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# EXPRESSION OF THE SECRETED FACTORS NOGGIN AND BONE MORPHOGENETIC PROTEINS IN THE SUBPENDYMAL LAYER AND OLFACTORY BULB OF THE ADULT MOUSE BRAIN

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**Abstract** - The antagonism between noggin and the bone morphogenetic proteins (BMPs) plays a key role during CNS morphogenesis and differentiation. Recent studies indicate that these secreted factors are also widely expressed in the postnatal and adult mammalian brain in areas characterized by different types of neural plasticity. In particular, significant levels of noggin and BMP expression have been described in the rodent olfactory system. In the mammalian forebrain, the olfactory bulb (OB) and associated subependymal layer (SEL) are documented as sites of adult neurogenesis. Here, using multiple approaches, including the analysis of noggin-LacZ heterozygous mice, we report the expression of noggin and two members of the BMP family, BMP4 and BMP7, in these regions of the adult mammalian forebrain. We observe that along the full extent of the SEL, from the lateral ventricle to the olfactory bulb, noggin and BMP4 and 7 are mainly associated with the astrocytic glial compartment. In the OB, BMP4 and 7 proteins remain primarily associated with the SEL while strong noggin expression was also found in cells located in different OB layers (i.e. granule, external plexiform, glomerular layers). Taken together our data lead us to hypothesize that within the SEL the antagonism between noggin and BMPs, both produced by the glial tubes, act through autocrine/paracrine inductive mechanisms to maintain a neurogenetic environment all the way from the lateral ventricle to the olfactory bulb. In the OB, their expression patterns suggest multiple regulatory roles on the unusual neural plasticity exhibited by this region.

**Key words:** adult neurogenesis, subventricular zone, olfactory system, glial tubes, neurogenetic niche, cytokines.

The fundamental roles played during vertebrate embryonic development by the bone morphogenetic proteins (BMPs) and their modulators such as noggin have become well established in recent years (for review see Hogan, 1996; Mehler et al., 1997). In early embryogenesis, these secreted proteins are involved in neurulation and in subsequent dorso-ventral patterning within

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Abbreviations: BMP, bone morphogenetic protein;  $\beta$ -gal,  $\beta$ -galactosidase; BSA, bovine serum albumin; GFAP, glial fibrillary acidic protein; LV, lateral ventricle; OB, olfactory bulb; PB, phosphate-buffered saline; PFA, paraformaldehyde; PSA-NCAM, polysialylated neural cell adhesion molecule; PVDF, polyvinylidenedifluoride; RE, rostral extension; SEL, subependymal layer; SVZ, subventricular zone; TBS, Tris-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside.

the neural tube (Hogan, 1996; Dale et al., 1999; Bachiller et al., 2000; Briscoe and Ericson, 2001). Later in development, BMPs, and their regulators, receptors and antagonists, are expressed within the germinal zones of the CNS where they play instructive and inductive roles in lineage specification (Mehler et al., 1997; Li and Lo Turco, 2000; Stull et al., 2001). Recent studies have shown that different BMPs and noggin are still present in the postnatal and adult mammalian CNS (Valenzuela et al., 1995; Lim et al., 2000; Peretto et al., 2002; Fan et al., 2003). Strong noggin mRNA expression has been demonstrated in neurons of the olfactory bulb (OB) such as the tufted and mitral cells (Valenzuela et al., 1995). Notable levels of noggin mRNA have been also described in neurons of the piriform cortex, hippocampus, cerebellar Purkinje cells and ependymal cells (Valenzuela et al., 1995; Lim et al., 2000; Fan et al., 2003). 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; Fan et al., 2003). Recently, we have demonstrated that BMP4, 6 and 7 are widely distributed in the mouse rostral forebrain (Peretto et al., 2002). During embryonic development they are present in the peripheral olfactory mucosa, OB and subventricular zone (SVZ), and during postnatal and young-adult life, they are observed in the OB mitral cells and subependymal layer (SEL). Whereas the role/s of BMPs and noggin in the adult brain is/are still largely unexplored, our previous data (Peretto et al., 2002), together with recent reports (Shou et al., 1999, 2000; Lim et al., 2000; Coskun et al., 2001; Coskun and Luskin, 2002), support a potential role for these proteins in anatomical and functional plasticity of the adult brain. This view is strengthened by the observation that BMPs and their antagonists and modulators are associated with highly dynamic systems outside the nervous system, and in particular to restricted zones housing stem cells, such as the stromal cell system (Deans and Moseley, 2000), gonads, skin and gut of both vertebrates and invertebrates (reviewed by Spradling et al., 2001). Thus, BMPs, noggin and other BMP-related factors should be included within the group of molecules which participate to create a microenvironmental "niche" that controls the activity of stem cells and their progeny (Spradling et al., 2001).

It is well known that like the hippocampus, the OB of adult rodents retains high structural and functional neural plasticity, principally in terms of neurogenesis of the two main classes of OB interneurons: the granule and periglomerular cells (Luskin 1993; Lois and Alvarez-Buylla, 1994). During embryonic development these cell populations derive from the lateral ganglionic eminence (Wichterle et al., 1999; Corbin et al., 2001) and in adulthood continue to be supplied by the proliferative activity of multipotent stem cells located in the SEL. The SEL is also referred to as the adult SVZ, having originated from the embryonic SVZ (Boulder Committee, 1970). The SEL corresponds to a tissue domain which directly underlies the monolayer of ependymal cells lining the lateral ventricles (SEL-LV), and extends along the olfactory peduncles with its rostral extension (SEL-RE) reaching the center of the OB (SEL-OB). Within the SEL two principal components have been described, highly proliferating/migrating neuroblasts and glial tubes (Lois et al., 1996; Jankovski and Sotelo, 1996; Peretto et al., 1997). The neuroblasts are organized as polysialylated neural cell adhesion molecule (PSA-NCAM)-positive tangentially oriented chains of cells (Bonfanti and Theodosis, 1994; Rousselot et al., 1995; Peretto et al., 1999). This organization is lost when the chains reach the OB at which point the individual neuroblasts migrate radially

to the granule and glomerular cell layers (Bonfanti and Theodosis 1994; Hack et al., 2002). The astrocytic glial cell bodies and processes form a dense meshwork generating a system of channels, called glial tubes, which envelop the migrating chains during their displacement to the OB. The glial tubes progressively disappear within the OB as the chains disaggregate into single cells (Peretto et al., 1997). Recent studies suggest that the glial tubes, rather than being directly involved in cell migration (Wichterle et al., 1997), act as a physical barrier to separate the area of cell genesis and migration from the mature brain parenchyma, and are potentially involved in providing a stem cell niche and the stem cell reservoir of the SEL (Doetsch et al., 1999; Wichterle et al., 1999; Lim et al., 2000). In fact, SEL astrocytes express particular morphological and molecular features some of which are frequently found in embryonic and postnatal radial glia (Peretto et al., 1997, 1999; Alves et al., 2002) that are implicated in creating molecular barriers (i.e. tenascin-c, Jankovski and Sotelo, 1996), while others are strictly related to neurogenetic processes (expression of EphB receptors and ligands, Conover et al., 2000, expression of BMP proteins and receptors, Lim et al., 2000; Coskun et al., 2001; Coskun and Luskin, 2002; Peretto et al., 2002). Additional strong evidence supporting this role for glia in inducing adult neurogenesis comes from the demonstration that the protein noggin, expressed in ependymal cells of the LV, can create a neurogenetic niche in the SEL by antagonizing the BMPs present in the SEL astrocytes (Lim et al., 2000).

In the work reported here, we have used  $\beta$ -galactosidase ( $\beta$ -gal) expression by the noggin-LacZ “knock-in” heterozygous adult mouse (McMahon et al., 1998; Lim et al., 2000) as a reporter to monitor the distribution and cell localization of noggin within the rostral forebrain, with particular attention to the SEL and the OB. This distribution was compared with that of BMP7 and BMP4 previously demonstrated to be expressed in this region (Peretto et al., 2002). Our results demonstrate strong expression of noggin, BMP4 and BMP7 throughout the prosencephalon. Within the SEL, noggin and BMPs appear largely associated with the astrocytes forming the glial tubes. The presence of noggin protein and mRNA in the SEL region and in different layers of the OB has been confirmed, by means of immunohistochemistry, in situ hybridization and RT-PCR. Our results lead us to hypothesize that the antagonism between these factors acts through autocrine/paracrine inductive mechanisms that maintains a neurogenetic environment in the SEL all the way from the LV to the OB.

## **EXPERIMENTAL PROCEDURES**

### **Tissue preparation**

All the procedures involving animals were approved by the University of Maryland School of Medicine’s Institutional Animal Care and Use Committee and were carried out on young/adult (1–2 months old) mice. These experiments conform to international guidelines on the ethical use of animals and were designed to minimize the numbers, and discomfort, of the mice used. For immunohistochemistry and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) staining at least 10 noggin-LacZ “knock in” heterozygous mice, and 10 wild type 129S1/SvImJ mice were analyzed. Founder noggin-lac-Z hemizygous mice were generously provided by A. McMahon (Harvard University, Cambridge, MA, USA) (McMahon et al., 1998). The mice were maintained by mating hemizygous mice to wild-type mice of

the background strain, 129S1/SvImJ (Jackson Laboratories, Bar Harbor, ME, USA). Noggin lac-Z mice were identified by the blue color generated on incubating ear punches in X-gal reagent (Cummings et al., 2000). In situ hybridization (four animals) and RT-PCR (15 animals) analyses were performed on CD-1 strain mice (Charles River, Calco, Italy). Mice were deeply anesthetized with a ketamine/xylazine solution (100 mg/kg body weight: 33 mg/kg body weight) and transcardially perfused with ice cold saline solution (0.9% NaCl) followed by a freshly prepared solution of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. For X-gal histochemistry, the perfused brains were separated sagittally into two halves. One was cut coronally into ca. 2 mm slices and the other half was cut into a few sagittal slabs. For immunohistochemistry and in situ hybridization tissues were postfixed (2- 4 h) in the same fixative, cryoprotected in ascending sucrose solutions (7.5%, 15%, 30%), quick frozen in cold isopentane (-80 °C), cut in serial coronal or sagittal sections (10 -25 µm), and stored in 0.01 M PB. For RT-PCR, mice were anesthetized as described above, brains promptly removed and thick coronal sections (250 µm) cut on a vibratome. Under a high magnification dissecting microscope, different levels along the extent of the SEL (SEL-LV, SEL-RE, SEL-OB), the septum and OB granule layer were carefully micro-dissected.

### **Immunohistochemistry**

Sections were processed for immunohistochemistry either by using the biotin-avidin system or immunofluorescence. All incubations were run at room temperature and washes were done in 0.01 M phosphate buffered saline (PBS) for 15 min. Tissue sections were treated overnight with the following primary antibodies diluted in PBS with 0.1%- 0.5% Triton X-100 and 1% non-immune serum of the same donor species as the secondary antiserum: rabbit-anti-β-gal (1:4000; 5Prime-3Prime, Inc., Boulder, CO, USA); mouse-anti-glial fibrillary acidic protein (GFAP; 1:200; Boehringer, Mannheim, Germany); goat-anti-noggin (1 µg/ml; R&D Inc., Minneapolis, MN, USA; lot FCD09); rabbit-anti-GFAP (1:800; Sigma, St. Louis, MO, USA); goat-anti-BMP7 and goat-anti-BMP4 (1:100; Santa Cruz, CA, USA); mouse-anti-PSA-NCAM (1:1000; G. Rougon, Marseille, France). The next day sections were rinsed in PBS and, for the biotin-avidin system, incubated with the appropriate biotinylated secondary antibodies for 1 h (anti-goat 1:250 for BMP4 or 1:1000 for noggin, or antirabbit 1:250; Vector Laboratories, Peterborough, UK), rinsed, and incubated in avidin- biotin complex (1:400; Vector Laboratories, Inc.). The reaction product was visualized with 0.15 mg/ml 3,3' diaminobenzidine in PBS with 0.01% H<sub>2</sub>O<sub>2</sub>. Sections were then serially mounted onto Superfrost Plus slides (Fisher, Pittsburgh, PA, USA), air dried, dehydrated in graded alcohols, cleared in xylenes, and coverslipped using D.P.X. mounting medium (Aldrich, Milwaukee, WI, USA). For dual immunofluorescence, after incubation with the primary anti-sera, sections were washed and incubated with appropriate solutions of secondary anti-rabbit Cy3, anti-mouse Cy2, anti-mouse Cy3, anti-goat Cy2 (all diluted 1:800; Jackson ImmunoResearch, West Grove, PA, USA). Sections were then coverslipped with an anti-fade mounting medium (Dako Corporation, Carpinteria, CA, USA).

Control reactions included: (i) omission of the primary antibodies, (ii) incubation of sections in BMP antisera pre-absorbed with 10 µM of the respective immunogenic peptides, (iii) incubation with rabbit-anti-β-gal of equivalent sections from wild type 129S1/SvImJ mice, (iv) noggin antibody

specificity has been evaluated by absorbing the antibody with 1 µg/ml of recombinant human noggin protein (Pepro Tech. Inc. Rody Hill NJ), for either immunocytochemistry or Western blot analysis.

### **Western blot**

Protein samples were prepared from OB by homogenization in 10 volumes Tris-lysis buffer (50 mM Tris-HCl, 2 mM DTT, 1 M NaCl) and protease inhibitors (10 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) following by sonication. After centrifugation for 1 h at 100,000xg the supernatants were aspirated and protein concentrations determined by the Bradford method (BioRad, Hercules, CA). Extracts (40 µg) were separated in 10 -20% SDS-polyacrylamide gel electrophoresis (Ready Gels; BioRad) and electro-transferred to polyvinylidenedifluoride (PVDF) membranes at 100 V for 1 h in ice. The membranes were blocked for 1 h in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T) containing 1% bovine serum albumin (BSA) and then were incubated overnight at 4 °C with primary noggin antibodies (0.1 µg/ml in TBS-T with 1% BSA). The membranes were then washed three times for 10 min in TBS-T and incubated for 1 h at room temperature with donkey anti-goat HRP-labeled secondary antibodies (1:40,000 dilution in TBS-T with 1% (BSA); Jackson Immunoresearch). The final wash was in TBS-T three times for 10 min and twice in TBS for 15 min. The immunoreactive protein bands were visualized using Super Signal West Pico enhanced chemiluminescence (Pierce, Rockford, IL, USA).

### **X-gal histochemistry**

Sagittal and coronal slabs were incubated, for about 2.5 h at room temperature, in X-gal substrate solution (1 mg/ml) containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl<sub>2</sub>, 0.01% sodium deoxycholate and 0.02% Nonidet P-40 (Cummings et al., 2000). When the color development was complete, the tissue was rinsed with cold PBS and the reaction was stopped by transferring the tissue to 4% buffered PFA. Sections were then photographed on a Wild dissecting microscope equipped with a 35 mm camera back. Equivalent material from wild type mice did not generate any histochemical reaction under these conditions.

### **RT-PCR**

The expression of noggin, BMP4 and BMP7 was evaluated by a semi-quantitative RT-PCR. Total RNA was extracted by the acidic phenol method (Chomczynski and Sacchi, 1987). Aliquots (1 µg) of total RNA were transcribed by the MMLV Reverse Transcriptase (APBiotech, Piscataway, NJ, USA). Genomic DNA contamination was tested by incubating the RNA in the absence of reverse transcriptase. Aliquots of the cDNA were amplified by TaqDNA polymerase (RedTaq; Sigma, St. Louis, MO, USA) polymerase chain reaction, 30 cycles. The amount of cDNA to be amplified for each sample was standardized by a previous amplification reaction for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Primers were selected from the mouse sequences available in GenBank (<http://www.ncbi.nlm.nih.gov/entrez/>) by means of Primer3 (<http://www.genome.wi.mit.edu/cgi-bin/primer/primer3>) and were as follows: for noggin (accession number U79163), forward

CAGATGTGGCTGTGGTCAC, reverse GCAGGAACACTTACACTCGG, size 261 bp, from base 682–942; for BMP4 (X56848) forward CTCCAAGAATCATGGACTG, reverse AAAGCAGAGCTCTCACTGGT, 468 bp, from 305 to 772; for BMP7 (NM007557), forward CCTGGGCTTACAGCTCTCTG, reverse GGTGGCGTTCATGTAGGAGT, 415 bp from 726 to 1122.

## **Probes**

The 261 bp cDNA fragment of the noggin sequence was subcloned in pGEM-T (Promega, Italia, Milano, Italy), the antisense probe was generated using T7 RNA-polymerase following restriction digestion of the clone with PstI endonuclease and the sense (control) probe by SP6 RNA-polymerase following digestion of the clone with ApaI endonuclease. The riboprobes were labeled by transcription in the presence of DIG-UTP using the DIG RNA Labeling Kit (Roche Diagnostics, Monza, Italy).

Mouse 28S ribosomal RNA, a 1.3 kb fragment (Mariani-Costantini et al., 1998) was used as a control of RNA integrity.

## ***In situ* hybridization**

Hybridization was performed essentially as previously described (Dati et al., 1996). Briefly, slides were rehydrated for 5 min in PBS, treated for 5 min at 37 °C with 5 µg/ml Proteinase K (Sigma), acetylated and then incubated overnight at 42 °C with 1 ng/ml of DIG-labeled riboprobe in a mixture containing 60% deionized formamide, 2x SSC, 1 mg/ml yeast tRNA, 100 µg/ml herring sperm DNA, 5x Denhardt's solution, 1 U/ml RNase inhibitor (RNAGuard, APBiotech). The next day slides underwent an RNase A (Sigma) treatment, 20 µg/ml in 10 mM Tris pH 8.0, 0.5 M NaCl, 30 min at 37 °C, and a stringency wash, 0.1x SSC, 10 min at 65 °C followed by 10 min at room temperature. The slides were then incubated overnight with an anti-DIG antibody conjugated with alkaline phosphatase (Roche) diluted 1:5000. The alkaline phosphatase activity was revealed by the DIG Nucleic Acid Detection Kit (Roche).

## **Preparation of figures**

Light photomicrographs were digitized using a Phase One PowerPhase digital camera (Phase One, Denmark) fitted to a Leica DMRX microscope (Leica Microsystems Inc., Deerfield, IL, USA). Analysis and digital photography of the double immunofluorescence was carried out using a laser-scanning Olympus Fluoview confocal system (Olympus Italia, Milano, Italy). Digital images were brightness, color and contrast balanced, assembled into montages using Corel-Draw 11 (Corel Corporation, USA). Plates of photomicrographs were printed using a Fuji Fujix Pictography 3000 printer (Fuji Photofilm USA, Carlstadt, NJ, USA).

## **RESULTS**

To visualize the cell type/s expressing noggin protein in the adult mouse prosencephalon we used a previously generated transgenic model obtained by a gene replacement strategy in which most of the noggin coding region wasm

replaced by the  $\beta$ -gal coding region of *Escherichia coli* (McMahon et al., 1998). This placed lac-Z under the control of the noggin promoter so that the noggin-lac-Z hemizygous mice express  $\beta$ -gal in the same embryonic and postnatal structures where noggin expression has been identified by in situ hybridization (Valenzuela et al., 1995; McMahon et al., 1998). We confirmed this by Western blotting and immunocytochemistry using anti-noggin antibody and by RT-PCR.

### **$\beta$ -Gal activity in the forebrain of the adult brain as a reporter of noggin expression**

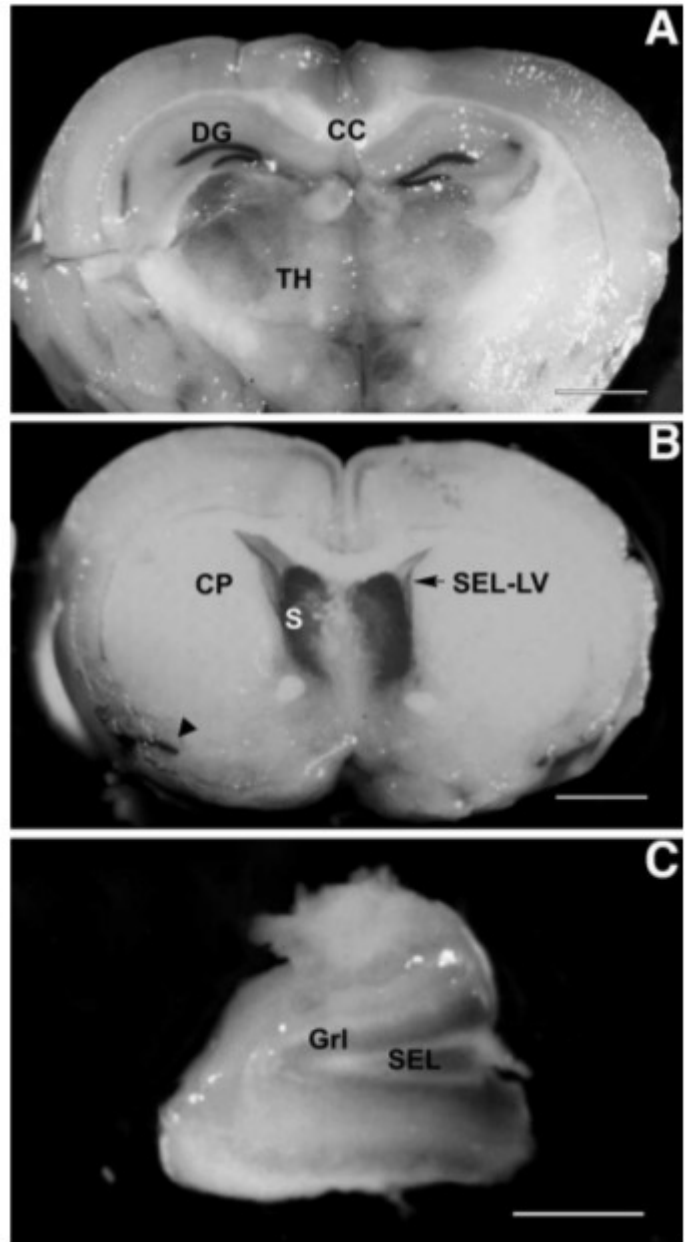
Abundant Lac-Z expression, as a reporter of noggin expression, was visualized throughout the whole brain in thick coronal and parasagittal brain sections (ca. 2 mm) of young/adult mice (1-2 months old) visualized by X-gal histochemistry. The staining was particularly strong in restricted areas such as the hippocampal dentate gyrus, piriform cortex, septal area, and OB (Fig. 1).

$\beta$ -Gal immunostaining was used for higher resolution analysis within the anterior prosencephalon, with particular attention to the SEL and OB. Parasagittal and coronal sections (12-25  $\mu$ m) cut from the LV to the OB were examined with immunofluorescence and avidin- biotin staining techniques. Strong expression of  $\beta$ -Gal was found throughout the full extent of the SEL and in the OB (Fig. 2A-F). At the level of the LV  $\beta$ -gal staining was identified in the SEL and in ependymal cells lining the ventricular cavities (Figs. 2B, 3A, C). More anteriorly, along the olfactory peduncle the staining was found primarily associated with the SEL-RE (Fig. 2A, C). Within this area, thick immunoreactive septa delineate the SEL perimeter and in part enter within the SEL itself giving rise to a web-like structure resembling the glial tubes (Figs. 2C; 3D, F, G, I, J, L). The staining appeared dense and diffuse, often preventing identification of clearly distinguishable cell bodies and processes (Figs. 2, 3). In the closely surrounding tissues, some positive cells with thin astrocytic-like processes were also observed (not shown). Some neuronal-like positive cells were also observed in the anterior olfactory nucleus (not shown). Double immunofluorescence performed with PSA-NCAM as a marker of the migrating neuroblasts, and GFAP as a marker of the glial tubes, confirmed that, from the LV to the SEL-OB, the  $\beta$ -gal protein is mostly localized in the SEL glial compartment (Fig. 3D-I).  $\beta$ -Gal expression was never observed in the PSA-NCAM-positive migrating neuroblasts, neither during their tangential chain migration (Fig. 3A-F) nor when they spread out radially as individual elements through the OB (not shown).

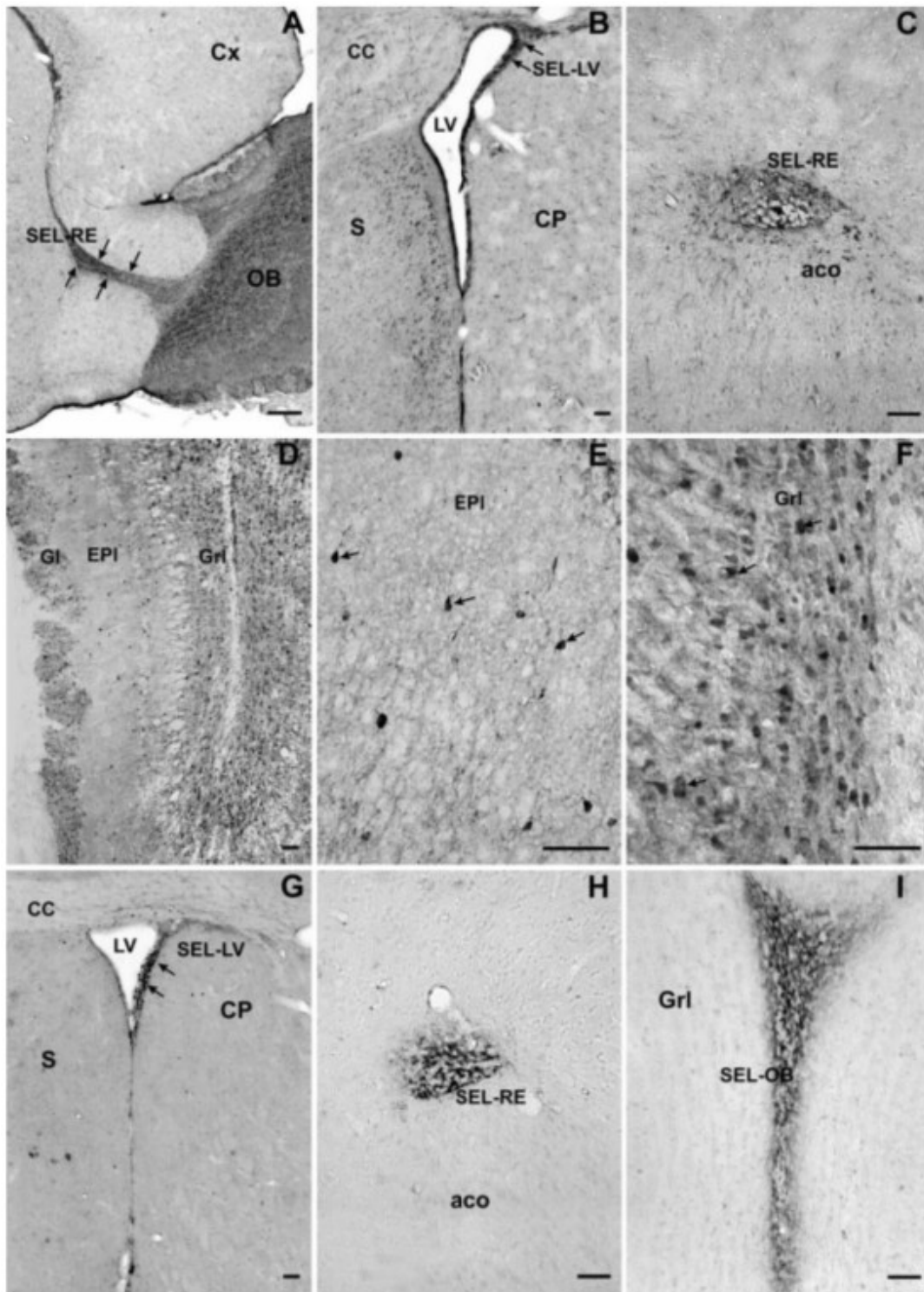
In the OB a dense and diffuse  $\beta$ -gal immunostaining is present throughout diverse layers (Fig. 2A, D-F).

Within the SEL-OB the staining is less intense, compared with that observed in more posterior SEL regions, and is still associated with the glial tubes that, at this level, are progressively disappearing. A strong immunoreaction was found in granule cells of both the main and accessory OB, in putative tufted neurons localized at different depth of the external plexiform layer and in some mitral cells. Within the glomerular layer, a diffuse reaction was observed in the neurites constituting the glomerulus and in some juxtglomerular neurons. No staining was found in the olfactory nerve layer. In equivalent sections from control wild-type mice we never observed any  $\beta$ -gal staining.

**Fig. 1.** X-gal histochemical analysis of noggin-lacZ mouse brain slabs. (A, B) Coronal sections, (C) parasagittal section. X-gal staining is visible in different areas of the adult forebrain, some of which are characterized by neural plasticity, such as the dentate gyrus of the hippocampus (A) and the granule and the SEL of the OB (C). Abundant staining is present in the SEL-LV (arrow), septum and piriform cortex (arrowhead) (B). No staining was observed in tissue from wild-type mice. CC, corpus callosum; CP, caudate putamen; DG, dentate gyrus; Grl, granule cell layer; S, septum; TH, thalamus. Scale bars=2 mm A, B; C, 1 mm.

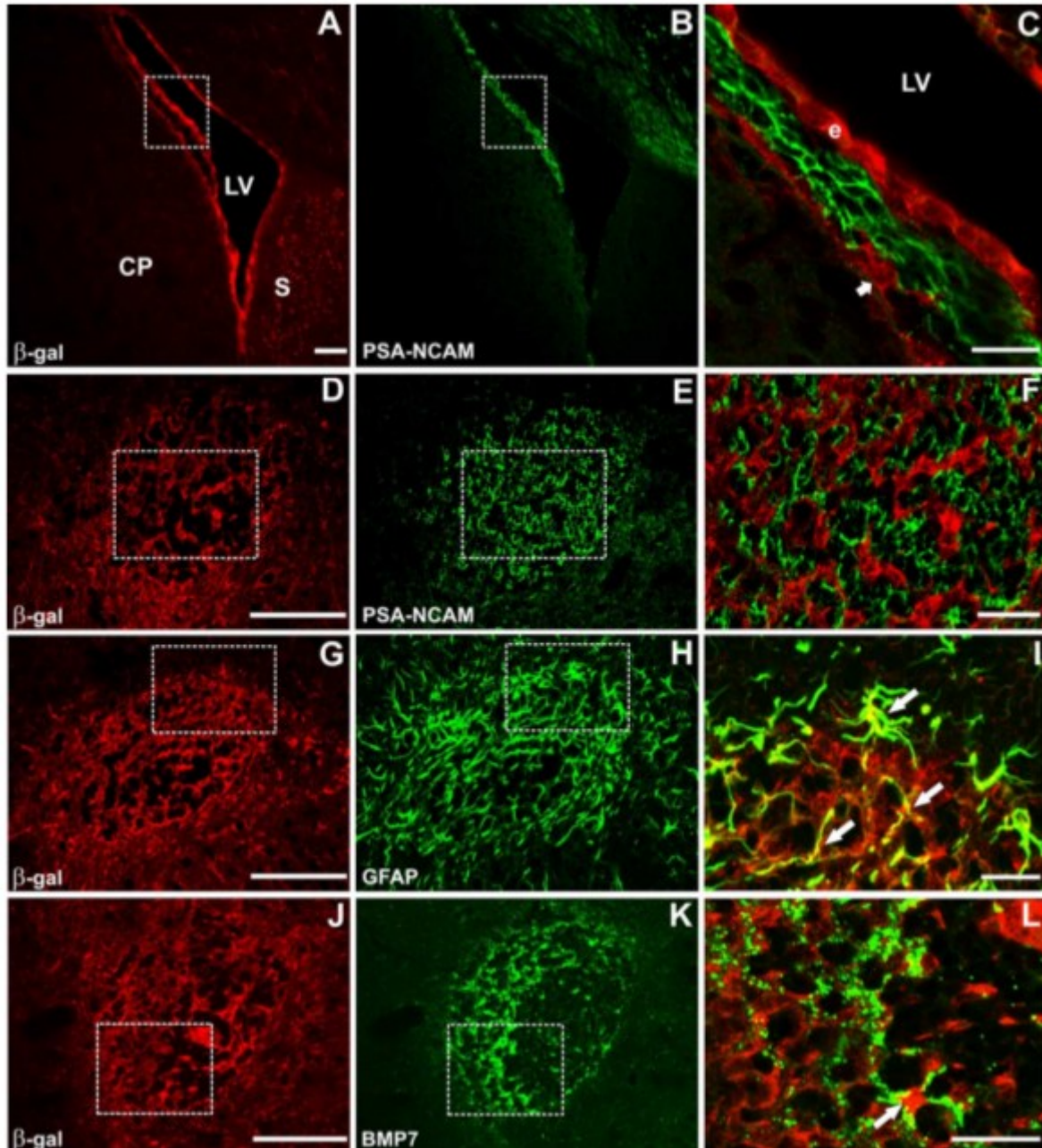






**Fig. 2.**  $\beta$ -gal and BMP7 immunohistochemical localization in the adult mouse rostral forebrain. (A) Parasagittal section showing  $\beta$ -gal protein expression in the adult SEL-RE (arrows) and OB. (B, C) Coronal sections cut respectively at the level of the LV and olfactory peduncle. Strong staining is associated with the SEL-LV area (arrows in B) and SEL-RE (C).  $\beta$ -Gal positive staining is also present in the ependymal monolayer and septum (B). Within the OB (D-F)  $\beta$ -gal is expressed in the glomerular, external plexiform, and granule cell layers. (E) Higher magnification of (D) showing positive cells in the external plexiform layer (arrows). (F) Diffuse staining is present in numerous granule cells, some of which show stronger staining (e.g. arrows). (G-I) BMP7 is associated with the whole SEL extension from the LV (arrows in G) to the OB, where it mainly restricted to the SEL area (I). aco, anterior commissure; CC, corpus callosum; CP, caudate putamen; Cx, cerebral cortex; EPI, external plexiform layer; Gl, glomerular layer; Grl, granule cell layer; S, septum. Scale bars=200  $\mu$ m, A; B-I, 50  $\mu$ m.

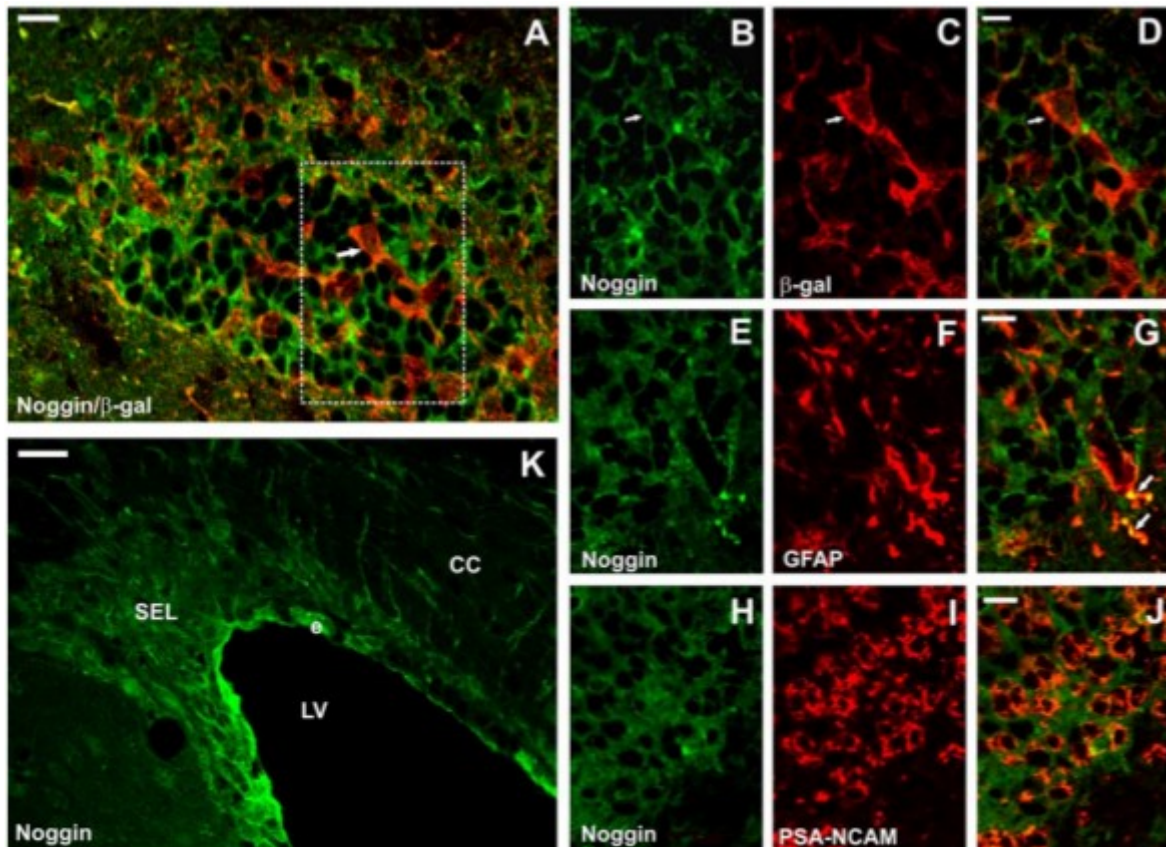
The use of a knock-in reporter-gene model to study protein expression is a powerful tool; however, ectopic expression of the reporter gene may occur confounding the interpretation. In order to address this question and to further confirm the presence of noggin protein in the SEL we performed immunocytochemical and Western blot analyses of noggin protein.



**Fig. 3.** Double immunostaining between  $\beta$ -gal, BMP7, and markers of the SEL compartment in adult mice.  $\beta$ -Gal (A, D, G, J), PSA-NCAM (B, E), GFAP (H), BMP7 (K) immunoreactivity in coronal sections. (A-C) LV; D-L olfactory peduncle. Double immunostaining for  $\beta$ -gal and anti-PSA-NCAM showed no overlap of expression (A-F). At the level of the LV an intense  $\beta$ -gal immunoreactivity (A, C) is present in the ependymal monolayer (e) and in glial-like cells of the SEL (arrow in C). (G-I) A high degree of colocalization is observed in double staining between  $\beta$ -gal and GFAP (arrows in I). (J-L) Only a partial overlap was found in dual labeling for  $\beta$ -gal and BMP7 (arrow in L). (C, F, I, L) Higher magnifications of a simultaneous view of the pictures in the same row obtained from 1  $\mu$ m optical section. Scale bars=50  $\mu$ m, A, B, D, E, G, H, J, K; C, F, I, L, 20  $\mu$ m.

## **Noggin protein detection in the SEL confirms the specificity of the Noggin Lac-Z model**

The immunohistochemical detection of noggin was performed on sections cut along the rostral prosencephalon of adult mice, where we observed a strong immunoreactivity in the same areas positive for  $\beta$ -gal staining in the Lac-Z model (not shown). Noggin appears to be abundantly expressed in the SEL (Fig. 4). In this region the staining appears localized to cell bodies and processes; moreover, immunoreactivity is also present in extracellular spaces (Fig. 4A, B, E, H, K), Confocal microscopic analyses on sections treated for double staining with anti-noggin and anti- $\beta$ -gal (see Fig. 4A-D) showed that in the SEL area all the  $\beta$ -gal positive cells express noggin, thus excluding ectopic  $\beta$ -gal expression in this region. However, the extent of the staining obtained with anti-noggin was larger than that observed with anti- $\beta$ -gal. Noggin and GFAP immunoreactivities were colocalized (Fig. 4E-G), confirming the astroglial cells as the source of noggin. To a lesser extent, we also observed



**Fig. 4.** Relationships between noggin,  $\beta$ -gal, GFAP and PSA-NCAM in the SEL-RE, and noggin protein localization in the SEL-LV. (A-D) Noggin and  $\beta$ -gal colocalize in cells of the SEL-RE (see arrow in B-D). (E-G) Glial GFAP-positive processes are strongly immunopositive for noggin (arrows). (H-J) A partial overlap occurs between noggin and PSA-NCAM immunoreactivities. D, G, and J have been obtained examining 1  $\mu$ m optical section. (K) Noggin protein is abundant in the ependyma (e) and in the SEL-LV region. High levels of immunofluorescence are identifiable in the ependymal cells and in astroglial-like cell processes in the SEL-LV and corpus callosum (CC). Scale bars=12  $\mu$ m, A; B-D, 6  $\mu$ m; E-J, 8  $\mu$ m; K, 20  $\mu$ m.

a partial overlap between noggin and PSA-NCAM (Fig. 4H-J). This partial colocalization with PSA-NCAM immunopositive migrating cells was never observed in the case of  $\beta$ -gal expression, and may be the consequence of secretion and diffusion of noggin protein. PSA-NCAM antibody recognizes the polysialyl- enriched extracellular portion of NCAM, thus a partial colocalization between PSA-NCAM and Noggin may be due to overlapping of these two extracellular antigens.

We have demonstrated the specificity of the antinoggin antibody in both Western blots and in immunohistochemistry by blocking with 1  $\mu$ g/ml recombinant human noggin protein (Fig. 5).

### **Noggin co-localizes with BMP4 and BMP7**

We have previously analyzed the expression of different BMPs within the mouse olfactory system during embryonic and postnatal life (Peretto et al., 2002). Our results demonstrated a discrete spatial and temporal expression of these cytokines within the olfactory system and in particular, we observed expression of BMP4 and 7 in the embryonic and postnatal SVZ. Here we have

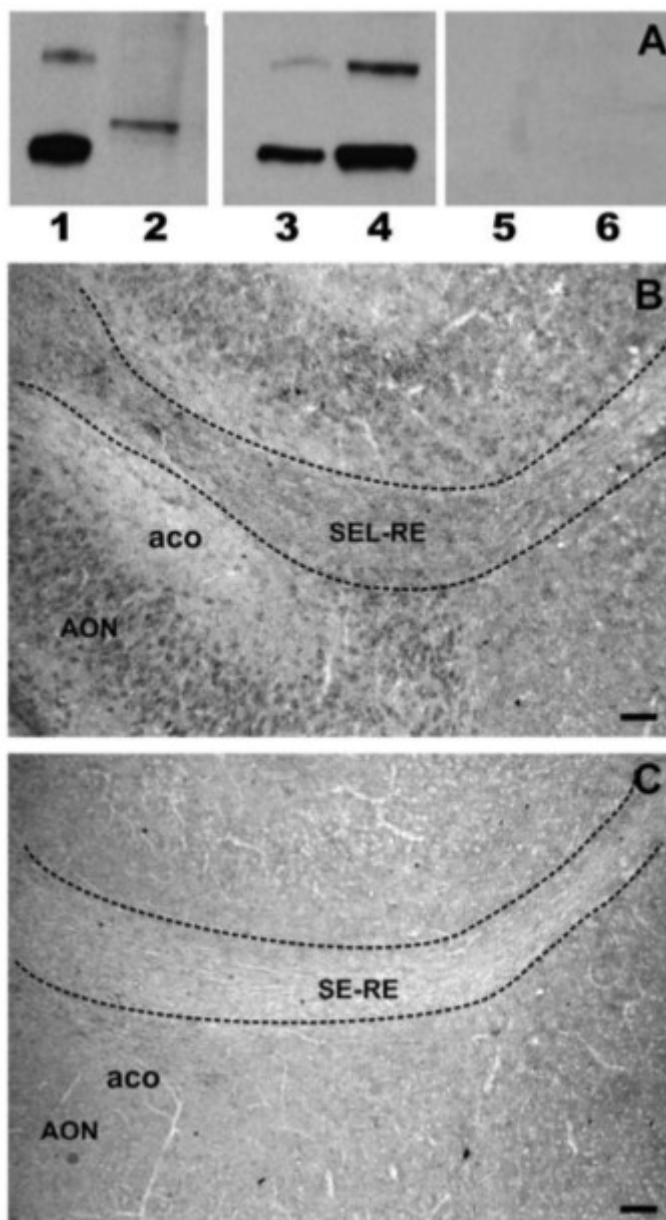
characterized the distribution of these two BMPs, within the extent of the SEL and in the OB of young/adult mice, in comparison to that of their antagonist/modulator noggin.

Single immunostaining obtained with anti-BMP4 and anti-BMP7 showed very similar patterns of distribution within the SEL and OB. In the SEL, from the LV to the OB, both BMP4 (not shown) and 7 (Figs. 2G-I, 3K) give rise to a dense and diffuse meshwork-like immunostaining resembling the appearance of the glial tubes. Double immunoreactions performed with antibodies to BMPs GFAP and PSA-NCAM (not shown), demonstrated no colocalization with the chains of migrating neuroblasts, and only a partial overlap with the glial GFAP-positive SEL compartment, confirming our previous results (Peretto et al., 2002). Comparison of the staining of BMP7 with that of  $\beta$ -gal in sections visualized with double immunofluorescence showed only partial colocalization of these molecules (Fig. 3J-L). Although anti-BMP4 and 7 each gave a strong signal in the SEL region of the OB (Fig. 2I), they gave only a very faint signal in the olfactory nerve and glomerular layers (not shown).

### **Noggin mRNA expression in the SEL and OB**

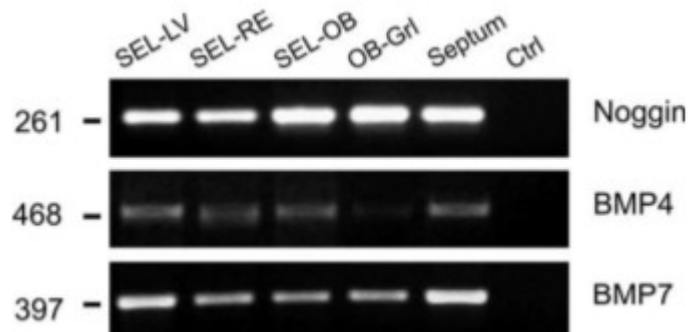
To further confirm the presence of noggin, BMP4, and BMP7 in the SEL and OB regions, RT-PCR analyses were performed on tissue microdissected from thick coronal sections cut at different levels of the SEL (SEL-LV, -RE, -OB) and from the OB of wild-type adult mice (see Experimental Procedures for details).

As a positive control, microdissected septum tissue, that contains a significant level of noggin mRNA (Valenzuela et al., 1995), was included in our experiments. We observed the presence



**Fig. 5.** Specificity of anti-noggin antibody. (A) Western blot analysis; (B and C) immunohistochemistry. (A) Lanes 1, 3, 5: 5 ng recombinant human noggin. Lane 2: 40  $\mu$ g of SDS protein extract from OB spiked with 5ng recombinant human noggin. Lanes 4, 6: 40  $\mu$ g of SDS protein extract from OB. After electrophoresis and transferring onto PVDF membranes lanes 1- 4 were incubated with R & D goat anti-noggin antibody (0.1  $\mu$ g/ml), lanes 5 and 6 were incubated with R & D goat anti-noggin antibody preincubated with 1  $\mu$ g/ml of noggin protein to block the antibody. All samples were run simultaneously. Note the presence of a small amount of noggin dimer and the elimination of the immunoreactivity by blocking the antiserum. (B, C) Consecutive parasagittal sections incubated respectively with B) R & D goat anti-noggin antibody (1  $\mu$ g/ml) and C) R & D goat anti-noggin antibody preincubated with 1  $\mu$ g/ml of noggin protein to block the antibody. In B a strong immunoreactivity was observed both in the SEL-RE and in the anterior olfactory nucleus. No staining is observed in the same areas in the section treated with the blocked antibody. *aco*, anterior commissure; AON, anterior olfactory nucleus.

of noggin and BMP mRNAs in all specimens examined (Fig. 6). In situ hybridization for noggin mRNA was performed on adult wild-type animals in coronal sections through the rostral telencephalic region. Noggin mRNA was evident in several prosencephalic areas including cells of the septum and piriform cortex (not shown).



**Fig. 6.** Expression of noggin, BMP4 and BMP7 mRNAs in the SEL and OB of wild-type adult mice. RT-PCR analysis of microdissected tissue fragments obtained from different levels of the SEL and from the OB showed expression of noggin, and BMP4/7 mRNAs in the SEL-LV,-RE, -OB. The septum was chosen as a positive control (Valenzuela et al., 1995).

Within the SEL a reproducible in situ signal was present from the LV to the

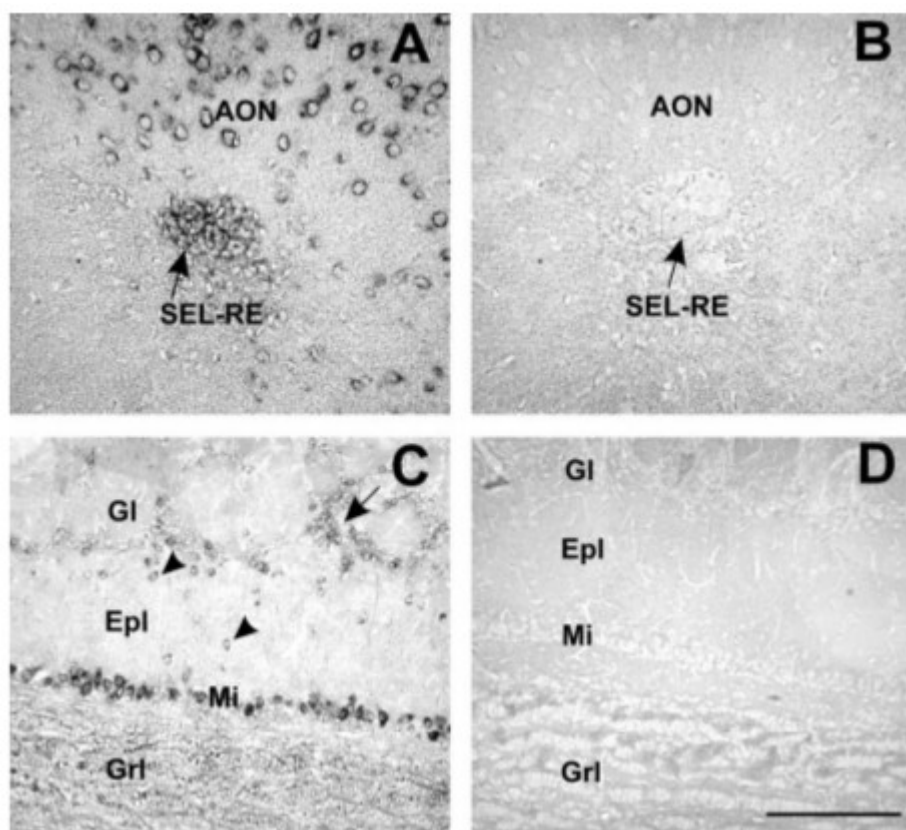
OB. At the level of the LV, strong signal was found also associated with the ependymal cells (not shown). These data confirmed our observation of lacZ expression in these sites as a reporter of noggin protein expression. The pattern of staining obtained in the SEL-RE (Fig. 7A) is reminiscent of the glial tubes. An intense labeling was also observed in cells of the anterior olfactory nucleus (Fig. 7A). Within the OB itself, the noggin anti-sense probe showed a pattern of expression that was similar to that seen for  $\beta$ -gal protein. Thus, a weak in situ signal was found associated with granule cells, while strong staining was found in putative tufted and juxtglomerular cells and in mitral cells (Fig. 7C). Incubation with the noggin sense probe gave only background levels of hybridization, confirming the specificity of the analysis (Fig. 7B, D).

## DISCUSSION

In the last decade the discovery of neural stem cells in restricted regions of the adult mammalian brain has opened exciting new opportunities for research in neuroscience (see for review Bonfanti et al., 2001). In addition to the characterization of the location and properties of these stem cells, major efforts are under way to identify the mechanisms that regulate their activity. As a result a new concept, the "niche," has been proposed (Lim et al., 2000; Spradling et al., 2001). The niche is considered to be a subset of cells and extracellular substrates that create the microenvironment capable of indefinitely sequestering one or more stem cells and that functions to control their selfrenewal and expansion in vivo (Spradling et al., 2001). Knowledge of the stem cells' microenvironment is, therefore, fundamental for understanding how a stem cell can proliferate, choose its fate and eventually integrate into mature tissue.

### Noggin and BMPs are components of the stem cell niche

It is striking that strong similarities have been described between the niches present in different tissues in both vertebrates and invertebrates (reviewed in Spradling et al., 2001). From the molecular point of view, several candidate



**Fig. 7.** In situ hybridization for *noggin* mRNA on coronal sections cut at the level of the olfactory peduncle and OB. (A) *Noggin* mRNA expression is present in the SEL-RE (arrow) and in cells of the anterior olfactory nucleus. (C) Within the OB, mitral, external plexiform and glomerular layers show numerous labeled cells. A weak reaction was also found in the granule cell layer. In adjacent sections treated with the sense probe (B, D) no hybridization is present. Arrow in B indicates the SEL-RE area. Arrow and arrowheads in C indicate stained cells respectively in the glomerular and external plexiform layers. AON, anterior olfactory nucleus; Epl, external plexiform layer; Gl, glomerular layer; Grl, granule cell layer; Mi, mitral cell layer.

niche components have been proposed. Members of the transforming growth factor- $\beta$  family such as the BMPs and their antagonist/modulator *noggin* have been described as critical components of this stem cell niche microenvironment (Xie and Spradling, 1998, 2000; Coskun et al., 2001; Lawson et al., 1999; Kulesa et al., 2000; Lim et al., 2000; Panchision and McKay, 2002). In addition to their morphogenetic roles during development (Hogan, 1996; McMahan et al., 1998; Dale et al., 1999; Bachiller et al., 2000), the balance between BMPs, *noggin* and other BMP antagonists/modulators such as *chordin*, seems to regulate the proliferation and specification of neural cell precursors. Indeed, their expression has been widely reported in the embryonic germinal zones where undifferentiated precursor cells exhibit temporal and site-specific responsiveness to these proteins (Gross et al., 1996; Li et al., 1998; Mabie et al., 1999; Mehler et al., 2000). Furthermore, the antagonism between *noggin*, expressed in the developing neocortex, and BMPs may regulate the differentiation of neocortical neurons in vivo (Bachiller et al., 2000; Li and LoTurco, 2000).

### **Noggin and BMPs are expressed in the adult SEL**

The presence of these proteins has been also described in many regions of the adult rodent brain that are characterized by different types of neural plasticity, suggesting similar and/or additional roles in the adult CNS (Valenzuela et al., 1995; Coskun et al., 2001; Charytoniuk et al., 2000; Lim et al., 2000; Peretto et al., 2002). Consistent with these reports we have observed the presence of *noggin* as well as BMP4 and 7 in the rostral forebrain of young/adult mice. The analysis of *noggin* localization in the SEL-OB system was mainly performed by means of a well-characterized model, the *noggin-lacZ* knock-in mouse (McMahan et al., 1998; Lim et al., 2000). The results obtained in the SEL were compared with the immunohistochemical detection of *noggin*, demonstrating the absence of ectopic  $\beta$ -gal expression in the transgenic model. However, differences in the patterns of staining have been found, namely  $\beta$ -gal expression appears to be more restricted compared with *noggin*. This result suggests that  $\beta$ -gal expressed under the control of the *noggin* promoter does not mimic the characteristic secretory behavior of the native *noggin* protein. Moreover, a significant level of  $\beta$ -gal expression reporting the presence of *noggin*, was seen in multiple areas such as the hippocampus, piriform cortex, septum, and OB, where different types of

neural plasticity have been reported (Peretto et al., 1999; Bernier et al., 2002; Garcia, 2002). A strong expression of  $\beta$ -gal protein was found to be specifically associated with the SEL throughout its extent and in the OB, regions marked by adult neurogenesis due to the presence of multipotent stem cells (for review see Peretto et al., 1999).

### **Noggin and BMPs are associated with the SEL glial compartment**

Within the SEL, the staining is clearly associated with the glial compartment. Our previous results demonstrated that BMP4 and 7 immunostaining also partially overlap with markers of the glial tubes but were never associated with the migrating neuroblasts (Peretto et al., 2002). In this study, a colocalization was observed between BMP4, BMP7 and  $\beta$ -gal. Thus, our results demonstrate that the astrocytes forming the glial tubes can produce BMP4, 7 and their natural antagonist noggin. The diffuse staining observed for noggin immunolabeling in the SEL region supports a mechanism of production and a subsequent secretion of noggin from the astroglial component. Lim and colleagues (2000) demonstrated the presence of both BMPs, their receptor and noggin within the periventricular area, and proposed a model in which the antagonism between these factors can regulate neurogenesis in the adult SVZ, consistent with the existence of similar regulatory mechanisms during development and adulthood. In their model, noggin, which they reported to be specifically associated with ependymal cells, creates a neurogenetic environment in the adjacent SEL by blocking endogenous BMP signaling. We have observed expression of noggin in the ependymal cells too, but in addition we found a similar intensity of staining in the SEL all the way from the LV to the OB. The reason for this difference is unclear but may relate to the sensitivity of the staining techniques (X-gal staining versus anti- $\beta$ -gal immunostaining). Our RT-PCR analysis performed on wild-type mice, confirmed the presence of noggin mRNA at different levels of the SEL extent. Although, we cannot exclude that our microdissected fragments also contained small portions of the tissue surrounding the SEL, the intensity of the signal obtained was comparable to that of the septum, a region where high levels of noggin mRNA has been previously described (Valenzuela et al., 1995). This was confirmed by our in situ hybridization analysis demonstrating the presence of a high level of noggin mRNA in the rostral forebrain and in particular in the SEL-LV and OB.

We can now hypothesize, as previously suggested by Lim and colleagues (2000) at the level of the LV, that the antagonism between noggin and BMPs regulates adult neurogenesis all the way from the LV to the OB. This implies that the SEL represents a quite homogeneous system along its entire extension. In support of this idea, and in addition to the anatomical evidence (see Peretto et al., 1999 for review), the existence of adult neural stem cells has recently been shown along the entire SEL area up to the core of the OB (Gritti et al., 2002), thus demonstrating that the SEL-RE is not simply the migratory pathway for the progeny of periventricular stem cells, as previously thought, but is also a source of stem cells. The expression of noggin and BMPs along the SEL from the LV to the OB may also indicate that BMP signaling can exert multiple regulatory roles on adult neurogenesis. Studies addressing neurogenetic mechanisms within the postnatal SVZ suggest that SEL neuroblasts express the cell-cycle inhibitor P19 INK4d (see Coskun and Luskin, 2002). This gene product is dynamically regulated along the migratory

pathway and enables these cells to show the unusual characteristic of retaining the capacity to divide during migration. The dynamic expression of P19 INK4d, which appears higher at the level of the SEL-OB, seems to be regulated by the BMPs, since inhibition of BMP signaling, by injecting replication-deficient retrovirus encoding a dominant negative bone morphogenetic protein receptor-Ia, results in down-regulation of P19 INK4d and initiation of a new cell cycle. This result suggests that in vivo BMP signaling must be dynamically regulated along the SEL migratory route, consistent with our demonstration of the presence of the negative BMP-modulator, noggin, from the LV to the OB.

Furthermore, it has been shown that astrocytes present in adult neurogenic regions such as the SEL and the hippocampal formation exhibit features that allow these glial cells to regulate processes of neurogenesis. (Song et al., 2002), demonstrated that in vitro, astrocytes can direct the proliferation, as well as the fate, of hippocampal stem cells, inducing their progeny to become neurons rather than glia. These data are consistent with previous reports showing that contact with fragments, or monolayers, of astrocytes can modulate proliferation, fate and differentiation of stem cells from the cortex of developing rat and adult SVZ (Temple and Davis, 1994; Lim and Alvarez-Buylla, 1999). It seems that only younger astrocytes exhibit these characteristics (Smith et al., 1990). In this context, it is worth noting that SEL astrocytes retain some features of immature radial glia-like cells (Jankovski and Sotelo, 1996; Lois et al., 1996; Peretto et al., 1997, 1999), and subpopulations of these glial cells could act as quiescent stem cells (Doetsch et al., 1999). Thus, our results demonstrating that the SEL glial compartment can express both BMPs and noggin, which are key niche components, reinforce the emerging view that astrocytes have an active regulatory role in zones of neurogenesis in adults.

### **Noggin and BMPs are expressed in the OB**

In the OB, noggin-LacZ expression was found in all OB layers. In particular, a strong signal was observed in mature granule cells, external plexiform layer, juxtglomerular cells and olfactory glomeruli dendritic terminals.

Nevertheless, a slight difference was observed in the expression of noggin mRNA and  $\beta$ -gal protein. Most mitral cellular bodies were stained in the ISH analysis but only some of them were labeled in the immunocytochemical analyses of the noggin-lacZ heterozygous mice. This may reflect differential mRNA vs. Protein turnover and/or subcellular compartmentalization. Thus, our data suggest that  $\beta$ -gal protein is restricted to the dendrites of the mitral cells based on the strong noggin- LacZ expression found in the OB glomeruli. A differential expression of mRNA vs. protein in the mitral cell layer has also been reported in other studies (Baker et al., 2001). Alternatively, this discrepancy could be due to a lack of expression of  $\beta$ -gal in some mitral cells. High levels of noggin and BMPs in the OB are justified considering the peculiar characteristic of this region. Adult OB represents a site of persistent plasticity, both at the level of the glomeruli, where a continuous renewal of the olfactory receptor neurons axons can lead to reorganization within the glomerular neuropil (Graziadei and Monti Graziadei, 1978), and in the granule cell layer, the principal site of adult OB neurogenesis (Luskin 1993; Bonfanti and Theodosis, 1994; Lois and Alvarez-Buylla, 1994). Unlike noggin, the expression of BMP4 and 7 in the OB was mainly restricted to the SEL region.

This result indicates that, in the more external layers of the OB, the balance observed between these proteins along the SEL changes and noggin protein is predominant and/or antagonizes others members of the BMPs (i.e. high amounts of BMP6 is present in the OB glomeruli, Peretto et al., 2002). In conclusion, the expression and localization of BMP4, 7 and noggin reported here, lead us to hypothesize that: i) within the SEL, the glial compartment acts through an autocrine or paracrine inductive mechanism between noggin and BMPs to maintain a neurogenetic environment, reinforcing suggestions of a role of the astrocytic glia in creating the neurogenetic niche all along the SEL extension; ii) within the OB, the persistence of high levels of noggin in different types of neurons suggests an involvement of this protein in the multiple processes of neuronal plasticity peculiar to this structure.

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