Glia Induce Dendritic Growth in Cultured Sympathetic Neurons by Modulating the Balance between Bone Morphogenetic Proteins (BMPs) and BMP Antagonists

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Dendritic growth in cultured sympathetic neurons requires specific trophic interactions. Previous studies have demonstrated that either coculture with glia or exposure to recombinant bone morphogenetic proteins (BMPs) is both necessary and sufficient to induce dendrite formation. These observations led us to test the hypothesis that BMPs mediate glial-induced dendritic growth. In vitro hybridization and immunocytochemical studies indicate that the spatiotemporal expression of BMPs, -6, and -7 in rat superior cervical ganglia (SCG) is consistent with their proposed role in dendritogenesis. In vitro, both SCG glia and neurons were found to express BMP mRNA and protein when grown in the presence or absence of the other cell type. However, addition of ganglionic glia to cultured sympathetic neurons causes a marked increase in BMP proteins coincident with a significant decrease in follistatin and noggin. Functional assays indicate that glial-induced dendritic growth is significantly reduced by BMP7 antibodies and completely inhibited by exogenous noggin and follistatin. These data suggest that glia influence the rapid perinatal expansion of the dendritic arbor in sympathetic neurons by increasing BMP activity via modulation of the balance between BMPs and their antagonists.

Key words: BMPs; BMP antagonists; noggin; follistatin; dendrites; sympathetic neurons; neuron–glia interactions

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It has been reported that BMP receptors are expressed in developing sympathetic ganglia (Zhang et al., 1998). Thus, as a first test of the hypothesis that BMPs mediate glial-induced dendritic growth, we examined BMP, noggin, and follistatin expression in superior cervical ganglia (SCG) neurons and glia. To assess the function of BMPs in glial-induced dendritic growth, we interfered with BMP activity in neuron–glia cocultures using function-blocking BMP antibodies or recombinant BMP antagonists. Our data indicate that glial-induced dendritic growth is mediated by increased BMP activity resulting from simultaneous upregulation of BMP levels and downregulation of noggin and follistatin levels. Thus, glia regulate dendritic growth by modulating the relative balance between BMPs and BMP antagonists.

MATERIALS AND METHODS

Materials. Purified human recombinant BMPs (2, 4, 5, 6, and 7) were provided by Creative Biomolecules (now Curis, Cambridge, MA). Affinity-puriﬁed polyclonal antibody (Ab) speciﬁc for BMP2, -4, -5, and -6 and their corresponding blocking peptides were purchased from Research Diagnostics (Flanders, NJ). The speciﬁcity of these antibodies was veriﬁed with Western blot analyses demonstrating that these antibodies did not cross-react with inappropriate BMP family members and that blocking peptide inhibited binding to the appropriate recombinant BMP. Two different monoclonal antibodies (mAbs), 12G3 and 1B12, which react with different epitopes on the BMP7 molecule (W. K. Jones, Creative Biomolecules/Curis, personal communication), and polyclonal Ab raised against recombinant human BMP7 were generous gifts from Creative Biomolecules (Curis). It has been demonstrated that neither mAb cross-reacts with BMP2 or -4 (Vukicevic et al., 1994). Both mAbs were tested in all function-blocking and immunocytochemistry experiments described in Results and were found to yield comparable results. The polyclonal BMP7 Ab, which was used for Western blot analysis, did not cross-react to any signiﬁcant extent with recombinant BMP2, -4, -5, or -6. Xenopus noggin protein (Lamb et al., 1993) was the generous gift of Drs. José de Jesús and Richard Harland (University of California at Berkeley). Regeneron Pharmaceuticals generously provided rat mAb RP57-16 to human noggin. There is only one amino acid change between human and rodent noggin (McMahen et al., 1998), and this amino acid does not lie within the region recognized by mAb RP57-16. Recombinant human follistatin (B4384) was obtained through the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases via Dr. A. F. Parlow (Torrance, CA); mouse anti-human follistatin mAb was purchased from R & D Systems (Minneapolis, MN). Antibody that recognizes Smad-1 was purchased from Upstate Biotechnology (Lake Placid, NY).

Tissue culture. Sympathetic neurons were dissociated from the SCG of embryonic day 21 (E21) or postnatal day 1–2 (PN1–2) Holtzmann rats (Harlan Sprague Dawley, Rockford, IL) according to previously described methods (Higgins et al., 1991). Cells were plated onto one of two different substrates: (1) glass coverslips or 35 mm plastic culture dishes (Boehringer Mannheim) or (2) 25 mm molded Aclar dishes coated with reagents that antagonize BMP function on glial-induced dendritic growth were performed with cultures grown on collagen I. All other experiments were performed with cells grown on both types of substrates with each yielding qualitatively similar results. However, micrographs show only cultures grown on the poly-lysine/collagen IV substrate because this provided superior optical conditions. All cells were maintained in serum-free medium (Higgins et al., 1991) containing β-NGF (100 ng/ml; Harlan Bioproducts, Madison, WI). To eliminate endogenous non-neuronal cells from cultures, cytotoxic β-d-arabinofuranoside (1 μM; Sigma) was added to the culture medium for 48 hr beginning on day 2. Purified populations of glial cells were prepared from sympathetic ganglia using Countess counterstaining/immunocytochemistry (Higgins et al., 1998) and maintained in serum-free medium (Higgins et al., 1991) supplemented with heregulin-β1 (100 ng/ml; R & D Systems).

Morphological analyses. Neuronal morphology was assessed in cultures immunostained with mAbs shown previously to react selectively with dendrites of sympathetic neurons (Lein et al., 1995). These included mAbs AF20 (Sigma) and SM1-32 (Stemberger Immunocytochemicals, Baltimore, MD), which are speciﬁc for MAP2, and mAb SM1-32 (Stemberger Immunocytochemicals) to nonphosphorylated forms of the M and H neuroﬁlament subunits. All antibodies were localized by indirect immunoﬂuorescence using previously described procedures (Lein et al., 1995). Data in the text are presented as the mean ± SEM, and statistical signiﬁcance was determined using ANOVA followed by Fisher’s LSD multiple comparison test.

Cell viability assay. Cell viability was determined using the Live/Dead Viability/Cytotoxicity Kit (Vaughan et al., 1995) from Molecular Probes (Eugene, OR). Viable cells were distinguished from nonviable cells on the basis of ﬂuorescence: live cells cleave membrane-permeable calcein AM to yield cytoplasmic green ﬂuorescence; in contrast, nonviable cells take up the membrane-impermeable ethidium homodimer-1, which labels nucleic acids with red ﬂuorescence. Using MetaMorph Imaging software (Universal Imaging, West Chester, PA), the number of viable and nonviable cells was determined in 10 random ﬁelds (5 ﬁelds per culture) at 200× magniﬁcation.

Immunocytochemistry. Anti-BMP mAbs were used to localize BMP proteins in cells cultured from SCG as well as frozen tissue sections of SCG harvested from perinatal (E20, PN1, PN7) and adult rats. To detect total (cellular) BMP expression, cells were incubated for 4 hr with antibodies to recombinant BMPs. Cultures were ﬁxed in 4% paraformaldehyde and then permeabilized with methanol at −20°C (Sigma) before addition of anti-BMP Ab (10 μg/ml). To detect BMPs associated only with the cell surface and extracellular spaces, cultures were incubated for 1 hr at 4°C in serum-free L15 medium containing anti-BMP Ab (10 μg/ml) and 5% BSA before ﬁxation with 4% paraformaldehyde. Immunoreactivity was visualized by indirect immunoﬂuorescence as described previously (Lein et al., 1995). The speciﬁcity of the anti-BMP antibodies used in these studies was conﬁrmed by preincubating each primary mAb with its speciﬁc blocking peptide or with peptides immunogenic for a different BMP family member before reaction with cultures. For all anti-BMP antibodies reported herein, preincubation with the speciﬁc blocking peptide but not with nonspeciﬁc BMP peptides reduced immunoreactivity to background levels.

To localize BMPs in intact tissues, sympathetic ganglia were ﬁxed in 4% paraformaldehyde for 24 hr at 4°C and then equilibrated in 20% sucrose solution. Cryostat sections (10 μm) were rinsed in PBS and then incubated in blocking solution (PBS, pH 7.4, 5% BSA, 0.3% Triton X-100) for 1 hr before reaction with anti-BMP Ab (10 μg/ml) for 1 hr. Primary antibody was localized using the Vectastain ABC–Peroxidase kit according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA).

In situ hybridization. The probe constructs used to detect BMP transcripts were generated using highly divergent sequences primarily in the pro-region. Specific constructs included pO455–8, a 270 bp fragment of murine BMP6, and pO319–3, a fragment that encompasses amino acids 63–263 of the pro region and the first 25 amino acids of the N-terminal domain of the mature polypeptide of murine BMP7 (Ozkanay et al., 1992). To detect BMP mRNA in cultured cells, digoxigenin-labeled antisense and sense riboprobes were generated by in vitro transcription according to the manufacturer’s instructions (Promega, Madison, WI). Cultures were ﬁxed for 10 min in 4% paraformaldehyde after 4–5 d in culture, and in situ hybridization was performed under high stringency conditions as described previously (Zhai et al., 1997). Signal was detected with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) using nitroblue tetrazolium (Boehringer Mannheim) as substrate.

To detect mRNA in tissue sections, SCG harvested from perinatal (E20, PN1, PN7) and adult rats were ﬁxed in 4% paraformaldehyde for 24 hr at 4°C and then equilibrated in 20% sucrose solution. Cryostat sections (10 μm) were mounted on Fisherbrand Superfrost/Plus microscope slides (Fisher Scientiﬁc, Pittsburgh, PA). In situ hybridization with 35S-labeled cRNA probes was performed as described previously (Tedge, 1991). Prehybridization, hybridization, and high-stringency washes were performed at 50°C. For microscopic analyses, sections were dipped in NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with HPLC water. Sections were exposed for 3 weeks at 4°C under ultraviolet light. Autoradiographs were dipped in Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY) and then washed in 0.1 M sodium thiosulfate for 10 min, followed by 2× SSC washes for 10 min each. Exposure times were 2–4 weeks at −70°C. Autoradiographs were then developed in Kodak D19 emulsion and counterstained with 3% aqueous cresyl violet.
Table 1. Specific PCR primer sequences with expected size of PCR products used in RT-PCR analyses of mRNA from SCG and cultured SCG cells

<table>
<thead>
<tr>
<th>BMP target</th>
<th>Sense primer sequence (5’ to 3’ direction)</th>
<th>Antisense primer sequence (5’ to 3’ direction)</th>
<th>Expected number of base pairs</th>
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</thead>
<tbody>
<tr>
<td>BMP2</td>
<td>CCAGGTTAGTGACTCAGAACAC</td>
<td>CATCTTTGGTGCAAAGACCTGC</td>
<td>200</td>
</tr>
<tr>
<td>BMP4</td>
<td>TCTGTCCTCCCGTCCTAATG</td>
<td>CTGAAATCTGGACGACCTTTT</td>
<td>414</td>
</tr>
<tr>
<td>BMP5</td>
<td>TTATGC AAAAGGAGGCTT GG</td>
<td>CATGCTCCCGTCGACTGT</td>
<td>200</td>
</tr>
<tr>
<td>BMP6</td>
<td>CAGACCGTGGATGAGGAA</td>
<td>TCTGACACGTGACAGTACATA</td>
<td>420</td>
</tr>
<tr>
<td>BMP7</td>
<td>ATTTCACGGTCGAGACGAG</td>
<td>TGGAAAGATCAAACGCGAAC</td>
<td>412</td>
</tr>
<tr>
<td>Noggin</td>
<td>GAGCAAGAAGCTGAGAGG</td>
<td>GTCGAGGTACAGACATGGGA</td>
<td>192</td>
</tr>
<tr>
<td>Follistatin</td>
<td>CCGAACCCCTCATTCTTCCAG</td>
<td>GCCAACCTTGAAATCTCCCTA</td>
<td>200</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>GTAACCCTGGTAACCGATT</td>
<td>CCATACCAATCGGTAGTACCG</td>
<td>150</td>
</tr>
</tbody>
</table>

Immunoprecipitation and Western blot analyses. Conditioned medium was collected and concentrated ~5× in the presence of 100 µg/ml PMSF and 300 µg/ml aprotinin (Sigma) by centrifugal filtration at 6000 × g using membranes with a molecular weight cutoff of 10 kDa. Adherent cells were rinsed with ice-cold PBS and then triturated in ice-cold lysis buffer (1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml PMSF, and 300 µg/ml aprotinin). Cell lysates were microcentrifuged at maximal speed for 5 min, and the protein concentration of the resultant supernatant was determined using the Bradford assay (Bio-Rad, Hercules, CA). For immunoprecipitation, supernatants volume-adjusted to contain equal amounts of protein were incubated with BMP-specific antibodies (each at 10 µg/ml) and Protein A/Protein-G Sepharose beads (Pierce, Rockford, IL) at 10 µl/ml for 1 hr at 4°C. The beads were then washed successively in buffer C (50 mM Tris, pH 8.0, 500 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 0.02% NaAzide), background buffer, and buffer E (10 mM Tris, pH 7.5, 0.1% NP-40) followed by extraction with 8 M guanidine HCl in Tris buffer (10 mM, pH 7.4). Immunoprecipitates or cell lysates and conditioned medium samples containing equivalent amounts of protein were resolved by 12% SDS-PAGE under reducing conditions and then electroblotted onto polyvinylidene difluoride membranes. Blots were blocked at room temperature for 1 hr in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing 5% dried fat-free milk and then incubated overnight at 4°C in TBS-T containing 0.5% milk and primary Ab (0.5 µg/ml for antibodies against BMP2, -4, -5, and -6, and follistatin mAb; 1 µg/ml for anti-BMP7 polyclonal Ab; 20 µg/ml for noggin mAb; 100 µg/ml aprotinin (Sigma) by centrifugal filtration at 10,000 × g for 5 min, and the protein concentration of the supernatant was determined using the Bradford assay (Bio-Rad, Hercules, CA)). Blots were washed twice with TBS-T containing 0.5% milk and then incubated at room temperature for 2 hr in TBS-T containing 0.5% milk and HRP-conjugated secondary Ab. Subsequently, blots were washed three times as described above and visualized using enhanced chemiluminescence detection method (ECL, Amersham Biosciences, Arlington Heights, IL). Blots of cell lysates were stripped and reprobed using antibodies specific for α-tubulin (1:10,000; Sigma). To quantify data, films were scanned using an HP ScanJet ADF scanner and HP Precision ScanPro software, and band density was determined as arbitrary absorption units using the MacBas software program (version 2.31, Fuji Film).

RNA isolation and analyses. Total RNA was extracted from freshly harvested SCG using Trizol (Invitrogen, Carlsbad, CA) and from cultured cells using RNasey (Qiagen, Valencia, CA) as specified by the manufacturer. RNA samples (3 µg) were reverse transcribed using random primers at annealing temperatures of 65°C (You-Prime-the-First-Strand kit, Amersham Biosciences, Piscataway, NJ). Resultant cDNA was amplified by PCR using primers described in Table 1. Preliminary experiments in which product formation was determined as a function of cycle number were used to select the cycle number for each primer set that corresponded to the upper end of the linear response range. Amplification programs were set using annealing temperatures ranging from 55 to 62°C, depending on the primer set, for 1 min and denaturing temperature of 94°C for 1 min; the Mg2+ concentration was 1.5 mM. As a negative control, each sample was run through PCR without previous reverse-transcription. After synthesis, PCR products were subjected to 1% agarose gel electrophoresis and found to have the expected sizes (Table 1).

RESULTS

BMP translational and transcriptional products are expressed in sympathetic ganglia during periods of maximal dendritc growth

Preliminary RT-PCR analyses of total RNA isolated from perinatal rat SCG detected transcripts for BMP5, -6, and -7, low levels of BMP2 mRNA, but no BMP4 mRNA; thus subsequent studies focused on the 60A BMP subfamily members. Using 18S rRNA as an internal standard, comparative RT-PCR analyses of BMP mRNA in SCG of animals of varying ages indicated that mRNA for BMP6 and -7 is present in SCG during periods of initial and maximal growth of dendrites in sympathetic neurons (E21 to PN1). However, transcript levels are significantly downregulated during later stages of dendritic maturation (PN21) and are not detectable in SCG of adult animals (Fig. 1). Previously we reported a similar temporal pattern of mRNA expression for BMP5 (Beck et al., 2001). Immunocytochemical analyses indicate that protein levels of BMP5, -6, and -7 follow a similar pattern of developmental expression. As illustrated in a sample immunostained with BMP7 mAb (Fig. 2A), significant BMP immunoreactivity is present in SCG of PN1 rat pups. Labeling is evident throughout the cellular and extracellular spaces of the SCG but is clearly excluded from neuronal nuclei. BMP7 immunoreactivity is also observed throughout the SCG of E20 and PN7 pups but is significantly downregulated in the adult SCG. Similar spatiotemporal expression patterns are observed in sections of SCG immunostained with antibodies selective for BMP5 or -6 (data not shown). In situ hybridization analyses indicate that SCG also express mRNA for BMP6 and -7 (Fig. 2D,E,G).
Consistent with BMP protein expression, BMP mRNA label is distributed throughout the ganglia, and the densest labeling is observed in SCG from E20 embryos, less dense labeling is observed in PN7 SCG, and negligible labeling is observed in the adult SCG. These data indicate that transcriptional and translational products for BMP5, -6, and -7 are present in the SCG at times corresponding to initial growth and rapid expansion of the dendritic arbor.

**BMPs are synthesized by SCG glia and neurons in vitro**

The BMP localization studies in intact ganglia clearly indicate that the spatiotemporal pattern of BMP expression is consistent with a proposed role for BMPs in regulating dendritic growth in SCG neurons. To determine which cell type(s) might produce and bind BMPs in developing SCG, we assessed both surface and cytoplasmic immunoreactivity of BMP proteins in cultured SCG cells. Neuron–glia cocultures derived from embryonic SCG and grown for 1–2 weeks in vitro before immunostaining exhibit significant levels of endogenous BMP5, -6, and -7. The distribution of secreted BMPs was determined by reacting cultures with BMP antibodies before fixation and permeabilization. As illustrated in a neuron–glia coculture immunostained for BMP7, neuronal somata exhibit diffuse surface immunoreactivity, and neuronal processes are clearly delineated by punctate staining (Fig. 3 A, B). Surface staining of glial cells is difficult to detect; however, BMP7 immunoreactivity is clearly observed in most if not all ganglionic glial cells in cocultures permeabilized before reaction with BMP7 mAb (Fig. 3 C, D). Glial BMP7 staining is evident throughout the cytoplasm but is excluded from the nucleus (Fig. 3 C). Neuronal somata and processes also react strongly with BMP7 mAb in permeabilized cultures (Fig. 3 C). Neuronal staining appears brighter than glial staining, attributable in part to the fact that the soma of neurons is thicker than that of cultured glial cells. Similar patterns of immunoreactivity were observed in neuron–glia cocultures immunostained with Ab selective for BMP5 (Beck et al., 2001) and BMP6 (Fig. 3 E). To determine whether neuronal immunoreactivity for BMPs reflects uptake of BMPs originating from glial cells or possible neuronal production of BMPs, BMP7 immunoreactivity was assessed in neurons cultured in the absence of glial cells. Although not as intense as in neuron–glia cocultures (Fig. 3 C), significant staining with BMP7 mAb is observed in neurons permeabilized before reaction with BMP7 mAb (Fig. 3 G). However, little to no surface immunoreactivity is observed in neurons reacted with BMP7 mAb after permeabilization (Fig. 3 H).

These observations suggested that neurons were capable of producing BMPs; however, an alternative interpretation is that neuronal immunoreactivity reflects BMPs internalized by neurons before dissociation for culture. To determine whether both neuronal and glial cell types are capable of BMP synthesis, cocultures were analyzed by in situ hybridization using probes specific for BMP6 or -7. Transcripts for BMP7 (Fig. 4 A, B) and BMP6 (Fig. 4 C) are present throughout the cytoplasm of ganglionic glial cells. Neurons cocultured with glial cells also exhibit heavy somatic labeling for BMP7 (Fig. 4 A) and BMP6 (Fig. 4 C) mRNA. This latter observation was not expected because sympathetic neurons grown in the absence of glial cells typically do not extend dendrites (Tropea et al., 1988) (see Fig. 8). Therefore, we examined the expression of mRNA for BMP6 and -7 in neurons grown in the absence of ganglionic

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**Figure 2.** BMP protein and transcript are present in sympathetic ganglia of perinatal rat pups during the time of rapid growth and expansion of the dendritic arbor. A, B, Bright-field micrographs of HRP reaction product in frozen sections from SCG of a PN1 rat pup. A, Sections immunostained with mAb 12G3 raised against BMP7 exhibit significant HRP reaction product throughout the neuronal cytoplasm and in extracellular spaces; in contrast, labeling is not observed in neuronal nuclei. B, Labeling is not observed in control sections treated only with the secondary antibody. C, D, Bright-field (C) and dark-field (D) micrographs (200×) of a SCG from a PN7 rat pup hybridized with 35S-labeled riboprobes and counterstained with cresyl violet. Sections hybridized with BMP7 antisense probe (C, D) show significant labeling throughout most of the ganglion. Higher magnification (400×) dark-field images of SCG from a PN7 rat hybridized with 35S-labeled BMP7 antisense (E) or BMP6 antisense (G) probes exhibit diffuse distribution of grains. The grain density in sections labeled with BMP7 or BMP6 antisense probes (3.2 × 10^6 ± 0.14 × 10^6 grains/cm² and 3.3 × 10^6 ± 0.05 × 10^6 grains/cm², respectively) is significantly greater than the grain density observed in sections hybridized with BMP7 sense probe (F) (0.6 × 10^6 ± 0.06 × 10^6 grains/cm²) or BMP6 sense probe (H) (0.73 × 10^6 ± 0.06 × 10^6 grains/cm²). Scale bar: 200× magnification, 50 μm; 400× magnification, 25 μm.
glial cells. Even under these culture conditions, neurons exhibit significant labeling for BMP7 (Fig. 4B) and BMP6 (Fig. 4D) mRNA.

**BMP expression is upregulated in neuron–glial cocultures**

The in situ hybridization data suggest that both neurons and glia transcribe BMP mRNA but do not indicate whether both cell types translate and secrete BMPs, nor do these data reveal whether coculturing neurons with glia alters patterns of BMP protein expression. To address these questions, we used Western blot analyses to compare levels of BMP2, -4, -5, -6, and -7 in cell lysates and conditioned medium of SCG neurons and glia grown in the absence or presence of each other. The specificity of the antibodies used in these studies is illustrated in Figure 5. All cultures expressed BMP5, -6, and -7 but not BMP2 and -4 (Fig. 6). A representative blot probed with BMP7 mAb (Fig. 5B) illustrates that conditioned medium and cell lysates of neuron–glia cocultures typically yield one or two bands with the higher
and lower molecular weight bands corresponding to the dimeric and monomeric forms of the mature BMP7 protein, respectively. Significantly less BMP7 is present in purified neuronal cultures, and it is mostly in the dimeric form (Fig. 5B). In this sample, BMP7 appears to be mostly absent from the conditioned medium; however, this pattern was not observed consistently in all samples from purified neuronal cultures (Fig. 6). Purified glial cultures express both the dimeric and monomeric forms of BMP7, which in all samples were found predominantly in the conditioned medium. Densitometric analyses of Western blots of cell lysates and conditioned medium from neurons and glial cocultures (far left lane), purified neuronal cultures (middle lane), or purified cultures of ganglionic glial cells (far right lane) were probed using antibody selective for BMP7. Equal amounts of protein were loaded into all wells, and blots of cell lysates were also probed for α-tubulin. The levels of both the monomeric (~17 kDa) and dimeric (~30 kDa) forms of BMP7 varied between the different culture conditions.

**Figure 5.** Western blot analyses of BMPs in cocultures of sympathetic neurons and ganglionic glial cells. A. The specificity of the antibodies used for Western blot analysis was evaluated by reacting blots of recombinant BMP2, -4, -5, -6, or -7 (50 ng) with antibodies raised against each of these BMPs. Each BMP antibody (0.5 μg/ml) reacted only with the BMP against which it was raised, and this interaction was inhibited by preincubation of the antibody with specific blocking peptide (2.5 μg/ml). B. Cell lysates and conditioned medium from neuron and glial cocultures (far left lane), purified neuronal cultures (middle lane), or purified cultures of ganglionic glial cells (far right lane) were probed using antibody selective for BMP7. Equal amounts of proteins were loaded into all wells, probed with antibodies selective for BMP7, -6, -5, -4, or -2 (0.5 μg/ml), and quantified by densitometry. Bars represent levels of total (combined dimeric and monomeric) BMP. C. Blots of cell lysates were also probed for α-tubulin. Data are presented as the mean ± SEM (n = 3 per condition). *p < 0.01 versus corresponding value for neuron–glia cocultures.

**Figure 6.** BMPs are present in significantly greater concentrations in cocultures of sympathetic neurons and ganglionic glial cells relative to cultures of neurons only or glial cells only. A, B. Densitometric analyses of Western blots of cell lysates (A) and conditioned medium (B) from cocultures of sympathetic neurons and ganglionic glia, cultures of neurons only, or cultures of ganglionic glial cells only. Equal amounts of proteins were loaded into all wells, probed with antibodies selective for BMP7, -6, -5, -4, or -2 (0.5 μg/ml), and quantified by densitometry. Bars represent levels of total (combined dimeric and monomeric) BMP. C. Blots of cell lysates were also probed for α-tubulin. Data are presented as the mean ± SEM (n = 3 per condition). *p < 0.01 versus corresponding value for neuron–glia cocultures.

Coculture with glia decreases levels of surface BMP complexed with BMP antagonists

In addition to altering BMP expression, BMP signaling can be regulated by modulating expression of soluble BMP antagonists that bind BMPs to prevent functional interactions between BMPs and their cognate receptors. Preliminary RT-PCR analyses using primers specific for noggin, follistatin, Cerberus, or gremlin indicated that noggin and follistatin transcripts are the predominant BMP antagonists detected in perinatal rat SCG. Noggin and follistatin mRNA are abundantly expressed in intact SCG at E20 and then decrease from PN3 to PN7 (Fig. 7A). RT-PCR analyses of purified cultures of neurons or glia indicate that both (Fig. 6C). These data suggest that neurons are capable of producing BMPs, but the level of production in neurons cultured in the absence of glia is significantly less than that observed in neuron–glia cocultures.
cell types express noggin and follistatin mRNA and that coculture does not appear to significantly alter levels of mRNA for either antagonist (Fig. 7B). To determine the effects of coculture on levels of BMP complexed to antagonist, blots of proteins immunoprecipitated from neurons cultured in the absence or presence of glial cells were probed with antibodies to either follistatin or noggin. Consistent with earlier observations, BMP levels were increased by coculture (data not shown); however, levels of BMPs complexed to noggin and follistatin were significantly decreased by coculture (Fig. 7C).

Figure 7. Expression of the BMP antagonists noggin and follistatin in SCG cells. A, Noggin and follistatin mRNA were detected by RT-PCR in total RNA extracted from rat SCG at E20 and PN3, which correspond to times of initial dendrite extension (E20) and rapid expansion of the dendritic arbor (PN3) in these neurons. At later developmental times when the dendritic arbor is undergoing maximal expansion (PN7), transcript levels for both noggin and follistatin decrease. B, RT-PCR analyses of total RNA from cultured SCG cells indicate that both neurons and glial cells express noggin and follistatin mRNA, and these levels are not altered significantly by coculture. C, Protein immunoprecipitated with BMP antibodies from neuron–glia cocultures or neuron monocultures probed with antibodies specific for noggin or follistatin. Coculture of neurons with glial cells significantly decreases levels of BMPs complexed to noggin and follistatin relative to levels present in neuronal monocultures.

Figure 8. Reagents that block BMP function inhibit glial-induced dendritic growth. Sympathetic neurons were cocultured with ganglionic glial cells in the absence or presence of mAb 12G3 raised against BMP7 (15 μg/ml) or an isotype-matched control antibody (15 μg/ml), or varying concentrations of the BMP antagonists noggin and follistatin. After 12–14 d of exposure to these reagents, cultures were immunostained for MAP-2, and dendritic growth was quantified as the percentage of neurons with dendrites (A) and the number of dendrites per neuron (B). Data are presented as the mean ± SEM (n ≥ 70 per condition). *p < 0.001 versus neuron–glia cocultures grown in the absence of BMP function-blocking reagents; †p < 0.001 versus neurons cultured in the absence of glia.

Glia-induced dendritic growth is blocked by antagonists of BMP function

Sympathetic neurons dissociated from the SCG of embryonic rats were maintained in serum-free medium, and ganglionic non-neuronal cells were eliminated from all cultures during the first few days in vitro by exposure to an antimitotic agent. After 5–7 d in vitro, neuronal cultures were reseeded with highly purified populations of glial cells originally derived from sympathetic ganglia. Consistent with previous studies (Tropea et al., 1988), within 2 weeks after adding glial cells to cultures, 75–85% of the neuronal cell population had extended one to three distinct dendrites; in contrast, neurons maintained in the absence of glia had very little dendritic growth (Fig. 8).

To test the hypothesis that BMPs mediate glial-induced dendritic growth, BMP activity was inhibited using either function-blocking BMP7 mAb or soluble antagonists of BMP action. These reagents were initially added to the culture medium 3 d after reseeding with ganglionic glial cells, and the medium was continuously replenished with the appropriate reagent at each subsequent medium exchange. All cultures were analyzed for
dendritic growth at the end of the third week in vitro. In the presence of the function-blocking anti-BMP7 mAb (Vukicevic et al., 1994), the percentage of neurons with dendrites and the number of dendrites per neuron were reduced by 56 and 62%, respectively (Fig. 8A,B). Similar results were obtained using BMP7 mAb that recognizes a different epitope of BMP7 (data not shown). Concentrations of anti-BMP mAb >15 μg/ml did not cause further inhibition of dendritic growth. Isotype-matched control antibody at similar concentrations had no inhibitory effect on either parameter of dendritic growth.

The observation that mAbs selective for BMP7 did not completely block glial-induced dendritic growth is not surprising because BMP5 and -6 are also present in these cocultures, and both have been reported to promote dendritic growth in sympathetic neurons (Guo et al., 1998; Beck et al., 2001). Because function-blocking antibodies selective for these additional BMPs are not available, we tested the hypothesis that multiple BMPs mediate glial-induced dendritic growth by exposing neuron–glia cocultures to exogenous noggin or follistatin. Preliminary studies indicated that the dendrite-promoting activity of recombinant BMPs mediate in cultured sympathetic neurons (10–20 ng/ml) (Lein et al., 1995; Guo et al., 1998) was significantly inhibited by both BMP antagonists in a concentration-dependent manner [see also Beck et al. (2001)]. Noggin (500 ng/ml) blocked the dendrite-promoting activity of BMP2, -4, -5, -6, and -7 at concentrations that induce robust dendritic growth in cultured sympathetic neurons (10–20 ng/ml) (Lein et al., 1995; Guo et al., 1998) was significantly inhibited by both BMP antagonists in a concentration-dependent manner [see also Beck et al. (2001)]. Noggin (500 ng/ml) blocked the dendrite-promoting activity of BMP2 and -4 almost completely (97% inhibition) and significantly reduced the effect of BMP5, -6, and -7 (~80% inhibition). Follistatin (10 μg/ml) strongly antagonized the effects of BMP5, -6, and -7 on dendritic growth (85–95% inhibition) and significantly decreased that of BMP2 and -4 (60–75% inhibition).

The addition of noggin or follistatin to neuron-glial cocultures significantly reduced both the percentage of neurons with dendrites (Fig. 8A) and the number of dendrites per neuron (Fig. 8B). This inhibition of glial-induced dendritic growth was concentration dependent, with maximal effects observed at 500 ng/ml for noggin and 10 μg/ml for follistatin. These concentrations are comparable with those previously reported to block BMP effects on other developmental endpoints in cultured neurons (Ai et al., 1999; Li and LoTurco, 2000; Lim et al., 2000). A combined exposure to maximally effective concentrations of noggin and follistatin reduced dendritic growth to levels observed in sympathetic neurons cultured in the absence of glia.

Morphometric analyses of cultures by phase-contrast microscopy indicated that treatment with noggin or follistatin had no effect on the density or viability of neurons or glia. Thus, in all cultures examined, the axonal plexus appeared intact, glial cells were confluent across the dish, and neuronal somata were surrounded by glia. To confirm these observations, neuronal cell number and total cell viability were assessed in neuron–glia cultures grown in the absence or presence of BMP antagonists. The number of neurons per 35 mm dish of neuron–glia cocultures (n = 3 per condition) exposed to 500 ng/ml noggin (21,712 ± 1987 neurons per 35 mm dish), 10 μg/ml follistatin (17,498 ± 1699), or noggin and follistatin at maximal concentrations (18,317 ± 2454) did not differ significantly from that of cocultures grown in the absence of BMP antagonists (20,049 ± 3541). Similarly, BMP antagonists did not alter the number of viable cells (which includes neuronal and glial cells) as determined by incorporation of calcein and ethidium-homodimer-1 dyes. When expressed as a percentage of control (cocultures not exposed to BMP antagonists), the number of viable cells in cocultures exposed to 500 ng/ml noggin was 96 ± 2%; 10 μg/ml follistatin, 105 ± 6%; noggin and follistatin, 98 ± 4%. These data suggest that the inhibition of dendritic growth by these BMP antagonists does not result from compromised neuronal or glial cell viability.

Addition of glia to neuronal cultures increases nuclear translocation of Smad-1 in neurons but not glia

Increased BMP activity in neuron–glia cocultures could directly induce dendritic growth in neurons via interaction with neuronal BMP receptors or indirectly via interaction with glial BMP receptors. BMP ligand binding to their receptors activates Smad transcription factors, which subsequently translocate from the cytoplasm to the nucleus. To determine which cell type BMPs activated in neuron–glia cocultures, the distribution of Smad-1 immunoreactivity was assessed in purified cultures of neurons or glia and in neuron–glia cocultures (Fig. 9). Consistent with previous observations (Guo et al., 2001), Smad-1 immunoreactivity was restricted primarily to the cytoplasm in purified cultures of neurons. However, as observed when sympathetic neurons are treated with recombinant BMPs (Guo et al., 2001), when cocultured with glia, most neurons also exhibited Smad-1 immunoreactivity in the nucleus. In glial cells grown in purified culture, Smad-1 was localized predominantly in the cytoplasm, and coculturing with neurons did not alter this pattern of Smad-1 immunoreactivity.

DISCUSSION

Dendritic growth can be induced in sympathetic neurons by coculture with glial cells or treatment with BMPs (Tropea et al., 1988; Lein et al., 1995; Guo et al., 1998). The following observations suggest a causal link between these phenomena. First, the spatiotemporal expression pattern of BMPs in SCG correlates with periods of dendritic growth in vivo. In SCG,
dendritic growth begins prenatally, and maximal expansion of the dendritic arbor occurs during postnatal weeks 1 and 2 (Rubin, 1985; Voyvodic, 1987). Transcriptional and translational products for BMP5, -6, and -7 were present at significant levels throughout the ganglia from E20 through PN7. Others have reported that transcripts for BMP2, -5, and -7 can be isolated from E14 sympathetic ganglia (Kobayashi et al., 1998). These data, together with observations that mRNA for BMP type IA and type II receptors are expressed in perinatal sympathetic ganglia (Zhang et al., 1998), are consistent with a role for BMPs in regulating the initiation and rapid expansion of the dendritic arbor in sympathetic ganglia.

BMP5, -6, and -7 were also detected in blots of cell lysates and conditioned medium from neuron–glia cocultures; in contrast, analyses using BMP2- or BMP4-specific antibodies yielded negative data. These data suggest that multiple members of the 60A BMP subfamily are synthesized and secreted by neuron–glia cocultures. Immunocytochemical analyses of cocultures indicate that BMP5, -6, and -7 are localized to glial cells, but neurons also display significant immunoreactivity for these BMPs, raising the question of which cell type(s) actually synthesizes BMPs. Two lines of evidence indicate that both cell types are capable of transcribing and translating BMPs. First, as revealed by in situ hybridization, glia as well as neurons cultured in the presence or absence of glia express BMP6 and -7 transcripts at significant levels. Second, BMP5, -6, and -7 proteins are detected by immunocytochemical and Western blot analyses in purified cultures of either cell type.

There are reports that mRNA for BMPs is detected in CNS neurons during development (Wall et al., 1993; Tomizawa et al., 1995). However, the observation that sympathetic neurons contain mRNA for BMP6 and -7 and are apparently capable of translating this mRNA and secreting the resultant protein product was unexpected because previous studies demonstrated that these neurons require exposure to either glia or exogenous BMPs to initiate dendritic growth (Tropea et al., 1988; Lein et al., 1995). There are at least two plausible explanations for these seemingly discrepant observations: (1) the addition of large numbers of glial cells to low-density cultures of sympathetic neurons increases BMPs to biologically active concentrations, an explanation that would be consistent with previous reports of weak dendritic growth in sympathetic neurons cultured at very high cell densities in the absence of glia (Bruckenstein and Higgins, 1988), and (2) coculture decreases levels of BMP antagonists. Our data suggest that both mechanisms may underlie glial-induced dendritic growth. Comparative Western blot analyses of BMP concentrations in neuron–glia cocultures versus purified neuronal populations indicate significant upregulation of BMP expression in the former. Increased BMP levels in neuron–glia cocultures may reflect specific upregulation of BMP7 synthesis mediated by neuron–glia interactions or simply an increased density of BMP-expressing cells in cocultures. Our data support the former interpretation in that an equal amount of protein was loaded into all lanes, and α-tubulin levels did not differ across cell lysate samples. However, we cannot determine from these studies whether the increased BMP levels result from upregulated synthesis in glia or neurons, or both.

There is increasing evidence that BMP signaling in the developing nervous system is regulated not only by expression of BMPs and their receptors but also by soluble BMP antagonists (McMahon et al., 1998; Sela-Donenfeld and Kalcheim, 1999; Li and LoTurco, 2000; Smith and Graham, 2001). Transcripts for both noggin and follistatin were detected in intact SCG and in purified cultures of neurons. Coculture with glia had no appreciable effect on mRNA expression for these BMP antagonists. However, adding glia to neuronal cultures did significantly decrease levels of BMPs complexed to noggin and follistatin. Although these data do not directly demonstrate that coculture with glia decreases protein levels of these BMP antagonists, there are two reasons to suspect that this may be true. First, the volume of extract used for immunoprecipitation was adjusted such that equivalent amounts of protein were processed for each culture condition. Second, noggin and follistatin bind BMPs with greater affinity than BMP receptors (Holley et al., 1996; Zimmerman et al., 1996; Fainsod et al., 1997; Iemura et al., 1998), so it is unlikely that the extracellular compartment contains significant levels of antagonist not complexed to BMPs. The mechanism(s) by which glia cause downregulation of noggin and follistatin is not known. Furthermore, these observations stand in contrast to the current paradigm of a negative feedback loop in which BMPs induce expression of BMP antagonists (Gazzerro et al., 1998; Pereira et al., 2000). However, this paradigm was established using non-neural systems, principally osteoblasts, and other studies of the developing nervous system have demonstrated that paraxial mesoderm in the dorsal neural tube functions to downregulate noggin expression in the presence of physiologically active concentrations of BMPs (Sela-Donenfeld and Kalcheim, 2000).

On the basis of these data, we propose that glia induce dendritic growth in sympathetic neurons by increasing BMP activity via two mechanisms: (1) increased synthesis of BMPs and (2) decreased expression of noggin and follistatin. The most direct support for this hypothesis is the finding that glial-induced dendritic growth in cultured sympathetic neurons is significantly reduced by function-blocking BMP antibodies and completely blocked by the BMP antagonists noggin and follistatin. The observation that glial-induced dendritic growth is effectively blocked by three different agents that inhibit BMP activity via different molecular mechanisms suggests that their inhibitory effects result from a direct block of BMP function and not from nonspecific molecular interactions or cross-reactivity. A potential difficulty with this interpretation is that follistatin is known to avidly bind not only BMPs but also activin (Nakamura et al., 1990). However, earlier studies have shown that neither activin nor other members of the TGF-β superfamily such as GDNF, dorsalin, TGF-β1, or -β2, induce dendritic growth in sympathetic neurons (Lein et al., 1995), suggesting that dendrite-promoting activity is restricted to the BMP subfamily.

The data obtained from the function-blocking studies suggest that multiple BMPs mediate the dendrite-promoting activity of glia. Thus, BMP7 mAbs caused significant but not complete inhibition of dendritic growth. One explanation is that the BMP7 mAbs exhibit a 10-fold lower binding affinity for BMP7 relative to BMP7 receptors (P. Kaplan, unpublished observations). However, because BMP2, -5, and -6 have also been reported to promote dendritic growth in sympathetic neurons (Guo et al., 1998; Beck et al., 2001), an additional explanation is that neuron–glia cocultures express multiple BMPs. This interpretation is consistent with observations that noggin and follistatin, which block multiple BMPs, cause a greater inhibition of glial-induced dendritic growth. Moreover, our immunocytochemical and Western blot analyses demonstrate that neuron–glia cocultures express mRNA and protein not only for BMP7 but also BMP5 and -6.
One question not answered by the functional studies of the role of BMPs in dendritic growth is whether BMPs directly induce dendritic growth via interaction with neuronal receptors or indirectly via activation of glia to produce a dendrite-promoting stimulus. BMP binding to surface receptors causes translocation of the transcription factor Smad-1 from the cytoplasm to the nucleus. Analysis of the subcellular localization of Smad-1 immunoreactivity in neuron–glia cocultures indicated that >70% of the neurons, but <20% of the glia, exhibited nuclear Smad-1 staining. These data suggest that BMPs interact directly with neuronal receptors to induce dendritic growth. This interpretation is consistent with previous reports that addition of recombinant BMPs to purified cultures of neurons is sufficient to elicit robust dendritic growth (Lein et al., 1995; Guo et al., 1998).

Neuronal cell division predominates during the early formation of the SCG (E14.5–18.5), whereas glial cell populations begin dividing rapidly later in development and continue expanding in number in the postnatal ganglion (Hendry, 1977). Thus, we propose the following model. During the early formation of the SCG, neurons synthesize and secrete both BMPs and BMP antagonists, and the balance is such that the antagonists effectively inhibit the dendrite-promoting activity of the BMPs. However, as glial cell populations begin to proliferate, the levels of BMPs begin to increase, whereas the levels of BMP antagonists begin to decrease. Thus the balance is shifted such that BMP antagonists become saturated, and BMPs released from inhibition are able to activate neuronal receptors resulting in dendritic growth. Future work will be aimed at identifying the mechanism(s) by which neuron–glia interactions stimulate BMP synthesis and downregulates noggin and follistatin expression.

REFERENCES


