamine (2 mM), human transferrin

(100 μg/ml), putrescine (100 μM), sodium selenite (30 nM), and insulin (5 μg/ml) and the culture was continued for 6 more days, with one medium change 3 d after the addition of the serum-free medium. BDNF, NT-3, NGF, TGFα, or EGF (50 ng/ml each) were added on the day of plating and replenished twice, at the time of initiating the culture in chemically defined medium and 3 d later. On day 6, the cultures were fixed in 4% paraformaldehyde-PBS pH 7.4 for 30 min at room temperature and processed for combined immunohistochemical detection of TH and p75-NGFR (see above). Following completion of the immunohistochemical reactions, immunopositive cells were counted in each well and classified as either TH-, p75-NGFR-, or TH/p75-NGFR positive. To assess the viability of the cultures, phase-contrast photomicrographs were usually taken on day 6. In addition, the neuronal composition of the cultures was immunohistochemically assessed by the identification of NSE-positive cells.
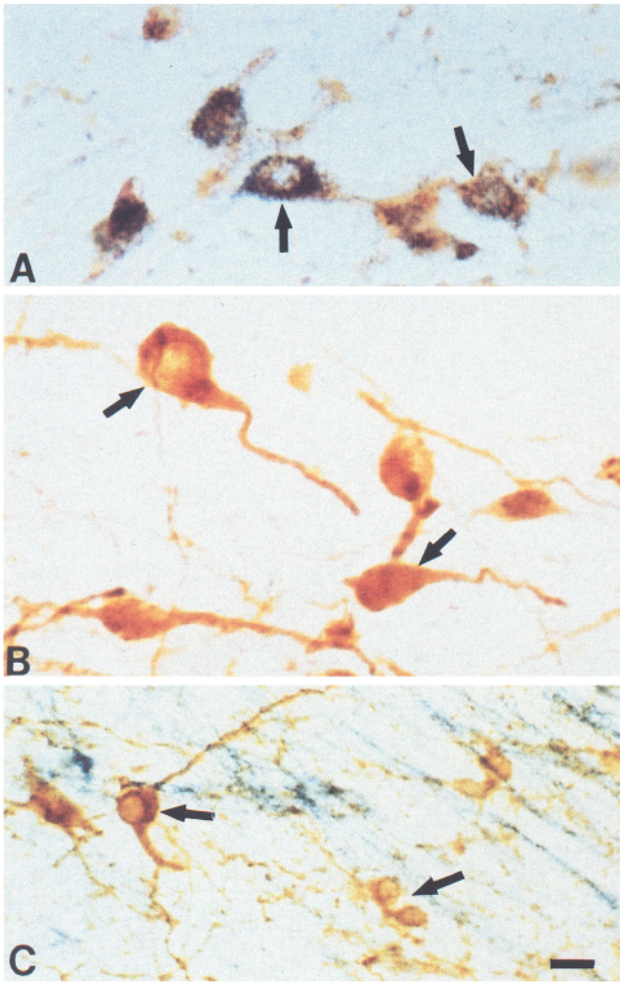
Figure 6. Double-label immunohistochemistry illustrating that some, but not all, dopaminergic neurons in the hypothalamus express the NGFR. *A, Arrows* show a population of TH-positive neurons (*brown*) in the zona incerta that are also NGFR-positive (*punctate blue*). *B* and *C, Arrows* depict TH-positive neurons in both the zona incerta (*B*) and the arcuate nucleus (*C*) that are NGFR-negative. None of the tuberoinfundibular (arcuate nucleus) DA neurons was found to be p75-NGFR positive. Scale bar, 7 μm.

Statistics

The effect of different growth factors on neuronal survival was analyzed using a one-way analysis of variance followed by the Student-Neuman-Keuls multiple comparison test for unequal replications.

Results

Localization of p75-NGFR protein in the forebrain and hypothalamus

Figure 1 depicts the immunohistochemical distribution of neurons expressing p75-NGFR in the forebrain and hypothalamus of immature female rats. The majority of these p75-NGFR–positive neurons were localized to the more rostral sections containing the diagonal band of Broca (Figs. 1A, 2A) and the medial and lateral preoptic areas (Fig. 1B). Positive cells were also seen scattered within the para- and periventricular regions of the rostral hypothalamus (Fig. 1C). More circumscribed groups of p75-NGFR–positive neurons were detected in the ventrolateral hypothalamic area of the intermediate region of the hypothalamus and in the zona incerta of the dorsal hypothalamus (Fig. 1D). p75-NGFR was identified on the soma of these neurons, as well

as on their unipolar, bipolar or multipolar processes (Fig. 2B,C). In the tuberoinfundibular area, intense staining was observed on the ependymal (tanycyte) cells lining the third ventricle, as well as on some of their processes within the median eminence, but not in neurons (Fig. 2D). Additionally, nerve fibers bearing p75-NGFR immunoreactivity were observed in all regions studied.

Localization of p75-NGFR mRNA and trkA mRNA in the forebrain and hypothalamus

Hybridization histochemistry using p75-NGFR and *trk*A antisense RNA probes verified the ability of these probes to detect p75-NGFR and *trk*A mRNAs in magnocellular neurons of the diagonal band of Broca, which was used as a positive control region (Figs. 3A, 4A, respectively). p75-NGFR mRNA was also found in a subset of neurons of the zona incerta (Fig. 3B), suggesting that the immunoreactive p75-NGFR material detected in neurons of this region (Figs. 1D, 2B) indeed corresponds to the protein product of the p75-NGFR gene. In contrast, none of the neurons of the zona incerta expressed detectable levels of *trk*A mRNA (not shown). A similar situation was observed in the periventricular region; while some cells scattered along the walls of the third ventricle were found to be p75-NGFR mRNA positive, no *trk*A mRNA-containing cells were detected in the same region.

A subset of neurons, detected in the same area of the ventrolateral hypothalamus that contains p75-NGFR immunoreactive cells (Fig. 1D), was found in adjacent sections to express both p75-NGFR mRNA (Fig. 3C) and *trk*A mRNA (Fig. 4B), suggesting that these cells are sensitive to NGF. In contrast to the correspondence between p75-NGFR immunoreactive material and p75-NGFR mRNA expression observed in neurons of the zona incerta, periventricular area and lateroventral hypothalamus, the intense p75-NGFR immunoreactivity detected in tanycytes of the median eminence (Fig. 2D), was not accompanied by significant p75-NGFR mRNA hybridization (Fig. 3D). This discrepancy was previously noted by others (Koh et al., 1989). Tanycytes also appeared devoid of *trk*A mRNA (Fig. 4C).

Identification of specific neuronal phenotypes expressing p75-NGFR

Using double-label immunohistochemistry we identified specific types of neurons in the forebrain and hypothalamus that bear NGFR. For a positive control, we confirmed the presence of p75-NGFR–bearing neurons containing choline acetyltransferase (CAT) immunoreactivity within the diagonal band of Broca, as described previously (Koh and Loy, 1988). These CAT-positive neurons were often found intermingled among other p75-NGFR-positive neurons which were CAT-negative (Fig. 5A).

With regard to peptidergic neurons, some, but not all, galanin (GAL)-containing neurons in the paraventricular nucleus were NGFR-positive (Fig. 5B). Interestingly, a population of interleukin-1α (IL-Iα)-containing neurons, which share the same anatomical distribution with NGFR-positive neurons in the extreme lateral preoptic area, were observed to be p75-NGFR negative (Fig. 5C). Neuropeptide Y (NPY)-positive neurons in the cortex were found to express NGFR (Fig. 5D), whereas those NPY neurons within the arcuate nucleus of the hypothalamus did not (Fig. 5E). Furthermore, while a subpopulation of luteinizing hormone-releasing hormone (LHRH)-containing neurons in the medial septum (Fig. 5F), diagonal band of Broca and around the organum vasculosum of the lamina terminalis were p75-NGFR
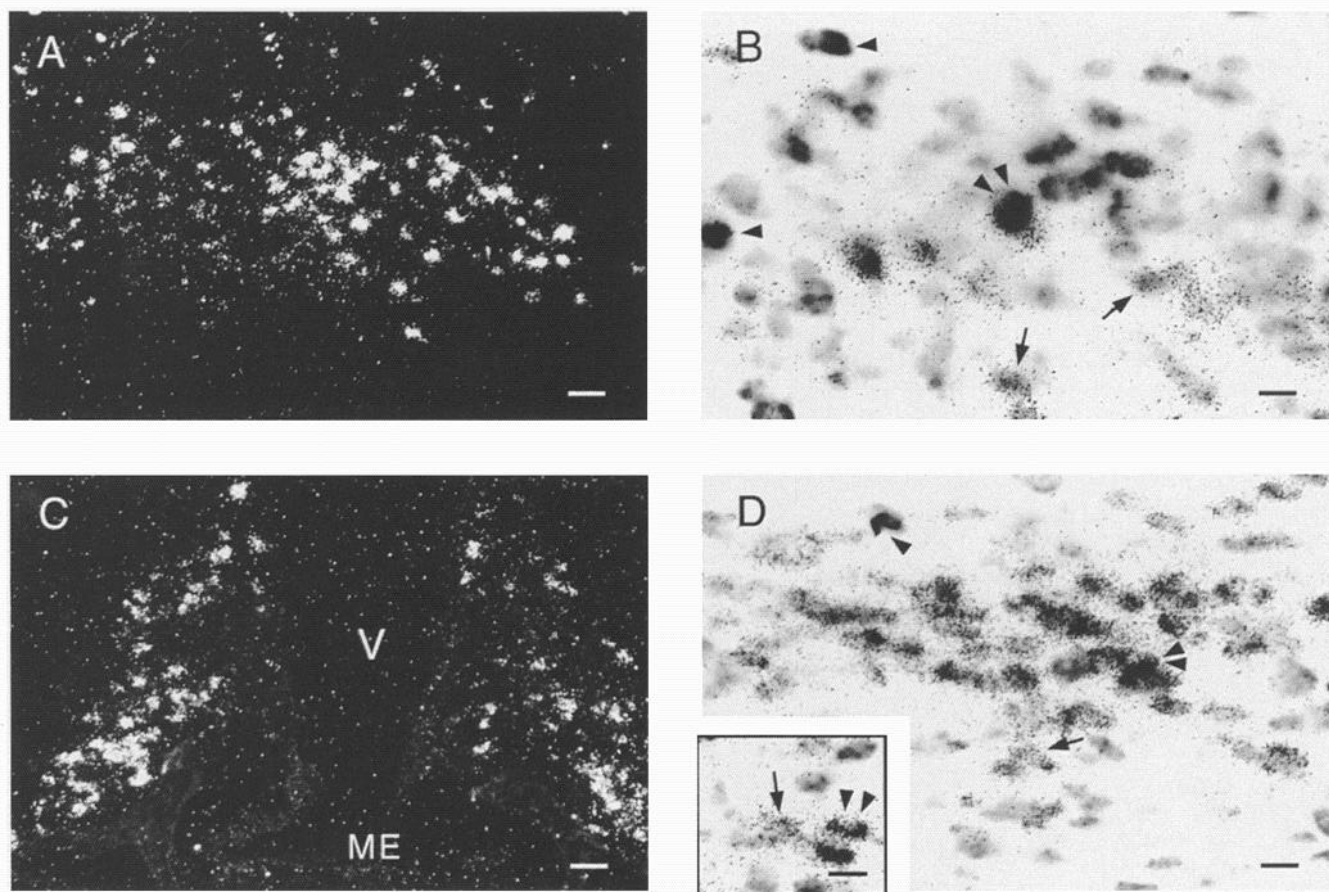
Figure 7. Detection of the messenger RNAs encoding *trk*B (the receptor for BDNF) and *trk*C (the receptor for NT-3) in hypothalamic dopaminergic neurons by double hybridization histochemistry. The two main groups of hypothalamic dopaminergic neurons, located either in the zona incerta (*A*) or arcuate nucleus (*C*) were identified by the detection of TH mRNA, utilizing a ^{35}S-labeled antisense RNA probe. *B* shows that most TH neurons of the zona incerta do not express *trk*C mRNA. TH mRNA was detected as before; *trk*C mRNA was detected using a digoxygenin-labeled cRNA probe complementary to the intracellular domain encoding region of *trk*C mRNA. *Single arrows*, cells expressing only TH mRNA; *single arrowheads*, cells expressing only *trk*C mRNA; *double arrowheads*, cell coexpressing TH and *trk*C mRNA. *D* shows that many TH mRNA positive cells of the zona incerta express *trk*B mRNA. *Trk*B mRNA was detected with a digoxygenin-labeled cRNA probe complementary to the intracellular domain encoding sequence of *trk*B mRNA, while TH was again detected as before. *Single arrow*, TH mRNA-only expressing cells; *single arrowheads*, *trk*B mRNA-only expressing cells; *double arrowheads*, TH neurons coexpressing TH and *trk*B mRNA. Scale bars: *A* and *C*, 20 μm; *B* and *D*, 5 μm.

positive, other LHRH neurons within this region were devoid of the receptor (Fig. 5*G*).

p75-NGFR is differentially expressed in two regionally segregated groups of dopaminergic neurons

The hypothalamus contains four groups of dopaminergic neurons; of these, the major two groups are located in the tuberoinfundibular-arcuate nucleus region and the zona incerta, respectively (Björklund and Nobin, 1973; Björklund et al., 1975). The third group, located in the rostral hypothalamus, is less well defined as it is composed of cells scattered along the periventricular region (Björklund and Nobin, 1973; Björklund et al., 1975). The fourth group extends from the dorsal hypothalamus to the caudal thalamus. According to the nomenclature proposed by Fuxe et al. (1969), these neuronal subsets are referred to as groups A13 (zona incerta), A12 (arcuate nucleus), A14 (rostral periventricular group), and A11 (caudal hypothalamic-thalamic group). They can be readily identified by immunohistochemical detection of TH (Fig. 6) or by *in situ* hybridization using probes that recognize TH mRNA (Fig. 7*A,C*).

We found that NGFR is differentially expressed in the two major DA groups of the hypothalamus. In the zona incerta some

of the TH-positive neurons were found to also be p75-NGFR positive (Fig. 6*A*), whereas other TH neurons in this area were p75-NGFR negative (Fig. 6*B*). Conversely, in the tuberoinfundibular region none of the TH-positive neurons in the arcuate nucleus contained p75-NGFR immunoreactivity (Fig. 6*C*).

Differential expression of trkB and trkC mRNAs in dopaminergic neurons of the zona incerta and tuberoinfundibular regions

Double *in situ* hybridization revealed that while a few TH mRNA-positive neurons in the zona incerta contain *trk*C mRNA (Fig. 7*B*, double arrowheads), most TH mRNA-expressing neurons do not appear to express the *trk*C gene (Fig. 7*B*, arrows). Some *trk*C mRNA-containing neurons were TH mRNA-negative (Fig. 7*B*, single arrowhead). In contrast, a substantial fraction of DA neurons of the zona incerta exhibit readily detectable levels of *trk*B mRNA (Fig. 7*D*, double arrowheads). Nevertheless, not all TH mRNA-containing neurons in this region express *trk*B mRNA (Fig. 7*D*, arrow), and some *trk*B mRNA-positive neurons did not contain TH mRNA (Fig. 7*D*, arrowhead). The inset depicts adjacent neurons containing only TH mRNA (arrow) or both TH and *trk*C mRNAs (double arrowhead).
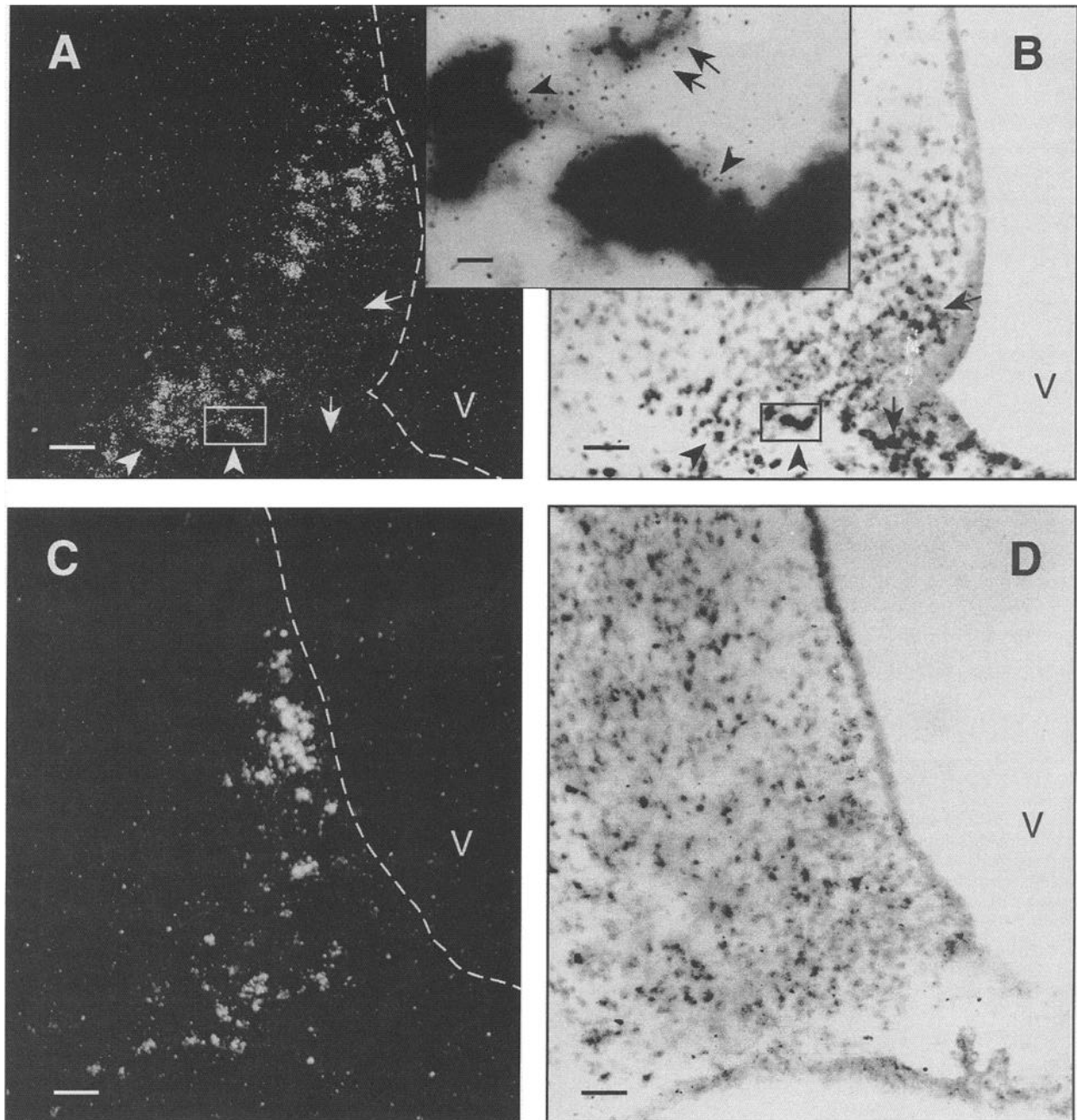
Figure 8. Differential expression of *trk*B and *trk*C mRNAs in DA neurons of the tuberoinfundibular region. *A*, TH mRNA-positive cells shown using dark-field illumination. *B*, The same section depicted in *A* shows under bright-field illumination that some TH mRNA-positive cells in the ventral portion of the arcuate nucleus (*arrowheads* in *A* and *B*) also contain *trk*C mRNA. Other *trk*C mRNA-containing neurons are TH mRNA-negative (*arrows* in *A* and *B*) (scale bars, 20 μm). The *inset* is an enlargement of the *boxed area* in *A* and *B* and shows at a higher magnification TH mRNA positive cells expressing *trk*C mRNA (*arrowheads*) and TH mRNA positive cells devoid of *trk*C mRNA (*double arrow*) (scale bar, 2 μm). *C*, A different section of the median eminence-arcuate nucleus depicting TH mRNA-containing neurons. *D*, The same section depicted in *C* shows under bright-field illumination the absence of detectable *trk*B mRNA in tuberoinfundibular TH mRNA-positive cells (scale bars, 20 μm).

In marked contrast with the zona incerta, the vast majority of dopaminergic neurons of the tuberoinfundibular-arcuate region had undetectable levels of *trk*B mRNA (Fig. 8*C,D*). Some of them, however, particularly those more ventrally located exhibited *trk*C mRNA hybridization signal (Fig. 8*A,B*, inset).

BDNF enhances the survival of p75-NGFR containing dopaminergic neurons in culture

To determine the physiological importance of the differential expression of *trk* receptors in hypothalamic DA neurons, we treated hypothalamic cultures with different neurotrophins, including NGF, BDNF, and NT-3. TGFα and EGF were also tested in consideration of the finding that both growth factors have been shown to be trophic for mesencephalic dopaminergic neurons (Casper et al., 1991; Alexi and Hefti, 1993). The results demonstrated that BDNF facilitates the survival of TH neurons expressing p75-NGFR, but not of those devoid of p75-NGFR (Fig. 9). Thus, BDNF may be predominantly trophic for A13 DA neurons of the zona incerta since these cells represent the major group of hypothalamic DA neurons expressing the *trk*B
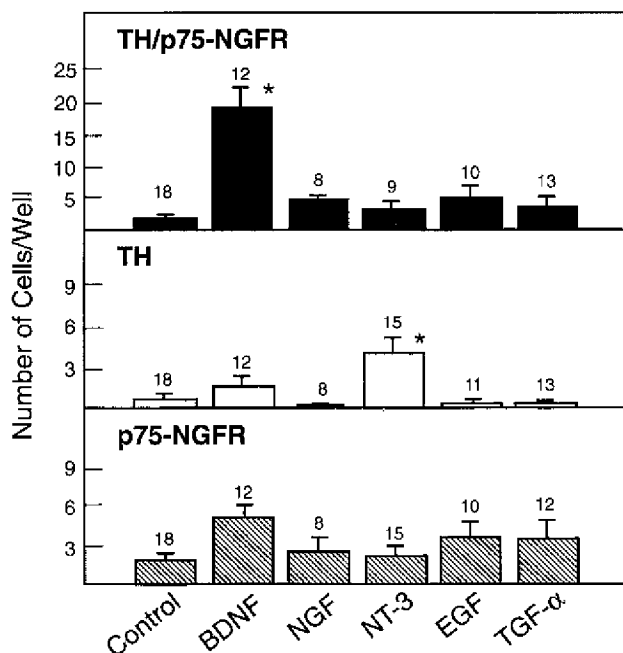
Figure 9. Effect of brain derived neurotrophic factor (*BDNF*) and neurotrophin 3 (*NT-3*) on the survival of hypothalamic dopaminergic neurons in culture. Hypothalami from 18 d old rat fetuses were mechanically dissociated and seeded on eight well Lab-Tek plastic culture chamber slides (Nunc Inc.) at a density of 50,000 cells per well in DEEM-F12 medium containing 7.5% fetal calf serum. After overnight culture, the medium was replaced by a serum-free medium (see Materials and Methods) containing the different growth factors tested (each at 50 ng/ml) and the culture was continued for 6 more days, with one medium change and growth factor replenishment 3 d after addition of the serum-free medium. On day 6, the cultures were fixed in 4% paraformaldehyde–PBS pH 7.4 (30 min at room temperature) and processed for combined immunohistochemical detection of tyrosine hydroxylase (*TH*) and the low-affinity nerve growth factor receptor (*p75-NGFR*). *TH/p75-NGFR*, Neurons immunopositive for both TH and p75-NGFR; *TH*, neurons immunopositive only for TH; *p75-NGFR*, neurons immunopositive only for p75-NGFR. *Numbers on top of bars* are number of wells per group; *, *p* < 0.01 versus control wells.

gene. Although we did not analyze the *in vivo* expression of *trk*B mRNA in A14 rostral periventricular DA cells, they do contain p75-NGFR (Fig. 1) and thus their survival may have also been promoted by BDNF. On the other hand, the survival of TH neurons lacking p75-NGFR was facilitated, though to a lesser extent, by NT-3 (Fig. 9). Since most hypothalamic p75-NGFR–negative TH neurons are located in the tuberoinfundibular region and some of them express *trk*C mRNA, this *in vitro* effect of NT-3 suggests that NT-3 may support the survival of at least a fraction of A12 DA neurons. None of the other growth factors tested had any effect on the survival of the DA neurons analyzed. Figure 10*A* illustrates the general aspect of 6 d old cultures, as assessed by phase-contrast microscopy. That a substantial fraction of the total population of viable cells correspond to neurons is demonstrated by the abundance of NSE-positive cells detected in these cultures (Fig. 10*B*). Figure 11 shows examples of DA neurons expressing p75-NGFR and cultured in the presence of BDNF (*A* and *B*), TH-negative neurons containing p75-NGFR alone, also cultured in the presence of BDNF (*C*) and TH- immunopositive neurons devoid of p75-NGFR, cultured in the presence of NT-3 (*D*).

Discussion

The hypothalamus contains a great diversity of neuronal phenotypes, ranging from those that utilize classical neurotransmitters for their synaptic communications to those that secrete neuropeptides into the portal vasculature to regulate hypophyseal function. Not surprisingly, given the low prevalence of p75-NGFR mRNA in the hypothalamus (Koh et al., 1989; Lu et al., 1989; Ojeda et al., 1990), only some of the neuronal populations identified in the present study were found to express p75-NGFR. The finding that the receptor could be detected in only a small subset of cells within a given population of peptidergic neurons was, however, unexpected. Thus, p75-NGFR was detected in only some galanin-containing neurons of the periventricular region and a small fraction of LHRH neurons of the preoptic region. Additionally, p75-NGFR was detected in some vasoactive intestinal peptide (VIP)-containing neurons of the suprachiasmatic nucleus. Since the presence of p75-NGFR may facilitate and/or enhance the biological actions of neurotrophins exerted via activation of *trk* receptors (Berg et al., 1991; Benedetti et al., 1993; Davies et al., 1993), it may be speculated that, within each peptidergic phenotype, those neurons expressing p75-NGFR may be the most responsive to a particular neurotrophin, should they have the appropriate *trk* receptor. Experiments to define the type of *trk* receptor expressed by hypothalamic p75-NGFR–containing peptidergic neurons and their relative responsiveness to neurotrophins are required to assess the validity of this notion.

Of the peptidergic neurons identified, those producing LHRH are the best characterized both functionally and morphologically. They play a central role in directing sexual development (Ojeda and Urbanski, 1994) and mature reproductive function (Silverman et al., 1994). Despite their importance, nothing is known about the trophic factors that they may require for either survival, differentiation or maintenance of their differentiated functions. Although our results clearly show the presence of p75-NGFR in a subset of LHRH neurons, we can not conclude that these cells are responsive to neurotrophins. p75-NGFR is not essential for neurotrophin action (Ibáñez et al., 1992, and references therein; Jing et al., 1992), a concept derived from *in vitro* experiments, but supported by the lack of central neuronal deficits observed in animals carrying a targeted mutation of the p75-NGFR gene (Lee et al., 1992) and by the present finding of hypothalamic DA neurons that do not express p75-NGFR and yet are responsive to NT-3 (see below). In fact, neurotrophins do not appear to be trophic for embryonic LHRH neurons in culture (unpublished observations), and GT1-7 cells—a well-characterized LHRH neuronal cell line (Mellon et al., 1990)—do not contain detectable levels of either *trk*A, B, or C mRNA (Zhou et al., 1994, and unpublished observations). Thus, the physiological significance of the presence of p75-NGFR in a subset of LHRH neurons remains to be ascertained.

A much more conclusive picture emerged from the finding that p75-NGFR is differentially expressed in the two main groups of hypothalamic DA neurons, because in this case we were also able to demonstrate the differential expression of *trk* genes in these cells, as well as the ability of neurotrophins to differentially promote the survival of p75-NGFR–containing and p75-NGFR–devoid hypothalamic DA cells in culture. The hypothalamus contains four DA cell groups (Björklund and Nobin, 1973): a rostral periventricular group (A14) composed of cells scattered in the periventricular region and extending from the
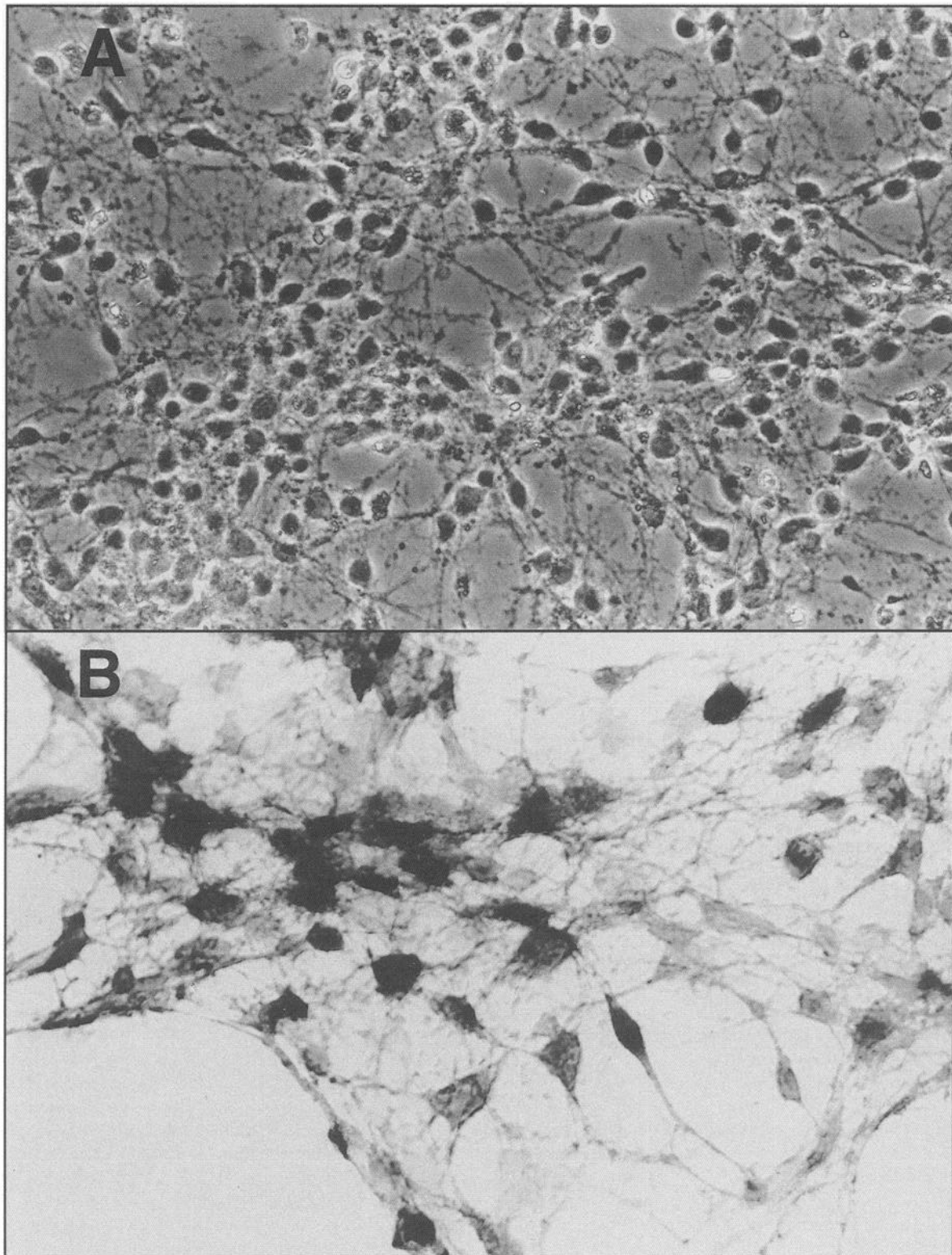
Figure 10. *A*, Phase-contrast photomicrograph of a typical hypothalamic culture prepared from 18 d old rat fetuses, after 6 d in neuronal defined medium. Notice the presence of numerous neuron-like cells and their extensive arborization (159×). *B*, Immunohistochemical identification of NSE-positive cells demonstrates the presence of a substantial number of neurons in the same cultures (1080×).

level of the anterior commissure to the rostral border of the median eminence, a cell group located in the arcuate nucleus (A12) that constitute the tuberoinfundibular dopaminergic system, a dorsal group (A13) which is located in the medial zona incerta, and a caudal group (A11), which contains both DA and noradrenergic neurons and extends diffusely from the posterior hypothalamic area to the caudal thalamus (Björklund and Nobin, 1973; Björklund et al., 1975). Of these four groups, the DA
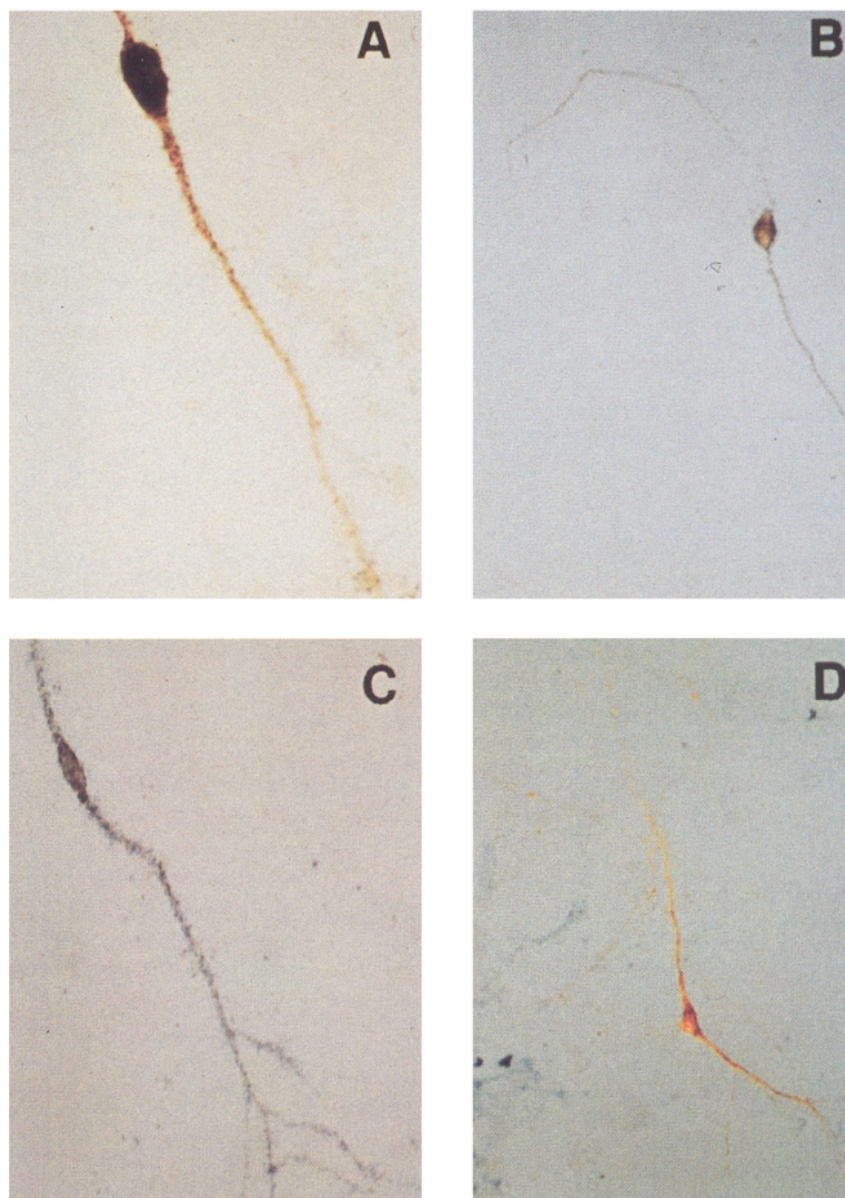
Figure 11. Representative hypothalamic neurons in culture expressing either both TH and p75-NGFR immunoreactivities (*A, B*), only p75-NGFR (*C*) or only TH (*D*). TH immunoreactivity has a brown color and smooth appearance. p75-NGFR immunoreactivity has a blue color and a crystal-like appearance. The neurons depicted correspond to cultures treated with BDNF (*A–C*) or NT-3 (*D*). All panels, 1560×.

neurons of the zona incerta and arcuate nucleus are the most prominent, not only because they are anatomically more circumscribed (Björklund and Nobin, 1973; Björklund et al., 1975), but also because there is a greater knowledge of the functions in which they are involved. Tuberoinfundibular DA neurons play a fundamental role in the control of prolactin release from the anterior pituitary (Neill and Nagy, 1994). They release DA into the hypothalamic portal vasculature, which then transport the catecholamine to the pituitary gland where it directly affects hormone secretion (Kordon et al., 1994; Neill and Nagy, 1994). Dysfunction of the tuberoinfundibular DA system results in unrestricted prolactin secretion (hyperprolactinemia) and infertility. Since tuberoinfundibular DA neurons also regulate the secretion of other pituitary hormones such as gonadotropins and growth hormone (Reichlin, 1985; Fink, 1988), it is clear that they play an important role in both reproductive function and somatic development.

The zona incerta, on the other hand, is involved in a wider spectrum of activities as neurons in this region contribute to the control of food and water intake (Rowland et al., 1979; Brown and Grossman, 1980; Dalton and Grossman, 1982), nociception (Kaelber and Smith, 1979), visual discrimination (Legg, 1979), sexual behavior (Wilson et al., 1991) and gonadotropin release (McKenzie et al., 1984; Sanghera et al., 1991; Wilson et al., 1991). This diversity of functions is, in fact, predicted by the complexity of the terminal fields innervated by zona incerta DA neurons, as they project diffusely to the anterior and dorsal hypothalamic areas, the dorsomedial nucleus of the hypothalamus and the posterior hypothalamus (Björklund et al., 1975; Ricardo, 1981). In contrast to A13 and A12 groups of DA neurons, not much is known about the functions of the rostral A14 and the caudal A11 groups, perhaps because of their more scattered distribution. While the caudal A11 group projects to the spinal cord, the rostral A14 group innervates the preoptic area, the periventricular region, the suprachiasmatic nucleus, and the lateral septal nucleus (Björklund et al., 1975).

In the present study we observed a striking difference in neurotrophin receptor expression consisting of the absence of p75-

NGFR in tuberoinfundibular A12 neurons as opposed to the rostral A14 and dorsal A13 groups, in which both immunoreactive p75-NGFR protein and p75-NGFR mRNA were detected. Clearly, however, not all zona incerta A13 or rostral A14 DA neurons express p75-NGFR, suggesting that cellular diversity of neurotrophin receptor gene expression in the hypothalamus occurs even in spatially circumscribed neuronal groups, which—because of their common localization and neurotransmitter phenotype—may be anticipated to originate from the same progenitor cells. Interestingly, this diversity is not limited to p75-NGFR, as it is also prominent for *trk*B and *trk*C receptors. As in the case of p75-NGFR, zona incerta DA neurons, but not tuberoinfundibular A12 DA neurons, express *trk*B mRNA. Also, *trk*B mRNA was not detected in all zona incerta DA neurons. The pattern of expression of *trk*C is more complex since *trk*C mRNA was seen in some zona incerta DA neurons, as well as in some of the most ventrally located tuberoinfundibular DA neurons. We did not analyze the expression of *trk* receptor genes in the rostral A14 group of DA cells, but noticed occasional expression of *trk*B mRNA in these cells.

The *in vitro* experiments clearly demonstrate that DA cells—identified by their TH immunoreactivity—respond differently to BDNF or NT-3, according to the presence of absence of p75-NGFR. Thus, BDNF selectively enhances the survival of TH/p75-NGFR-positive cells, whereas NT-3 has no effect on these cells, but exhibits a significant survival promoting effect on TH cells devoid of p75-NGFR. Consistent with the absence of *in vivo* expression of *trk*A in hypothalamic DA neurons, no survival or trophic effect of NGF on TH immunoreactive neurons was detected in culture, indicating that, as shown for mesencephalic DA neurons (Knüsel et al., 1991; Hyman et al., 1994), hypothalamic DA neurons do not require NGF for survival.

It would be difficult to argue that the presence or absence of p75-NGFR determines the responsiveness of DA cells to BDNF or NT-3. One can assume, however, that TH/p75-NGFR positive cells in culture correspond to DA cells of the A13 zona incerta/A14 rostral group and that TH positive/p75-NGFR negative *in vitro* mainly reflect the A12 tuberoinfundibular group detected *in vivo*. Based on this correspondence, the *in vitro* results argue in favor of the notion that BDNF supports the survival of A13 and perhaps A14 DA neurons, whereas NT-3 is trophic for both the subsets of A13 zona incerta and A12 tuberoinfundibular DA neurons expressing the *trk*C gene. This view is not only consistent with the observation that mesencephalic DA neurons expressing p75-NGFR are responsive to BDNF (Hyman et al., 1991), but may also provide an explanation for the conflicting observations concerning the responsiveness of these neurons to NT-3 (Knüsel et al., 1991; Hyman et al., 1994). If mesencephalic DA neurons are as heterogeneous in their sensitivity to neurotrophins as their hypothalamic counterparts, detection of selective NT-3 effects will depend on the size of the subset of cells bearing *trk*C receptors.

The presence of BDNF in the anterior hypothalamus, preoptic area and ventromedial nucleus (Friedman et al., 1991), which contain terminal fields for the A13 zona incerta and A14 rostral DA neurons suggest that these regions have target cells involved in supporting the survival and differentiated functions of A13 and A14 DA groups. In turn, the reported involvement of the zona incerta in feeding behavior (Kendrick and Baldwin, 1986; Rowland et al., 1979; Brown and Grossman, 1980) and the observation that mice with a null mutation of the *trk*B gene do not display feeding behavior (Klein et al., 1993), suggest that *trk*B

receptors are important for the development and maintenance of hypothalamic zona incerta DA neurons.

In a broader context, our results suggest that survival of neurons with an identical neurotransmitter phenotype and located within the same brain region or even within the same cellular group, is not determined by the same intercellular signalling events. It rather appears that "identical" neurons may follow different developmental options, which specify the class of trophic molecules the cells will require to survive. Since an obvious difference among regionally circumscribed neurons of identical phenotypes resides in the targets they innervate, it is conceivable that target cells themselves play a role in determining the type of neurotrophic receptor expressed by the innervating neurons. The molecular processes underlying this induction may be similar to those by which cells of an "equivalent" group become committed to a particular identity in response to signals provided by cells that are not part of the group (Greenwald and Rubin, 1992). Manifestation of this heterogeneity in trophic signal dependency would maximize the functional integrity of particular subsets of innervating-target neurons. On the other hand, loss of trophic support to a subset of neurons within a group endowed with the same neurotransmitter phenotype would only lead to the death of those neurons affected, without compromising other functions exerted by the rest of the group. Such a safety mechanism may be especially important for neuronal groups with such diverse functions as the DA neurons of the tuberoinfundibular region and zona incerta of the hypothalamus.

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