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BDNF-modulated Spatial Organization of Cajal–Retzius and GABAergic Neurons in the Marginal Zone Plays a Role in the Development of Cortical Organization

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The present study utilizes nestin-BDNF transgenic mice, which offer a model for early increased brain-derived neurotrophic factor (BDNF) signalling, to examine the role of BDNF in the development of cortical architecture. Our results demonstrate that the premature and homogeneous expression of BDNF, while preserving tangential migration from the ganglionic eminence to the cortex, impairs the final radial migration of GABAergic neurons, as well as their integration in the appropriate cortical layers. Moreover, Cajal–Retzius (CR) cells and GABAergic neurons segregate in the cortical marginal zone (MZ) in response to BDNF signalling, leading to an alternating pattern and a columnar cortical organization, within which the migration of different neuronal populations is specifically affected. These results suggest that both CR and GABAergic neurons play a role in directing the radial migration of late-generated cortical neurons, and that the spatial distribution of these cells in the MZ is critical for the development of correct cortical organization. In addition, reelin secreted by CR cells in the MZ is not sufficient to direct the migration of late-born neurons to the upper cortical layers, which most likely requires the presence of reelin-secreting interneurons in layers V–VI. We propose that in addition to modulating reelin expression, BDNF regulates the patched distribution of CR and GABAergic neurons in the MZ, and that this spatial distribution is involved in the formation of anatomical and/or functional columns and convoluted structures.

Keywords: BDNF, Cajal-Retzius cells, cerebral cortex, GABAergic neurons, neuronal migration, reelin

Introduction

The formation of cortical layers requires neurons to exit the cell cycle at specific times, adhere to and migrate along different substrates, identify their final destination, and then settle. The first cortical neurons that are generated form the preplate, which is split into a superficial marginal zone and a deep subplate by successive migrating neurons that form cortical layers II–VI between them, in an inside-out sequence (Caviness, 1982; Polleux *et al.*, 1997). Although the birth date of a neuron defines its position within the cortex, pyramidal neurons and interneurons have distinct origins, and follow specific developmental programs. Pyramidal glutamatergic neurons are generated in the cortical ventricular neuroepithelium and migrate radially along glial fibers to the cortex (Rakic, 1990; Nadarajah *et al.*, 2001). GABAergic interneurons are generated in the medial, lateral and caudal ganglionic eminences, migrating tangentially to the cortex, and then radially to reach their appropriate cortical layers (Anderson *et al.*, 2001; Nery *et al.*, 2002; Ang *et al.*, 2003)

The best-characterized disorder of neuronal migration occurs in the reeler mutant mouse, which displays an inversion of the normal inside-out order of cortical lamination (Caviness, 1982). The gene disrupted in reeler mice codes for a large extracellular matrix glycoprotein, reelin, which is secreted by Cajal–Retzius (CR) cells (D'Arcangelo *et al.*, 1995; Ogawa *et al.*, 1995). The reelin pathway involves the mouse homolog of the *Drosophila* gene *Disabled* (*Dab1*), which encodes an intracellular phosphoprotein (Sheldon *et al.*, 1997; Howell *et al.*, 1999), as well as two families of reelin receptors: (i) the structurally related very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) (D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999); and (ii) the cadherin-related neuronal receptor (CNR) proteins (Senzaki *et al.*, 1999). Migration defects different from those associated with reeler have been described in engineered mutations of the cyclin-dependent kinase *CDK5*, and its regulatory subunit *p35* (Gilmore *et al.*, 1998; Kwon and Tsai, 1998). Moreover, mutations in *LIS1* and *XLIS/DCX* account for the majority of human classical lissencephalies, as well as the double cortex syndrome (Pilz *et al.*, 1998).

The first cues about the mechanisms responsible for guiding tangentially migrating neurons to the cortex have now been revealed. Secreted semaphorins, acting through neuropilin receptors, repel GABAergic neurons from the ganglionic eminence and the striatum, thereby directing tangential migration to the cortex (Marin *et al.*, 2001; Tamamaki *et al.*, 2003). In addition, the chemokine stromal cell-derived factor 1 (SDF-1) regulates interneuronal, but not CR or pyramidal neuron, migration in the developing neocortex (Stumm *et al.*, 2003).

Defects in the structure and chemical composition of the cortical marginal zone (MZ) are often associated with cortical malformations. The MZ is limited superficially by the meningeal basement membrane, and is mainly composed of CR neurons, the earliest generated and differentiated cortical neurons, located in the most superficial tier. In mice, CR neurons secrete reelin, utilize glutamate as a neurotransmitter, and can be identified by calretinin (Calr) expression (del Rio *et al.*, 1995; Alcantara *et al.*, 1998). Although their exact origin remains a subject of controversy, the caudomedial wall of the telencephalic vesicle has been proposed as the primary source (Takiguchi-Hayashi *et al.*, 2004). The second large population in the MZ consists of GABAergic interneurons, which are located in the inner tier of the MZ, below the CR neurons. They are principally generated in the ganglionic eminences over an extended period of embryogenesis, with most comprising migrating neurons *en route* to the cortical plate. GABAergic neurons express at least one of the two isoforms (65 or 67) of the enzyme glutamate decarboxylase (GAD),

and most also express calbindin (Calb) during the embryonic and early postnatal period (Anderson *et al.*, 2001; Ang *et al.*, 2003). GABAergic neurons also express reelin, but late in development (Alcantara *et al.*, 1998). Finally, a third population of early-generated 'pioneer neurons' has recently been described (Morante-Oria *et al.*, 2003); this population does not express reelin or GABA, but can be identified by TAG1 expression and possesses a subpallial origin.

Neurotrophins are a family of peptide growth factors regulating neuronal survival, synaptic modulation, and axon growth and branching. Brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT4) are also known to be involved in the control of neuronal migration (reviewed in Huang and Reichardt, 2003). Studies of null BDNF and TrkB mutant mice indicate subtle requirements for BDNF in the development of embryonic cortical neurons, which increase postnatally (Alcantara *et al.*, 1997; Gorski *et al.*, 2003). However, application of NT4 and BDNF to the embryonic cortex, either *in vitro* or *in vivo*, produces heterotopies in the MZ that appear to result from aberrant migration from the ganglionic eminence (Brunstrom *et al.*, 1997; Brunstrom and Pearlman, 2000). Moreover, ectopic overexpression of BDNF prior to the onset of endogenous expression downregulates reelin and produces a polymicrogyric cortex with disorganized CR cells and aberrant cortical lamination (Ringstedt *et al.*, 1998). During embryonic development, CR neurons express BDNF and NT4 along with their receptor TrkB (Fukumitsu *et al.*, 1998), whereas GABAergic neurons only express TrkB (Gorba and Wahle, 1999). Both neuronal cell types exhibit morphological, neurochemical and physiological changes in response to BDNF/NT4, including accelerated dendritogenesis, synaptogenesis and spontaneous activity (Marty *et al.*, 1996; Aguado *et al.*, 2003; Wirth *et al.*, 2003). Moreover, BDNF and NT4 stimulate cortical interneuron migration through PI3-kinase signalling (Polleux *et al.*, 2002).

To address the role of BDNF in the development of cortical architecture, we have generated an *in vivo* gain-of-function model that overexpresses BDNF under the control of the nestin promoter (nestin-BDNF; Ringstedt *et al.*, 1998). Using this mouse model, we show that under these conditions GABAergic neurons reach the cortex by tangential migration, but fail to integrate within their appropriate cortical layer, subsequently forming ectopies. In the MZ, ectopic clusters of GABAergic neurons occupy the gyri, and segregate from CR cells, which occupy the sulci, leading to columnar, cell-type-specific disturbances in cortical lamination. Taken together, our data reveal that BDNF is involved in the final phase of interneuron migration and layer recognition, and that GABAergic and CR neurons in the MZ have specific and differentiated roles both in guiding neuronal migration and in organizing anatomical columns and cortical lamination.

Materials and Methods

Animals

Nestin-BDNF transgenic mice were generated as previously described (Ringstedt *et al.*, 1998). The day of oocyte transplantation was considered embryonic day 0 (E0) and the offspring (at E14, E16 or E18) were screened for founders by polymerase chain reaction. In addition, every founder was considered an independent transgenic line. P0 reeler mice (Orleans strain) were obtained by breeding homozygous (–/–) mice, identified by their ataxic behaviour. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Tissue Collection

After deep anaesthesia of the dams, embryos were extracted and fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.3. Embryos older than E14 were transcardially perfused with the above fixative. After postfixation, the brains were embedded in paraffin, or cryoprotected in 30% sucrose and frozen on dry ice. Coronal sections 40 μm thick were collected from frozen tissue in a cryoprotectant solution, and stored at –30°C until later use.

In Situ Hybridization

In situ hybridization was performed on free-floating sections, essentially as previously described (Alcantara *et al.*, 1998). Riboprobes were labelled with digoxigenin-dUTP (Roche Diagnostics) by *in vitro* transcription. cDNA fragments from the 5′ region of mouse reelin (2 kb), mouse DAB1 (2.3 kb), mouse semaphorin 3A (2 kb), mouse semaphorin 3F (917 bp), rat neuropilin 1 (1.1 kb), rat neuropilin 2 (1.1 kb), and rat GAD67 (3.2 kb) were transcribed using T3 polymerase; fragments from rat GAD65 (2.3 kb), rat robo1 (1 kb) and rat robo2 (1.7 kb) were transcribed using T7 polymerase; a 1.1 kb fragment from mouse gap43 was transcribed using SP6 polymerase (Ambion, TX). Labelled antisense cRNA was added to the prehybridization solution (250–500 ng/ml) and hybridization was carried out at 60°C overnight. Sections were then washed under increasing stringency. Following hybridization, sections were blocked in 10% normal goat serum and incubated overnight with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics, 1:2000). Sections were developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Life Technologies, Gaithersburg, MD), mounted on gelatinized slides, and coverslipped with Mowiol™. Control hybridizations, including those with sense digoxigenin-labelled riboprobes or pre-treated with RNase A digestion, prevented alkaline phosphatase staining above background levels.

BrdU Birthdating

Thymidine analog 5-bromo-2′-deoxyuridine (BrdU; Sigma) was injected intraperitoneally on gestational days E11, E14 and E16 into pregnant females at a concentration of 50 μg/g body wt. On E18, pups were perfused and processed as described above. Incorporated BrdU was then detected by immunohistochemistry.

Immunohistochemistry

Sections from different developmental stages, some previously hybridized with the different riboprobes, were immunolabelled with polyclonal antibodies against calbindin D28k and calretinin (1:8000 and 1:3000 respectively, Swant); B3 antibody against DAB1 (1:500–1000, Howell); GFAP (1:2000, Vector), GAP-43 (1:100000, Schrama) and monoclonal antibodies against nestin (1:500, Pharmigen); BrdU (1:100, Dako), G10 and CR50 against reelin (1:5000 and 1:2000 respectively; Goffinet, Nakajima). Sections were then incubated with secondary antibodies (1:200, Vector) followed by a streptoavidin–peroxidase complex (1:400, Amersham). The enzymatic reaction was developed with diaminobenzidine (DAB) and H_2O_2. Omission of the primary antibodies or addition of blocking peptides prevented DAB staining.

Western Blotting

Soluble proteins from the forebrains of three BDNF-overexpressing mice and three control littermates were separated by 10% SDS–PAGE and electro-transferred to a nitrocellulose membrane. Membranes were incubated with a polyclonal antiserum against DAB1 B3 (1:1000, Howell). A polyclonal antibody against CDK5 (1:500, Santa Cruz) was used as a control for protein loading. Protein signal was detected using the ECL chemiluminescent system (Amersham). Densitometric analysis, standardized to CDK5, was performed using TotalLab v. 2.01 software.

Photography

Micrographs were captured with a Zeiss Axiophot or Nikon Eclipse 800 microscope. Images were assembled in Adobe Photoshop (v. 6.0), with adjustments for contrast, brightness and colour balance to obtain optimum visual reproduction of data.

Quantitative Analysis of Cell Position in the Cerebral Cortex

We used a general linear model (GLM) to estimate and compare the distances migrated by BrdU-labelled neurons in the cerebral cortex of

wild-type (WT) and nestin-BDNF sulci and gyri. Fisher's least significant difference (LSD) procedure was used to discriminate between the means. Three E18 mice injected at E14, and two injected at E16, were analysed for each genotype (WT and nestin-BDNF). Ten-micron sections of paraffin-embedded brains were cut in the coronal plane and immunostained using anti-BrdU alone, or double-stained with anti-Calb or -Calr antibodies. The position of BrdU-positive neurons was analysed in four or five sections (spaced 100 μm apart) from the parietal cortex of each mouse. Photographs from processed sections were captured using a Nikon Eclipse 800 microscope and a Diagnostic Instruments Inc. Spot JR video camera, and then imported directly into Photoshop. Ten bins of equal size were assigned along the radial axis of the cerebral cortex and arranged in the following orientation: bin 1 at the pial surface and bin 10 at the subventricular zone (SVZ). The number of BrdU-positive neurons in each bin was determined as the average percentage of cells in a 185 × 111 μm^2 area. Error bars reflect the standard deviation of the means.

Results

The Development of Polymicrogyria in Nestin-BDNF Mice Is Correlated to the Segregation of GABAergic and CR Neurons in the MZ

We first analysed the distribution of GABAergic interneurons and CR cells in the MZ during embryonic development from E12 to E18, close to the maximal survival time of nestin-BDNF mice. Interneurons were identified by the presence of Calb and/or the two isoforms (65 and 67) of glutamic acid decarboxylase (GAD), which is the biosynthetic enzyme of γ-aminobutyric acid (GABA), while CR cells were identified by Calr and/or reelin expression.

Rodent MZ exhibits a homogeneous bilayered organization in which CR cells occupy the superficial half, the inner half being occupied by GABAergic neurons. In nestin-BDNF mice, the early development of CR cells is preserved, exhibiting normal morphology and distribution at E14 and earlier stages. Subsequently, at E16, CR cells began to reorganize in the MZ, leaving empty areas, forming cell clusters, and sending their axons abnormally deep into the cortical wall (Fig. 1*A–F*).

As with CR cells, GABAergic neurons were normally distributed in the MZ until E14 (not shown), but from E16 onwards the first ectopies were clearly recognizable. In both transgenic and control mice, GABAergic neurons delineated three migratory pathways along the MZ, upper-intermediate (IZ) and SVZ zones, indicating that tangential migration from the ganglionic eminences to the cortex was maintained, or even increased, in transgenic mice (Fig. 1*G–J*). However, wedge-shaped clusters of GABAergic interneurons emanating from the MZ and SVZ were prominent in the transgenic cortex (Fig. 1*H,J,L*), suggesting a failure in both ventricle- and pia-directed radial migration into the cortical plate (CP).

At E18, the cerebral cortex was polymicrogyric, and enlarged CR cells formed aggregates in the MZ of the sulci (Fig. 2*A,B*), a distribution consistent with that of a previous description (Ringstedt *et al.*, 1998). Hypertrophied CR cells expressed high levels of Calr, lost their monopolar and horizontal morphology (adopting more heterogeneous shapes and random orientation), and extended their profuse axonal plexuses abnormally deep into the superficial layers of the CP (Fig. 2*A,B,G,H,K–N*). At E18, the initial clustering of GABAergic neurons in the MZ of nestin-BDNF mice was exacerbated. Neuronal heterotopies were distributed in a mosaic pattern: GABAergic neurons accumulated in the gyri, whereas clusters of CR cells occupied the sulci. GABAergic neurons were also present in the IZ and

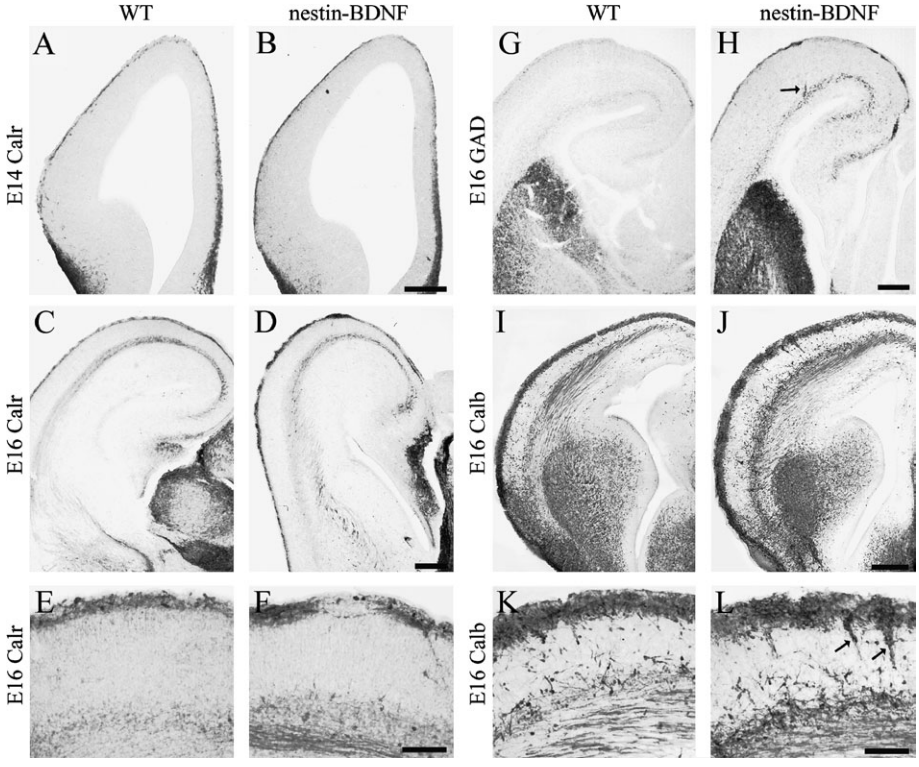

Figure 1. Distribution of GABAergic and CR neurons at the onset of BDNF-induced malformation. Coronal sections immunostained for Calr at E14 (*A*, *B*) and E16 (*C*, *D*) of wild-type (WT) and nestin-BDNF mice, exhibiting the beginnings of CR cell clustering and segregation. E16 coronal sections hybridized with GAD65/67 antisense riboprobes (*G*, *H*), or immunostained for Calb (*I–L*), displaying GABAergic neuronal clusters in the MZ and IZ. Wedge-shaped ectopies of GABAergic neurons are indicated with an arrowhead. Scale bars: 100 μm (*A–D*, *G–J*); 250 μm (*E*, *F*, *K*, *L*).

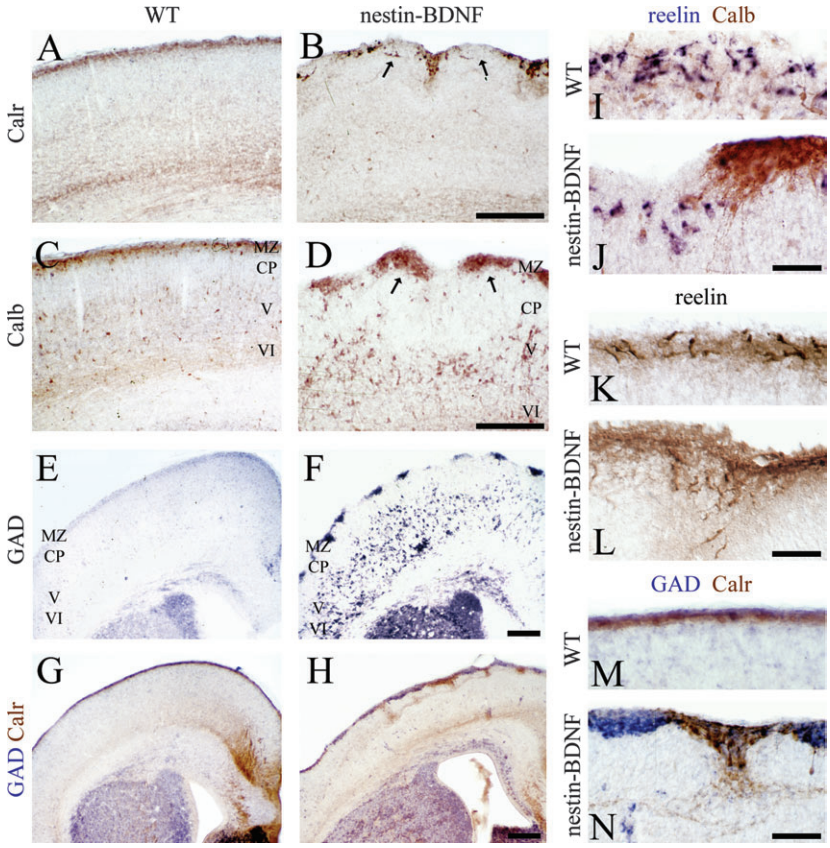

Figure 2. Segregation of GABAergic and CR neurons in the MZ at the end of the embryonic period in nestin-BDNF mice. E18 coronal sections immunostained for Calr (*A*, *B*) and Calb (*C*, *D*), or hybridized with GAD65/67 riboprobes (*E*, *F*), and double-labelled with Calr antibodies (*G*, *H*), revealing the alternated distribution of GABAergic and CR neurons in the MZ of the transgenic cortex. Areas lacking CR cells (*B*) surrounded by GABAergic neuronal ectopies (*D*) in the MZ are indicated by an arrowhead. High-magnification photomicrographs of the MZ in sections hybridized with an antisense probe for reelin and double-labelled with Calb antibodies (*I*, *J*), immunostained with reelin antibodies (*K*, *L*), or double-labelled with GAD65/67 riboprobes and Calr antibodies (*M*, *N*), showing roughly normal reelin mRNA and protein expression in CR cells, which are almost completely segregated from GABAergic neurons. Scale bars: 250 μm (*A–D*, *E–H*); 50 μm (*I–N*).

deep CP, but only formed cellular columns that traversed the cortical wall in the gyri (Figs 2*C–H*, 6*B*). This columnar distribution of migrating GABAergic neurons suggests that different guidance signals emanate from the MZ of the sulci and gyri. Moreover, GAD mRNA and Calb were overexpressed in interneurons, which also exhibited heterogeneous shapes and orientations, with enlarged soma, dendrites and axonal arbours (Figs 2*C,D,I,J*, 6*D–E*).

In summary, despite the early initial activation of the nestin promoter, BDNF-induced cortical polymicrogyria does not develop until the late gestational period (E16–18). Early ectopic overexpression of BDNF produces a dramatic distortion of the bilaminar organization of GABAergic neurons and CR cells, resulting in a patchy distribution of the two cell types in the MZ. The time course of this segregation starts long after the initial splitting of the preplate, and correlates strongly with the development of polymicrogyria. This spatial and temporal correlation is suggestive of a causal relationship between the initial cellular segregation in the MZ and the subsequent folding, as CR cells accumulate in the sulci and GABAergic neurons in the crown of the gyri.

Alterations in the Radial Glial Palisade in Nestin-BDNF Mice

The segregation of GABAergic and CR cells in the MZ of nestin-BDNF mice suggests the possibility of columnar differences in the organization of the CP. As the integrity of the radial glial scaffold is a prerequisite for the correct migration of cortical neurons, we decided to analyse glial organization in the transgenic embryos. To achieve this, we placed crystals of the lipophilic tracer DiI on the subpial surface of E18 cortex to trace the radial organization of the embryonic glia (Voigt, 1989). In the WT cerebral cortex, DiI staining revealed homogeneous radial distribution of labelled fibres, whereas a marked radial heterogeneity was evident in the nestin-BDNF cortex, in which columns of vertically arranged, DiI-labelled fibers appeared separated by empty areas (Fig. 3*A,B*). As DiI only diffuses in cells that are in direct contact, the empty areas might reveal a lack of radial glia in this area, or a retraction and absence of attachment between the glial endfeet and the pial membrane.

We next analysed the distribution of nestin-positive fibers during development. Nestin, an intermediate filament protein expressed by precursor cells in the developing cortex, including radial glia, similarly delineated radial glial processes in both the WT and transgenic cortex (Fig. 3*C–H*). In the WT cortex, nestin-positive processes followed more or less tangential trajectories from the VZ/SVZ to the SP, adopting a clear radial orientation when penetrating the CP, and ending in an apical tuft in the MZ. In the transgenic cortex, vertical radial glial structures were clearly seen at E14, following roughly normal trajectories when traversing the CP (Fig. 3*C,D*). However, the perfect arrangement of parallel fibres in the CP was

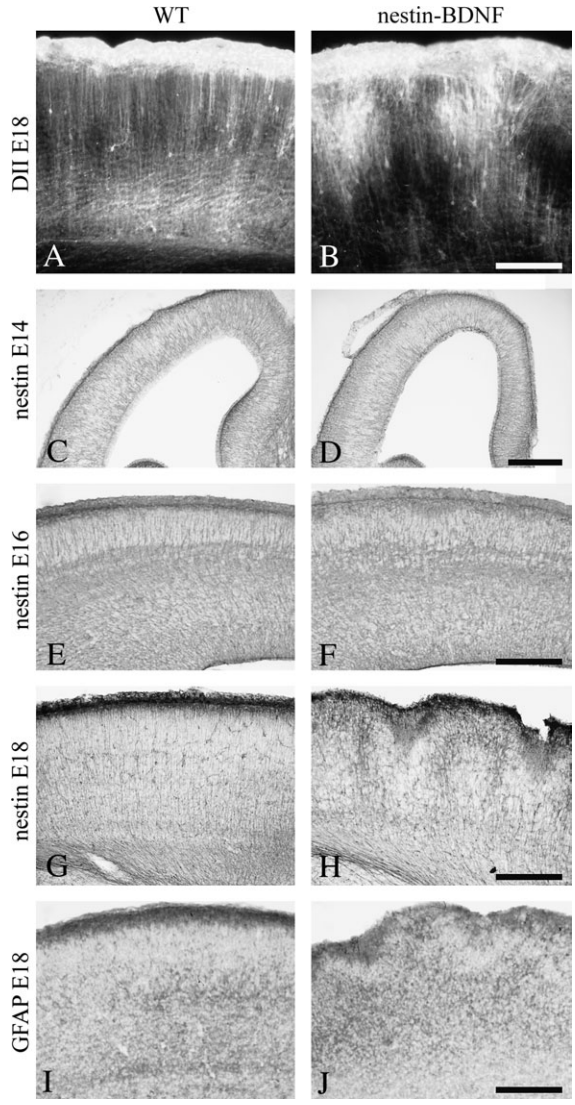

Figure 3. Organization of glial cells. Coronal sections of the E18 cerebral cortex in WT (*A*) and nestin-BDNF mice (*B*), displaying the patchy distribution of radial fibres labelled after the placement of a DiI crystal on the pial surface of the transgenic brain. Coronal sections immunostained for nestin at E14 (*C*, *D*), E16 (*E*, *F*) and E18 (*G*, *H*), showing the progressive misplacement of cortical radial glia fibres in nestin-BDNF mice. (*I*, *J*) Mature astrocytes labelled with GFAP antibodies at E18, exhibiting abnormal distribution in the MZ of gyral areas in transgenic mice. Scale bars: 250 μm.

subtly distorted at E16, and more dramatically at E18, at which time the structure of the apical tufts in the MZ differed between gyri and sulci. Thin nestin-positive processes, probably corresponding to terminal glial tufts, invaded the MZ of the sulci, where CR cells were located. In contrast, thick radial processes invaded the gyri, where GABAergic neurons accumulated, without a clear terminal tuft in the MZ (Fig. 3*E–H*). In summary, these data suggest that the radial organization of the radial glia is roughly preserved in the sulci, whereas glial fibres thicken in the gyri without forming a typical terminal tuft in the MZ.

At E18, astrocytes, identified by GFAP expression, were abundant in the WT MZ, where they contributed to the 'meningoglial barrier' at the brain surface, but were scarce in the CP. In transgenic mice, mature GFAP-positive astrocytes were correctly located in the superficial MZ, but extended to deeper positions below the ectopic mass of GABAergic cells in the gyri (Fig. 3*I–J*). No major changes in GFAP expression were found in the CP, indicating roughly normal maturation of the astrocytic population.

BDNF Overexpression Differentially Affects Distinct Neuronal Populations

To determine whether BDNF overexpression differentially affects the development of different neuronal populations in the cerebral cortex, we compared the distribution of GAD-expressing neurons with that of the widely distributed growth-associated protein GAP-43, the expression of which has been correlated with the capacity for axon growth and synaptic remodelling, and with the expression of some guidance cues for migrating neurons, dendrites and axons (Shen *et al.*, 2002).

At E18, GABAergic interneurons were distributed throughout the developing cortical layers in the WT cortex, although concentrated in layer V and the MZ. In the transgenic cortex, GAD-expressing neurons formed heterotopic clusters throughout the region between the SVZ and layer V, and in the MZ. The upper, and still not well-defined, layers of the CP were mostly devoid of GAD-expressing neurons (Fig. 4*A,B*). GAP-43 mRNA was widely expressed at low levels throughout the WT cerebral cortex. However, in nestin-BDNF mice, GAP-43 expression dramatically increased in layers V and VI (Fig. 4*C,D*), exhibiting a non-overlapping distribution with GAD expressing neurons. This result indicates that, despite GAP-43 overexpression, the migration of early-generated pyramidal neurons remained mostly intact. In fact, GAP-43 expression in the embryonic mutant cortex closely resembled the normal postnatal and adult patterns, in which it is strongly expressed in long projecting pyramidal neurons in layers V and VI (Feig, 2004). GAP-43 protein is expressed in cortico-fugal axons, and in thalamo-cortical (Maier *et al.*, 1999) and serotonergic afferents (Donovan *et al.*, 2002). In WT, GAP-43 positive axons occupied the IZ and MZ at E16, with very few entering the CP. At E18, they were similarly present throughout the cortical layers. GAP-43-positive fibres exhibited a similar distribution in transgenic mice, except for the presence of empty areas in the MZ at E16, and columnar differences resembling the alternating distribution of CR and GABAergic neurons at E18. The GAP-43-positive fibre arrangement resembled the WT organization in the sulci, while it had a broad distribution in the gyri, where the fibres invaded the upper CP. Moreover, labelled axons in the IZ and internal capsule showed increased fasciculation and formed thick bundles (Fig. 4*E–H*). These results indicate that BDNF induces GAP-43 overexpression in cortico-subcortical projection neurons without dramatic changes in their laminar position. Moreover, GAP-43-labelled axons, including serotonergic and thalamocortical afferents, exhibited increased fasciculation and columnar differences in their distribution.

We subsequently analysed changes in the expression of guidance cues involved in the migration of cortical GABAergic neurons. At E18, neuropilins 1 and 2 (NP1, NP2) were widely expressed from the VZ to the MZ in the WT cerebral cortex, with some intensely labelled neurons located in layer V. Although both receptors were similarly expressed in the transgenic cortex, NP1 in particular, high-expressing neurons were more broadly distributed in the gyri, where they occupied the entire cortical thickness, than in the sulci (Fig. 4*I,J*). The distribution of sema3A in the WT cortex was similar to that of NP1, with the exception of high-expressing neurons, which were also found scattered in layer VI and in the most superficial

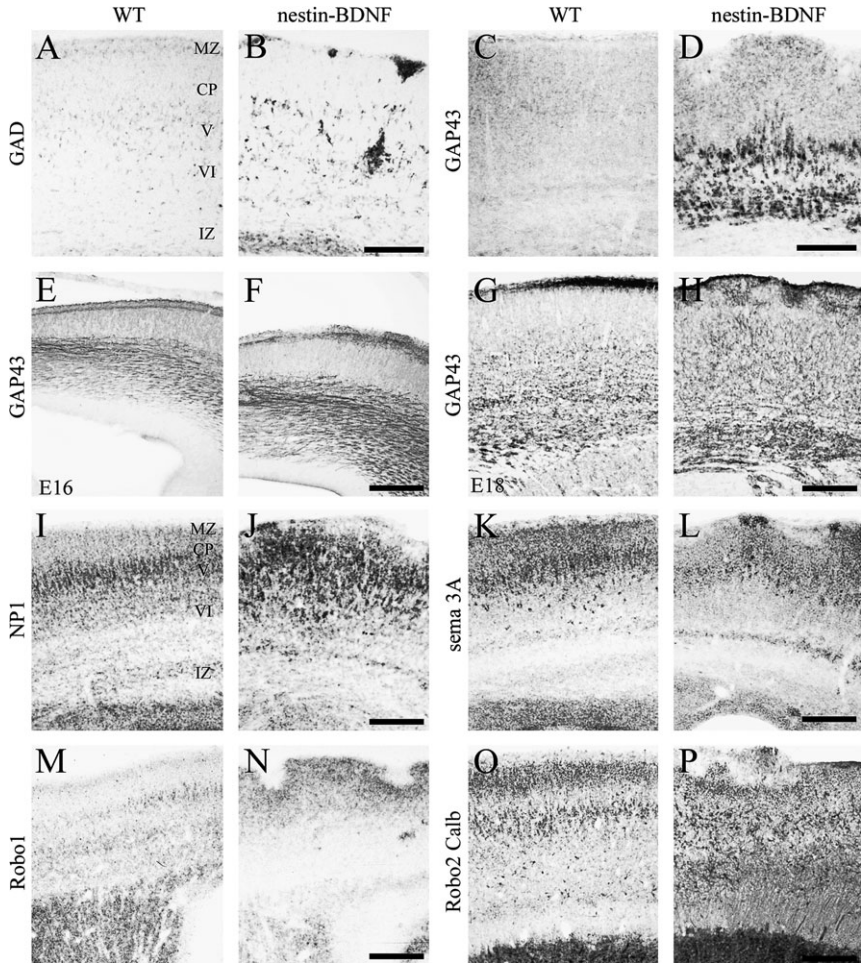

Figure 4. Effects of BDNF overexpression on different neuronal populations. (*A*, *B*) GAD65/67 ISH at E18, exhibiting ectopies of GABAergic neurons in the MZ and deep cortical layers of nestin-BDNF mice. (*C*, *D*) GAP-43 ISH at E18, showing upregulation of mRNA expression in the SP and layer V–VI pyramidal neurons of nestin-BDNF mice, and preserved lamination of the deep cortical layers. GAP-43 immunopositive fibres occupy the IZ and MZ at E16 in WT (*E*) and nestin-BDNF (*F*), demonstrating increased fasciculation in the IZ and gaps in the MZ of the mutant. (*G*, *H*) GAP-43 immunopositive fibers at E18, showing increased fasciculation and differential organization in the sulci compared with the gyri of nestin-BDNF mice (*H*). (*I*–*L*) ISH with antisense riboprobes for neuropilin 1 (NP1; *I*, *J*) and semaphorin 3A (sema3a; *K*, *L*). Clustering of high-expressing sema3a neurons in the MZ (*L*) is accompanied by increased NP1 expression in all cortical layers (*J*) in transgenics. (*M*, *N*) robo1 ISH and (*O*, *P*) double-labelling with robo2 riboprobe (blue) and Calb antibodies (brown), showing increased robo1 and 2 expression in the upper cortical layers of nestin-BDNF mice; expression is reduced in these animals in layers IV and V, where GABAergic neurons accumulate. Scale bars: 200 μm.

part of the CP. In the transgenic cortex, as with GABAergic neurons, these intensely labelled cells accumulated in the gyri, particularly in the crown, indicating their probable GABAergic phenotype (Fig. 4*K*,*L*). No clear differences were found in Sema3F distribution, which was widely expressed at low levels in both WT and transgenic cerebral cortex (not shown).

Slit/Robo interactions are involved in the regulation of dendritic development and in the guidance of callosal axons (Sang *et al.*, 2002; Shu *et al.*, 2003). The Slit receptors Robo 1 and 2 exhibited similar laminar distributions in the E18 cerebral cortex, with more labelled cells in the IZ, SP, layer V and in the most superficial tier of the CP. In the transgenic cortex, however, Robo1 and 2 expression increased in the upper half of the CP, corresponding to layer IV and the forming layers II–III, the destination of callosal pyramidal neurons, and did not overlap with areas containing GABAergic neurons (Fig. 4*M*–*P*).

Taken together, these results indicate that BDNF induces increased expression of genes linked to dendritic and axonal growth and guidance, with limited effects on the laminar position of early-generated pyramidal neurons.

Differences in Radial Migration Between Sulci and Gyri

The segregation of GABAergic and CR neurons in the MZ of nestin-BDNF mice, in the gyri and sulci respectively, led us to hypothesize that a columnar distribution of putative specific guidance cues, emanating from both cell types, might differentially affect subpopulations of migrating neurons. To explore this possibility, we injected BrdU in pregnant dams at different gestational ages: E11, to label SP and MZ neurons; E14, to label neurons destined to occupy layers V and IV; and E16, for neurons destined to occupy the upper cortical layers. We then analysed the laminar arrangement of labelled neurons at E18 in coronal sections of the WT cortex, and in the gyri and sulci of the nestin-BDNF transgenic cortex (Fig. 5).

E11 BrdU-labelled nuclei exhibited the same bilaminar distribution in nestin-BDNF mice as in WT, indicating, as expected, that the SP and MZ were correctly split by CP neurons in the transgenic cortex (Fig. 5*C*,*D*). To analyse the laminar fates of cells labelled at E14 and E16, the cortex was divided into ten radial bins of equal size; bin 1 was located at the MZ and bin 10 at the SVZ. Binning facilitates laminar analysis of

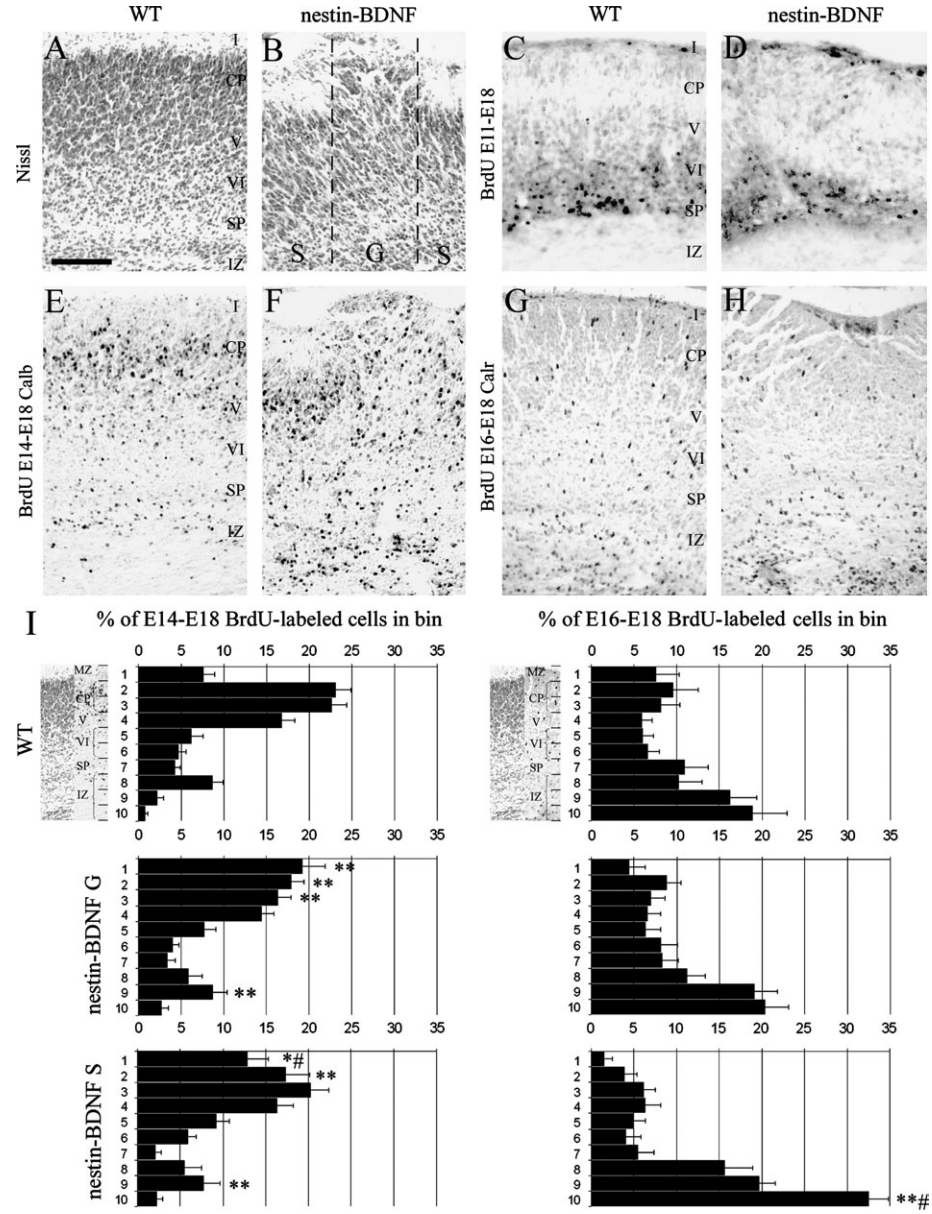

Figure 5. Columnar changes in the laminar fate of late generated neurons in nestin-BDNF mice. Nissl-stained E18 coronal sections of WT (*A*) and nestin-BDNF mice (*B*), indicating the sulci (S) and Gyri (G). (*C*, *D*) BrdU injection at E11 and BrdU immunocytochemistry at E18 reveals normal preplate splitting in nestin-BDNF mice. (*E*, *F*) BrdU injection at E14 and immunocytochemistry at E18 for BrdU (black) and Calb (brown); and (*G*, *H*) BrdU injection at E16 and immunocytochemistry at E18 for BrdU (black) and Calr (brown), reveal columnar differences in the laminar distribution of labelled cells in transgenic (*F*, *H*) versus WT (*E*, *G*) cortex. (*I*) Quantitative analysis of the E18 laminar distribution at E18 of cells labelled with BrdU at different stages. E14-labelled cells in nestin-BDNF mice accumulate in the MZ, more in the gyri than in the sulci, and in the IZ. E16-labelled cells accumulate in the deep IZ in the sulci of nestin-BDNF mice. $*P \leq 0.05$ and $**P \leq 0.01$ with respect to wild type; $\#P \leq 0.05$ between gyri and sulci, using the LSD test. Scale bar: 200 μm.

immature or malformed cortex without a clear cortical lamination. Histograms (Fig. 5*I*) illustrate the average percentage of BrdU-labelled cells at E14 and at E16 in a given radial bin for the three structures compared. Additionally, the mean distance of BrdU-labelled cells from the pia was calculated for the three structures: WT cortex, and gyri and sulci of nestin-BDNF transgenics. The distribution of BrdU-labelled cells by bin was compared using a general linear model (GLM), a procedure similar to the analysis of variance (ANOVA) model (Searle, 1987). This analysis demonstrated that the distribution of E14 BrdU-labelled cells by bin was significantly different among the three structures ($P < 0.0001$). The main differences were a dramatic increase in the proportion of labelled cells in the MZ of the nestin-BDNF cortex (especially in the gyri) and in the IZ, and a 33% reduction in the total number of E14 BrdU-labelled cells restricted to the sulci ($P \leq 0.001$). The same GLM analysis revealed that the mean distance from the pial surface of E16 BrdU-labelled cells was significantly different among the three structures ($P = 0.0009$), due to a dramatically increased proportion of labelled cells in the deep IZ/SVZ of the sulci ($P \leq 0.001$), but without affecting the total number of labelled cells.

These results indicate that radial migration is differentially affected in sulci and gyri. The presence of CR cells alone in the MZ of the sulci is not sufficient to maintain the normal radial distribution of BrdU-labelled cells, suggesting that normal

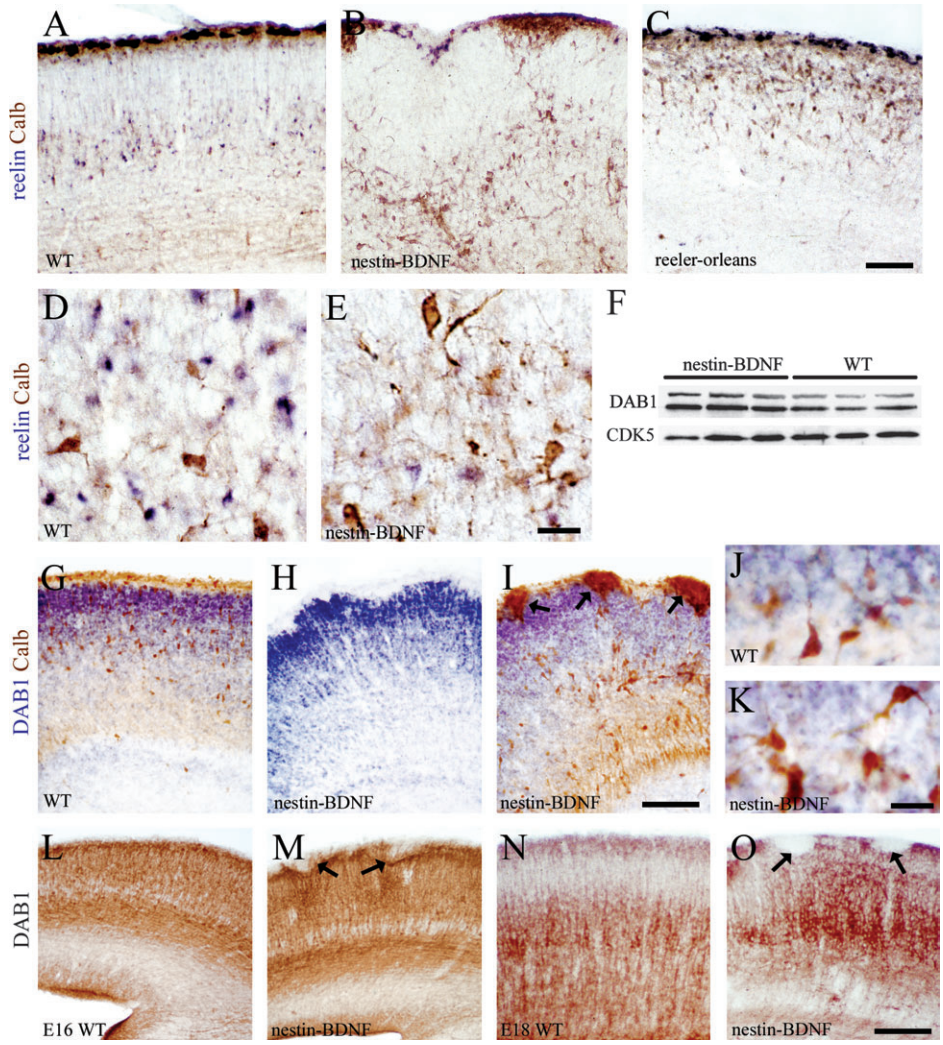

Figure 6. Reelin signalling components in the transgenic cerebral cortex. Coronal sections of the cerebral cortex hybridized with an antisense probe for reelin (blue) and double-labelled with Calb antibodies (brown) at E18 of WT (*A*, *D*), nestin-BDNF (*B*, *E*), and P0 reeler Orleans strain mice (*C*), a mutation in which reelin mRNA is expressed and translated but the protein is not secreted. Reelin and GABAergic neuron distribution in nestin-BDNF mice is completely different from that seen in the reeler malformation. Note the marked reduction in reelin expression in cortical plate interneurons in nestin-BDNF mice (*D*, *E*). (*F*) Western blot showing an increase in the total DAB1 protein content in the forebrain of three nestin-BDNF mice compared with three WT. As CDK5 protein remains unchanged, it was used as a loading control. (*G–K*) DAB1 ISH (blue) and Calb immunohistochemistry (brown) at E18, and DAB1 immunoreactivity at E16 (*L*, *M*) and at E18 (*N*, *O*), showing lack of DAB1 expression in Calb-positive interneurons, and increased DAB1 immunoreactivity in pyramidal neurons. Note the exclusion of DAB1-labelled dendrites from GABAergic neuronal ectopies in nestin-BDNF mice (arrows). Scale bars: (*A–C*, *G–I*, *L–O*) 200 μm; (*D*, *E*, *J*, *K*) 50 μm.

laminar cortical organization requires CR cells and GABAergic neurons, and that their segregation in the MZ leads to columnar differences in the radial distribution of neuron subpopulations generated at different ages.

Reelin Pathway Involvement in the Development of Polymicrogyria

In addition to the reduced expression in CR cells (Ringstedt *et al.*, 1998), our results revealed a dramatically reduced, or even absent, reelin expression in the CP (Fig. 6*A–B*,*D–E*), where it is normally expressed by GABAergic neurons. Moreover, the segregated structure of the MZ and the columnar arrangement of the cortical lamination characteristic of the nestin-BDNF mouse was completely different from that of the reeler mouse, in which the main defect is the failure of preplate splitting, resulting in a superficial superplate that retains the bilaminar organization of GABAergic and CR cells (Fig. 6*A–C*). To assess the contribution of altered reelin signalling in late embryonic development, as well as the formation of polymicrogyria, we focused on Dab1, an 80 kDa cytoplasmic signalling protein involved in relaying the reelin signal.

Western blot analyses revealed two main isoforms of DAB1 in the telencephalon of both WT and nestin-BDNF mice. The levels of both isoforms increased 2-fold in the nestin-BDNF forebrain compared with WT (Fig. 6*F*). As increases in DAB1 protein content are directly associated with low reelin expression (Howell *et al.*, 1999), we employed ISH and immunocytochemistry to determine whether local increases in DAB1 protein were also associated with areas of low reelin availability.

At E18, DAB1 mRNA was strongly expressed in the most superficial and immature CP, decreasing to low levels in the deep cortical layers, IZ and VZ, a laminar distribution that was maintained in the transgenic cortex. The MZ was completely devoid of DAB1 mRNA expression, indicating that DAB1 was not

expressed by CR cells or GABAergic neurons. This observation was corroborated by double-staining with Calb, which revealed an almost complete absence of double-labelled cells in the CP, including the clusters of GABAergic cells in the MZ (Fig. 6*G–K*). In the WT E16–18 cortex, DAB1 protein was found in the soma, dendrites and axons of most cortical neurons, but especially in layer V pyramidal neurons and in radial glia. In the nestin-BDNF cortex, DAB1 expression slightly increased in the CP below the GABAergic clusters in the MZ at E16, and at E18 its expression dramatically increased in pyramidal neurons, essentially in layer V, thereby exhibiting a columnar periodicity roughly corresponding to gyral and sulcal distribution (Fig. 6*L–O*). A general DAB1 increase in layer V pyramidal neurons at E18 is consistent with the nearly absent reelin expression by CP GABAergic neurons at that time (Fig. 6*D,E,N,O*). The laminar distribution of DAB1 immunoreactivity was preserved, although large layer V pyramidal cells were more dispersed in the transgenic cortex. This diffuse immunoreactivity in the MZ most likely results from the dendritic apical tufts of pyramidal cells and glial end feet. Moreover, DAB1-positive structures avoided entering the clusters of GABAergic cells in the MZ of the transgenic cortex (Fig. 6*M,O*).

Considered as a whole, our data demonstrate that rather than effecting a global change, regional laminar and columnar differences in reelin expression by CR cells and GABAergic neurons in the nestin-BDNF mouse induce increments of DAB1 protein expression in subsets of pyramidal cells, corresponding spatially to local decreases in reelin availability.

Discussion

In this study we employed a gain-of-function model, in the form of nestin-BDNF transgenic mice, to investigate the role of BDNF in the development of cortical architecture. We have shown that the premature expression of BDNF preserves tangential migration, but impairs the final radial migration of GABAergic neurons and their integration in the appropriate cortical layers. Moreover, CR cells and GABAergic neurons segregate in the MZ, leading to a columnar cortical organization within which the migration of different neuronal populations is specifically affected.

Effects of BDNF on the Tangential Migration of GABAergic Neurons

GABAergic interneurons enter the cortex by tangential migration, and after reaching their primary destination, follow ventricle- and pia-directed radial migrations to assume positions alongside isochronically generated, radially derived neurons (Anderson *et al.*, 2001; Ang *et al.*, 2003; Tanaka *et al.*, 2003). The initial tangential migration of GABAergic cells from the GE is preserved, or even increased, in nestin-BDNF mice. However, the presence of ectopic GABAergic neurons in the MZ, IZ and deep cortical layers suggests impaired final radial migration and layer recognition.

We found that reelin expression was nearly abolished in GABAergic neurons. However, unlike pyramidal neurons, the migration of GABAergic neurons is not dependent on reelin (Hevner *et al.*, 2003; Hammond *et al.*, 2004): neuronal ectopies are never present in the reeler mouse, and although misplaced, GABAergic neurons are still able to migrate radially into the reeler cortex (Hevner *et al.*, 2004). Thus, it is unlikely that altered reelin expression is responsible for the impaired radial migration and layer recognition of GABAergic neurons observed in nestin-BDNF mice.

During embryonic and early postnatal development, BDNF is expressed at low levels in the cerebral cortex, where it may stimulate chemotaxis and tangential interneuron migration to the cortex (Behar *et al.*, 1997; Polleux *et al.*, 2002). After the migratory period is completed, BDNF acts as a sensor of activity, since rapid activity-dependent tuning is necessary for the maintenance of cortical dendrites and active synapses (Lein *et al.*, 1999; Lein and Shatz, 2000; Gorski *et al.*, 2003). Thus, the presence of excess BDNF might impair the ability of GABAergic neurons to adopt their final position in the cortex by accelerating dendrite and synapse formation in an activity-independent manner.

Segregation of CR and GABAergic Neurons in the MZ Induces Columnar Differences in Radial Migration

In nestin-BDNF mice, the radial migration of neurons destined to occupy layers II–IV is severely impaired. Moreover, migration of GABAergic neurons through the CP is only partially preserved in the gyri, where GABAergic cells are present in the MZ (illustrated in Fig. 7).

We have shown that local changes in reelin availability are translated into focalized increases in Dab1 protein levels in pyramidal neurons (Fig. 6). Although ectopic reelin expression does not alter cell migration in the neocortex, in a reeler background it rescues preplate splitting, but not cortical lamination (Magdaleno *et al.*, 2002). Despite its critical role in the development of cortical lamination, the function of reelin itself remains a subject of controversy, and attractive or repulsive activities have so far failed to be demonstrated (Jossin and Goffinet, 2001). Reelin may arrest neuronal migration by binding to $\alpha 3\beta 1$ integrin, which mediates the switch from the gliophilic to the neurophilic mode of neuronal interaction (Anton *et al.*, 1999; Dulabon *et al.*, 2000). Moreover, reelin signalling directly affects radial glial elongation, basement membrane attachment and neurochemical maturation (Soriano

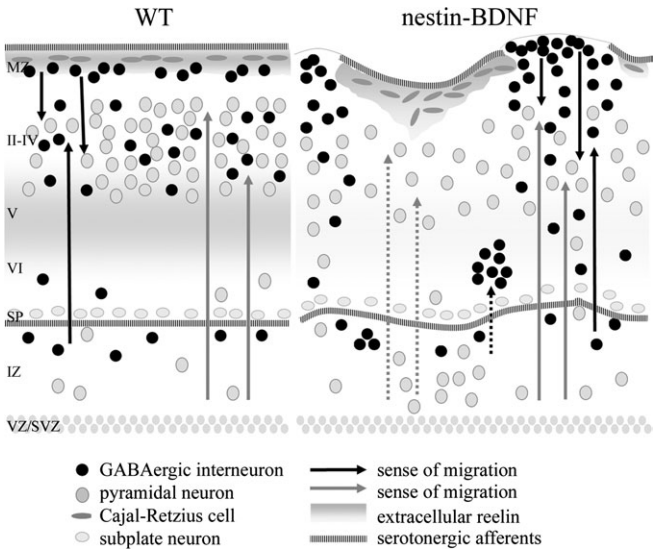

Figure 7. Schematic representations of the cortical organization and laminar fate of late-generated neurons, normally destined to occupy layers II–IV in the WT cortex, and in the sulcal and gyral areas of nestin-BDNF mice. Dotted arrows indicate impaired migration.

et al., 1997; Hartfuss *et al.*, 2003; Luque *et al.*, 2003). Local depletion of reelin might account for the distorted apical tufts observed in gyral regions of nestin-BDNF mice; however, we cannot rule out an autocrine effect of BDNF, as nestin-expressing radial glia also express functional TrkB receptors (Barnabe-Heider and Miller, 2003; Rose *et al.*, 2003). The absence of CR cells generates a cortical structure that is consistent with a defect in the terminal phase of glial-guided migration in the gyri, as occurs in the cerebral cortex of null-mutants for SPARC-like 1, a radial glial surface antigen with anti-adhesive properties (Gongidi *et al.*, 2004). However, the presence of CR cells alone is not sufficient to drive the normal radial migration of late-born neurons (Fig. 5*G–I*). Extensive crosstalk between reelin, CDK5 and Lis1 signalling has recently been demonstrated (Assadi *et al.*, 2003; Beffert *et al.*, 2004). Local changes in DAB1 phosphorylation in migrating pyramidal neurons and altered crosstalk between these pathways might account for the impaired pyramidal cell migration observed in the sulci, despite the presence of reelin-expressing CR neurons in the MZ and the roughly normal distribution of radial glia. The different effects of BDNF overexpression on the radial migration of pyramidal and non-pyramidal neurons most likely reflect different guidance mechanisms, including reelin- and glia-dependent radial migration for pyramidal neurons, and reelin- and glia-independent radial migration for GABAergic neurons.

Taken together, our results indicate that both CR and GABAergic neurons play a role in directing radial migration, and that their spatial organization in the MZ might be critical for the development of correct cortical lamination. Moreover, reelin secreted by interneurons in layers V–VI is most likely necessary for the migration of late born neurons to the upper cortical layers as well.

Role of BDNF in the Development of Cellular Organization in the MZ and the Formation of Cortical Columns

Columnar organization is characteristic of some specialized cortical structures. In the visual cortex, their establishment is independent of sensory experience (Crowley and Katz, 2002), and is sculpted by GABA circuits (Hensch and Stryker, 2004). Recent studies using flattened whole-mount preparations of the telencephalic vesicle have revealed a dynamic mosaic pattern in the embryonic MZ, with superimposed territories of CR and GABAergic neurons moving in the same direction, though rarely touching (Soria and Fairen, 2000; Ang *et al.*, 2003). Regularly spaced clusters of CR cells have been reported in the immature presubicular cortex, and have been linked to the formation of vertical arrays of CP neurons that are preferentially localized by avoiding reelin-rich zones, which form intercolumnar spaces (Nishikawa *et al.*, 2002). The formation of this columnar organization also requires the integrity of serotonergic afferents, which contact CR cells at E17 (Janusonis *et al.*, 2004). BDNF modulates serotonergic function (Lyons *et al.*, 1999), while during the formation of a different specialized columnar structure, the barrel cortex, serotonin and TrkB signalling cooperate in the segregation of layer IV granular neurons (Vitalis *et al.*, 2002).

During embryonic stages, GABA itself acts as a chemoattractant, initially stimulating the motility of GABA-containing cells via $GABA_B$ receptors, and then inhibiting migration via $GABA_A$ receptors, as neurons are arranged into primitive layers (Behar *et al.*, 1996, 1998, 2000). BDNF increases GABA synthesis and release, and regulates $GABA_A$ receptor expression and activity (Aguado *et al.*, 2003; Mizoguchi *et al.*, 2003; Jovanovic *et al.*, 2004). BDNF also increases glutamate release by pyramidal and CR cells, as well as AMPA receptor subunit expression and activity (Matsumoto *et al.*, 2001; Pascual *et al.*, 2001; Nagano *et al.*, 2003). Calb-positive IZ neurons express calcium-permeable AMPA receptors, the activation of which induces neurite retraction in migrating interneurons (Metin *et al.*, 2000; Poluch *et al.*, 2001). Moreover, glutamate antagonizes the excitatory and chemotactic effects of GABA, as well as acting through NMDA receptors to stimulate embryonic cortical neuronal migration (van den Pol *et al.*, 1998; Behar *et al.*, 1999).

We have identified a high sema3A-expressing neuronal population that accumulates in the MZ in response to BDNF overexpression, strongly indicative of a GABAergic phenotype. Sema3A is involved in dendritic morphogenesis in pyramidal neurons (Niclou *et al.*, 2003; Fenstermaker *et al.*, 2004), and BDNF modulates the response of pyramidal cells to sema3A (Dontchev and Letourneau, 2002).

Considered as a whole, these observations suggest a possible mechanism for BDNF regulation of differential neuronal guidance under physiological conditions. In the late migratory period, increased BDNF levels induced by neuronal activity may modulate the response to positional cues, while exacerbation of the patched distribution of CR and GABAergic cells in the MZ may generate alternating areas enriched in glutamate and GABA respectively, leading to the formation of anatomical and functional columns.

The Formation of Polymicrogyria

Polymicrogyria reflects the presence of an abnormally high number of small gyri with altered cortical lamination, for which both genetic and epigenetic origins have been described (Dambska and Laure-Kamionowska, 2001; Piao *et al.*, 2004). Recent evidence has highlighted the persistence of reelin-expressing CR neurons in all types of human polymicrogyria, as occurs in the experimentally induced condition in rodents (Super *et al.*, 1997; Eriksson *et al.*, 2001). Increased numbers of CR cells have also been reported in the MZ of the pax6 mutant mouse (Stoykova *et al.*, 2003), and pax6 mutations have been linked to polymicrogyria (Mitchell *et al.*, 2003). CR cells also express the extracellular protein fukutin, which is involved in severe congenital muscular dystrophy and associated polymicrogyria (Sasaki *et al.*, 2000). Moreover, neonatal ablation of CR cells by local application of domoic acid disrupts neuronal migration of late-generated neurons (Super *et al.*, 2000). We have found, however, that clusters of CR cells are associated with impaired migration of late-generated neurons, whereas GABAergic neuronal clusters in the MZ and the absence of CR cells are related to a lack of laminar specificity, rather than to blocked migration. Domoic acid, the AMPA receptor agonist used by Super *et al.* (2000), probably depletes the MZ of CR and GABAergic neurons, as both cell types expresses AMPA receptor subunits, which explains the differences observed in the present study.

The radial unit hypothesis postulates that convolutions may be formed by changes in cortical precursor proliferation and by the addition of radial units or minicolumns, with a net increase in the cortical surface (Rakic, 1988, Haydar *et al.*, 1999). Taken together, our data suggest that the formation of a polymicrogyric

cortex may result from impaired neuronal migration below the areas enriched in those CR cells forming the sulci, although we cannot rule out the possibility that regional variations in precursor proliferation or survival contribute to the formation of sulci and gyri.

We propose that the subtle spatial reorganization of distinct cellular populations of the MZ may produce columnar differences in the migration of neurons destined to occupy the upper cortical layers, thereby leading to the formation of microcolumnar structures and/or convoluted patterns.

Supplementary Material

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

Notes

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