BDNF/MAPK/ERK–Induced BMP7 Expression in the Developing Cerebral Cortex Induces Premature Radial Glia Differentiation and Impairs Neuronal Migration

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During development of the mammalian nervous system, a combination of genetic and environmental factors governs the sequential generation of neurons and glia and the initial establishment of the neural circuitry. Here, we demonstrate that brain-derived neurotrophic factor (BDNF), one of those local acting factors, induces Bone Morphogenetic Protein 7 (BMP7) expression in embryonic neurons by activating Mitogen-Activated Protein Kinase/Extracellular signal-Regulated Kinase signaling and by the negative regulation of p53/p73 function. We also show that intraventricular injection of BMP7 at midgestation induces the early differentiation of radial glia into glial precursors and astrocytes and the expression of mature glial markers such as the antiadhesive protein SC1. As a result of this precocious radial glia maturation, the laminar distribution of late-born pyramidal neurons is altered, most likely by the termination of radial glia ability to support neuronal migration and the early neuronal detachment from the glial rail. We propose a mechanism for BDNF regulation of BMP7 in which local activity-driven BDNF-induced BMP7 expression at the end of neurogenesis instructs competent precursors to generate astrocytes. Such a mechanism might ensure synchronic neuronal and glial maturation at the beginning of cortical activity.

Keywords: astrocytogenesis, cortical development, neurotrophins

Introduction

In the developing cerebral cortex, radial glial cells act both as precursors of excitatory pyramidal glutamatergic neurons (Gotz and Huttner 2005; Guillemot 2005) and as migratory scaffolds for the radial migration of newly generated neurons (Rakic 1990; Nadarajah and Parnavelas 2002). After the completion of neurogenesis, radial glia transform into cortical astrocytes (Hunter and Hatten 1995; Hartfuss et al. 2001). γ-aminobutyric acid (GABAergic) inhibitory interneurons originate at ganglionic eminences and migrate tangentially to the cortex (Anderson et al. 2001; Ang et al. 2003). Independently of their origins, neurons generated at the same time roughly converge in the same cortical layer, following an inside-out sequence of positioning.

Genetic programs regulate the early steps of mammalian cortical development, and, as development proceeds, sensory experience and electrical activity are the driving forces that match glial and neuronal numbers and finely tune the structural and functional refinement of cortical circuits (Zhang and Poo 2001; Fox and Wong 2005; Spitzer 2006).

It is well established that transcription of brain-derived neurotrophic factor (BDNF) mRNA is robustly induced by neuronal activity in late stages of cortical development, and this activity-regulated production of BDNF is needed for postnatal neuronal survival and to balance excitatory and inhibitory synapses in cortical networks (Lu 2003; Nagappan and Lu 2005; Pattabiraman et al. 2005). BDNF and its receptor TrkB play key roles in neural development and plasticity (Huang and Reichardt 2001; Lu et al. 2005). BDNF expression is subjected to fine temporal and spatial regulation, and some of its functions rely on its ability to act as a sensor of activity. For instance, activity-dependent regulation of BDNF is required for the development of cortical inhibition but not for the survival or differentiation of GABAergic neurons (Hong et al. 2008). Loss-of-function studies on animal models have shown subtle BDNF requirements during embryonic central nervous system (CNS) development that increase postnatally (Alcántara et al. 1997; Gorski et al. 2003). However, early embryonic exposure to increased BDNF alters cell fate, neuronal migration, and synaptic function in the cerebral cortex (Brunstrom et al. 1997; Ringstedt et al. 1998; Aguado et al. 2003; Alcántara et al. 2006). Therefore, altered BDNF expression during critical developmental periods may result in cortical malformations and excitatory/inhibitory imbalance and compromise cognitive function in the adult. In support of this notion, aberrant levels of BDNF are associated with neurodevelopmental disorders (Tsai 2005; Chang et al. 2006; Lu and Martinowich 2008) and epilepsy (Scharfman 2005).

The mechanism of activity-dependent induction of BDNF has been extensively investigated. However, less is known about the genes that are targets of BDNF regulation during late embryonic cortical development. In order to identify such genes, we injected BDNF into the brain of mice at defined times during embryonic development, and we monitored changes in the expression level of a selected group of genes that were represented in a customary DNA microarray. By using this approach, we found that expression of bone morphogenetic protein 7 (BMP7) was upregulated by BDNF.

Here, we demonstrate that BDNF induces neuronal BMP7 expression during embryonic development, both in vivo and in vitro, through the Mitogen-Activated Protein Kinase/Extracellular signal-Regulated Kinase (MAPK/ERK) pathway and that this expression is partially mediated by blockage of the transcriptional activity of the p53 family of transcription factors. Exposure to increased BMP7 induced a premature transformation of radial glia into astrocytes that altered neuronal radial migration. Finally, we propose a physiological role for BDNF regulation of BMP7 during corticogenesis.

Materials and Methods

Animals and Injection in Uterus

Experiments were designed to minimize the number of animals used in the procedure. All animal protocols were approved by the Institutional Animal Care and Use Committee in accordance with Spanish and European Union regulations.
For the injection in murine brains in uterus, pregnant OF1 females carrying embryonic day 14 (E14) embryos (with E0 being the day the vaginal plug) were anesthetized with Ketamine/Valium (150 μg/g, 5 μg/g, intraperitoneal) and the uterine horns were exposed. Two microliters of recombinant human BMP7 (1 μg, PeproTech, London, UK), recombinant human BDNF (1 μg, PeproTech, London, UK), vehicle, or DNA expression vectors (6–10 μg) were delivered into the lateral ventricles of the embryos via intrauterine injection, followed by electroperoration in the case of vectors. The uterus was returned to the abdominal cavity, and the embryos were allowed to develop normally. Embryos were sacrificed at E15, E16, or E18 and used for protein or mRNA extraction, cell culture, or Immunohistochemistry (IHC).

To collect tissue for IHC analysis, embryos were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, and their brains were postfixed for 8–12 h, cryoprotected, and kept frozen. Coronal sections of 40-μm thickness were collected in a cryoprotective solution and stored at −30 °C for further use.

In Uterus Electroporation
Electroporation was performed in uterus as previously described (Tabata and Nakajima 2001). pEF1-GFP vector or a mixture of pEF1-BDNF (mBDNF cDNA inserted into pEF1 vector) and pEF1-GFP vectors at a 4:1 ratio were injected in the lateral ventricle of E14 mouse embryos. The head of the in utero embryo was held by a tweezer-type electrode (CUY650-5; Nepagene, Ichikawa, Japan), and electronic pulses (34 V for 50 ms) were discharged 4 times at 950-ms intervals with a CUY21E electroporator (Nepagene). The embryos returned to the abdominal cavity to allow normal development.

5-Bromo-2-deoxyuridine Birthdating
Thymidine analog 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, St Louis, MO) was injected intraperitoneally into pregnant females at E14 at a concentration of 50 mg/kg body weight, 3 h after BMP7 injection to the embryos. At E18, embryos were perfused and processed as described above. Incorporated BrdU was then detected by IHC.

mRNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction
Dissected cerebral cortices of E18 mice were collected and individually frozen in RNA later and stored at −80 °C until use. mRNA was purified with the RNeasy Protect Mini Kit (Qiagen, Alameda, CA) and was treated with DNase I to eliminate genomic DNA traces. The mRNA concentration and integrity were analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Synthesis of cDNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster city, CA). For real-time polymerase chain reaction (RT-PCR), TaqMan PCR assays (TaqMan Gene Expression Assay, Applied Biosystems) for mouse BMP7 and glyceraldehyde-3-phosphate dehydrogenase (as the endogenous reference) were performed from the cDNA obtained from 6 ng of RNA, in triplicate, on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Standards were prepared using cDNA from control E18 mouse RNA. Fluorescent signal was captured using the Sequence Detector Software (SDS version 1.9; Applied Biosystems)

Cell Culture
Primary cultures were prepared from E15–E16 mice neocortex. Briefly, embryonic cortices were dissected out and dissociated by trypsin-ethylenediaminetetraacetic acid (Biological Industries, Kibbutz Beit Haemek, Israel) and DNase I (Sigma-Aldrich) treatment for 10 min, followed by mechanical disruption. To obtain enriched neuronal cultures, the dissociate was plated in a 10-cm culture dish for 1 h at 37 °C in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% normal horse serum (NHS) (Gibco, Auckland, New Zealand). Embryonic cortical cells were then recovered from the supernatant and seeded on 6- and 24-well plates containing slides coated with poly-D-lysine (Sigma-Aldrich) in serum-free Neurobasal medium (Gibco, Paisley, UK) supplemented with B27 (Gibco, Paisley, UK). By using these conditions, we obtained a neuron-enriched culture, in which few glial cells were retained. To obtain primary glial cultures, we used a similar protocol, in which P0-P1 cortical cell suspensions were plated directly onto uncoated 6- and 24-well plates in DMEM with 10% NHS. Once the cells reached confluence, they were dissociated and plated again in order to eliminate remnant neurons. We only used passages 1–3. Three to 4 days after plating, serum-free neuronal cultures or glial cultures that were serum starved for 24 h were treated with 75 ng/mL BMP7 (R&D) or 10–200 ng/mL BDNF (PeproTech, London, UK) for the indicated time periods (1 h–4 days).

Cortical organotypic cultures were performed using 300-μm thick slices from E17 embryonic cortex exposed to agarose beads preabsorbed with BMP7, BDNF, or bovine serum albumin (BSA) and cultured for 2 days.

Pharmacological Treatments
In some experiments, E15–E16 primary neuronal cultures grown in serum-free medium were treated after 1-5 days in vitro with pharmacological inhibitors of the BDNF-TrkB signaling pathway. We treated neuronal cultures with the TrkB inhibitor K252a (0.6 μM Sigma-Aldrich), the MAPK/ERK Kinase 1-2 (MEK1-2)-specific inhibitor U0126 (10 μM; Calbiochem, San Diego, CA), or the PI3-kinase inhibitor wortmannin (0.1 μM, Sigma-Aldrich). We also used the p35 transcriptional inhibitor cyclic p[11]-[10]M (Sigma, Tallinn, Estonia), which inhibits the binding of the inhibitor MIDM2 to p35. All inhibitors were applied 1 h before applying 100 ng/mL BDNF (PeproTech, London, UK) for 1 or 6 h. All experiments were carried out at least 3 times and BMP7 mRNA levels were analyzed by RT-PCR.

Immunofluorescence of Culture Cells and Tissues and Western Blot Analysis
For immunofluorescence for primary cultures or tissue sections, sections that had been blocked for 1 h were incubated with primary antibodies at 4 °C overnight and subsequently with secondary antibodies conjugated to fluorophores Alexa488, Alexa555, or Alexa647 (1:500, Molecular Probes, Eugene, Oregon). In some cases, sections were incubated with biotinylated secondary antibodies (1:200, Vector, Burlingame, CA) and subsequently with a streptavidin-peroxidase complex (1:400, Amersham, Buckinghamshire, UK) and the enzymatic reaction was developed with diaminobenzidine (DAB, Sigma-Aldrich) and H2O2. TO-PRO-3 iodide (1:500, Molecular Probes, Eugene, Oregon) was used to stain nuclei. Cells and sections were counterstained with Mouse Anti-GFAP (Calbiochem).

For western blot analysis, protein extracts were obtained from primary cultures or from cerebral cortex and proteins in total extracts were separated by SDS-PAGE and electro-transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked and incubated firstly with primary antibodies overnight at 4 °C, and then with their corresponding secondary antibodies conjugated antibodies (1:3000; Santa Cruz Biotechnology, San Diego, CA). Protein signal was detected using the ECL chemiluminescent system (Amersham, Buckinghamshire, UK). Densitometric analysis, standardized to actin as a control for protein loading, was performing using ImageJ software.

Primary antibodies against the following proteins were used: actin (1:2000, Santa Cruz Biotechnology), BLBP (1:3000; Chemicon, Hampshire, UK), Brn1 (1:100, Santa Cruz Biotechnology), BrdU (1:200; GE Healthcare, Buckinghamshire, UK), calbindin (1:3000; Swant, Bellinzona, Switzerland), glial-fibrillary acidic protein (GFAP) (1:3000; Dako, Glostrup, Denmark), nestin (1:500; BD Pharmingen, Franklin Lakes, NJ), TuJ1 (1:1000; Covance, Berkeley, CA), SC1 (Secreted Protein, Acidic and Rich in Cysteines-like 1 [SPARC-like 1], 1:100; Secreted Protein, Acidic and Rich in Cysteines-like 1 [SPARC-like 1], 1:100; Santa Cruz Biotechnology), calretinin (1:2000; Swant), reelin (1:400, Chemicon), Ki-67 (1:100; Abcam, Cambridge, UK), phospho-AKT 308 (1:500; Cell Signalling, Danvers, MA), phospho-ERK 1/2 (1:1000, Sigma-Aldrich), TBR2/eomes (1:500, Abcam).

For BMP7, we used 3 different polyclonal antibodies: BMP7 N19 and L19 antibodies (1:100; Santa Cruz Biotechnology) gave stronger ICC staining, and BMP7 antibody from PeproTech gave a clearer signal in western blots. F9 cell lysate (Santa Cruz Biotechnology) and recombinant BMP7 protein were used as positive controls, and blocking peptides were used for negative controls.
**Quantitative Analysis of Cell Position in the Cerebral Cortex**

We used a general linear model that is similar to an analysis of variance model to compare the position of labeled neurons in the cerebral cortex. Fisher's least significant difference (LSD) procedure was used to discriminate between the means. Three to 8 mice were analyzed per condition (untreated mice and injected with vehicle, BDNF, or BMP7). The position of Calb+, BrdU+, Ki-67+, Thr2 + Ki-67, and BRN1 + BrdU double-labeled cells was analyzed at E18 in 3–4 coronal sections (spaced by 200 µm) from the parietal cortex of each mouse. Images from immunostained sections were captured and then imported into Photoshop. A 1665-µm wide vertical strip along the radial axis of the cerebral cortex was divided into 10 bins of equal size arranged in the following orientation: bin 1 at the pial surface and bin 10 at the ventricle. For representative purposes, bins were grouped into cortical plate (CP, bins 1–3), corresponding to the marginal zone (MZ) and developing layers II-IV, layers V-VI (bins 4–6), subplate/intermediate zone (SP-IZ, bins 7–8) and ventricular/subventricular zone (VZ/SVZ, bins 9–10), or VZ (bin 10) and cortical parenchyma (bins 1–9). The number of labeled cells in each zone was determined as the average percentage of labeled cells with respect to the total strip. Error bars reflect the standard deviation of the means.

**Results**

**BDNF Induces BMP7 Expression during Cerebral Cortex Development in Vivo**

To identify BDNF-regulated genes during corticogenesis, we injected BDNF into the lateral ventricle of E14 mouse embryos in uterus and collected cerebral cortex tissue at E18 for gene expression analysis. A low-density microarray was designed containing 25 gene members of the transforming growth factor (TGFβ) signaling cascade. As a control for selectivity in the BDNF injection assays, we also injected Neurotrophin 4 (NT4), the second neurotrophin that preferentially acts through TrkB receptor (Reichardt 2006), and SDF1α, a chemokine not related to TrkB signaling pathway. As negative controls, we used noninjected animals and animals injected with vehicle (sham). The microarray results revealed that out of the 25 members of the TGFβ family, only BMP7 expression was significantly increased at E18 in the cerebral cortex of BDNF-injected mice (1.49-fold increase) as compared with intact, sham and SDF1α-injected mice. BMP7 was also increased in NT4-injected cortices although to a lesser extent (1.22-fold increase) (Supplementary Fig. 1).

To corroborate this finding, we performed RT-PCR analysis on a different group of E18 cerebral cortices obtained under the same conditions. Consistent with our microarray data, 4 days after direct intraventricular injection, BDNF and NT4 elicited a significant increase in BMP7 mRNA in the cerebral cortex compared with intact and sham operated animals (Fig. 1A). To determine whether the rise in BMP7 mRNA was correlated with increased protein levels, a third group of embryos treated similarly with BDNF were harvested 24 or 48 h after injection and analyzed by western blot. A significant increase in the 17-kDa mature form of BMP7 protein was found after 24 (not shown) and 48 h (Fig. 1B). Taken together, these results indicate that TrkB activation mediated by BDNF or NT4 elicits long-lasting BMP7 mRNA and protein expression changes in the embryonic cerebral cortex in vivo.

To further define the mechanism by which BDNF induces BMP7 expression, we used a model of focal BDNF overexpression. E14 cortices were focally transfected with a murine BDNF expression vector or a GFP control plasmid by electroporation in uterus. As vector incorporation was restricted to one cerebral hemisphere, we used the contralateral hemisphere as an untransfected control. We analyzed the extent of BDNF overexpression in the transfected cortices by IHC. GFP-transfected and the GFP-untransfected hemispheres showed the normal pattern of BDNF expression at E18, characterized by low intensity in the VZ, lower CP (layers VI-V) and the upper CP, and weak expression in IZ (Fig. 1C). BMP7 expression was stronger in the areas transfected with the BDNF vector (Fig. 1D). In transfected areas, intensely labeled individual BDNF-positive cells were found scattered throughout the cortex, particularly in the VZ and deeper regions. In general, BDNF-transfected cells accounted for a small percentage of the total cellular content of the affected area.

We next analyzed the expression of BMP7 protein in adjacent sections to those immunostained with BDNF. Control areas expressed low levels of BMP7, mainly localized to the most mature cortical layers and the MZ (Fig. 1E). In contrast, in the region transfected with BDNF vector, BMP7 labeling increased dramatically in the upper CP (Fig. 1F). BMP7 was not induced in the VZ or IZ despite increased BDNF expression there. The overwhelming number of BMP7-overexpressing cells in the CP compared with BDNF-transfected cells indicates that BMP7 expression is induced in a paracrine fashion in cortical postmigratory neurons.

**BDNF Induce Neuronal but not Glial BMP7 Expression in Vitro**

To identify the cell type responsible for BDNF-dependent BMP7 expression, we cultured cerebral cortices from E15–E16 embryonic mice in serum-free medium. E15–E16 cortical cultures were mainly composed of neurons, neural progenitors, and a few mature glial cells (Supplementary Fig. 2). Primary cortical cultures were harvested at different times after treatment with 100 ng/mL of BDNF. Analysis of BMP7 mRNA expression by RT-PCR showed an early rise in BMP7 mRNA levels 6 h after BDNF treatment (Fig. 2A) that correlated with an increase in protein levels (Fig. 2B). Moreover, BMP7 induction by BDNF is dose dependent (Supplementary Fig. 3), showing a linear relation at BDNF concentrations up to 50 ng/mL and reaching a plateau at 50 ng/mL that is maintained up to 200 ng/mL. These data would be consistent with a direct effect of BDNF/TrkB signaling on BMP7 transcription. The reduced glial content of E15–E16 cortical cultures indicates that neurons were the most likely source of this increase in BMP7 mRNA in response to BDNF.

To examine whether BDNF also induced BMP7 expression in glial cells, we performed pure glial cultures from newborn mice and analyzed their BMP7 expression by RT-PCR. BMP7 mRNA was expressed at similar levels in serum-starved glial cultures and neuronal cortical cultures. However, BDNF treatment did not induce an increase in BMP7 mRNA in pure glial cultures (Fig. 2C).

**BDNF Induces BMP7 Expression through the MAPK/ERK Signaling Cascade and p53 Signaling**

Neurons mainly express the full-length catalytic form of the BDNF receptor TrkB. Thus, we investigate pharmacologically whether BDNF-dependent BMP7 induction was mediated by TrkB protein tyrosine kinase activity. Figure 3 shows the effect
of pretreating serum-free cortical cultures with the selected inhibitors for 1 h immediately preceding BDNF 1- or 6-h incubation. BMP7 mRNA levels were determined by RT-PCR. K252a compound is a potent protein kinase blocker that prefers Trk receptors. Pretreatment with K252a completely abolished BMP7 induction in cortical cultures treated with BDNF (Fig. 3B). To further dissect the TrkB signaling cascade involved in BDNF-dependent BMP7 expression, we focused on the PI3K/AKT pathway, mainly related to neuronal survival, and the MAPK/ERK pathway, which is involved in neuronal differentiation and synaptic plasticity (Chao 2003; Reichardt 2006). In order to test the involvement of these pathways in BDNF-mediated BMP7 upregulation, we used the specific inhibitors wortmannin (inhibitor of PI3K) and U0126 (that selectively inhibits MEK) in cortical primary cultures (Fig. 3D,E). Each inhibitor, individually or in combination, slightly reduced the basal levels of BMP7 mRNA in neuronal cultures. However, while PI3K inhibitor did not significantly affect BDNF-dependent BMP7 expression, MEK inhibitor completely abolished BMP7 induction by BDNF (Fig. 3D). These results indicate that BDNF-dependent BMP7 induction is mediated by direct activation of TrkB and MAPK/ERK signaling.

A recent study identified the p53 family of transcription factors (p53, p63, and p73) as transcriptional corepressors of BMP7 (Laurikkala et al. 2006). Furthermore, neurotrophins and ERK promote neuronal survival in part by decreasing p53 activation (Wade et al. 1999; Wu 2004; Miller and Kaplan 2007). Thus, we examined whether BDNF-dependent BMP7 induction involves a reduction in the transcriptional activity of p53. If so, pharmacological blockade of p53/p73-dependent transcription with pifithrin-α (Davidson et al. 2008) would induce BMP7 expression. Otherwise, pharmacological activation of p53/p63/p73 with nutlin-3, which blocks their binding to MDM2 (Vassilev et al. 2004), would reduce BMP7 expression. Pretreatment with 10 μM cyclic pifithrin-α or 10 μM nutlin-3 did not affect the basal levels of BMP7 mRNA, but, as expected, they modulated BDNF-dependent BMP7 expression in opposite ways (Fig. 3E,F). Pretreatment with pifithrin-α induced a 26% increase, whereas nutlin-3 decreased BMP7 expression by 30% after 6 h of BDNF treatment. These results indicate that the p53 family of transcription factors corepresses BMP7 transcription and that BDNF activation of the ERK pathway induced BMP7 expression in part by releasing this repression (Fig. 8A).

**BMP7 Affects Radial Neuronal Migration**

We then explored the physiological consequences of the rise in BMP7 levels. First, we analyzed the effect of BMP7 exposure on the laminar organization of the cerebral cortex. E14 progenitors...
were labeled by BrdU administration 3 h after the intraventricular injection of BMP7 or vehicle. The number and position of labeled cells were examined at E18. In vehicle-injected animals, the greatest number of BrdU+ cells was found in the CP corresponding to developing layers IV–II. In contrast, mice injected with BMP7 showed altered distribution of labeled cells, with a significant increase in the percentage of BrdU+ cells in the IZ, together with a lower percentage of labeled cells in the upper CP (Fig. 4A–C). Despite their altered laminar distribution, the total number of BrdU+ cells was not significantly different in BMP7-treated (130 ± 22 cells) and sham-operated animals (149 ± 26 cells per 1665-µm wide strip), indicating that neurogenesis was not affected by BMP7 treatment.

Alterations in the radial distribution of birthdated neurons as described here might be caused by a change in the laminar fate of late-generated neurons or by a defect in the machinery of neuronal migration. To investigate whether BMP7 affects the laminar fate or the migratory machinery, we analyzed the number and position of neurons double-labeled with BrdU and BRN1, a protein specific to layer II–V glutamatergic neurons (McEvilly et al. 2002). This colocalization experiment revealed that at E18 substantial numbers of E14 labeled BrdU+ cells also expressed BRN1 both in vehicle- and BMP7-treated cortices (Fig. 4E–H). In vehicle-injected animals, most double-labeled neurons were in the CP and in the VZ and SVZ. In contrast, in BMP7-injected animals, double-labeled neurons accumulated in the SP and IZ, with a marked reduction in the number of double-labeled neurons in the CP (Fig. 4D–H). The laminar distribution of BrdU-labeled neurons expressing BRN1 was identical to that of single BrdU-labeled cells. No significant differences in total number of double-labeled cells were found between BMP7-treated (41 ± 12 cells) and vehicle-treated (46 ± 14 cells per 1665-µm wide strip) cortices or in the total number of BRN1+ neurons (257 ± 40 in sham vs. 254 ± 43 BMP7) suggesting that the migratory machinery rather than the laminar fate was altered by BMP7.

Cortical glutamatergic neurons migrate on radial glia fibers, whereas GABAergic neurons use different substrates for migration (Rakic 1990; Ang et al. 2003; Kriegstein and Noctor 2004). To address whether BMP7 also affects the laminar position of GABAergic neurons, we used calbindin immunostaining. The number and laminar distribution of calbindin-positive GABAergic neurons in the cerebral cortex was analyzed at E18 in animals injected at E14 with vehicle or BMP7. BMP7-injected animals were used as a positive control for altered interneuron migration (Fig. 5A–D). The number and laminar position of GABAergic neurons remained unaltered after vehicle (74 ± 18 cells) or BMP7 injection (80 ± 10 cells per 1665-µm wide strip). In contrast, the total number of calbindin-positive neurons increased significantly in BMP7-treated cortices (92 ± 17 cells per 1665-µm wide strip, 99% LSD test), and their laminar position had shifted to the deeper layers V–VI. Furthermore, BMP7 did not show any attractive or repulsive effect on GABAergic neurons when agarose beads preabsorbed with BMP7 were placed on E17 cortical organotypic cultures (Fig. 5E–G). Taken together, these results indicate that early overexposure to BMP7 impairs the radial migration of pyramidal neurons but not that of GABAergic interneurons.

**BMP7 Affects Radial Glia Organization**

Radial migration in the cerebral cortex is dependent on the integrity of radial glia and the expression of several cell surface or extracellular factors that regulate neuron-glial adhesion. A frequent cause of defective radial migration involves reelin, an extracellular matrix protein secreted by Cajal–Retzius cells in the MZ. The lack of reelin gives rise to the reeler phenotype of inverted lamination (D’Arcangelo et al. 1995), in part by affecting radial glia integrity (Hartfuss et al. 2003). Using calretinin to identify Cajal–Retzius cells, we found that they were similarly arranged in the MZ of vehicle- and BMP7-injected mice (Fig. 6A,B). Reelin immunostaining in BMP7-injected mice also showed normal distribution (Fig. 6C,D). These results suggest that the impaired migration observed in these mice cannot be explained by defects in the organization of Cajal–Retzius cells or deficits of reelin.

A second possibility is that BMP7 directly affects radial glia phenotype or integrity, as BMPs promote astrocytogenesis from neural progenitors (Yanagisawa et al. 2001). We then analyzed the expression of several markers of radial glia and astrocytic maturation. Nestin is an intermediate filament expressed in neural progenitors and radial glia (Hartfuss et al. 2001). At E18, nestin staining was intense in the VZ lining the ventricle,
where it strongly labels radial glia cell bodies and other progenitors located in this area. In addition, nestin-positive fibers spanning the cortical wall from the VZ to the pia lined the entire radial glial palisade (Fig. 6E). Mice injected with BMP7 showed reduced nestin immunoreactivity in the VZ, where radial glia somas are located. Distorted positive fibers and isolated nestin-positive cell bodies were also frequent in the SVZ and IZ (Fig. 6F).

We next analyzed the expression of brain lipid-binding protein (BLBP), also a marker for subsets of radial glia and differentiating astrocytes (Feng et al. 1994; Feng and Heintz 1995; Hartfuss et al. 2001). In vehicle-injected cortices, BLBP labeled radial glia with a pattern that closely resembled the nestin distribution. In addition, a few ramified BLBP-positive cells were found scattered throughout the cortical wall (Fig. 6G). As occurs with nestin, BMP7 injection also reduced BLBP staining in radial fibers in the IZ and deep cortical layers and increased the number of BLBP-labeled cells scattered throughout the cortex (Fig. 6H). The changes in nestin and BLBP distribution observed in BMP7-injected cortices are consistent with an early transformation of radial glia to the astrocytic lineage.

We used IHC to detect the expression of astrocytic maturity markers as SPARC-like 1 (SC1) and GFAP. SC1 is an extracellular protein that is involved in the final neuronal detachment from radial glia at destination and is also expressed in mature astrocytes (Mendis et al. 1996; Lively and Brown 2007). In control animals, SC1 labeling was found in the entire CP (Fig. 6I). In the animals treated with BMP7, SC1 immunoreactivity was similarly distributed through the cortex but was increased, especially in layers VI–V (Fig. 6J). Similarly, SC1 protein content increases in primary cortical cultures treated with BMP7 (Supplementary Fig. 4). On the other hand, GFAP is a final marker for astrocyte maturation that is weakly expressed in the developing rodent cerebral cortex (Sancho-Tello et al. 1995). Agarose beads preabsorbed with BSA or BMP7 were deposited on organotypic cultures from E17 cortices. After...
2 days in culture, GFAP expression was not affected by BSA beads, whereas BMP7 beads showed more intense GFAP staining and the presence of ramified astroglia in their vicinity (Fig. 6K,L). Similarly, the number of GFAP-positive cells increased in primary cortical cultures treated with BMP7 (Supplementary Fig. 2).

Taken together, these results indicate that BMP7 induces a precocious radial glia-to-astrocyte transformation and increased expression of SC1 protein in the embryonic cerebral cortex.

**BMP7 Effects on VZ and SVZ Progenitors**

SVZ progenitors constitute a second proliferating population mostly derived from radial glia that appears at E13 and increases at the end of neurogenesis (Malatesta et al. 2003; Noctor et al. 2004). To determine if BMP7 alters the distribution of progenitors in this secondary germinal region, we determined the position of all progenitor cells at E18 using antibodies against Ki-67 nuclear antigen, a protein that is present during all active phases of the cell cycle but absent from resting cells (Scholzen and Gerdes 2000). This is also a good way to estimate the persistence of radial glia in the VZ, as in rodents all radial glial cells are cycling and express Ki-67 (Hartfuss et al. 2001). The total number of proliferating cells was similar in sham- and in BMP7-injected cortices (from 77 ± 15 to 116 ± 24 cells per 1665-μm wide strip). However, we found significant differences in the laminar distribution of cycling cells. In E18 sham-operated cortices, Ki-67-positive cycling progenitors were mainly found in the VZ (58%), while in BMP7-injected cortices, this percentage was reduced to 44% (99% LSD test) (Fig. 7A–C).

To determine if BMP7 treatment affects progenitor subtypes, we performed a double immunofluorescence with Ki-67 and T-brain gene-2 (TBR2) that is specifically expressed intermediate (basal) progenitor cells (IPCs), a type of neurogenic progenitors (Englund et al. 2005). We calculated the ratio of IPCs respect to the total progenitor pool by dividing the number of Ki-67+TBR2 cells into the total number of Ki-67 cells. What we found was that in E18 sham-operated cortices 84 ± 15% of Ki-67 cells were double-labeled with TBR2 while in BMP7-injected cortices the percentage of Ki-67 + TBR2 double-labeled cells was significantly reduced (55 ± 9%, 99% LSD test).

Attending to their laminar distribution, Ki-67+TBR2 progenitors were present in roughly normal proportions in the VZ (bin 10) while reduced through the SVZ and cortical parenchyma (bins 1–9) (Fig. 7D–F).

Taking together, our data suggest that BMP7 does not affect the total number of cortical progenitors but accelerates the transformation of radial glia into SVZ progenitors. Moreover,
the reduction of Ki-67 + TBR2 intermediate neurogenic progenitors respect to the total progenitor pool is suggestive of a bias from neurogenesis to gliogenesis. Although due the complexity of this process, further work will be needed to confirm this hypothesis.

**Discussion**

Our results in vivo and in vitro support 3 main conclusions. First, in the developing cerebral cortex, TrkB ligands BDNF and NT4 induce BMP7 expression in neurons through MAPK/ERK signaling, probably involving blockage of repressor activity from p53/p63/p73 transcription factors. Second, the rise in BMP7 at midgestation induces radial glia to begin their transformation into astrocytes. Third, as a result of this precocious radial glia transformation, radial neuronal migration is impaired, and cortical lamination is altered. Together, these findings support a developmental mechanism by which, at the end of corticogenesis, activity-driven rises in BDNF induce BMP7 expression in cortical neurons that in turn locally instructs competent precursors to generate astrocytes. Such a mechanism might ensure simultaneous neuronal and glial maturation at the beginning of cortical activity (Fig. 8B).

Our results indicate that neurons are the main factors responsible for BDNF-dependent BMP7 expression in vitro. Neuronal pattern of BMP7 expression was also observed in vivo in the cerebral cortex after BDNF transfection at E14 or at P0 (not shown). However, we cannot rule out the possibility that in vivo some glial cells or other cell types such as capillary endothelial cells, a recently identified source of BMP7 in the cerebral cortex (Imura et al. 2008), might also account for their upregulation, as cerebral endothelium also expresses and responds to BDNF (Guo et al. 2008). The differences in the induction of BMP7 by BDNF in neurons and glia might rely on the distinct TrkB isoforms that they express. Differential splicing of TrkB mRNA generates the full-length TrkB, which is mainly expressed in neurons, and several truncated isoforms (TrkB-t) predominant in glial cells (Cheng et al. 2007). Signaling is also different and TrkB activates PI3K/AKT, MAPK/ERK and PKC signaling pathways, whereas TrkB-t isoforms that lack kinase activity do not (Chao 2003; Reichardt 2006). By analyzing the activation of TrkB signaling pathways, we have shown that BDNF-dependent BMP7 expression requires the activation of TrkB and MAPK/ERK pathway but not that of PI3K/AKT, as the Trk inhibitor K252a and the ERK1/2 and ERK5 inhibitor U0126 but not the PI3K inhibitor wortmannin blocked BMP7 induction by BDNF.

Activated ERK phosphorylates a number of transcription factors, including p53, which in turn induce or repress the transcription of downstream genes (Chang et al. 2003; Wu 2004). A recent study has identified a p53-responsive element in intron 1 of the BMP7 gene (Yan and Chen 2007). Mutations in p53 that abrogate its DNA binding or N-terminally truncated isoforms of p63 (Δp63) and p73 (Δp73) that fail to trans-activate p53-dependent gene expression induce BMP7 expression in several systems (Laurikkala et al. 2006; Yan and Chen 2007). This indicates that full-length p53 family members repress transcription of BMP7. In agreement with these
findings, our results showed that pharmacological blockage of p53/73 transcriptional activity synergizes with BDNF in the induction of BMP7 transcription, whereas pharmacological activation of p53/73 partially reverted it. Our results also point to a basal and a regulated mechanism for BMP7 transcription, as basal BMP7 expression was not completely abolished by any of our pharmacological manipulations. Additional transcriptional activators may be required for regulation by BDNF, and p53 family members might contribute to repression.

Trk-mediated MAPK/ERK activation contributes to neuronal survival and differentiation by decreasing activation of the p53 pathway (Wade et al. 1999; McCubrey et al. 2007). Moreover, Δp73 and Δp63 isoforms are induced in the developing nervous system by Trk (Pozniak et al. 2000) and BMP7 (Laurikkala et al. 2006) signaling, respectively. This induction facilitates a regulatory loop between TrkB signaling and BMP7 transcriptional regulation by blocking the activation of p53 family members and by inducing the expression of their dominant negative truncated forms.

Our findings indicate that increased BMP7 levels at midgestation arrests the migration of glutamatergic neurons destined for the upper cortical layers. BDNF alters the laminar fate of glutamatergic neurons (Fukumitsu et al. 2006) and impairs radial neuronal migration by reducing reelin expression in Cajal–Retzius cells and cortical interneurons (Ringstedt et al. 1998; Alcántara et al. 2006). Our data indicate that BMP7 mainly affects the machinery for gliophilic radial migration, as E14-labeled ectopic neurons maintained the expression of transcription factors characteristic of their birthdates, and the laminar fate and tangential migration of cortical interneurons was preserved, at least at the early ages we studied.

Defective radial migration is caused by alteration of radial glia morphology or cell adhesion and adhesion-modulating proteins. Reelin and SC1 are extracellular matrix proteins controlling gliophilic migration. Although the mode of reelin action in neuronal migration is still controversial, a "detach-and-go" model in which reelin regulates detachment from radial glia and somal translocation has recently been proposed (Cooper 2008). In the present study, we found preserved cellular organization of the MZ after the intraventricular injection of BMP7, including reelin expression and distribution. We also failed to detect a local effect of BMP7 on Cajal–Retzius cells when applying BMP7-preabsorbed beads directly to the MZ in organotypic cultures (not shown). Our results indicate that alterations in Cajal–Retzius cell organization or in reelin expression are not the principal responsible of the migration.
arrest caused by BMP7, although we cannot completely rule out their involvement. On the other hand, SC1 is an antiadhesive protein of the SPARC-related family that regulates the interaction of cells with the ECM and that has been implicated in neuronal detachment at the end of migration (Gongidi et al. 2004). BMP7-dependent increases of SC1 expression in the CP as shown here might induce the early detachment of migrating neurons from the glial rail as they approach the CP, resulting in ectopic accumulation in the IZ similar to that observed in BMP7-treated cortices.

**Figure 7.** BMP7 effects in VZ and SVZ progenitors. Ki-67 immunostaining showing the distribution of the cycling progenitor pool in sham-operated (A) and BMP7-injected animals (B). (C) Histogram showing the displacement of Ki-67 progenitors from the VZ (bin 10) to more basal positions (bin 1–9). (D, E) Figures show the distribution of IPCs double-stained with Ki-67 and Tbr2 in sham-operated (D) and BMP7-injected (E) animals. (F) Histogram showing different distribution of IPCs between sham-operated and BMP7-injected animals. **Significant difference \( P < 0.01 \), LSD test. Error bars reflect the standard deviation. Scale bar, 80 μm.

**Figure 8.** Model of BMP7 activation by BDNF. (A) Pathway of activation. (B) Model of physiological role for BDNF-dependent BMP7 expression during development.
In addition to serving as a radial scaffold for neuronal migration, radial glia originate neurons, IPCs and glial restricted progenitors of the SVZ, and postnatally evolve into astrocytes with a precise although overlapping temporal sequence (Ihrke and Alvarez-Buylla 2008; Malatesta et al. 2008), a dynamic process regulated by crosstalk with embryonic neurons (Hatten 1985; Miller and Gauthier 2007). This process occurs in 2 sequential steps: First, notch ligands secreted by young neurons induce the expression of nuclear factor I, which promotes the demethylation of astrocyte-specific genes in neural precursors (Namihira et al. 2009), and second, glyogenic cytokines secreted by subsequent neuronal waves might then act on these demethylated glial promoters, committing competent neural precursors to the astrocyte lineage (Barnabe-Heider et al. 2005). Secreted BMPs induce astrocytogenesis and astroglial maturation from competent neural progenitors through the induction of inhibitory transcription factors of the Inhibitor of DNA binding (ID) family. ID factors antagonize proneural basic Helix-Loop-Helix protein function and induce GFAP promoter in late embryos (Yanagisawa et al. 2001; Miller and Gauthier 2007). Our data are consistent with a precocious radial-glia-to-astrocyte transformation induced by BMP7. The loss of radially and reduction of nestin expression in radial glia, together with the greater number of BLBP-positive cells in the cortical parenchyma and the rise in SC1 expression, indicate that glioblasts increase at expenses of radial glia after BMP7 treatment. In addition, GFAP and ID1 transcription factor expression were also induced in E16 cortical cultures after BMP7 treatment (Supplementary Fig. 4).

The total number of cycling progenitors at E18 is not significantly altered by BMP7 treatment; however, progenitors are displaced from the VZ to more basal positions. Double-labeling experiments using TBR2, a specific marker for IPCs (Englund et al. 2005), showed a reduction of TBR2 progenitors in the SVZ respect to the total progenitor pool. IPCs are considered neurogenic transit amplifying progenitors; thus, our data are compatible with the notion of an early radial glia transformation to progenitors of the glial lineage induced by BMP7. However, due to the complex dynamics of germinal matrices during corticogenesis, further work and long-term analysis of changes in neuronal and glial populations will be needed to sustain this assumption.

Taken together, our results indicate that the migration arrest observed in BMP7-treated animals is the result of precocious transformation of radial glia to astrocyte and a change in the extracellular matrix composition that promotes neuron-glia detachment by increasing the expression of antiadhesive factors such as SC1.

At the end of neurogenesis, sensory experience and electrical activity shape the functional and structural architecture of the CNS by regulating the transcription of a large set of genes including BDNF and its receptor TrkB (Hughes et al. 1999; Nagappan and Lu 2005; Hong et al. 2008). Activity-driven BDNF expression promotes neuritogenesis, the formation of new synapses, and the development of cortical inhibition (Bonhoeffer 1996; Aakalu et al. 2001; Kohara et al. 2007), and by binding to TrkB+ isoforms, BDNF also directs cortical progenitors to a glial cell fate (Cheng et al. 2007).

On the basis of our results, we propose a model in which BDNF-dependent BMP7 expression constitutes a local regulatory system. At the end of corticogenesis, BMP7 induction by BDNF through the TrkB and MAPK/ERK/p53 pathway might synchronize neuronal survival and differentiation with astrocytic maturation on the arrival of incoming axons and the beginning of cortical activity.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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**References**


