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BMP mRNA and Protein Expression in the Developing Mouse Olfactory System

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ABSTRACT

The bone morphogenetic proteins (BMPs) play fundamental roles during the organization of the central nervous system. The presence of these proteins has also been demonstrated in regions of the adult brain that are characterized by neural plasticity. In this study, we examined the expression of BMP4, 6, and 7 mRNAs and proteins in the murine olfactory system. The olfactory system is a useful model for studying cell proliferation and neural differentiation because both of these processes persist throughout life in the olfactory epithelium (OE) and olfactory bulb (OB). Our results demonstrate a differential expression of BMP4, 6, and 7 in the embryonic, postnatal, and adult olfactory system. In particular, BMP4 and BMP7 showed similar immunostaining patterns, being expressed in the olfactory region from the earliest stages studied (embryonic day 15.5) to adulthood. During development BMPs were expressed in the OE, olfactory bulb nerve layer, glomerular layer (GL), mitral cell layer (MCL), and subventricular zone. During the first postnatal week of life, BMP4 and 7 immunoreactivity (-ir) was particularly evident in the GL, MCL, and in the subependymal layer (SEL), which originates postnatally from the subventricular zone. In adults, BMP4 and 7 immunostaining was present in the GL and SEL. Within the SEL, BMP4 and 7 proteins were expressed primarily in association with the astrocytic glial compartment. BMP6-ir was always found in mature olfactory receptor neurons and their axonal projections to the OB. In summary, these data support the hypothesis that BMPs play a role in the morphogenesis of the olfactory system during development and in its plasticity during adulthood.

Indexing terms: neural plasticity; olfactory glomerulus; subventricular zone; transforming growth-factor- β

The bone morphogenetic proteins (BMPs) are a subclass of ligands that are members of the transforming growth factor- β (TGF β) superfamily of cytokines. This large group of molecules includes more than 20 members, which participate in a multitude of biological processes. BMPs exert their biological function by interacting with serine/threonine kinase receptors, including BMP receptor type-I and type-II (BMPRI and II). They play important roles throughout neural development, acting in cell proliferation, survival, lineage commitment, apoptosis, and differentiation. In early embryogenesis BMPs together with BMP-related factors, such as their natural antagonists/modulators noggin, chordin, tollid, and follistatin, are involved in neurulation

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and in subsequent dorsoventral patterning within the neural tube (Hogan, 1996; Dale et al., 1999; Bachiller et al., 2000). Later during development, BMPs play instructive and inductive roles in lineage elaboration within the peripheral and central nervous system (Mehler et al. 1997). The expression of BMPs and their receptors has been demonstrated within the embryonic germinal ventricular (VZ) and subventricular (SVZ) zones of the brain. In vivo and in vitro studies have shown that the undifferentiated precursor cells of the VZ and SVZ exhibit temporal and site-specific responsiveness to different members of the BMPs (Gross et al., 1996; Li et al., 1998; Mabie et al., 1999; Mehler et al., 2000). BMPs are also implicated in regional neuronal survival (Mehler et al., 1997; Shou et al., 1999), induction and maintenance of specific neuronal phenotypes (Lopez-Coviella et al., 2000), dendritic growth (Le Roux et al., 1999; Withers et al., 2000), and gap junctional communication (Bani Yaghoub et al., 2000). Furthermore, several reports suggest a role for these cytokines in neural plasticity. Transcripts for BMP type-I and type-II receptors are upregulated in neurons of the dentate gyrus after cortical injury in the same hemisphere (Ebendal et al., 1998), and sympathetic, hippocampal, and cerebral cortical neurons exposed to BMP7 in vitro exhibited enhanced dendritic growth without altering rates of cell survival (Lein et al., 1995; Le Roux et al., 1999; Withers et al., 2000). Taken together, these results demonstrate the involvement of BMPs in the development and differentiation of many cell types in the nervous system.

The aim of this study was to investigate the presence of BMP4, 6, and 7 in the developing and adult murine olfactory system. In rodents, the olfactory epithelium (OE) and olfactory bulb (OB) begin developing during the prenatal period. Morphologic and functional maturity of the OE and OB is reached during the first postnatal weeks, and a high degree of neural plasticity is retained in these regions into adulthood. Indeed, it is well known that, in the OE and OB of rodents, neurogenesis persists throughout life (Altman, 1969; Bayer, 1983; Brunjes and Frazier, 1986). The olfactory receptor neurons (ORNs) are capable of being replaced as a consequence of the mitotic activity of undifferentiated precursor cells located in the basal layers of the OE (Thornhill, 1970; Graziadei and Metcalf, 1971; Graziadei and Monti Graziadei, 1979; Calof et al., 1998). The ORNs project their axons to the OB, where they establish synapses with the dendrites of mitral/tufted and juxtglomerular cells in regions of neuropil called OB glomeruli. The replacement of ORNs can lead to reorganization of the neural circuits within the OB glomeruli (Graziadei and Monti Graziadei, 1978).

Recently, cell renewal occurring in the adult OB of rodents has been related to the migration of newly generated cells from the forebrain subependymal layer (SEL) (Luskin, 1993; Lois and Alvarez-Buylla, 1994). The SEL consists of a remnant of the embryonic subventricular zone that persists in the adult forebrain (Boulder Committee, 1970). In rodents, the newly formed cells migrate to the OB as immature elements along the SEL, within a channel system of astrocytic glial cells and processes called glial tubes (Lois et al., 1996; Peretto et al., 1997). After arrival in the OB, the SEL neuroblasts differentiate into granule and periglomerular cells (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Alvarez-Buylla et al., 2000).

Previous studies demonstrated that in cultures of embryonic OE, BMPs exert both positive and negative concentration-dependent effects on neurogenesis. Thus, high levels of BMP2, 4, and 7 inhibit neurogenesis, whereas low levels of BMP4 promote ORN survival. In addition, ligand-specific activity has been reported, e.g., BMP4 blocks the activity of noggin. Because noggin inhibits neurogenesis in this model, exogenous BMP4 results in an enhancement of neurogenesis in OE cultures (Shou et al., 1999, 2000). In addition, in vivo studies of the mouse OB during neonatal development have shown that mitral cells express BMPR-I and -II mRNA (Zhang et al., 1998). Finally, the abundant expression of several BMPs and their receptors has been reported in the OB and hippocampus of adult rat and mouse (Charytoniuk et al., 2000). These observations indicate that the expression of BMPs and/or related factors is maintained in regions of the postnatal and adult nervous systems that are characterized by continuing neural plasticity. The involvement of these proteins in modulating central nervous system (CNS) plasticity is further confirmed by studies suggesting that BMPs and their natural antagonist, noggin, participate in the regulation of neurogenesis of adult OB interneurons (Coskun et al., 2001; Lim et al., 2000). Our

results demonstrate a differential expression of BMP4, 6, and 7 in the embryonic, postnatal, and adult olfactory system. These data support the involvement of the BMPs in multiple actions that regulate developmental and neurogenetic events and provide a framework for future functional investigations.

MATERIALS AND METHODS

Western blot analysis

Olfactory bulbs and epithelia were dissected from CD-1 mice at specified ages and homogenized at 4°C in 50 mM Tris-HCl, pH 7.5, containing 2 mM dithiothreitol, 0.2 mM phenylmethyl sulfonyl fluoride, 5 µg/ml leupeptin, 10 µg/ml aprotinin, and centrifuged at 100,000 x *g* for 1 hour. Protein concentration of the supernatants was determined by the Bradford method (Bio-Rad, Hercules, CA). Equal amounts of protein in denaturing sample-buffer were separated by sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis on 10–20% ReadyGels (Bio-Rad) and electroblotted to Immobilon-P membranes (Millipore, Bedford, MA). Blocking was for 1 hour at room temperature or overnight at 4°C in 25 mM Tris-HCl, pH 7.4, 0.8% NaCl; 0.02% KCl, 0.05% Tween-20, and 1% bovine serum albumin (TBST/BSA). Western analyses were done with goat anti-BMP4, BMP6, or BMP7 (1:500 in TBS-Tween-BSA solution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hour at room temperature. Anti-BMP4 and 6 antibodies were directed against peptides at the amino terminus of the BMP4 and BMP6, respectively. Anti-BMP7 was directed against a peptide corresponding to an amino acid sequence near the carboxyl terminus of BMP7. After multiple rinses in TBST/BSA, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-goat antiserum (1:50,000 in TBST/BSA) for 1 hour at room temperature. Washes were in TBST and in TBS alone. The reaction was visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), and images were collected on Kodak X-OMAT film. Control reactions included (1) omission of primary antibodies, and (2) incubation of membranes in BMP antisera preabsorbed with 2 µg/ml of their respective peptide (Santa Cruz Biotechnology, Inc.).

Tissue preparation

Embryos. Timed pregnant CD-1 female mice (the morning after coitus was E0.5) were deeply anesthetized with ketamine/xylazine (100 mg/kg body weight/33 mg/kg body weight), and embryos were removed and perfused transcardially with ice-cold 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PFA). Embryo heads were removed, post-fixed in 4% PFA for several hours to overnight, embedded in 10% gelatin/0.1 M sodium phosphate buffer, pH 7.4 (PB), and further fixed and cryoprotected in 2% PFA with 30% sucrose/PB overnight at 4°C. Embedded tissue was then quick-frozen in cold isopentane (-70°C) and sectioned coronally on a cryostat at 35 µm for immunohistochemistry. Embryos used for in situ hybridization were perfused as above, post-fixed in 4% PFA, cryoprotected in 30% sucrose/PB overnight at 4°C, quick-frozen and sectioned at 8 µm. Sections for in situ hybridization (ISH) were mounted onto slides precoated with a 2% solution of 3-aminopropyltriethoxysilane and stored at -70°C until further processing (for details, see Schmale and Behrens, 1995).

Postnatal and adult stages. Brains and olfactory epithelia were obtained from CD-1 mice on postnatal days 2 (P2), P7, P14, P21, and adult. For each age group, data were analyzed from at least three different animals, including both sexes, from at least two different litters. Animals were deeply anesthetized with ketamine/xylazine (100 mg/kg body weight / 33 mg/kg body weight) and then perfused transcardially with ice-cold 0.9% NaCl (1 to 7 minutes) followed by 4% buffered PFA (20 to 100 ml). After dissection, brains and olfactory epithelia were post-fixed overnight at 4°C in the same fixative, cryoprotected in ascending sucrose solutions (7.5%, 15%, 30%), quick-frozen in cold isopentane (-70°C), and sectioned on a cryostat for immunohistochemistry (10 µm). Coronal

sections were serially collected onto Superfrost Plus slides (Fisher, Pittsburgh, PA). All procedures involving animals were approved by the University of Maryland School of Medicine's Institutional Animal Care and Use Committee.

Immunohistochemistry

Embryos. Sections were processed for conventional free-floating biotin-avidin system immunohistochemistry by using goat anti-BMP4, BMP6, or BMP7 (Santa Cruz Biotechnology, Inc.; dilutions: 1:100, 1:500, and 1:250, respectively) or goat anti-olfactory marker protein (OMP; dilution: 1:20,000; Keller and Margolis, 1975). Briefly, sections were incubated in 1% H₂O₂ in Tris buffered saline (TBS: 0.05 M Tris/HCl, pH 7.2, 0.25 M NaCl), to block endogenous peroxidases, rinsed in TBS, and transferred to blocking solution containing 0.1% gelatin, 5% normal horse serum (Vector Laboratories, Inc.), and 0.5% Triton X-100. Sections were then incubated in primary anti-serum diluted in TBS with 3% normal horse serum and 0.2% Triton X-100 overnight at 4°C. The next day, sections were rinsed in TBS, placed in biotinylated anti-goat secondary antiserum (1:200; Vector Laboratories, Inc.), rinsed, and incubated in avidin-biotin complex (ABC, 1:400; Vector Laboratories, Inc.). The reaction product was visualized with 0.15 mg/ml 3,3'-diaminobenzidine in TBS with 0.01% H₂O₂. Sections were then serially mounted onto Superfrost Plus slides (Fisher), air-dried, dehydrated in graded alcohols, cleared in xylenes, and cover-slipped by using D.P.X. mounting medium (Aldrich, Milwaukee, WI).

Postnatal and adult stages. In sections processed for conventional ABC immunohistochemistry on slides, the following primary antisera were used: goat anti-BMP4, BMP6, or BMP7 (dilutions: 1:100, 1:100, and 1:150, respectively; Santa Cruz Biotechnology, Inc.). All incubations were run at room temperature. Briefly, sections were rinsed in 0.1 M sodium phosphate-buffered saline (PBS), pH 7.4, then incubated overnight with primary antibodies diluted in PBS containing 0.1% Triton X-100 and 1% non-immune serum of the same donor species of the secondary antiserum. The next day, sections were rinsed in PBS and treated with biotinylated anti-goat secondary antiserum (1:200; Vector Laboratories, Inc.) for 1 hour, rinsed, and incubated in avidin-biotin complex (1:400; Vector Laboratories, Inc.) for 1 hour. After rinses in PBS and in 0.05 M TBS, pH 7.4, the reaction product was visualized with 0.15 mg/ml 3,3'-diaminobenzidine in TBS with 0.01% H₂O₂.

For dual immunofluorescence primary antibodies were used at the following concentrations: goat anti-BMP4, BMP6, or BMP7 (dilutions: 1:80, 1:60, and 1:80, respectively; Santa Cruz Biotechnology, Inc.); rabbit anti-gial fibrillary acidic protein (anti-GFAP; 1:500; DAKO Corporation, Carpinteria, CA); anti-PSA-NCAM (a monoclonal immunoglobulin [Ig] M antiserum raised against the capsular polysaccharides of meningococcus group B that share α -2,8-PSA residues with PSA-NCAM; 1:1,000; generously provided by Dr. G. Rougon, University of Marseille); monoclonal IgG anti-growth associated protein 43 (anti-GAP43; 1:150; F. Hoffmann-La Roche, Ltd., Basel, Switzerland); rabbit anti-olfactory marker protein (anti-OMP; 1:5,000; Keller and Margolis, 1975). Double labeling was carried out following the single-label protocol. Sections were incubated in primary antisera overnight, rinsed, and transferred to secondary antisera conjugated with either CY3 or biotin (diluted 1:800 and 1:200, respectively; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 90 minutes. When biotinylated secondary antisera were used, the sections were incubated in a tertiary streptavidin conjugated to CY2 (1:800; Jackson ImmunoResearch Laboratories, Inc.) for 1 hour. Sections were then cover-slipped with an anti-fade mounting medium (DAKO Corporation). Control reactions included (1) omission of the primary antibodies, and (2) incubation of sections in BMP antisera preabsorbed with 10 μ M of the respective peptides.

In situ hybridization

Probe preparation. BMP4 was a 467-bp polymerase chain reaction fragment generated by using 5'-CTGGGAAGAATCATGGACTG-3' as the forward and 5'-AAAGCAGACCTCTCACTGGT-3' as the reverse primers, and subcloned into pGEM-T. Linearization with *ApaI* or *BstXI* and transcription with T7 or Sp6 polymerases gave the sense or antisense digoxigenin (DIG) -11-UTP-labeled riboprobes, respectively (Schmale and Behrens, 1995; Behrens et al., 2000). BMP6 was an 893-bp EcoRI-SacI fragment cloned into pBS-SK generously provided by B. Hogan. Linearization with EcoRI or SacI and transcription with T3 or T7 polymerases gave the sense or anti-sense DIG-11-UTP-labeled riboprobes, respectively, as above.

Tissue processing. The details of this procedure are exactly as described previously (Schmale and Behrens, 1995; Behrens et al., 2000) and are briefly summarized here. Sections were immersed in 4% paraformaldehyde, 1x PBS, 1 mM MgCl₂ (pH 7.2) for 5 minutes; rinsed with 1x PBS (pH 7.4); ultraviolet-crosslinked with an energy of 120 mJ/cm²; rinsed again with 1x PBS (pH 7.4). The tissue was then treated with 0.2 M HCl for 10 minutes, with 1% Triton X-100 in 1x PBS (pH 7.4) for 2 minutes, and acetylated in 0.1 M triethanolamine (pH 8.0), 0.25% acetic anhydride. After a brief rinse in 1x PBS (pH 7.4), sections were submerged in prehybridization buffer (50% formamide, 0.75 M NaCl, 25 mM Pipes, 25 mM EDTA, 5x Denhardt's reagent, 0.2% SDS, 250 µg/ml yeast tRNA, 250 µg/ml herring sperm DNA; pH 6.8) freshly heated to 85°C for 10 minutes and incubated for 5 hours at 55°C in a humid chamber containing 50% formamide. Hybridization was done overnight at 55°C in a humid chamber with 50% formamide in hybridization buffer (25–80 ng/ml DIG-labeled riboprobes, and 100 mg/ml dextran sulfate in pre-hybridization buffer freshly heated to 85°C for 10 minutes). Stringency washes were done as follows: 3 x 5 minutes 2x SSC at room temperature, 30 minutes RNase-buffer (10 µg/ml RNase A, 0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA; pH 7.5) at 37°C, 15 minutes 0.4x SSC at room temperature, two times 0.4x SSC at 55°C. For immunohistochemical detection of DIG-labeled probes, the specimens were incubated at room temperature for 5 minutes in buffer 1 (150 mM NaCl, 100 mM maleic acid; pH 7.5), 60 minutes in buffer 1 containing 1% blocking reagent (Roche), 60 minutes in buffer 1 containing 1% blocking reagent, with 1:750 diluted anti-DIG-alkaline phosphatase, followed by two 30-minute washes in buffer 1. For colorimetric detection, slides were equilibrated in 100 mM Tris-HCl, 100 mM NaCl, 50mM MgCl₂; pH 9.5, and incubated in the same buffer containing 25 mg/ml 5-bromo-4-chloro-3-indoxyl phosphate, and 50 mg/ml nitroblue tetrazolium overnight. After stopping the color reaction with TE-buffer, sections were mounted by using a water-based mounting medium (Dako fluorescent mounting medium).

Preparation of Figures

Light photomicrographs were digitized by using a Phase One PowerPhase digital camera (Phase One, Denmark) fitted to a Leica DMRX microscope (Leica Microsystems Inc., Deerfield, IL). Analysis and digital photography of the double immunofluorescence was carried out by using a FluoView Olympus confocal microscope (Olympus, Melville, NJ) equipped with krypton and argon ion lasers and standard excitation and emission filters for the visualization of CY2 and CY3. Double-labeling visualization was performed by combining the visualization of one chromogen with the visualization of the second antigen with a different chromogen. Digital images were adjusted linearly, cropped, and assembled in Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA). Plates of photomicrographs were printed by using a Fuji Fujix Pictography 3000 printer (Fuji Photofilm USA, Carlstadt, NJ).

RESULTS

Western blot analysis

As an initial assessment of the presence of the BMPs in the olfactory system and to confirm the specificity of the antisera, extracts prepared from the OB and OE of post-natal and adult animals were immunoblotted with antibodies directed against BMP4, BMP6, or BMP7. The BMP4 antibody resulted in a band of approximately 30 kDa both in OB and OE extracts (Fig. 1B). The BMP6 antibody recognized a single band with a molecular weight of slightly less than 30 kDa. Protein preparations from animals at different ages (P10, P17, adult) revealed a gradual increase in the expression of BMP6 both in the olfactory bulb and epithelium (Fig. 1A). The BMP7 antibody identified a major band of approximately 46 kDa (Fig. 1C). Preincubation of each antiserum with the appropriate immunogenic peptide (2 μ g/ml) completely eliminated all immunoreactivity, confirming the specificity of these antisera.

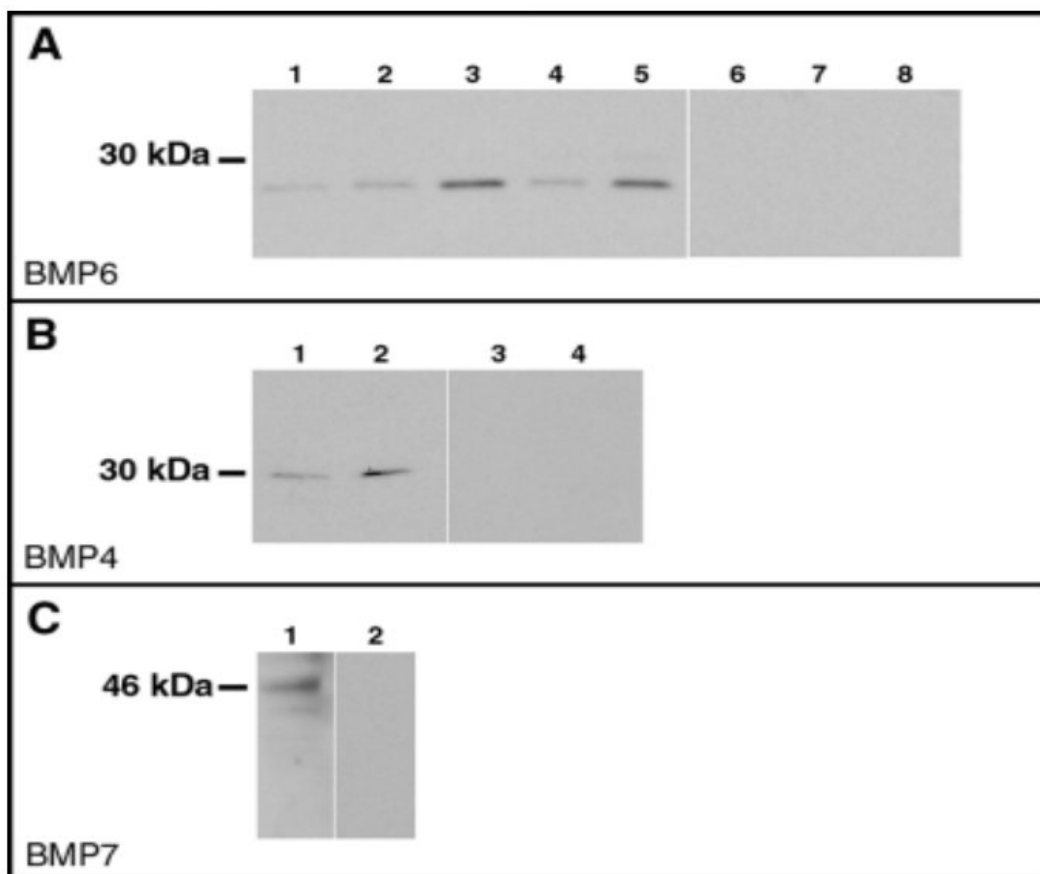


Fig. 1. Western blot analysis of bone morphogenetic protein (BMP) expression during the postnatal development of the olfactory system. Mouse tissue extracts (A: 20 μ g/lane; B: 34.5 μ g/lane; C: 30 μ g/lane;) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with different BMP antisera. A: Tissue extracts from olfactory bulbs (lane 1, 6: postnatal day [P] 10; lane 2, 7: P17; lane 3, 8: adult) and from olfactory epithelium (lane 4: P10; lane 5: adult) probed with anti-BMP6 (lanes 1–5) or with the antiserum pretreated with the appropriate peptide to block immunoreactivity (lanes 6 – 8). B: Tissue extracts from adult olfactory bulbs (lanes 1, 3) or from olfactory epithelia (lane 2, 4) probed with anti-BMP4 (lanes 1, 2) or with the antiserum blocked with the appropriate BMP4 peptide (lanes 2, 4). C: Tissue extracts from P17 olfactory bulbs probed with anti-BMP7 (lane 1) or with the antiserum blocked with its peptide (lane 2).

BMP4 localization changes during development

From E15.5 to E17.5, a diffuse BMP4 immunoreactivity (-ir) was identified in connective tissue around the developing OB and below the OE in the lamina propria (data not shown). At E19.5,

BMP4 immunostaining was less pronounced in the connective tissue and became visible in the developing OB, where it was identified in the olfactory nerve layer, glomerular layer, in the SVZ and in the emerging granule cell layer that surrounds the olfactory ventricle (Fig. 2C). Nonspecific staining was present along the mucus/cilia layer of the olfactory epithelium and in the meninges surrounding the OB (see Fig. 2C,D). This staining, visible in sections processed with and without primary antisera, was attributed to background. At this age, in situ hybridization with the BMP4 antisense probe labeled cells in the OE and in the mitral cell layer of the OB (Fig. 2A), illustrating the presence of BMP4 mRNA in ORN and mitral cell populations. Incubation with the BMP4 sense probe gave only background levels of hybridization, confirming the specificity of the analysis (Fig. 2B).

During the first postnatal week (P1–P7), BMP4-ir was particularly evident in the glomerular layer of the OB, where glomeruli were seen as intensely labeled globular structures (Fig. 2D). Strong immunostaining was also observed in the outer part of the internal plexiform layer and in the mitral cell layer, whereas the olfactory nerve layer and the external plexiform layer were less intensely stained. BMP4 expression was also identified in the SEL, which represents the remnant of the embryonic SVZ (see Fig. 2D).

The pattern of BMP4-ir at postnatal day (P) 14 resembled that seen at P7. At this stage, the immunostaining in the SEL was more intense and appeared in a web-like distribution (Fig. 2E). In adult animals, BMP4-ir was completely absent from the internal and external plexiform layers and very faint in the olfactory nerve and glomerular layers. Strong BMP4 immunostaining persisted, however, in the SEL (Fig. 2F). As in the Western blots, preincubation of the anti-BMP4 with the immunogenic peptide (10 μ M) completely abolished the immunoreactivity, confirming the specificity of the staining (data not shown).

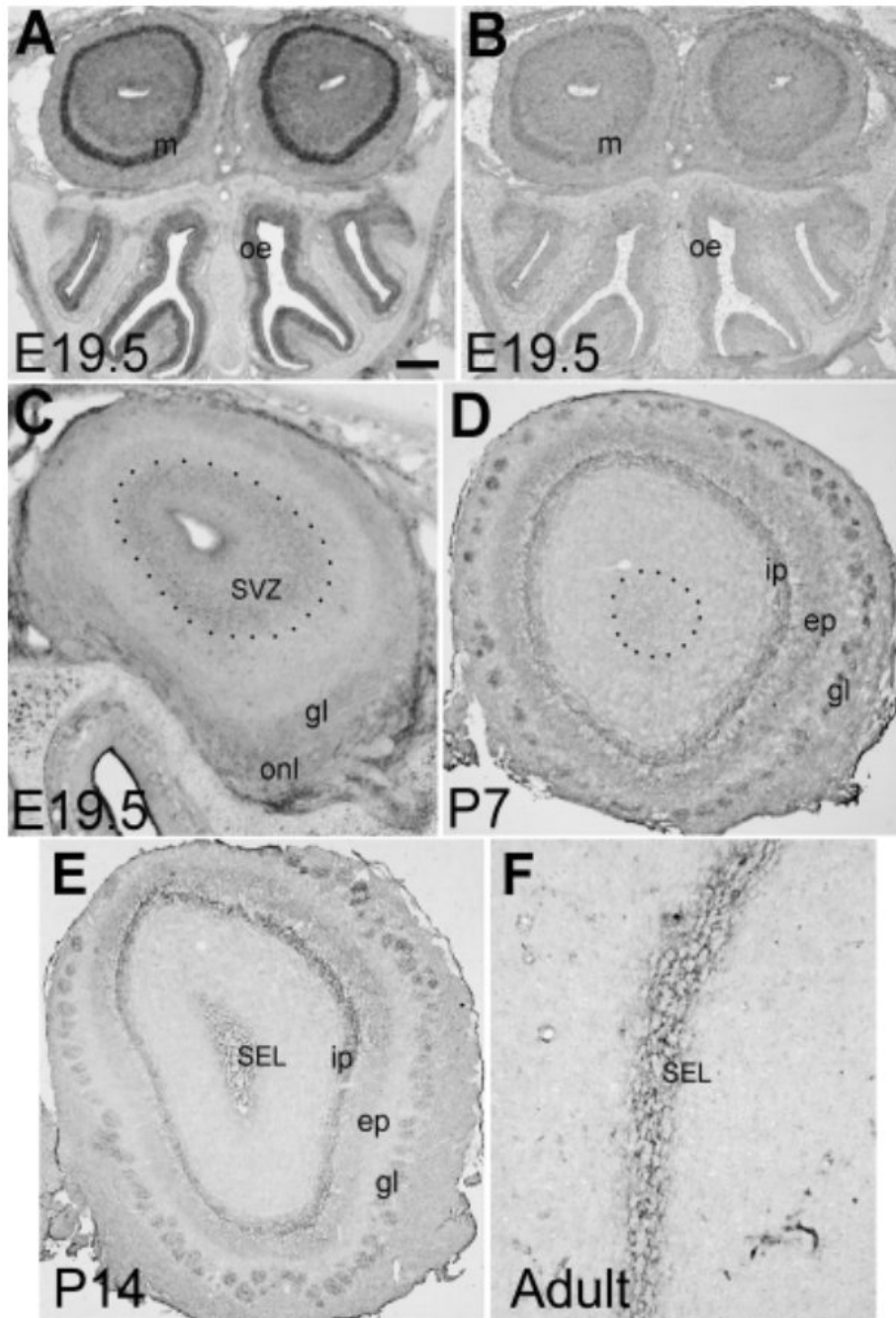


Fig. 2. Distribution of bone morphogenetic protein-4 (BMP4) mRNA and BMP4 immunoreactivity (-ir) in the olfactory system during development. Adjacent sections of an embryonic day (E) 19.5 olfactory system treated for in situ hybridization by using antisense BMP4 probe (A) and sense probe (B). A: In the olfactory epithelium (oe), labeled cells are visible in all neuronal layers of the epithelium, whereas BMP4 mRNA expression is restricted to the mitral cell layer (m) of the bulb. B: Use of the BMP4 sense probe resulted in background levels of hybridization only, confirming the probe specificity. C–F: Coronal sections of mouse OB immunostained with anti-BMP4 antisera. C: At E19.5 BMP4-ir is localized to connective tissue around the developing olfactory bulb (OB), in the olfactory nerve layer (onl), and in the emerging glomerular layer (gl). Labeling in the subventricular zone (SVZ) surrounding the olfactory ventricle is also visible. D: At postnatal day (P) 7, immunostaining is widely distributed in different OB layers: stronger in the glomeruli (gl) and in the internal plexiform layer (ip), weaker in the onl, and in the external plexiform layer (ep). The dotted line in C and D indicate the SVZ/SEL area. SEL, subependymal layer. E: At P14, BMP4-ir distribution is similar to that seen at P7, moreover strong immunostaining in the SEL appears. F: In the adult, the staining is restricted to the SEL; some endothelial cells around blood vessels are also immunopositive. Section thickness = 8 μ m in A,B, 35 μ m in C, 10 μ m in D–F. Scale bar in A = 200 μ m in A,B, 100 μ m in C, 125 μ m in D,E, 50 μ m in F.

BMP7 is expressed in olfactory nerves, the glomerular layer, and SVZ/SEL

The patterns of BMP4-ir and BMP7-ir were very similar during the early development of the olfactory system. From E15.5 to E17.5 BMP7 immunostaining was found primarily in regions of connective tissue around the bulb and, at E17.5, faint staining was also visible in tissue surrounding the olfactory ventricle (Fig. 3A). At E19.5 and P2 BMP7-ir was still present, but less intense, in areas of connective tissue, whereas stronger immunostaining was identified in cells located in the external part of the SVZ. At these perinatal ages, BMP7-ir began to appear in the developing glomerular layer (Fig. 3B). From P21 to adult-hood, BMP7-ir was evident in the olfactory nerve and glomerular layers. Intense immunoreactivity for BMP7 was also observed in the SEL (Fig. 3C). Like BMP4 immunostaining, BMP7-ir within the SEL exhibited a mesh-work appearance with no clearly distinguishable stained cell bodies. As seen with BMP4-ir, nonspecific staining was found in the mucus/cilia layer of the OE and in the meninges surrounding the OB (Fig. 3A, B).

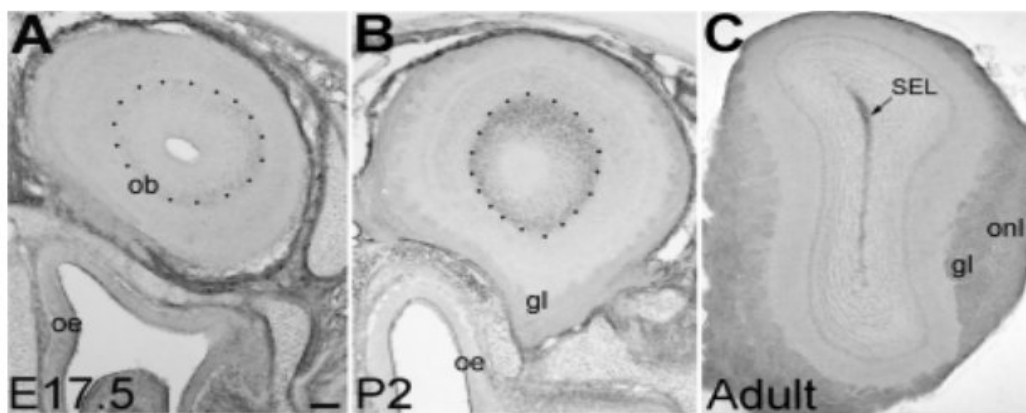


Fig. 3. Bone morphogenetic protein-7 (BMP7) immunoreactivity (-ir) in the olfactory bulb at different ages. A,B,C: Coronal sections. A: At embryonic day (E) 17.5, BMP7 immunostaining is visible in the connective tissue around the bulb (ob) and in cells of the subventricular zone (SVZ) within the bulb. B: At postnatal day (P) 2, BMP7-ir is similar to that observed for BMP4 in perinatal age (see Fig. 2C), i.e., connective tissue around the bulb, olfactory nerve layer (onl), glomerular layer (gl), and postnatal SVZ are stained. C: In the adult, BMP7-ir is still present in the onl and gl and is also localized strongly in the SEL and very weakly in the mitral cell layer. The dotted lines in A and B indicate the SVZ/subependymal layer region. Section thickness \approx 35 μ m in A–C. Scale bar in A \approx 100 μ m in A,B, 50 μ m in C.

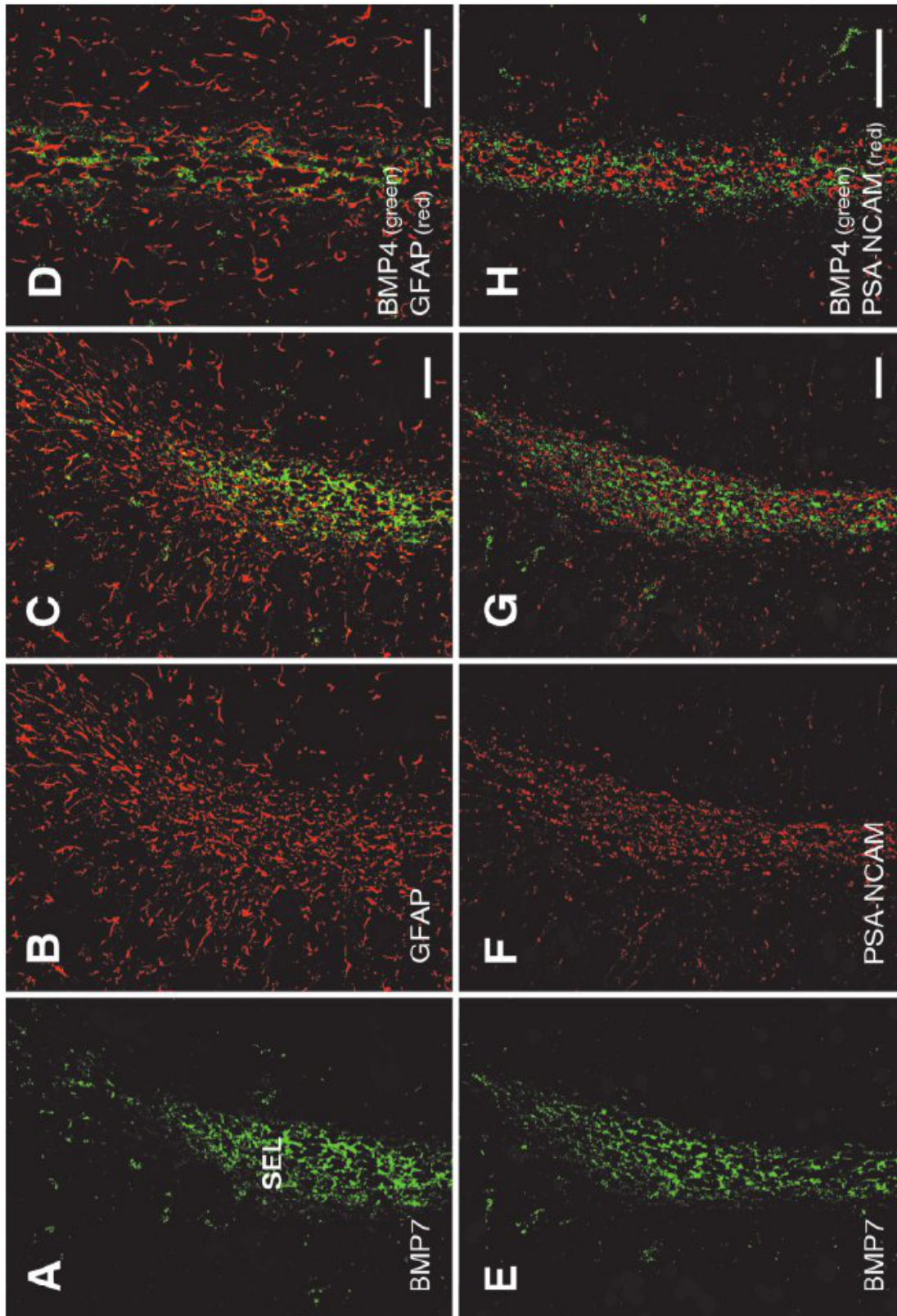


Fig. 4. Immunoreactivity for bone morphogenetic protein-7 (BMP7) or BMP4 and other markers in the subependymal layer (SEL) of adult olfactory bulb. Coronal sections. A–C: Double immunostaining for BMP7 and glial fibrillary acidic protein (GFAP). C: In the simultaneous view of BMP7 immunoreactivity (-ir) and GFAP-ir, only a partial overlap is detectable. D: A simultaneous view of BMP4-ir and GFAP-ir reveals a pattern of overlap that is similar to BMP7/GFAP immunostaining. E–G: Double immunostaining for BMP7 and PSA-NCAM. G: The simultaneous view of BMP7-ir and PSA-NCAM-ir shows no overlap of expression. H: Similarly, no overlap is observed in the simultaneous view of BMP4-ir and PSA-NCAM-ir. The similarity in overlap between BMP4 and 7 and these neuronal and glial markers concurs with our observation that BMP4 and BMP7 share the same expression pattern. Scale bars \bar{c} 50 μ m in C (applies to A–C), in D, in G (applies to E–G), in H.

Which component of the SEL expresses BMP4/7?

Strong BMP4 and BMP7 immunostaining appeared in the SEL of the P14 olfactory bulb. Because the antisera for BMP4 and BMP7 were produced in the same species, double-labeling immunostaining was not conducted. Examination of coronal sections from rostral forebrain showed that, within the SEL, the immunoreactivity for BMP4 and BMP7 had very similar times of onset and a meshwork-like distribution (see Figs. 2F, 3C). Therefore, we will refer to this immunostaining as BMP4/7-ir in the SEL. To identify the SEL cellular compartment labeled by BMP4/7-ir, double immunostaining with GFAP (marker of the astrocytic component) and PSA-NCAM (marker for the migrating cells) was performed (see Fig. 4). Image analysis of the double labeling was carried out with confocal microscopy by (1) examining the sum of optical sections scanned through a 10- μm thickness (as in Fig. 4C,G), or by (2) examining single 1- μm optical sections (as in Fig. 4D,H). The results obtained by using these two methods appeared comparable. Double immunostaining for GFAP and BMP4 or BMP7 revealed an overlap in the glial meshwork (Fig. 4C,D). The overlap was only partial, however, and at higher magnification it was possible to observe that the BMP4-ir or BMP7-ir was often at the edge of GFAP-immunopositive processes. This distribution was detected all along the SEL from the lateral ventricle to the olfactory bulb. Double immunolabeling with PSA-NCAM and BMP4 or BMP7 revealed no overlap in any regions of the SVZ at any age examined (Fig. 4G,H). However, the meshwork-like BMP4/7 immunostaining among the PSA-NCAM-positive cells was very evident in the SEL of the OB.

BMP6 is restricted to the olfactory nerve and glomeruli

During development, BMP6-ir is present in the OE from E15.5 to adulthood in the upper third of the OE and in the axon bundles coursing through the lamina propria toward the OB (see Fig. 5A). From E15.5 to E17.5, BMP6-ir was visible in the olfactory nerve and nerve layer of the OB. At E17.5 in the OE region, strong staining was observed in association with axon bundles below the mucosa (Fig. 5A). At E19.5, the antisense probe for BMP6 hybridized to the most superficial olfactory receptor neurons in the epithelium (Fig. 5C), indicating that BMP6 mRNA is present in these cells. Incubation of adjacent sections with the BMP6 sense probe resulted in background levels of hybridization (Fig. 5D).

After birth, BMP6 immunostaining in the bulb was found in the emerging glomerular layer as well as in the olfactory nerve layer. In these layers, strong BMP6-ir was observed in early postnatal olfactory bulb tissue, e.g., at P7 (Fig. 5E); however, by adulthood, some glomeruli exhibited more intense levels of immunostaining than others (Fig. 5F). Incubation of tissue sections with BMP6 antisera preabsorbed with the appropriate immunogenic peptide (10⁻⁶ M) completely abolished the immunoreactivity leaving only some nonspecific staining in cartilage (see Fig. 5B).

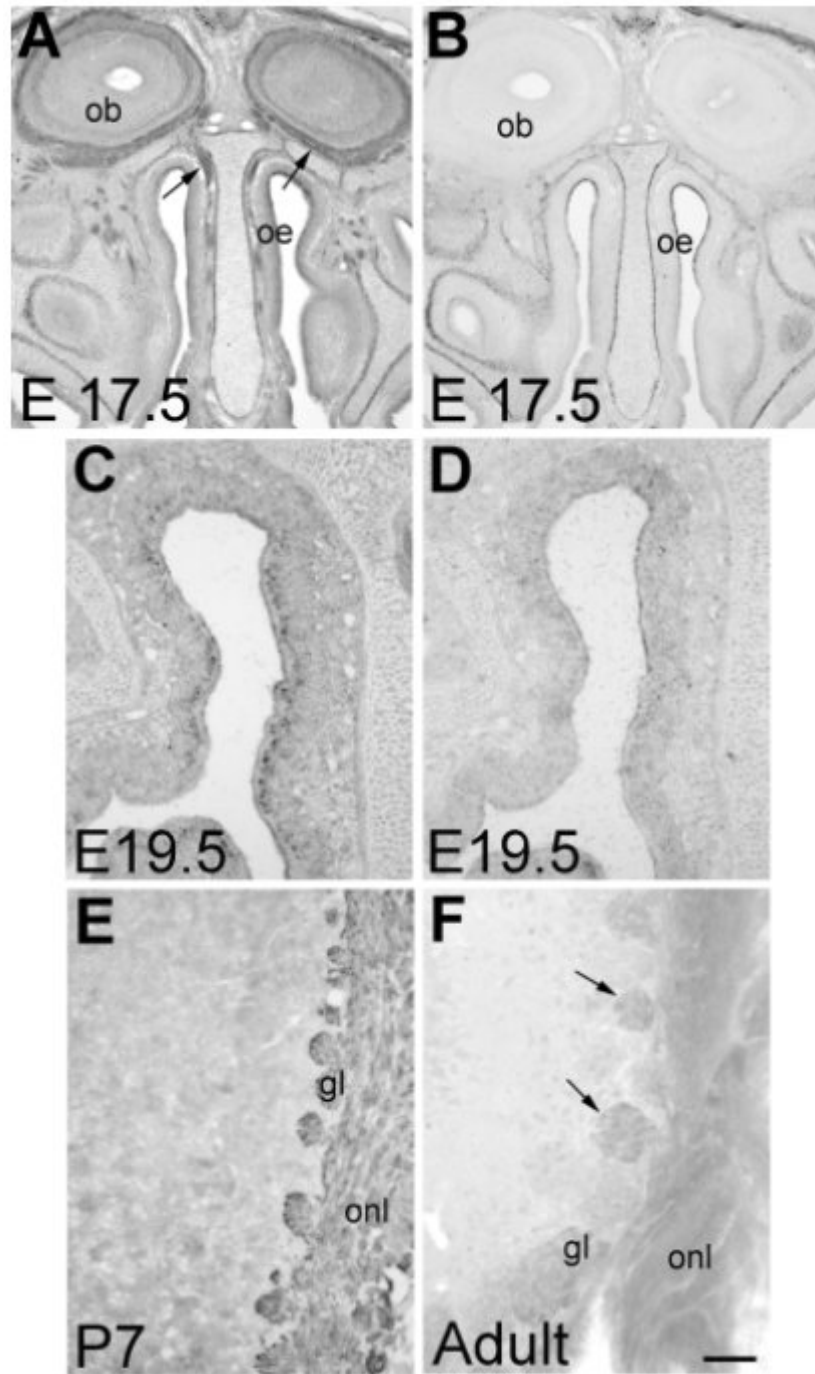


Fig. 5. Distribution of bone morphogenetic protein-6 (BMP6) immunoreactivity and BMP6 mRNA. A,B: Adjacent sections of an embryonic day (E) 17.5 olfactory system treated with anti-BMP6 antiserum (A) or with the antiserum absorbed with its peptide (B). A: Arrows indicate BMP6-ir in the olfactory nerves surrounding the bulbs (ob) and in the axon bundles below the olfactory epithelium (oe). The subventricular zone (SVZ) in the bulb is also weakly stained. B: In preabsorbed control sections, immunostaining in the axon bundles, nerve layer, and glomerular layer is blocked. Nonspecific immunoreactivity remains around cartilage and in the vomeronasal organ. C,D: E19.5 adjacent coronal sections of olfactory epithelium treated for in situ hybridization with antisense (C) and sense probes (D). C: BMP6-mRNA expression is visible in olfactory receptor neurons in the most superficial layer of the epithelium. D: In tissue treated with the sense probe, no hybridization is present. E,F: BMP6-ir in coronal sections of olfactory bulb. E: At postnatal day 7, a strong immunostaining is evident in the olfactory nerve layer (onl) and in all glomeruli (gl). F: In the adult olfactory bulb, the staining appeared to decrease in intensity. In the gl, BMP6-ir exhibits a patchy distribution: some glomeruli show stronger immunostaining (arrows) than others. Section thickness = 35 μ m in A,B,F, 8 μ m in C,D, 10 μ m in E. Scale bar in F = 225 μ m in A,B, 100 μ m in C,D, 65 μ m in E,F.

BMP6-ir is predominantly colocalized with OMP-ir

To identify which component(s) of the OE express BMP6, we performed double immunofluorescence reactions with BMP6 and GAP43 (a marker for immature ORNs; Verhaagen et al., 1989; Schwob et al., 1992) or OMP (a marker for mature ORNs; Margolis, 1972) in the OE and OB of young adult mice. Double immunostaining with BMP6 (Fig. 6A,D) and GAP43 (Fig. 6B,E) antisera revealed a minimal overlap; only a few dendrites extending from GAP43-immunopositive neurons, and some rare cell bodies (arrow, Fig. 6F), appeared double labeled (Fig. 6C,F). Furthermore, there appeared to be incomplete overlap in BMP6-ir and GAP43-ir between axons coursing through the large nerve bundles (Fig. 6C,F). Double immunofluorescence with BMP6 and OMP antisera showed a more extensive overlap of OMP and BMP6 in the nerve bundles compared with that seen in sections double-labeled for BMP6 and GAP43 (Fig. 6C,I). In addition, many neurons double-labeled for BMP6 and OMP were present in the upper third of the OE (Fig. 6I,L), and strong BMP6-ir was found in the most superficial part of the epithelium corresponding to the sustentacular cell layer. These data suggest that BMP6 is expressed in mature ORNs, their apical dendrites, and perhaps sustentacular cells.

In the OB, BMP6-ir was expressed in the olfactory nerve and glomerular layers (Fig. 7A,D). Dual labeling with BMP6 and GAP43 antisera was observed in the olfactory nerve layer and in a few discrete regions within glomeruli (Fig. 7A–C). These foci were concentrated mostly in the caps of the glomeruli, where olfactory axons enter, and around the edges. In the OB, BMP6 and OMP expression patterns were very similar, both appeared homogeneously distributed in the olfactory nerve layer and throughout the glomerular layer (Fig. 7D–F). The overlap of BMP6-ir and OMP-ir was almost complete, although BMP6-ir appeared more intense around the edges of glomeruli compared with the center (Fig. 7D). This observation is interesting and may relate to the compartmental organization of the olfactory glomerulus that has been observed with other markers (Kosaka et al., 1998; Kasowski et al., 1999). In addition, some axons in the olfactory nerve layer stained positively for either OMP or BMP6 alone. This result concurs with our observation in the axon bundles of the epithelium (see Fig. 6I) and indicates heterogeneity of coexpression of OMP and BMP6. All the results regarding the expression and distribution of BMP4, 6, and 7 mRNA and protein are summarized in Table 1.

DISCUSSION

In this study, we have analyzed the expression of BMP4, 6, and 7 mRNA and protein in the mouse olfactory system during embryonic and postnatal development and in adulthood. BMPs represent a subclass of ligands of the TGF β superfamily of cytokines that exhibit a multitude of biological effects during development and regeneration. We focused our attention on the olfactory system, because it retains a high degree of neural plasticity throughout life both in the OE (where ORNs can be continuously replaced) and in the OB (where olfactory glomerular reorganization and protracted neurogenesis occurs). Our results demonstrate a precise spatial and temporal expression of these cytokines in both the OE and OB. These data are consistent with the growing literature regarding the multitude of roles played by BMPs in regulating mechanisms of neurogenesis, differentiation, and rearrangement of neural synaptic circuits in the embryonic CNS and in particular areas of the adult brain.

BMP4 and BMP7 in the olfactory mucosa and in the OB

Although BMP4 and 7 are considered members of separate subgroups of BMPs (Kingsley, 1994) and double-immunofluorescence experiments were not performed because both the antibodies were raised in the same donor species, our data show that, in the olfactory system, these proteins share similar patterns of expression during embryonic and postnatal life. Our *in situ* hybridization and immunohistochemical analyses demonstrate that the changes in BMP4 and 7 localization during

development occur in parallel. In the early stages of embryonic development, a diffuse immunoreactivity for these secreted factors was identified in the connective tissue around the developing OB and below the OE (see Fig. 3A). At E19.5, ISH showed that BMP4 mRNA is present in the OE and in the MCL of the olfactory bulb. At this same age, BMP4 and 7 immunostaining became evident in the olfactory bulb nerve layer (ONL) and in the forming glomerular layer (GL). These data suggest that, during embryonic development, both ORNs and mitral cells can produce and transport these proteins along their neuritic processes.

The presence of BMP4 and 7, together with BMP6 (see below), suggests a role for these factors in the ontogenesis of the OE. This suggestion is further supported by two recent studies (Shou et al., 1999, 2000) demonstrating that BMP2, 4, and 7 are expressed in the embryonic OE region. *In vitro*, high concentrations of BMP2, 4, or 7 inhibit neurogenesis by acting on an early stage progenitor in the ORN lineage by means of inactivation of the transcription factor MASH-1 (Shou et al., 1999), which is required for ORN production *in vivo* (Guillemot and Joyner, 1993). On the other hand, at concentrations below the threshold for inhibition of neurogenesis, BMP4 increases neurogenesis by promoting the survival of newly generated ORNs (Shou et al., 2000).

As noted above, the pattern of BMP-ir changes during the first postnatal days of life. In particular, between P1 and P7, we found an increase of BMP4 and 7 in the olfactory glomeruli and a concomitant reduction of immunoreactivity in the ONL. This temporal rise of BMP-ir in the olfactory glomeruli suggests an involvement of these proteins in processes regulating the axonal targeting of ORNs and glomerular maturation. Indeed, it is interesting to note that olfactory axons coalesce into glomeruli before birth; however, the MC apical dendrites initially arborize over several glomeruli during the first postnatal week of life and then become restricted to individual glomeruli (Malun and Brunjes, 1996; Bailey et al., 1999). The rearrangements of the MC dendrites and the subsequent synaptic stabilization between these dendrites and the ORN axons might be driven by the activity of secreted factors such as the BMPs. The involvement of BMPs and their receptors in the process of dendritic formation and maturation is well documented in other systems (Lein et al., 1995; Le Roux et al., 1999; Withers et al., 2000). In particular, Withers and colleagues working on cultures of hippocampal neurons demonstrated that BMP7 acts as a growth factor selective for dendritic development. Addition of BMP7 to cultured hippocampal neurons results in a rapid and profound acceleration of dendritic growth and enhances synaptogenesis. Since the presence of BMPs and their receptors have been described *in vivo* in the adult hippocampus and OB (Charytoniuk et al., 2000), regions where the dendritic arbor continues to be remodeled throughout life (Graziadei and Monti Graziadei, 1979; Stanfield and Trice, 1988), these authors suggest a role for BMP7 in modulating this type of plasticity. Furthermore, enhancement of dendritic growth due to the BMPs has been demonstrated for sympathetic (Lein et al., 1995) and cerebral cortical (Le Roux et al., 1999) neurons.

Our results, showing a precise temporal and spatial expression of BMP4 and 7, are consistent with a possible involvement of these secreted proteins in mechanisms regulating the glomerular synaptic organization both during ontogenesis and adulthood. These reports are consistent with our results showing (1) the presence of BMP4 and 7 in the olfactory bulb glomerular layer from the early postnatal period to adulthood, and (2) a peak of expression of these proteins in the olfactory glomeruli during the first weeks of postnatal life, concomitant with extensive remodeling of the mitral cell dendritic arbors. In fact, the synaptic contacts between the axons of ORNs and dendrites of mitral and tufted cells are capable of being remodeled throughout life.

Finally, the decrease of BMP immunoreactivity in the ONL observed during the early postnatal period could indicate that, at this stage, the mitral cells are the principal source of BMP7 and 4. Alternatively, these proteins may be produced by the ORNs with subsequent transport along their axons and release at the level of the presynaptic terminals, as are some neurotrophins (Altar and DiStefano, 1998). This latter possibility is also supported by our Western blot data, indicating persistence of BMP4 in the OE region of adult animals. Evaluation of these alternatives awaits analysis of BMP secretion patterns.

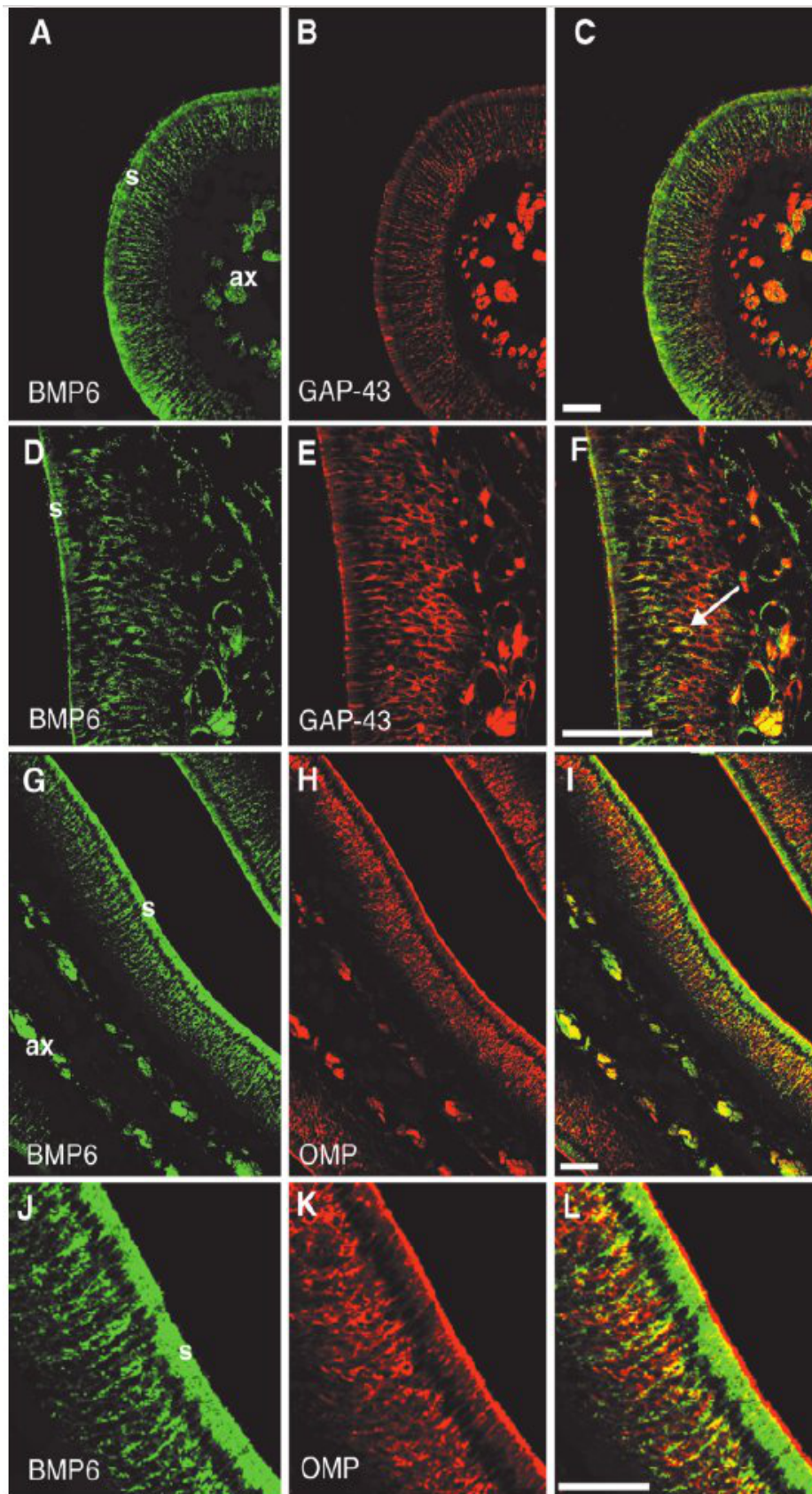


Fig. 6. Immunoreactivity for bone morphogenetic protein-6 (BMP6) and other antigens in olfactory epithelium of a postnatal day 21 mouse (coronal sections). A–F: Double immunostaining of BMP6/ GAP43. GAP43, growth associated protein 43. G–L. Double immunostaining of BMP6/OMP. OMP, olfactory marker protein. C,F,I,L: Simultaneous views of BMP6 immunoreactive (-ir) and GAP43-ir (C,F) and of BMP6-ir and OMP-ir (I,L) illustrate the partial overlap of BMP6/GAP43 expression and the greater overlap of BMP6/OMP expression. s, sustentacular cell layer; ax, olfactory axons. Scale bars = 50 μ m in C (applies to A–C), in F (applies to D–F), in I (applies to G–I), in L (applies to J–L).

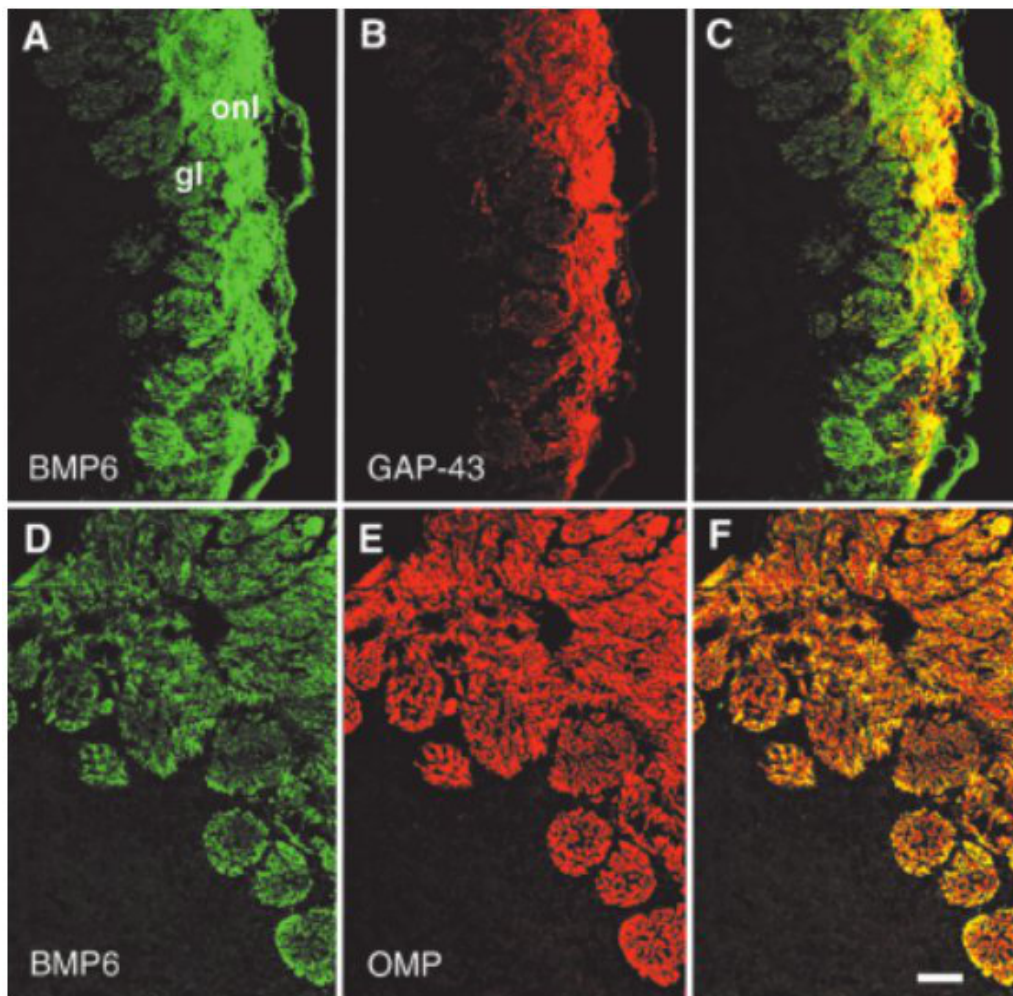


Fig. 7. Immunoreactivity for bone morphogenetic protein-6 (BMP6) and other antigens in the olfactory bulb of a postnatal day 21 mouse (coronal sections). A–F: Dual labeling for BMP6/GAP43 (A–C), and for BMP6/OMP (D–F). GAP43, growth associated protein 43; OMP, olfactory marker protein. C: Simultaneous view of BMP6 immunoreactivity (-ir) and GAP43-ir shows that these antigens are both expressed along the olfactory nerve (onl) but are not colocalized in glomeruli (gl). F: Simultaneous view of BMP6-ir and OMP-ir demonstrates complete overlap in these expression patterns. Scale bar = 50 μ m in F (applies to A–F).

TABLE 1. BMPs Distribution in the Olfactory System ¹

	Olfactory epithelium region		Olfactory bulb						
	OE	OS	ONL	GL	EPL	ML	IPL	GRL	SVZ/SEL
BMP4/BMP7									
Prenatal E15.5–E19.5	++ (ISH)	+ (ICC)	++ (ICC)	+ (ICC)	+ (ICC)	+++ (ISH)	–	–	++ (ICC)
Postnatal P1–P20	–	–	+ (ICC)	+++ (ICC)	+ (ICC)	+ (ICC-ISH)	++ (ICC)	–	++ (ICC)
Young/adult P30–P90	–	–	++ wb ³	+ (ICC)	–	–	–	–	+++ (ICC)
	+++ wb ²		+ (ICC)	+					
			+++ wb ²						
BMP6									
Prenatal E15.5–E19.5	++ (ICC-ISH)	+++ (ICC)	+++ (ICC)	+ (ICC)	–	–	–	–	–
Postnatal P1–P20	++ (ICC)	++ (ICC)	+++ (ICC)	+++ (ICC)	–	–	–	–	–
	++ wb		++ wb						
Young/adult P30–P90	++ (ICC)	++ (ICC)	++ (ICC)	++ (ICC)	–	–	–	–	–
	+++ wb		+++ wb						

¹Identification of bone morphogenetic protein 4 (BMP4), BMP7, and BMP6 in the olfactory system of prenatal (E15.5–E19.5), postnatal (P1–P14), and young/adult (P30–P90) mice. Because BMP4 and BMP7 share similar patterns of localization, they were reported in the same tab. These results were obtained by using immunocytochemistry (ICC), in situ hybridization (ISH), and Western blot (wb) analysis. For details see the text. OE, olfactory epithelium; OS, olfactory submucosa; ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; ML, mitral cell layer; IPL, internal plexiform layer; GRL, granule cells layer; SVZ/SEL, subventricular zone/subependymal layer.

²Related to BMP4.

³Related to BMP7.

BMP4/7 expression in the SVZ/SEL of mice

We have identified BMP4-ir and BMP7-ir in the germinative SVZ within the embryonic OB beginning as early as E19.5. These observations are in agreement with several *in vivo* and *in vitro* studies showing that BMPs and their receptors are present in the embryonic ventricular and subventricular germinal zones where they play temporal and spatial-specific roles in modulating the proliferation, survival, and differentiation of the neural precursor cells. BMP2, 4, 6, 7, and 13 inhibited cell proliferation and promoted neuronal differentiation of neocortical precursors in the VZ (Li et al., 1998). BMP4 and 7, and BMP receptors are expressed in the SVZ precursor cells. The addition of BMP2, 4, 6, or 7 to cultured, dissociated SVZ precursor cells resulted in astroglial lineage commitment (Gross et al., 1996). We have demonstrated strong expression of BMP4 and 7 in the postnatal and adult SEL. This region is interposed between the lateral ventricles and the OB and originates during the first days of postnatal life from the SVZ after the occlusion of the olfactory ventricle (Boulder Committee, 1970). The SEL of adult rodents shows an interesting organization (glial tubes and chains of migrating newly formed cells) and is considered the source of the newly generated neuroblasts of the OB (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Peretto et al., 1999). During postnatal life, the expression of BMP4/7 within the SEL of the OB increases, and from approximately P14, a web-like distribution, resembling glial tubes, can be recognized.

It is interesting to note that, in the SEL of neonatal rats, neither glial tubes nor chains of migrating neuroblasts are detectable before the end of the second postnatal week of life (personal observations). Moreover, in adults, double immunostaining for BMP4 and 7 with markers for both the glial tubes and migrating neuroblasts showed that these cytokines are not associated with one specific SEL component (see Fig. 4). However, dual labeling with BMP4/7 and GFAP antibodies indicated a partial colocalization among the astrocytic cells of the glial tubes (see Fig. 4C,D). Taken together, these data support our hypothesis that BMP4/7 is secreted by SEL glia as early as the second week of postnatal life, when the typical adult compartmentalization occurs in this region. This postulated secretion of BMPs by the astrocytic glia within the SEL suggests that BMP4/7 participate in the regulation of adult neurogenesis, which is consistent with different studies showing the presence of BMPs and their receptors in the SEL region, and that the glial tubes can secrete extracellular matrix molecules that may also be involved in regulating proliferation, migration, differentiation, and fate of the SEL neuroblasts (Gates et al., 1995; Jankovski and Sotelo 1996; Coskun et al., 2001; Lim et al., 2000). It is noteworthy that Lim et al., 2000, have demonstrated that the activity of the BMPs in the SEL of the lateral ventricle is modulated by the action of noggin, one of their natural antagonists, which is secreted by the ependymal cells lining the ventricles. Their concomitant action creates a niche for adult neurogenesis. Ongoing studies are needed to elucidate the role of BMPs in the most anterior parts of the SEL and the possible presence of antagonists or modulatory factors.

BMP6 expression in the OE and OB

The first occurrence of BMP6 in the OE was identified at E15.5, and high levels of expression in this area and in the OB are preserved into adulthood. Both mRNA and protein expression were found in the upper third of the olfactory mucosa. BMP6-ir was also identified in axon fascicles below the mucosa and coursing toward the OB, suggesting that mature ORNs express BMP6. This suggestion was further confirmed in adult animals by double immunostaining for BMP6 and specific markers for immature and mature ORNs (see Figs. 6, 7). A strong colocalization between BMP6-ir and OMP-ir was found in the OE and OB. OMP is expressed only by mature olfactory receptor cells and is present in their perikarya, dendrites, axons, and terminals in the OB (Margolis, 1972; Graziadei et al., 1980; Farbman and Margolis, 1980). Whereas a partial colocalization between BMP6 and GAP43 (a marker for immature ORNs) was found in the olfactory axon bundles located below the olfactory mucosa and within the ONL, only a few double-immunopositive BMP6/GAP43 ORNs were observed in the OE. These data confirm that BMP6 is primarily

associated with mature ORNs and suggests a role for this protein in the process of maturation of the ORNs, e.g., the switch from GAP43 to OMP expression. As described for BMP4 and 7, the persistence of BMP6-ir in the OE and in the OB glomeruli might be also related to synaptic regulation of the olfactory glomerular circuits.

In conclusion, the patterns of distribution of BMP4, 6, and 7 in the OE and bulb described in this study support the idea that these factors play a role in the morphogenesis of the olfactory system both during development and during the regenerative activity present in adult animals. Within this context, it is particularly intriguing to consider the roles played by several BMP antagonists such as noggin, a putative modulator of neurogenesis and specification (McMahon et al., 1998; Lim et al., 2000). Future studies will be directed at ascertaining the postulated roles of these proteins in olfactory system plasticity, particularly in the functional organization of the olfactory glomeruli.

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