Non-Cell-Autonomous Regulation of GABAergic Neuron Development by Neurotrophins and the p75 Receptor

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Basal forebrain GABAergic and cholinergic circuits regulate the activity of cholinergic projections to the cortex and hippocampus. Because these projections influence cortical development and function, the development of basal forebrain excitatory and inhibitory neurons is critical for overall brain development. We show that the neurotransmitter phenotype of these neurons is developmentally regulated by neurotrophins and the p75 receptor. Neurotrophins (nerve growth factor and brain-derived neurotrophic factor) increased the number of both cholinergic and GABAergic neurons in neonatal basal forebrain neuron cultures from the region of the medial septum. However, the p75 receptor is required only for neurotrophin-dependent expansion of the GABAergic, not the cholinergic, population. Neurotrophin-induced GABAergic development can be rescued in *p75*^{−/−} cultures by expression of a p75 rescue construct in neighboring cells or by treatment with medium collected from neurotrophin-treated wild-type cultures. Because p75 is not expressed in basal forebrain GABAergic neurons, this defines a new, non-cell-autonomous mechanism of p75 action in which ligand binding results in release of a soluble factor that modifies neurotrophin responses of nearby neurons. p75 is also required for the maintenance of basal forebrain GABAergic neurons *in vivo*, demonstrating that p75-mediated interactions between cholinergic and GABAergic neurons regulate the balance of excitatory and inhibitory components of basal forebrain circuits.

Key words: basal forebrain; neurotransmitter phenotype; neurotrophins; p75 receptor; cholinergic neurons; GABAergic neurons

Introduction

Basal forebrain cholinergic neurons send projections to the cortex and hippocampus where they regulate neuronal development and activity (Semba, 2000; Nishimura et al., 2002; Hohmann, 2003). Inhibitory GABAergic neurons in the basal forebrain coproject with cholinergic neurons (Freund and Meskenaite, 1992; Gritti et al., 1997) and exhibit extensive local collaterals onto cholinergic neurons (Zaborszky and Duque, 2000). In turn, cholinergic neurons form reciprocal connections onto the GABAergic neurons (Brauer et al., 1998), defining a local circuit for the regulation of cholinergic output. Thus, mechanisms that control the balance between cholinergic and GABAergic neurons in the basal forebrain may be critical to the development and function of cortical and hippocampal circuitry.

Neurotrophins have long been known to regulate the development of basal forebrain cholinergic neurons (BFCNs). Nerve growth factor (NGF) increases the expression and ac-

tivity of choline acetyltransferase (ChAT) (Hatanaka et al., 1988) and vesicular acetylcholine transporter (VACHT) (Pon-grac and Rylett, 1998; Berse et al., 1999) and increases cholinergic neuron number *in vitro* (Hatanaka et al., 1988). Brain-derived neurotrophic factor (BDNF) enhances cholinergic marker expression and supports survival of postnatal basal forebrain neurons (Nonomura and Hatanaka, 1992; Nonomura et al., 1995; Ward and Hagg, 2000). The effects of neurotrophins on basal forebrain GABAergic neurons are less clear. Although NGF may increase GABAergic neuron numbers in some culture systems (Arimatsu and Miyamoto, 1991), intraventricular infusion in adult rats showed no effects of NGF or BDNF on numbers of GABAergic neurons (Koliatsos et al., 1994). However, target ablation of basal forebrain projections resulted in a partial loss of GABAergic neurons (Plaschke et al., 1997), suggesting a requirement for target-derived factors for GABAergic neuron development or survival.

Although many effects of neurotrophins are mediated through Trk receptors, the lower-affinity p75^{NTR} receptor (p75) also participates in neurotrophin signaling (Dechant and Barde, 2002). In the basal forebrain, p75 is localized to the cholinergic, but not GABAergic, neurons (Hartikka and Hefti, 1988; Heckers et al., 1994). Studies of the basal forebrain of mice lacking p75 have reported either an increased number of BFCNs (Van der Zee et al., 1996; Yeo et al., 1997; Naumann et al., 2002) or a decrease or no change in number (Hagg et al., 1997; Peterson et al., 1997, 1999; Ward and Hagg, 1999; Greferath et al., 2000). Hence, it is likely that p75 plays a role in regulating basal forebrain neuron

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development, although specific effects on neuronal subpopulations, specifically the GABAergic population, are not clear.

We examined the influence of neurotrophins on the acquisition of cholinergic and GABAergic properties in basal forebrain cultures and investigated the role of the p75 receptor. We demonstrate that neurotrophins promote the expression of both cholinergic and GABAergic neuronal phenotypes and have identified a p75-dependent non-cell-autonomous mechanism for the regulation of GABAergic development in the basal forebrain. These experiments demonstrate a novel role for p75 in establishing the developmental balance of cholinergic and GABAergic neuron numbers.

Materials and Methods

Dissociated basal forebrain neuron culture. Basal forebrain cultures were prepared from neonatal wild-type or *p75*^{−/−} mice (Lee et al., 1992) (The Jackson Laboratory, Bar Harbor, ME). Animal protocols were approved by the Brandeis University Institutional Animal Care and Use Committee. The brains were removed and collected in cold artificial CSF (in mM: 126 NaCl, 3 KCl, 2 MgSO₄, 1 NaH₂PO₄, 25 NaHCO₃, 10 dextrose, and 2 CaCl₂). Meninges surrounding the brains were removed, and the cerebral hemispheres were spread laterally to expose the basal forebrain, in which the septal area (containing basal forebrain neurons from the medial septum (MS) and the horizontal and vertical limbs of the diagonal band of Broca) was dissected as described previously (Hatanaka et al., 1988). Dissected tissues were minced, enzymatically dissociated with papain (Worthington Biochemical, Lakewood, NJ), and triturated with sterile Pasteur pipettes. The resulting cell suspensions were diluted in a culture medium consisting of minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Invitrogen), 2% penicillin/streptomycin (Invitrogen), 2% B27 supplement (Invitrogen), 1% L-glutamine (Invitrogen), and 30 mM dextrose. The neurons were plated (18,000–20,000 cells/cm²) onto previously established monolayers of cortical astrocytes (see below). In the cultures for transfection experiments, 40,000 cells/cm² neurons were plated onto astrocytes. Basal forebrain cultures were incubated in the absence (control condition) or presence of 50 ng/ml NGF (Upstate Biotechnology, Lake Placid, NY) and 50 ng/ml BDNF (gift from Regeneron, Tarrytown, NY) during the entire culture period and maintained in a 37°C/5% CO₂ incubator. One day after plating the cells, 1 μM cytosine arabinofuranoside (AraC) (Sigma, St. Louis, MO) was added to the cultures to reduce non-neuronal cell division. Half of the culture medium was replaced every 2–3 d.

Astrocyte cultures were prepared by plating postnatal day 0–3 mouse visual cortical cells (20,000 cells/cm²) in the same medium as used for culturing basal forebrain neurons but excluding the B27 supplement. The cultures became a confluent monolayer by 10 d, at which time neurons were plated. Cultured astrocytes have been reported to express neurotrophins (Rudge et al., 1996); thus, these cultures may contain low levels of endogenous neurotrophins that could vary with the final glial density obtained in different experiments.

Conditioned medium (CM) was collected from basal forebrain cultures prepared from wild-type or *p75*^{−/−} mice, with or without NGF and BDNF (50 ng/ml each) treatment. Half of the culture medium was collected every 2 d, filtered through a 0.45 μm filter (Corning, Corning, NY), and stored at −80°C.

Immunocytochemistry. Basal forebrain cultures were fixed in 4% paraformaldehyde 2, 6, or 10 d after plating. Cells were permeabilized with 0.1% Nonidet P-40 (Sigma) in PBS for 10 min and blocked in 5% horse serum for 1 h at room temperature. Cells were incubated at 4°C overnight in primary antibody diluted in 1% horse serum/PBS. Primary antibodies used were mouse anti-microtubule-associated protein 2 (MAP2) (1:2000; Sigma) or chicken anti-MAP2 (1:2000; Chemicon, Temecula, CA), rabbit anti-glutamic acid decarboxylase (GAD) 65/67 (1:2000; Chemicon), mouse anti-GAD67 (1:2000; Chemicon), goat anti-VACHT (1:4000; Chemicon), rabbit anti-active caspase 3 (1:100; Chemicon), rabbit anti-bromodeoxyuridine (BrdU) (1:800; Megabase, Lincoln, NE), and rabbit anti-human p75 receptor intracellular epitope (1:500; Pro-

mega, Madison, WI). Primary antibodies were detected after incubation with AMCA (aminomethylcoumarin acetate), FITC-, rhodamine-, or cyanine 5 (Cy5)-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature.

Stained cells were viewed using an Olympus Optical (Center Valley, PA) IX-70 inverted fluorescent microscope fitted with UV, FITC, rhodamine, and Cy5 filters. Images were captured using an Orca-ER CCD digital camera (Hamamatsu, Shizuoka, Japan) and Openlab software (version 4.0.2; Improvision, Lexington, MA).

To evaluate cell proliferation, 1 mM BrdU (Roche Diagnostics, Mannheim, Germany) was added for the last 8 h of the culture period. AraC was not added to these cultures. Cells were fixed as described, washed, treated with 2N HCl in PBS for 10 min at room temperature, washed, and treated with 0.1 M Na₂B₄O₇·10H₂O for 10 min at room temperature. Cultures were immunostained as described above.

In the cultures with double staining of GAD/MAP2 and VACHT/MAP2, the percentage of GABAergic and cholinergic neurons was determined by counting the numbers of GAD-positive (GAD⁺) or VACHT⁺ cells, divided by the numbers of MAP2⁺ cells, in the central strip of each culture well. When counting the cells, researchers were blind to the conditions of the experiment. The resulting cell counts were limited by the intensity of the fluorescence staining, the sensitivity of the viewer's eyes, and the optics of the microscope. Duplicate wells for each condition were counted for a minimum of three experiments per condition. For control purposes, each experiment included the culture processed in the absence of the primary antibody.

For the measurement of soma sizes, images of double-stained GAD⁺ neurons and their corresponding MAP2 stain or triple-stained VACHT⁺, MAP2⁺, and p75⁺ neurons were taken using a 40× objective with 0.85 numerical aperture. Using NIH ImageJ software, neuronal cell bodies were outlined by hand for the MAP2 stain with the freehand selection tool and measured for area.

Western blots. Lysates were isolated from 2 and 10 d cultures, and protein was assayed using the Bio-Rad (Hercules, CA) Protein Assay with a BSA standard. Fifteen micrograms of protein and molecular weight standards were run on 7.5% separating gels and electroblotted to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were probed with primary antibodies against GAD (1:7000; Chemicon) or β-actin (1:15,000; Sigma) for 2 h at room temperature. After washing, the blots were incubated in HRP-conjugated secondary antibodies (1:10,000; Jackson ImmunoResearch) for 1 h. Immune complexes were visualized by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA) and exposed to film. The densities on the film of ~42 kDa (β-actin) and 65 kDa (GAD65) immunoreactive bands were quantified with background subtraction using Quantity One software (Bio-Rad). All data are obtained as arbitrary units normalized to β-actin.

Gene transfection. Human p75 was expressed in basal forebrain cultures prepared from *p75*^{−/−} mice using Lipofectamine 2000 reagent (Invitrogen). Cells were transfected 1 d after plating with pEGFP-C1 (control; Clontech, Palo Alto, CA), with pEGFP-C1 and pCMV5A, an expression vector containing a human p75 cDNA, or with pEGFP-C1 and p75-105 (Yan and Chao, 1991), encoding a p75 protein deficient in ligand binding (p75 constructs were gifts from Dr. Moses V. Chao, New York University School of Medicine, New York, NY). Expression of p75 was detected by staining with an anti-p75 antibody (Promega) that recognized the intracellular domain of human p75 protein.

Brain slice preparation, imaging, and analyses. Adult wild-type (C57BL/6J) and *p75*^{−/−} mice (Lee et al., 1992) at 3 or 10–14 months of age were anesthetized (with chloroform and then injected with ketamine, xylazine, and acepromazine mix) and killed by transcardiac perfusion with saline and 4% paraformaldehyde in phosphate buffer, pH 7.4. Brains were rapidly dissected out, postfixed overnight at 4°C, and preserved in 30% sucrose at 4°C. Coronal slices (40 μm thick) containing the MS were made on a vibratome and collected in PBS, beginning where the corpus callosum first begins to cross until the anterior commissure crosses. Alternating slices were immunostained for cholinergic and GABAergic neurons using a free-floating procedure. Sections were blocked and permeabilized in solution containing 0.1% NP-40 with 10% donkey serum

in PBS for 30 min at room temperature on a shaker at low setting. They were then incubated overnight in the same solution plus primary antibody. After washing three times in PBS (10 min each), the primaries were visualized using FITC- (or Cy2-) and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch). For GABAergic neuron stains, the antibodies used were rabbit anti-GABA (1:4000; Sigma) and mouse anti-neuronal-specific nuclear protein (NeuN) (1:1000; Chemicon) for quantifying total neuron numbers. To identify cholinergic neurons, a goat polyclonal antibody to ChAT was used (1:500, AB144P; Chemicon). To assess costaining of p75 and cholinergic markers, slices were costained for ChAT and p75 (rabbit polyclonal against human p75, 1:500; Promega). Slices were washed three to four times in PBS, mounted on Superfrost plus slides in *n*-propyl gallate, and stored at 4°C until imaged.

The medial septal region of each brain slice was imaged using the 10× objective on a Leica (Nussloch, Germany) SP2 confocal microscope, taking a series (or stack) of images simultaneously for GABA or ChAT and NeuN stains at 2 μ m intervals through the slice. These image series were then analyzed using NIH ImageJ software. Image stacks were z-projected into one image for each stain using the maximum intensity values for each stack to allow for viewing all cell somas through the slice in one image. The region of the MS was defined and outlined by hand using the freehand tool for the NeuN image and then copied onto the corresponding GABA or ChAT image. The MS region is visually apparent in the NeuN stain from surrounding tissue by its specific cell density and morphology. (The neuronal somas appear more clustered together and have a common directionality.) The total MS area was measured in pixels and converted to square micrometers. Then, the total number of neurons (NeuN⁺ cells) and the number of either GABAergic (GABA⁺) or cholinergic (ChAT⁺) cells within each MS were counted by hand using the Pointpicker function and identifying positive cells by eye while blinded to condition. The number of GABAergic and cholinergic neurons per total number of cells within the region was then computed and averaged across slice and then across mice. In addition, each brain slice was viewed under low magnification and assigned an anatomical coordinate value as “distance from bregma” based on anatomical landmarks present in the slice and by comparison to slices in the mouse brain atlas (Paxinos and Franklin, 2001). These coordinates were then used to further examine if differences in ratios of cholinergic and GABAergic neurons change along the anteroposterior (rostrocaudal) axis of the MS.

An inverted Olympus Optical IX-81 scope equipped with epifluorescence and automatic focus drive was used to image cortical areas of three wild-type and three *p75*^{−/−} brains used previously for imaging the MS. A 438.55 × 334.13 μ m region of the somatosensory cortex was imaged at 2 μ m intervals using Volocity software (Improvision). These image series were then analyzed using NIH ImageJ to quantify the ratio of GABAergic neurons in the cortex in the same manner as described above for the MS.

Statistics. Significance was analyzed by Student's *t* tests or ANOVA followed by *post hoc* tests using StatView software (Abacus Concepts, Berkeley, CA).

Results

Neurotrophins increase the number of cholinergic and GABAergic neurons

We examined the regulation of anterior basal forebrain cholinergic and GABAergic neuron numbers by neurotrophins using a well defined *in vitro* system (Hartikka and Hefti, 1988; Ha et al., 1999; Lopez-Coviella et al., 2005) in which neurons from the neonatal medial septum and diagonal bands of Broca were dissociated and cultured on astrocyte monolayers. We used immunolabeling for VACHT to identify cholinergic neurons and GAD65/67 to label GABAergic neurons (Fig. 1A). Cultures were treated with or without 50 ng/ml of both NGF and BDNF to examine overall population effects of neurotrophins. Treatment was for 2, 6, and 10 d followed by immunostaining. Labeling for MAP2 was used to identify all neurons in the cultures. In the absence of added neurotrophins, ~30% of the neurons expressed VACHT, a number that did not change significantly over the

culture period (Fig. 1B). At 2 d, ~20% of the neurons expressed a GABAergic phenotype, increasing to >30% by 6 d in culture (Fig. 1C). The percentage of cholinergic neurons was significantly increased by neurotrophin treatment (Fig. 1B), as was the percentage of GABAergic neurons (Fig. 1C). In addition, neurotrophins significantly increased soma size for both cholinergic and GABAergic neurons (Fig. 1D). Control cultures without primary antibody did not show immunoreactivity (data not shown). There was no significant change in the total number of MAP2-immunopositive cells with neurotrophin treatment (data not shown).

Neurotrophin-dependent increases in cholinergic and GABAergic neurons could be a consequence of increased proliferation or neuronal survival, or recruitment of neurons to a cholinergic or GABAergic phenotype. Birthdating of rat BFCNs has shown that developing neurons withdraw from the cell cycle by embryonic day 17 (E17) (Semba and Fibiger, 1988; Brady et al., 1989), suggesting that neuronal proliferation in our neonatal cultures is unlikely to underlie the effects of neurotrophins. We tested whether a neurotrophin-responsive proliferative cell population could give rise to neurons by pulsing neurotrophin-treated and untreated cultures with BrdU for 6 h before fixation. We found that none of the MAP2⁺ neurons were BrdU labeled (Table 1), demonstrating that neurotrophin-dependent increases did not result from proliferation. We also examined whether neurotrophins promoted the preferential survival of young cholinergic or GABAergic neurons. Cultures were labeled for active caspase 3, a marker of apoptosis, after a 12 h, 2 d, or 6 d treatment with neurotrophins. Fewer than 2.5% of the neurons were immunolabeled for caspase 3 at any time point (Table 1), and the percentage was not affected by neurotrophins. The absence of proliferative or survival effects suggests that neurotrophins promote the expression of a cholinergic or GABAergic phenotype in VACHT-negative/GAD-negative neurons.

Neurotrophin response of GABAergic neurons requires p75

Basal forebrain neurons maintain expression of the p75 neurotrophin receptor through postnatal development. We investigated whether p75 regulates neurotransmitter phenotypes in cultured basal forebrain neurons from the medial septal region. We prepared cultures from neonatal mice deficient in the p75 receptor (*p75*^{−/−}) (Lee et al., 1992) and quantified the number of cholinergic and GABAergic neurons when treated with or without NGF and BDNF. Neurotrophins significantly increased the percentage of cholinergic neurons at both 2 and 6 d (Fig. 2A), indicating that p75 was not required for the neurotrophin effect on cholinergic expression. In contrast, we found no neurotrophin-dependent increase in GABAergic neuron number in *p75*^{−/−} cultures (Fig. 2B). The number of GABAergic neurons in both neurotrophin-treated and untreated cultures increased between 2 and 6 d, reaching a maximal number that was similar to neurotrophin-treated wild-type neurons. This increase was not dependent on the presence of neurotrophins, suggesting that p75 plays a role in limiting GABAergic neuron number in the absence of neurotrophins. A comparison with neurotrophin responses in wild-type cultures (Fig. 2C,D) shows that p75 is necessary for neurotrophin-dependent increases in GABAergic but not cholinergic neurons.

Although p75 is necessary for neurotrophin-dependent increases in GABAergic neuron number, not all neurotrophin responses require p75. GABAergic soma size was increased after neurotrophin treatment in both wild-type (Fig. 1D) and *p75*^{−/−} (Fig. 3A) cultures. In addition, Western blot analysis revealed

neurotrophin-dependent regulation of GAD65 protein levels (Fig. 3B). These results indicate that p75 is selectively required for neurotrophin-dependent recruitment of neurons to a GABAergic neurotransmitter phenotype but is not required for other aspects of their development, including the regulation of GAD expression in neurons already expressing a GABAergic phenotype.

Expression of p75 in cholinergic neurons

The requirement for p75 in establishing the GABAergic phenotype was surprising because p75 expression has been reported to be restricted to the cholinergic population (Hartikka and Hefti, 1988; Heckers et al., 1994). In contrast, Trk receptors are expressed by both GABAergic and cholinergic neurons (Kordower et al., 1994). We therefore examined the expression of p75 in cholinergic and GABAergic neurons in our neonatal cultures. Cultures were quadruple labeled with VACHT and GAD to identify cholinergic and GABAergic neurons, respectively, with p75 to identify neurotransmitter phenotypes coexpressing the neurotrophin receptor and with MAP2 to mark all neurons in the culture (Fig. 4A). We found that p75 was exclusively expressed in the VACHT-expressing population *in vitro*. We also examined the expression of p75 in cholinergic neurons *in vivo*. Coronal sections through adult mouse medial septum were stained for ChAT and p75 (Fig. 4B). We found that p75 was exclusively expressed in the ChAT-expressing cholinergic neurons. It appeared that all ChAT-expressing neurons in the medial septum coexpressed p75, although expression levels of p75 were somewhat variable. This expression pattern is specific to the cholinergic neurons of the basal forebrain in that ChAT-expressing neurons in other brain regions such as the striatum did not coexpress p75 (data not shown).

p75 promotes neurotrophin-dependent GABAergic development

The p75 expression pattern in basal forebrain cultures and *in vivo* raised the possibility that p75 acted in a non-cell-autonomous manner to regulate neurotrophin-dependent acquisition of a GABAergic phenotype. To explore this possibility, we overexpressed p75 in individual basal forebrain neurons prepared from *p75*^{−/−} mice and examined the neurotransmitter phenotype of surrounding neurons. We transfected cultures with green fluorescent protein (GFP) or GFP and human p75 1 d after plating the neurons. In addition, some neurons were transfected with GFP and *p75-105*, a p75 mutant that lacks a ligand binding component (Yan and Chao, 1991). In pilot experiments, we found that 94% of neurons trans-

Figure 1. Neurotrophins promote the development of cholinergic and GABAergic neurons in basal forebrain cultures. Basal forebrain neurons were immunostained for MAP2 (green) and for GAD or VACHT (red) markers for GABAergic or cholinergic neurons, respectively. **A**, Double-stained images show a subset of basal forebrain neurons that are GABAergic or cholinergic. Scale bar, 30 μ m. **B**, **C**, After 2, 6, and 10 d in cultures in control (CON, open bars) or 50 ng/ml NGF and BDNF (NT, filled bars) the percentage of VACHT⁺ (**B**) or GAD⁺ (**C**) cells that colabeled with MAP2 was measured. Values are given as mean percentage $\pm$ SEM of the number of VACHT⁺ or GAD⁺ cells/the number of MAP2⁺ cells (at least 4 independent experiments in each condition). **D**, The soma area of VACHT⁺ and GAD⁺ neurons was measured at 2 d in cultures ($n = 4$ experiments for cholinergic neurons and $n = 3$ for GABAergic neurons). * $p < 0.05$, ** $p < 0.01$, unpaired t test.

fected with two constructs coexpressed both markers, indicating that GFP-positive cotransfected neurons were likely to be p75 positive (Fig. 5A and data not shown). Transfected neurons were cultured in the presence or absence of neurotrophins and, after a 7 d culture period, the number of GAD⁺ neurons within a 300- μ m-diameter circle around the transfected neuron was determined (Fig. 5B). The cultures were double labeled with MAP2 to determine the percentage of GABAergic

Table 1. Neurotrophin effects on cell proliferation and death in basal forebrain cultures

	CON	NT
Percentage of BrdU ⁺ neurons		
2 d (<i>n</i> = 3)	0	0
Percentage of active caspase 3 ⁺ neurons		
12 h (<i>n</i> = 3)	0.7 ± 0.2	0.4 ± 0.1
2 d (<i>n</i> = 3)	1.4 ± 0.6	1.6 ± 0.4
6 d (<i>n</i> = 4)	2.3 ± 0.5	2.4 ± 0.9

Percentage of MAP2⁺ cells that contained with BrdU or active caspase 3 in basal forebrain cultures treated with (NT) or without (CON) neurotrophins. Values are given as mean ± SEM. For each experiment, two wells of cultures, each containing at least 600 MAP2⁺ cells, were analyzed.

Figure 2. p75 is required for GABAergic, but not cholinergic, neurotrophin responses. Basal forebrain neurons from *p75*^{-/-} mice were cultured with (NT, filled bars) or without (CON, open bars) neurotrophins. The percentage of VACHT⁺ (A) or GAD⁺ (B) cells that colabeled with MAP2 was measured (values are mean ± SEM; *n* = a minimum of 6 independent experiments), **p* < 0.05, unpaired *t* test. C, A comparison of wild-type (*p75*^{+/+}) and *p75*^{-/-} cultures shows that neurotrophins significantly increase cholinergic neurons in both cultures. D, Neurotrophins increase GABAergic neuron percentage in *p75*^{+/+}, but not *p75*^{-/-}, cultures (values are mean ± SEM percentage of NT relative to CON condition). **p* < 0.05, ***p* < 0.01, unpaired *t* test.

neurons in close proximity to *p75* or control transfected neurons. There was an average of 16.5 ± 4.7 MAP2⁺ cells in individual circles. Neurotrophin treatment significantly increased the percentage of GABAergic neurons in the proximity of *p75*-overexpressing neurons (Fig. 5C, GFP + *p75*). In contrast, there was no neurotrophin-dependent increase in GABAergic neurons near neurons expressing GFP alone or the ligand binding-deficient *p75* variant (Fig. 5C, GFP + *p75*-105). *p75*-105-expressing neurons did not confer neurotrophin responsiveness on nearby neurons, although in the absence of added neurotrophins there was a small, but not significant, increase in the percentage of GABAergic neurons that may reflect a ligand-independent activity of this variant (Yang et al., 2002). We also measured the percentage of cholinergic neurons in the circled area surrounding transfected neurons. There was a significant increase in cholinergic neuron percentage in neurotrophin-treated cultures in the proximity of each type of transfected neuron (Fig. 5D). These results confirm that p75 is not required for the neurotrophin-dependent acquisition of a cholinergic phenotype by basal forebrain neurons and demonstrate a non-cell-autonomous effect of p75 in the regulation of GABAergic properties.

A**Soma area of GABAergic neurons** **B****GAD65 protein level**

Figure 3. GABAergic neurotrophin responses in the absence of p75. A, Soma area of GAD⁺ neurons derived from *p75*^{-/-} cultures. Neurotrophin treatment (NT) is significantly different from the control (CON) at both 2 and 6 d in cultures (mean ± SEM; *n* = 3). ***p* < 0.01, ****p* < 0.001, unpaired *t* test. B, Western analysis of GAD65 protein in basal forebrain cultures from *p75*^{+/+} and *p75*^{-/-} mice. Neurotrophin treatment (NT) increased GAD65 protein levels at 10 d in cultures in both *p75*^{+/+} and *p75*^{-/-} conditions compared with control (CON). The protein level was normalized to β-actin. Values are given as mean ± SEM percentage of intensity of GAD65 band intensity of NT relative to CON condition (*n* = 6). **p* < 0.05, unpaired *t* test.

p75 mediates neurotrophin-dependent GABAergic development via secreted factors

The non-cell-autonomous effects of p75 could be mediated through cell–cell interactions, sequestration or presentation of local factors, or the p75-mediated, neurotrophin-dependent release of soluble factors by cholinergic neurons. We distinguished these possibilities by treating *p75*^{-/-} basal forebrain neurons with conditioned medium generated from neurotrophin-treated wild-type cultures (CM-WT/NT). We found that CM-WT/NT was sufficient to restore the neurotrophin-dependent increase in GABAergic neurons in *p75*^{-/-} cultures (Fig. 6A). Residual neurotrophins in the CM-WT/NT apparently had reduced activity after storage, freezing, and thawing of the conditioned medium (see Materials and Methods) because fresh neurotrophins added to the CM-WT/NT-treated *p75*^{-/-} cultures resulted in a significant increase in GABAergic neuron number.

Conditioned medium obtained from *p75*^{-/-} cultures (CM-*p75*KO/NT) did not restore neurotrophin effects on GABAergic neuron number (Fig. 6B), indicating that p75 is required for the production of the conditioned medium factor(s). In addition to p75

expression, added neurotrophins are also required for production of the conditioned medium activity. Conditioned medium generated in wild-type cultures in the absence of NGF and BDNF did not restore the GABAergic neurotrophin response to $p75^{-/-}$ cultures (Fig. 6B). These data suggest that neurotrophins act on p75-expressing cells to induce a soluble activity that permits neighboring, p75-negative neurons to respond to neurotrophins by expressing a GABAergic phenotype.

p75 promotes neurotrophin-dependent GABAergic development *in vivo*

We have shown that p75 expression in the medial septum is restricted to cholinergic neurons both *in vitro* and *in vivo* (Fig. 4). We therefore asked whether p75 has a non-cell-autonomous effect on GABAergic development in the animal by examining GABAergic neuron number in the medial septum of $p75^{-/-}$ mice. We analyzed brain slices through the medial septum from adult wild-type and $p75^{-/-}$ mice (3 or 10–14 months of age; see Materials and Methods). Slices were immunostained for GABA or ChAT and the neuronal nuclear marker NeuN to label the GABAergic neurons, cholinergic neurons, and total population of neurons. Slices were imaged on a confocal microscope, and the medial septal region was outlined based on images of the NeuN stain. GABAergic neurons and total neurons were counted in the identified region, and the ratio of GABAergic neurons to total neurons was determined. The proportion of GABAergic neurons was lower in $p75^{-/-}$ mice compared with wild-type age-matched 10- to 12-month-old animals (Fig. 7A) but not in 3-month-old mice (data not shown). There was no significant difference in total neuron density between the wild-type and $p75^{-/-}$ mice (Fig. 7B).

We also asked whether the decrease in GABAergic neurons in the $p75^{-/-}$ mice was generalized in different brain regions by examining a region of the somatosensory cortex that does not receive projections from the medial septum and was contained within the same slices that were imaged for the medial septum. In adult animals, the neurons in this region did not express p75 (data not shown). There was no difference in GABAergic neurons in somatosensory cortex between age-matched 10- to 12-month-old $p75^{-/-}$ and wild-type mice (Fig. 7C), consistent with the idea that p75-expressing cholinergic neurons play a role in setting the number of GABAergic neurons in the medial septal region.

Finally, we examined whether there were differences in medial septal cholinergic neurons in the $p75^{-/-}$ and wild-type mice. Initial analysis showed a trend toward increased cholinergic neuron ratios in the 10- to 14-month-old $p75^{-/-}$ ani-

Figure 4. p75 is expressed by cholinergic neurons. Cholinergic neurons from the medial septal region of the basal forebrain exclusively express p75. **A**, Cultures treated with neurotrophins were fixed at 6 d and quadruple immunostained for p75 (red), VACht (green), GAD67 (yellow), and MAP2 (blue). In this image set, there is one $p75^{+}/VACht^{+}$ cholinergic neuron surrounded by non-p75-expressing, $GAD67^{+}$ GABAergic neurons. Scale bar, 25 μ m. **B**, Coronal brain sections (40 μ m thick) through the medial septum of a 13-month-old wild-type female mouse were costained for p75 (green) and ChAT (red). All $p75^{+}$ neurons in this region colabel for the cholinergic marker ChAT. Scale bar, 100 μ m.

mals that was not significant when averaged across all slices through the septum. However, when we arranged the slices into groups based on their anteroposterior (rostrocaudal) position, we found a significantly greater proportion of cholinergic neurons in the more posterior (caudal) slices through the medial septum in $p75^{-/-}$ mice (Fig. 7D). Because p75 is expressed in cholinergic neurons in both anterior and posterior regions, this suggests that developmental cues vary across the basal forebrain resulting in region-specific requirements for p75 in GABAergic development.

Figure 5. Exogenous p75 expression modulates neurotrophin responses of surrounding neurons. **A**, Coexpression of GFP (green) and human p75 (yellow) in a basal forebrain neuron derived from a p75^{-/-} mouse. **B**, p75^{-/-} basal forebrain cultures were transfected with GFP (green) and p75 and immunostained for GAD (red) and MAP2 (blue) 6 d later. A 300- μ m-diameter circle was drawn around the image of an individual GFP⁺ cell, and the numbers of GAD⁺ and MAP2⁺ neurons in the circle were counted. **C**, The percentage of GABAergic neurons in the circle was calculated for neurons transfected with GFP, GFP and human p75, or GFP and the variant human p75-105 and cultured with (NT, filled bars) or without (No NT, open bars) neurotrophins. Values are given as mean $\pm$ SEM percentage of the number of GAD⁺ cells/the number of MAP2⁺ cells ($n = 6$). **D**, The percentage of cholinergic neurons were measured in the surrounding area for neurons transfected with GFP, GFP and human p75, or GFP and the variant human p75-105 (mean $\pm$ SEM; $n = 6$). * $p < 0.05$, unpaired t test.

Discussion

Local circuits involving GABAergic and cholinergic neurons contribute to the output of basal forebrain projections, contributing to the development and function of cortical circuits.

We have shown that neurotrophins and the p75 receptor regulate the development and relative number of medial septal region basal forebrain cholinergic and GABAergic neurons. Neurotrophins increase the numbers of both neuron types, although only the cholinergic neurons express the p75 neurotrophin receptor. Loss of p75 does not affect neurotrophin-dependent increases in cholinergic neurons *in vitro* but limits neurotrophin-dependent development of GABAergic neurons. This non-cell-autonomous effect is mediated via release of a soluble factor that promotes the neurotrophin-dependent acquisition of a GABAergic phenotype. Loss of p75 *in vivo* results in a loss of GABAergic neurons and a region-specific increase in cholinergic neurons. Thus, cell–cell interactions involving neurotrophins and p75 regulate neuronal subpopulations within the medial septum and alter the balance of cholinergic and GABAergic neurons in basal forebrain circuits.

Neurotrophins and GABAergic phenotypes

Extensive work in the basal forebrain has shown that neurotrophins induce cholinergic markers (Li et al., 1995; Pongrac and Rylett, 1998; Berse et al., 1999), promote the survival of BFCNs (Hatanaka et al., 1988; Nonomura and Hatanaka, 1992; Nonomura et al., 1995), and increase cholinergic neuron number (Hatanaka et al., 1988; this study). In addition, we found that neurotrophins influence GABAergic development, regulating neuron number and soma size. Thus, neurotrophins regulate the neurotransmitter identity of both major classes of basal forebrain neurons.

New cholinergic or GABAergic neurons could arise by differentiation of a proliferating precursor population, by increased neuronal survival, or by acquisition of phenotypic markers by preexisting neurons. The lack of significant differences in total neuron number or cell division suggests that neurotrophins did not promote the development of new neurons. This is consistent with birthdating studies showing cell cycle withdrawal of basal forebrain neurons during rat embryonic development, with cholinergic neurons born by E17 (Semba and Fibiger, 1988; Brady et al., 1989). Thus, cholinergic and GABAergic increases involve the acquisition or maintenance of phenotypic properties. This is unlikely to reflect a switch from a cholinergic to a GABAergic phenotype because both populations increase after neurotrophin treatment.

Rather, neurotrophins may act on noncholinergic, non-GABAergic neurons (Gritti et al., 2006) to promote these neurotransmitter properties.

The lack of change in total neuron number suggests that neu-

Figure 6. Production of a neurotrophin modulatory activity requires p75 and neurotrophins. **A**, $p75^{-/-}$ basal forebrain neurons were cultured in conditioned medium prepared from neurotrophin-treated wild-type basal forebrain cultures (CM-WT/NT). Neurotrophins (NT, filled bars) induced a significant increase in the percentage of GAD⁺ neurons compared with control (No NT, open bars) in CM-WT/NT-treated, but not untreated, $p75^{-/-}$ cultures. Values are given as mean $\pm$ SEM; neuron medium, $n = 4$; CM-WT/NT, $n = 5$. **B**, When conditioned medium was made from $p75^{-/-}$ cultures (CM-p75KO/NT) or from wild-type cultures in the absence of added neurotrophins (CM-WT/no NT), there was no significant change in the percentage of GAD⁺ neurons in $p75^{-/-}$ cultures in response to neurotrophins (mean $\pm$ SEM; $n = 3$). $p < 0.05$, unpaired t test.

rotrophins are not acting as trophic factors for these basal forebrain subpopulations. This fits with studies showing that neurotrophins act as survival factors for mature basal forebrain cholinergic neurons (Burke et al., 1994; Koliatsos et al., 1994) but not young, postnatal neurons (Hatanaka et al., 1988; Nonomura and Hatanaka, 1992). Conversely, several studies show that NGF acts to promote the expression and activity of cholinergic pathway components, including ChAT and VAcHT (Hatanaka et al., 1988; Pongrac and Rylett, 1998; Berse et al., 1999), supporting the idea that neurotrophins promote phenotypic properties of cholinergic, and presumably GABAergic, neurons. Expression of cholinergic markers is first seen at approximately E20 in the rat (Bender et al., 1996), several days after the neurons have been born (Semba and Fibiger, 1988). This suggests a period of phenotypic plasticity after the birth of basal forebrain neurons during which the development of cholinergic or GABAergic pheno-

Figure 7. p75 expression is necessary to maintain GABAergic neuron numbers *in vivo*. Altering 40 μ m slices through adult mouse MS from wild-type and $p75^{-/-}$ mice were costained for GABA or ChAT and NeuN. Three-dimensional image series were maximum intensity z-projected to generate a composite image. The MS was visually identified and outlined by hand using the NeuN stain image and copied onto the corresponding GABA or ChAT image. **A**, GABA⁺ and NeuN⁺ neurons were counted on each image within the defined MS region, and GABAergic neuron percentages were generated. There was a significantly lower percentage of GABA⁺ neurons in adult $p75^{-/-}$ mice compared with wild types ($n = 5$, ranging from 10 to 12 months old; $p < 0.05$, unpaired t test). **B**, There was no difference in the average number of neurons (NeuN⁺ cells) in the MS observed between genotypes. **C**, No difference in GABAergic neuron percentages was observed between $p75^{-/-}$ and $p75^{+/+}$ mice in a region of somatosensory cortex ($n = 3$). **D**, The percentages of ChAT⁺ neurons in brain slices through the MS were not significantly different when averaged across all slices. When slices were grouped by their rostral (anterior; ~ 1.18 – 1.10 mm from bregma) and caudal (posterior; ~ 0.86 – 0.74 mm) position in the brain, we observed a significantly greater percentage of ChAT⁺ neurons in $p75^{-/-}$ mice in the caudal slices ($n = 7$; aged 10–14 months old; $p < 0.05$, unpaired t test).

types is influenced by the availability of local or target-derived factors.

Our results suggest that postmitotic neurons in the neonatal medial septum retain the potential to develop into either cholinergic or GABAergic neurons. These immature neurons could be committed to the expression of one or the other phenotype or, alternatively, could be plastic in regard to their final neurotransmitter phenotype. Interestingly, this possibility is supported by studies showing that the expression of LIM homeodomain factor L3 (Lhx8, Lhx7) (Mori et al., 2004; Bachy and Retaux, 2006) is necessary for the specification of cholinergic neurons and repression of a GABAergic phenotype in basal forebrain neurons. In fact, suppression of L3 results in GABAergic development in a neuronal cell line that normally differentiates into cholinergic neurons (Manabe et al., 2005).

p75 receptors in the development of basal forebrain neurons

Whereas p75 is expressed in BFCNs (Sobreviela et al., 1994), most effects of neurotrophins are mediated via Trk receptors (Knusel et al., 1992; Fagan et al., 1997). p75 has been implicated in the regulation of BFCN number, although analysis of $p75^{-/-}$ mice have produced conflicting data (Van der Zee et al., 1996; Peterson et al., 1997, 1999; Yeo et al., 1997; Ward and Hagg, 1999; Greferath et al., 2000; Naumann et al., 2002). Some differences in pre-

vious studies could reflect differences in effects of p75 loss along the rostrocaudal axis. We found an increase in the percentage of cholinergic neurons in more caudal regions of the medial septum, with no changes seen in rostral regions. The reasons for these regional differences are not clear but could reflect differences in the timing or availability of trophic factors and the expression of Trk and p75 receptors.

Although p75 limits the number of cholinergic neurons *in vivo*, we saw no difference in the neurotrophin response of cultured wild-type and $p75^{-/-}$ BFCNs. Because cultures were derived from neonatal animals whereas the number of cholinergic neurons *in vivo* was analyzed in adult mice, this suggests that there may be developmental changes in p75 function in the basal forebrain. A number of ligands and interacting proteins regulate p75 function, resulting in discrete effects on cell death, survival, and neuronal function (Dechant and Barde, 2002). Developmental changes in the expression of these p75-interacting proteins could provide an explanation for the late effects of p75 in limiting cholinergic neuron numbers *in vivo*.

In contrast to the cholinergic system, the role of p75 in the development of GABAergic basal forebrain neurons has not been addressed. At first glance, this seems an unnecessary question because these neurons do not express p75 (Heckers et al., 1994) (Fig. 3). In culture, we found a complete loss of neurotrophin response in GABAergic neurons derived from the $p75^{-/-}$ mice, suggesting a possible role for p75 in GABAergic development. Interestingly, GABAergic neurons still develop in $p75^{-/-}$ cultures, attaining levels similar to that of neurotrophin-treated wild-type cultures within 6 d. This suggests that p75 may normally act to suppress or delay GABAergic development and that, *in vitro*, this effect can be overcome by high levels of exogenous neurotrophins.

Examination of the medial septum of $p75^{-/-}$ mice shows that p75 also acts *in vivo* to regulate the number of GABAergic neurons. Adult $p75^{-/-}$ mice had a smaller percentage of medial septal neurons expressing GABA than strain-matched wild-type animals. Interestingly, we observed this loss in mature adult mice (10–12 months) but not in younger animals (3 months; data not shown), suggesting a role for p75 in the maintenance of the GABAergic phenotype in mature animals. The lack of change in younger animals suggests that p75 is not required for the initial establishment of the GABAergic system. This is consistent with our *in vitro* finding that the number of neonatal GABA neurons is similar in neurotrophin-treated wild-type and $p75^{-/-}$ cultures (at 6 d), although the neurotrophin responses and the timing of development are altered. The same mature adult p75-deficient animals that had fewer medial septal GABAergic neurons showed the same or greater percentage of cholinergic neurons. Thus, these data define p75 as a regulator of cholinergic–GABAergic balance in the medial septum.

Non-cell-autonomous mechanisms involving interactions between different cell types have been implicated in neuronal development (Hiramoto et al., 2000; Helmbacher et al., 2003). In the peripheral nervous system, interactions between developing sympathetic neurons and surrounding non-neuronal cells result in the release of a factor that induces TrkA expression in the neurons (Verdi et al., 1996). The lack of p75 expression in wild-type GABAergic neurons and the requirement for p75 for neurotrophin-dependent GABAergic development also suggest a non-cell-autonomous mechanism. Analysis of p75 knock-out neurons and p75 overexpression suggests that p75 in cholinergic neurons is both necessary and sufficient for exogenous neurotrophins to regulate increases in GABAergic neuron number. This

non-cell-autonomous expression of p75 results in the release of a soluble factor that influences some GABAergic neurotrophin responses, including the timing of recruitment of new neurons to a GABAergic phenotype. A similar situation is seen in a non-cell-autonomous pathway of motor neuron development (Helmbacher et al., 2003). Met, the tyrosine kinase receptor for hepatocyte growth factor, is required for the recruitment of motor neurons to a *pea3* (polyoma enhancer activator 3)-positive pool, although it is not expressed in the *pea3*⁺ neurons. Interestingly, met is not required for the initiation of *pea3* expression but for the expansion of the *pea3*⁺ population. Similarly, in the basal forebrain, p75 is required for the neurotrophin-dependent expansion of the GABAergic population. This suggests that p75-mediated interactions between cholinergic and GABAergic neurons regulate the ratio of cholinergic to GABAergic neuron number within local basal forebrain circuits, potentially influencing the level of neurotransmission of basal forebrain projections.

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