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31. The pulsed-field gel was run under conditions that emphasize the smaller chromosomes: 0.9% agarose, 0.5× tris-borate EDTA, 60- to 120-s switch, 6 V/cm, 120°C, 24 hours, 15°C, run on a CHEF DRIII (Biorad). Cells were grown on YEPD. Chromosomes were prepared as described (*27*).
32. N. Abu-Absi and F. Srienc helped with the FACS analysis. S. Scherer, J. Beckerman, H. Chibana, and S. Grindle contributed helpful criticism, and J. Berman and S. Scherer provided comments on the manuscript. C. Hull and A. Johnson generously provided the sequence of the *MTLα* allele and the organization of the *MTL* region before publication. We are very grateful to D. Gartner and the College of Biological Sciences Imaging Center (University of Minnesota) for help with the figures. Supported by grants AI16567, AI35109, and AI46351 from NIH (P.T.M.).

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Induction and Maintenance of the Neuronal Cholinergic Phenotype in the Central Nervous System by BMP-9

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Bone morphogenetic proteins (BMPs) have multiple functions in the developing nervous system. A member of this family, BMP-9, was found to be highly expressed in the embryonic mouse septum and spinal cord, indicating a possible role in regulating the cholinergic phenotype. In cultured neurons, BMP-9 directly induced the expression of the cholinergic gene locus encoding choline acetyltransferase and the vesicular acetylcholine transporter and up-regulated acetylcholine synthesis. The effect was reversed upon withdrawal of BMP-9. Intracerebroventricular injection of BMP-9 increased acetylcholine levels in vivo. Although certain other BMPs also up-regulated the cholinergic phenotype in vitro, they were less effective than BMP-9. These data indicate that BMP-9 is a differentiating factor for cholinergic central nervous system neurons.

The signals that determine and maintain specific neurotransmitter phenotypes are poorly understood. BMPs—members of the transforming growth factor–β (TGF-β) superfamily of growth and differentiation factors (*1*)—play critical roles in the regulation of neurulation and dorsoventral patterning during gastrulation and of neurogenesis (*2–7*) and forebrain formation (*8*) during later developmental stages. BMPs promote the survival and phenotypic maturation of neurons in the peripheral nervous system and of lineage-restricted neuronal progenitor cells in the central nervous system (CNS) (*9–19*). Here we show that BMP-9, a relatively uncharacterized member of the BMP family, with hepatogenic, osteogenic, and hemopoietic properties (*20–22*), is a potent inducer of the cholinergic phenotype in the CNS.

We determined the expression of BMP-9 in the CNS during mouse development (*23*). On embryonic day 14 (E14), the highest abundance of BMP-9 mRNA was found in the septum and spinal cord (Fig. 1A). The resemblance of this expression pattern to that of mature cholinergic neurons suggested that BMP-9 may influence the development of these cells. We thus treated primary cells derived from the septal area of E14 mice with human recombinant BMP-9 and measured acetylcholine (ACh) in the cultures (*24*) (Fig. 1, B and C). BMP-9 increased ACh content of these cells in a time- (Fig. 1B) and concentration-dependent (Fig. 1C) fashion [median effective concentration (EC_{50}) = 3 ng/ml]. After an initial 24-hour lag period after the addition of BMP-9 (10 ng/ml), ACh levels rose monotonically until 72 hours and then tended to level off. Untreated cultures maintained low levels of ACh throughout the study, indicating that cholinergic neurons initially present in the cultures did not degenerate in the absence of BMP-9. To determine the specificity of BMP-9 action, we treated the cells for 96 hours with other members of the BMP family of proteins. In the absence of BMPs, ACh levels were low (6.4 ± 1.3 pmol/plate), and the addition of BMPs (10 ng/ml) increased the levels of this neurotransmitter to varied extents. BMP-6, BMP-7, and BMP-12 caused a fivefold increase in cellular ACh levels. BMP-2 and BMP-4 increased ACh levels 13- and 14-fold, respectively. BMP-9 was the most effective among the factors tested, increasing the cellular ACh content 20-fold. Moreover, this effect of BMPs was not shared by TGF-β_1, which did not substantially affect the levels of ACh.

BMP-9 also altered the morphology of neural cultures. Whereas untreated cells grew in uniformly dispersed monolayers, cells exposed to BMP-9 tended to grow in characteristic round clusters and extended long and numerous processes (Fig. 1D). These neuronal clusters were positive for βIII-tubulin (an early marker for neurons during development) and concurrently also expressed choline acetyltransferase (ChAT), the ACh-synthesizing enzyme (*25*) (Fig. 1E). Although we also observed neuronal markers in untreated cells, ChAT immunofluorescence was almost completely absent (Fig. 1E). We then repeated double-label experiments with antibodies against βIII-tubulin and the vesicular acetylcholine transporter (VAChT) protein, another cholinergic marker. In most cases, the βIII-tubulin–positive neurons in these clusters were also stained for VAChT (Fig. 1F). Experiments with antibodies against tyrosine hydroxylase (TH) and glutamic acid decarboxylase (GAD)—markers for catecholaminergic and γ-aminobutyric acid–ergic neurons, respectively—revealed no positive staining for these proteins in cells treated with BMP-9, and only a slight increase in TH immunoreactivity was observed by Western blot (*26*). The greatest responsiveness to BMP-9 occurred in cells derived from brain regions that most abundantly express BMP-9 (Fig. 1A) and contain high numbers of cholinergic neurons (Fig. 2A).

Determination of cell fate by extrinsic factors, including BMPs, is typically dependent on the stage of development (*27*). Cholinergic neurons are among the first to leave the mitotic cycle in the mouse basal forebrain (*28*), and cholinergic neurogenesis begins in a caudorostral progression from E11 until E18 (*28, 29*). We studied the effects of BMP-9 on ACh content in septal cultures obtained at E11, E14, and E18. The amount of ACh in the control cultures increased with the gestational age between E11 and E18 (Fig. 2B). BMP-9 increased ACh levels in all of these cultures. However, the response to BMP-9 was the highest in cells originating from E14 embryos, precisely the time during embryogenesis when cholinergic differentiation peaks (*28*) (Fig. 2B).

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Mouse embryos were injected with a single dose of BMP-9 (6 ng) into the cerebral ventricles on E14 and E16, and brain ACh levels were analyzed 2 days later (*24*). BMP-9 significantly increased ACh levels in the forebrain by 70 and 30% measured on E16 and E18, respectively (Fig. 2C). However, BMP-9 had no significant effect in older animals (i.e., mice treated on E18 or E19 or on postnatal days 1 to 3).

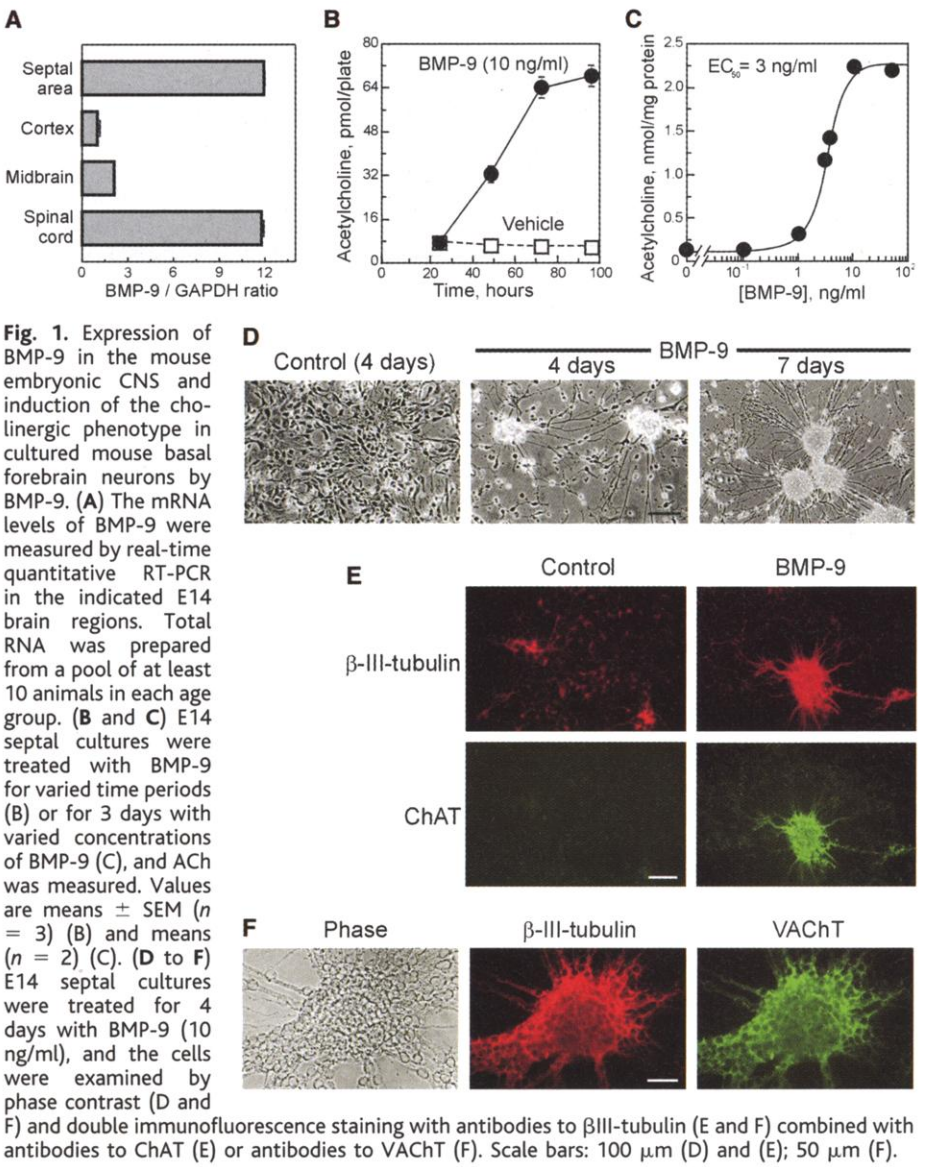

Fig. 1. Expression of BMP-9 in the mouse embryonic CNS and induction of the cholinergic phenotype in cultured mouse basal forebrain neurons by BMP-9. (**A**) The mRNA levels of BMP-9 were measured by real-time quantitative RT-PCR in the indicated E14 brain regions. Total RNA was prepared from a pool of at least 10 animals in each age group. (**B** and **C**) E14 septal cultures were treated with BMP-9 for varied time periods (B) or for 3 days with varied concentrations of BMP-9 (C), and ACh was measured. Values are means ± SEM (n = 3) (B) and means (n = 2) (C). (**D** to **F**) E14 septal cultures were treated for 4 days with BMP-9 (10 ng/ml), and the cells were examined by phase contrast (D and F) and double immunofluorescence staining with antibodies to βIII-tubulin (E and F) combined with antibodies to ChAT (E) or antibodies to VAChT (F). Scale bars: 100 μm (D) and (E); 50 μm (F).

mRNA levels of ChAT and VAChT were measured in septal cultures by Northern blot. The ChAT and VAChT genes reside in an evolutionarily conserved single genomic locus (Fig. 3A), an organization that is thought to permit coordinated regulation of their expression (*30*). In the absence of BMP-9, levels of both ChAT and VAChT mRNA (*31*) were very low or undetectable in cells derived from E14 embryos after 4 days in culture. Addition of BMP-9 substantially increased the expression of ChAT and VAChT mRNA (Fig. 3B), indicating that BMP-9 induced the expression of the cholinergic gene locus.

To determine if BMP-9 up-regulates the cholinergic phenotype by acting directly on responsive cells, we determined the effect of this BMP on ChAT and VAChT expression in a murine septal cell line, SN56T17 (*30*, *32*, *33*). BMP-9 increased the expression of ChAT and VAChT mRNA in these cells (*31*) (Fig. 3B). This action of BMP-9 was quantitatively smaller in SN56T17 cells than in primary neurons, presumably because the former are already committed cholinergic cells with high basal expression of ChAT and VAChT. Moreover, BMP-9 increased the abundance of endogenous ChAT mRNA originating from the M promoter (Fig. 3C) and stimulated the expression of a luciferase reporter gene driven by a 4.8-kb fragment of the ChAT gene containing this promoter (Fig. 3, A and D) (*30*, *33*). These results show that up-regulation of the cholinergic phenotype by BMP-9 occurs in a homogenous population of competent cells, supporting the notion that in primary brain cultures BMP-9 also acts directly on cholinergic precursor cells and not by inducing diffusible factors originating from other cell types. Moreover, the data point to the presence of a BMP-9–responsive region within the cholinergic gene locus.

Studies in animals have shown that when cholinergic neurons are deprived of their targets and/or trophic factors, they lose their ChAT marker but do not die (*34*). This suggests that expression of the cholinergic phe-

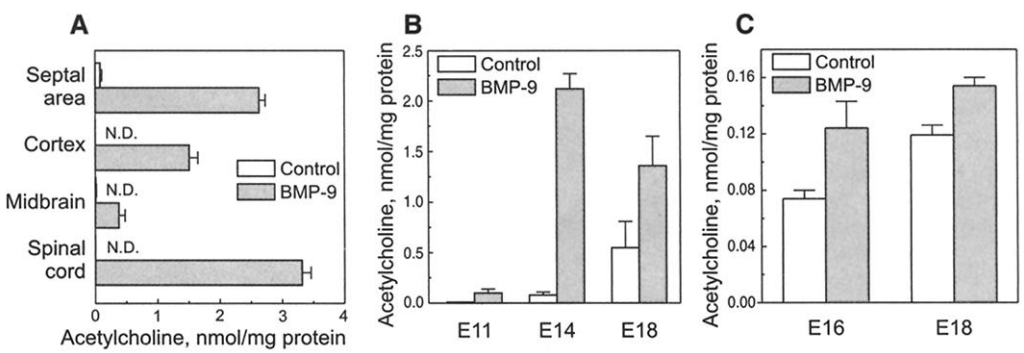

Fig. 2. In vitro and in vivo induction of the cholinergic phenotype by BMP-9. (**A**) Brain region specificity in E14 cultures from the CNS regions indicated. N.D., not detected. (**B**) Developmental stage specificity in septal area cultures obtained at E11, E14, and E18. (**C**) Advanced maturation of the cholinergic phenotype in vivo. (A and B) Primary cultures were treated for 3 days with BMP-9 (10 ng/ml) and ACh was measured. (C) Mouse embryos were injected intracerebroventricularly with 6 ng of BMP-9, and brain tissue was processed for ACh content 2 days after (*24*). Values are means ± SEM (n = 5). BMP-9 significantly increased the levels of ACh at E16 and E18 (two-way analysis of variance with Fisher's post hoc test, $P < 0.01$ and $P < 0.05$, respectively).

notype is not a fully intrinsic property of these neurons but rather that its maintenance continues to depend on extrinsic signals. Cells were cultured for 3 days in the presence of BMP-9 and subsequently grown in the presence or absence of this BMP for another 3-day period (Fig. 4A). Cells treated continuously with BMP-9 maintained their high ACh content, whereas in cells deprived of BMP-9, ACh levels were reduced by 80%, indicating that BMP-9 was necessary for the maintenance of the induced cholinergic phenotype. A 3-day treatment of the cells with BMP-9 after a deprivation period resulted in a twofold up-regulation of ACh synthesis, indicating that they retain their responsiveness to BMP-9.

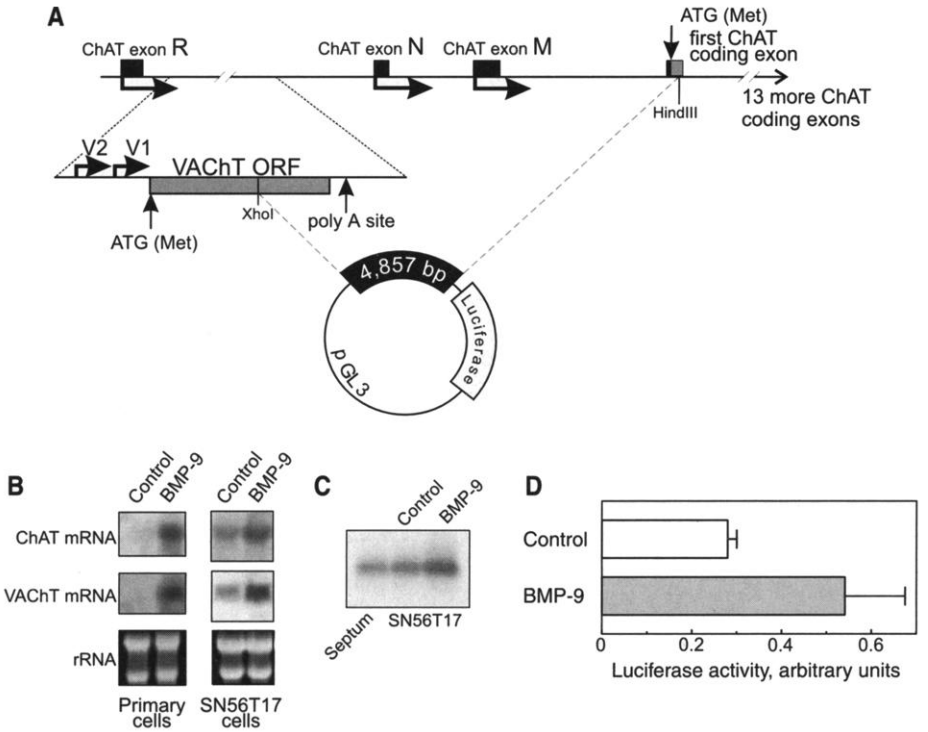

Fig. 3. Induction of the cholinergic gene locus expression by BMP-9. (**A**) Organization of the cholinergic gene locus and the reporter gene construct used in (D). The noncoding exons R, N, and M are shown as black boxes and the coding sequences are in gray. The intronless open reading frame (ORF) of VAChT resides between exons R and N of ChAT. For the reporter gene assays, the 4857–base pair Xho I–Hind III fragment of the murine gene was inserted into the pGL3-Basic luciferase plasmid. (**B**) E14 septal cultures or SN56T17 cells (*32*) were treated with BMP-9 (10 ng/ml) for 3 days, and ChAT and VAChT mRNA was determined by Northern blotting. (**C**) BMP-9 up-regulates the expression of the M exon of ChAT. SN56T17 cells were treated for 2 days with BMP-9 (10 ng/ml), and the expression of the M exon was determined by RT-PCR. Adult mouse septum is also shown as positive control. (**D**) Up-regulation of luciferase expression driven by the ChAT promoter in SN56T17 cells treated for 2 days with BMP-9 (10 ng/ml).

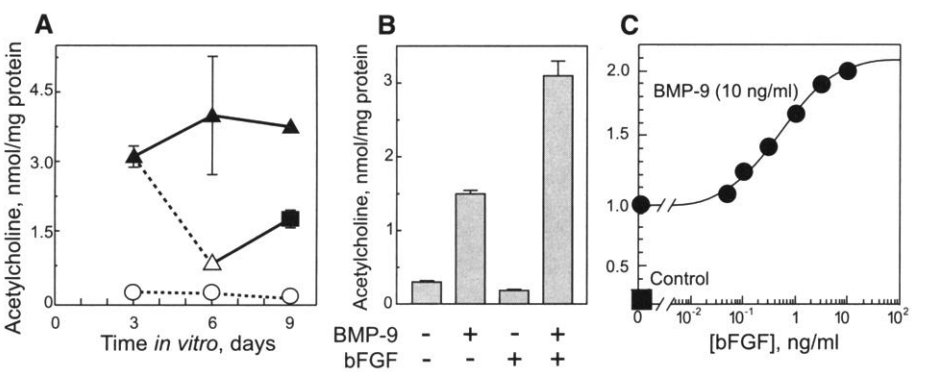

Fig. 4. Requirement of BMP-9 for the maintenance of the induced cholinergic phenotype and potentiation by bFGF of the induction of the cholinergic phenotype evoked by BMP-9. (**A**) ACh was measured in septal cultures from E14 mice treated with BMP-9 (10 ng/ml) (▲) for 3, 6, and 9 days or for 3 days followed by a 3-day withdrawal of BMP-9 (△) and then an additional 3-day period with BMP-9 (■). Controls received no BMP-9 (○). (**B** and **C**) E14 septal cultures were treated for 3 days with (B) BMP-9 (10 ng/ml) and/or bFGF (10 ng/ml) or (C) in the presence of BMP-9 with varying concentrations of bFGF, and ACh was measured. The values are means ± SEM (n = 4).

There is evidence for an interaction between basic fibroblast growth factor (bFGF) and BMP signaling in the developing nervous system (*35*, *36*). FGF promotes the proliferation of progenitor cells, preventing their exit from the cell cycle and contributing to the specification of progenitor cell identity (*37*). BMPs, in contrast, tend to reduce cell proliferation, promoting cell differentiation and cell lineage restriction. During development, these apparently opposing effects may contribute to a specific cell fate (*38*). We measured the response of septal cells to BMP-9 in the presence and absence of bFGF. Whereas bFGF had no effect on ACh synthesis by itself, BMP-9 alone was sufficient to increase ACh levels by more than fourfold as compared with controls (Fig. 4B). However, in the presence of bFGF, the effect of BMP-9 on ACh content was potentiated, and the extent of potentiation by bFGF was concentration-dependent (Fig. 4C). These data are consistent with the notion that bFGF stimulates proliferation of cholinergic progenitor cells that are induced by BMP-9 to express the cholinergic phenotype.

Our results suggest that the rate of maturation of cholinergic neurons may be enhanced by BMP-9 and that BMP-9 most likely acts on cells committed to become cholinergic or on cells with restricted fates. Alternatively, cells from E11 and E18 cultures or those in older animals, in vivo, that are less responsive to BMP-9 may produce antagonists that bind, and thereby inactivate, BMP-9, a mode of regulation that has been observed for other BMPs (*6*–*8*, *35*, *39*).

It is likely that, depending on brain region and developmental stage, various BMPs will regulate the expression of other neurotransmitter phenotypes. Indeed, there is evidence that BMPs modulate neurotransmitter expression in sympathetic (*16*–*19*, *40*), spinal cord (*41*), mesencephalic (*42*, *43*), and striatal (*44*) neurons in vitro. However, in contrast to the current study, some of these effects of BMPs were either indirect and mediated by diffusible factors released by glial cells (*42*, *45*) or due to their action as survival factors (*45*).

Our results point to a developmental function for BMP-9 and suggest a model in which BMP-9, synthesized locally in the vicinity of the developing cholinergic neurons, acts to induce the expression of the cholinergic gene locus in these cells. This action of BMP-9 as a cholinergic differentiation factor suggests its potential use in the treatment of diseases that affect cholinergic neurons.

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23. BMP-9 mRNA was measured in the septum, cortex, mesencephalon, and spinal cord dissected from E14 mice and snap-frozen in liquid nitrogen. Total RNA was prepared with a Quiagen RNeasy Mini Kit. Each sample contained RNA prepared from a pool of at least 10 animals to minimize animal-to-animal variations. cDNA was prepared with a Superscript Preamplification System (Gibco-BRL, Gaithersburg, MD). Real-time semiquantitative polymerase chain reaction (PCR) was performed with the SYBR Green PCR Reagents and an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). BMP-9 mRNA levels were normalized to levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR primers used were as follows: BMP-9: forward primer, 5′-TCCCCACCGACTTGTTCTTC-3′; reverse primer, 5′-GAGAGTCAGCTGGGAGCTTGA-3′; GAPDH: forward primer, 5′-TGTGTCCGTCGTGGATCTGA-3′; and reverse primer, 5′-CCTGCTTCACCACCTTCTTGA-3′. Sequencing of PCR products confirmed their identity.
24. Dissociated cells obtained from E11 to E18 mice were plated on poly-L-lysine/laminin–coated tissue culture dishes and incubated with Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum and basic fibroblast growth factor (bFGF) (20 ng/ml) to maintain high numbers of precursor cells. BMP-9 and other TGF-β family members (Genetics Institute) were added immediately after plating and every 24 hours until the cells were collected for analysis. Intracerebroventricular injections (1 μl) of BMP-9 (6 ng) and vehicle, both containing bFGF (20 ng), were performed in embryos at E14 and E16. The animals were anesthetized with isofluorane. After a midventral laparotomy, the uterus was externalized, and the embryos were injected through the uterine wall, into the CNS ventricular system, by means of a 33-gauge needle attached to a microcannula. This procedure was carried out under a surgical microscope. After the injections, the uterus was repositioned into the abdominal cavity, which was closed at various planes with absorbable and nonabsorbable surgical suture. Animals were allowed to recover and then were killed 2 days later by CO_2 inhalation. Brains were dissected and placed flat in ice-cold physiological salt solution. The forebrain was obtained by means of a coronal cut performed under microscope, 2.5 mm from the frontal pole at the level of the thalamus, including the telencephalic vesicles. ACh content of the forebrain was measured by high-performance liquid chromatography (*30*). All experiments with animals were approved by the Boston University Institutional Animal Care and Use Committee.
25. Immunofluorescence studies were performed with goat affinity-purified polyclonal antibodies for ChAT (1:100), TH (1:200), GAD (1:1000), and VAChT (1:500) and a mouse monoclonal antibody against βIII-tubulin (1:500) (all from Chemicon), done in parallel with negative and positive controls. Briefly, cells plated on Biocoat chamber slides (Falcon) or cover-glass coated with poly-D-lysine and laminin were fixed in methanol for 5 min at −10°C and incubated at room temperature with 0.1% Triton-X 100 solution. The cells were washed with phosphate-buffered saline, blocked for 30 min with 1% bovine serum albumin (BSA), and incubated overnight with primary antibodies in 1% BSA and for 40 min with the appropriate fluorescent Alexa-conjugated secondary antibodies (Molecular Probes). Slides were mounted with Prolong Antifade (Molecular Probes) and visualized on an epifluorescence-equipped microscope.
26. I. López-Coviella, B. Berse, R. Krauss, R. S. Thies, J. K. Blusztajn, data not shown.
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31. Total RNA was extracted from the cells with the acid guanidinium thiocyanate–phenol-chloroform method. Northern blotting was performed as described (*30*). Signal intensities were quantified directly from the blots with a PhosphoImager 400E and ImageQuant software (Molecular Dynamics). To control for total mRNA content and lack of degradation, we stripped the blots and subsequently hybridized them with a mouse GAPDH cDNA probe.
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33. Transfections and reporter plasmid experiments were carried out on mouse cholinergic septal SN56T17 cells. The cells were maintained in DMEM containing 10% fetal bovine serum and gentamicin (50 μg/ml). Reverse transcription (RT)–PCR for the M exon of ChAT was performed with the Access RT-PCR system from Promega. The forward primer (5′-GGGGTGGCTGGTTTGCTTGCAGTCA-3′) was designed specifically for detection of transcripts originating at the M promoter, and the reverse primer (5′-GGGGGCACTGGCAACTTAGGTAAG-3′) was derived from the coding region of the ChAT gene. After PCR, amplification products were subjected to Southern blotting with a ChAT-specific probe and visualized by autoradiography. To make a reporter construct, we inserted a 4.8-kb Xho I–Hind III fragment of the ChAT promoter (a gift from J. Naciff) upstream of the luciferase coding region in the plasmid pGL3-Basic from Promega. SN56T17 cells were transfected with LipofectAMINE (Gibco-BRL), and luciferase activity was measured with the Luciferase Assay System from Promega.
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