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BDNF Regulates Reelin Expression and Cajal-Retzius Cell Development in the Cerebral Cortex

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Summary

Cajal-Retzius (CR) cells of the cerebral cortex express receptors for the neurotrophin brain-derived neurotrophic factor (BDNF) and downregulate expression of the extracellular matrix protein Reelin during early postnatal development, coincident with the onset of cortical BDNF expression. During this period, mice lacking BDNF have elevated levels of Reelin in CR cells. Acute BDNF stimulation of cortical neuron cultures and overexpression of BDNF in the developing brain of transgenic mice prior to the onset of endogenous production causes a profound, dose-dependent reduction of Reelin expression in CR cells. In addition, overexpression of BDNF produces gaps and heterotopias in the marginal zone and disorganization and aggregation of cortical CR cells and induces several other malformations, including aberrant cortical lamination, similar to the phenotype of *reeler* mutant mice, which lack Reelin. These results demonstrate a role for BDNF on cortical CR cells and identify Reelin as a direct effector of this neurotrophin during brain development.

Introduction

Neuronal migration and lamination in developing cerebral cortex depend upon secreted signals, including the extracellular matrix protein Reelin, which is produced by a transient subpopulation of pioneer neurons, the Cajal-Retzius (CR) cells (D'Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995; Pearlman and Sheppard, 1996; Del Rio et al., 1997; Soriano et al., 1997). CR cells are first observed during early cortical development in a primordial layer known as the preplate. The subsequent migration of neurons from the subventricular zone into the cortex divides the preplate into an inner layer, the subplate, and an outer layer, or marginal zone, rich in CR cells. Successive waves of newly born neurons migrate into the cortex, passing through the subplate, as well as all previous layers of the cortical plate, until they reach the marginal zone, which remains the outermost layer of the cortex. This process generates the characteristic "inside-out" organization of the layers in the cortical plate, with younger neurons in the more external layers and older neurons in the more internal layers (Gilmore and Herrup, 1997).

Reelin, an early marker of CR cells, is necessary for the formation of the inside-out layer organization of the cerebral cortex. In *reeler* mutant mice, which lack expression of Reelin (D'Arcangelo et al., 1995; Hirotsune et al., 1995), cortical layering is grossly abnormal: migrating neurons fail to split the preplate and instead line up below it, in the same order as that in which they are born (Caviness, Jr., 1982; Goffinet, 1984; Caviness, Jr., et al., 1988). As a result, the *reeler* cortex lacks an identifiable subplate as well as a proper marginal zone, and cortical layering is reversed, as compared with normal animals. The *reeler* mutation also causes abnormalities in the hippocampus (Caviness and Sidman, 1973; Stanfield and Cowan, 1979), the cerebellum (Goffinet et al., 1984), and the olfactory bulb (Wyss et al., 1980), indicating that Reelin plays a crucial role in the histogenesis of several laminated structures in the mammalian brain. The molecular mechanisms by which Reelin regulates cell migration are, however, unknown. Mutations in the mouse gene *mdab*, encoding a target of Src-like protein tyrosine kinases, produce a phenotype that is indistinguishable from *reeler* (Howell et al., 1997; Sheldon et al., 1997), suggesting that the protein encoded by *mdab* may be part of a downstream signaling pathway for Reelin.

During postnatal brain development, and concomitant with the completion of cortical lamination, Reelin expression is downregulated in cortical CR cells (Schiffmann et al., 1997). Downregulation of Reelin expression is followed by the disappearance of CR cells, which in the murine neocortex occurs during the second and third postnatal weeks (Derer and Derer, 1990; Del Rio et al., 1995). However, the actual fate of CR cells has been controversial: cell dilution, neuronal death, and transformation into nonpyramidal neurons have been proposed as possible outcomes (Parnavelas and Edmunds, 1983; Del Rio et al., 1995, 1996). Morphological, histochemical, and fate analyses have presented evidence for postnatal CR cell death in the cerebral cortex (Derer and Derer, 1990; Del Rio et al., 1995, 1996), although it is unclear whether all or only part of the CR cell population is eliminated by this process. It has also been proposed that some CR cells remain in the adult brain (Liu et al., 1996). The factors and molecular mechanisms that regulate CR cell development and Reelin expression are totally unknown.

The neurotrophins are a family of structurally and functionally related polypeptides that control the differentiation, survival, and maintenance of developing and adult vertebrate neurons (Lewin and Barde, 1996). Targeted disruption of individual neurotrophin genes results in the nearly complete ablation of distinct subpopulations of peripheral neurons (Crowley et al., 1994; Ernfors

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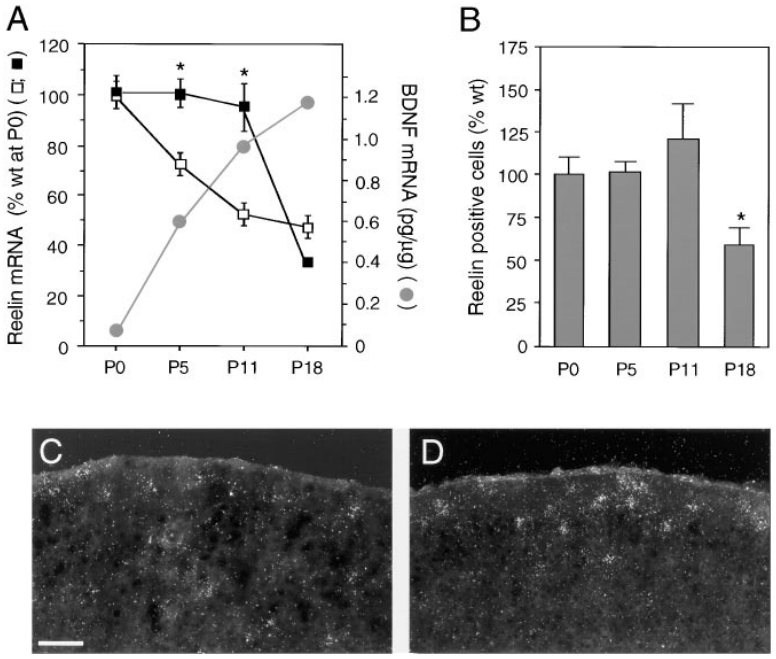

Figure 1. Elevated Reelin Expression in Cortical CR Cells of Mice Lacking BDNF

(A) Quantification of Reelin mRNA levels per cell in postnatal marginal zone cells of wild-type (wt) (open squares) and BDNF$^{-/-}$ (closed squares) mice. Results are presented as percentage of wild-type at P0 (mean ± SD of more than 60 cells examined). Superimposed in the graph is the expression profile of BDNF mRNA in the cerebral cortex (grey circles), adapted with permission from Timmusk et al. (1994).

(B) Number of Reelin-expressing cells in marginal zone of BDNF$^{-/-}$ mice. Values are presented as percentage of wild-type ($n = 5$). *$p < 0.01$.

(C and D) Dark field autoradiograms of Reelin mRNA in situ hybridization through the cortex of P11 wild-type (C) and BDNF$^{-/-}$ (D) mice. Scale bar, 200 μm.

et al., 1994a, 1994b, 1995; Fariñas et al., 1994; Jones et al., 1994), underscoring the importance of neurotrophins as physiological target-derived survival factors in the peripheral nervous system. In contrast, the survival of many subpopulations of central neurons known to express neurotrophin receptors and to respond to exogenous neurotrophins in vitro and in vivo is not compromised by disruption of neurotrophin genes. Initial studies on developing central neurons of neurotrophin knockout mice were hampered by the relatively short survival times of these animals, typically of a few days after birth. By extending the survival of these animals for up to 2–3 weeks and combining several gene mutations, more recent studies have revealed that neurotrophins, in particular brain-derived neurotrophic factor (BDNF) and its receptor tyrosine kinase TrkB, are necessary for the survival of neurons in several regions of the postnatal brain, including the hippocampus, cortex, basal forebrain, and cerebellum (Minichiello and Klein, 1996; Alcantara et al., 1997; Fagan et al., 1997; Schwartz et al., 1997). Nevertheless, the reductions in neuron number reported so far have been relatively mild, and in no case has mutation of a neurotrophin or *trk* gene resulted in the complete disappearance of an anatomically or chemically distinct subpopulation of central neurons. Taken together, the available data on the developmental roles of neurotrophins and their receptors in the central nervous system suggest the existence of redundant survival pathways or alternative roles for neurotrophins in central neurons, such as in the control of gene expression and cell fate.

The work presented here addresses the role of BDNF in the development and function of cortical CR cells. For this purpose, we have examined CR cells in BDNF knockout mice during the initial period of expression of this neurotrophin in the postnatal cerebral cortex and in transgenic mice overexpressing BDNF prior to the onset of endogenous production. These studies were complemented by experiments on Reelin-positive cortical neurons in culture and, together, demonstrate a role for BDNF as a negative regulator of Reelin expression and a signal for CR cell development.

Results

Elevated Reelin Expression in Cortical CR Cells of Mice Lacking BDNF

Previous studies (Marty et al., 1996; Brunstrom et al., 1997) and our own data (see below) indicate that CR cells express TrkB receptors and have the capacity to respond to BDNF, suggesting that this neurotrophin could act directly on these cells to regulate gene expression and survival. During normal development, very low BDNF expression can first be detected in the rodent brain at embryonic day 13 (E13), with little change until the second postnatal day (P2), after which BDNF levels increase rapidly, with a peak at P14 (Friedman et al., 1991; Timmusk et al., 1994). This pattern of expression is even more pronounced in the cerebral cortex, where BDNF is virtually undetectable before birth (Timmusk et al., 1994). BDNF upregulation coincides with the time course of decline in Reelin levels and the disappearance of CR cells that occur during the normal development of the postnatal rodent brain (Derer and Derer, 1990; Schiffmann et al., 1997), suggesting that BDNF could be involved in several of these events.

We examined Reelin mRNA expression in CR cells of the marginal zone in brains of wild-type and BDNF$^{-/-}$ mice at P0, P5, P11, and P18 by in situ hybridization. Levels of Reelin mRNA expression per cell were quantified by image analysis of hybridized tissue sections. No difference in the levels of Reelin mRNA per cell could be detected between wild-type and BDNF$^{-/-}$ mice at P0 (Figure 1A), when levels of BDNF mRNA in normal cerebral cortex are still below detection levels (Timmusk et al., 1994). However, elevated levels of Reelin mRNA

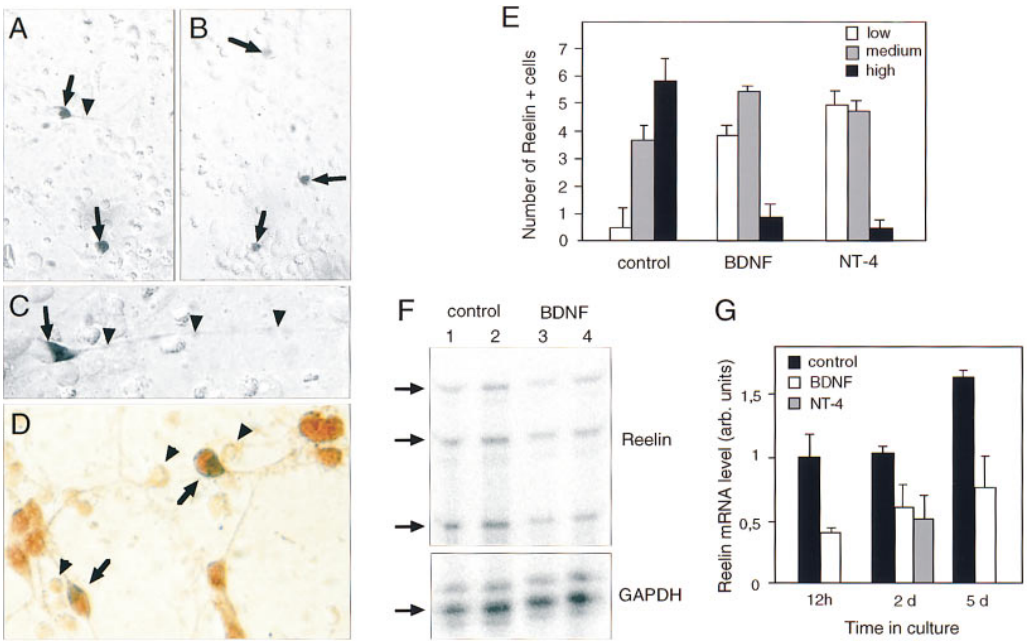

Figure 2. BDNF Downregulates Reelin Expression in Dissociated Cultures of Cortical Neurons

(A–C) Phase contrast photomicrographs of dissociated cultures of E16 rat cortical neurons stained with an anti-Reelin monoclonal antibody. Arrows indicate Reelin-positive neurons. Note that the staining is confined to one half of the cell soma.

(A) Control culture.

(B) Culture treated with 50 ng/ml BDNF for 5 days.

(C and D) Close-up view of a Reelin-expressing neuron from untreated cultures. Reelin immunoreactivity was also found in the axon, in a punctated pattern (arrowheads).

(D) Double-staining immunohistochemistry of Reelin (grey) and TrkB (red) in neuronal cultures. Arrows indicate double-labeled cells; note the characteristic staining pattern of Reelin, distinct from that of TrkB, which extended over the whole cytoplasm. Arrowheads denote several unlabeled cells.

(E) Quantification of Reelin immunoreactivity in dissociated cultures of cortical neurons after second treatments with BDNF and NT-4. Reelin staining intensity of 10 cells in each of six different wells ($n = 6$) was scored by an observer who was blind to the treatments made to the cultures. Similar results were obtained by a second observer. Results are presented as the average number of cells scoring low, medium, and high in Reelin intensity (see Experimental Procedures). All factors were tested at 50 ng/ml.

(F) Autoradiogram of an RPA for Reelin mRNA of dissociated cultures of cortical neurons treated for 5 days with 50 ng/ml BDNF. The bottom panel shows the corresponding signal for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used to normalize for different amounts of RNA. Lanes 1, 2 , 3, and 4 correspond to duplicate experiments.

(G) Phosphorimager quantitation of RPA for Reelin mRNA of dissociated cultures treated with neurotrophins. Values (average of two experiments ± SD) are expressed in arbitrary units and standardized to the signal obtained with the GAPDH riboprobe.

were seen in marginal zone cells of BDNF$^{-/-}$ mice, compared with wild-type, at both P5 and P11 (Figures 1A, 1C, and 1D), coincident with the onset of BDNF mRNA expression in normal cortex (Timmusk et al., 1994). Thus, while Reelin expression declined steadily in wild-type mice, high levels were maintained in the cortex of BDNF knockout mice during the first two postnatal weeks, indicating a protracted period of Reelin expression in the absence of BDNF. During this period, no significant differences in the number of cells expressing Reelin mRNA could be seen between wild-type and BDNF$^{-/-}$ mice (Figure 1B). However, a significant decrease in the number of Reelin-positive cells was observed in the marginal zone of BDNF$^{-/-}$ mice at P18, about the longest survival time of these animals (Figure 1B). At this time, the surviving Reelin-positive cells in the marginal zone of BDNF$^{-/-}$ mice expressed lower (i.e., comparable to wild-type) levels of Reelin mRNA (Figure 1A), perhaps a consequence of the process of the degeneration of these cells in the absence of BDNF. Together, these data suggested that BDNF is required for the developmental downregulation of Reelin expression in cells of the marginal zone and that, in addition, this neurotrophin may be a survival factor for a subpopulation of Reelin-positive cells at later stages (≥P11) of postnatal development.

BDNF Downregulates Reelin Expression in Cortical Neurons in Culture

We investigated further the possibility that Reelin is a direct target gene of BDNF by studying the responses of cortical neurons in culture to acute BDNF treatment. Dissociated cultures of E16 rat cerebral cortex were exposed to BDNF for either 12 hr, 2 days, or 5 days, after which Reelin expression was studied by RNase protection assay (RPA) and immunocytochemistry. At this time of development, all Reelin-positive neurons in the cerebral cortex are confined to the marginal zone. An anti-Reelin monoclonal antibody stained several neurons of a characteristic bipolar morphology in these cultures (Figures 2A and 2B). Intriguingly, staining in the axons was punctated (Figure 2C), and in the cell body,

Reelin staining was confined to one half of the cell soma (Figure 2C). Importantly, double-staining experiments indicated that Reelin-positive cells in these cultures expressed the BDNF receptor TrkB (Figure 2D). BDNF treatment reduced Reelin staining in these cultures at all three time points tested without affecting the total number of Reelin-positive cells (Figure 2E and data not shown). Blind scoring of staining intensity by two independent observers showed that BDNF increased the proportion of cells expressing low levels of Reelin, whereas it decreased the proportion of highly expressing cells (Figure 2E). Neurotrophin-4 (NT-4), another neurotrophin agonist of the TrkB receptor, had a comparable effect on BDNF (Figure 4E), while neurotrophin-3 (NT-3), which predominantly acts via the TrkC receptor, was not different from control (data not shown). BDNF also downregulated Reelin mRNA expression in these cultures at all three time points to about 50% of control levels, as assessed by RPA (Figures 2F and 2G). The relatively rapid kinetics of this regulation suggest direct effects of BDNF on Reelin-expressing cells. A comparable downregulatory effect on Reelin mRNA was also seen after the second treatment with NT-4 (Figure 2G). Combined, these data suggest that activation of TrkB receptors by either BDNF or NT-4 can directly regulate Reelin expression in developing neurons of the cerebral cortex.

Reduced Reelin Expression in CR Cells of Transgenic Mice Overexpressing BDNF

Our data on BDNF$^{-/-}$ mice and cortical neurons in culture indicated that Reelin is a direct effector of BDNF during normal development, and, although in vivo this neurotrophin would normally act on Reelin expression postnatally, our in vitro data clearly showed that BDNF is also capable of affecting Reelin levels in embryonic neurons. These observations prompted us to investigate the effects of a premature exposure to BDNF on Reelin expression, CR cell development, and cortical lamination in the brains of transgenic mice overexpressing BDNF prior to the onset of endogenous production. *nestin*–BDNF transgenic mice overexpress BDNF under the control of the promoter and enhancer regions of the *nestin* gene, an intermediate filament gene widely expressed in neuronal precursors and developing neurons throughout the central and peripheral nervous systems (Zimmerman et al., 1994; Dahlstrand et al., 1995). Mouse embryos expressing the *nestin*–BDNF transgene have a normal external appearance throughout gestation but die shortly before or right after birth of what appears to be a cardiorespiratory malfunction (T. R. and C. F. I., unpublished data). Most transgenic embryos show significantly higher levels of BDNF mRNA in the brain than control littermate animals, primarily in the ventricular zone and cerebral cortex (Figure 3). The peak of BDNF mRNA expression in the brain is seen between E14.5 and E15.5; levels of expression vary by a factor of ~10 between different transgenic embryos, probably owing to differences in transgene integration site and copy number, as each embryo arises from an independent pronuclear injection. At E14.5, BDNF protein levels in brain homogenates, as determined by two-site enzyme immunoassay, vary between 10 and 60 ng/g tissue in different transgenic embryos, while the level of BDNF in total brain homogenates from control animals is in the order of 1–2 ng/g tissue.

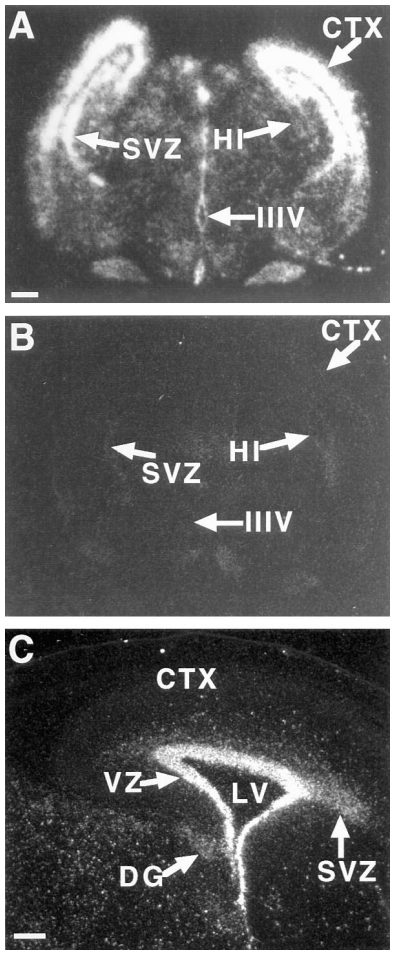

Figure 3. Elevated Expression of BDNF mRNA in the Brain of *nestin*–BDNF Transgenic Mice

(A) Coronal section of the E15.5 brain of a *nestin*–BDNF transgenic mouse hybridized in situ with an antisense BDNF riboprobe.
(B) Coronal section of an E15.5 control brain.
(C) Sagittal section of an E15.5 transgenic brain. (A) and (B) were prepared simultaneously under comparable conditions. Abbreviations: SVZ, subventricular zone; HI, hippocampus; CTX, cerebral cortex; VZ, ventricular zone; DG, dentate gyrus; LV, lateral ventricle; IIIV, third ventricle. Scale bar, 400 μm.

We examined the expression of Reelin mRNA and protein in the brains of *nestin*–BDNF transgenic mice by in situ hybridization and immunohistochemistry. In contrast to control mice, which showed a continuous band of cells expressing high levels of Reelin mRNA (Figures 4A and 4C), labeling was lower and sparser in the marginal zone of E18.5 transgenic mice (Figures 4B and 4E). In agreement with the mRNA data, levels of Reelin protein were greatly reduced in the marginal zone of transgenic mice (Figures 4G and 4H). At higher magnification, several cells expressing Reelin mRNA formed clusters in the marginal zone (Figures 4E and 4F). The

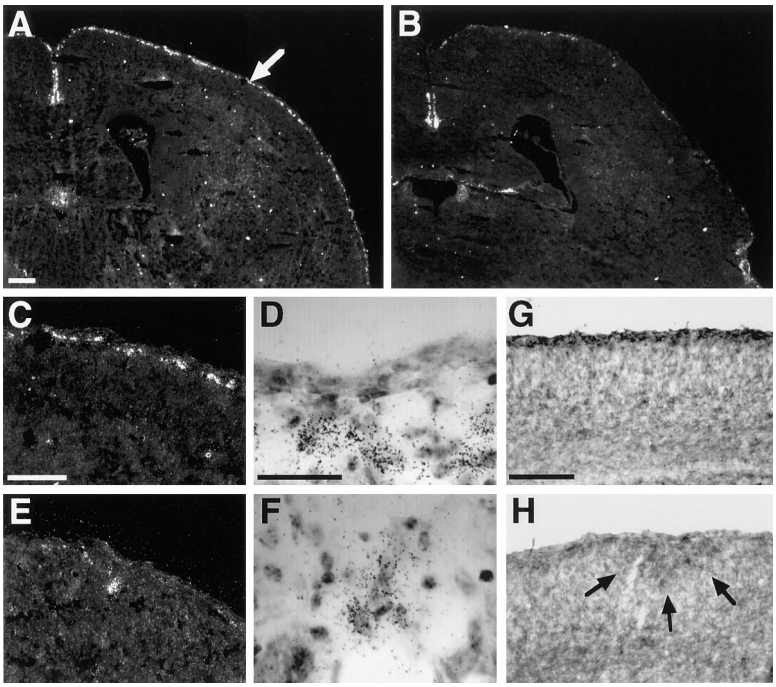

Figure 4. Reduced Reelin Expression in CR Cells of Transgenic Mice Overexpressing BDNF

(A and C) In situ hybridization of coronal sections through a normal E18 mouse brain. Note the uniform labeling of Reelin mRNA in the marginal zone (arrow). Scale bar, 400 μm.

(B and E) Lower and sparse Reelin mRNA labeling in the marginal zone of E18.5 transgenic mice.

(D) Reelin mRNA–expressing cells in normal cortex. Scale bar, 80 μm.

(F) Reelin mRNA–expressing cells of aberrant morphology in invaginations of the marginal zone in transgenic mice.

(G) Reelin immunohistochemistry in normal cortex. Scale bar, 400 μm.

(H) Reduced Reelin protein levels in transgenic cortex. Arrows point to an invagination in the transgenic marginal zone. The presence of marginal zone cells in this segment of transgenic cortex was confirmed independently by calretinin and cresyl violet staining (data not shown).

low levels of Reelin mRNA expression in the cortex of transgenic mice could not satisfactorily be accounted for by a decrease in the number of cells expressing Reelin mRNA, as this was not significantly reduced, compared with controls (data not shown). We therefore quantified the level of Reelin mRNA per cell and found, on average, a 50% reduction of expression in cells of E18 transgenic mice, a value that was in good agreement with the data that we obtained in vitro. Quantification of the levels of BDNF mRNA in the same transgenic embryos revealed a strong negative correlation between BDNF and Reelin mRNA levels in CR cells of *nestin*–BDNF mice (Figure 5), thus bringing further support to the notion that BDNF is a negative regulator of Reelin expression.

Morphological Abnormalities in the Cortex of *nestin*–BDNF Transgenic Mice

Next, we investigated the effects of BDNF overexpression and Reelin downregulation in the brains of *nestin*–BDNF mice. Histological analysis of sections from E18.5 transgenic brains revealed a grossly aberrant cortical architecture. Fifteen of 17 transgenic animals analyzed at this age showed multiple microgyric sulcus formations consisting of invaginations of the marginal zone into the underlying cortical plate (Figure 6B). The invaginations distorted the normal radial architecture of the underlying cells, which instead oriented themselves perpendicular to the sides of the sulci. The depth and abundance of the invaginations varied among different transgenic animals and correlated with the level of BDNF mRNA expressed in each embryo. Cells within these invaginations were found to express mRNA for the BDNF receptor TrkB (Figure 6E) and expressed reduced levels of Reelin protein (Figure 4H, arrows). In a few cases, heterotopic collections of cells were observed in the marginal zone (Figure 6D), similar in appearance to those previously reported to be induced by exogenous administration of NT-4 (Brunstrom et al., 1997). In addition to invaginations and heterotopias, portions of the cortex of transgenic mice were devoid of marginal zone (Figure 6C). Less pronounced and less abundant defects were also seen in the brains of E15.5 transgenic mice, while

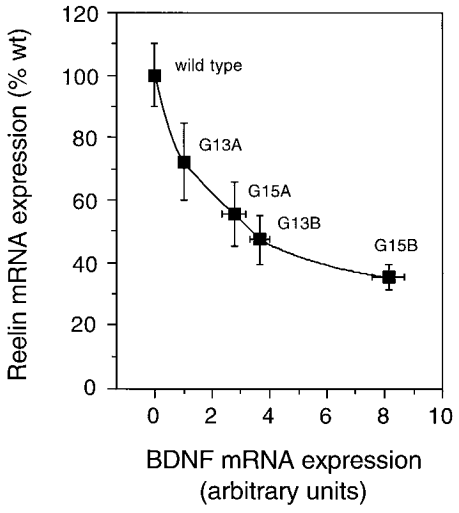

Figure 5. Correlation between Reelin and BDNF mRNA Expression in Marginal Zone Cells of E18 Transgenic Mice Overexpressing Different Amounts of BDNF

The level of Reelin mRNA per cell in marginal zone cells is plotted against the corresponding BDNF mRNA levels per cell measured in the same brains (see Experimental Procedures for details). Reelin mRNA levels are expressed relative to those of wild-type mice; BDNF mRNA levels are in arbitrary units. Wild-type and four transgenic embryos are indicated. Values are expressed as mean ± SEM of more than 60 cells analyzed.

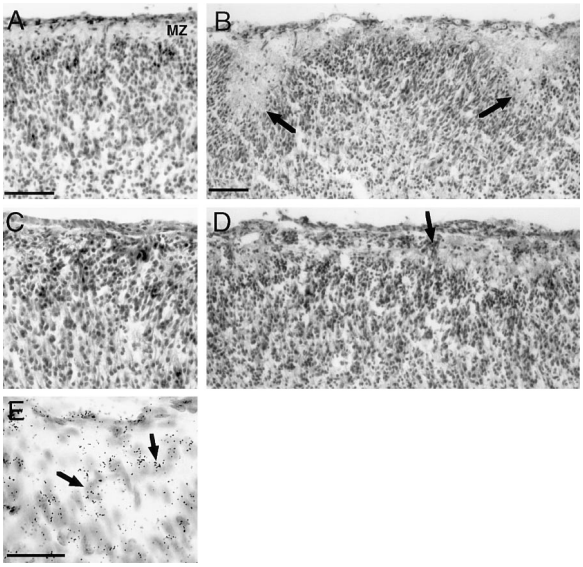

Figure 6. Morphological Abnormalities in the Cortex of *nestin*–BDNF Transgenic Mice

(A) Cresyl violet staining of a section through the cortical plate of a normal E18.5 mouse brain. The marginal zone (MZ) is indicated. Scale bar, 200 μm.

(B) Cortical plate in an E18.5 *nestin*–BDNF mouse. Note the pronounced invaginations of the marginal zone (arrows). Scale bar, 200 μm.

(C) Portion of the cortical plate of a transgenic brain. Note the absence of marginal zone.

(D) Heterotopic collection of cells in the marginal zone of a transgenic mouse brain (arrow).

(E) TrkB mRNA expression in cell bodies within invaginations of the marginal zone in E15.5 transgenic mice (arrows). Scale bar, 80 μm.

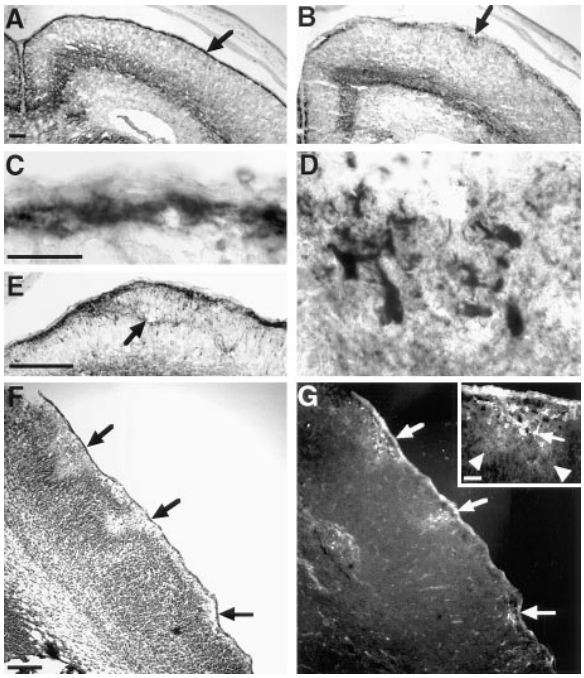

Figure 7. Abnormal Organization and Morphology of CR Cells in *nestin*–BDNF Transgenic Mice

(A) Calretinin immunohistochemistry of a section through a normal E18.5 brain. Note the pronounced labeling in the marginal zone (arrow). Scale bar, 400 μm.

(B) Discontinuous calretinin labeling in the marginal zone of E18.5 transgenic mice.

(C) Close-up view of calretinin-positive cells in the marginal zone of control mice. Note the horizontal arrangement of CR cells and their processes. Scale bar, 80 μm.

(D) Close-up view of calretinin-positive cells from E18.5 transgenic mice (area indicated by arrow in [B]). Note the aberrant organization and morphology of CR cells.

(E) A less affected transgenic animal showing a more uniform distribution of calretinin labeling, but with loose bundles of calretinin-positive fibers entering into the cortical plate (arrow).

(F) Cortical plate of a transgenic mouse brain with marginal zone invaginations (arrows), visualized by cresyl violet staining. Scale bar, 400 μm.

(G) Calretinin staining of a section adjacent to the one shown in (F), visualized by immunofluorescence. Note the correspondence between marginal zone invaginations and clusters of calretinin-positive cells (arrows).

(Inset) Calretinin-positive cell bodies and fibers fill the cavity of the invagination (arrowheads). Scale bar, 400 μm.

no abnormalities were seen at E12.5 (data not shown), indicating a progressive increase in the severity of the phenotype over time. No cortical malformations were seen in *nestin*–NT-3 mice, which overexpress another member of the neurotrophin family (Ringstedt et al., 1997), indicating that the effects seen with BDNF were not caused by nonspecific overexpression of any neurotrophic factor.

Abnormal Organization and Morphology of CR Cells in Transgenic Mice Overexpressing BDNF

Because one of the major cell types in the marginal zone is the CR cell, we examined the expression of the CR cell marker calretinin in sections of E18 transgenic brains (Figure 7). In contrast to control animals, calretinin labeling in the marginal zone of transgenic mice was discontinuous, with stretches of marginal zone devoid of calretinin-positive cell bodies and aggregations of CR cells of abnormal morphology in microgyric sulci (Figure 7D). In some cases, loose bundles of calretinin-positive fibers entering into the cortical plate were also seen in transgenic brains (Figure 7E). Invaginations in the marginal zone of transgenic mice were filled with calretinin-positive cell bodies and fibers (Figure 7G). Double labeling of calretinin and Reelin in these sections revealed colocalization of these two proteins in the CR cells of control and transgenic mice (Figure 8). Invaginations of the transgenic marginal zone showed clusters of Reelin-positive cells flanked by areas devoid of Reelin or calretinin staining (Figure 8). Despite the paucity of calretinin staining, the marginal zone of transgenic mice contained normal numbers of calretinin-positive neurons.

Aberrant Lamination of the Cerebral Cortex of *nestin*–BDNF Transgenic Mice

Finally, we examined cortical organization in transgenic mice overexpressing BDNF. Immunostaining for microtubule-associated protein-2 (MAP-2), a marker of differentiated neurons, was found in a characteristic layered pattern in the cerebral cortex of control E18 mice in

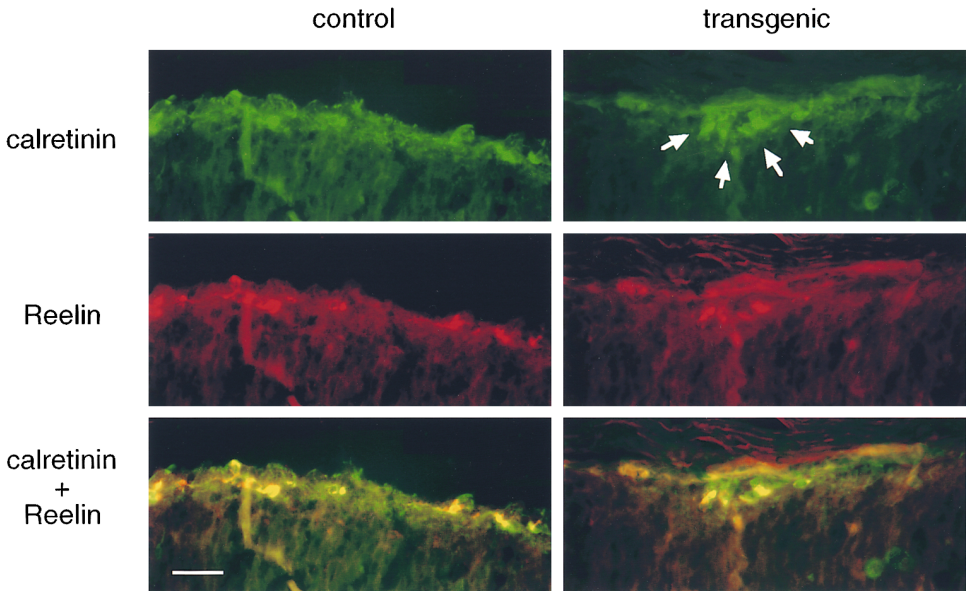

Figure 8. Colocalization of Reelin and Calretinin in Invaginations of the Marginal Zone in Transgenic Mice

Shown are sections through the marginal zone of E18.5 brains stained with anticalretinin (green) and anti-Reelin (red) antibodies. The lower panels show the merging of the two labelings (calretinin + Reelin); double-labeled cells appear yellow. The layer of background signal overlying the marginal zone in the section of transgenic brain corresponds to autofluorescence from the pial surface, which remained attached to the brain during preparation of the tissue. Scale bar, 120 μm.

which the marginal zone, the subplate, and an intermediate layer (possibly future layer V) could be clearly distinguished (Figures 9A and 9C). In contrast, transgenic cerebral cortex showed a more uniform distribution of MAP-2 labeling without clearly delineated layers (Figures 9B and 9D). Although a cortical subplate could be distinguished in the majority of the transgenic animals examined by MAP-2 immunostaining, this was often distorted, with pronounced waves and occasional gaps (Figure 9B).

The process of cortical lamination was investigated directly by examining at E18 the fate of cells that had been pulse labeled with bromodeoxyuridine (BrdU) at either E13.5 or E15.5. Labeling with BrdU at E13.5 resulted in a broad band of BrdU-positive cells in the central and deeper parts of the cortical plate in E18 normal mice, with few or no cells in the outer layers of the cortex or in the marginal zone (Figure 10B). In contrast, several BrdU-labeled cells in transgenic mice were found in more external cortical layers (Figure 10F), suggesting that these might contain cells originally destined for the cortical plate. In both normal and transgenic animals, the neuroepithelium in the ventricular zone was largely devoid of labeled cells (Figures 10A and 10E). In E18 control animals that had been labeled at E15.5, a well-defined band of BrdU-labeled cells was found in the outer layers of the cortical plate close to the marginal zone, while few labeled cells were seen in the neuroepithelium (Figures 10C and 10D). In contrast, fewer BrdU-labeled cells, scattered throughout the cortical plate,

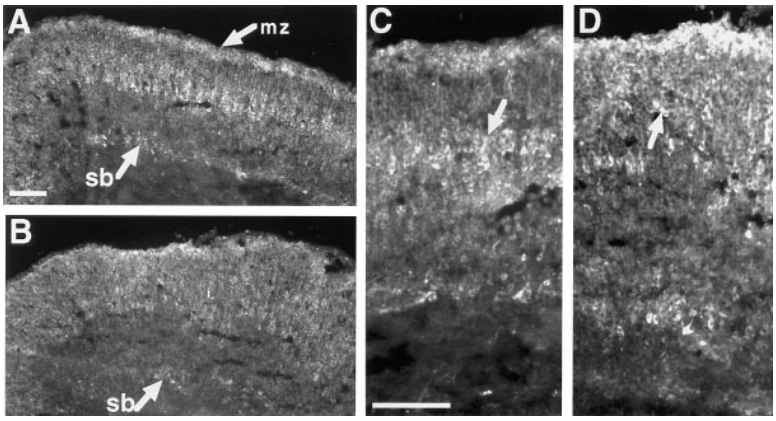

Figure 9. Cortical Malformations in Transgenic Mice Overexpressing BDNF

(A–D) MAP-2 immunohistochemistry in sections of E18 mouse brain.

(A) In control mice, labeling in the marginal zone (mz), the subplate (sb), and in an intermediate layer (possibly future layer V) can be clearly distinguished. Scale bar, 400 μm.

(B) In the cortex of transgenic mice, MAP-2 labeling is uniform, without clearly delineated layers. Labeling in the subplate (sb) was often distorted, with pronounced waves and occasional gaps.

(C) High magnification of the view shown in (A); note the laminar arrangement of cells in an internal cortical layer (arrow). Scale bar, 400 μm.

(D) High magnification of the view shown in (B); note the distorted organization of MAP-2–positive cells in the cortex of transgenic mice (arrow).

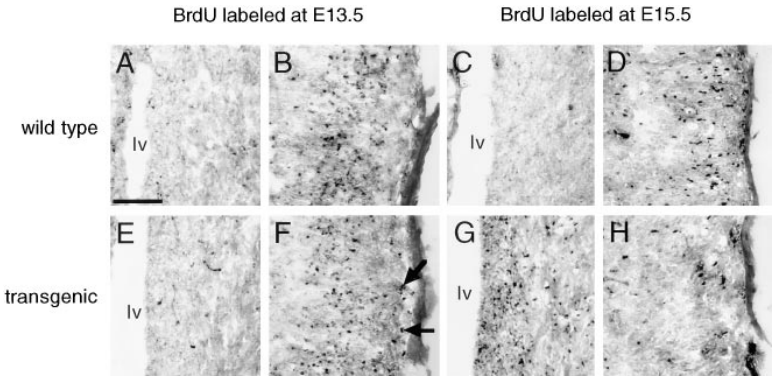

Figure 10. Aberrant Cortical Lamination in *nestin*–BDNF Mice

Analyses of BrdU-labeled cells in developing cortex of E18 wild-type and *nestin*–BDNF mice. Control (A–D) and transgenic (E–H) mouse brains were pulse labeled with BrdU at E13.5 (A, B, E, and F) or E15.5 (C, D, G, and H) and analyzed at E18.5.

(A, C, E, and G) Neuroepithelium in the ventricular and intermediary migratory zones.

(B, D, F, and H) Cortical plate and marginal zone. Note the presence of BrdU-labeled cells in the marginal zone of transgenic mice that had been labeled at E13.5 (F, arrows). Note also BrdU-labeled cells in the neuroepithelium and intermediary migratory zone in transgenic mice that had been labeled at E15.5 (G). Abbreviation: lv, lateral ventricle. Scale bar, 200 μm.

were found in E18 transgenic mice that had been labeled at E15.5 (Figure 10H), while dense labeling was found in the neuroepithelium and intermediary migratory zone in these animals (Figure 10G). These results indicated a disturbed lamination of the cortical plate in transgenic mice, with several early born neurons reaching out all the way into the marginal zone, and many later born neurons that did not migrate into the outer layers of the cortex and remained instead in deeper layers. These abnormalities are similar to the phenotype of *reeler* mice, which are deficient in Reelin expression (Caviness, Jr. et al., 1988; Ogawa et al., 1995).

Discussion

We have investigated activities of the neurotrophin BDNF in the developing central nervous system using animal models with either deficient or increased BDNF signaling, as well as dissociated neuronal cultures, and found an unexpected regulatory interaction between this neurotrophin and the extracellular matrix protein Reelin. Reelin expression was found to be under the negative control of BDNF, both in vivo and after acute stimulation in vitro, suggesting that Reelin is one direct effector of this neurotrophin during brain development.

Reelin, which is required for the normal lamination of the cortex during embryonic development, is downregulated during normal postnatal development as cortical lamination and brain maturation are being completed. This downregulation of Reelin expression is dependent on BDNF, as it was significantly delayed in the absence of this neurotrophin, suggesting that BDNF acts as a cortical maturation factor. The abnormalities observed in the brain of *nestin*–BDNF transgenic mice may therefore be the consequence of the premature expression of a maturation signal in an otherwise immature brain. Many of the defects observed in these mice, including aberrant lamination and inverted cortical layering of the cerebral cortex, resemble the phenotype of *reeler* mutant mice, and some of these could therefore be the result of the profound reduction in Reelin levels produced by BDNF overexpression. *nestin*–BDNF mice are not, however, a precise phenocopy of the *reeler* mutation, as they show several other features not present in *reeler* mice, including invaginations of the marginal zone and altered morphology and disorganization of CR cells. These effects, on the other hand, appear to be the result of the direct action of BDNF on CR cells, which were found to express TrkB receptors, suggesting that this neurotrophin is capable of affecting several aspects of the development of these cells, such as gene expression, migration, differentiation, and survival. Although the level of Reelin expression per cell is lower in transgenic mice overexpressing BDNF, double-labeling experiments indicate that the invaginations of the marginal zone do contain aggregations of Reelin-expressing CR cells, which as such could represent localized sites of greater Reelin production, as compared with regions of the marginal zone devoid of invaginations. This localized concentration of Reelin-expressing cells could perhaps promote the formation of invaginations in the marginal zone in BDNF-overexpressing mice.

Although less abundant, the heterotopias observed in *nestin*–BDNF mice are similar to those described by Brunstrom et al. (1997) after exogenous administration of purified NT-4 to organotypic slice cultures and embryos in utero. Interestingly, that study found exogenous application of BDNF to be much less effective than NT-4 at inducing heterotopias (Brunstrom et al., 1997), which could be due to differences in the way cells respond to these two factors. These authors ruled out increased proliferation or decreased cell death as mechanisms of generation of NT-4–induced heterotopias and proposed that NT-4 and, to a lesser extent, BDNF promoted tangential migration into the marginal zone of excess neurons cogenerated with normal marginal zone cells. A similar mechanism could be the origin of the heterotopias seen in the marginal zone of *nestin*–BDNF transgenic mice.

In addition, the fact that E13 BrdU-labeled cells were seen in the marginal zone of transgenic mice suggests that some of the heterotopias observed in these animals might contain cells originally destined for the cortical plate. In the same study, Brunstrom and colleagues also reported invaginations of the marginal zone after treatment with NT-4, but not BDNF, of organotypic slice cultures that were filled with heterotopic collections of cells (Brunstrom et al., 1997). In contrast, invaginations in *nestin*–BDNF mice did not appear to contain additional cells and were instead rich in calretinin-positive cellular

processes. The ability of BDNF to induce CR cell aggregation and marginal zone invaginations in *nestin*–BDNF mice may be due to the endogenous and prolonged expression of this factor in the brains of transgenic animals, compared with the more acute treatments made by Brunstrom and colleagues.

Although developmental downregulation of Reelin expression during the first 2 postnatal weeks was prevented in the absence of BDNF, in the third postnatal week, cells expressing Reelin appear to require BDNF for survival. Death of CR cells at equivalent stages has previously been reported in organotypic slice cultures (Del Rio et al., 1996). In the absence of BDNF, the surviving neurons expressed normal (i.e., reduced) levels of Reelin, suggesting that BDNF acts as a survival factor for the same subpopulation that normally downregulates Reelin expression during postnatal development. It is therefore possible that, upon the completion of cortical lamination, this neurotrophin induces a change of fate in a subpopulation of Reelin-positive, BDNF-dependent CR cells. In addition to downregulation of Reelin, BDNF may also induce morphological changes or migration of these cells, as it does in embryonic CR cells of *nestin*–BDNF transgenic mice, and could trigger expression of other gene products. In this context, BDNF has been shown to promote morphological differentiation and expression of calbindin in small cortical GABAergic neurons (Pappas and Parnavelas, 1997) and expression of calretinin in hippocampal CR cells (Marty et al., 1996), as well as phenotypic differentiation of GABAergic neurons in the striatum (Mizuno et al., 1994).

In conclusion, the evidence presented here indicates that Reelin is one direct effector of BDNF during postnatal brain development. In this regard, BDNF appears to function as an intrinsic determinant of cortical maturation, acting as a negative regulator of Reelin expression and a signal for CR cell development. We propose that this neurotrophin is an important component in a developmental program that controls the organization of laminated structures in the mammalian brain.

Experimental Procedures

Transgenic Mice

The generation of $BDNF^{-/-}$ mice has been described previously (Ernfors et al., 1994b). The *nestin*–BDNF construct consisted of a region extending 5.8 kb upstream from the initiation codon of the mouse *nestin* gene (Zimmerman et al., 1994), followed by a 1 kb fragment from the fifth exon of the mouse BDNF gene containing the complete BDNF protein coding sequence, a 300 bp SV40 polyadenylation signal, and 5.4 kb of *nestin* gene downstream sequence, including introns 1, 2, and 3 (Zimmerman et al., 1994). The construct was injected into fertilized mouse oocytes that were subsequently transplanted into pseudopregnant females. Transgenic embryos were identified by PCR.

Cell Culture

Dissociated cultures of embryonic rat cortex were prepared as described previously (Lu and DiCicco-Bloom, 1997), with the exception that embryos were taken on the sixteenth day of gestation, and cells were plated at high density (1.5×10^6 cells/cm^2) to minimize death. Recombinant neurotrophins BDNF, NT-3, or NT-4 (Regeneron Pharmaceuticals) were added at 50 ng/ml at the time of plating. Cultures were maintained in serum-free, defined media (N2) for 12 hr for 2 or 5 days; at these times, the cultures consisted of >90% small, round, phase-bright process-bearing cells. For immunocytochemistry, cultures were fixed in 4% paraformaldehyde and processed as described. Scoring of staining intensity was done by two independent observers who were blind to the treatments made to the cultures. Cells showing weak but distinct signs of labeling above background were considered as having "low" staining intensity; cells showing saturated levels of staining (maximally stained cells) were considered as having "high" staining intensity; cells with labeling intermediate between these two categories were considered as having "middle" staining.

Histological Techniques

BrdU labelings were made by injecting pregnant females with 0.15 ml of 1 mg/ml BrdU at the indicated times of gestation. Histological techniques were otherwise performed as previously described (Ringstedt et al., 1997). ^{33}P- and ^{35}S-labeled antisense oligonucleotides were used for detection of Reelin and TrkB mRNAs, respectively, by in situ hybridization. For BDNF in situ hybridization, a ^{35}S-labeled riboprobe complementary to the mouse BDNF coding sequence was used. The preparation of wild-type and transgenic tissues for in situ hybridization was totally comparable. Each transgenic brain was sectioned simultaneously with a control brain, so that each glass slide contained one section from a transgenic brain and an analogous section from a control brain of the same age. The quantitations in each transgenic brain were normalized relative to the levels observed in the control section present in the same glass slide and were therefore processed simultaneously and under the same conditions. Anti-Reelin monoclonal antibodies were kindly provided by Dr. André Goffinet; anti-calretinin polyclonal antibodies were from Swant Swiss Antibodies; anti-MAP-2 polyclonal antibodies were from Peninsula Laboratories; anti-BrdU antibodies were from Sigma. Anti-TrkB polyclonal antiserum, directed against the extracellular domain of rat TrkB (TrkB out), was kindly provided by Dr. David Kaplan. Marginal zone cell counts (total, calretinin-positive and Reelin-positive) were determined by counting all cells in two 90° sectors of marginal zone, from the midline to the most lateral aspect, in coronal sections at the level of the anterior and posterior hippocampus, respectively.

Image Analysis

The staining intensity of mRNA in tissue sections hybridized in situ was assessed using an automated procedure implemented in the IPLab Spectrum software package (Signal Analytics, Vienna, VA). For *nestin*–BDNF transgenic animals and their controls, dark field images were taken with a digital camera at constant aperture, exposure, and contrast settings. To separate grains from background noise, the images were sharpened by applying an Erode filter followed by a Dilate filter, and then segmented into foreground and background by a threshold that was held constant throughout all measurements. Individual cells were then outlined manually, and the total foreground area was recorded. These measurements could not be done blind to the genotype of the animals, owing to the obvious phenotype of the transgenic animals. The gene-targeted mice and their littermate controls were assessed independently by the same procedure, except that the Erode/Dilate steps were replaced by a single application of the 3 x 3 Sharpen filter, which appeared better at separating the grains from the background. These measurements were done by an observer who was blind to the genotype of the mice. Measurements over unstained cells using the same procedure were used to correct for background.

RNase Protection Assay and Enzyme Immunoassay

Cultures were homogenized in guanidine isothiocyanate (GITC) and β-mercaptoethanol. For RNA extraction, 1/10 volume of 2 M sodium acetate (pH 4.0) was added, followed by phenol/chloroform extraction and ethanol precipitation. A riboprobe was made from a cDNA fragment of the mouse *reelin* gene (GenBank accession number AA268883) with a kit of reagents from Promega. RPA was performed on 5 μg of the total RNA with a kit of reagents from Ambion, according to the instructions of the manufacturer. RPA gels were analyzed in a Storm 840 phosphorimager with ImageQuant software (Molecular Dynamics). Enzyme immunoassay for BDNF was performed as previously described (Nawa et al., 1995).

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References

Alcantara, S., Frisen, J., Delrio, J.A., Soriano, E., Barbacid, M., and Silossantiago, I. (1997). TrkB signaling is required for postnatal survival of CNS neurons and protects hippocampal and motor neurons from axotomy-induced cell death. J. Neurosci. *17*, 3623–3633.

Brunstrom, J.E., Grayswain, M.R., Osborne, P.A., and Pearlman, A.L. (1997). Neuronal heterotopias in the developing cerebral cortex produced by neurotrophin-4. Neuron *18*, 505–517.

Caviness, V.J., and Sidman, R.L. (1973). Retrohippocampal, hippocampal and related structures of the forebrain in the *reeler* mutant mouse. J. Comp. Neurol. *147*, 235–254.

Caviness, Jr., V.S. (1982). Neocortical histogenesis in normal and reeler mice: a developmental study based upon [3H]thymidine autoradiography. Dev. Brain Res. *4*, 293–302.

Caviness, Jr., V.S., Crandall, J.E., and Edwards, M.A. (1988). The reeler malformation: implications for neocortical histogenesis. In Development and Maturation of Cerebral Cortex, A. Peters and E.G. Jones, eds. (New York: Plenum), pp. 59–89.

Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K.S., Pittsmeek, S., Armanini, M.P., Ling, L.H., McMahon, S.B., Shelton, D.L., Levinson, A.D., et al. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell *76*, 1001–1011.

Dahlstrand, J., Lardelli, M., and Lendahl, U. (1995). nestin mRNA expression correlates with the CNS progenitor cell state in many, but not all, regions of developing CNS. Dev. Brain Res. *84*, 109–129.

D'Arcangelo, G., Miao, G.G., Chen, S.C., Soares, H.D., Morgan, J.I., and Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant *reeler*. Nature *374*, 719–723.

Del Rio, J.A., Martinez, A., Fonseca, M., Auladell, C., and Soriano, E. (1995). Glutamate-like immunoreactivity and fate of Cajal-Retzius cells in the murine cortex as identified with calretinin antibody. Cereb. Cortex *5*, 13–21.

Del Rio, J.A., Heimrich, B., Super, H., Borrell, V., Frotscher, M., and Soriano, E. (1996). Differential survival of Cajal-Retzius cells in organotypic cultures of hippocampus and neocortex. J. Neurosci. *16*, 6896–6907.

Del Rio, J.A., Heimrich, B., Borrell, V., Forster, E., Drakew, A., Alcantara, S., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K., et al. (1997). A role for Cajal-Retzius cells and reelin in the development of hippocampal connections [see comments]. Nature *385*, 70–74.

Derer, P., and Derer, M. (1990). Cajal-Retzius cell ontogenesis and death in mouse brain visualized with horseradish peroxidase and electron microscopy. Neuroscience *36*, 839–856.

Ernfors, P., Lee, K.-F., Kucera, J., and Jaenisch, R. (1994a). Lack of neurotrophin-3 leads to deficiences in the peripheral nervous system and loss of limb proprioceptive afferents. Cell *77*, 503–512.

Ernfors, P., Lee, K.F., and Jaenisch, R. (1994b). Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature *368*, 147–150.

Ernfors, P., Vandewater, T., Loring, J., and Jaenisch, R. (1995). Complementary roles of BDNF and NT-3 in vestibular and auditory development. Neuron *14*, 1153–1164.

Fagan, A.M., Garber, M., Barbacid, M., Silossantiago, I., and Holtzman, D.M. (1997). A role for TrkA during maturation of striatal and basal forebrain cholinergic neurons *in vivo*. J. Neurosci. *17*, 7644–7654.

Fariñas, I., Jones, K.R., Backus, C., Wang, X.Y., and Reichardt, L.F. (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature *369*, 658–661.

Friedman, W., Olson, L., and Persson, H. (1991). Cells that express brain-derived neurotrophic factor mRNA in the developing postnatal rat brain. Eur. J. Neurosci. *3*, 688–697.

Gilmore, E.C., and Herrup, K. (1997). Layers of complexity. Curr. Biol. *7*, R231-R234.

Goffinet, A.M. (1984). Events governing organization of postmigratory neurons. Studies on brain development in normal and *reeler* mice. Brain Res. *319*, 261–296.

Goffinet, A.M., So, K.F., Yamamoto, M., Edwards, M., and Caviness, V.J. (1984). Architectonic and hodological organization of the cerebellum in *reeler* mutant mice. Brain Res. *318*, 263–276.

Hirotsune, S., Takahara, T., Sasaki, N., Hirose, K., Yoshiki, A., Ohashi, T., Kusakabe, M., Murakami, Y., Muramatsu, M., Watanabe, S., et al. (1995). The *reeler* gene encodes a protein with an EGF-like motif expressed by pioneer neurons. Nat. Genet. *10*, 77–83.

Howell, B.W., Hawkes, R., Soriano, P., and Cooper, J.A. (1997). Neuronal position in the developing brain is regulated by mouse disabled-1. Nature *389*, 733–737.

Jones, K.R., Fariñas, I., Backus, C., and Reichardt, L.F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell *76*, 989–999.

Lewin, G., and Barde, Y.-A. (1996). Physiology of neurotrophins. Annu. Rev. Neurosci. *19*, 289–317.

Liu, Y., Fujise, N., and Kosaka, T. (1996). Distribution of calretinin immunoreactivity in the mouse dentate gyrus. I. General description. Exp. Brain Res. *108*, 389–403.

Lu, N., and DiCicco-Bloom, E. (1997). Pituitary adenylate cyclase–activating polypeptide is an autocrine inhibitor of mitosis in cultured cortical precursor cells. Proc. Natl. Acad. Sci. USA *94*, 3357–3362.

Marty, S., Carroll, P., Cellerino, A., Castren, E., Staiger, V., Thoenen, H., and Lindholm, D. (1996). Brain-derived neurotrophic factor promotes the differentiation of various hippocampal nonpyramidal neurons, including Cajal-Retzius cells, in organotypic slice cultures. J. Neurosci. *16*, 675–687.

Minichiello, L., and Klein, R. (1996). TrkB and TrkC neurotrophin receptors cooperate in promoting survival of hippocampal and cerebellar granule neurons. Genes Dev. *10*, 2849–2858.

Mizuno, K., Carnahan, J., and Nawa, H. (1994). Brain-derived neurotrophic factor promotes differentiation of striatal GABAergic neurons. Dev. Biol. *165*, 243–256.

Nawa, H., Carnahan, J., and Gall, C. (1995). BDNF protein measured by novel enzyme immunoassay in normal brain and after seizures: partial dissaccordance with mRNA levels. Eur. J. Neurosci. *7*, 15–35.

Ogawa, M., Miyata, T., Nakajima, K., Yagyu, K., Seike, M., Ikenaka, K., Yamamoto, H., and Mikoshiba, K. (1995). The *reeler* gene–associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron *14*, 899–912.

Pappas, I.S., and Parnavelas, J.G. (1997). Neurotrophins and basic fibroblast growth factor induce the differentiation of calbindin-containing neurons in the cerebral cortex. Exp. Neurol. *144*, 302–314.

Parnavelas, J.G., and Edmunds, S.M. (1983). Further evidence that Retzius-Cajal cells transform to nonpyramidal neurons in the developing rat visual cortex. J. Neurocytol. *12*, 863–871.

Pearlman, A.L., and Sheppard, A.M. (1996). Extracellular matrix in early cortical development. Prog. Brain Res. *108*, 117–134.

Ringstedt, T., Kucera, J., Lendahl, U., Ernfors, P., and Ibáñez, C.F. (1997). Limb proprioceptive deficits without neuronal loss in transgenic mice overexpressing neurotrophin-3 in the developing nervous system. Development *124*, 2603–2613.

Schiffmann, S.N., Bernier, B., and Goffinet, A.M. (1997). Reelin mRNA expression during mouse brain development. Eur. J. Neurosci. *9*, 1055–1071.

Schwartz, P.M., Borghesani, P.R., Levy, R.L., Pomeroy, S.L., and Segal, R.A. (1997). Abnormal cerebellar development and foliation in BDNF$^{-/-}$ mice reveals a role for neurotrophins in cns patterning. Neuron *19*, 269–281.

Sheldon, M., Rice, D.S., D'Arcangelo, G., Yoneshima, H., Nakajima, K., Mikoshiba, K., Howell, B.W., Cooper, J.A., Goldowitz, D., and Curran, T. (1997). *Scrambler* and *yotari* disrupt the *disabled* gene and produce a *reeler*-like phenotype in mice. Nature *389*, 730–733.

Soriano, E., Alvarado, M.R., Dumesnil, N., Del Rio, J.A., and Sotelo, C. (1997). Cajal-Retzius cells regulate the radial glia phenotype in the adult and developing cerebellum and alter granule cell migration. Neuron *18*, 563–577.

Stanfield, B.B., and Cowan, W.M. (1979). The development of the hippocampus and dentate gyrus in normal and *reeler* mice. J. Comp. Neurol. *185*, 423–459.

Timmusk, T., Belluardo, N., Persson, H., and Metsis, M. (1994). Developmental regulation of brain-derived neurotrophic factor messenger RNAs transcribed from different promoters in the rat brain. Neuroscience *60*, 287–291.

Wyss, J.M., Stanfield, B.B., and Cowan, W.M. (1980). Structural abnormalities in the olfactory bulb of the Reeler mouse. Brain Res. *188*, 566–571.

Zimmerman, L., Lendahl, U., Cunningham, M., McKay, R., Parr, B., Gavin, B., Mann, J., Vassileva, G., and McMahon, A. (1994). Independent regulatory elements in the *nestin* gene direct transgene expression to neural stem cells or muscle precursors. Neuron *12*, 11–24.