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# BMPs inhibit neurogenesis by a mechanism involving degradation of a transcription factor

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**Bone morphogenetic proteins (BMPs), negative regulators of neural determination in the early embryo, were found to be potent inhibitors of neurogenesis in olfactory epithelium (OE) cultures. BMPs 2, 4 or 7 decreased the number of proliferating progenitor cells and blocked production of olfactory receptor neurons (ORNs). Experiments suggested that this effect was due to an action of BMPs on an early-stage progenitor in the ORN lineage. Further analysis revealed that progenitors exposed to BMPs rapidly (< 2 h) lost MASH1, a transcription factor known to be required for the production of ORNs. This disappearance was due to proteolysis of existing MASH1 protein, but new gene expression was required to trigger it. The data suggest a novel mechanism of BMP action, whereby the induced degradation of an essential transcription factor results in premature termination of a neuronal lineage.**

Neurogenesis—the process whereby neuronal progenitor cells proliferate and differentiate into postmitotic neurons—is tightly regulated in the vertebrate[1]. Although many studies have focused on molecular signals that stimulate neurogenesis[2], signaling molecules that inhibit it are just as likely to be important in regulating neuron number. Strong evidence for regulation of neurogenesis through inhibitory signals has come from studies of the rodent olfactory epithelium. If olfactory receptor neurons of the OE are killed, neuronal progenitors proliferate and generate new ORNs[3–5], a response that tissue culture studies suggest is due to loss of an ORN-derived inhibitory signal that normally suppresses neurogenesis[6]. Similar 'feedback regulation' of neuron production has also been reported in larval *Xenopus* retina[7].

The bone morphogenetic proteins (BMPs) are good candidates for molecules that act as negative regulators of neurogenesis. Indeed, BMPs seem to function as inhibitory signals from the earliest stages of vertebrate neural development: during embryonic neural induction, endogenous BMP4 promotes acquisition of epidermal fate and suppresses neural fate in developing ectoderm[8,9]. In cultures of embryonic brain, BMPs have also been shown to inhibit proliferation[10,11] and to induce apoptosis[10,12] of progenitor cells.

A powerful general approach for exploring molecular mechanisms underlying the regulation of neurogenesis is offered by tissue culture assays of mouse OE[13]. For example, we have exploited such assays not only to demonstrate that fibroblast growth factors are positive regulators of neurogenesis, but also to identify the lineage stages at which they and other positive regulators (such as the transcription factor MASH1) exert their effects[5,6,14,15]. Because BMPs and their receptors are expressed in embryonic OE and/or olfactory placode[16–19] (J.S. & A.L.C., unpublished results), we were prompted to test BMPs for possible effects on OE neurogenesis. Our findings indicate that BMPs 2, 4 and 7 strongly inhibit OE neurogenesis, and that they exert this inhibitory action on neuronal progenitor cells at a specific stage in the ORN lineage. In addition, we report a novel mechanism of BMP action, whereby ligand binding to target cells induces proteolytic degradation of a transcription factor whose activity is known to be required for neuronal development.

## Results

To assess BMP effects on neurogenesis, we first used a neuronal colony-forming assay. When purified OE neuronal progenitor cells are cultured at clonal density on feeder layers of mitotically inactivated fibroblasts for over six days, colonies containing both undifferentiated neuronal progenitors and differentiated ORNs develop[6]. Addition of BMP4 (10 ng per ml) at the time of cell plating completely blocked development of such neuronal colonies (**Table 1**). Interestingly, BMP4 had no significant effect on any of the other (non-neuronal) colony types that develop in these assays, indicating that the observed inhibitory effect of BMP4 on neuronal colony development was not due to toxicity of the added protein. Equivalent effects were obtained with BMP2, a close homologue[20] of BMP4 (**Table 1**). We also tested BMP7, a member of the 60A subfamily of BMPs and hence more distantly related[20] to BMP4. In embryonic brain development, BMP7 has been reported to have a stimulatory, rather than an inhibitory, effect on progenitor cell proliferation[10,21]. However, in our OE neuronal colony-forming assays, BMP7 was equivalent to BMPs 2 and 4 in its inhibitory effects (**Table 1**).

The ORN lineage is complex, with at least two stages of progenitor cells interposed between neuronal stem cells and postmitotic ORNs. The neuronal colony-forming cell is hypothesized to be the neuronal stem cell[6,22]. When cultured on feeder cell layers, this cell can continue to produce downstream progenitors and ORNs for up to two weeks[23,24]. However, in OE explant cul-

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tures grown in defined medium, neuronal stem cells initially give rise to downstream progenitor cells but subsequently become undetectable. Stem cells may die or simply cease dividing, as factors such as fibroblast growth factors are known to be necessary for their survival and/or proliferation[14]. The progeny of the stem cell is thought to be the MASH1$^+$ neuronal progenitor, which undergoes one to two rounds of division to give rise to MASH1-negative immediate neuronal precursors[5] (INPs). INPs divide one to two times *in vitro* (cell cycle length, ~17 h), quantitatively giving rise to post-mitotic ORNs[14,25].

Because development of cells at each stage in the ORN developmental pathway is regulated[5,6,14,15,26], it was important to know at which stage(s) BMPs exert their inhibitory effect. To address this, we used neuronal colony-forming assays with BMP4 added at different times following cell plating, grew the cultures a total of six days and then counted neuronal colonies. When BMP4 was added early (0–2 days after plating), neuronal colonies were greatly decreased in number (**Fig. 1a**). However, addition of BMP4 at three days or later after initiation of the cultures produced no significant effect. Because early addition of BMP4 was required for inhibition of neuronal colony formation, we asked how rapidly BMP4 acts. To accomplish this, we added BMP4 for the first 24 hours *in vitro*; then cultures were washed and allowed to grow for the remaining five days of the assay. Exposure of cultures to BMP4 for only 24 hours following plating was sufficient to dramatically inhibit neuronal colony development (**Fig. 1b**). Thus, the effect of BMP4 on neurogenesis is very rapid. Moreover, its effect is likely to be exerted on neuronal progenitors rather than post-mitotic ORNs, because ORNs are detectable in colonies only after about four days *in vitro*, when progenitor cells have had sufficient time to undergo several divisions and generate neurons[6]. Potential targets of BMP4 action thus included the neuronal colony-forming cell or the cell type thought to be its progeny, the MASH1-expressing progenitor, or the progeny of the MASH1$^+$ progenitor, the immediate neuronal precursor of ORNs.

If BMPs act on neuronal progenitor cells, we reasoned that the effects of BMPs might involve changes in progenitor cell proliferation. To address this we used explant cultures of OE, in which the proliferation kinetics of cells are easily quantified[14,25]. OE explants were cultured for 20 hours in BMP4, with [$^3$H]thymidine added for the last 6 hours of culture to detect cells in S phase. Addition of BMP4 dramatically decreased the number of neuronal cells incorporating [$^3$H]thymidine (**Fig. 2a**), indicating that BMP4 inhibits proliferation of neuronal progenitor cells.

Because late addition of BMP4 to colony-forming assays had no effect on neuronal colony numbers in our experiments, it seemed unlikely that BMPs would have adverse effects on the survival of ORNs. To test this directly, we cultured OE explants for 48 hours, with BMP4 and [$^3$H]thymidine added for the final 24 hours. We focused our analysis on ORNs that had been generated in the first 24 hours of the culture period; these could be distinguished by expression of NCAM (a marker for ORNs[25]) and lack of [$^3$H]thymidine labeling, indicating that they were already post-mitotic at the time of BMP4 addition. This was important, because effects of BMP4 on ORNs generated after the first 24 hours of culture could be secondary to its effects on progenitor cell proliferation (**Fig. 2a**). The total number of [$^3$H]thymidine-negative ORNs per OE explant was virtually identical in cultures grown in the presence or absence of BMP4 (**Fig. 2b** and **c**), indicating that BMP4 had no effect on neuronal survival.

Apparently, BMPs inhibit olfactory neurogenesis not by impairing ORN survival, but by decreasing numbers of proliferating neuronal progenitor cells. Because our previous studies showed that expression of the transcription factor MASH1 marks an early stage of neuronal progenitor in the ORN lineage[5], and studies of a targeted mutation in the *Mash1* gene showed that MASH1 is essential for both survival of neuronal progenitors and development of ORNs *in vivo*[22,26,27], we sought to determine if MASH1-expressing progenitors are affected by BMPs. We cultured OE explants for eight hours (when the number of MASH1-expressing cells in explant cultures is high[5]) in the presence or absence of BMP4. When cultures were stained with an antibody to MASH1[28], MASH1 immunoreactivity was virtually abolished by BMP4: 6.5 ± 1.3% (s.e.) of migratory cells exhibited MASH1

**Table 1. Inhibition of neuronal colony formation by BMPs.**

| | Number of neuronal colonies (% control value) | Number of non-neuronal colonies (% control value) |
|---|---|---|
| BMP4 (*n* = 4) | 0 | 77.6 ± 25.7 (s.d.) |
| BMP2 (*n* = 2) | 0 | 67.6 ± 6.4 (s.d.) |
| BMP7 (*n* = 2) | 0 | 96.6 ± 20.1 (s.d.) |

Neuronal colony-forming assays were done with progenitor cells plated in the absence (control) or presence of BMP4 (10 ng per ml), BMP2 (10 ng per ml) or BMP7 (10 ng per ml) for the entire culture period. Neuronal and non-neuronal colonies were counted in each condition, for a minimum of two independent experiments (*n*). Numbers of colonies were expressed as a percentage of the control value for the same experiment, and percentages averaged for all experiments in a given condition (s.d., standard deviation). No neuronal colonies were observed when BMPs were added. Actual numbers of colonies under control conditions: BMP4 experiments, neuronal, 26.3 ± 19.9 (s.d.); non-neuronal, 71.8 ± 33.3 (s.d.). BMP2 experiments, neuronal, 15.5 ± 2.1 (s.d.); non-neuronal, 48.5 ± 7.8 (s.d.); BMP7 experiments: neuronal, 57 ± 32.5 (s.d.); non-neuronal, 177 ± 50.9 (s.d.).

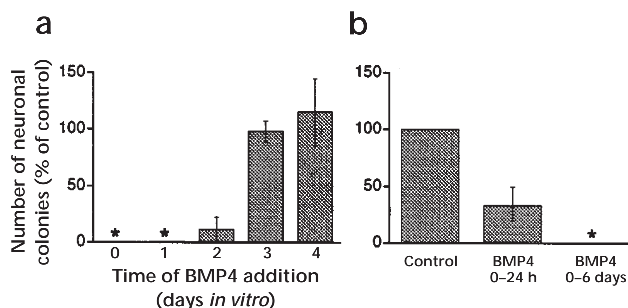


**Fig. 1.** Inhibition of neuronal colony formation requires early addition of BMP4. **(a)** Neuronal colony-forming assays were done as described for Table 1, except that BMP 4 (10 ng per ml) was added to half the plates at 0, 1, 2, 3 or 4 days after progenitor cells were plated, and was present for the rest of the culture period. After six days *in vitro*, neuronal colonies were counted and that value expressed as a percentage of the control value for the same experiment. Data are plotted as mean ± range for 2–3 independent experiments in each test condition. Asterisks indicate that no neuronal colonies ever developed under these conditions. **(b)** Neuronal colony-forming assays were done in the presence or absence of BMP4 (10 ng per ml). After 24 h, plates were rinsed in calcium- and magnesium-free Hank's balanced salt solution and re-fed with growth medium; BMP4 was not replenished in half of the plates to which it had originally been added (BMP4 0–24 h). After six days *in vitro*, neuronal colonies were counted and the data expressed as described above. Error bars indicate the range of values obtained in three independent experiments; asterisk indicates that no neuronal colonies ever developed in the BMP4 0–6 days condition.

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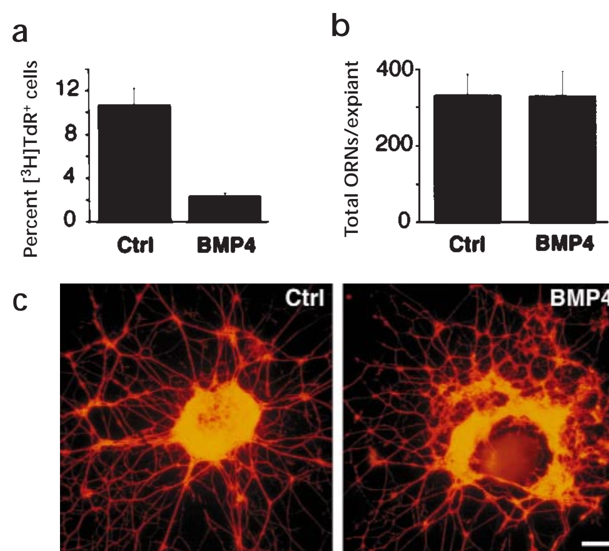


**Fig. 2.** Effects of BMP4 on OE neuronal progenitor cells and ORNs. **(a)** OE explants were cultured for a total of 20 h in the presence or absence of BMP4 (10 ng per ml), with 1.5 µCi per ml [$^3$H]thymidine added for the final 6 h. The percentage of cells that were [$^3$H]thymidine-positive ([$^3$H]TdR$^+$) was determined as the fraction of total migratory cells surrounding each explant that had > 5 silver grains over the nucleus. Approximately 5,000 migratory cells were counted in each condition. Data are plotted as mean ± s.e. **(b)** OE explants were cultured for a total of 48 h, with BMP4 (10 ng per ml) added to half the cultures and [$^3$H]thymidine (0.1 µCi per ml) added to all the cultures for the last 24 h. Total cell number, the number of cells expressing neural cell adhesion molecule (NCAM, a marker for post-mitotic ORNs[14,25]), the number of [$^3$H]thymidine-positive cells, and the number of cells that were positive for both NCAM and [$^3$H]thymidine were counted for each explant in both conditions; the area of the explant body was also measured using NIH Image 1.61. Cells that were NCAM-positive but [$^3$H]thymidine-negative were considered to be ORNs that had been generated during or before the first 24 h of the culture period, before BMP4 addition. The number of these ORNs was counted for each explant in each condition; for comparison, these values were normalized to an average explant area of 25,000 µm$^2$ (approximate mean explant area). Approximately 4,000 migratory cells (> 95% of which were NCAM-positive ORNs) were counted in each condition. Data are plotted as the mean of values obtained from two independent experiments; error bars indicate root mean square of the standard errors **(c)** Fluorescence photomicrographs of OE explants from the experiments described in **(b)**. Red color indicates NCAM immunostaining. Scale, 50 µm.

immunoreactivity in control cultures, whereas only 0.41 ± 0.19% (s.e.) of migratory cells did so in BMP4-treated cultures. To test whether BMP4 causes MASH1$^+$ cells to die[10,12], we cultured OE explants for eight hours with or without BMP4 (20 ng per ml) and processed them for MASH1 immunocytochemistry and DNA fragmentation *in situ* (TUNEL assay[15]) to detect apoptotic cells. Death of MASH1-expressing cells was an unlikely explanation for decreased MASH1 immunoreactivity in BMP4-treated cultures, as very little apoptotic cell death occurred with or without BMP4: 1.97 ± 0.91% (s.e.) of migratory cells were TUNEL-positive in control cultures, and 1.92 ± 0.22% (s.e.) were TUNEL-positive in BMP4-treated cultures. Furthermore, no MASH1$^+$ cells were TUNEL-positive in either condition.

Further investigation showed that the effect of BMPs on MASH1 expression is very rapid. Exposing explant cultures to BMP4 for the last two hours of an eight-hour culture period was sufficient to dramatically decrease MASH1 immunoreactivity in progenitor cells (**Fig. 3a**). (BMP2 and BMP7 also abolished MASH1 immunoreactivity within two hours; data not shown.) We confirmed that effects on MASH1 expression were due specifically to the action of BMP4 (as opposed to, for instance, a contaminant in the recombinant protein preparation) by showing that the effect could be blocked by the specific BMP antagonist, noggin[29](**Fig. 3a**). We also cultured explants for six hours and then exposed them to BMP4 for varying periods. The number of MASH1-immunoreactive cells was decreased by 50% after 60 minutes of BMP4 treatment, and the decrease was maximal by 2 hours (**Fig. 3b**). Photomicrographs of cultures treated for two hours with BMP4 and then immunostained to detect MASH1 are shown in Fig. 4a.

The rapidity of BMP4's effect on MASH1 suggested that BMPs act directly on MASH1-expressing progenitor cells to decrease the levels of MASH1 protein within these cells. We did two types of control experiments to determine if MASH1 protein levels were decreased in cultures treated for two hours with BMP4. The first was to test whether the apparent loss of MASH1 immunoreactivity might be an artifact of its redistribution within the cell. MASH1 protein is normally concentrated in the nucleus; if BMPs caused MASH1 translocation into the cytoplasm, dilution of the protein might lower the immunofluorescent signal below the limits of detection by eye. Control and BMP4-treated cultures were therefore processed for MASH1 immunostaining in the normal manner and imaged using a digital cooled CCD camera. Fluorescence over the entire cell body (not just the nucleus) was measured for individual, randomly chosen, migratory neuronal progenitor cells, and cells were binned according to fluorescence intensity. The resulting frequency histogram (**Fig. 4b**) shows a small subpopulation of cells with very high fluorescence intensities (> 45 units per cell) in control cultures; these cells were also judged by eye to be MASH1-immunoreactive. In BMP-treated cultures, however,

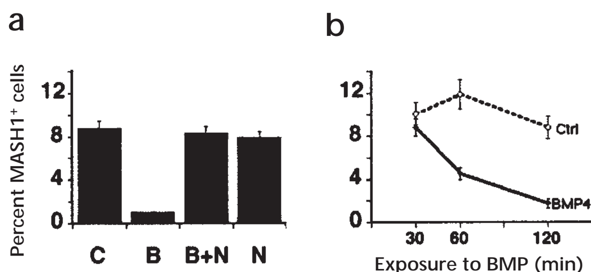


**Fig. 3.** BMP4 causes a rapid decrease in MASH1 immunoreactivity in OE neuronal progenitors. **(a)** OE explants were cultured for six hours, then exposed to vehicle (C; culture medium), BMP4 (B, 20 ng per ml), noggin (N, 150 ng per ml, a fivefold molar excess) or BMP4 plus noggin (B + N, 20 ng per ml and 150 ng per ml, respectively) for an additional 2 h (total, 8 h in culture). BMP4, noggin, and the mixture were held as 10× stocks on ice for 1 h before addition. The total number of migratory cells and the number of these that were MASH1-positive were counted for each explant, and the percentage of MASH1-positive migratory cells was expressed on a per-explant basis. Data are plotted as mean ± s.e. of values obtained from a minimum of 20 explants (~3,000 migratory cells) in each condition. **(b)** OE explants were cultured for 6 h, then exposed to BMP4 (10 ng per ml) for 30, 60 or 120 min; control cultures in which vehicle alone (culture medium) was added were also taken at each time point. Data are plotted as described in **(a)**.

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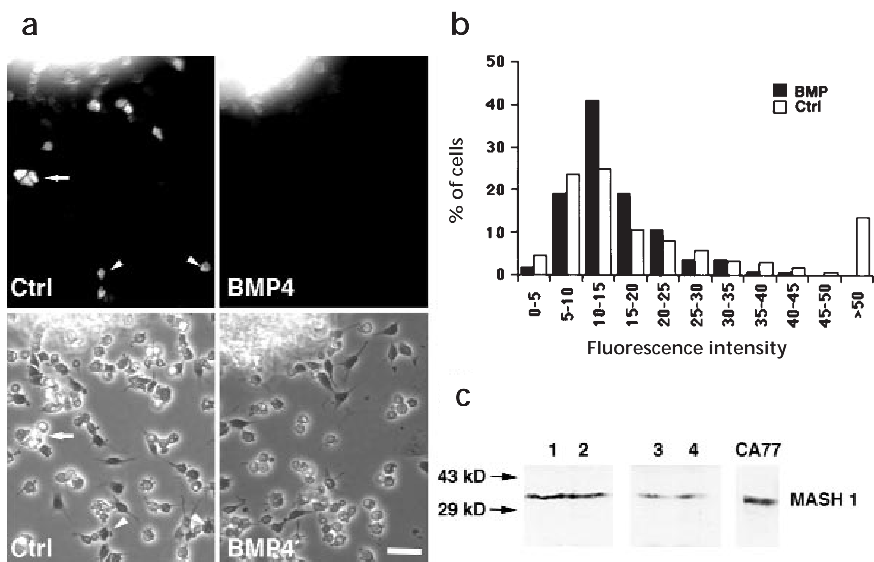


**Fig. 4.** BMP-mediated decrease in MASH1 immunoreactivity is due to loss of MASH1 protein. **(a)** Fluorescence and phase-contrast photomicrographs of explant cultures grown for a total of eight hours *in vitro*, with or without BMP4 (20 ng per ml) added for the final two hours. In control conditions (Ctrl), arrow indicates a cluster of migratory neuronal progenitor cells expressing MASH1; arrowheads indicate examples of individual MASH1-positive cells. In BMP4 (BMP4), no cells have detectable MASH1 immunofluorescence. Scale, 20 µm. **(b)** OE explants were grown for a total of eight hours, with half the cultures exposed to BMP4 (10 ng per ml) for the final two hours, and processed for MASH1 immunoreactivity. Fluorescence intensities of 235 randomly chosen, migratory neuronal progenitor cells in each condition were determined. Cells were binned according to their total fluorescence level (expressed in integer units; *x*-axis), and the number of cells that fell within each bin was graphed as the percentage of total cells imaged for a given growth condition (*y*-axis). **(c)** OE suspension cultures were prepared as described[15] and cultured for a total of eight hours, with or without BMP4 (20 ng per ml) for the final two hours. Sample preparation and immunoblotting procedures are described in Methods. Lanes 1–4 were each loaded with 120 µg of total cellular protein. MASH1 was detected as a doublet of approximately 33 kD. Densitometric analysis of band intensity using NIH Image 1.61 revealed a substantial reduction (80%) in the amount of MASH1-immunoreactive protein present in cultures treated with BMP4 (lanes 3 and 4) versus controls (lanes 1 and 2). 70 µg of total cellular protein from CA77 cells (a rat thyroid C cell line expressing high levels of MASH1[48]) was used as a positive control for MASH1 detection.

this population was absent, indicating that MASH1-immunoreactive protein is indeed lost, and not simply redistributed within cells. We also assessed levels of MASH1 expression by immunoblotting. Cultures were lysed, lysates were electrophoresed on SDS-polyacrylamide gels and proteins were transferred electrophoretically to nitrocellulose membrane and probed with an anti-MASH1 monoclonal antibody[28]. The results (**Fig. 4c**) indicate that addition of BMP4 to OE cultures for two hours caused an 80% reduction in the amount of MASH1 protein.

These findings, taken together with the rapidity of the BMP effect (**Fig. 3b**), suggested that the mechanism of action of BMPs might be at the level of MASH1 protein degradation. Indeed, cycloheximide-block experiments supported this idea by showing that the half-life of MASH1 protein is sufficiently long (> 4 hours) in these cells that a complete cessation of *Mash1* transcription or translation could not produce the rapid loss of MASH1 protein observed after treatment with BMPs. For these experiments, cultures were grown for six hours with cycloheximide (5 µg per ml) added to half the cultures for an additional two, four or six hours. On counting the percentage of migratory cells in each condition that were MASH1-positive, we found that, after 4 hours in cycloheximide, the number of MASH1-positive cells decreased by only 20% (control, 6.40 ± 0.67%, mean ± s.e.; cycloheximide, 5.16 ± 0.53%, mean ± s.e.

Because ligand-stimulated targeting of proteins for proteasome-mediated proteolytic degradation is an important means of regulating many cellular processes[30], we hypothesized that the proteasome pathway might be involved in the BMP-mediated loss of MASH1 in OE neuronal progenitors. To test this, we assayed pharmacological inhibitors of the proteasome pathway for their ability to abolish BMP-induced MASH1 degradation. Lactacystin or MG132, both highly selective inhibitors that react with active sites of the proteasome and block proteolytic cleavage[31,32], were used. Both inhibitors completely blocked degradation of MASH1 following two-hour incubation of cultures in BMP2 (**Fig. 5a**). Thus, these data indicate that BMPs bind to OE neuronal progenitor cells and stimulate rapid degradation of MASH1 protein via the proteasome pathway.

It is generally thought, however, that BMPs exert their effects on target cells at a transcriptional level, because BMP receptor activation results in phosphorylation of cytoplasmic effector proteins called Smads that then translocate to the nucleus and initiate new gene expression[33]. To test whether the BMP effect on MASH1

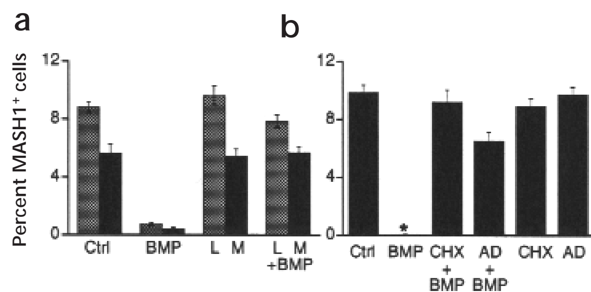


**Fig. 5.** MASH1 degradation is via proteasome-mediated proteolysis; downregulation of MASH1 immunoreactivity by BMP4 is dependent on transcription and translation. **(a)** OE explants were cultured for a total of eight hours with BMP2 (BMP, 20 ng per ml) added to half the cultures for the final two hours. thirty minutes before BMP addition, lactacystin (L, 10 µM[49]), MG132 (M, 10 µM[49]) or control vehicle (Ctrl, 0.1% DMSO in culture medium) was added. Data were evaluated and plotted as described in Fig. 3a. **(b)** OE explants were grown for six hours in culture, then exposed to control vehicle (Ctrl, 0.05% ethanol in culture medium), cycloheximide (CHX, 5 µg per ml) or actinomycin D (AD, 5 µg per ml) for 30 minutes. BMP4 (BMP, 20 ng per ml) was then added to half the cultures in each condition for an additional 2 h for a total of 8.5 h *in vitro*. Data were evaluated and plotted as described in Fig. 3a.

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degradation required the induction of new gene expression, we used pharmacological agents to inhibit RNA and protein synthesis. Either cycloheximide or actinomycin D (which by themselves had no effect on MASH1 expression) abolished loss of MASH1 resulting from two-hour exposure to BMP4 (**Fig. 5b**). Thus, although a major consequence of BMP binding to OE neuronal progenitor cells is rapid proteolytic degradation of MASH1, new gene expression is required to trigger this event.

## Discussion

These results indicate that BMPs 2, 4 and 7 have a potent anti-neurogenic effect on OE cultures: BMPs markedly decrease the number of proliferating neuronal progenitor cells by acting on progenitors at an early stage in the ORN lineage. A transcription-dependent yet very rapid consequence of BMP action is the proteolytic degradation of the essential transcription factor MASH1 in early-stage progenitors.

These *in vitro* consequences of BMP action parallel the effects of loss of MASH1 on the OE *in vivo*[27] strikingly well. In mice homozygous for a targeted disruption of the *Mash1* gene, extremely few ORNs arise. Cells that express transcripts from the (disrupted) *Mash1* gene are found in the OE, but the OE is abnormally thin and exhibits an abnormally high rate of apoptosis[22,26]. No increase in size of any non-neuronal cell population is apparent in the OE[26,27], suggesting that the neuronal lineage is simply terminated by cell death at or after the *Mash1*-expressing stage. Consistent with this view, inhibition of apoptosis can rescue neurogenesis in *Mash1*$^{-/-}$ OE cells to some extent[22].

In a similar manner, when OE cultures are treated with BMPs, the ORN lineage also apparently terminates. Development of neuronal colonies is completely inhibited, but there is no increase in the numbers of any non-neuronal colony type (**Table 1**), suggesting that neuronal progenitor cells are not driven into a non-neuronal fate. As in *Mash1*$^{-/-}$ animals, there is a marked decrease in the overall number of proliferating progenitor cells (**Fig. 2a**). Even the cell death found in the OE of *Mash1*$^{-/-}$ animals is phenocopied by BMP treatment of OE cultures: although short-term (8 h) exposure to BMPs does not elicit cell death (see Results), 20-h BMP4 exposure results in a substantial (57%) increase in the number of apoptotic migratory neuronal cells in OE explant cultures (data not shown).

These similarities, taken together with the observation that BMPs cause rapid disappearance of MASH1 from OE cultures (**Fig. 3b**), suggest that the destruction of MASH1 could account for the anti-neurogenic effect of BMPs. Proof of this hypothesis would involve demonstrating that blockade of MASH1 proteolysis renders OE cell resistant to inhibition of neurogenesis by BMPs. Unfortunately, the only currently available tools for blocking MASH1 proteolysis are general proteasome inhibitors, and proteosomes also control key elements in cell cycle progression, such as cyclin levels[30]. Not surprisingly, we find that the lowest concentrations of MG132 and lactacystin that block BMP-induced MASH1 proteolysis also directly arrest neuronal progenitor cell division in OE cultures, making it impossible to test for a rescue of neurogenesis (J.S. & A.L.C., data not shown). Testing whether MASH1 degradation has a causal role in the antineurogenic effect of BMPs will most likely require other approaches, such as the identification of mutant forms of MASH1 that fail to become targeted for degradation by BMPs, and the introduction of such mutant protein into OE progenitor cells.

Indeed, the question of how MASH1 becomes targeted for proteolysis by BMPs is an intriguing one. It is widely accepted that BMP signaling involves the receptor-mediated phosphorylation of Smad proteins, which translocate to the nucleus and trigger specific gene expression[33]. Both cycloheximide and actinomycin D abolished the effects of BMP4 on MASH1 expression by OE neuronal progenitor cells (**Fig. 5b**), strongly suggesting that new gene expression is required to trigger MASH1 proteolysis. Because degradation of MASH1 is rapid (50% complete one hour after BMP addition; **Fig. 3b**), it is reasonable to speculate that a gene directly activated by BMP signaling may be the primary effector of MASH1 proteolysis (for instance, a gene that encodes an enzyme that post-translationally modifies MASH1, marking it for destruction). Differences among various cell types in the genes that are downstream targets of BMP signaling most likely explain why BMPs can trigger degradation of MASH1 protein in olfactory neuronal progenitor cells but can actually maintain *Mash1* expression in some neural crest cells[34].

However, little is known about the genes directly activated by Smads, especially in neuronal progenitor cells, in which most of the known effects of BMPs (such as induction of apoptosis and/or changes in cell fate) occur many hours or even days following BMP exposure[10,12,34–37]. In several cases, it has been suggested that BMPs exert their effects through the induction of transcriptional activators, such as the homeobox-containing genes *Msx-1* and *Msx-2*, which then elicit further changes in gene expression and alter cell fate[10,12,38]. Our study indicates, however, that BMP signaling can lead relatively directly to the loss of transcriptional activators, and that this may be an important means of effecting rapid changes in cell function. In this light, it is interesting that a recent study has shown a role for the post-translational inactivation (although not destruction) of the helix-loop-helix transcription factor HES-1 in mediating the actions of nerve growth factor in neuronal differentiation[39].

The present study provides the first evidence, for any cell type, that BMP signaling may be mediated via targeting of molecules to the proteasome pathway. Recently, however, TGF-β signaling was shown to modulate proteasome-mediated proteolysis of RhoB[40]. Furthermore, it is well established that cytokines trigger proteasome-mediated proteolysis of cytoplasmic I-κB, which then releases NFκB to enter the nucleus and activate gene expression[41], a process essential for development[42,43]. Proteolytic degradation via the proteasome pathway appears to be a major mechanism by which β-catenin levels are regulated in the Wnt signaling pathway as well[44].

One question not resolved by our work is whether BMPs are solely or partly responsible for the feedback inhibition of neurogenesis in the OE that we have described *in vitro* and that is presumed to occur *in vivo*[6]. Current evidence suggests that this inhibition is mediated by a heat-labile macromolecule(s)[23], but whether it is a BMP that has been detected in the OE (for instance, see J.S., P.C.R. & A.L.C. *Soc. Neurosci. Abstr.* **23**, 120.7, 1997), a novel BMP or an unrelated molecule remains to be determined.

A second question is whether the mechanism underlying anti-neurogenic action of BMPs in the OE can be generalized to other parts of the nervous system in which BMPs have been reported to inhibit cell proliferation[10,11] and to stimulate apoptosis[10,12]. Because MASH1 is expressed in several regions of the developing CNS, it will be interesting to determine whether BMPs cause MASH1 degradation in neural tissues other than the OE. Unlike the OE, however, most of the CNS does not absolutely require MASH1 for neuronal development[27], suggesting that MASH1 functions may be redundantly carried out by other, related transcriptional activators[45]. It will therefore be interesting to learn whether BMPs exert effects on the levels of these other factors as well.



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## Methods

Purification of OE neuronal progenitor cells from E14.5–15.5 Rosa26 transgenic mice (Jackson Labs) and culture methods for neuronal colony-forming assays were as described[6] with two modifications: OE stromal cell feeder layers (derived from outbred CD-1 embryos) were mitotically inactivated by γ-irradiation (3300 rad), and calcium concentration in the culture medium was increased to 1.4 mM. Except where noted, cultures were re-fed with fresh medium (with BMPs or other agents, if appropriate) every other day, then fixed and processed for X-gal staining after a total of six days. Quantitative analysis of neuronal and non-neuronal colonies was as described[6]. OE explant cultures using tissue purified from E14.5–15.5 CD-1 mice (Charles River) were generated as described[5], and grown on polylysine/merosin-coated coverslips[14] in defined, serum-free low-calcium culture medium containing 5 mg per ml crystalline bovine serum albumin[15] (ICN).

Explant cultures were fixed with 10% formalin in phosphate-buffered saline containing 5% sucrose for 15–30 minutes. Anti-MASH1 immunocytochemistry[5] and anti-NCAM immunocytochemistry[14] were done as described. For cultures incubated in [$^3$H]thymidine, coverslips were dehydrated and dipped in NTB2 emulsion (Kodak) diluted 1:1 in water, then exposed for 48 h (for proliferation assays) or 7 days (for ORN survival assays) at –80°C, developed in D-19 developer, and nuclei stained with Hoechst 33258 (bisbenzimide; 1 μg per ml). TUNEL staining to detect apoptotic cells was done as described[15].

For quantitative analysis of immunofluorescence, cultures were processed for MASH1 immunoreactivity and individual cells were imaged under rhodamine optics. Images for each cell (over the entire cell) were acquired as raw data files using a cooled CCD digital camera (Diagnostic Instruments SP100, 1315 × 1035 pixel resolution). Raw data files were imported to Adobe Photoshop 4.0 for image analysis. Fluorescence intensities of individual cells were calculated as the sum of the pixel intensities for each cell (with background pixel intensities subtracted).

For immunoblots, OE suspension cultures were collected by centrifugation (2000 × g, 1 min), lysed in SDS gel loading buffer[46], boiled for 10 min and centrifuged to remove debris; then urea was added to 8 M. Proteins were separated on 14% SDS-PAGE and transferred to nitrocellulose membrane using standard procedures[46]. The membrane was incubated in undiluted A42B7 anti-MASH1 hybridoma supernatant[28] overnight at 4°C, washed in phosphate-buffered saline, briefly blocked with 1% blocking reagent (Boehringer-Mannheim) and incubated with affinity purified rabbit anti-mouse IgG1 (1:1,000 dilution, Dako) for 30 min at room temperature. After washing, the blot was incubated in horseradish peroxidase-conjugated anti-rabbit antibody (1:5,000, Amersham) for one hour at room temperature. Following extensive washes in buffer (0.1 M Tris, pH 7.5), the membrane was dipped in ECL™ chemiluminescence substrate and exposed to Hyperfilm™ (Amersham) for 20 min at room temperature. The blot was then stripped in 62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol for 1 h at 55 °C, rinsed, and re-probed with a rabbit antiserum to β-tubulin[47] to ensure that an equal amount of cellular protein had been loaded on each lane (data not shown).

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1. Caviness, V. S., Takahashi, T. & Nowakowski, R. S. Numbers, time and neocortical neuronogenesis: a general developmental and evolutionary model. *Trends Neurosci.* **18**, 379–383 (1995).
2. Temple, S. & Qian, X. bFGF, neurotrophins, and the control of cortical neurogenesis. *Neuron* **15**, 249–252 (1995).
3. Schwartz-Levey, S., Chikaraishi, D. M. & Kauer, J. S. Characterization of potential precursor populations in the mouse olfactory epithelium using immunocytochemistry and autoradiography. *J. Neurosci.* **11**, 3556–3564 (1991).
4. Schwob, J. E., Szumowski, K. E. M. & Stasky, A. A. Olfactory sensory neurons are trophically dependent on the olfactory bulb for their prolonged survival. *J. Neurosci.* **12**, 3896–3919 (1992).
5. Gordon, M. K., Mumm, J. S., Davis, R. A., Holcomb, J. D. & Calof, A. L. Dynamics of MASH1 expression *in vitro* and *in vivo* suggest a non-stem cell site of MASH1 action in the olfactory receptor neuron lineage. *Mol. Cell. Neurosci.* **6**, 363–379 (1995).
6. Mumm, J. S., Shou, J. & Calof, A. L. Colony-forming progenitors from mouse olfactory epithelium: Evidence for feedback regulation of neuron production. *Proc. Natl. Acad. Sci. USA* **93**, 11167–11172 (1996).
7. Reh, T. A. & Tully, T. Regulation of tyrosine hydroxylase-containing amacrine cell number in larval frog retina. *Dev. Biol.* **114**, 463–469 (1986).
8. Hawley, S. H. B. *et al.* Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction. *Genes Dev.* **9**, 2923-2935 (1995).
9. Wilson, P. A. & Hemmati-Brivanlou, A. Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376**, 331–336 (1995).
10. Furuta, Y., Piston, D. W. & Hogan, B. L. Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* **124**, 2203-2212 (1997).
11. Li, W., Cogswell, C. A. & LoTurco, J. J. Neuronal differentiation of precursors in the neocortical ventricular zone is triggered by BMP. *J. Neurosci.* **18**, 8853–8862 (1998).
12. Graham, A., Francis-West, P., Brickell, P. & Lumsden, A. The signaling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. *Nature* **372**, 684–686 (1994).
13. Calof, A. L., Mumm, J. S., Rim, P. C. & Shou, J. in *The Neuron in Tissue Culture* (ed. Haynes, L.) 23–44 (Wiley, Chichester, 1999).
14. DeHamer, M., Guevara, J., Hannon, K., Olwin, B. & Calof, A. L. Genesis of olfactory receptor neurons: Regulation of progenitor cell divisions by fibroblast growth factors. *Neuron* **13**, 1083–1097 (1994).
15. Holcomb, J. D., Mumm, J. S. & Calof, A. L. Apoptosis in the neuronal lineage of the mammalian olfactory epithelium: regulation *in vivo* and *in vitro. Dev. Biol.* **172**, 307–323 (1995).
16. Dewulf, N. *et al.* Distinct spatial and temporal expression patterns of two type I receptors for bone morphogenetic proteins during mouse embryogenesis. *Endocrinology* **136**, 2652–2663 (1995).
17. Helder, M. N. *et al.* Expression pattern of Osteogenic Protein-1 (Bone Morphogenetic Protein-7) in human and mouse development. *J. Histochem. Cytochem.* **43**, 1035–1044 (1995).
18. Zhang, D., Mehler, M. F., Song, Q. & Kessler, J. A. Development of bone morphogenetic protein receptors in the nervous system and possible roles in regulating trkC expression. *J. Neurosci.* **18**, 3314–3326 (1998).
19. Wu, D. K. & Oh, S.-H. Sensory organ generation in the chick inner ear. *J. Neurosci.* **16**, 6454–6462 (1996).
20. Kingsley, D. M. The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.* **8**, 133–146 (1994).
21. Arkell, R. & Beddington, R. S. P. BMP-7 influences pattern and growth of the developing hindbrain of mouse embryos. *Development* **124**, 1–12 (1997).
22. Calof, A. L., Mumm, J. S., Rim, P. C. & Shou, J. The neuronal stem cell of the olfactory epithelium. *J. Neurobiol.* **36**, 190–205 (1998).
23. Calof, A. L. *et al.* Factors regulating neurogenesis and programmed cell death in mouse olfactory epithelium. *Ann. NY Acad. Sci.* **855**, 226–229 (1998).
24. Pixley, S. K. CNS glial cells support *in vitro* survival, division, and differentiation of dissociated olfactory neuronal progenitor cells. *Neuron* **8**, 1191–1204 (1992).
25. Calof, A. L. & Chikaraishi, D. M. Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor *in vitro. Neuron* **3**, 115–127 (1989).
26. Cau, E., Gradwohl, G., Fode, C. & Guillemot, F. Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* **124**, 1611–1621 (1997).
27. Guillemot, F. *et al.* Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* **75**, 463–476 (1993).
28. Lo, L. C., Johnson, J. E., Wuenschell, C. W., Saito, T. & Anderson, D. J. Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. *Genes Dev.* **5**, 1524–1537 (1991).
29. Zimmerman, L. B., De Jesus-Escobar, J. M. & Harland, R. M. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599–606 (1996).
30. Weissman, A. M. Regulating protein degradation by ubiquitination. *Immunol. Today* **18**, 189–198 (1997).
31. Rock, K. L. et al. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**, 761–771 (1994).
32. Fenteany, G. *et al.* Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268**, 726–731 (1995).
33. Kretzschmar, M. & Massague, J. SMADs: mediators and regulators of TGF-β signaling. *Curr. Opin. Genet. Dev.* **8**, 103–111 (1998).
34. Lo, L. C., Sommer, L. & Anderson, D. J. MASH1 maintains competence for BMP2-induced neuronal differentiation in post-migratory neural crest cells. *Curr. Biol.* **7**, 440–450 (1997).

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35. Varley, J. E., Wehby, R. G., Rueger, D. C. & Maxwell, G. D. Number of adrenergic and Islet-1 immunoreactive cells is increased in avian trunk neural crest cultures in the presence of human recombinant Osteogenic Protein-1. *Develop. Dynam.* **203**, 434–447 (1995).
36. Gross, R. E. *et al.* Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. *Neuron* **17**, 595–606 (1996).
37. Shah, N. M., Groves, A. K. & Anderson, D. J. Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. *Cell* **85**, 331–343 (1996).
38. Marazzi, G., Wang, Y. & Sassoon, D. Msx2 is a transcriptional regulator in the BMP4-mediated programmed cell death pathway. *Dev. Biol.* **186**, 127–138 (1997).
39. Ström, A., Castella, P., Rockwood, J., Wagner, J. & Caudy, M. Mediation of NGF signaling by post-translation inhibition of HES-1, a basic helix-loop-helix repressor of neuronal differentiation. *Genes Dev.* **11**, 3168–3181 (1997).
40. Engel, M. E., Datta, P. K. & Moses, H. L. RhoB is stabilized by transforming growth factor β and antagonizes transcriptional activation. *J. Biol. Chem.* **273**, 9921–9926 (1998).
41. Alkalay, I. *et al.* Stimulation-dependent IκBa phosphorylation marks the NF-κB inhibitor for degradation via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA* **92**, 10599–10603 (1995).
42. Kanegae, Y., Tavares, A. T., Belmonte, J. C. I. & Verma, I. M. Role of Rel/NF-κB transcription factors during the outgrowth of the vertebrate limb. *Nature* **392**, 611–614 (1998).
43. Bushdid, P. B. *et al.* Inhibition of NF-κB activity results in disruption of the apical ectodermal ridge and aberrant limb morphogenesis. *Nature* **392**, 615–618 (1998).
44. Aberle, H., Bauer, A., Stappert, J., Kispert, A. & Kemler, R. β-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* **16**, 3797–3804 (1997).
45. Lee, J. E. Basic helix-loop-helix genes in neural development. *Curr. Opin. Neurobiol.* **7**, 13–20 (1997).
46. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual.* (Cold Spring Harbor Press, Cold Spring Harbor, New York, 1989).
47. Kim, S., Magendantz, M., Katz, W. & Solomon, F. Formation of the chicken erythrocyte marginal band *in vivo*: orgins of a cytoplasmic microtubule structure. *J. Cell Biol.* **104**, 51–59 (1987).
48. Clark, M. S., Lanigan, T. M., Page, N. M. & Russo, A. F. Induction of a serotonergic and neuronal phenotype in thyroid C-cells. *J. Neurosci.* **15**, 6167–6178 (1995).
49. Magae, J. *et al.* Transcriptional squelching by ectopic expression of E2F-1 and p53 is alleviated by proteasome inhibitors MG-132 and lactacystin. *Oncogene*


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