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Opposing effects of bone morphogenetic proteins on neuron production and survival in the olfactory receptor neuron lineage

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SUMMARY

In olfactory epithelium (OE) cultures, bone morphogenetic proteins (BMPs) can strongly inhibit neurogenesis. Here we provide evidence that BMPs also promote, and indeed are required, for OE neurogenesis. Addition of the BMP antagonist noggin inhibited neurogenesis in OE-stromal cell co-cultures. *Bmp2*, *Bmp4* and *Bmp7* were expressed by OE stroma, and low concentrations of BMP4 (below the threshold for inhibition of neurogenesis) stimulated neurogenesis; BMP7 did not exhibit a stimulatory effect at any concentration tested. Stromal cell conditioned medium also stimulated neurogenesis; part of this effect was due to the presence within it of a noggin-binding factor or factors. Studies of the pro-neurogenic effect of BMP4 indicated that it did not increase progenitor cell proliferation, but rather promoted survival of newly generated olfactory receptor neurons. These findings indicate that BMPs exert both positive and negative effects on neurogenesis, depending on ligand identity, ligand concentration and the particular cell in the lineage that is responding. In addition, they reveal the presence of a factor or factors, produced by OE stroma, that can synergize with BMP4 to stimulate OE neurogenesis.

Key words: BMP, Mouse, Olfactory epithelium, Neurogenesis, Olfactory receptor neuron, Neuron survival, Neuronal progenitor cell

INTRODUCTION

Accumulating evidence indicates that bone morphogenetic proteins (BMPs), the largest group of ligands in the transforming growth factor β superfamily, have important roles in vertebrate neurogenesis. Although an early embryonic requirement for *Bmp2* and *Bmp4* function (Winnier et al., 1995; Zhang and Bradley, 1996) has so far precluded extensive genetic analysis of their roles in mammalian neurogenesis, other studies indicate that these factors regulate neurogenesis in important ways. During embryonic neural induction, endogenous BMP4 promotes acquisition of epidermal, rather than neural, fate by developing ectoderm (Hawley et al., 1995; Wilson and Hemmati-Brivanlou, 1995). At later stages of development, BMP2 and BMP4 have been shown to have anti-neurogenic effects in several systems, where they inhibit proliferation and/or induce apoptosis of neural progenitor cells (Furuta et al., 1997; Golden et al., 1999; Graham et al., 1994; Li et al., 1998; Mabie et al., 1999; Shou et al., 1999; Song et al., 1998). In some systems, however, BMP2 and BMP4 have been reported to have positive effects on neurogenesis, for example by promoting neuronal differentiation (Li et al., 1998; Reissmann et al., 1996; Schneider et al., 1999; Shah et al., 1996; Varley and Maxwell, 1996; Varley et al., 1995). BMP6 and BMP7, members of a separate BMP subfamily (Kingsley, 1994), have been reported to have both positive (Arkell and Beddington, 1997; Furuta et al., 1997; Jordan et al., 1997) and negative (Li et al., 1998; Shou et al., 1999) effects on neurogenesis. Given the complexity of the systems studied, it is not known whether the same cells are targeted by opposing effects of BMPs, or whether the disparate effects of BMPs reflect actions on different cell types or lineage stages.

In the olfactory epithelium (OE), cell types and lineage stages have been identified, upon which both extrinsic and intrinsic factors act to regulate the generation of olfactory-receptor neurons (ORNs; reviewed by Calof et al. (1998). This system was used recently to establish one mechanism by which BMPs inhibit neurogenesis: BMP2, BMP 4 and BMP7 all act on an early stage progenitor in the ORN lineage, which expresses the transcription factor MASH1 (ASCL1 – Mouse Genome Informatics; Gordon et al., 1995). Exposure of these neuronal progenitors to BMPs targets pre-existing MASH1 protein for degradation via the proteasome pathway, resulting in a decrease in progenitor cell proliferation and premature termination of this neuronal lineage (Shou et al., 1999). Since genetic studies indicate that MASH1 is required for ORN production in vivo (Guillemot and Joyner, 1993), these results suggest that BMP-mediated degradation of MASH1 is an important mechanism by which ORN number is regulated.

In the present study, we have sought to investigate whether endogenous BMPs play a role in the feedback inhibition of neurogenesis that is thought to regulate neuron number in the OE (Calof et al., 1996; Mumm et al., 1996). Our experiments led to the surprising discovery that, despite their profound

anti-neurogenic actions, BMPs are actually required for neurogenesis to take place in OE cultures. In addition, BMP4, but not BMP7, was found to stimulate neurogenesis when applied at low concentration; this effect was due to promotion of survival of newly generated ORNs. Thus, BMPs exert both ligand-specific and concentration-dependent effects on OE neurogenesis. Moreover, these opposing effects are exerted at different cell stages in the neuronal lineage.

MATERIALS AND METHODS

Materials

Recombinant human BMPs were from Genetics Institute (Cambridge, MA). Recombinant *Xenopus* noggin protein was from Richard Harland (Berkeley, CA). Crystallized bovine serum albumin (BSA), clinical reagent grade BSA (CRG-BSA), and [^{3}H]thymidine ([^{3}H]thymidine; 60-80 Ci/mmol) were from ICN. Tissue culture reagents were from Gibco/Life Technologies, and reagents for in situ hybridization from Boehringer Mannheim.

Tissue culture

Neuronal colony-forming assays

Purification of OE neuronal progenitor cells from E14.5-16.5 (date of vaginal plug detection = E0.5) Rosa26 mice (B6,129-GtRosa26; Jackson) and isolation and culture of OE stromal cell feeder layers were performed as described (Shou et al., 1999). Any agents (BMPs, noggin, etc.) were added when co-cultures were initiated, and cultures were re-fed every other day. Assays were fixed, processed for X-gal staining after 6 days in vitro, and numbers of neuronal and non-neuronal colonies counted. For comparison, colony numbers were standardized to 96,000 input cells (the number normally plated in a 96-well plate for each test condition (cf. Mumm et al. (1996)); this number was then normalized to the control value in a given experiment. The average number of neuronal colonies counted in control conditions ranged from 36-84; for non-neuronal colonies, from 172-231.

OE explant assays

OE explants were isolated from E14.5-15.5 CD-1 mice (Charles River) and cultured on coverslips as described (DeHamer et al., 1994) in serum-free, low-Ca^{2+} culture medium containing either 5 mg/ml BSA (LCM + 5 medium; Calof et al.,1995) or 1 mg/ml CRG-BSA (LCM + 1 medium; DeHamer et al., 1994). BMPs added to the culture medium were replenished every day.

Stromal cell conditioned medium

Stromal cell conditioned medium (SCM) was prepared from cultured stromal cells that had been mitotically inactivated by γ-irradiation (3300 rads) and plated at 6 x 10^6 cells per 100 mm dish. The day after plating, cells were rinsed with PBS and fed 5 ml of LCM + 5 medium with $CaCl_2$ level increased to 1.4 mM (Shou et al., 1999). After 2 days' incubation, SCM was collected, filter sterilized (0.2 μm) and stored in aliquots at –80°C until used. A given plate of stromal cells was used for 1-2 rounds of SCM collection.

RT-PCR

Nasal turbinates, purified OE, cultured stromal cells and purified OE neuronal cells were isolated from CD-1 mice as described (Calof et al., 1995; Calof and Lander, 1991; Mumm et al., 1996), and total RNA extracted using Ultraspec (BIOTECX). For each sample, 20-30 μg RNA was added to RT buffer (Promega) and treated with 5 units of RNase-free DNase (Promega; 1 hr, 37^0C). 5 μg of DNase-treated RNA was added to Ready-To-Go You-Prime First-Strand Beads (Pharmacia) with 100 pmol random hexamers (Life Technologies) to make cDNA, and PCR performed using Qiagen Taq DNA polymerase. Primer sequences for *Bmp2* and *Bmp4* were from (Johansson and Wiles, 1995); primer sequences for *Bmp7* were from (Gross et al., 1996). Primer sequences for *Nog* (noggin) and *Hprt* were: *Nog*, 5′-GGCCAGCACTATCTACACAT-3′ (forward), 5′-TCTGTAACTTCCTCCTCAGC-3′ (reverse); *Hprt*, 5′-GCTGGTGAAAAGGACCTCT-3′ (forward), 5′-CACAG-GACTAGAACACCTGC-3′ (reverse). PCR products were verified either by Southern blot or by subcloning into pCR2.1 (Invitrogen) and sequencing.

In situ hybridization

Non-radioactive in situ hybridization using digoxigenin-labelled probes was performed on cryostat sections (12-18 μm) of fresh-frozen (*Bmp2*, *Bmp4*, *Ncam*) or paraformaldehyde-fixed (*Bmp7*; 4% paraformaldehyde in PBS) OE from CD-1 embryos (E14.5-15.5) or adult male mice as described previously (Birren et al., 1993). Probes used were: 1.2 kb mouse *Bmp2*-coding region (Lyons et al., 1989); 1550 bp full-length mouse *Bmp4* cDNA (Winnier et al., 1995); 900 bp mouse *Bmp7* cDNA (Furuta et al., 1997); and a 391 bp fragment of mouse *Ncam*-coding region (Barthels et al., 1987). Probes were hydrolyzed to approx. 250 bp by incubation in 80 mM $NaHCO_3$, 120 mM Na_2CO_3 at 60°C. Bound probe was visualized using sheep anti-DIG antibodies and BCIP/NBT.

Noggin-mediated depletion of stroma-conditioned medium

20 μl purified recombinant *Xenopus* noggin (0.5 mg/ml in 10 mM HEPES, 1 mM EDTA, 10% glycerol, pH 7.6) was biotinylated using 1.5 μl NHS-LC-Biotin (1 mg/ml in DMSO; Pierce) according to manufacturer's instructions. Reactants were mixed with 60 μl of 10 mM sodium phosphate buffer (pH 6.8) containing 800 μg/ml BSA, and free biotin removed by passing through a NAP^{TM}-5 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer, pH 6.8. 45 μl of biotinylated noggin (~824 ng noggin, assuming complete recovery) was incubated for 2 hours with 200 μl of a 50% slurry of streptavidin agarose beads (2.8 mg/ml streptavidin per ml packed gel; Life Technologies). Unbound noggin was removed by extensive rinsing, and beads incubated with 10 ml of SCM overnight at 4°C. Mock-depleted SCM was made using streptavidin agarose that had not been incubated with biotinylated noggin. To confirm that noggin beads could deplete BMPs from solution, 200 μl of noggin-streptavidin-agarose bead slurry was incubated with 1.25 ml LCM + 5 medium containing 20 ng/ml BMP4 (9 hours, 4°C), and this medium used in assays to detect BMP-induced degradation of MASH1 protein in OE neuronal progenitor cells (Shou et al., 1999). The results indicated that noggin depletion was capable of removing >99% of the BMP4 present in a 20 ng/ml solution (data not shown).

RESULTS

Exogenous noggin blocks neurogenesis in OE neuronal colony-forming assays

We have used in vitro neuronal colony-forming assays to quantify effects of cell interactions and growth factors on neurogenesis in the OE. These assays are performed by culturing purified OE neuronal progenitor cells at low density on feeder layers of stromal fibroblasts for 6-7 days, then counting the number of neuronal colonies that develops as an index of neurogenesis. Recently, it was found that BMP2, BMP4 or BMP7, applied to OE neuronal colony-forming assays, at concentrations of 10-20 ng/ml, strongly inhibits neurogenesis (Shou et al., 1999). Since an earlier study had shown that addition of a large excess of ORNs to colony-forming assays also resulted in inhibition of neurogenesis (Mumm et al., 1996), we were interested in determining if

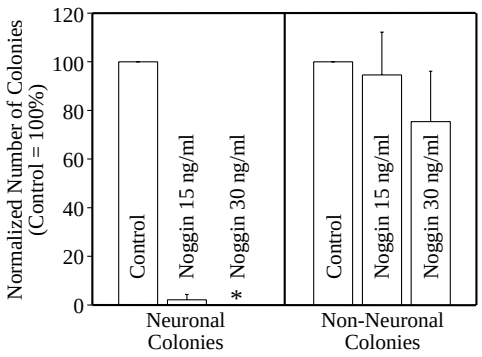


Fig. 1. Inhibition of neuronal colony formation by noggin. Numbers of neuronal and non-neuronal colonies were normalized to the control (no noggin added) value in a given experiment. These values were expressed as the mean for two independent experiments in a given condition (±range). Asterisk indicates that no neuronal colonies were observed when cultures were treated with 30 ng/ml noggin.

BMPs might constitute all or part of this ORN-derived inhibitory signal. We planned to test this by determining whether the soluble BMP antagonist, noggin (Zimmerman et al., 1996), could block the inhibitory effect of ORNs in colony-forming assays. Before beginning these experiments, however, it was necessary to perform control studies first in which noggin itself was tested for effects on OE neurogenesis.

To our surprise, noggin alone had a strong inhibitory effect. As shown in Fig. 1, at a concentration of 30 ng/ml, noggin completely blocked development of neuronal colonies; at 15 ng/ml, it was almost as effective, with neuronal colony development only about 2% of the level seen in control cultures. There was no significant effect of noggin on development of any of the other, non-neuronal colonies that develop in these assays, indicating that the inhibition of neurogenesis was not due to a nonspecific (e.g. toxic) effect of the recombinant noggin preparation. Since the only known function of noggin is to bind to and inactivate BMPs (Chang and Hemmati-Brivanlou, 1999; Zimmerman et al., 1996), the results of these experiments suggested that one or more noggin-binding BMPs is already present in our neuronal colony-forming assays, and that this or these BMPs are required for allowing the normal (control) level of neurogenesis observed in these cultures.

Expression of *Bmps* and *Noggin* in OE and its underlying stroma

Noggin binds BMP2 and BMP4 with high affinity (K_d=19 pM), and BMP7 to a lesser degree (Zimmerman et al., 1996). To determine if *Bmp2*, *Bmp4* and *Bmp7*, as well as *Nog*, are normally expressed in OE or OE stroma, we first used RT-PCR to look for their transcripts in tissue taken from E14.5-15.5 embryos, the age at which OE is taken for in vitro assays. As shown in Fig. 2A, mRNAs for *Bmp2*, *Bmp4* and *Bmp7* were all present in nasal turbinates. However, only *Bmp4* and *Bmp7* were expressed in OE proper, while stromal fibroblasts appeared to express all three BMPs. Since *Bmp4* and *Bmp7* were expressed in pure OE, we further refined our analysis by determining if these BMPs were expressed by neuronal cells. A purified neuronal cell fraction, consisting of NCAM-expressing ORNs and NCAM-negative neuronal progenitors, was prepared (Calof and Lander, 1991; Mumm et al., 1996), and RT-PCR performed. Fig. 2A shows that both *Bmp4* and *Bmp7* were indeed expressed by OE neuronal cells. *Nog*, however, was expressed by neither OE proper nor cultured OE stromal cells.

To learn more about the expression pattern of BMPs in the OE, in situ hybridization for *Bmp2*, *Bmp4* and *Bmp7* to tissue sections of E14.5-15.5 mouse OE was performed. As a positive control, a probe for *Ncam*, a marker for ORNs (Calof and Chikaraishi, 1989), was used. The data (Fig. 2B) are consistent with the RT-PCR results and reveal different expression patterns for the three BMPs. All three were expressed in stroma underlying the OE. *Bmp4* was expressed in scattered cells throughout the OE, in a pattern overlapping, but more extensive, than that seen with *Ncam*, which was expressed exclusively in ORNs. In contrast, *Bmp7*-expressing cells were located in patches in the basal region of the OE. No expression of *Bmp2* above background levels was seen in OE proper. Interestingly, these same general patterns of expression appeared to be maintained in adult animals: *Bmp2* was not expressed in OE proper, and *Bmp7* only in scattered small patches of cells in basal OE (data not shown). *Bmp4*, however, continued to be widely expressed in the neuronal cell layers of the OE (Fig. 2C).

BMP4, but not BMP7, exerts a stimulatory effect on neurogenesis at low concentration

The finding that *Bmp2*, *Bmp4* and *Bmp7* were expressed by stromal cells (Fig. 2), taken together with the observation that adding noggin to colony-forming assays inhibits neurogenesis (Fig. 1), suggested that BMPs made by stromal cells might act as positive regulators of OE neurogenesis. To test whether this is plausible, neuronal colony-forming assays were performed in which BMP4 or BMP7 was added at concentrations spanning several orders of magnitude. The results are shown in Fig. 3. As observed previously (Shou et al., 1999), either BMP4 or BMP7 at a concentration of 10 ng/ml profoundly inhibited development of neuronal colonies. However, when cultures were grown in 0.1 ng/ml BMP4, a consistent, significant increase was observed in the number of neuronal colonies; lower or higher concentrations of BMP4 failed to show this stimulatory effect (Fig. 3A). Interestingly, although BMP7, like BMP4, inhibited neuronal colony formation at 10 ng/ml, no significant stimulatory effect of BMP7 was observed at any concentration tested (Fig. 3B). In addition, there was no significant effect of either BMP4 or BMP7 on the number of non-neuronal colonies that developed in these assays (Fig. 3C,D), at any concentration tested, indicating that the observed effects of BMPs were specific to neuronal progenitors and/or neurons.

Conditioned medium from cultured OE stromal cells contains two independent pro-neurogenic activities

Stromal cell feeder layers are an essential component of OE neuronal colony-forming assays; without them, neuronal colonies fail to develop (Mumm et al., 1996). One possible explanation for this observation is that stromal cells produce soluble factors that promote neurogenesis. If this is correct, then supplementation of neuronal colony-forming assays with molecules released by stromal cells might be expected to

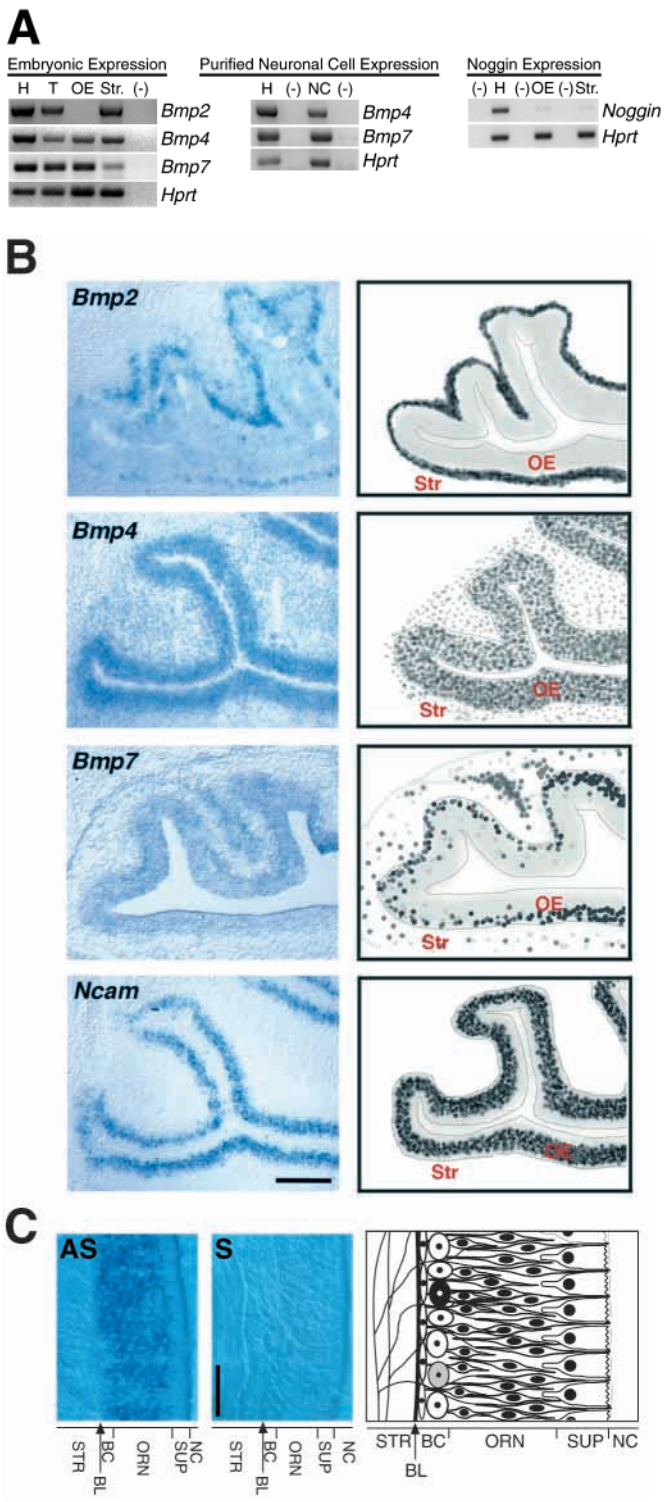


Fig. 2. Expression of *Bmps* and *Noggin* in the olfactory epithelium (OE). (A) RT-PCR analysis. (Left) RT-PCR was performed on total RNA isolated from purified OE, cultured stromal cells (Str.), and E 14.5-15.5 nasal turbinates (T). Total RNA from E12 heads (H) served as a positive control. No-RT controls (–) contained RNA, primers and water only. Sizes of products for *Bmp2*, *Bmp4*, *Bmp7*, *Nog* and *Hprt* are 720bp, 566bp, 253bp, 416bp and 249bp, respectively. (Middle) RT-PCR of total RNA from E12 mouse heads (H) and purified OE neuronal cells (NC). (Right) RT-PCR of total RNA from purified OE (OE), cultured stromal cells (Str.), and E12 mouse head (H). Size of *Nog* product is 249bp. (B) In situ hybridization analysis (embryonic). Frozen sections of E14.5-15.5 CD-1 OE were hybridized with antisense RNA probes to BMPs and *Ncam*; sense probe controls (not shown) showed no staining. Right panels are schematic drawings summarizing expression patterns of BMPs and *Ncam* in olfactory epithelium (OE). Str, stroma. (C) In situ hybridization analysis (adult). Localization of *Bmp4* transcripts in frozen sections of adult OE (AS ,antisense probe; S, sense (control) probe). Schematic drawing on the right shows major cell layers in the OE. OE proper is separated from stroma (STR) by a basal lamina (BL). Within the basal cell layers (BC) of the OE, a layer of flat horizontal basal cells underlies globose neuronal progenitors, which include stem cells (black), MASH1+ progenitors (gray) and INPs (white). Several layers of postmitotic olfactory receptor neurons (ORN) lie atop progenitor cells and are capped by the supporting cell layer (SUP) adjacent to the nasal cavity (NC) (Calof et al., 1998). Scale bars: 200 μm in B; 50 μm in C.

stimulate neurogenesis above the levels seen with stromal cell feeder layers alone. To test this idea, serum-free medium conditioned for 2 days over cultured stromal cells (stromal cell conditioned medium, SCM) was collected and used to supplement growth medium in neuronal colony-forming assays. Addition of SCM resulted in a large (65%) increase in the number of neuronal colonies that developed (Fig. 4A), but had no effect on the number of non-neuronal colonies that developed (data not shown).

We then asked whether the pro-neurogenic effect of SCM could be attributed to the presence within it of BMPs. To address this, noggin was biotinylated, coupled to streptavidin agarose beads, and retention of its ability to bind BMP4 was verified. These beads were then used to deplete SCM of any noggin-binding BMPs (‘depleted SCM’). As a control, SCM was incubated with streptavidin beads that had not been pre-bound with biotinylated noggin (‘mock-depleted SCM’). Both types of SCM were then tested in neuronal colony-forming assays. The results, shown in Table 1, indicate that treatment of SCM with immobilized noggin resulted in a partial loss (mean=26.8%) of pro-neurogenic activity. Since the identical treatment with noggin-streptavidin beads was capable of depleting >99% of the BMP4 present in a 20 ng/ml solution (see Materials and Methods), the results of these experiments suggest that noggin-binding BMPs account for part, but not all, of the stimulatory effect of SCM on OE neurogenesis.

To test further whether the pro-neurogenic activities of SCM and low-dose BMP4 are distinct from one another, neuronal colony-forming assays were performed in which cultures were fed with 0.1 ng/ml BMP4, alone or in combination with SCM. As shown in Fig. 4B, 50% SCM in combination with 0.1 ng/ml BMP4 had a significantly greater stimulatory effect on neuronal colony formation than either SCM or 0.1 ng/ml BMP4 alone. In contrast, the effect of low-dose BMP4 itself was not additive: 0.2 ng/ml BMP4 did not have a significantly larger effect than 0.1 ng/ml BMP4. The synergistic action of 0.1 ng/ml BMP4 and SCM provides strong evidence that these two agents act independently to stimulate neurogenesis. No synergy of BMP4 and SCM in promoting development of non-neuronal colonies was observed (data not shown).

Since noggin inhibits (Fig. 1), while SCM promotes (Fig. 4), OE neurogenesis, we sought to determine the effect of combining noggin and SCM. Colony-forming assays were therefore performed in which SCM was added in the absence

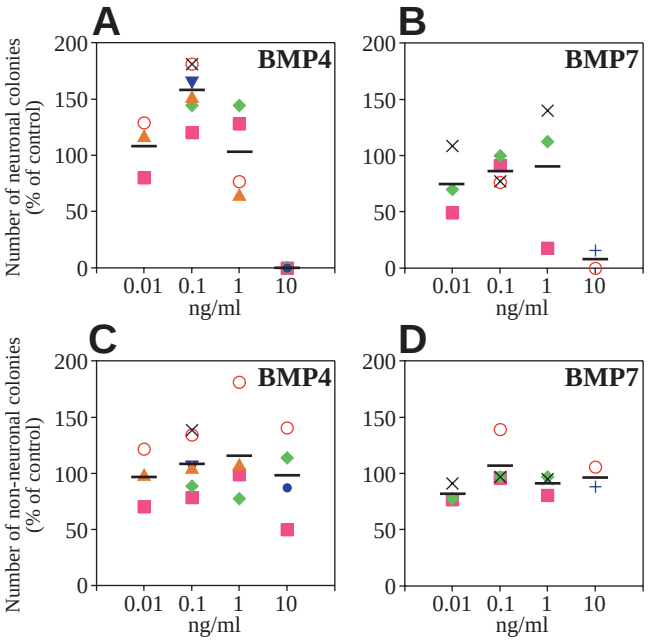


Fig. 3. BMP4, but not BMP7, has a stimulatory effect on neuronal colony formation at low concentration. Neuronal colony-forming assays were performed and numbers of neuronal and non-neuronal colonies expressed as a percentage of the control (no BMP added) value for the same experiment (cf. Fig. 1). In each graph, different symbols indicate values obtained in independent experiments; the means of these values, for each concentration tested, are plotted as horizontal black bars. (A) Addition of 0.1 ng/ml BMP4 resulted in a significant (P<0.05, paired t-test) increase in the number of neuronal colonies compared to control cultures; 10 ng/ml BMP4 was inhibitory. (B) BMP7 showed no stimulatory effect at any concentration tested, but was inhibitory at 10 ng/ml. (C,D) Neither BMP4 nor BMP7, at any concentration, showed a significant effect on the number of non-neuronal colonies that developed.

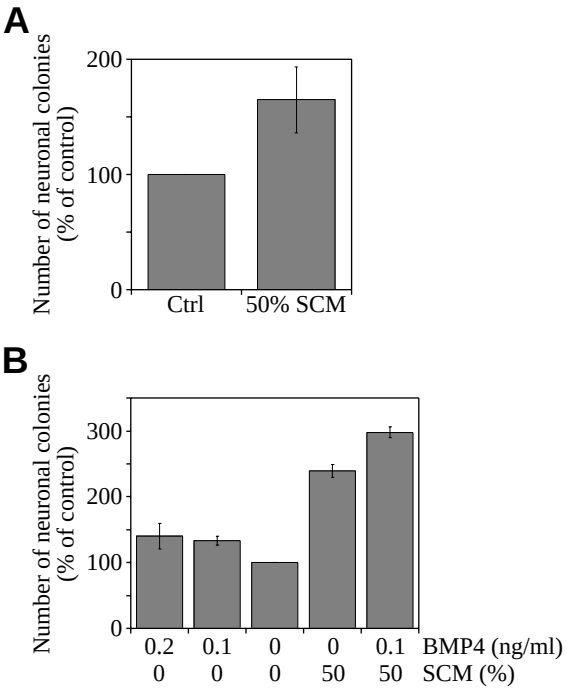


Fig. 4. Effects of SCM on OE neurogenesis. **(**A) Supplementation of growth medium to 50% SCM resulted in a large increase in the number of neuronal colonies. Error bars indicate s.e.m. for four independent experiments. SCM had no effect on the number of non-neuronal colonies (data not shown). (B) 0.1 ng/ml BMP4 acts synergistically with SCM to stimulate neurogenesis. Error bars indicate range for two independent experiments. ANOVA followed by Dunnett's test (for multiple comparisons against a single control; Glantz, 1992) indicated that 50% SCM and 50% SCM + 0.1 ng/ml BMP4 treatment groups differed significantly from control and BMP4 alone groups (P<0.05); 50% SCM + 0.1 ng/ml BMP4 yielded a significant increase in the number of neuronal colonies compared with 50% SCM alone (one-way ANOVA; P<0.05). No treatment had a significant effect on the number of non-neuronal colonies (data not shown).

Table 1. Noggin-mediated depletion of stromal cell conditioned medium

	Growth medium	Number of neuronal colonies	% Stimulation (increase over control)	Decrease in % stimulation (by) depleting SCM)
Exp. 1	Control	38.2	0	
	Mock-depleted SCM	76.3	100.0	
	Depleted SCM	64.9	70.0	30.0
Exp. 2	Control	34.6	0	
	Mock-depleted SCM	161.5	366.6	
	Depleted SCM	131.5	279.9	23.6

Stromal cell conditioned medium, depleted using either biotinylated noggin immobilized on streptavidin agarose (Depleted SCM) or streptavidin agarose alone (Mock-depleted SCM), was added to a final concentration of 50% of the total volume of growth medium in neuronal colony-forming assays.

or presence of 30 ng/ml noggin (Fig. 5). As before, SCM was found to strongly stimulate formation of neuronal colonies. In the presence of noggin, however, almost no neuronal colonies developed, even when SCM was present. These data indicate that noggin-binding factors (presumably, BMPs) are required for OE neurogenesis, and that the stimulatory effect of SCM cannot overcome this requirement.

The pro-neurogenic effect of low-dose BMP4 is not due to stimulation of neuronal progenitor cell proliferation

A simple explanation for the pro-neurogenic effect of low-dose BMP4 would be that it results from stimulation of neuronal progenitor cell proliferation. To assess this directly, we turned to explant cultures of purified OE, in which proliferation of migratory neuronal progenitor cells can be quantified easily (Calof and Chikaraishi, 1989; DeHamer et al., 1994). In addition, since no stromal cells are present in explant cultures, direct actions of exogenous agents on OE neuronal cells can be assessed (DeHamer et al., 1994). Three stages of progenitor cells in the ORN lineage can be analyzed in OE explant cultures: putative neuronal stem cells (DeHamer et al., 1994); their progeny, MASH1-expressing progenitors (Gordon et al., 1995); and immediate neuronal precursors (INPs), the cells that give rise directly to ORNs (Calof and Chikaraishi, 1989; DeHamer et al., 1994). We used three different OE explant culture paradigms to assess proliferation of each of these three progenitor cell types.

In the first set of tests, OE explants were cultured for a total

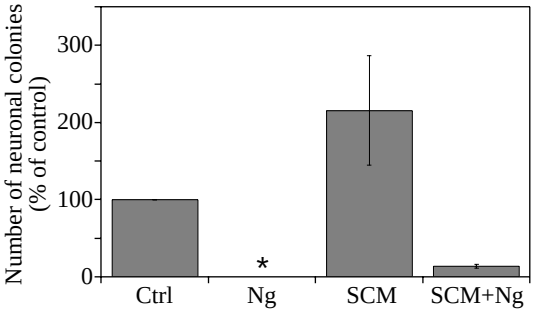


Fig. 5. Noggin-binding factor(s) are required for OE neurogenesis and this requirement cannot be overcome by SCM. Neuronal colony-forming assays were fed with noggin (Ng; 30 ng/ml), plus or minus 50% SCM. Error bars indicate range for two independent experiments. Asterisk indicates that no neuronal colonies developed in the noggin-only condition.

of 20 hours, with or without BMP4 added at concentrations spanning several orders of magnitude; for the final 6 hours of the culture period, [^{3}H]thymidine was added to all cultures in order to label progenitor cells. The percentage of migratory neuronal cells that had incorporated [^{3}H]thymidine was then determined. This assay has been used to detect inhibitory effects of high concentrations of BMPs on MASH1-expressing progenitors (Shou et al., 1999). Indeed, the results, plotted in Fig. 6A, show a large decrease in the number of [^{3}H]thymidine-positive migratory cells (neuronal progenitors) in cultures treated with 10 ng/ml BMP4. However, at no concentration tested did BMP4 treatment result in an increase in the labelling index of migratory cells, consistent with a lack of any stimulatory effect of BMP4 on progenitor cell proliferation.

INPs comprise the majority of proliferating cells in explant cultures and are the terminal progenitor cell stage in the ORN lineage (Calof and Chikaraishi, 1989). In previous studies, we used a 48-hour explant assay to identify growth factors that stimulate neurogenesis by increasing the number of rounds of division undergone by INPs (DeHamer et al., 1994). To determine if low-dose BMP4 stimulates neurogenesis by this same mechanism, we cultured OE explants in the presence or absence of BMP4 for a total of 48 hours, with [^{3}H]thymidine added for the final 24 hours to label INPs undergoing extended proliferation. As shown in Fig. 6B, 0.1 ng/ml BMP4 did not increase INP proliferation. A low dose of BMP7 (1 ng/ml) also failed to promote an increase in the number of proliferating INPs. In contrast, cultures treated with FGF2 showed a large increase in INP proliferation in this assay, as observed previously (DeHamer et al., 1994).

Studies of FGF action on OE neurogenesis have shown that FGFs stimulate neurogenesis not only by promoting INP proliferation, but also by stimulating proliferation and/or survival of rare progenitor cells, thought to be neuronal stem cells of the OE (DeHamer et al., 1994). This activity can be assessed using a 4-day OE explant assay, in which stimulation of putative stem cells is detected by the presence (in FGF-treated cultures) of a small fraction of explants that contain large numbers of proliferating neuronal progenitors (DeHamer et al., 1994). To determine if low-dose BMP4 might exert such a stimulatory effect, OE explants were cultured in the presence or absence of 0.1 ng/ml BMP4 for a total of 96 hours, with

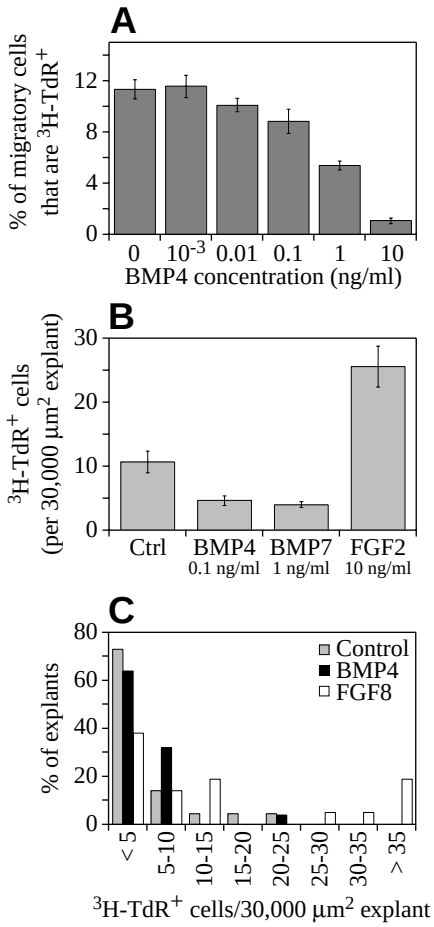


Fig. 6. BMP4 does not stimulate neuronal progenitor cell proliferation. (A) OE explants were cultured for a total of 20 hours in LCM + 5 medium, with 1.5 μCi/ml [^{3}H]thymidine added for the final 6 hours. Cultures were fixed and processed for autoradiography, and the percentage of migratory cells with ≥ 5 silver grains over their nuclei determined. Data are expressed as mean ± s.e.m. for 20 randomly chosen fields in each condition. (B) OE explants were cultured for a total of 48 hours in LCM + 1 medium, with 0.1 μCi/ml [^{3}H]thymidine added for the final 24 hours. To evaluate labelling indices, the number of [^{3}H]thymidine-positive migratory cells surrounding each explant was counted and divided by the area of the explant (measured using NIH Image 1.61). For comparison, labelling indices were normalized to an average explant area of 30,000 μm^2. Data are expressed as mean ± s.e.m. for 30 explants in each condition. (C) OE explants were cultured for a total of 96 hours in LCM + 1 medium, with growth factors (BMP4 concentration, 0.1 ng/ml; FGF8 concentration, 50 nM) replenished every 24 hours and 0.1 μCi/ml [^{3}H]thymidine added for the final 24 hrs. Explant labelling indices were evaluated as described in B, and explants binned according to their normalized labelling indices. The *y*-axis is percent of total explants, in a given condition, with labelling indices of a given magnitude. Twenty explants were analyzed for each condition.

[^{3}H]thymidine added for the final 24 hours to label proliferating neuronal progenitors. As shown in Fig. 6C, no explants exhibiting high numbers of proliferating progenitors were present in either control or BMP4-treated cultures. Cultures treated with FGF8, in contrast, showed the expected stimulatory effect (Calof et al., 1997; DeHamer et al., 1994).

Thus, low-dose BMP4 does not promote OE neurogenesis by stimulating proliferation of putative neuronal stem cells in OE cultures.

BMP4, but not BMP7, promotes survival of newly-generated ORNs

The results discussed above suggest that low-dose BMP4 achieves its pro-neurogenic effect via a mechanism other than promoting proliferation of OE neuronal progenitor cells. Even though no increase in progenitor cell proliferation was observed in 4-day explant assays, we nevertheless noted that treatment with 0.1 ng/ml BMP4 had a positive effect on the appearance of OE explants. Specifically, there were substantially more healthy-looking ORNs surrounding explants in cultures treated with 0.1 ng/ml BMP4, compared with control cultures, at 4 days in vitro. To quantify this, we cultured OE explants with or without 0.1 ng/ml BMP4 for 4 days, and then fixed and processed them for immunoreactivity for NCAM, a marker for postmitotic ORNs (Calof and Chikaraishi, 1989). As shown in Fig. 7A, very few NCAM-positive ORNs were observed in control cultures at 4 days (as expected, since cultured ORNs die within 2-3 days in the absence of appropriate trophic support; Holcomb et al., 1995). In contrast, there were large numbers of NCAM-expressing ORNs surrounding explants in cultures treated with 0.1 ng/ml BMP4. When the total number of migratory, NCAM-positive ORNs surrounding individual explants was counted for each culture condition, it was found that most explants in control cultures had very few ORNs surrounding them (Fig. 7B). However, the majority (86%) of explants treated with 0.1 ng/ml BMP4 had large numbers of NCAM-expressing ORNs surrounding them.

One likely explanation for this finding is that BMP4 promotes survival of ORNs. To test this idea, a pulse-chase approach was used to follow a cohort of newly generated ORNs in OE explant cultures. [^{3}H]thymidine was used to label S-phase INPs in explants between 16 and 20 hours in vitro. Newly generated ORNs, derived from [^{3}H]thymidine-labelled INPs, were distinguished by their expression of NCAM and the presence of silver grains over their nuclei. Survival of these ORNs was assessed every day over the course of an additional 3-day culture period, by determining the total number of migratory cells that were doubly-positive for [^{3}H]thymidine and NCAM at each timepoint in each condition. As shown in Fig. 8A, the number of [^{3}H]thymidine$^+$, NCAM$^+$ ORNs was relatively constant between 2 and 3 days in culture, but then dropped sharply after 3 days in control cultures. However, no decline in neuron number was observed in cultures treated with 0.1 ng/ml BMP4. In contrast, when BMP7 was tested at 1 ng/ml, a concentration below the threshold for its anti-neurogenic effect (Fig. 3B), a decrease in neuron number between 3 and 4 days in vitro, similar to that observed in control cultures, was observed (Fig. 8B).

The simplest explanation for these results is that low-dose BMP4 indeed has a positive effect on ORN survival. The alternative interpretation, that BMP4 accelerates neuronal differentiation of INPs (cf. Li et al., 1998), cannot explain this effect, because INPs differentiate rapidly even in the absence of exogenous factors. Typically, nearly all INPs generate NCAM$^+$ ORNs within 24 hours of [^{3}H]thymidine incorporation (Calof and Chikaraishi, 1989; DeHamer et al., 1994). Thus, in Fig. 8A, any acceleration of differentiation elicited by BMP4 could, at best, only slightly elevate the number of NCAM$^+$, [^{3}H]thymidine-labelled ORNs at 1 day after labelling; at later timepoints any such effect would necessarily disappear. As it happens, even within the time period from 10 to 24 hours after [^{3}H]thymidine incorporation by INPs, we have failed to observe any significant effect of BMP4 on NCAM acquisition and neuronal differentiation (J. Shou, P. C. Rim and A. L. Calof, unpublished observations).

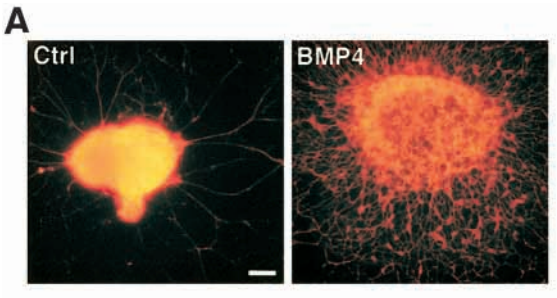
DISCUSSION

BMP4 exerts opposing, concentration-dependent actions on different cell stages in the ORN lineage

We have shown previously that BMP4 and BMP7, when applied to OE neuronal colony-forming assays at high (10-20 ng/ml) concentrations, inhibit neurogenesis (Shou et al., 1999). In the present study, it was found that noggin, a BMP antagonist that binds both BMP4 and BMP7, also inhibits neurogenesis when added to neuronal colony-forming assays (Fig. 1). Further experiments revealed that while high concentrations of BMP4 or BMP7 were anti-neurogenic, treatment with a low concentration (0.1 ng/ml) of BMP4 actually increased neurogenesis. BMP7, in contrast, did not stimulate neurogenesis at any concentration tested (Fig. 3). Taken together, these results indicate that BMPs exert concentration-dependent, ligand-specific effects on OE neurogenesis.

Evidence from several systems indicates that the nature of target tissue responses to the closely related group of BMP ligands, BMP2 and BMP4, and their *Drosophila* homolog decapentaplegic (DPP), can depend on the concentration of ligand to which tissues are exposed. For example, increasing concentrations of *dpp* transcripts drive development of progressively more dorsal structures in the *Drosophila* embryo (Ferguson and Anderson, 1992). In developing *Xenopus* embryos, exposure of ectoderm to BMP4 promotes epidermal differentiation and suppresses neural fate in this tissue, with expression of epidermal genes turning on (and neural gene expression turning off) as ligand concentration increases (Wilson et al., 1997). Similarly, experiments with chick embryos provide evidence that different concentrations of BMP4 can specify distinct subtypes of mesoderm (Tonegawa et al., 1997).

Despite providing evidence for concentration-dependent effects of BMP4 on tissue patterning, however, such studies have not resolved the question of concentration-dependent BMP4 action at the cellular level. Does the same cell have one response to BMP4 at a low concentration, and a distinct response to BMP4 at a higher concentration? Or are differences in overall tissue development the result of actions of different concentrations of BMP4 on distinct cell types, with subsequent changes in tissue patterning evolving from altered interactions among affected cells? The results of the present study suggest that, at least in the OE model system, the latter hypothesis is correct. Tests to determine the cell stage at which low-dose BMP4 exerts its positive action on neurogenesis failed to reveal a stimulatory effect on proliferation of neuronal progenitor cells (Fig. 6). Instead, the low concentration of BMP4 was



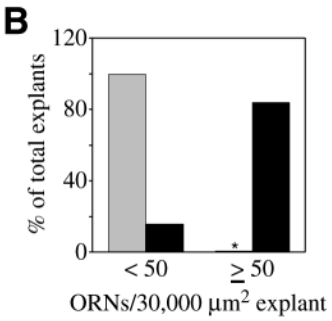


Fig. 7. Low-dose BMP4 has a direct effect on olfactory neurogenesis. OE explants were cultured for a total of 96 hours in the presence or absence of 0.1 ng/ml BMP4 in LCM + 1 medium, and cultures fixed and processed for NCAM immunostaining. The total number of NCAM-positive ORNs surrounding each explant was counted, and this number divided by the area of the explant. For comparison, this value was normalized to an average explant area of 30,000 μm^2. (A) Fluorescence photomicrographs of OE explants in the two conditions. Scale bar: 50 μm. (B) Explants were binned into two groups: those with ≥50 NCAM$^+$ ORNs and those with <50 NCAM$^+$ ORNs per 30,000 μm^2 explant. The number of explants that fell within each group was expressed as the percent of total explants counted for a given growth condition. Control, gray bar; BMP4, black bar. Asterisk indicates there were no explants with ≥50 NCAM$^+$ ORNs in control cultures.

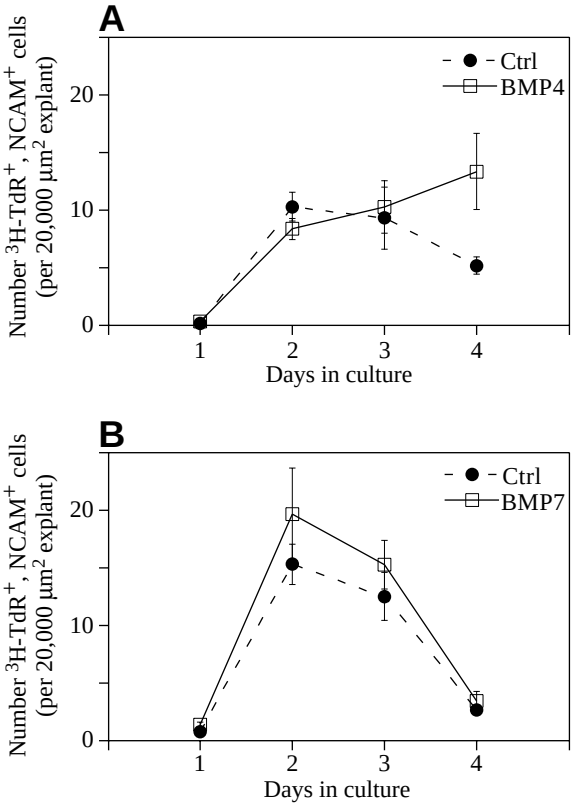


Fig. 8. Low-dose BMP4 promotes survival of newly-generated ORNs. OE explants were cultured in LCM +1 medium for a total of 96 hours, in the absence (Ctrl) or presence of BMP4 (0.1 ng/ml) or BMP7 (1 ng/ml). 16 hours after plating, cultures were pulsed with 3 μCi/ml [^{3}H]thymidine for 4 hours, then either fixed or chased with fresh medium containing 50 μM cold thymidine. At 1 day intervals, cultures were fixed and processed for NCAM immunostaining and autoradiography. To calculate survival of ORNs generated from [^{3}H]thymidine-labelled progenitors, the total number of migratory cells that were doubly positive for [^{3}H]thymidine and NCAM was counted for individual explants at each timepoint in each condition, and that number divided by the explant area. For comparison, labelling indices were normalized to an average explant area of 20,000μm^2. Data are graphed as mean±s.e.m. for 20 explants analyzed in each condition at each timepoint. (A) A significant difference in the numbers of surviving ORNs was observed between control and BMP4-treated cultures at day 4 (P=0.02, Student's t-test; Glantz, 1992). (B) BMP7 had no significant effect on ORN survival.

shown to promote survival of newly generated ORNs (Figs 7, 8); BMP7 had no positive effect on ORN survival (Fig. 8). Since BMP4, but not BMP7, stimulates neurogenesis in neuronal colony-forming assays (Fig. 3), and BMP4, but not BMP7, promotes survival of ORNs (Fig. 8), it is reasonable to propose that the effects of low-dose BMP4 on ORN survival are responsible for its stimulatory effect on neurogenesis. In contrast, the inhibitory effect of BMP4 on OE neurogenesis, evident when tissue is exposed to a higher dose of ligand (10-20 ng/ml; Fig. 3), is exerted at the level of an early neuronal progenitor cell (Shou et al., 1999). Thus, the opposing actions of different concentrations of BMP4 on neurogenesis in the OE are exerted on cells at different stages in the ORN lineage.

Mechanism of ligand-dependent action of BMPs on OE neurogenesis

What is the mechanism by which BMP4 and BMP7 exert different effects at low doses but the same effect at high doses? One possibility is that the effects of these different BMPs are the result of activation of distinct receptors in their different target cell types. Evidence from genetic studies in *Caenorhabditis elegans* and *Drosophila* supports the idea that cellular response may be dictated by the identity of the Type I BMP receptor that is activated (Krishna et al., 1999; Nguyen et al., 1998). It is known that the Type IA and IB BMP receptors are expressed in OE; interestingly, the OE is one of the few regions in the developing nervous system to express high levels of the Type IB BMP receptor (Dewulf et al., 1995; Zhang et al., 1998). Although BMP4 binds to both BMPR-IA and BMPR-IB with equal affinity, current knowledge suggests that BMP7 binds to and signals primarily through BMPR-IB (Massague, 1996; ten Dijke et al., 1994). Since both BMP4 and BMP7 are able to exert a high-dose anti-neurogenic effect, but only BMP4 is able to exert a low-dose pro-neurogenic effect, one possibility is that the anti-neurogenic effect is mediated by a receptor through which both ligands signal (BMPR-IB), whereas the pro-neurogenic effect is mediated by a receptor through which only BMP4 signals (BMPR-IA). Indeed, this model, in which distinct effects of BMPs would result from activation of distinct receptor types in target cells, is supported by evidence from studies showing that different Type I BMP receptors regulate differentiation of specific cell types during

skeletal development (Chen et al., 1998; Kawakami et al., 1996; Zou et al., 1997).

Identity of the stromal cell-conditioned medium factor(s)

Factor(s) produced by stromal cells are known to be required for the generation of ORNs in neuronal colony-forming assays in vitro. Indeed, adding an excess of stromal cells to these assays results in an increase in neurogenesis (Mumm et al., 1996). In the present study, it was found that medium conditioned over cultured stromal cells (SCM) also stimulates neurogenesis in neuronal colony-forming assays, by approximately twofold (Figs 4, 5). Three observations prompted us to test whether the stimulatory activity of SCM could be accounted for by the presence within it of noggin-binding BMPs: (1) addition of noggin to neuronal colony-forming assays completely blocks neurogenesis (Fig. 1); (2) BMP4 at low concentrations stimulates neuronal colony formation (Fig. 3); and (3) stromal cells express *Bmp4*, which binds noggin with high affinity (Fig. 2; Zimmerman et al., 1996). However, when we used noggin-coated agarose beads to deplete SCM of BMPs, only 27% of its stimulatory activity could be removed (Table 1). This finding, taken together with the observation that the stimulatory activities of low-dose BMP4 and SCM can act synergistically (Fig. 4B), suggests that there are at least two pro-neurogenic factors in SCM: a noggin-binding factor (presumably, BMP4); and a second factor(s) that acts independently of low-dose BMP4, and that either does not bind noggin, or binds noggin with a lower affinity than BMP4, and so could not be removed from SCM in our experiments. The identity of the second factor is unknown at present, but candidates include Wnts such as *Wnt8*, which is known to promote neural induction in *Xenopus* embryos (Baker et al., 1999); and relatively uncharacterized BMPs such as *Gdf7*, which is required for the development of a subset of interneurons in the developing central nervous system (Lee et al., 1998). Interestingly, GDF7 has been reported to be antagonized by noggin (Lee et al., 1998), although no information is available on the affinity with which noggin and GDF7 bind.

Role of BMPs in regulating OE neurogenesis in vivo

What are the relative concentrations of BMP2, BMP4 and BMP7 in the OE, and how might these BMPs regulate morphogenesis of this tissue and the generation (and regeneration) of neurons within it? If mRNA levels are predictive of protein levels, results from our in situ experiments indicate that the highest net concentration of BMPs in the embryonic nasal region should be in stroma subjacent to OE, where *Bmp2*, *Bmp4* and *Bmp7* are all expressed (Fig. 2B). Since high concentrations of BMP2, BMP4 and BMP7 are anti-neurogenic in vitro (Fig. 3; Shou et al., 1999), this would predict that olfactory neurogenesis in vivo should be inhibited within stroma. This raises an interesting issue, since cultured stromal fibroblasts promote, and indeed are required for, olfactory neurogenesis in vitro (see above and Mumm et al., 1996). One possibility is that, in vitro, concentrations of stroma-derived BMPs may be quite low, since neuronal progenitors are cultured on monolayers of stromal fibroblasts in a large volume of medium. Thus, in culture, neurogenesis would be permitted due to low levels of BMPs, and might in fact be promoted by the low level of BMP4 produced by the stromal cell monolayer (cf. Figs 3, 4 and Table 1). In vivo, in contrast, high levels of BMPs might act to prevent expansion of OE into BMP-rich regions of stroma, by inhibiting neurogenesis and inducing apoptosis of olfactory progenitors (Shou et al., 1999). Indeed, a similar role has been proposed for BMP-induced apoptosis in regulating regional morphogenesis in several developing tissues, such as lung (Bellusci et al., 1996), limb (Zou and Niswander, 1996; Macias et al., 1997), and, significantly, forebrain (Furuta et al., 1997) and rhombencephalic neural crest (Graham et al., 1994).

It is also possible that BMP protein levels are controlled in a manner not reflected strictly by mRNA levels. For example, binding of BMPs to extracellular matrix may be important (Reddi, 1995, 1997). In vivo, the OE is separated from underlying stroma by a basal lamina, which can be identified immunohistochemically as early as embryonic day 14 (Fig. 2C; Julliard and Hartmann, 1998). This basal lamina may serve as a diffusion barrier, preventing stroma-derived BMPs from ever reaching cells within the OE (cf. Dowd et al., 1999). Another possibility is that, in vivo, regional expression of BMP antagonists regulates the levels of BMPs to which OE neuronal progenitors are exposed. Although there is no detectable expression of *Nog* in OE or stroma (Fig. 2), expression of two other BMP antagonists, chordin and follistatin (Iemura et al., 1998; Piccolo et al., 1996), can be detected in these tissues by RT-PCR (R. C. Murray and A. L. Calof, unpublished observations). The role of chordin, if any, in OE neurogenesis is unknown at present. It is noteworthy, however, that addition of even high levels of follistatin (up to 200 ng/ml) has no effect on neurogenesis in neuronal colony-forming assays (J. Shou and A. L. Calof, unpublished observations).

The OE is one of the few neural tissues in which neurogenesis and neuronal regeneration persist in adult animals, and these processes are thought to be regulated by cell-cell interactions. Under normal conditions, proliferation of neuronal progenitors within the OE proceeds at a low basal rate. However, when ORNs are induced to die, e.g. by ablation of their synaptic target tissue the olfactory bulb, neurogenesis is induced and progenitor cells proliferate rapidly. This high rate of induced neurogenesis is maintained until new ORNs are generated and the epithelium is restored (Calof et al., 1996). Such observations have led to the hypothesis that ORNs normally produce a signal that feeds back to inhibit neurogenesis by their own progenitors (Mumm et al., 1996). When ORNs are lost, progenitors would be released from feedback inhibition and able to proliferate. The results reported here suggest a way in which BMP4 could act as the signaling molecule mediating this feedback inhibition: *Bmp4* is expressed within the neuronal layers of the OE (Fig. 2), strongly suggesting that ORNs produce BMP4. If this is the case, then when ORNs are induced to die, the overall level of BMP4 to which progenitors are exposed would decrease, since the number of BMP4-producing cells in the OE would be reduced. This decrease in BMP4 should permit an increase in progenitor cell proliferation and favor production of new ORNs. These newly generated ORNs, in turn, would begin to produce BMP4, which would serve both to support their own survival, and, as ORN numbers (and thus BMP4 levels) increase, to suppress further progenitor cell proliferation and thus prevent overgrowth of the OE. In this way, the opposing

actions of BMP4 on OE neuronal progenitors and ORNs should act in concert to maintain proper neuron number in the epithelium. This suggestion, that BMP4-mediated regulation of neurogenesis constitutes an autoregulatory pathway in vivo, is intriguing in view of the fact that in most of the mammalian nervous system, neurogenesis gradually slows during development as increasing numbers of differentiated neurons are produced by progenitors residing in germinative neuroepithelia (Caviness et al., 1995; Kauffman, 1968). Our observations in the OE system suggest that this widespread phenomenon may be explained, at least in part, by the accumulation of neuron-derived factors (such as BMP4) that have dual actions: inhibiting proliferation of neuronal progenitors, but promoting the survival of neurons themselves.

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