Published in final edited form as:

Brain Res. 2008 December 30; 1246: 19–28. doi:10.1016/j.brainres.2008.09.085.

Differential modulation of nerve growth factor receptor (p75) and cholinergic gene expression in purified p75-expressing and non-expressing basal forebrain neurons by BMP9

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Abstract

The synthesis of acetylcholine and its release from basal forebrain cholinergic neurons (BFCN) that innervate the cerebral cortex and hippocampus are considered essential processes for normal learning, memory and attention. We have developed a purification and cell culture method of BFCN in order to examine the regulation of their cholinergic phenotype. Cells isolated from the septal region of late embryonic mice were purified by fluorescence-activated cell sorting based on their expression of the nerve growth factor receptor (p75), a surface marker for mature BFCN. Consistent with previous reports, p75-positive (p75+) cells were enriched in choline acetyltransferase (ChAT) and the high-affinity choline transporter (ChT), as measured by reverse transcriptase PCR. In culture, these cells maintained their gene expression of p75, ChAT and ChT, while p75-negative (p75-) cells had a low expression of these genes. Incubation of the cells with BMP9 not only increased p75 and ChAT gene expression in p75- cells, but also augmented the expression of these genes in p75+ cells. Conversely, BMP9 decreased ChT gene expression in p75+ cells and had no such effect in p75- cells. Immunostaining confirmed that p75 protein expression was modulated by BMP9 in a similar way as p75 mRNA, and also revealed that only a subset of p75- cells respond to BMP9 in this manner. These data suggest that mature BFCN in culture may express their cholinergic phenotype in the absence of exogenous trophic input, but that BMP9 can further modulate this phenotype. Moreover, BMP9 induces the cholinergic phenotype in a set of basal forebrain noncholinergic neurons or precursor cells.

Keywords

basal forebrain cholinergic neurons; bone morphogenetic proteins; neurotrophin receptor; fluorescent-activated cell sorting

Introduction

The long axon basal forebrain cholinergic neurons (BFCN) that project to the hippocampus and cerebral cortex regulate multiple processes including attention, learning, memory (Sarter and Parikh, 2005) and sleep (Jones, 2005). The cell bodies of these neurons reside in the medial septum, the diagonal band of Broca and the nucleus basalis magnocellularis (Dutar et al.,

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1995; Fibiger, 1982; Mesulam et al., 1983). The degeneration or malfunction of BFCN is thought to contribute to the pathophysiology of memory deficits associated with Alzheimer's Disease (Davies and Maloney, 1976). Study of the development of BFCN is fundamental to the understanding of learning and memory, and may help in the development of new treatments for diseases affecting these processes.

In the mouse, cholinergic neurons first arise in the basal forebrain at approximately embryonic day (E)11, and express cholinergic markers by E16 (Sweeney et al., 1989). These cells extend efferents toward hippocampal and cortical targets, with innervation occurring during the first postnatal weeks (Sweeney et al., 1989). In the septum and the nucleus of the diagonal band, 90% of cholinergic neurons express the low affinity nerve growth factor receptor (aka. neurotrophin receptor, p75), and 90% of p75-expressing (p75+) cells are cholinergic neurons (Dawbarn et al., 1988; Hartikka and Hefti, 1988; Heckers et al., 1994; Lin et al., 2007). Furthermore, p75 is one of the earliest markers expressed in these neurons (Koh and Loy, 1989). Acetylcholine (ACh) is synthesized from choline and acetyl-coenzyme A by the enzyme choline acetyltransferase (ChAT) (Tucek, 1985), a *sine qua non* marker for cholinergic neurons. The choline transporter (ChT, SLC5A7) is a transmembrane protein, co-localized with ChAT at the nerve terminals, that binds choline with high affinity and transports it into the cell (Okuda and Haga, 2003). Although choline from alternative sources, including low affinity uptake and phosphatidylcholine breakdown, may be used for ACh synthesis, uptake of choline by ChT is thought to be the rate-limiting step in the synthesis of ACh in most cases (Haga, 1971). Because very few noncholinergic cells express ChT, it is also considered a marker of cholinergic neurons.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β superfamily of polypeptides that influence many processes such as developmental tissue patterning, apoptosis, cell proliferation, adhesion, positioning and fate decisions in a variety of organs (Massague, 2000; Massague et al., 2000; Massagué, 1998). Specialized functions for BMPs in CNS development include regulation of forebrain formation where a balance between BMP inductive actions and active repression of BMP signals is at play (Liu and Niswander, 2005). Our previous studies implicated BMP9 as a cholinergic differentiation factor in the developing septum (Lopez-Coviella et al., 2000; Lopez-Coviella et al., 2002; Lopez-Coviella et al., 2005). For example, *in vivo* administration of BMP9 into the cerebral ventricles of developing mouse embryos significantly increased the ACh levels in the forebrain. Furthermore, *In vitro* application of this factor to primary septal cells resulted in increased ChAT expression and ACh production; the latter potentiated by addition of bFGF (Lopez-Coviella et al., 2000). All previous *in vitro* studies of BFCN have examined the heterogeneous septal cell population, however BFCN comprise only a minority population in the septum (Greferath et al., 2000a; Naumann et al., 2002).

We have developed a method to isolate and culture BFCN utilizing fluorescence-activated cell sorting (FACS) in order to address fundamental questions concerning the development and nature of these neurons. Both purified BFCN and the noncholinergic cell fraction were responsive to BMP9. We show that the expression of cholinergic markers in purified BFCN persists in basal culture medium without additional trophic input, and that BMP9 further modulates this expression. BMP9 also increased ChAT and p75 expression in noncholinergic cells, however increased p75 surface protein was detected on less than half of these cells, suggesting that only a distinct subset of noncholinergic cells can respond to BMP9 in this manner.

Results

Conditions for the isolation and culture of sorted septal neurons

In order to study p75⁺ septal neurons in isolation, a method for the dissociation, immunostaining, cell sorting and culture of these cells was developed (Schnitzler et al., 2008). The choice of proteolytic enzyme was found critical to the detection of cell surface p75. Though digestion with papain results in higher cell yield, we were unable to detect surface p75 on cells digested with this protease (A.C. Schnitzler, unpublished observations). In contrast, trypsin appears to protect detectability of p75, and therefore it was used as the dissociation enzyme in the isolation method.

Non-neuronal cells can adhere to tissue culture-treated plastic without additional extracellular matrix factors, whereas neurons require additional coating such as poly-d-lysine and laminin for attachment. Therefore, in order to prevent the outgrowth of non-neuronal cells in the septal cultures, the dissociated septal cells were briefly incubated on uncoated tissue culture dishes prior to sorting. As shown in Figure 1A, the adherent fraction of cells was depleted of the cholinergic neuron markers ChAT and ChT whereas the non-adherent cells express these markers. The high level of colony stimulating factor 1 receptor mRNA in the adherent fraction suggests these are infiltrating myeloid lineage cells. Note that some of these cells apparently express p75.

Freshly isolated cells were initially plated in DMEM with 10% FBS and 20 ng/mL bFGF that has been shown to potentiate the effects of BMP9 on ACh production (Lopez-Coviella et al., 2000). Neurobasal medium (NB) with B27 supplement was then used to culture the septal neurons because it is a chemically defined medium developed specifically for the culture of embryonic neurons (Brewer et al., 1993). The cytostatic agent cytosine arabinoside (AraC) was included when the culture medium was changed from DMEM to NB, preventing the outgrowth of any remaining non-neuronal-appearing cells without affecting neuronal morphology or viability (data not shown).

Characterization of FACS-sorted p75-expressing and non-expressing septal cells

Prior to the isolation of p75⁺ septal cells, the specificity of the anti-p75 antibody to be used for the purification was confirmed. p75 protein was clearly detectable on cultured septal cells from wild-type mice by immunostaining, while absent from cells from p75-deficient mice (a gift from Dr. Susan Birren at Brandeis University; see Schnitzler et al. (2008)). FACS was then utilized to purify freshly isolated septal cells based on their expression levels of p75. Septal cells from E17 mice were isolated as described above and in the Methods, immunostained for surface p75 and analyzed by flow cytometry. The cells were sorted into the four fractions R1 to R4 with low to high p75 surface levels, respectively (Figure 1B). Typically, 5–7% of septal cells express high levels of p75 (R4), yielding a highly pure (>95%) single-cell suspension of approximately 10,000 healthy, p75⁺ BFCN per E17–18 septum, or $4 - 5 \times 10^5$ cells per experiment (Supplemental Figure 1). In order to confirm that sorting septal cells based on p75 expression enriched for cholinergic neurons, the ACh content of freshly isolated p75⁺ and p75[−] cells was analyzed by HPLC. p75⁺ cells averaged 3.7 fold higher ACh levels per cell than p75[−] cells (data not shown).

Analysis by RT-PCR revealed that p75 mRNA levels were high in the R4 gate, minimally present in R3 and absent in the negative gates R1 and R2, confirming that p75⁺ septal cells can be enriched for using this technique (Figure 1C). The expression of various neuronal markers was also evaluated in septal cells with varied levels of p75 expression. The cholinergic markers ChAT and ChT were highly enriched in the p75⁺ (R4) fraction, consistent with previous reports (Lopez-Coviella et al., 2005).

p75+ and p75- cells are responsive to BMP9

It is not known whether p75+ BFCN in the developing septum maintain their phenotype in a cell-autonomous manner, or if they require signals from the septal microenvironment. In order to address this question, freshly sorted cells were cultured in basal medium for 3 days, followed by evaluation of cholinergic gene expression by RT-PCR. p75+ cells maintained high levels of p75, ChAT and ChT mRNA in comparison to p75- cells (Figure 2). In light of these results, we were interested in whether p75+ BFCN could respond to a factor that induces the cholinergic phenotype in the forebrain. Interestingly, the addition of BMP9 to the culture medium significantly increased both p75 ($p = 0.047$) and ChAT ($p = 0.010$) gene expression in p75+ cells in a dose-dependent manner up to 40% at the highest concentration, while ChT expression was decreased by 40% ($p = 0.013$, Figure 2). p75- cells exhibited an even more robust increase in p75 ($p < 0.001$) and ChAT ($p = 0.029$) expression in response to BMP9 (up to two-fold). ChT expression in these cells was not significantly affected by BMP9 ($p = 0.445$). As a positive control for the responsiveness of the sorted cells to a BMP signal, the effect of BMP9 on the expression of Id3 was evaluated (Hollnagel et al., 1999). BMP9 induced Id3 expression to a similar extent in p75- and p75+ cells (5-fold, $p = 0.008$ and 0.014 , respectively). The expression of all genes examined also significantly differed between the two cell types (p values < 0.02).

A distinct subset of p75- septal cells is responsive to BMP9

In order to confirm that BMP9-induced p75 gene expression corresponds to a higher level of p75 protein, E17 septal cells sorted based on p75 expression were cultured for 3 days in the presence or absence of BMP9 then analyzed by immunocytochemistry (Figure 3). Indeed, p75 protein was readily detectable in p75+ cells cultured in basal medium, while p75- cells had little detectable p75. Consistent with its effect on p75 mRNA, BMP9 treatment resulted in appreciably increased levels of p75 protein in the initially p75- cells and had little effect on p75+ cells. The staining patterns of p75 and β -III-tubulin, an early neuronal differentiation marker, demonstrated p75 expression in neurons as well as the highly neuronal nature of these cultures (Figure 3).

The immunostaining pattern of p75- cells treated with BMP9 suggested that only a subset of cells in this population respond by increasing p75 levels. To estimate the proportion of responding cells, p75- cells from E17 septa were sorted (Figure 4A) and cultured with or without BMP9 for 3 days. The cells were then briefly trypsinized, stained for p75 and analyzed by flow cytometry. Both the percent of cells in the p75+ gate and the mean fluorescence intensity (MFI) in this fraction were increased with BMP9 treatment (Figure 4B and C; 23.3% to 41.9% of cells, and 40.1 MFI to 73.4 MFI). As suggested by immunocytochemistry, BMP9 induced higher levels of p75 in only a subset of cells. The portion of p75- cells decreased from 77.2% in the untreated culture to 58.7% with BMP9, indicating that approximately a quarter of the p75- cells were responsive to BMP9 in these cultures.

The subset of p75- cells responding to BMP9 by increasing p75 expression could be either progenitor cells or noncholinergic neurons. In order to distinguish between these possibilities, we evaluated the effect of BMP9 on β -III-tubulin. In the whole septal cell population, BMP9 treatment did not alter β -III-tubulin protein levels (Figure 5A). Likewise, β -III-tubulin gene expression was not altered in either sorted p75- or p75+ neurons (Figure 5B), suggesting that an already neuronal set of cells in the p75- population responds to BMP9.

Discussion

There is a paucity of known cell surface markers that identify the different neurotransmitter-expressing subsets of neurons in the CNS. We have taken advantage of p75, one such marker that has a high level of co-expression with intracellular markers of BFCN, and FACS in order

to purify BFCN based on their p75 expression. We show that p75⁺ and p75[−] cells from the septum are enriched and depleted in cholinergic markers, respectively. Moreover, these cells can be cultured under conditions favorable to neurons and are responsive to trophic input *in vitro*.

Perhaps surprisingly, cholinergic neurons in isolation did not require trophic input beyond that provided by NB with B27 supplement to maintain their levels of p75, ChAT and ChT expression. This observation suggests that once acquired, the cholinergic phenotype of basal forebrain neurons may be maintained in a cell-autonomous manner. Alternatively, components of the NB or B27 supplement may be maintaining the expression of cholinergic markers in the p75⁺ cells. NB is based on the DMEM formulation and has been optimized for the culture of embryonic neurons (Brewer et al., 1993). The B27 supplement contains components, notably insulin and retinyl acetate, that may contribute to the maintenance of cholinergic gene expression that we observed in p75⁺ neurons. IGF-I and IGF-II have been shown to increase ChAT activity in rat and mouse septal cultures (Knusel et al., 1990; Konishi et al., 1993). Insulin has also been shown to increase ChAT activity in rat septal cultures, though supraphysiological concentrations were required for a significant effect (Knusel et al., 1990). A role for retinoic acid in cholinergic neuron development has been more clearly established. For example, ChAT activity and mRNA, as well as ACh synthesis are increased in primary neurons and SN56 cells by various retinoids, the latter effect mediated by the retinoic acid receptor alpha (Berse and Blusztajn, 1995; Kobayashi et al., 1994; Malik et al., 2000; Pedersen et al., 1995). Indeed, a consensus sequence for a retinoic acid response element exists within the cholinergic gene locus that encodes both ChAT and the vesicular ACh transporter.

Components of NB, or endogenous factors, may also be responsible for the slight increase in p75 protein on sorted p75[−] cells when cultured in NB medium. Alternatively, cells already set to express p75 but lacking detectable surface p75 at the time of sorting, may have been collected into the p75[−] fraction. Nonetheless, we show that BMP9 induces a greater number of cells to express p75 at a higher level of protein, indicating that BMP9 is acting on a set of cells that do not express the marker in the untreated cultures. Our observations that only a portion of p75[−] cells responds to BMP9 by increasing p75 expression suggest that the late embryonic septum harbors a distinct subset of cells that can be induced to become cholinergic. This population is likely a set of noncholinergic or uncommitted neurons, as BMP9 was not found to modulate the expression of β -III-tubulin.

It has not been clear whether BMP9 exerts its effects on cells already committed to the cholinergic phenotype, a precursor population, or both. The increase in Id3 expression in p75⁺ and p75[−] septal cells cultured with BMP9 indicates that both cell subsets are responsive to BMP signals. Although basal Id3 expression in untreated cultures was very low in comparison to BMP9-induced Id3 expression, untreated p75⁺ BFCN did express 3-fold higher levels of Id3 than untreated p75[−] cells. These data suggest that Id3 expression may be a feature of the BFCN phenotype and that, perhaps, a low level of an endogenous autocrine BMP-mediated signal is present in the p75⁺ cultured BFCN. In light of the search for better treatments of diseases involving cholinergic deficits, the ability of BMP9 to increase p75 and ChAT gene expression in both purified BFCN and noncholinergic septal cells is a significant finding. Improved techniques for delivery of proteins directly to the CNS, for example by gene delivery (Tuszynski et al., 2005), is making trophic factors a feasible treatment option. A factor with the ability to increase the cholinergic potential of both surviving cholinergic neurons and noncholinergic neighbors is a highly desirable treatment candidate.

The importance of high-affinity choline uptake for the production of ACh has long been established, and is highlighted by the neonatal lethality of mice deficient in the choline transporter ChT (Ferguson et al., 2004). Although we did not test the actions of BMP9 on ChT

protein levels, our current data show that BMP9 reduces the expression of ChT mRNA in BFCN. Depending on the trophic input and context, *in vitro* studies reveal that both coordinated and inverse changes in ChAT and ChT expression are possible (Berse et al., 2005; Lecomte et al., 2005; Madziar et al., 2005). Moreover, ChAT haploinsufficient (*Chat*^{+/-}) mice that have an approximately 50% reduction in ChAT levels express 50 – 100% more ChT protein than *Chat*^{+/+} mice, likely accounting for the normal levels of ACh in these animals. Similarly, ChT protein levels are increased by 60% in the striatum of acetylcholinesterase knockout mice possibly because, as a result of slow hydrolysis of ACh, they have less free choline available for uptake from the synaptic cleft (Volpicelli-Daley et al., 2003). Thus, the modulation of ChT may be a mechanism to maintain proper neurotransmission in response to changes in other cholinergic components.

The neurotrophin receptor p75 is widely expressed during early stages of CNS development, but becomes increasingly more restricted into adulthood, maintaining its expression on BFCN (Koh and Loy, 1989). The induction of not only ChAT, but also a marker so closely associated with BFCN is strong evidence that BMP9 induces a differentiation pathway for these neurons. Involved in cell death, survival and axon elongation, p75 likely has a multifunctional role on BFCN (Metsis et al., 1992); though the exact nature of this role remains unclear. In mice deficient in p75, both increased and decreased numbers of BFCN have been reported (Greferath et al., 2000b; Naumann et al., 2002; Peterson et al., 1999; Yeo et al., 1997). Albeit conflicting, these data suggest a role for p75 in regulating the number of these neurons during development. In contrast, the presence of p75 greatly enhances the affinity of the neurotrophins for their respective Trk receptors that mediate survival signals (Bibel et al., 1999; Hempstead et al., 1991). Interestingly, a recent report demonstrates that neurotrophins (nerve growth factor and brain-derived neurotrophic factor in combination) increase the number of both cholinergic and GABAergic neurons in neonatal septal cell cultures, and the expression of full-length p75 in these cultures is required for the GABAergic expansion (Lin et al., 2007). These findings imply that the expression of p75 on cholinergic neurons in the forebrain is an important mechanism for establishing or maintaining neurotransmitter balance. In light of these functions of p75 and its induction by BMP9, it will be important to evaluate the effect of BMP9 on basal forebrain neuron responses to neurotrophins. For example, recent work suggests that other BMPs enhance and complement the protective effects of neurotrophins on BFCN during stress conditions (Nonner et al., 2001; Nonner et al., 2004).

These data suggest that BMP9 induces the differentiation of BFCN, as characterized by p75 and ChAT expression, by acting on a set noncholinergic neurons to direct them towards the cholinergic phenotype. Moreover, although mature BFCN can maintain their phenotype in the absence of BMP9, they also respond to this trophic input by modulating their cholinergic properties. The ability of BMP9 to augment the cholinergic phenotype in distinct cell subsets not only suggests multiple roles for BMPs in the development and regulation of the cholinergic phenotype, but also broadens its potential to treat cholinergic deficits.

Experimental Procedures

Unless otherwise noted, all reagents and chemicals were obtained from Sigma-Aldrich (St. Louis MO). Timed-pregnant CD-1 mice were obtained from Charles River Laboratories (Wilmington MA). See Schnitzler et al. (2008) for a detailed protocol of the septal cell isolation, FACS and culture methods outlined below.

Septal cell isolation

The basal forebrain septal region of embryonic mice was dissected as follows (Hefti et al., 1989): Pregnant mice (gestational stages are specified in the text) were euthanized by carbon dioxide inhalation. Following removal of the uterus, tissue was kept in ice-cold, sterile

Hibernate E (BrainBits, Springfield IL) medium for the duration of the dissection. The olfactory bulbs were removed and vertical incisions were made along the borders of the septum, as guided by the anterior horns of the lateral ventricles and the thalamus, to free the septum from the brain. The meningeal membranes were removed from the excised septa.

Minced septa from approximately 50 fetuses were digested in 1.5 mL of 0.17% trypsin (Invitrogen, Carlsbad CA) at 37°C in the presence of 33 U/mL DNase for 10 min. After placing the septa on ice, the protease solution was aspirated and replaced by 0.5 mL 4% BSA/PBS, 1 mL 10% FBS (heat inactivated, Invitrogen) in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), and 2 mL Hibernate E medium containing 100 U/mL DNase. Single cell suspensions were achieved by repetitively pipetting of septal pieces through fire-polished, siliconized glass pipets (Hartikka and Hefti, 1988), followed by centrifugation for 5 min through a 4% BSA/PBS cushion. The cells were resuspended in ice-cold 0.2% BSA/PBS and incubated in un-coated tissue culture dishes for 20 minutes at room temperature. Non-adherent cells were rinsed from the dishes with 0.2% BSA/PBS, and filtered through sterile, 40 µm nylon mesh filters. Cells were centrifuged for 5 minutes and resuspended as indicated below for FACS or culture.

FACS of primary septal cells

Cells were stained at a concentration of 20×10^6 cells per mL in 0.2% BSA/Hibernate E medium with 5 µg/mL anti-p75 NGFR affinity-purified antibody (ATS, San Diego CA) for 35 min at 4°C. Cells were washed with 0.2% BSA, and incubated with an Alexa 488-conjugated anti-rabbit-IgG secondary antibody (1:500 dilution, Molecular Probes) for 25 minutes at 4°C. Cells were washed, and either resuspended in 0.2% BSA and 1% paraformaldehyde (PFA) in PBS prior to analysis on a FACScan (Becton Dickinson, Franklin Lakes NJ), or 0.2% BSA in Hibernate E medium prior to sorting on a MoFlo cell sorter (DakoCytomation, Denmark). For FACScan analysis, all samples were analyzed through the forward/side scatter whole cell gate. Instrument parameters for each experiment were set using cells stained with secondary antibody alone. Sorted cells were gated through the forward/side-scatter whole cell gate, the single cell gate as determined by pulse width, and the propidium iodide (1 µg/mL) negative gate. Cells were collected into 20% FBS/Hibernate E medium. Data were acquired and analyzed using Summit software (DakoCytomation), or WinMDI 2.8 software (Joseph Trotter, The Scripps Institute).

Septal cell culture

Cells were plated at a density of 1.25×10^5 cells per cm² on poly-D-lysine/laminin-coated tissue culture-ware in 10 % FBS/DMEM supplemented 20 ng/mL bFGF (PeproTech, Rocky Hill NJ), 50 U/mL penicillin and 50 µg/mL streptomycin (pen/strep; Invitrogen). Sixteen hours after plating, culture medium was changed to Neurobasal medium (NB; Invitrogen) containing 2% B-27 (Invitrogen) supplement, 20 ng/mL bFGF, 2 µM cytosine arabinoside hydrochloride (AraC), 250 µM L-glutamine, 250 µM GlutaMax (Invitrogen) and pen/strep. At this time BMP9 (a gift from Wyeth, Cambridge, MA) was added at the indicated concentrations, and every 24 hours subsequently for the duration of the culture. Cultured cells to be analyzed by FACS were rinsed with PBS and trypsinized for 5 minutes at 37°C. Subsequently, 0.5 mL 4% BSA/PBS, 1 mL 10% FBS/DMEM, and 2 mL Hibernate E medium containing 100 U/mL DNase was added to inhibit the protease reaction, and the cells were briefly pipetted through fire-polished, siliconized glass pipets. Finally, the cells were centrifuged through a 4% BSA/PBS cushion, and immunostained as described above.

Reverse transcriptase PCR

RNA for RT-PCR was isolated using an RNAqueous-micro kit according to the manufacturer's instructions (Ambion, Austin, TX). RNA was quantified using the RiboGreen reagent

(Molecular Probes, Carlsbad CA). RT-PCR was performed using the SuperScript One-Step with Platinum *Taq* kit (Invitrogen). Each reaction included 1.2 – 2.2 mM MgSO₄ and 10 – 50 ng of RNA. Reaction conditions were optimized for each primer set such that product amplification fell within the linear range of both cycle number and RNA concentration. The following primer sets were used: ChAT sense 5'-TGA GGA GGT GCT GGA CTT ACC-3', anti-sense GCA CAC CAG AGA TGA GGC TG-3'; p75-NGFR sense 5'-CAC CAC CTC CAG AGC GAG ACC TCA TAG, anti-sense 5'-GAA CAT CAG CGG TCG GAA TG-3'; ChT sense 5'-CGA AAC CTA TGC GTT CCA AG-3', anti-sense 5'-GCT GAG GAT GAA GAG AGG ACC-3'; Id3 sense 5'-GGG TAA GTG AAG AGG GCT GG-3', anti-sense 5'-GAC GAC ATG AAC CAC TGC TAC TC-3'; β -actin sense 5'-CAC AGC TGA GAG GGA AAT C-3', anti-sense 5'-TCA GCA ATG CCT GGG TAC-3'. The RT-PCR reaction products were electrophoresed through 1.8% agarose or 10% acrylamide gels at 100 V, visualized with ethidium bromide on an ImageStation 440CF (Kodak, Rochester NY) and analyzed with Kodak 1D Image Analysis software.

Immunocytochemistry

For immunocytochemical studies cells were grown on 12-mm poly-D-lysine/laminin coated coverslips (Becton Dickinson) that were pre-incubated with 10% FBS/DMEM for one hour prior to the addition of 7.5×10^4 cells per coverslip. After culturing as described above, coverslips were rinsed with PBS then fixed with 3% PFA/PBS for 10 minutes at room temperature. Cells were permeabilized with 100% MeOH for 10 min on ice, or with 0.1% Triton X-100 for 10 min at room temperature, then stained with primary antibody in 0.2% BSA and 0.1% saponin in PBS overnight at 4°C. Anti- β -3-tubulin (Chemicon) and anti-p75 (ATS) were used at a 1:200 dilution. Cells were washed 3 times for 3–5 min with 0.2% BSA in PBS and stained with Alexa-conjugated, species-specific anti-IgG secondary antibodies (Molecular Probes, 1:500 dilution) for 1 h at room temperature. Cells were washed twice with PBS, followed by one rinse in water. Coverslips were mounted using VectaShield Mounting Medium (Vector, Burlingame CA), and analyzed on a Nikon (Melville, NY) inverted fluorescence microscope equipped with the appropriate excitation and emission filters. Exposure settings were optimized with the brightest sample in an experiment. Images were captured with a Spot RT-KE camera and software (Diagnostic Instruments, Sterling Heights MI) using the same exposure and image processing settings for all samples in an experiment.

Immunoblotting

Cells were lysed in buffer containing 137 mM NaCl, 20 mM Tris-HCl pH 8.0, 1% NP-40, 10% glycerol, 1% protease inhibitor cocktail (Sigma P8340) and 1mM PMSF. Four μ g of each sample was electrophoresed through 10% Bis-Tris pre-cast gels (Invitrogen) and transferred to blotting membrane using the iBlot system (Invitrogen). Membranes were blocked with 4% milk in PBS/0.1% Tween (PBS-T), washed with PBS-T and probed with anti- β -3-tubulin (1:2000) or anti- β -actin (1:2000) followed by species-specific anti-IgG-HRP (BioRad, 1:5000). Reactive bands were detected with SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford IL) and captured with an ImageStation 440CF and Kodak 1D Image Analysis software.

Statistics

The statistical significance of BMP9-induced changes in gene expression was determined by one-way ANOVA for each cell type and gene. Two-way ANOVA was also used to evaluate the effect of BMP9 and cell type on gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Yanhui Deng of the Boston University Medical Center Flow Cytometry Core Facility and Stephen Kwok of the Tufts University Flow Cytometry Core for providing expertise in fluorescence activated cell sorting. This work was supported by NIA grant AG009525.

ABBREVIATIONS

BFCN	basal forebrain cholinergic neurons
ChAT	choline acetyltransferase
ChT	choline transporter
ACh	Acetylcholine
BMP	Bone morphogenetic proteins
FACS	fluorescence-activated cell sorting
NB	Neurobasal medium
AraC	cytosine arabinoside
MFI	mean fluorescence intensity

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Figure 1.

Isolation of p75⁺ BFCN via FACS. (A) Dissociated E18 septal cells were incubated in uncoated culture dishes for 20 minutes at room temperature, followed by analysis of the adherent and non-adherent fractions by RT-PCR. (B) Non-adherent, E18 septal cells were stained for p75 (anti-p75 primary antibody, anti-IgG-488 secondary antibody) and sorted based on p75 expression level into fractions R1-R4 (top panel). Cells stained with secondary antibody alone are shown in the bottom histogram. (C) RNA from fractions R1-R4 was analyzed by RT-PCR for expression levels of the indicated genes.

Figure 2.

Effects of BMP9 on p75+ and p75- cell gene expression. E17 p75+ and p75- septal cells were initially cultured in DMEM/10% FBS overnight, then switched to NB plus B27 supplement and cultured for three days in the presence or absence of BMP9 at the indicated concentrations. RNA was isolated and analyzed by RT-PCR. Plotted values are average $\pm$ SEM sample band intensities that are normalized to β -actin ($n = 4$). Representative gels are shown in the lower panel.

Figure 3.

p75⁻ septal cells respond to BMP9 by increasing p75 protein levels. E17 p75⁺ and p75⁻ septal cells were cultured for three days in the presence or absence of BMP9 (100 ng/ml). Cells were fixed with 3% paraformaldehyde, permeabilized with methanol and stained with anti-p75 and anti-IgG-594, or anti-β-3-tubulin and anti-IgG-488 antibodies. BF = bright field. Images were captured with a 20x objective lens.

Figure 4.

A distinct set of p75⁻ septal cells responds to BMP9. (A) E17 septal cells were sorted based on p75 expression (black line = anti-p75 primary antibody and anti-IgG-488 secondary antibody, grey fill = secondary antibody only). (B) The p75⁻ cells were cultured overnight in DMEM/10%FBS, followed by culture in NB plus B27 supplement in the presence or absence of BMP9 (20 ng/mL). After 72 hours, the cells were trypsinized, immunostained for p75 expression and analyzed by flow cytometry (black line = untreated cells, dotted line = BMP9 treated cells, grey fill = secondary antibody only).

Figure 5.

β-3-tubulin is not modulated by BMP9 in late embryonic septal cell cultures. E18 unsorted septal cells, and E17 sorted p75⁻ and p75⁺ septal cells were treated for 3 days in the presence or absence of BMP9 at the indicated concentrations. (A) Cell lysates from eight individual dishes of unsorted septal cells treated with or without BMP9 were analyzed by immunoblot for β-3-tubulin and β-actin protein levels. (B) RNA from sorted p75⁻ and p75⁺ septal cells was analyzed by RT-PCR for β-3-tubulin and β-actin gene expression.