BMP4 supports noradrenergic differentiation by a PKA-dependent mechanism

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Received for publication 27 May 2005, revised 10 August 2005, accepted 11 August 2005
Available online 13 September 2005

Abstract

Differentiation of neural crest-derived noradrenergic neurons depends upon signaling mediated downstream of BMP binding to cognate receptors and involving cAMP. Compiled data from many groups suggest that neurogenesis and cell type-specific noradrenergic marker gene regulation is coordinated through the expression and function of the basic helix–loop–helix DNA binding protein HAND2 and the homeodomain DNA binding protein Phox2a. However, information detailing how BMP-mediated signaling and signaling through cAMP are coordinated has been lacking. We now provide compelling data suggesting that differentiation of noradrenergic sympathetic ganglion neurons depends upon both canonical and non-canonical pathways of BMP-mediated signaling. The non-canonical pathway involves the activation of protein kinase A (PKA) independent of cAMP. This is a novel mechanism in neural crest-derived cells and is necessary to support neurogenesis as well as aspects of DBH promoter regulation involving HAND2 phosphorylation and dimerization. The expression of transcripts encoding HAND2 and Phox2a is regulated via canonical BMP signaling and thus affects both neurogenesis and cell type-specific gene expression. Interestingly, cAMP- and MAPK-mediated signaling modulate specific target sites in both the canonical and non-canonical BMP pathways. Activity of MAPK is required for HAND2 transcription and thus affects neurogenesis. Signaling affected by cAMP is necessary for the transcription of Phox2a as well as regulation of DBH promoter transactivation by Phox2a and HAND2. We suggest a comprehensive model that shows how BMP- and cAMP-mediated intracellular signaling integrate neurogenesis and cell type-specific noradrenergic marker gene expression and function.

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Keywords: HAND2; dHAND; BMP4; Neural crest; cAMP/PKA; Neurotransmitter specification; Phox2; DBH; Neurotransmitter transcriptional regulation

Introduction

Lineage segregation of neural crest-derived precursors occurs as a consequence of integrating cell extrinsic instructive cues with intrinsic networks of intracellular signaling cascades and transcriptional regulation. Identification of factors influencing the generation of neural crest-derived noradrenergic sympathetic ganglion neurons has received significant attention. Neural crest-derived cells that will differentiate as noradrenergic neurons receive instructive signals from the neural tube (Teillet and Le Douarin, 1983; Howard and Bronner-Fraser, 1985, 1986; Stern et al., 1991; Groves et al., 1995) and from the dorsal aorta where they localize and ganglionate (Reissmann et al., 1996; Schneider et al., 1999; Howard et al., 2000; reviewed in Goridis and Rohrer, 2002; Howard, 2005). Although the relevant neural tube-derived factor has not been identified (Howard and Gershon, 1993), an essential role for aorta-derived bone morphogenetic protein growth factors (BMP) in the differentiation of noradrenergic neurons is well established (Varley et al., 1995, 1998; Shah et al., 1996; Reissmann et al., 1996; Schneider et al., 1999; Howard et al., 2000). BMP4 induces expression of a transcription factor network that includes the homeodomain DNA binding proteins Phox2a, Phox2b, and the basic helix–loop–helix (bHLH) DNA binding protein HAND2 (Howard et al., 2000). We and others have provided evidence suggesting that the coordinated expression and function of HAND2 and Phox2a specify multiple components of a sympathetic noradrenergic phenotype (reviewed in Goridis and Rohrer, 2002; Howard, 2005). Both neurogenesis and cell type-specific gene expression depend upon signaling mediated downstream of BMP (Reissmann et al., 1996; Lo et al., 1999; Howard et al., 2000; reviewed in Goridis and Rohrer, 2002; Howard, 2005). Although the relevant neural tube-derived factor has not been identified (Howard and Gershon, 1993), an essential role for aorta-derived bone morphogenetic protein growth factors (BMP) in the differentiation of noradrenergic neurons is well established (Varley et al., 1995, 1998; Shah et al., 1996; Reissmann et al., 1996; Schneider et al., 1999; Howard et al., 2000). BMP4 induces expression of a transcription factor network that includes the homeodomain DNA binding proteins Phox2a, Phox2b, and the basic helix–loop–helix (bHLH) DNA binding protein HAND2 (Howard et al., 2000). We and others have provided evidence suggesting that the coordinated expression and function of HAND2 and Phox2a specify multiple components of a sympathetic noradrenergic phenotype (reviewed in Goridis and Rohrer, 2002; Howard, 2005). Both neurogenesis and cell type-specific gene expression depend upon signaling mediated downstream of BMP (Reissmann et al., 1996; Lo et al., 1999; Howard et al.,
2000; Dubreuil et al., 2002) and likely involve translocation of Smad1 to the nucleus (Wu and Howard, 2001). Although BMP4 induces expression of transcripts encoding Phox2b and HAND2, it is not known if this is a direct effect on gene transcription or whether intracellular signaling molecules recruited to non-canonical BMP-mediated signaling pathways are required intermediates.

A role for signaling downstream of cyclic AMP (cAMP) in the generation of noradrenergic neurons has been established (Maxwell and Forbes, 1990; Dupin et al., 1993; Shah et al., 1996; Lo et al., 1999) although the proximal signal activating cAMP and the downstream effects of its stimulation remain unclear. Initially, it was shown that chick embryo extract (CEE) induces differentiation of noradrenergic neurons (Howard and Bronner-Fraser, 1985, 1986) as well as stimulating adenylate cyclase activity (Dupin et al., 1993). These studies suggested that extrinsic signals induced the differentiation of noradrenergic neurons in vitro and the involvement of cAMP was inferred by demonstrating that direct activation of adenylate cyclase with forskolin induced expression of TH (Dupin et al., 1993). A more complicated picture emerged as it became clear that the response to cAMP is dose-dependent with low levels of cAMP supporting biosynthesis of norepinephrine while higher concentrations are inhibitory (Maxwell and Forbes, 1990; Bilodeau et al., 2000). These data suggested that cAMP might act as a switch in neuronal precursor cells to regulate their differentiation into noradrenergic neurons by affecting expression of transcripts encoding Phox2a and MASH1 (Maxwell and Forbes, 1990; Bilodeau et al., 2000). Although these early studies suggested that cAMP-mediated signaling had an impact on specification of neurotransmitter phenotype, questions relating to neurogenesis and the function of BMP remained exigent.

For differentiation of sympathetic noradrenergic ganglion neurons, both BMP and cAMP have demonstrated significant roles. The expression and function of Phox2a may be the focal point linking BMP- and cAMP-mediated signaling to noradrenergic cell type-specific gene expression. The specification of neurotransmitter identity depends in part upon cAMP (Lo et al., 1999). In rodent neural crest-derived stem cells, expression of the catecholaminergic marker gene, tyrosine hydroxylase (TH), is dependent upon both cAMP and Phox2a. Interestingly, expression of the noradrenergic marker gene, dopamine-β-hydroxylase (DBH) and pan-neuronal genes, requires both BMP and cAMP (Lo et al., 1999). These data suggest that Phox2a, alone, is sufficient to induce expression of catecholaminergic cell type-specific marker genes (Lo et al., 1998; Howard et al., 2000) but is not sufficient to support expression of noradrenergic or pan-neuronal genes (Lo et al., 1998). Although this study provided evidence for a cAMP-dependent function of Phox2a in specifying neurotransmitter identity, it left open the question of linkage between the function of BMP and cAMP in neuronal differentiation. This link may be based in the function(s) of HAND2.

These data suggest that the integrated expression and function of TH and DBH necessary for norepinephrine biosynthesis require the coordinated expression of several environmentally-derived signals as well as transcriptional regulators in addition to Phox2a. HAND2 has been identified as an essential determinant of the noradrenergic phenotype (Howard et al., 1999, 2000) as well as a co-transcriptional regulator of DBH expression (Xu et al., 2003; Rychlik et al., 2003). In total, these data suggest multiple effects of cAMP in neurogenesis and expression and function of DBH. Because of the central role of Phox2 proteins and HAND2 in both neurogenesis and noradrenergic cell type-specific gene expression (reviewed in Goridis and Rohrer, 2002; Rohrer, 2003; Howard, 2005), we undertook to determine at what points along the path of differentiation as noradrenergic neurons neural crest-derived cells require BMP and/or cAMP-dependent signaling for differentiation and/or synthesis of norepinephrine. Our results suggest that BMP4-mediated intracellular signaling involves both canonical and non-canonical pathways. We present evidence that neurogenesis and noradrenergic marker gene expression is integrated, downstream of BMP4, by cAMP. We provide a comprehensive model suggesting that BMP4 is an instructive signal to neural crest-derived cells via its canonical signaling pathway. This aspect of signaling supports transcription of HAND2 that is required for neurogenesis and differentiation as noradrenergic neurons. Additionally, BMP4 provides a permissive signal, via a non-canonical pathway, supporting neurogenesis and DBH function; components of each of these pathways are influenced by cAMP.

Materials and methods

Neural crest cell culture

Neural crest cells were obtained from stage 13–14 (Zacchei, 1961) quail embryos (Coturnix coturnix japonica) as previously described (Howard and Bronner-Fraser, 1985, 1986; Howard et al., 1995, 1999; Wu and Howard, 2001). Briefly, fertile quail eggs (Bear Bayou Quail Farm, Channelview, TX) were incubated at 38°C for 43–47 h. Using electrolytically sharpened tungsten needles, a fragment containing the last eight developed somites and associated neural tube, was surgically removed from embryos. The tissue blocks were then incubated in 0.5% collagenase A (Roche, Indianapolis, IN) for 12 min at room temperature, and gentle trituration used to release the neural tube. The neural tubes were washed and collected in fresh growth medium (as described below) and plated on 35-mm tissue culture plates coated with 24 µg/ml fibronectin (BD Bioscience, Bedford, MA). After 14–16 h in culture, the neural tube explants were removed from the dish, leaving behind the neural crest cells that had migrated from the explant onto the dish. To document complete removal of neural tube cells, plates were visually screened for the presence of motoneurons on culture day two; no dishes containing motoneurons were used in the studies reported in this paper. At the time the explants were removed, growth medium was changed and every other day thereafter, for the entire culture period.

Growth media and reagents

Neural crest cell cultures were fed with Eagle’s minimal essential medium (Invitrogen Corp., Carlsbad, CA) supplemented with 15% v/v horse serum (Omega Scientific Inc., Tarzana, CA) and either 2% or 10% 11-day chicken embryo extract (CEE, 2% or 10%, v/v) prepared as previously described (Howard and Bronner-Fraser, 1985). In some experiments, BMP4 and/or pharmacological inhibitors or analogs of cAMP or PKA were added to growth medium. BMP4 (R&D Systems, Minneapolis, MN) was added to a final concentration of 10 ng/ml at the time of plating, and with each change of growth media, unless otherwise stated. To inhibit the activity of adenylate cyclase, 2’,5’-dideoxyadenosine (dda) (Calbiochem, La Jolla, CA) or SQ 22,536 (Sigma, St. Louis, MO) was added to growth medium in DMSO at a
final concentration of 200 μM. To inhibit the activity of PKA, either H-89 (10 μM) (Calbiochem, La Jolla, CA) or KT 5720 (1 μM) (Sigma, St. Louis, MO) was added to neural crest cells at the time of plating and every second day thereafter, unless otherwise stated. Inhibitors of cAMP and PKA were reconstituted in DMSO (0.1%–0.4% final concentration); in vehicle control experiments, DMSO was found to have no effect upon cell growth, viability, or noradrenergic differentiation (Howard, unpublished data). The cAMP analog, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP, Sigma, St. Louis, MO), was used at a final concentration of 1 μM.

Catecholamine histofluorescence

Cells synthesizing and storing norepinephrine were detected by the formaldehyde induced fluorescence (FIF) method of Falck and Torp (1962) as previously described (Howard and Bronner-Fraser, 1985; Howard and Gershon, 1993; Howard et al., 1999). Briefly, the growth medium is aspirated from culture plates and the cells are heat fixed under a stream of hot air. The cells are exposed to formaldehyde vapor at 80°C for 1 h, cover-slipped in mineral oil, and visualized using a catecholamine filter set. To assess the differentiation and phenotypic expression of the noradrenergic marker, NE, the number of cells showing NE histofluorescence (FIF) was counted in a minimum of 10 randomly chosen microscope fields from each explant on a culture plate. For each growth condition or treatment, a minimum of nine explants from three separate platings were counted, unless otherwise stated.

Assay of protein kinase A activity

The activity of protein kinase A (PKA) was determined in neural crest cells derived from 15–20 explants grown in 2% or 10% CEE-medium with or without added BMP4 (10 ng/ml) and added dda (200 μM) or H-89 (10 μM). To determine the effect of added 8-Br-cAMP on levels of PKA activity, neural crest-derived cells were grown in 2% CEE or 10% CEE-medium in the presence or absence of added 8-Br-cAMP (1 μM). For those studies where the effect of pharmacological intervention was being tested, neural crest-derived cells were maintained in culture for a period of 7 days. Cells were harvested by scraping and homogenization on ice in extraction buffer (modified Radio-immunoprecipitation buffer (RIPA)) containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of each of the following protease inhibitors: leupeptin, aprotinin, and sodium vanadate. The activity of PKA was determined using the SigmaTECT cAMP-Dependent Protein Kinase (PKA) Assay System, according to manufacturer’s directions (Promega, Madison, WI).

Accumulation of cAMP

The ability to mobilize cAMP in neural crest-derived cells was determined using an 125I-cAMP RIA kit according to manufacturer’s directions (Amersham Bioscience, Piscataway, NJ). For each determination, neural crest-derived cells, from 15–20 neural tube explants, were pooled and cAMP extracted after 7 days in culture. cAMP was extracted by treating the cultures with 300 μl ice-cold 70% ethanol for 12–16 h at −20°C. For each dish, extracted material was transferred to a 1.5 ml centrifuge tube and spun at 14,000 g (13,000 rpm) for 10 min at 4°C. The supernatant was recovered in a fresh tube. Pellets were resuspended in 200 μl of the RIA buffer. The amount of cAMP in each sample was determined according to the manufacturer’s instructions and based on a standard curve with known amounts of cAMP ranging from 25 fmol/tube to 1600 fmol/tube. Each sample was measured in duplicate.

Semi-quantitative RT-PCR

Total cellular RNA was purified from the outgrowth of 15 to 20 primary neural crest explants (per point) using the RNeasy Mini kit (QIAGEN Inc., Valencia CA) according to manufacturer’s directions. Following RNase-free DNase I (QIAGEN Inc., Valencia CA) treatment, 1 μg purified cellular RNA was used as template for first strand synthesis using 1 unit of SuperScript reverse transcriptase (Invitrogen Corp., Carlsbad, CA), 0.5 mM each dNTP, 3 mM MgCl₂, 10 mM DTT, 2 units RNaseOUT ribonuclease inhibitor (Invitrogen Corp., Carlsbad, CA), and 5 μM random hexanucleotides in a final volume of 20 μl. First-strand synthesis was carried out for 50 min at 42°C. The DNA–RNA hybrids were denatured at 75°C for 15 min and then cooled on ice for 5 min prior to PCR amplification. Equivalent aliquots of first strand cDNA were amplified using primers specific for HAND2, Phox2a, or chicken β-actin. The final reaction contained 0.2 mM dNTPs, 0.4 μM of each primer, 1.5 mM MgCl₂, and 1 unit Platinum Taq polymerase (Invitrogen Corp., Carlsbad, CA). For the amplification of HAND2, the cycler protocol was 94°C, 4 min, then 27 cycles of: step 1, 94°C, 75 s, step 2, 55°C, 75 s, step 3, 72°C, 75 s, and a final elongation step, 72°C, 10 min. For the amplification of Phox2a, the cycler protocol was 94°C, 4 min, followed by 31 cycles of 94°C, 60 s, 55°C 60 s, 72°C, 60 s, and 72°C 10 min. Upon completion of the PCR reaction, amplicons were maintained at 4°C until fractionation in a 1.5% agarose gel. In pilot studies, we established that amplification of HAND2, Phox2a, or β-actin transcript was not saturated and that our assay was done in the linear range of amplification.

Quantitative real-time PCR

Changes in levels of transcript encoding HAND2 and β-Actin (as internal control) following treatment with BMP4 and/or dda and H-89 were established using RT-based real-time PCR as previously described (Zhou et al., 2004). cDNA samples equivalent to 50 ng of total RNA were incubated with forward and reverse primers (0.4 μM), Taqman probe (0.1 μM), and Taqman universal PCR master mix (Roche, Branchburg, NJ). The Taqman probes were labeled at the 5′ end with 6-FAM (carboxyfluorescein, reporter dye) and TAMRA (tetramethylrhodamine, quencher dye) at the 3′ ends. Selection of HAND2 and β-Actin gene-specific primers and probes was optimized using Applied Biosystems (Foster City, CA) Primer-Express software. Initially, a validation experiment was performed to demonstrate that the efficiencies of target (HAND2) and reference (β-actin) were approximately equal. For this purpose, a dilution series of 5 ng, 10 ng, 20 ng, 50 ng, and 100 ng of total RNA was used to define the appropriate input cDNA. The real-time RT-PCR reaction was performed essentially following the manufacturer’s protocol. Briefly, reaction mixture was prepared fresh for each real-time PCR run and contained 5.5 mM MgCl₂, 200 μM dNTP, 400 μM dUTP, 1 U UDG, 200 mM FAM-probe, and 400 mM of both forward and reverse primers, 1.5 unit of AmpliTaq Gold DNA polymerase in a final volume of 25 μl, and was analyzed in an ABI PRISM 5700 Sequence Detection System. Thermal cycling conditions were designed as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. All amplification reactions were performed in triplicate. The increase in generation of PCR product is monitored directly and is based on the threshold number of cycles (CT) required to produce a detectable change in fluorescence. Values of the threshold cycle, CT, were averaged from the values obtained from each reaction.

Relative levels of HAND2 cDNA \( \left( R_{\text{HAND2}} \right) \) in control and test samples (BMP4, H-89, and dda-treated cultures) were calculated from the difference in CT values \( \Delta C T = C T_{\text{control}} - C T_{\text{hand2}} \) for HAND2 amplifications \( \Delta C T_{\text{HAND2}} \) compared with those for the housekeeping gene, β-actin \( \Delta C T_{\text{Actin}} \) using Eq. (1).

\[
R_{\text{HAND2}} = \left( \frac{C T_{\text{HAND2}}}{C T_{\text{Actin}}} \right)
\]

In separate studies, the real-time PCR amplification efficiencies \( E \) were determined from the slope of CT vs. input log cDNA dilution where \( E = -10^{-1/\text{slope}} \). Values for amplifying HAND2 and β-Actin cDNAs were 2.01 and 2.28, respectively. It should be noted that we were unable to identify a functional probe and primer set for Phox2a. In independent studies, we have determined that we obtain similar results using our standard semi-quantitative PCR protocol and real-time PCR.
BrdU labeling

To identify proliferating cells, incorporation of the thymidine analog BrdU was performed on culture day 2. Neural crest-derived cells were incubated for 4 h in growth medium containing 50 μM 2-bromodeoxyuridine, washed in PBS, and fixed in 4% formaldehyde, freshly prepared from paraformaldehyde, for 1 h. For immuno staining of BrdU-labeled samples, cells were permeabilized in PBS containing 0.3% Triton X-100 and DNA was denatured using 2 N HCl at room temperature for 30 min. Acid was neutralized by incubation in 1 M borate buffer, pH 8.4, at room temperature for 30 min. Prior to incubation in primary antibody, non-specific binding was blocked by incubation in PBS containing 10% horse serum and 0.3% Triton X-100 for 30 min to 1 h at room temperature. Cells were incubated in anti-BrdU antibody (1:10, Developmental Studies Hybridoma Bank) in PBS containing 4% horse serum and 0.3% Triton X-100 overnight at 4°C. Following washing (PBS, 3 times 10 min), samples were incubated with species-specific secondary antibody conjugated to tetramethylrhodamine (KPL, Inc., Gaithersburg, MD) for 3 h at room temperature in the dark. Cells were washed in PBS (three times 10 min) following removal of the secondary antibody and mounted in anti-fade medium (Vector Laboratories, Burlingame, CA). To label cell nuclei, DAPI (4,6-diamidino-2-phenylindole) diluted 1:500 in PBS was added to the final wash for 10 min. Cells were visualized using an Olympus AX-70 fluorescence microscope.

Transient transfection

Neural crest-derived cells were transfected on culture day 2 using FUGENE 6 reagent (Roche Diagnostics Corp. Indianapolis, IN), according to manufacturer’s directions. Optimal conditions for transfection of neural crest-derived cells were determined testing both plasmid DNA concentration and ratio to FUGENE 6 reagent. After removal of serum from the growth medium, 0.5 μg of pFC-PKA plasmid DNA (Stratagene, La Jolla, CA) and 0.5 μg of pEGFP-N1 plasmid DNA (Clonetech, Palo Alto, CA) were added per 35 mm culture dish and maintained from 6–8 h; at this time, serum-free medium was replaced with complete growth medium. Cell morphology and differentiation of NE neurons were assessed on culture day 7.

The effect on HAND2-mediated synergistic activation of DBH by mutation of helix 1 (see below) was tested using HeLa cells in a DBH-luciferase reporter assay, as we have previously described (Xu et al., 2003). HeLa cells were plated at a density of 1 × 10^5 cells per well, in 12 well tissue culture plates. The ratio of DBH reporter construct (DBH-luc) to pRL-null (Promega Corp. Madison, WI) was maintained constant at 24:1. HAND2 or Phox2a expression plasmids were added at 150 ng/well. Total DNA (0.5 μg) was adjusted using pcDNA1. At 48 h following transfection, cells were harvested and luciferase reporter activity measured using a Dual-Luciferase Reporter Assay System (Promega Corp. Madison, WI). As an internal control for transfection efficiency, all transfections contained pRL-null encoding Renilla luciferase. All luciferase assays are reported as values normalized to 1 based on the ratio of Firefly to Renilla reporter activity in control and experimental transfections.

Mutagenesis

We have previously identified nucleotides encoding amino acids shown to be phosphorylated in a PKA-dependent manner in helix 1 of HAND2 (Finulli et al., 2003). Sites phosphorylated in HAND2 helix 1 by PKA were mutated using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), according to manufacturer’s directions. Threonine (T) and Serine (S) at amino acids 112 and 114 of HAND2, respectively, were mutated to Aspartic acid (HAND2ΔP) using the primer GGA GCC GCG CAG GGA TCA GGA TAT CAA CAG CGC CTT CGC. Mutation was confirmed by restriction digestion and DNA sequencing.

Data analysis

Data are presented as the mean ± SEM of normalized values, unless otherwise indicated. Determination of statistical significance is based on ANOVA and Bonferroni post hoc test, unless otherwise stated.

Results

cAMP/PKA signaling supports noradrenergic phenotypic expression

To determine if signaling mediated by cAMP could affect the differentiation of neural crest-derived NE neurons, we pharmacologically blocked cAMP-mediated signaling then determined the effect on expression of norepinephrine (NE) as a marker of noradrenergic differentiation. The cAMP pathway was blocked proximally using either deoxyoxygenosine (dda) or SQ22536 to inhibit adenylate cyclase or distally using H-89 or KT5720 to block protein kinase A (PKA) activity. Neural crest-derived cells were maintained in medium supplemented with 10% CEE in the presence or absence of 200 μM dda or SQ22536 or 10 μM H-89 or 1 μM KT5720 for 7 days and formaldehyde induced fluorescence (FIF) used to detect NE. In response to decreasing PKA activity, there was a significant reduction in the mean number of noradrenergic neurons detectable by FIF (Fig. 1A). The differentiation of noradrenergic neurons was significantly reduced from a mean of 66 ± 3 NE positive cells per field in medium supplemented with 10% CEE to 17 ± 6 or 18 ± 6 NE positive cells per field after addition in 10% CEE-medium of dda or H-89, respectively (Fig. 1A). This result suggested that signaling mediated by PKA was involved in the expression of NE in response to factors present in 10% CEE. To confirm this result and as a test for specificity of our pharmacological intervention, the experiment was repeated using SQ22536 or KT5720 to block adenylate cyclase or PKA, respectively. In response to blockade of adenylate cyclase, the number of differentiated noradrenergic neurons was significantly reduced from a mean of 110 ± 6 NE cells per field to 27 ± 5 NE cells per field while inhibition of PKA resulted in a significant reduction to 33 ± 5 NE cells per field. Overall, blockade of adenylate cyclase reduced the number of NE cells by approximately 75%, while blocking activated PKA resulted in the loss of about 72% of NE cells. These data suggest that factors in 10% CEE-medium induce expression of cAMP/PKA and that these signaling molecules influence the differentiation of noradrenergic neurons. Mechanistically, this result could reflect a change in the number of noradrenergic neurons or rather be a manifestation of alterations in the biosynthesis of NE.

To ask if reduction in the number of noradrenergic neurons observed in response to altered cAMP-mediated signaling was due to a change in the generation of additional neural crest-derived cells, we determined the cell division index in neural crest cells grown in the presence or absence of dda or H-89 (Fig. 1B). Neural crest explants were plated in 10% CEE-medium in the absence or presence of 200 μM dda or 10 μM H-89 and maintained for 2 days. Incorporation of BrdU was used to determine if pharmacological treatments used to affect a change in intracellular signaling by PKA influenced cell division in neural crest-derived cells. The proportion of neural crest-derived cells incorporating BrdU was not detectably affected by treatment with dda.
One implication of these results is that increasing PKA but rather to altered expression of NE supported by 10% inhibition of PKA is not due to altered generation of cells reduction in the number of noradrenergic neurons following maintained in 10% CEE (37°C) or H-89 (41°C) compared to control cells

Fig. 1. In 10% CEE-medium, blockade of cAMP signaling in neural crest-derived cells inhibits synthesis and storage of norepinephrine but has no effect on cell division. (A) Synthesis and storage of norepinephrine was used as a marker of noradrenergic differentiation. Neural crest cells were explanted in 10% CEE-medium in the presence or absence of pharmacological blockers of adenylate cyclase or PKA and the mean number of NE neurons developing after 7 days in culture was determined based on FIF. Generation of PKA by cAMP was inhibited proximally by blocking adenylate cyclase with 200 μM dda or 200 μM SQ22538. Blockade of adenylate cyclase significantly inhibited generation of cells expressing NE. To confirm signaling downstream of cAMP, PKA was inhibited using 10 μM H-89 or 1 μM KT5720. Blockade of PKA resulted in a significant reduction in the mean number of differentiated NE neurons. For each treatment, NE neurons were counted in the outgrowth from a minimum of 9 explants from 3 separate platings. The number of cells expressing NE was counted in 15–20 randomly selected microscope fields of cells in each neural crest explant. The mean number of neurons counted in each treatment condition has been normalized to control and is presented as the percentage of neurons compared to control values (100%). (B) To assess the potential function of CAMP-mediated signaling on cell proliferation, the percentage of neural crest-derived cells incorporating BrdU was determined on culture day 2. Neural crest explants were plated in 10% CEE-medium in the absence (control) or presence of 200 μM dda or 10 μM H-89 to inhibit adenylate cyclase or PKA, respectively. The percentage of dividing cells was determined by counting the total number of cells having incorporated BrdU as a fraction of total cells labeled with DAPI. The data suggest that signaling mediated by CAMP/PKA does not influence cell division. For each condition, a minimum of three neural crest explants were seeded per plate and five randomly chosen microscope fields per explant were counted. The experiment was repeated three times. **P < 0.01; ***P < 0.001.

should induce further differentiation of NE cells from neural crest-derived precursor cells.

In order to understand more fully the consequences on differentiation of noradrenergic neurons by altering PKA, neural crest-derived cells were treated with the membrane permeable analogue of cAMP, 8-bromo-cAMP (8-Br-cAMP) to elevate PKA (Fig. 2). Neural crest explants were plated under two growth medium conditions: cells were grown in 10% CEE-medium which is our standard control medium supporting the differentiation of NE neurons or in 2% CEE-medium which does not support the differentiation of NE neurons (Howard and Bronner-Fraser, 1985, 1986; Howard et al., 2000). Initially, PKA activity under these growth conditions was determined (Fig. 2A). Basal PKA activity in 10% CEE-medium is significantly higher (5 ± 1 pmol/min/μg protein) than in 2% CEE-medium (2 ± 0.2 pmol/min/μg protein) likely accounting, in part, for the lack of NE differentiation observed under this growth condition. The addition of 1 μM 8-Br-cAMP to neural crest-derived cells grown in either 2% or 10% CEE-medium for 7 days significantly increased PKA activity to 8 ± 0.3 pmol/min/μg protein or 9 ± 0.4 pmol/min/μg protein, respectively. The suggestion that PKA activity influences the differentiation of NE neurons from neural crest-derived precursor cells was confirmed by demonstrating a substantial increase in the number of neurons synthesizing NE in response to added 8-Br-cAMP (Fig. 2B). Neural crest-derived cells were grown in either 10% CEE-medium or 2% CEE-medium for 7 days in the presence or absence of 1 μM 8-Br-cAMP and FIF was used to determine effects on the generation of noradrenergic neurons. In 10% CEE-medium, the number of NE neurons per field increased from 35 ± 3 to 60 ± 5 while in 2% CEE-medium the number increased from 4 ± 2 to 15 ± 2. Although the absolute number of noradrenergic neurons is significantly greater in 10% CEE-medium compared to 2% CEE-medium, treatment with 8-Br-cAMP in medium supplemented with 2% CEE resulted in a greater fold enhancement of NE neuron numbers compared to 10% CEE-medium (Fig. 2B) and a similar parallel increase in the level of PKA activity (Fig. 2A). This result raises the possibility that additional factors induced by PKA and supported by 10% CEE but not 2% CEE, function as a trigger to promote either the differentiation of NE neurons or to enhance the generation of neural precursors derived from neural crest cells. Having demonstrated here that PKA has a role in the differentiation of NE neurons, we next reasoned that PKA activity might contribute to the transcriptional control of HAND2 and/or Phox2a (Fig. 2C). We used semi-quantitative RT-PCR to detect transcripts encoding HAND2 and Phox2a in neural crest cultures. Total cellular RNA was purified from the outgrowth of 20 to 25 neural crest explants maintained for 7 days in the absence or presence of 1 μM 8-Br-cAMP in 2% CEE-medium or 10% CEE-medium and levels of transcript encoding HAND2 and Phox2a were determined. Increasing PKA activity using 8-Br-cAMP resulted in a significant (P < 0.05) increase in transcript encoding HAND2 and Phox2a in both 2% CEE-medium (6.9-fold, 8.7-fold, respectively) and 10% CEE-medium (4.9-fold, 2.2-fold, respectively). These results indicate that PKA has a significant influence on...
the expression of essential determinants of a noradrenergic phenotype. We next tested the possibility that cell number was being affected by determining the cell division index (Fig. 2D). Incorporation of BrdU by neural crest cells grown in 10% CEE-medium (47 ± 3%) or 2% CEE-medium (47 ± 2%) was not significantly influenced by the addition of 1 μM 8-Br-cAMP to either 10% CEE-medium (49 ± 2%) or 2% CEE-medium (50 ± 2%) (Fig. 2D). This lack of effect on cell division index by increasing PKA activity is consistent with the idea that PKA promotes the differentiation of NE neurons rather than increasing the number of neural precursors. Since we and others (Howard et al., 2000; Wu and Howard, 2001; Lo et al., 1999; Dupin et al., 1993; Reissmann et al., 1996; Maxwell and Forbes, 1990; Bilodeau et al., 2000) have shown that both cAMP/PKA and BMP4 support the differentiation of noradrenergic neurons from neural crest-derived precursors, we were interested to know how these signaling pathways might interact. Additionally, aorta-derived BMP4 appears to be an endogenous factor responsible for induction of a transcription factor network, including HAND2 and Phox2a, required for the differentiation of NE neurons (Howard et al., 2000).

**BMP4 supports noradrenergic differentiation in a PKA-dependent manner but independent of cAMP**

BMP4, both in vitro (Varley et al., 1995; Reissmann et al., 1996; Varley et al., 1998) and in vivo (Schneider et al., 1999; Howard et al., 2000), supports the differentiation of neural crest-derived noradrenergic neurons. In tissue culture, addition of BMP4 to 2% CEE-medium is sufficient to support the differentiation of NE neurons in this culture condition. Using 8-Br-cAMP to elevate PKA activity supports a significant increase in transcripts encoding HAND2 and Phox2a in both 2% and 10% CEE-medium. Total cellular RNA was purified from the outgrowth of 20–25 primary neural crest explants and used for semi-quantitative RT-PCR. Data are presented as the ratio of ethidium bromide band intensity for HAND2 or Phox2a to chicken β-actin. Each value represents the mean of 3–4 individual platings. Statistical significance was determined based on one-way ANOVA and unpaired t test. The differentiation of NE neurons in response to increased levels of PKA activity (D) occurred independent of increasing cell division. Based on the incorporation of BrdU, cell numbers were not affected by increasing PKA activity in either 2% or 10% CEE-medium. For PKA assays, each bar represents 3–6 determinations with each sample being assayed in triplicate. For FIF, the mean number of NE cells was counted in 8–10 randomly chosen microscope fields in each of 6 to 9 primary neural crest explants from three individual platings. The cell division index was determined (see Materials and methods) by counting cells in 8–10 random microscope fields, per explant (9 explants) from three primary platings. *P < 0.05; **P < 0.01; ***P < 0.001.
neurons of blocking cAMP or PKA in the presence of added BMP4. Neural crest explants were plated in 2% CEE-medium supplemented with 10 ng/ml BMP4 in the absence or presence of dda or H-89 and FIF was performed after 7 days. Under control conditions in 2% CEE-medium, few if any noradrenergic neurons (3 ± 1 NE neurons/field) develop but addition of BMP4 dramatically increases differentiation (167 ± 9 NE neurons/field) of noradrenergic neurons (Fig. 3A). Addition of dda to block adenylate cyclase had no significant effect on the ability of BMP4 to support the differentiation of noradrenergic neurons (BMP4; 167 ± 9 vs. BMP4 + dda; 151 ± 9 NE neurons per field). Addition of H-89 to block PKA activity significantly reduced the number of noradrenergic neurons from 167 ± 9 neurons/field with added BMP4 to 4 ± 2 neurons/field, a number comparable to 2% CEE-medium alone. To test for specificity of pharmacological blockade, SQ22536 was used to block adenylate cyclase or KT5720 to block PKA with comparable results (Fig. 3B). Similar to treatment with dda, there was no significant reduction in the number of noradrenergic neurons developing in the presence of BMP4 + SQ22536 to block cAMP (BMP4; 122 ± 13 vs. BMP4 + SQ22536; 91 ± 5 NE neurons per field). However, addition of KT5720 significantly reduced the ability of BMP4 to support development of noradrenergic neurons (BMP4; 122 ± 13 vs. BMP4 + KT5720; 37 ± 7). These results raise the possibility that PKA is activated downstream of BMP4 but independently of cAMP. Although the mechanism of BMP4-mediated support of neurogenesis is not known, there are
several different choice points for either specification or cell type-specific gene regulation at which BMP4 might be active.

One possibility is that BMP4 supports the generation of additional neuronal precursor cells from neural crest-derived progenitor cells. To address this possibility, cell number was determined following incorporation of BrdU at culture day 2 in 2% CEE-medium supplemented with 10 ng/ml BMP4 in the presence or absence of dda or H-89 (Fig. 4A). There was no significant effect on cell number by addition of BMP4 or treatment with either dda or H-89 suggesting that rather than affecting an increase in neuronal precursor cells, BMP4 promotes neurogenesis; these data also indicate that neither cAMP nor PKA functions in the generation of noradrenergic neurons by influencing cell number. We asked if BMP4 influences neurogenesis by counting the number of cells expressing neuron specific β-tubulin (TUJ1 immunolabeling) as a function of the total number of neural crest-derived cells (DAPI labeling). The mean number of neurons (TUJ1+cells) was significantly increased (Fig. 4B) by addition of BMP4 from 4 ± 0.7% of total cells to 20 ± 1% of total cells. Although inhibition of cAMP using dda resulted in a small but significant decrease in neurons to 14 ± 1% of total cells, inhibition of PKA with H-89 significantly reduced neurogenesis to baseline levels (5 ± 0.9% of total cells). These data indicate that PKA is a downstream mediator of BMP4-supported neurogenesis and further suggest that PKA is activated independent of cAMP.

**PKA activity is increased downstream of BMP4 independent of cAMP-mediated signaling**

To directly test the idea that BMP4 activates PKA and does so without affecting activity of adenylate cyclase, we measured the accumulation of cAMP and PKA in neural crest-derived cells grown under conditions supporting different levels of neurogenesis. Neural crest-derived cells grown in the presence of BMP4 in the absence or presence of 200 μM dda or 10 μM H-89 and the activity of PKA (A) or accumulation of cAMP (B) was measured. Measurement of cAMP accumulation directly showed that added BMP4 does not affect a change in basal levels of cAMP present in 2% CEE-medium. Measurement of cAMP accumulation directly showed that added BMP4 does not affect a change in basal levels of cAMP present in 2% CEE-medium. (C) To demonstrate that activity of PKA was enhanced over short times in response to BMP4, we measured activity of PKA in neural crest-derived cells at 1 day in culture in 2% CEE-medium (control) or following a 5 min exposure to 10 ng/ml BMP4. At 5 min treatment with BMP4, PKA activity was significantly increased 2-fold. To demonstrate that signaling mediated by BMP4 could activate PKA, we used the BMP4 antagonist NOGGIN (D) to competitively inhibit BMP4 receptor binding on neural crest-derived cells and measured PKA activity. Treatment of neural crest-derived cells with Noggin resulted in a dose-dependent loss of PKA activity. PKA activity was reduced to basal levels present in 2% CEE at 50 ng/ml added NOGGIN. It should be noted that differentiation of NE neurons is inhibited ~100% by addition of NOGGIN (data not shown). These results suggest that BMP4-mediated signaling impinges on PKA in a cAMP-independent manner but requiring binding to its cognate receptor. For the measurement of PKA activity, each assay was done in triplicate from outgrowth of 20–25 primary neural crest explants; each experiment was repeated 3–6 times. For measurement of cAMP accumulation, each assay was performed in duplicate on cells derived from 20–25 primary neural crest explants; each experiment was repeated 4 times. *p < 0.05; **p < 0.01; ***p < 0.001 compared to control. ΔΔp < 0.01; ΔΔΔp < 0.001 compared to BMP4-treated culture.
of 10 ng/ml added BMP4 had significantly higher PKA activity (5 ± 0.7 pmol/min/μg protein) compared to cells grown in 2% CEE-medium alone (2 ± 0.2 pmol/min/μg protein). No significant reduction in PKA activity was observed (Fig. 5A) in the presence of added dda but treatment of cells with H-89 resulted in a significant reduction of PKA activity resulting in levels equivalent to baseline (1 ± 0.1 pmol/min/μg protein). Interestingly, the increase in PKA activity measured in response to added BMP4 was not secondary to increasing the accumulation of cAMP (Fig. 5B). The amount of accumulated cAMP in response to factors present in 2% CEE-medium (11 ± 0.4 fmol/μg protein) was not affected by addition of BMP4 (11 ± 1 fmol/μg protein). Activity of PKA is increased significantly after 5 min exposure of neural crest cells to BMP4 (Fig. 5C) suggesting a receptor-mediated event. The increase in PKA activity occurs secondary to BMP4 binding its receptor since antagonism of BMP4 using NOGGIN results in a dose-dependent loss of PKA activity (Fig. 5D) returning to baseline levels at 50 ng/ml added NOGGIN. In total, these data indicate that increased activity of PKA occurs downstream of BMP4-mediated intracellular signaling and independent of cAMP. These data raise the possibility that genes regulated secondary to BMP4 and required for differentiation of noradrenergic neurons are responsive to PKA. In addition, these data suggest differential effects of PKA depending upon whether PKA activity is increased secondary to cAMP or BMP4.

We have previously shown that signaling mediated by BMP4 either directly or indirectly induces expression of transcripts encoding HAND2 and Phox2a (Howard et al., 2000; Wu and Howard, 2001; cf. Mizuseki et al., 2003). To determine if this induction is dependent upon cAMP or PKA, neural crest-derived cells were grown in 2% CEE-medium in the absence or presence of 10 ng/ml added BMP4 and either dda or H-89 and relative levels of transcripts encoding HAND2 and Phox2a were assessed. The induction of transcripts encoding HAND2 mediated by BMP4 is not dependent upon generation of either cAMP or PKA (Fig. 6A). Interestingly, although the expression of transcript encoding Phox2a is not dependent upon BMP4-induced PKA, it is sensitive to cAMP (Fig. 6B) suggesting differential regulation in response to BMP4. To confirm these results, isobutylmethylxanthine (IBMX, 100 μM) was added to neural crest cell cultures to

Expression of transcripts encoding HAND2 and Phox2a is differentially influenced by cAMP and PKA in response to BMP4

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increase levels of cAMP, in the absence or presence of BMP4 (Bilodeau et al., 2000). In the presence of BMP4, elevation of cAMP affected a significant increase in transcripts encoding Phox2a (Fig. 6C) without detectably changing expression of transcripts encoding HAND2. Thus, as predicted, in the presence of BMP4, cAMP differentially affected expression of transcripts encoding HAND2 and Phox2a. These data suggest that PKA activity induced by BMP4 functions in aspects of noradrenergic differentiation not involving regulation of HAND2 transcription.

Over-expression of PKA is sufficient to promote differentiation of noradrenergic neurons

Having demonstrated that BMP4 increases the activity of PKA as well as supporting differentiation of noradrenergic neurons, we asked if over-expression of PKA could support differentiation of noradrenergic neurons from neural crest-derived cells. We tested this possibility by transiently transfecting neural crest-derived cells on culture day two using a plasmid constitutively expressing PKA (pFC-PKA) and co-transfecting with pEGFP-N1 to provide a visible marker of transfected cells. On culture day four, the morphology of cells expressing pEGFP was examined under Nomarski optics (Figs. 7A1 [control] and A3 [pFC-PKA]) or fluorescein optics (Figs. 7A2 [control] and A4 [pFC-PKA]) to determine if cells expressing EGFP displayed characteristics of neurons such as process outgrowth and rounded phase-bright cell bodies. On culture day seven, the number of noradrenergic neurons was counted following FIF (Fig. 7B); expression of pEGFP falls off beginning at culture day 4.5–5 obviating directly demonstrating that cells transfected with pFC-PKA express noradrenergic characteristics on culture day 7.

Based on assessment of morphological criteria, we concluded that over-expression of PKA was sufficient to support the differentiation of neural crest-derived cells into neurons (compare Figs. 7A2 and A4). Examination of neural crest cells under Nomarski optics (Fig. 7A3 [pFC-PKA]) revealed many cells with phase bright cell bodies characteristic of differentiated neurons (indicated by red arrows in A3 and A4); although cells with phase bright cell bodies were identified under control growth conditions (Fig. 7A1), these cells did not elaborate processes characteristic of neurons (Fig. 7A2). Identification of cells with phase-bright cell bodies (Fig. 7A3) as neurons was confirmed under fluorescein optics (Fig. 7A4) where cells expressing PKA also had elaborated processes characteristic of differentiated neurons; under control conditions, cells transfected with EGFP (indicated by red arrows in A3 and A4) did not acquire a neuronal morphology. This morphological analysis suggested that expression of PKA is sufficient to support neuronal differentiation of neural crest-derived cell in vitro. Constitutive expression of PKA also elicited a significant increase (3.2-fold) in the number of neurons (Fig. 7B) expressing NE from neural crest-derived cells maintained in 2% CEE-medium.

These data suggest that PKA activity is involved in the generation of noradrenergic neurons, subsequent to BMP4-mediated intracellular signaling but that this PKA activity does not influence transcription of HAND2 or Phox2a. We therefore tested the influence of MapK on expression of transcripts encoding HAND2 and Phox2a to determine if this arm of the intracellular signaling pathway (Wu and Howard, 2001) might influence this aspect of noradrenergic marker gene regulation (Fig. 8) downstream of BMP4. Neural crest explants were
plated in 2% CEE-medium in the presence or absence of 10 ng/ml added BMP4 with or without 7 μM PD98059 to inhibit MapK (Wu and Howard, 2001; Zhou et al., 2004). While growth in the presence of BMP4 significantly (P < 0.01) increased transcripts encoding HAND2 (~3.1-fold) and Phox2a (~2.5-fold; P < 0.05) (Fig. 8A) over that in control cells grown in 2% CEE-medium alone, inhibition of MapK blocked effects (P < 0.01) of BMP4 on HAND2 transcript expression (Fig. 8A); the reduction in expression of transcripts encoding Phox2a by PD98059 was not significant (P > 0.05). This result raised the possibility that MapK and PKA function in tandem to regulate different aspects of noradrenergic differentiation and NE marker gene expression. To test this possibility, we blocked MapK-mediated signaling using PD98059 and measured PKA activity in the presence and absence of added (10 ng/ml) BMP4 (Fig. 8B). When compared to neural crest-derived cells grown in 2% CEE-medium (2 ± 0.1 pmol ATP/min/μg protein), addition of BMP4 significantly increased PKA activity (4 ± 0.4 pmol ATP/min/μg protein) but this activity was not reduced in the presence of PD98059 (3 ± 0.3 pmol ATP/min/μg protein). Since blockade of MapK does not affect a change in PKA activity downstream of added BMP4, we suggest that PKA is regulated independently of MapK by BMP4 and that both MapK and PKA regulate aspects of BMP4-mediated differentiation of noradrenergic neurons from neural crest-derived precursors.

**PKA-dependent phosphorylation of HAND2 is crucial for biosynthesis of norepinephrine**

Dopamine-β-hydroxylase (DBH) is the biosynthetic enzyme required to convert dopamine to norepinephrine; Phox2a is a homeodomain DNA binding protein that regulates transcription of DBH (Swanson et al., 1997; Kim et al., 1998). We have shown previously that HAND2 synergistically promotes Phox2a-stimulated activity of the DBH promoter (Xu et al., 2003) implicating HAND2 in cell type-specific gene expression. We have also demonstrated that functional dimerization of HAND2 is dependent upon PKA-mediated phosphorylation of threonine 112 and serine 114 (Firulli et al., 2003) located in helix 1 of HAND2. To determine if this PKA-mediated post-translational processing of HAND2 has functional consequences for expression of norepinephrine, we mutated these sites in HAND2 abrogating their phosphorylation. We then tested the transcriptional activity of the mutant HAND2 (HAND2ΔP) protein at the DBH promoter (Fig. 9) using our standard DBH-luc reporter assay (Xu et al., 2003). HeLa cells were transiently transfected with HAND2, HAND2ΔP, and/or Phox2a, as indicated (Fig. 9) and promoter activity determined based on a dual luciferase assay (open bars). Basal promoter activity is only marginally affected by HAND2 alone but is significantly increased 4.6 ± 0.1-fold by Phox2a. Co-expression of HAND2 and Phox2a significantly increased DBH-reporter activity 2.6 ± 0.1-fold over Phox2a alone and 12.1 ± 0.1-fold over basal promoter activity. Interestingly, ~98% of the synergistic activation of DBH afforded by HAND2 is lost by preventing post-translational PKA-mediated phosphorylation of HAND2 in helix 1. However, co-expression of Phox2a and HAND2ΔP marginally activates DBH transcription over Phox2a alone suggesting that regulatory elements, in addition to phosphorylation, are required for the synergistic interaction of HAND2 and Phox2a at the DBH promoter. We tested the possibility that cAMP might influence the ability of HAND2 and Phox2a to regulate DBH (Fig. 9).

Enhancing cAMP expression by growing cells in the presence of 1 mM 8-Br-cAMP resulted in a significant increase of Phox2a driven DBH-luc reporter activity (~2.3-fold, P < 0.001) compared to Phox2a alone (Kim et al., 1994; Swanson et al., 1997; Xu et al., 2003). This cAMP-mediated increase in DBH transcription occurred under all treatment conditions with Phox2a and HAND2 suggesting that basal as well as activated transcription is cAMP-modified. As expected, if the DB1 enhancer CRE is mutated (DBH-CREM), basal transcription, and in response to Phox2a or HAND2 + Phox2a,
is abrogated (Fig. 9, blue bars). Interestingly, there is an apparent interaction of HAND2 and Phox2a at the CRE domain suggested by the small but consistently higher ($P < 0.001$) DBH-CREM promoter activity if HAND2 or HAND2Dp is present in the transcriptional complex with Phox2a. The significant decrease in DBH-luc reporter activity observed with HAND2Dp, even in the presence of cAMP, indicates that PKA-dependent phosphorylation of HAND2 is crucial for DBH activation.

**Discussion**

The molecular basis for specification and differentiation of noradrenergic neurons from neural crest-derived precursors is being elucidated. In the present study, we have focused on the consequences to neurogenesis and neurotransmitter biosynthesis of intracellular signaling downstream of BMP4 and cAMP in neural crest-derived precursor cells, in vitro. The major goal of these studies was to determine if BMP4-mediated signaling intersected with signaling through cAMP in order to ascertain if differentiation as neurons or synthesis of norepinephrine was specifically targeted by these molecules. Our data suggest that cAMP functions to coordinate BMP4-mediated signaling via both canonical (translocation of Smad1/4) and non-canonical (induction of PKA activity) pathways affecting neurogenesis (Fig. 10) and regulation of DBH promoter activity (Fig. 11). Our results account for coordinated expression of pan-neuronal and cell type-specific genes culminating in the differentiation of noradrenergic sympathetic neurons.

Impetus for the current studies centered around several sources of confusion in the literature regarding the requirement for cAMP to support neurogenesis or expression of neurotransmitter biosynthetic enzymes (Maxwell and Forbes, 1990; Lo et al., 1999; Bilodeau et al., 2000) necessary for the generation of noradrenergic sympathetic ganglion neurons. Since neuronal identity is determined by expression of both pan-neuronal characteristics and cell type-specific properties, identification of extrinsic signals and their function in expression of these features is a prerequisite to understanding
Having identified a transcription factor circuit (BMP → Phox2b → HAND2 → Phox2a) required for the differentiation of sympathetic noradrenergic neurons, we used these gene products and the consequences of their expression as the experimental focal point in which to understand the interplay between signaling downstream of BMP4 and cAMP. The most revealing aspect of these studies is that BMP4 induces PKA activity independent of cAMP. Signaling by PKA downstream of BMP4 affects neurogenesis and the function of HAND2; identification of this non-canonical intracellular signaling pathway has made it possible to propose a unifying model (Fig. 10) that provides a roadmap for generation of noradrenergic sympathetic ganglion neurons.

**cAMP modulates BMP-mediated signaling**

Previous studies implicated cAMP as a signaling molecule, acting either directly or indirectly with BMP, by demonstrating increased expression of TH in neural crest-derived cells in vitro in response to added cAMP (Maxwell and Forbes, 1990; Dupin et al., 1993; Lo et al., 1999; Bilodeau et al., 2000). These studies left open the question of what function cAMP served and how its function intersected with BMP-mediated responses. These culture studies suggested that an extrinsic signal activates cAMP (Lo et al., 1999) but this has not been confirmed in vitro or in vivo. Levels of cAMP measured in cells maintained in growth conditions permissive for differentiation of noradrenergic neurons (2% CEE-medium + BMP4) are equivalent to levels in cells grown in non-permissive conditions (2% CEE-medium) suggesting that cAMP alone is not sufficient to support differentiation of noradrenergic neurons. Neural crest-derived cells maintained in 2% CEE-medium express CASH1 but little if any Phox2b, HAND2, or Phox2a (Howard et al., 2000; Howard, unpublished observation). Lack of expression of the essential determinants of a noradrenergic phenotype (reviewed in Brunet and Pattyn, 2002) in 2% CEE-medium suggests that cAMP alone is not sufficient to support differentiation of noradrenergic neurons. Neural crest-derived cells maintained in 2% CEE-medium express CASH1 but little if any Phox2b, HAND2, or Phox2a (Howard et al., 2000; Howard, unpublished observation). Lack of expression of the essential determinants of a noradrenergic phenotype (reviewed in Brunet and Pattyn, 2002) in 2% CEE-medium suggests that cAMP alone is not sufficient to support differentiation of noradrenergic neurons. Neural crest-derived cells maintained in 2% CEE-medium express CASH1 but little if any Phox2b, HAND2, or Phox2a (Howard et al., 2000; Howard, unpublished observation). Lack of expression of the essential determinants of a noradrenergic phenotype (reviewed in Brunet and Pattyn, 2002) in 2% CEE-medium suggests that cAMP alone is not sufficient to support differentiation of noradrenergic neurons.
synthesis of norepinephrine, recapitulating the in vivo pattern of development (Groves et al., 1995; Reissmann et al., 1996; Schneider et al., 1999; Howard et al., 1999; Wu and Howard, 2001; Howard et al., 2000; Tsarovina et al., 2004). In this context, the function of cAMP is to modify or modulate the effects of intracellular signaling pathways induced by BMP. Our data show that cAMP-mediated signaling impacts both canonical and non-canonical BMP-mediated signaling pathways (Fig. 10). In the canonical pathway, expression of transcripts encoding Phox2a occurs downstream of HAND2 but requires cAMP. The cAMP-dependent influence on neurogenesis seen by us and others (Maxwell and Forbes, 1990, Dupin et al., 1993; Lo et al., 1999; Bilodeau et al., 2000) is thus indirect and involves regulation of Phox2a expression.

The identity of the extrinsic cue inducing cAMP-mediated PKA activity involved in regulation of Phox2a transcription is not yet known. In the non-canonical pathway, cAMP impacts DBH promoter regulation. Together, these data suggest that HAND2 and Phox2a couple aspects of neurogenesis and cell type-specific gene expression downstream of BMP but in a cAMP-dependent manner. Integration of canonical and non-canonical BMP-mediated signaling requires MapK activity as well. The expression of transcripts encoding HAND2 is dependent upon BMP both in vivo (Howard et al., 2000) and in vitro (Howard et al., 2000; Wu and Howard, 2001; Muller and Rohrer, 2002) and is modulated by signaling impinging upon MapK. The influence of MapK- and cAMP-mediated signaling on expression of transcripts encoding essential determinants of the noradrenergic phenotype suggests that canonical BMP-mediated signaling provides an instructive cue to neural crest-derived precursors for this aspect of differentiation.

Establishing that BMP4 induces PKA activity, and that the potential to support differentiation of noradrenergic neurons in vitro is correlated with the levels of PKA but not cAMP, helps to explain previous results from studies using cAMP (or other agents to increase PKA) as a mechanism to affect neuronal differentiation. For many of these studies, TH was used as a marker of noradrenergic differentiation (Dupin et al., 1993; Lo et al., 1998, 1999; Stanke et al., 1999; Bilodeau et al., 2000). Although mechanisms of TH regulation are not well defined, it is clear that many extrinsic signals can induce TH (Ghee et al., 1998; Quinn, 2002; Lewis-Tuffin et al., 2004). Recent data suggest that CREB is a focal point for transcriptional control of TH and is independent of the upstream inducing agent (Lonce and Ginty, 2002; Lewis-Tuffin et al., 2004). This may explain the previously reported lack of a consistent correlation between expression of TH, FIF, and transcripts encoding Phox2a generated by addition of cAMP (Maxwell and Forbes, 1990; Bilodeau et al., 2000). Our results suggest that PKA generated independently from cAMP is necessary for HAND2 expression and the integrated function of Phox2a. This suggests that signaling via the BMP-mediated non-canonical pathway provides a permissive signal coordinating neurogenesis and cell type-specific gene expression and function. The focal point for integration of BMP-mediated signaling may be the expression of HAND2 and integrated function of Phox2a.

Several lines of evidence indicate that Phox2a is required for expression of pan-neuronal genes but that it is insufficient alone to support neurogenesis (Lo et al., 1999; Stanke et al., 1999; Brunet and Pattyn, 2002). We have previously established the temporal and epistatic order of expression of Phox2b, HAND2, and Phox2a in avian embryos (Howard et al., 2000); we suggest that HAND2 induces expression of Phox2a and that their epistatic relationship places HAND2 upstream of Phox2a in the transcription factor network supporting differentiation of sympathetic noradrenergic neurons (Howard et al., 2000). Unlike Phox2a, ectopic expression of HAND2 is sufficient to induce TH, DBH, SCG10, NF, and biosynthesis of norepinephrine (Howard et al., 1999, 2000). This suggests that HAND2 and Phox2a together support neurogenesis. In addition, HAND2 can induce expression of Phox2b which is recognized as the determinant of autonomic neurons (reviewed in Brunet and Pattyn, 2002). Both Phox2b and Phox2a can bind to and transactivate the DBH promoter raising the formal possibility that it is Phox2b rather than Phox2a interacting with HAND2 that supports differentiation of noradrenergic sympathetic ganglion neurons. It is at this step, transcription of HAND2, where BMP-mediated canonical signaling intersects with non-canonical signaling. This cAMP-independent PKA activity is required for neurogenesis as well as for phosphorylation of HAND2 thus linking expression of pan-neuronal and cell type-specific genes. The function of BMP-mediated signaling does not impact generation of additional precursors but rather affects either specification or differentiation as noradrenergic neurons (and see Stanke et al., 1999).

**Synergistic transactivation of DBH promoter by HAND2 requires PKA-dependent phosphorylation in HAND2 dimerization domain**

Phox2a and HAND2 together can regulate DBH promoter activity establishing HAND2 as a transcriptional co-activator (Xu et al., 2003; Rychlik et al., 2003) and making transmitter biosynthesis a potential target for regulation by extrinsic molecules (Fig. 11). Interestingly, although Phox2a can bind to and transactivate both TH and DBH promoters (Zellmer et al., 1995; Yang et al., 1998; Kim et al., 1998), differential effects of cAMP-mediated signals raises the possibility that TH and DBH are not coordinately regulated; PKA synergistically enhances Phox2a activity at the DBH promoter but activates TH independent of Phox2a (Swanson et al., 1997). Thus, in trying to parse out differential influences of cAMP and/or PKA on neurotransmitter biosynthesis, we have focused on the DBH promoter. Our data indicate that DBH expression is regulated downstream of canonical BMP-mediated signaling but that promoter function is influenced by both cAMP and BMP-induced PKA activity in the non-canonical signaling pathway.

Transactivation of DBH by Phox2a is synergistically enhanced by HAND2; the activity of both HAND2 and Phox2a is elevated by cAMP. The CRE/AP1 site in the DB1 enhancer of DBH is critical for the synergistic activation of DBH by HAND2 (Xu et al., 2003); domain mapping studies suggest that the HLH domain of HAND2 is critical for interaction with
the CRE element in the DB1 enhancer (Xu et al., 2003). Our data suggest that the cAMP-dependent effects mediated at the CRE are separate from PKA-dependent phosphorylation effects on HAND2 that regulate HAND2 dimerization. Low but significant levels of stimulated DBH-CREM transactivation occurring in the presence of HAND2 and HAND2ΔP support the idea that cAMP and PKA influence different aspects of DBH promoter regulation; a logical extension of this conclusion is that HAND2 interacts with additional, as yet unidentified sites, in the DBH promoter. It is clear, however, that PKA-dependent phosphorylation of HAND2 is critical for regulation of DBH activity and thus synthesis of norepinephrine. Our current studies show that PKA-dependent phosphorylation of HAND2 regulates a critical component of HAND2 interaction in the transcription complex with Phox2a. Previous studies have shown that the DNA binding avidity of Phox2a is regulated by interactions with HAND2 (Adachi and Lewis, 2002; Rychlik et al., 2003, in press) as well as by PKA-dependent dephosphorylation. Our data thus suggest a model wherein the balance between dephosphorylation of dependent dephosphorylation. Our data thus suggest a model of Phox2b in synchronizing pan-neuronal and type-specific aspects of neurogenesis. Development 129, 5241–5253.


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