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Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development

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SUMMARY

Bone Morphogenetic Proteins (BMPs) play crucial roles in a variety of developmental processes, but their functions during early vertebrate brain development are largely unknown. To investigate this problem, we have compared by in situ hybridization the expression of five *Bmp* genes belonging to the *Drosophila Decapentaplegic* (*Bmp2* and *Bmp4*) and *60A* subgroups (*Bmp5*, *Bmp6* and *Bmp7*). Striking co-expression of these *Bmp*s is observed within the dorsomedial telencephalon, coincident with a future site of choroid plexus development. *Bmp* co-expression overlaps that of *Msx1* and *Hfh4*, and is complementary to that of *Bf1*. The domain of *Bmp* co-expression is also associated with limited growth of the neuroectoderm, as revealed by morphological observation, reduced cell proliferation, and increased local programmed cell death. In vitro experiments using explants from the embryonic lateral telencephalic neuroectoderm reveal that exogenous BMP proteins (BMP4 and BMP2) induce expression of *Msx1* and inhibit *Bf1* expression, a finding consistent with their specific expression patterns in vivo. Moreover, BMP proteins locally inhibit cell proliferation and increase apoptosis in the explants. These results provide evidence that BMPs function during regional morphogenesis of the dorsal telencephalon by regulating specific gene expression, cell proliferation and local cell death.

Key words: Bone Morphogenetic Proteins (BMPs), mouse embryo, dorsal forebrain, *Msx1*, *Bf1*, cell proliferation, cell death, choroid plexus development

INTRODUCTION

The vertebrate brain consists of a number of spatially and functionally distinct domains that arise within the fore-, mid- and hindbrain. Many genetic and experimental studies have provided insights into the molecular mechanisms underlying the metameric patterning and morphogenesis of the hindbrain and midbrain (reviewed by Lumsden and Krumlauf, 1996; Joyner, 1996). However, given its morphological complexity, little is known about the genetic programs involved in the patterning, growth and differentiation of the more anterior neural subdivision, the forebrain.

It is known that signals from the prechordal plate play an important role in the patterning and morphogenesis of the overlying forebrain neuroectoderm. For example, the product of the *Shh* gene regulates specification of ventral cell types in the forebrain neuroectoderm (Ericson et al., 1995), and the subdivision of the forebrain into bilateral compartments (Macdonald et al., 1995; Chiang et al., 1996). Within the neuroectoderm, members of the *Otx*, *Emx* and *Dlx* homeobox gene families are expressed in regionally restricted patterns (reviewed by Rubenstein et al., 1994). Mutations in some of these genes in mice (Matsuo et al, 1995; Qiu et al., 1996; Pellegrini et al., 1996; Yoshida et al., 1997) and humans (Brunelli et al., 1996) result in patterning and morphological abnormalities in regions including the forebrain. In addition, *Bf1*, encoding a member of the winged-helix family of transcription factors, is expressed specifically in the developing telencephalon and eye (Tao and Lai, 1992). The inactivation of this gene causes cell proliferation defects associated with premature neuronal cell differentiation, resulting in failure of expansion of the brain hemispheres (Xuan et al., 1995).

There is increasing evidence that secreted signaling molecules of the BMP subfamily of the TGFβ superfamily play versatile roles in many aspects of embryonic development, including neurogenesis (reviewed by Kingsley, 1994; Hogan, 1996). BMP functions in the dorsal-ventral patterning of the neuroectoderm have been inferred from the temporal and spatial patterns of *Bmp4* and *Bmp7* expression in the dorsal surface ectoderm and/or neural folds of the developing chick embryo. Moreover, the addition of BMP4 or BMP7 protein to undifferentiated intermediate neuroectoderm is sufficient to induce dorsal-specific neural markers (Liem et al., 1995). BMP4 has also been shown to be involved in *Msx* gene induction and apoptosis in presumptive neural crest cells from specific rhombomeres in chick hindbrain explants (Graham et al., 1994).

Several *Bmp* genes are expressed in localized patterns during embryonic mouse brain development (Jones et al., 1991; King et al., 1994; Lyons et al., 1995). However, genetic evidence for their role in neurogenesis is not available. For example, homozygous *Bmp2* and *Bmp4* mutant embryos die before or during

early neural development (Zhang et al., 1996; Winnier et al., 1995). In the case of *Bmp5*, *Bmp7* and *Bmp6* homozygous null mutants, no obvious neural phenotype has been observed so far, apart from the degeneration of the developing eye in *Bmp7* mutants (King et al., 1994; Dudley et al., 1995; Luo et al., 1995; Elizabeth Robertson, personal communication). This raises the possibility that the loss of a single *Bmp* gene is compensated for by others during early brain development.

To investigate BMP functions during mammalian forebrain development, we have studied the expression of five *Bmp* genes (*Bmp2*, *Bmp4*, *Bmp5*, *Bmp6* and *Bmp7*) in the developing mouse brain by in situ hybridization. This reveals a striking domain of *Bmp* co-expression in the dorsomedial telencephalon, which marks the future site of choroid plexus formation in the forebrain, and correlates with the developmental expression of other region specific genes. The domain of *Bmp* co-expression is also associated with reduced cell proliferation and regional apoptosis. Furthermore, functional studies demonstrate that local application of exogenous BMP proteins onto cultured explants of the lateral telencephalon induces dorsomedial phenotypes, as marked by *Msx1* and *Bf1* expression and changes in cell proliferation and programmed cell death. These results thus provide evidence that BMPs function in regional morphogenesis during forebrain development by regulating specific gene expression, cell proliferation and programmed cell death.

MATERIALS AND METHODS

Embryos

Embryos from ICR mice (Harlan Sprague-Dawley, Indianapolis) were processed for tissue sections or whole mount in situ hybridization as described below. Noon of the day of the vaginal plug is 0.5 day post-coitum (d.p.c.).

In situ hybridization

In situ hybridization using ^{35}S-UTP-labeled riboprobes and digoxigenin (DIG) UTP-labeled riboprobes was performed essentially as described by Hogan et al. (1994). The murine probes were as follows. A 1.2 kb fragment of *Bmp2* (Lyons et al., 1989), a 1 kb fragment of *Bmp4* (Jones et al., 1991), a 1.2 kb fragment of *Bmp5* encoding almost the entire coding region (a kind gift from Dr David Kingsley), a 900 bp fragment of *Bmp6* (Jones et al., 1991), a 860 bp fragment of *Bmp7* containing the region homologous to nucleotides 450-1314 of human *Bmp7*, a 1 kb fragment of *Msx1* (generously provided by Dr Robert Hill; Hill et al., 1989), a 1.2 kb fragment of *Bf1* (a kind gift from Dr Eseng Lai; Tao and Lai, 1992) and a 3′ 1.5 kb fragment of *Hfh4* (kindly provided by Dr Robert Costa).

Three dimensional reconstruction of in situ images

Only the neuroectoderm was selected from dark-field photomicrographs of the in situ hybridization for *Bmp4*, *Bmp6* or *Bmp7* on serial transverse sections of a 10.5 d.p.c. embryo. The orientation of each image was manually aligned using Photoshop 3.0 (Adobe) on a Macintosh computer. The images of 88 sections hybridized with each probe were reconstructed using Voxel View 2.5 (Vital Images, Fairfield, IA) running on an SGI Indigo^2 computer. The brain tissue was depicted as gray, and the in situ signal as red.

Analyses for cell proliferation and cell death

Pregnant mice were injected intraperitoneally with 2 ml/kg body weight of Cell Proliferation Labeling Reagent (Amersham) 2 hours before embryo dissection. Sections of BrdU-labeled embryos were processed for immunohistochemical analysis using an anti-BrdU monoclonal antibody (Cappel, #2272MBU). Immunohistochemistry and in situ hybridization using *Bmp* probes were performed on alternate sections to allow precise determination of proliferation rates in *Bmp*-expressing and non-expressing regions. The TUNEL method was used to detect apoptotic cells as described by Gavrieli et al. (1992).

Explant culture of telencephalic neuroectoderm

The heads of 10.5 d.p.c. embryos were dissected, and the surface ectoderm, optic stalk and frontonasal mass removed manually. The resulting tissues were treated with pancreatin/trypsin solution (Hogan et al., 1994) for 20-30 minutes on ice. The mesenchymal sheet surrounding the neuroectoderm was removed manually, and then pieces of lateral telencephalon of 200-500 μm in diameter were isolated. Shorter enzyme digestion was used for forebrain neuroectoderm from 3-5 somite stage embryos (8.0 d.p.c.). Isolated tissue pieces were cultured on a micropore filter (Costar, #110414) floating on culture medium. Protein-soaked beads (see below) were placed on the tissue pieces. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum, 1× non-essential amino acids (GIBCO), 1 mM sodium pyruvate (GIBCO) and 1× streptomycin/penicillin (GIBCO). Explants were BrdU-labeled for 2 hours using Cell Proliferation Labeling Reagent (Amersham) at a final dilution of 1:1000 before tissue collection. Tissues were processed for in situ analysis or immunohistochemistry as described above for embryos.

Preparation of protein carrying beads

Affi-Gel Blue Gel (Bio Rad, 100-200 mesh, #153-7302) beads were rinsed with PBS several times, and beads of 25-75 μm in diameter were manually collected under the dissection microscope. One hundred beads were soaked with 10 μl of BMP protein solution diluted at appropriate concentrations with PBS containing 0.1% bovine serum albumin (BSA) (BSA/PBS) in a microfuge tube for 1 hour at 37ºC. The BSA/PBS solution was used as a negative control. Siliconized tips and tubes were used for handling BMP proteins. Recombinant human BMP2 (batch #2D11J024), BMP4 (3178-163/3816-16), BMP6 (4739-73/B3D1) and BMP7 (3811-64B) were kindly provided by Genetics Institute (Cambridge, MA, USA).

RESULTS

Multiple *Bmp* family members are expressed in the early developing brain

The temporal and spatial patterns of expression of five *Bmp* family members in the mouse embryonic brain were studied by in situ hybridization. Transcripts of *Bmp*s are first detected in the anterior neuroectoderm at around the 5 somite stage (8.5 d.p.c.). At this time, *Bmp5* (arrowheads in Fig. 1C,H) and *Bmp7* (arrow in Fig. 1D, arrowhead in Fig. 1I) mRNA are present within the most anterior neuroectoderm. By contrast, *Bmp2* is exclusively expressed in the dorsal surface ectoderm immediately adjacent to the neuroectoderm (arrowheads in Fig. 1A,F), while *Bmp5* and *Bmp7* are weakly expressed throughout the head mesenchyme and dorsal surface ectoderm (Fig. 1C,D). Transcripts of *Bmp7* are also detected in the notochord and anterior foregut (Fig. 1D, arrowheads). *Bmp4* and *Bmp6* are not yet expressed at this anterior level (Fig. 1B,G,E,J).

At the 10-15 somite stage (9.0 d.p.c.), just before cephalic neural tube closure, transcripts of *Bmp4*, *Bmp5* and *Bmp7* are all present in the most anterior dorsal neuroectoderm (Fig. 1M-O), with expression of *Bmp7* extending more ventrally than

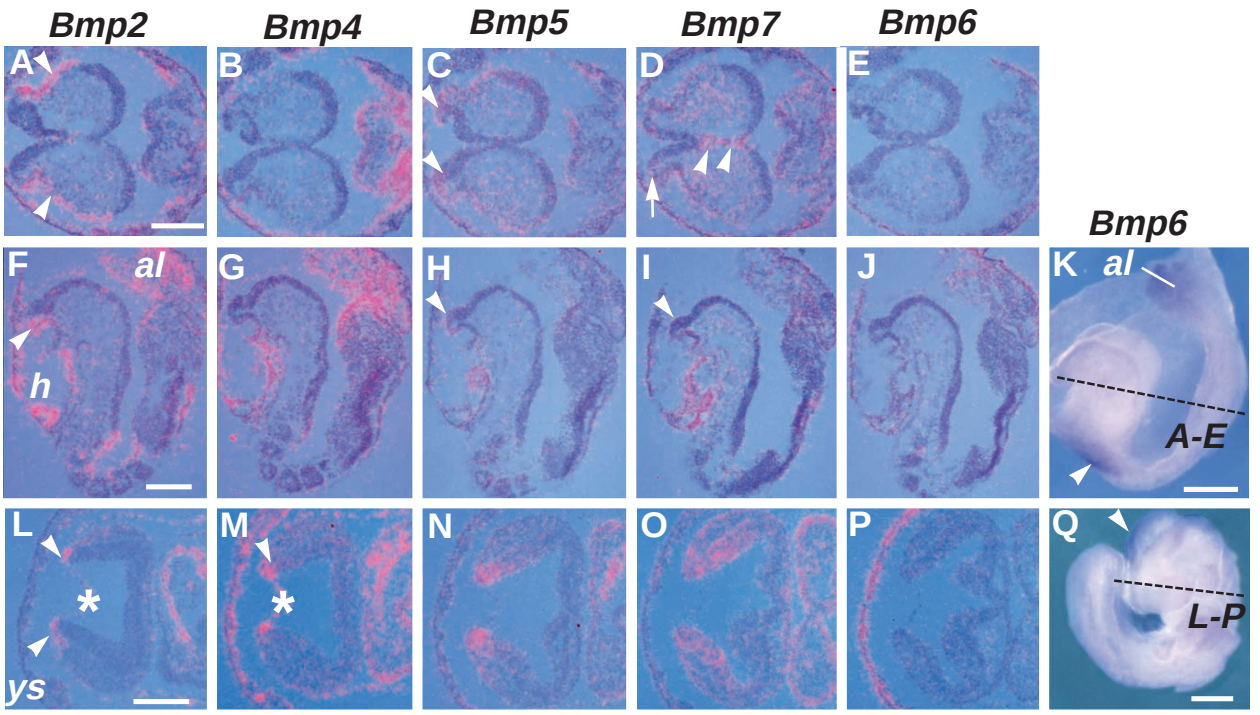


Fig. 1. Expression of *Bmp*s in the forebrain region before cephalic neural tube closure (8.5-9.0 d.p.c.). In situ hybridization of transverse (A-E) and sagittal (F-J) sections of 5-8 somite stage embryos (8.5 d.p.c.) (anterior is on the left). Approximate planes of the sections in A-E are indicated in K. Arrowheads indicate expression of *Bmp2* in the dorsal surface ectoderm (A,F), *Bmp5* in the most anterior neuroectoderm (C,H) and *Bmp7* in the anterior foregut and notochord (D). The arrow in (D) and arrowhead in (I), respectively, point *Bmp7* expression in the most anterior neuroectoderm. (K) Expression of *Bmp6* visualized by whole-mount in situ hybridization, showing localization of the transcripts in the foregut endoderm (arrowhead) and the allantois. (L-P) Expression of *Bmp* genes in forebrain regions of a 12 somite stage embryo (9.0 d.p.c.) (dorsal to the left). Approximate planes of the sections are indicated in (Q). Arrowheads indicate localization of *Bmp2* transcripts in the dorsal surface ectoderm adjacent to the neuroectoderm (L) and *Bmp4* expression within the dorsal neuroectoderm (M). Asterisks indicate expression of *Bmp2* (L), and *Bmp4* (M) in the amnion. (Q) Expression of *Bmp6* in a 15 somite stage embryo visualized by whole mount in situ hybridization. Transcripts of *Bmp6* are first detected at the telencephalon/diencephalon junction (arrowhead) at this stage. Abbreviations: al, allantois; h, heart; ys, yolk sac. Bar, 100 μm (A-E,F-J,K), 50 μm (L-P), 200 μm (Q).

Bmp5, and with *Bmp4* expression restricted to the most dorsal part (Fig. 1M, arrowhead). Although not detected at this anterior level (Fig. 1P), *Bmp6* begins to be expressed in the dorsal neuroectoderm around the junction between the prospective telencephalon and diencephalon (Fig. 1Q, arrowhead). *Bmp2* transcripts are present in the dorsal surface ectoderm only immediately adjacent to the neural fold (Fig. 1L), whereas *Bmp5* and *Bmp7* expression extends more laterally in the dorsal surface ectoderm (Fig. 1N,O).

At 9.5 d.p.c., after neural tube closure, *Bmp2* expression is dramatically downregulated in the dorsal surface ectoderm (Fig. 2A, arrowheads). Expression of *Bmp4*, *Bmp5* and *Bmp7* is maintained in the dorsal forebrain (Fig. 2B-D), and extends to the anteriormost dorsal roof of the telencephalon (Fig. 2H-J; arrowheads). *Bmp7* transcripts are detected in a relatively wider region than the other family members (Fig. 2D), consistent with their earlier distribution in the neuroectoderm. *Bmp6* expression in the dorsal midline extends posteriorly from the telencephalon/diencephalon boundary (Fig. 2E) through the entire body axis by this stage (Fig. 2F).

The domains of *Bmp* co-expression define a dorsomedial region of the forebrain

By 10.5 d.p.c., the telencephalic vesicles have begun to grow in size, expanding laterocaudally. At this time, a striking domain of *Bmp* co-expression is evident in the forebrain, defining a region that covers the dorsomedial part of the telencephalon and the dorsal midline of the anterior diencephalon (Fig. 3, also see Figs 5J-L, 6A-C). Expression of *Bmp2* is first detected within the neuroectoderm at this stage, overlapping with that of other *Bmp*s within the telencephalon (data not shown). Transcripts of multiple *Bmp*s are also seen in the dorsal hindbrain (Fig. 3, right 2 columns)

At 11.5 d.p.c., the expression pattern of *Bmp*s in the forebrain is basically similar to that seen one day earlier, but the co-expression domain becomes more tightly restricted as the telencephalon expands. This domain coincides with the medial walls of the lateral ventricles, corresponding to the prospective hippocampus and choroid plexus (Fig. 4A-E). The dorsal expression domain of *Bmp4*, *Bmp5*, *Bmp6* and *Bmp7* includes the roof of the diencephalon (Fig. 4A-D), extending to the anterior dorsal roof between the telencephalic hemispheres (Fig. 4F-I, arrowheads). By contrast, *Bmp2* expression is more caudally shifted within the dorsomedial telencephalon compared with the other genes (Fig. 4E), and is barely detectable in the dorsal midline (Fig. 4J).

At 13.5 d.p.c., *Bmp* co-expression is eventually restricted to the thin layer of the medial walls of the lateral ventricles, the

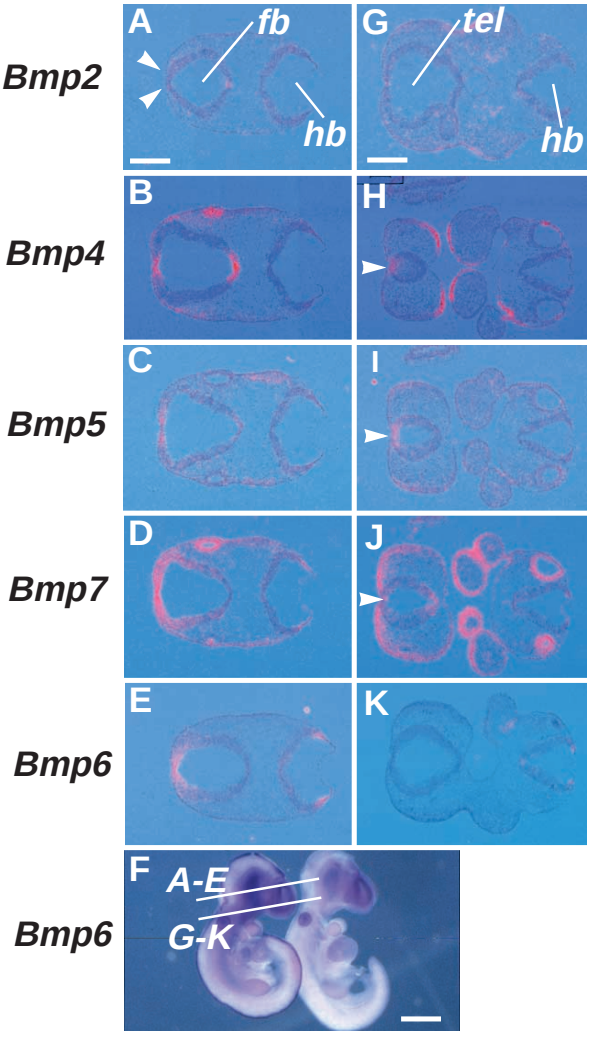


Fig. 2. Expression of *Bmp*s after cephalic neural tube closure (9.5 d.p.c.). In situ hybridization on transverse sections through the region of the telencephalon/diencephalon junction (A-E) and in the most anterior telencephalon (G-K) (anterior is on the left). Approximate planes of the sections are indicated in F. Note that *Bmp2* expression is dramatically downregulated in the dorsal surface ectoderm (arrowheads in A). (F) Embryos hybridized with antisense (left) and sense (right) *Bmp6* riboprobes (anterior on the right for the head region). Anterior extension of expression of *Bmp4* (H), *Bmp5* (I) and *Bmp7* (J) are indicated (arrowheads in H, I, J). Abbreviations: fb, forebrain; hb, hindbrain; tel, telencephalon. Bar, 200 μm (A-E,G-K), 400 μm (F).

fimbria (Fig. 4K-O). Strong expression of *Bmp4*, *Bmp5*, *Bmp6* and *Bmp7* is detected in the choroid plexus (Fig. 4K-N, arrows and arrowheads). *Bmp2* is barely detectable in the choroid plexus, but its expression coincides with a small part of the developing dentate gyrus of the hippocampus (Fig. 4O, arrowheads).

Correlation between co-expression of *Bmp*s and other region-specific genes in the telencephalon

There is increasing evidence to support the notion that BMPs induce the expression of specific genes in target tissues in various developmental systems. The expression of several

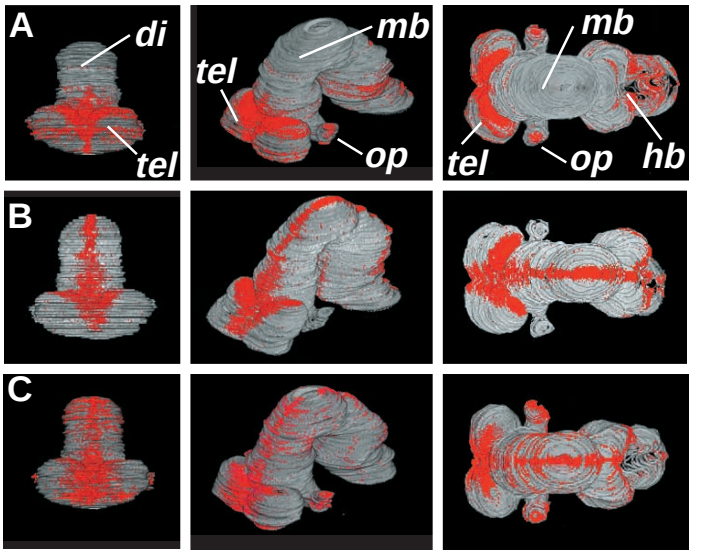


Fig. 3. Expression of *Bmp*s in the brain of a 10.5 d.p.c. embryo. Three dimensional reconstruction of the in situ hybridization images of *Bmp4* (row A), *Bmp6* (row B) and *Bmp7* (row C) viewed from the front, laterally from the front and from the top (left to right). Note that only expression in the neuroepithelium is shown. Red represents the in situ signals of the *Bmp*s. Abbreviations: di, diencephalon; mb, midbrain; hb, hindbrain; op, optic cup/stalk; tel, telencephalon.

genes was therefore examined in relation to that of *Bmp*s during forebrain development. Expression of *Msx1*, a member of the *Msh* homeobox gene family, was first examined, since this gene is induced by BMP signals in the spinal cord and hindbrain in chick embryos (Graham et al., 1994; Liem et al., 1995). At 8.5 d.p.c., *Msx1* is not yet expressed within the neuroectoderm of the prospective telencephalic region, although the gene is expressed in the dorsal aspect of the spinal cord posteriorly (Hill et al., 1989, and data not shown). At 9.5 d.p.c., *Msx1* expression is apparent in the dorsal midline of the telencephalon (Fig. 5A, arrowhead), but the transcript level is low compared with that in the posterior neural tube (e.g. in the dorsal midbrain). By contrast, high levels of *Msx1* expression are present in the surface ectoderm and underlying head mesenchyme (Fig. 5A). After 10.5 d.p.c., localization of *Msx1* is observed in the dorsomedial telencephalon (Fig. 5B, arrowheads), in a domain corresponding to that of *Bmp* co-expression (see Fig. 5J-L). *Msx1* expression extends to the anteriormost dorsal roof of the telencephalon (Fig. 5C, arrowhead) and throughout the dorsal midline of the entire neural tube posteriorly (Hill et al., 1989, and data not shown), including the dorsal hindbrain (Fig. 5B).

The winged helix gene, *Bf1*, is widely expressed during the development of the telencephalon (Tao and Lai, 1992). At 9.5 d.p.c., *Bf1* transcripts are present in the dorsal telencephalon (Fig. 5D, arrowheads), as well as in the lateral and ventral telencephalon at higher levels (Fig. 5D). By 10.5 d.p.c., however, *Bf1* expression has been excluded from the dorsomedial telencephalon (Fig. 5E) and the roof between the telencephalic hemispheres (Fig. 5F; Tao and Lai, 1992). When the patterns of expression of *Bmp*s and *Bf1* are compared at this stage, they appear almost complementary in the telencephalon (Fig. 5E,J-L).

At 13.5 d.p.c., a winged helix gene, *Hfh4*, is specifically expressed in the choroid plexus epithelium. This gene has been shown to be an upstream regulator of some genes encoding cerebrospinal fluid proteins, such as α2-microglobulin and insulin-like growth factor II (Lim et al., 1997). In the forebrain, expression of *Hfh4* is first detected at 9.5 d.p.c., only very weakly in the dorsal midline, around the telencephalon/diencephalon boundary (Fig. 5G, arrowhead). By contrast, higher levels of *Hfh4* transcripts are seen in the roof of the hindbrain (Fig. 5G). By 10.5 d.p.c., *Hfh4* expression increases around the dorsal midline of the forebrain (Fig. 5H), including the dorsal roof between the telencephalic hemispheres (Fig. 5I, arrowheads), and strong *Hfh4* expression is maintained in the hindbrain roof plate (Fig. 5H). Thus, the overall spatial expression domains of this gene strikingly resemble those of *Bmp*s in the telencephalon and anterior diencephalon (see Fig. 5J-L). These results support the hypothesis that *Bmp* transcription tightly correlates with choroid plexus differentiation.

Expression of *Bmp*s within the dorsal telencephalon is associated with limited expansion of the neuroectoderm due to reduced cell proliferation and regional cell death

As development proceeds beyond 10.5 d.p.c., the dorsomedial telencephalic neuroectoderm, where *Bmp*s are strongly expressed, remains thin, whereas the dorsolateral and ventral telencephalon expand rapidly to form the cerebral hemispheres (see Fig. 4A-E,K-O). This raises the possibility of a causal relationship between the expression of *Bmp*s and the limited expansion of the dorsomedial neuroectoderm.

To examine this question, we analyzed cell proliferation within the different regions of telencephalon of 10.5 d.p.c. embryos. *Bmp*s are already expressed in the dorsomedial telencephalon at this stage, preceding regional differences in the thickness of the telencephalic neuroepithelium, except in the dorsal midline (Figs 3, 6A-C). As summarized in Fig. 6I, the index of cell proliferation is significantly lower in the dorsal midline (Fig. 6I, region 2) where multiple *Bmp*s are expressed at higher levels (Fig. 6C). Also, in the medial walls of the telencephalon (Fig. 6I, region 3), there is significantly lower cell proliferation than in the lateral telencephalic neuroectoderm (Fig. 6, region 1). These results indicate that *Bmp* co-expression in the dorsomedial telencephalon is associated with reduced cell proliferation.

Apoptotic cell death is not evident throughout the telencephalon at this stage (Fig. 6L), except in a restricted region of the dorsal midline, from the telencephalon/diencephalon junction to the anterior dorsal telencephalic roof. Here, a significant number of TUNEL-positive cells can be detected (Fig. 6J,K, arrowheads), and this pattern of cell death appears coincident with co-expression of *Bmp*s and *Msx1* (see Figs 5, 6C).

Ectopic application of BMP4 protein to the lateral telencephalic neuroectoderm induces a dorsomedial phenotype in tissue explants

To test the role of BMPs during the development of the dorsal telencephalon more directly, we have employed an explant culture system. Pieces of the lateral telencephalic region, where *Bmp*s are not expressed, were collected from 10-10.5 d.p.c. embryos, and were cultured in the presence of beads soaked with purified recombinant human BMP proteins.

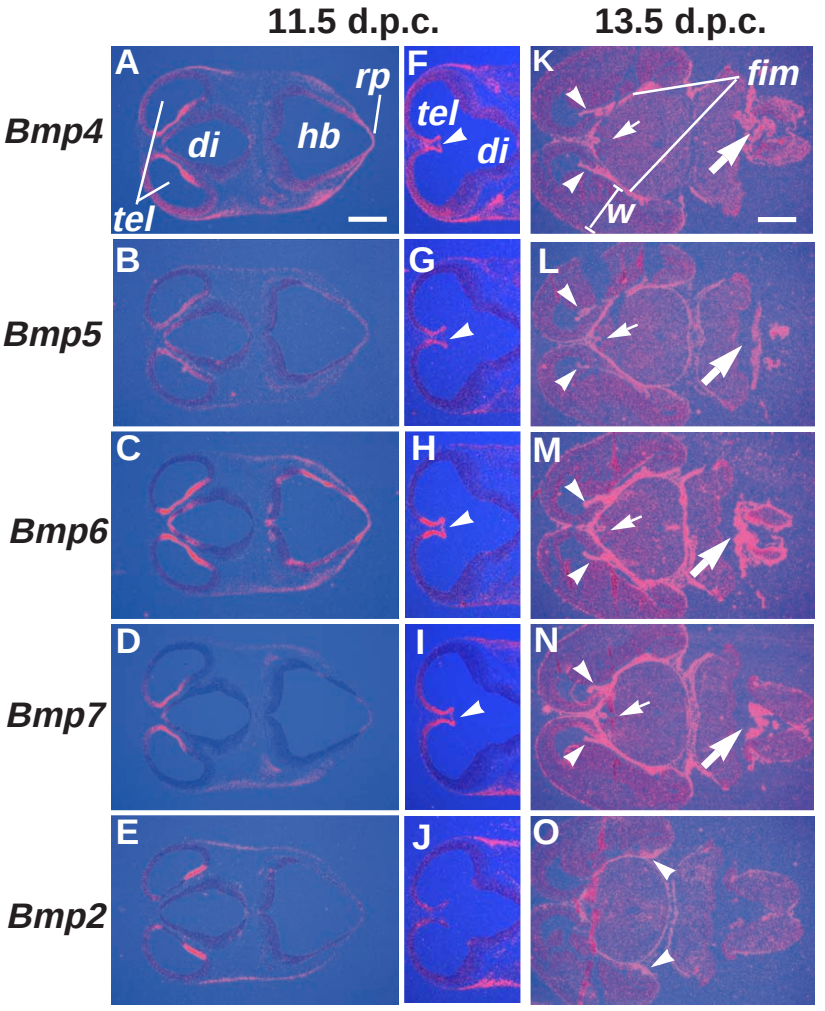


Fig. 4. Expression of *Bmp*s in brains of mid-gestation embryos. In situ hybridization on sections of an 11.5 d.p.c. (A-J) and a 13.5 d.p.c. brain (K-O). (A-E) Localization of *Bmp* transcripts to the dorsomedial telencephalon. *Bmp* expression is also detected in the dorsal diencephalon and the thin roof plate of the hindbrain. (F-J) Views at more anterior levels than A-E, showing expression domains of *Bmp*s extending to anterior regions, including the roof between cerebral hemispheres (arrowheads in F-I). *Bmp2* is only barely detectable in this region (J). (K-O) Localization of *Bmp* transcripts to the medial walls of the lateral ventricles and in the choroid plexus of the lateral ventricles (arrowheads in K-N), the 3rd and the 4th ventricles (small and large arrows in K-N). *Bmp2* is expressed in a small part of the developing dentate gyrus (arrowheads in O). Abbreviations: di, diencephalon; fim, fimbria; hb, hindbrain; rp, roof plate; tel, telencephalon; w, wall of ventrolateral telencephalon. Bar, 100 μm (A-J), 200 μm (K-O).

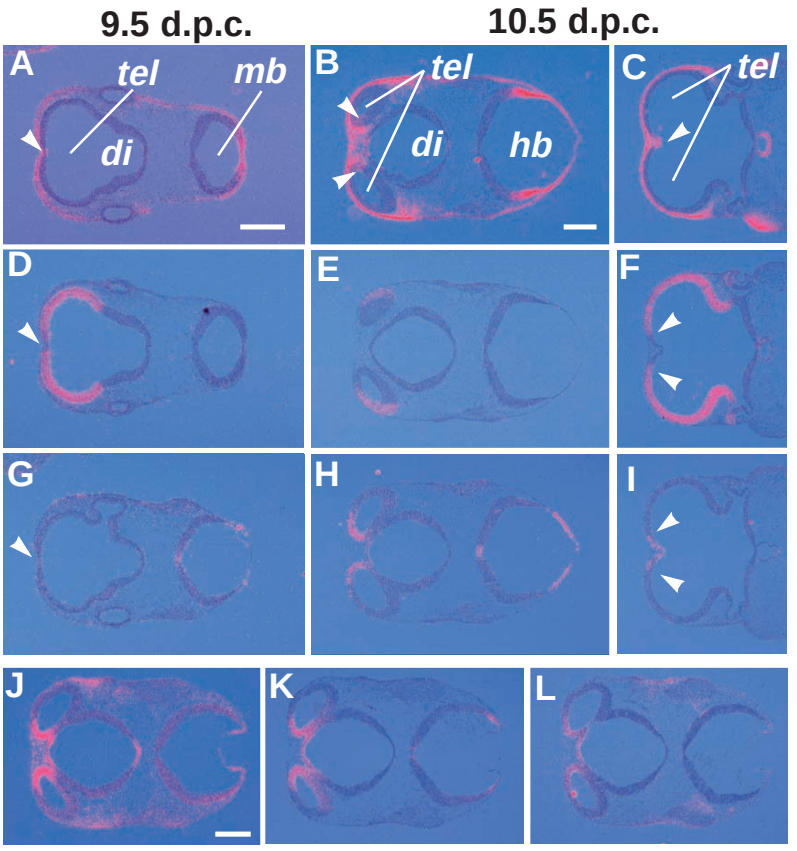


Fig. 5. Expression of region specific genes in comparison with *Bmp* genes at 9.5 d.p.c. and 10.5 d.p.c. (A-C) Expression of *Msx1* in the brains of 9.5 d.p.c. (A) and 10.5 d.p.c. (B,C) embryos. Within the neuroectoderm, transcripts are localized to the dorsal midline at 9.5 d.p.c. (arrowhead in A), and at 10.5 d.p.c., in the dorsomedial part (arrowheads in B) and dorsal midline of the telencephalon (arrowhead in C). *Msx1* is highly expressed in the surface ectoderm and head mesenchyme. (D-F) *Bf1* expression in sections close to the ones shown in A-C. *Bf1* transcripts are highly expressed throughout the telencephalon, with a slight downregulation in the dorsal midline at 9.5 d.p.c. (arrowhead in D). At 10.5 d.p.c., *Bf1* expression has disappeared from the dorsomedial region (E) and the dorsal midline of the telencephalon (arrowheads in F). (G-I) Localization of *Hfh4* transcripts in the brain of 9.5 d.p.c. (G) and 10.5 d.p.c. (H,I) embryos. Expression of *Hfh4* is only very weak in the dorsal midline at around the telencephalon-diencephalon boundary at 9.5 d.p.c. (arrowhead in G), while stronger signals are seen in the hindbrain. High levels of *Hfh4* transcripts are detected in the dorsomedial part (H) and dorsal midline of the telencephalon (arrowheads in I) at 10.5 d.p.c. (J-L) Expression of *Bmp4* (J), *Bmp6* (K) and *Bmp7* (L) in the brain of a 10.5 d.p.c. embryo. Note the strikingly complementary and similar expression patterns to those of *Bf1* and *Hfh4* (E and H, respectively). Abbreviations: di, diencephalon; hb, hindbrain; mb, midbrain; tel, telencephalon. Bar, 200 μm (A,D,G), (B,C,E,F,H,I), (J-L).

Initial experiments showed that BMP4 induces *Msx1* expression in this explant system in a dose dependent manner. Beads soaked with 200 ng/ml of BMP4 cannot induce *Msx1* expression within 24 hours of culture (beads: *n*=11) (Fig. 7A). BMP4 at 500 ng/ml can induce *Msx1* expression, but the extent of the induction is not consistent from bead to bead (*n*=14) (Fig. 7B). At 1 μg/ml, all of the beads induce *Msx1* expression at moderate levels (*n*=27) (Fig. 7C), and at the highest concentration tested, 2 μg/ml, induction occurs around all of the beads consistently at high levels (Fig. 7D,E), within a zone around 10-15 cell diameters from the beads. The expression of *Msx1* in these explants does not appear to form a gradient, but defines a discrete, high expression domain around the beads, suggesting the existence of a threshold of BMP4 protein required for the induction of *Msx1* expression. Under the same conditions, control BSA beads do not induce *Msx1*, even after 48 hours of culture (Fig. 7I). In the following experiments, BMP4 was used at a concentration of 2 μg/ml.

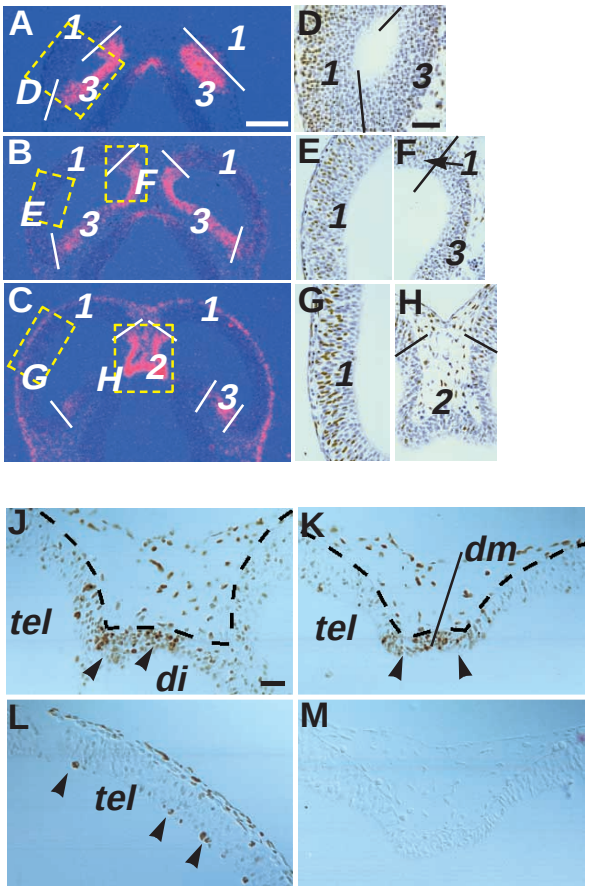
Since the expression patterns of *Bmp*s and *Bf1* are complementary within the telencephalon in vivo, we next tested the possibility that BMP4 inhibits *Bf1* expression in telencephalic neuroectoderm. In vivo, the region of neuroectoderm harvested for the explants already expresses *Bf1* at high levels (Fig. 5E,F). This expression is maintained after 24 hours of culture, and is not affected by the application of BSA beads (Fig. 7J). In contrast, a zone of *Bf1* downregulation is observed around each BMP4-soaked bead (Fig. 7F). This result demonstrates that BMP4 negatively regulates *Bf1* expression in the telencephalic neuroectoderm, and is consistent with the expression patterns of these genes seen in vivo.

Cell proliferation and apoptosis were then assayed in the explants. At 24 hours, inhibition of cell proliferation is observed around all the BMP4-carrying beads examined (*n*=35) (Fig. 7G). By contrast, BrdU-positive cells are distributed in substantial numbers randomly and uniformly throughout the explants cultured with control BSA-soaked beads (*n*=28) (Fig. 7K). When adjacent or nearby sections are analyzed for programmed cell death, apparent accumulation of TUNEL-positive cells is observed around the BMP4 beads, but not around control beads (Fig. 7H,L).

In time course experiments, expression of *Msx1* is first detected around only few of the beads at very low levels at 6 hours (Fig. 8B). By 12 hours, weak to moderate induction is observed around all the beads (*n*=15) (Fig. 8C), and by 18 hours, the level of *Msx1* expression is as high as at 24 hours (*n*=13) (Fig 8D, also see Fig. 7D). In contrast, suppression of *Bf1* expression is apparent only after 18 hours, in a zone similar to that of *Msx1* induction (Fig. 8E,H, and data not shown for 6 and 12 hours). A similar temporal analysis on cell proliferation and cell death shows that these changes in cell behavior begin later than *Msx1* induction (data not shown). Moreover, at 18 hours, when TUNEL-positive cells have increased within the zone of *Bf1* suppression (Fig. 8G), some BrdU-positive cells are still present in this zone (Fig. 8F).

Effect of other BMP proteins in the explant culture

In our explant culture system, BMP2 has a similar activity to BMP4, inducing *Msx1* (Fig. 9A) and cell death (data not shown), and inhibiting *Bf1* expression (Fig. 9B) and cell proliferation (data not shown), in a similar dose range. BMP7,



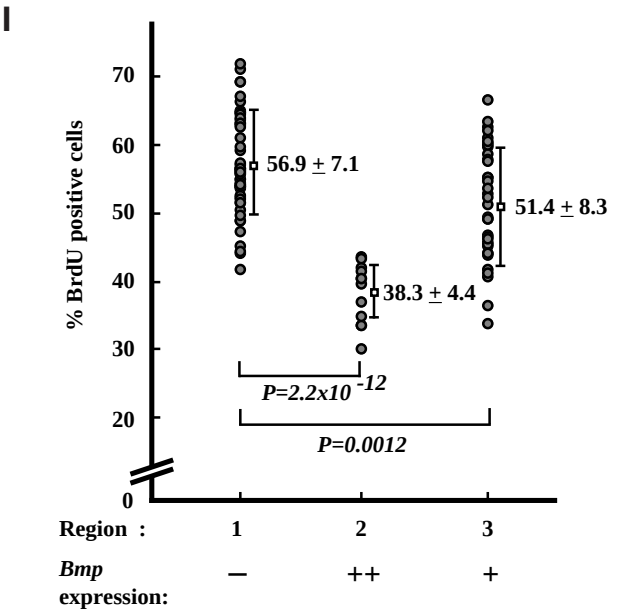


Fig. 6. Cell proliferation and cell death in the telencephalon of 10.5 d.p.c. embryos. (A-C) Expression of *Bmp6* (A), *Bmp5* (B), and *Bmp4* (C) in the telencephalon of a 10.5 d.p.c. embryo (dorsal to the top). The dorsal telencephalon is divided, according to the level of *Bmp* expression: (1) the lateral region, where *Bmp*s are not expressed; (2) the dorsal midline; and (3) the dorsomedial region, where *Bmp*s are expressed. (D-H) Examples of BrdU antibody staining in the regions shown in A-C. (I) Percentage of BrdU-positive cells in the neuroectoderm was determined by the number of positive cells/total cells in each region. The data are from a total of 41,400 cells counted on six different sections from each of three embryos. Each dot represents the value from one photomicrograph containing 200-500 cells. Mean values and standard deviations from each group are also shown. The differences in cell proliferation in *Bmp* non-expressing and expressing regions are statistically significant by Student's *t*-test ($P<0.01$). (J,K) Programmed cell death detected by TUNEL in the dorsal midline of the forebrain region, at the diencephalon/telencephalon junction (J) and the dorsal roof between telencephalic hemispheres (K) (arrowheads). The border between the head mesenchyme and neuroectoderm is indicated by dashed lines. (L) The lateral telencephalon in the same section as shown in K. TUNEL-positive cells are barely detectable in the lateral and ventral telencephalon at this stage (arrowheads). (M) A section near the one shown in K incubated with heat-inactivated terminal deoxynucleotidyl transferase as a negative control. Abbreviations: di, diencephalon; dm, dorsal midline of the telencephalon; tel, telencephalon. Bar, 200 μm (A-C), 50 μm (D-H), 25 μm (J-M).

however, does not induce *Msx1* nor suppress *Bf1*, even at 10 μg/ml (Fig. 9C,D). BMP7 also does not affect cell proliferation and apoptosis in explants (data not shown). When tested in explants of forebrain neuroectoderm from embryos at earlier stages (3-5 somites), BMP7 at 10 μg/ml induces *Msx1* expression at very low levels, while 2 μg/ml of BMP4 induces *Msx1* expression consistently at high levels in the same explant pieces (Fig. 9E). BMP6 does not induce any of these events at a concentration of 10 μg/ml in explants from either 10.5 d.p.c. or 8.0 d.p.c. embryos (Fig. 9F, and data not shown).

DISCUSSION

Expression of different *Bmp*s during forebrain development

In this study, we report a striking temporal and spatial co-localization of *Bmp* gene transcripts during anterior forebrain development. This co-localization may explain why no obvious abnormalities in embryonic brain development have been described so far in mice homozygous for null mutations in *Bmp5*, *Bmp7*, or *Bmp6* (King et al, 1994; Dudley et al., 1995; Luo et al., 1995; Elizabeth Robertson, personal communication). Our finding also raises the question of whether *Bmp*s regulate expression of one another in the neuroectoderm. However, individual BMP proteins did not induce other *Bmp* genes, nor did BMP4 induce its own expression in the explant system used in this study (data not shown).

There is evidence from chick spinal cord explants that BMP4 and BMP7 produced by the dorsal surface ectoderm function as dorsalizing signals to pattern the adjacent neuroectoderm, inducing expression of dorsal neural markers (Liem et al., 1995). The strong levels of *Bmp2* as well as weak levels of *Bmp5* and *Bmp7* RNA that we observe in the dorsal surface ectoderm in the cephalic region of the early mouse embryo (Fig. 1A,C,D) suggest that these BMPs are also involved in early patterning of the dorsal forebrain neuroectoderm. Thus, it may be *Bmp2* rather than *Bmp4* that plays the major role, fore example, in the specification of cephalic neural crest cells emigrating from this region at early somite stages (Osumi-

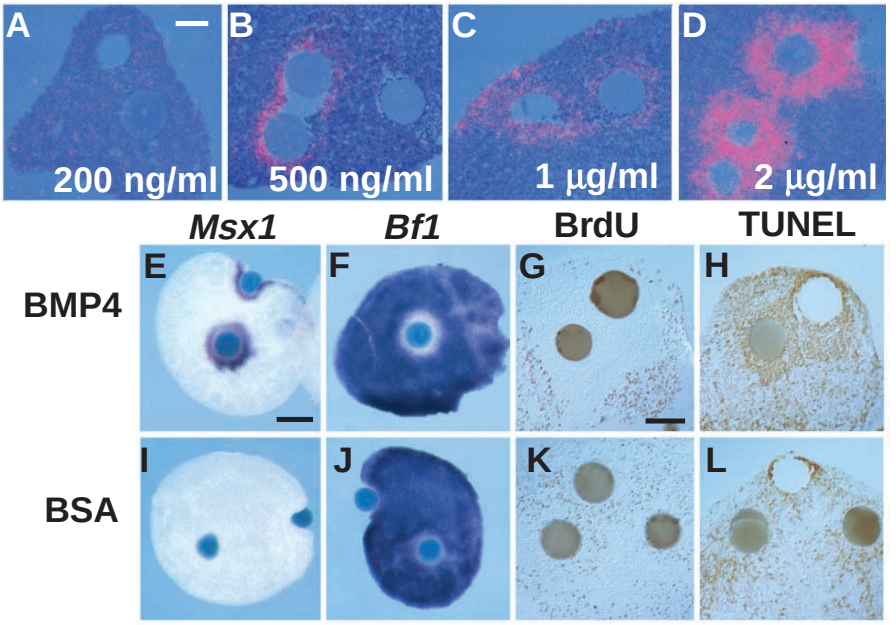


Fig. 7. Effect of BMP4 protein in explant cultures. (A-D) In situ hybridization on telencephalic explants showing dose dependency of the induction of *Msx1* expression by BMP4 protein after 24 hours of culture. (E,F) Whole mount in situ hybridization of the explants showing induction of *Msx1* expression (E) and suppression of *Bf1* expression (F) at 24 hours. (G) Suppression of cell proliferation by BMP4 in the explants at 24 hours. (H) In the adjacent section of G, significant accumulation of TUNEL-positive cells is notable around the BMP4-carrying beads. (I) BSA/PBS does not induce *Msx1* expression even after 48 hours of culture. (J) BSA/PBS has no effect for *Bf1* expression under the same conditions. (K) BSA/PBS beads do not affect cell proliferation. (L) A section near the one shown in K exhibiting random distribution of TUNEL-positive cells around the BSA/PBS beads. Bar, 50 μm (A-D), (E,E,H,I), (F,G,J,K).

Yamashita et al., 1994), since *Bmp4* is not yet expressed at this stage (Fig. 1B). Expression of *Bmp2* in a part of the forming dentate gyrus of the hippocampus is also intriguing (Fig. 4O). Although BMP2 showed similar activity to BMP4 in the explants, *Bmp2* may have a discrete function from other *Bmp*s in respect to hippocampus formation in vivo.

BMPs regulate cell death and expression of *Msx1* in the dorsal forebrain

Msx genes (reviewed by Davidson, 1995) have been implicated as downstream targets of BMPs in several developmental systems, such as the hindbrain, spinal cord, tooth, facial primordium and limb (Graham et al., 1994; Liem et al., 1995; Shimeld et al., 1996; Vainio et al., 1993; Barlow and West, 1997; Ganan et al., 1996). Two findings support the idea that *Msx1* is also downstream of BMPs in the telencephalon. First, *Msx1* expression in the developing dorsal telencephalon co-localizes with, and is preceded by, the expression of multiple *Bmp*s (Figs 1, 2, 5A-C,J-L). Second, ectopic *Msx1* expression is induced by BMP proteins in lateral telencephalic explants (Figs 7, 8A). It is intriguing that the induction of *Msx1* by BMP2/4 occurs in a discrete domain around beads in the explants, and does not appear to form a gradient, suggesting that a threshold level of BMP signaling is required for induction of *Msx1* transcription in the telencephalon.

We have also shown that BMP4 induces local apoptosis in explants (Figs 7H, 8G). This is consistent with the in vivo observation that in the dorsal midline of the anterior forebrain, where high level expression of *Bmp*s is detected (Figs 3, 6C), a significant number of the cells are undergoing programmed cell death by 10.5 d.p.c. (Fig. 6J,K). Furthermore, the zone of apoptosis is associated with the expression of *Msx1* both in vivo (Fig. 5C) and in vitro (Figs 7, 8). In several other developmental processes, expression of *Msx* genes is tightly correlated with BMP-mediated apoptosis. This has been demonstrated in the chick rhombencephalic neuroectoderm (Graham et al., 1994), as well as in the developing facial primordium (Barlow and West, 1997) and limb (Ganan et al., 1996) using ectopic application of BMP proteins. Conversely, downregulation of *Msx* gene expression is associated with the inhibition of interdigital cell death in the chick limb bud by blocking BMP signaling (Zou and Niswander, 1996). Thus, BMP-mediated apoptosis may occur by similar mechanisms in many developmental aspects, including the morphogenesis of the dorsal forebrain.

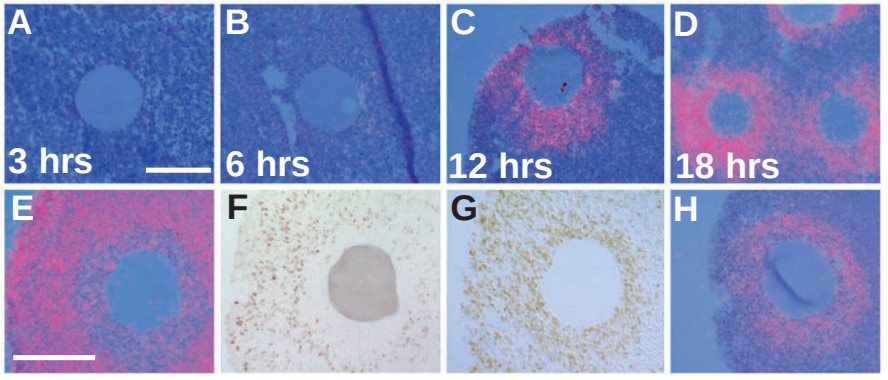


Fig. 8. Time course of BMP4 effects in explant cultures. (A-D) *Msx1* induction from 3 hours to 18 hours with beads soaked in 2 μg/ml of BMP4. (E-H) Four serial sections of an explant cultured with a bead carrying 2 μg/ml of BMP4 for 18 hours. Areas of *Bf1* downregulation (E), suppression of cell proliferation (F), apoptosis (G), and induction of *Msx1* (H) are shown. The intensity of the *Msx1* signal in D and H are not comparable since they represent different experiments. Bar, 50 μm (A-D), (E-H).

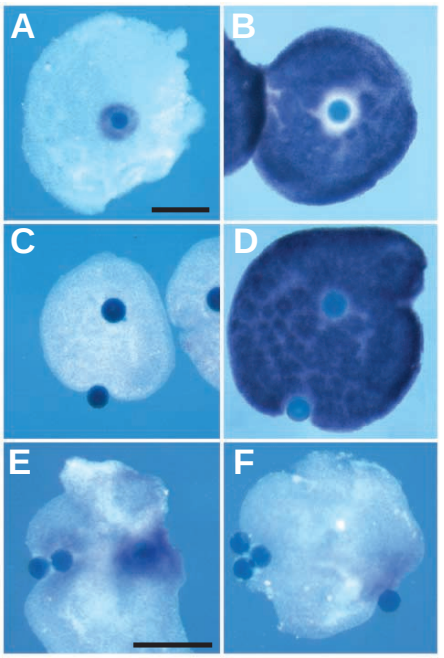


Fig. 9. Effects of other BMP proteins in explant culture. (A,B) Whole-mount in situ hybridization using *Msx1* (A) and *Bf1* (B) probes on explants cultured with beads carrying 2 μg/ml of BMP2. (C,D) BMP7 at 10 μg/ml does not induce *Msx1* expression (C) or *Bf1* suppression (D). (E) BMP7 can only induce low levels of *Msx1* expression in forebrain neuroectoderm explants from 8.0 d.p.c. embryos (2 beads on the left), whereas 2 μg/ml of BMP4 induces *Msx1* consistently at high levels (bead on the right). (F) BMP6 at 10 μg/ml does not induce *Msx1* expression on explants from 8.0 d.p.c. embryos (3 beads on the left) while *Msx1* induction is seen around the bead carrying 2 μg/ml of BMP4 on the right. Bar, 100 μm (A-D), (E,F).

BMPs and *Bf1* may function antagonistically during dorsal telencephalon development

The explant culture experiments demonstrate that BMPs induce a local suppression of cell proliferation in the telencephalic neuroectoderm (Figs 7G,8F). In vivo, the dorsal midline and the dorsomedial telencephalon, where multiple *Bmp* genes are expressed, have a reduced rate of cell proliferation, as shown in Fig. 6. Evidence from the targeted inactivation of the *Bf1* locus in the mouse shows that this forkhead/winged helix transcription factor is essential for continued proliferation of the cells in the telencephalon, and thus for the expansion of the brain hemispheres (Xuan et al., 1995). This suggests that the strictly complementary expression patterns of *Bmp*s and *Bf1* within the telencephalon at 10.5 d.p.c. (Fig. 5E,J-L) may, in part, be established by dorsally emanating BMP signals, locally inhibiting *Bf1* expression. This idea is supported by explant culture experiments, in which *Bf1* expression is downregulated by BMP4 in the lateral telencephalic neuroectoderm (Figs 7F, 9B). We thus speculate that *Bmp*s and *Bf1* function antagonistically during the morphogenesis of the telencephalon by differentially regulating cell proliferation.

Our in vitro studies suggest that suppression of *Bf1* in explants in response to BMPs occurs later than induction of *Msx1* (Fig. 8). This result may account for the in vivo observation that *Bmp* and *Bf1* expressions partially overlap in the dorsal telencephalon at 9.5 d.p.c. (see Figs 2, 5D-F), but are complementary by 10.5 d.p.c. (Fig. 5E,J-L). Suppression of cell proliferation and increase of apoptosis by BMP4 are also relatively late events in explants. Presently, we cannot fully exclude the possibility that the downregulation of *Bf1* transcripts in explants is a non-specific phenomenon due to cell death. However, substantial numbers of BrdU-labeled cells are present in the zone where *Bf1* downregulation occurs, and cells in this area still show high levels of *Msx1* expression (Fig. 8H). It is thus more likely that downregulation of *Bf1* is a consequence of BMP signaling rather than a non-specific effect due to extensive cell death.

Possible roles of BMPs in choroid plexus development and function

Co-expression of *Bmp*s correlates with the sites of choroid plexus differentiation (Figs 3, 4; Netsky and Shuangshoti, 1975; Thomas and Dziadek, 1993) and with *Hfh4* expression during early brain development (Fig. 5G-I). This raises the possibility that BMPs play a role in regulating the morphogenesis of the choroid plexus, a process that may require mechanisms such as restricted cell proliferation and/or local cell death to generate a thin monolayer of secretory columnar epithelium. In this study we have focused on the role of BMPs in the forebrain, but the same argument may hold for the hindbrain. In our attempts to test whether BMPs regulate choroid plexus specific genes, the expression of *Hfh4* is not induced by application of individual BMP proteins, or by combinations of these, in telencephalic explants (data not shown). One possibility still to be tested is that BMP proteins in heterodimeric combinations can induce *Hfh4* expression in the telencephalic neuroectoderm.

Expression of *Bmp*s in the choroid plexus also raises the possibility that the corresponding proteins are secreted into the cerebrospinal fluid. Secreted ligands might therefore be able to reach the ventricular layer of the lateral and ventral telencephalon, and function during later brain development. A few studies have described the expression of putative BMP receptor genes during embryogenesis. Among these, two type-I receptor genes, *Alk3* and *Alk6*, are widely expressed in the developing brain (Dewulf et al., 1995). Furthermore, it has been reported that BMPs can induce differentiation of the subventricular cells of the embryonic brain into particular glial cell types in culture (Gross et al., 1996).

So far, targeted inactivation of individual *Bmp* and receptor genes have not fully revealed the functional significance of BMPs during brain development. Generation of mice harboring compound mutations of multiple *Bmp*s and/or receptor genes, and tissue specific inactivation of these genes may be required to further elucidate this question. In combination with such genetic approaches, the explant culture system described here will potentially provide a useful paradigm to test functions of different individual BMPs or heterodimeric combinations during early forebrain development.

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