Neuron, Vol. 29, 353–366, February, 2001, Copyright ©2001 by Cell Press

Tbr1 Regulates Differentiation of the Preplate and Layer 6

Robert F. Hevner,* Limin Shi,* Nick Justice,* Yi-Ping Hsueh,† Morgan Sheng,† Susan Smiga,* Alessandro Bulfone,*# André M. Goffinet,§ Anthony T. Campagnoni,‡ and John L. R. Rubenstein*‖

*Nina Ireland Laboratory of Developmental Neurobiology
Department of Psychiatry
University of California, San Francisco
San Francisco, California 94143
†Howard Hughes Medical Institute and
Department of Neurobiology
Massachusetts General Hospital and
Harvard Medical School
Boston, Massachusetts 02114
‡Neuropsychiatric Institute
UCLA Medical School
760 Westwood Plaza
Los Angeles, California 90024
§Neurobiology Unit
FUNDP Medical School
B5000 Namur
Belgium

Summary

During corticogenesis, early-born neurons of the preplate and layer 6 are important for guiding subsequent neuronal migrations and axonal projections. Tbr1 is a putative transcription factor that is highly expressed in glutamatergic early-born cortical neurons. In *Tbr1*-deficient mice, these early-born neurons had molecular and functional defects. Cajal-Retzius cells expressed decreased levels of Reelin, resulting in a *reeler*-like cortical migration disorder. Impaired subplate differentiation was associated with ectopic projection of thalamocortical fibers into the basal telencephalon. Layer 6 defects contributed to errors in the thalamocortical, corticothalamic, and callosal projections. These results show that *Tbr1* is a common genetic determinant for the differentiation of early-born glutamatergic neocortical neurons and provide insights into the functions of these neurons as regulators of cortical development.

Introduction

Early-born cortical neurons (i.e., cells that become postmitotic and differentiate into neurons early in corticogenesis) are developmentally important for organizing the cellular and connectional architecture of the cerebral cortex. The earliest-born cortical neurons form the preplate, a layer of differentiated neurons superficial to the proliferative cells of the ventricular zone (reviewed in Allendoerfer and Shatz, 1994). Subsequent generations of postmitotic neurons migrate into the preplate and intercalate between the inner and outer cell populations, to form the cortical plate. Thus, the cortical plate splits the preplate into superficial and deep components, thereafter termed the marginal zone and subplate, respectively (Allendoerfer and Shatz, 1994). The cortical plate grows in an "inside-out" order, from layer 6, which contains the earliest-born cortical plate neurons, to layer 2, which contains the latest-born neurons (Angevine and Sidman, 1961; Caviness, 1982).

The preplate is thought to function primarily as a framework for further development of the cortex, organizing its laminar structure and some of its connections. In mice, preplate cells differentiate into at least two distinct types of neurons: Cajal-Retzius cells and subplate cells (though other classifications have also been proposed; see Meyer et al., 1998, 1999). Cajal-Retzius cells express Reelin and calretinin (del Río et al., 1995; Alcántara et al., 1998; Meyer et al., 1999) and have been implicated in controlling cell migrations (reviewed by Rice and Curran, 1999) and radial glia morphology (Supèr et al., 2000). Subplate cells are marked by expression of *golli-lacZ*, a transgene (Landry et al., 1998), and of calretinin (Fonseca et al., 1995). They have been implicated in guiding thalamocortical afferent axons into the cortex, and cortical efferent axons to their subcortical targets (McConnell et al., 1989, 1994; Ghosh et al., 1990; De Carlos and O'Leary, 1992; Ghosh and Shatz, 1993). However, the exact functions and origins of the preplate cells remain unclear, and their molecular specializations have only begun to be explored (Landry et al., 1998; Meyer et al., 1998, 1999; Rice and Curran, 1999; Gleeson and Walsh, 2000).

Tbr1 is a transcription factor gene of the T box family that is expressed soon after cortical progenitors begin to differentiate. It is highly expressed in early-born neurons of the preplate and layer 6 (Bulfone et al., 1995). Neurons in layer 6 are the source of corticothalamic axons (De Carlos and O'Leary, 1992) and have been implicated as pioneer neurons in the callosal projection (Koester and O'Leary, 1994). The high expression of *Tbr1* in the preplate and cortical plate suggested that *Tbr1* might play an important role in cortical development. To investigate this hypothesis, we examined the expression of Tbr1 protein in the developing cortex and studied mice with a loss-of-function mutation of the *Tbr1* gene (Bulfone et al., 1998).

We found that, in the *Tbr1* mutant cortex, early-born cells formed a preplate but did not express markers of Cajal-Retzius, subplate, or layer 6 neurons. In contrast, markers of later-born cortical layers were relatively normal. Furthermore, the cortex of *Tbr1* mutants displayed developmental abnormalities in the laminar organization of neurons, and in the guidance of cortical afferent and efferent axons. Other properties of cortical neurons such as neurotransmitter expression, cell death, and neuronal morphology were mostly unaffected. Our results point to specific developmental roles played by early-born

‖To whom correspondence should be addressed (e-mail: jlrr@cgl.ucsf.edu).
#Present address: Institute for Stem Cell Research, DIBIT, Hospital San Raffaele, Milan 20132, Italy.

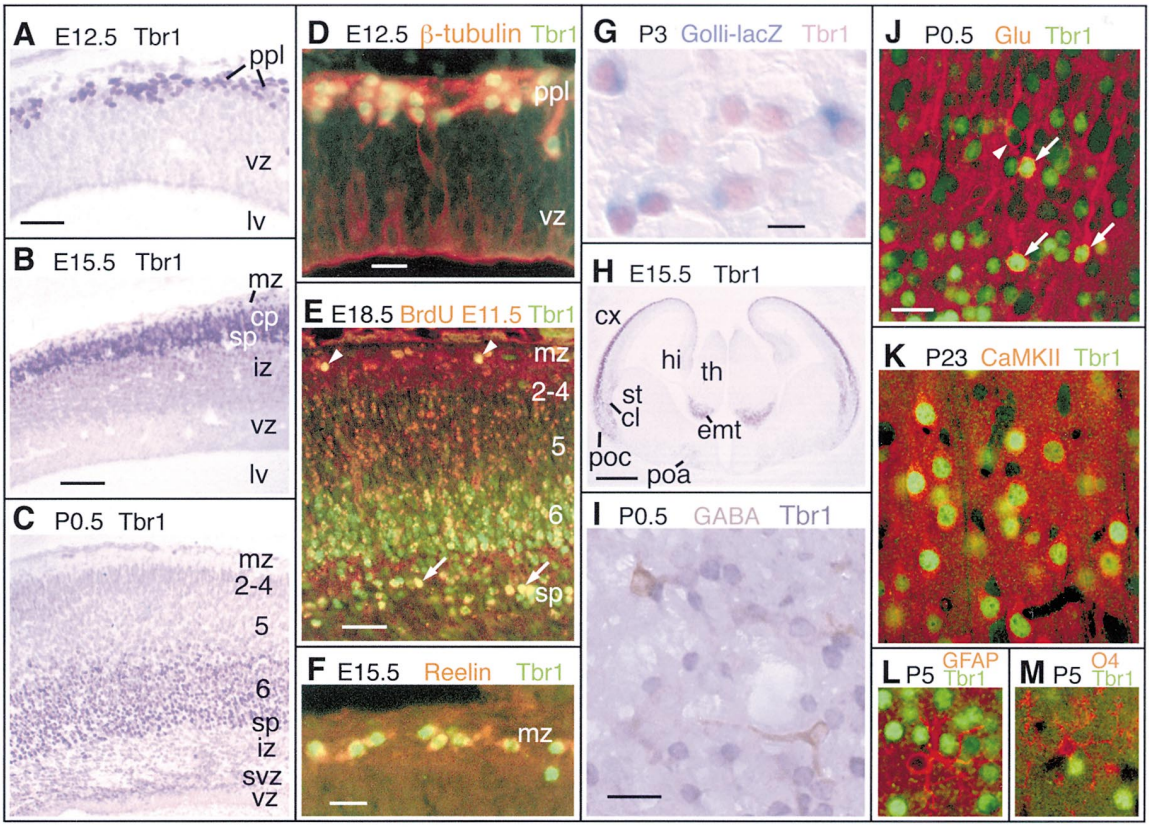

Figure 1. Tbr1 Protein Expression in the Developing Neocortex

(A–C) Tbr1 immunohistochemistry, E12.5 (A), E15.5 (B), P0.5 (C). cp, cortical plate; iz, intermediate zone; lv, lateral ventricle; mz, marginal zone; ppl, preplate; sp, subplate; svz, subventricular zone. 2–6, cortical layers. Scale bars: 50 μm (A), 100 μm (B and C).

(D–F) Two-color immunofluorescence. (D) Tbr1 (green) and βIII-tubulin (red), a neuronal marker, E12.5. (E) Tbr1 (green) and E11.5 BrdU (red), E18.5. Double-labeled cells (yellow) were found mainly in the marginal zone (arrowheads), subplate (arrows), and layer 6. (F) Tbr1 (green) and Reelin (red), a Cajal-Retzius cell marker, E15.5. Scale bars: 20 μm (D and F), 50 μm (E).

(G) Double labeling for Tbr1 (lavender, immunohistochemistry) and *golli-lacZ* expression (blue, β-galactosidase histochemistry), a subplate marker, P3. Coexpression was also seen at embryonic stages (data not shown). Scale bar: 10 μm.

(H) Tbr1 immunohistochemistry, E15.5. cl, claustrum; cx, neocortex; emt, eminentia thalami; hi, hippocampus; poa, preoptic area; poc, primary olfactory cortex; st, striatum; th, thalamus. Scale bar: 0.5 mm.

(I) Two-color immunohistochemistry for GABA (brown) and Tbr1 (gray), P0.5. Scale bar: 20 μm.

(J–M) Two-color immunofluorescence, confocal microscopy. (J) Tbr1 (green) and glutamate (red), P0.5. Most glutamatergic neurons expressed Tbr1 (arrows), but some did not (arrowhead). (K) Tbr1 (green) and CaMKII (red), a glutamatergic marker, P23. (L) Tbr1 (green) and GFAP (red), an astrocyte marker, P5. (M) Tbr1 (green) and O4 (red), an oligodendrocyte marker, P5. Scale bar: 20 μm.

cortical neurons and suggest novel mechanistic insights into the role of subplate cells in thalamocortical axon guidance.

Results

Tbr-1 Is Expressed in Cajal-Retzius Cells, Subplate Cells, and Glutamatergic Neurons

Tbr1 mRNA is expressed in postmitotic cells of the developing cortex from E10 through adulthood, with particularly high expression in the preplate and in layer 6 (Bulfone et al., 1995). To determine the types of cortical cells that express Tbr1 protein, we used immunohistochemistry for Tbr1 and for markers of specific cell types. Tbr1 protein was barely detectable at E10.5 (data not shown) but by E12.5 was strongly expressed in the preplate (Figure 1A). Consistent with its proposed role as a transcription factor, Tbr1 was only detected in cell nuclei. High expression was observed in the marginal zone, cortical plate, and subplate at E15.5 (Figure 1B) and in layer 6 and the subplate at P0.5 (Figure 1C). Cells in other cortical layers and in the intermediate zone expressed lower levels of Tbr1 protein (Figures 1B and 1C). No expression was detected in the ventricular zone. Tbr1 protein was also expressed in the olfactory bulbs and olfactory nuclei (Bulfone et al., 1998), the eminentia thalami (Figure 1H), the preoptic area/lateral hypothalamus region (Figure 1H), and the entopeduncular nucleus (data not shown). In adult mice (1 year old), Tbr1 expression was highest in neocortical layers 6 and 3 (data not shown). Interestingly, mitral cells did not express Tbr1 after the first postnatal month (data not shown), despite high expression at earlier ages (Bulfone et al., 1998).

In the E12.5 preplate, cells that expressed Tbr1 were identified as neurons on the basis of coexpression with βIII-tubulin (Figure 1D). The preplate contains cells that

differentiate into two distinct neuronal populations, namely Cajal-Retzius cells and subplate cells (Allendoerfer and Shatz, 1994). Both of these populations expressed high levels of Tbr1, as indicated by coexpression with Reelin (Figure 1F), a Cajal-Retzius cell marker (del Río et al., 1995; Alcántara et al., 1998; Meyer et al., 1999), and by coexpression with *golli-lacZ* transgene (Figure 1G), a subplate marker (Landry et al., 1998). Consistent with the birth dates of preplate cells between E10–E13 (Smart and Smart, 1977; Wood et al., 1992; Sheppard and Pearlman, 1997), high levels of Tbr1 were found in cells labeled with BrdU on E11.5, with survival to E18.5 (Figure 1E).

Most cortical neurons use either GABA (inhibitory interneurons) or glutamate (excitatory projection neurons) as their neurotransmitter. Tbr1 was expressed in glutamatergic neurons (Figure 1J), but not in GABAergic neurons (Figure 1I). The majority of glutamatergic neurons ($\geq$80%) in the neocortex expressed Tbr1 postnatally (P0, P5, or P23), though some did not (arrowhead, Figure 1J). Likewise, in the olfactory bulb and preoptic area, only glutamatergic cells expressed Tbr1, but not all glutamatergic cells did so (data not shown). Tbr1 also colocalized with CaM kinase II (CaMKII) (Figure 1K), a marker of cortical glutamatergic neurons at 3 postnatal weeks and later (Liu and Jones, 1996). Most, but not all, CaMKII-positive neurons were Tbr1 positive.

Tbr1 was not expressed in astrocytes labeled for glial fibrillary acidic protein (GFAP) (Figure 1L), nor in oligodendrocytes labeled for O4 (Figure 1M).

Tbr1 Mutant Mice Have a Cortical Malformation

To study *Tbr1* function, we generated mice with a targeted disruption of the gene, in which exons containing the T box (encoding the putative DNA binding domain) were deleted (Bulfone et al., 1998). No Tbr1 protein was detectable in homozygous mutants, by immunohistochemistry with antibodies against N-terminal or C-terminal Tbr1 sequences (data not shown). Previously, we reported that *Tbr1*$^{-/-}$ mice have small olfactory bulbs and lack olfactory bulb projection neurons (e.g., mitral cells) (Bulfone et al., 1998). Here, we show that *Tbr1*-deficient mice have severe defects of cortical development.

In neonatal *Tbr1* mutants, the cortex was approximately normal size (Figures 2A and 2B). (Since mutants die at P0–P3, studies were mostly limited to neonatal and earlier ages, though two mice were kept alive to 2–4 months by hand feeding; see below). Nissl staining revealed a complex cortical malformation, with distinct phenotypes in different regions. The piriform cortex was severely hypocellular (arrows, Figures 2C′ and 2D′). The neocortex was disorganized. In rostral areas, cortical layers did not match the normal pattern, and there was no subplate (Figure 2F'). In caudal areas, neuronal clusters were prominent (arrowheads, Figure 2G′). The clusters were largest at the caudal pole, in the entorhinal cortex (Figure 2E′). The laminar organization of the hippocampus was almost normal, except for small clusters in stratum pyramidale (data not shown).

Glutamate and GABA were expressed in the mutant cortex, but their distributions were altered. Glutamatergic neurons, which express Tbr1 (Figure 1J), were most abundant in layer 5 in P1.5 controls (Figures 2H and 2I). In the rostral cortex of mutants, glutamatergic neurons were more superficial than normal, but still had a layer-like distribution (Figure 2H′). In caudal areas, glutamatergic neurons formed large clusters (Figure 2I′). GABAergic cells, which do not express Tbr1 (Figure 1I), were also distributed abnormally. In P1.5 controls, GABAergic cells were concentrated in layer 5 (Figures 2J and 2K). In mutants, GABAergic cells were scattered among many layers in rostral cortex (Figure 2J′) and formed clusters in caudal cortex (Figure 2K′). The glutamatergic and GABAergic clusters mostly overlapped, as demonstrated by double-labeled immunofluorescence (data not shown).

Some fiber bundles were abnormal as well. The corpus callosum did not cross the midline, but formed a Probst bundle (pb, Figure 2C′) (Probst, 1901). Also, the anterior commissure lacked an anterior part (Figure 2C′), and the posterior part did not cross the midline. The hippocampal commissure crossed the midline, sometimes carrying aberrant callosal fibers (data not shown).

The severity of the cortical defects progressed with postnatal age. In two mutant mice that survived for 2 and 4 postnatal months (assisted by hand feeding), the cortex was thinned to about half the control thickness, while the hippocampus was not atrophic (data not shown). Barrels, which are somatosensory modules in the cortex, were not detectable in these animals by cytochrome oxidase histochemistry (data not shown).

Cell Birth, Migration, and Death in the *Tbr1*$^{-/-}$ Cortex

The disturbed laminar organization of the *Tbr1* mutant cortex suggested that cortical cell migrations were abnormal. To study migrations, we used bromodeoxyuridine (BrdU) birthdating. Normally, preplate cells are born earliest (from about E10 to E13), followed by cortical plate cells that populate the cortical layers in order from deepest (layer 6) to most superficial (layer 2) (Angevine and Sidman, 1961; Smart and Smart, 1977; Caviness, 1982; Wood et al., 1992; Sheppard and Pearlman, 1997).

To label preplate cells, we injected BrdU on E10.5 or E11.5. After survival to E15.5, E18.5, or P0.5, BrdU-labeled cells in control mice populated the marginal zone and subplate, as exemplified by BrdU injection at E10.5 with survival to E18.5 (Figures 3A1 and 3A2). In mutants, cells born on E10.5 occupied predominantly superficial positions, though labeled cells were more widely dispersed than normal (Figures 3A1′ and 3A2′). Similar results were obtained with BrdU injection at E11.5, and with survival to E15.5 or P0.5 (data not shown).

To label cortical plate cells, we injected BrdU on E13.5 (for deep layers) or on E16.5 (for superficial layers), and followed with survival to E18.5 or P0.5 (results were similar for both survival ages). In mutants, many E13.5-born cells were shifted to superficial positions, though a minority migrated to the deepest part of the cortex (Figures 3B1′ and 3B2′). In caudal areas, E13.5-born cells formed clusters deep in the cortex (arrows, Figure 3B2′). Cells born on E16.5 in controls migrated to superficial locations in the cortical plate, but mainly to deep positions in mutants (Figures 3C1–3C2′). In the caudal

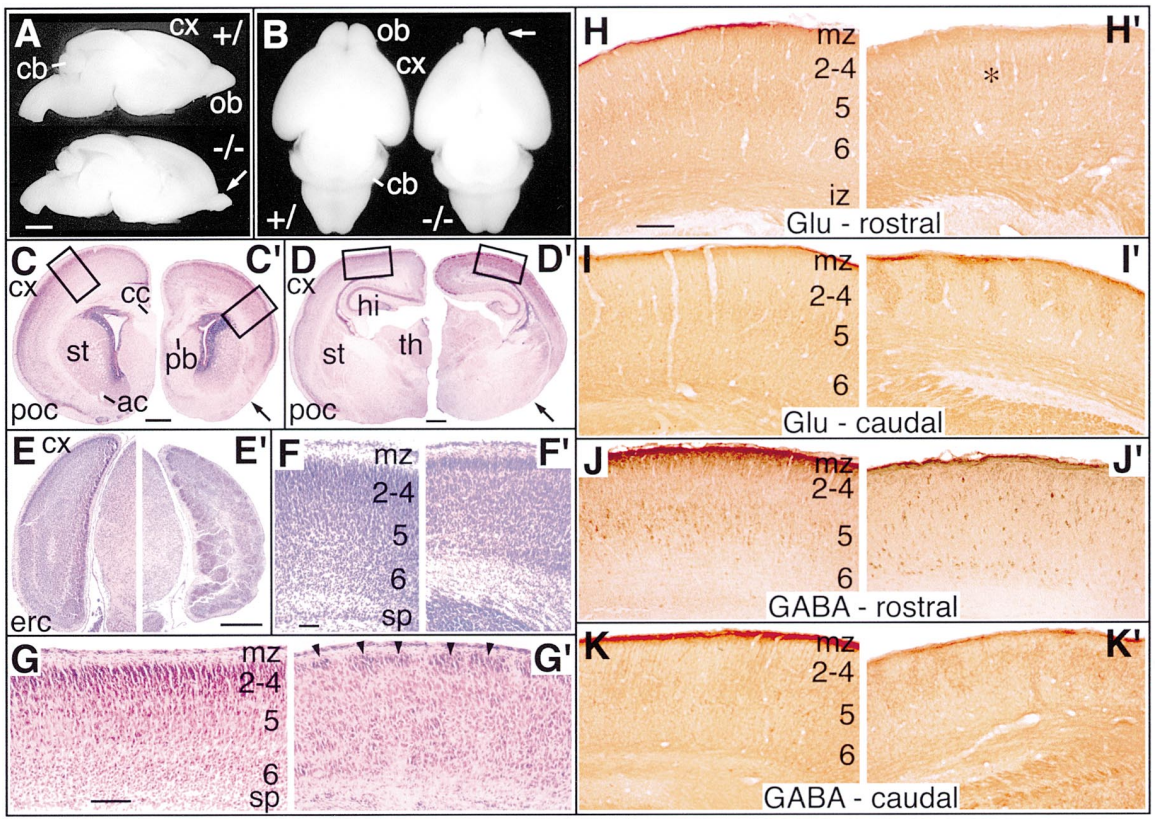

Figure 2. Cortical Malformation in *Tbr1* Mutants

Controls (C, D, E, F, G, H, I, J, and K), mutants (C′, D′, E′, F′, G′, H′, I′, J′, and K′).

(A and B) Medial (A) and dorsal (B) aspects of neonatal brains, P0.5. The olfactory bulbs (ob) were hypoplastic in mutants (arrows). cb, cerebellum; cx, cortex. Scale bar: 1 mm.

(C–G′) Cresyl violet histology (30 μm coronal sections), P1.5. Rostral (C and C′), middle (D and D′), and caudal (E and E′) levels. Boxes (C and C′) enlarged in (F and F′), boxes (D and D′) enlarged in (G and G′). Primary olfactory cortex (poc) was hypocellular (arrows), and neocortex (cx) had abnormal lamination. Neuronal clusters were seen in caudal neocortex (arrowheads) and entorhinal cortex (erc). The anterior commissure (ac) and corpus callosum (cc) did not cross the midline, and a Probst bundle (pb) was formed. hi, hippocampus; mz, marginal zone; st, striatum; th, thalamus. Scale bars: 0.5 mm (C, C′, D, D′, E, and E′), 100 μm (F, F′, G, and G′).

(H–I′) Glutamate immunohistochemistry (30 μm coronal sections), P1.5. Rostral (H and H′) and caudal (I and I′) levels. In controls, Glu-ir neurons were most abundant in layer 5. In mutants, Glu-ir neurons formed an ectopic superficial layer (*) in rostral cortex and clusters in caudal cortex. iz, intermediate zone.

(J–K′) GABA immunohistochemistry (30 μm coronal sections), P1.5. Rostral (J and J′) and caudal (K and K′) levels. GABA-ir neurons were most abundant in layer 5 in controls. In mutants, GABA-ir neurons were widely dispersed in rostral cortex but formed clusters in caudal cortex.

(H–K′) Scale bar: 100 μm.

cortex, some E16.5-born cells formed superficial clusters (arrows, Figure 3C2′). Nomarski optics showed that the clusters of E16.5-born cells contained mainly small neurons, while E13.5-born clusters contained larger neurons (data not shown).

Medial limbic structures such as the cingulate gyrus and hippocampus were exceptional, in that cell migrations appeared nearly normal by BrdU birthdating (data not shown). Below, we show that this disparity from other regions can be explained by differential changes in the expression of Reelin, an important regulator of cortical cell migrations.

To determine whether cell death was increased in the cortex of *Tbr1* mutants, we used the TUNEL method to stain apoptotic cells. Apoptosis was not increased in the neocortex of mutants at E14.5 or E16.5, but was increased at P0.5 (data not shown). These findings were consistent with the BrdU data suggesting that most cortical cells (including preplate neurons) survived to P0.5 (Figure 3), but also suggested that apoptosis contributed to cell loss between birth and 2–4 postnatal months (data not shown).

Defective Preplate Splitting and Reelin Downregulation in *Tbr1* Mutants

In view of the ubiquitous expression of Tbr1 in preplate neurons (Figure 1D) and the neuronal migration defects in *Tbr1* mutants, we hypothesized that the preplate might develop abnormally in *Tbr1* mutants. Nevertheless, the mutant preplate had a normal histological appearance at E12.5 and appropriately expressed βIII-tubulin, a neuronal marker (Figures 4A and 4A′). Furthermore, cells in the mutant preplate expressed *Tbr1* transcripts from the targeted allele (Figures 4B and 4B′). In contrast, *Tbr1*-expressing cells were absent from the primary olfactory cortex of E12.5 mutants (arrow, Figure 4B′).

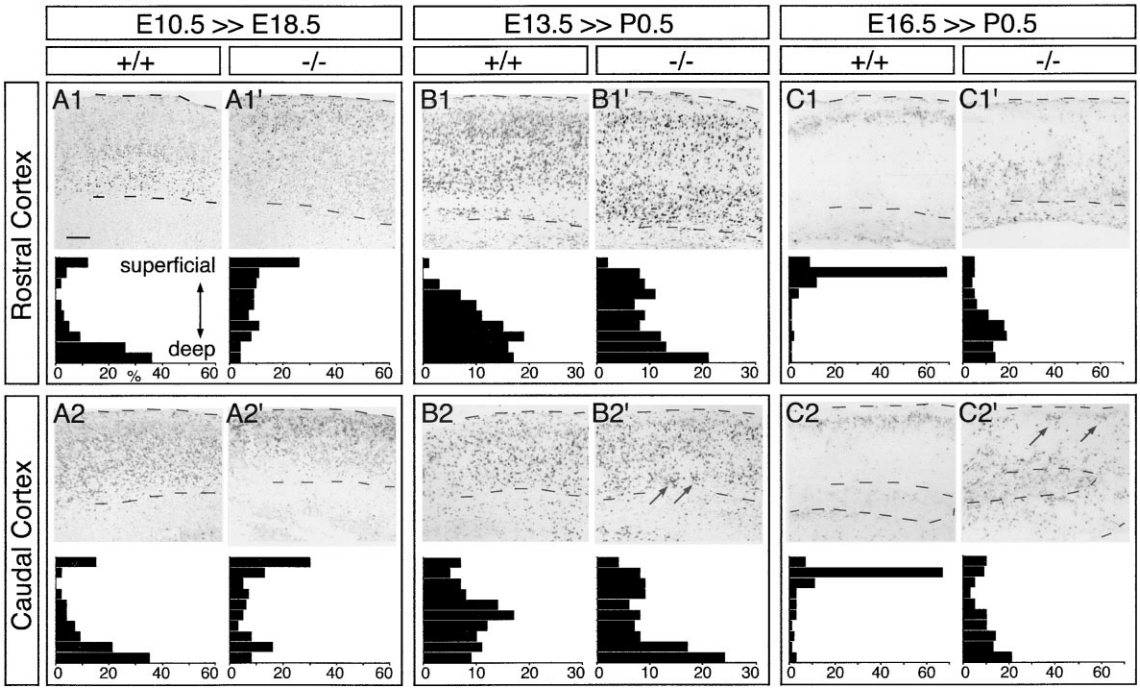

Figure 3. Bromodeoxyuridine (BrdU) Birthdating

Controls (A1, A2, B1, B2, C1, and C2), mutants (A1′, A2′, B1′, B2′, C1′, and C2′). Upper panels, BrdU immunohistochemistry; lower panels, bar graphs showing the radial distribution of heavily labeled cells (10 bins). Dashed lines, deep and superficial cortical boundaries.

(A1–A2′) E10.5 BrdU, E18.5. In controls, early-born cells populated the marginal zone, subplate, and layer 6, but in mutants, early-born cells were mostly superficial.

(B1–B2′) E13.5 BrdU, P0.5. BrdU-labeled cells shifted to more superficial positions and to the deepest cortical layer, where clusters were formed in caudal areas (arrows).

(C1–C2′) E16.5 BrdU, P0.5. BrdU-labeled cells were mostly shifted to deeper positions, though some superficial clusters were formed in caudal cortex (arrows, C2′).

Scale bar: 100 μm.

Although many features of the preplate appeared normal, its subsequent separation into marginal zone and subplate was defective. In controls, preplate splitting was visible at E14.5 by immunostaining for chondroitin sulfate proteoglycans (CSPGs), which labeled the marginal zone and the subplate (Figure 4C) (Bicknese et al., 1994); or by immunostaining for neurocan, which labeled mainly the subplate (Figure 4D) (Miller et al., 1995; Fukuda et al., 1997). In *Tbr1* mutants, CSPG and neurocan staining were predominantly superficial (Figures 4C′ and 4D′), indicating that the subplate had not separated from the marginal zone. This defect was not absolute, as partial splitting was seen in some foci (arrows, Figures 4C′ and 4D′). At E15.5, $MAP2^+$ preplate neurons were abnormally superficial in *Tbr1* mutants (Figures 4E and 4E′). Conversely, cortical plate neurons, labeled by TAG-1 immunostaining, occupied positions below the subplate in mutants, rather than above it as in controls (Figures 4F and 4F′).

The preplate splitting defect in *Tbr1* mutants was similar to the cortical phenotype in *reeler* mice (Caviness, 1982; Sheppard and Pearlman, 1997). This, and the fact that Tbr1 is expressed in Cajal-Retzius cells (Figure 1F), suggested that Tbr1 might control the expression of Reelin or other molecules in the Reelin signaling pathway. Expression of *Reelin* mRNA was indeed decreased in the preplate of *Tbr1* mutants, as observed at E12.5 (Figures 4G and 4G′). *Reelin* downregulation was region specific, affecting the paleocortex and lateral and dorsal neocortex, but not the medial (cingulate) cortex or the hippocampus (Figure 4G′). The intensity of *Reelin* mRNA expression in individual preplate cells was lower in *Tbr1* mutants than in controls, suggesting that Cajal-Retzius cells survived (Figures 3A1′ and 3A2′) and differentiated into neurons (Figure 4A′), but did not express adequate levels of *Reelin*. Western blotting showed that Reelin protein was also reduced (inset, Figure 4G′). *Reelin* mRNA expression remained low during embryonic development and the day of birth (Figure 4H′). At P0.5, cells with low-level Reelin expression appeared in the cortex of controls and mutants (arrowheads, Figures 4H and 4H′); these cells were probably GABAergic interneurons (Alcántara et al., 1998).

A second marker of Cajal-Retzius cells—calretinin (CR)—was also downregulated in *Tbr1* mutants (Figures 4I–4J′). In mice, CR is a marker of Cajal-Retzius cells in the marginal zone (del Río et al., 1995; Alcántara et al., 1998), and of subplate neurons deep to the cortex (Fonseca et al., 1995; Zhou et al., 1999). These CR^+ cells, as well as CR^+ thalamic axons in the subplate (Figures 4I–4J), were mostly lacking from the mutant neocortex (Figures 4I′ and 4J′), which contained only a few CR^+ cells in the marginal zone (arrowhead, Figure 4I′). In the hippocampus, the number of CR^+ Cajal-

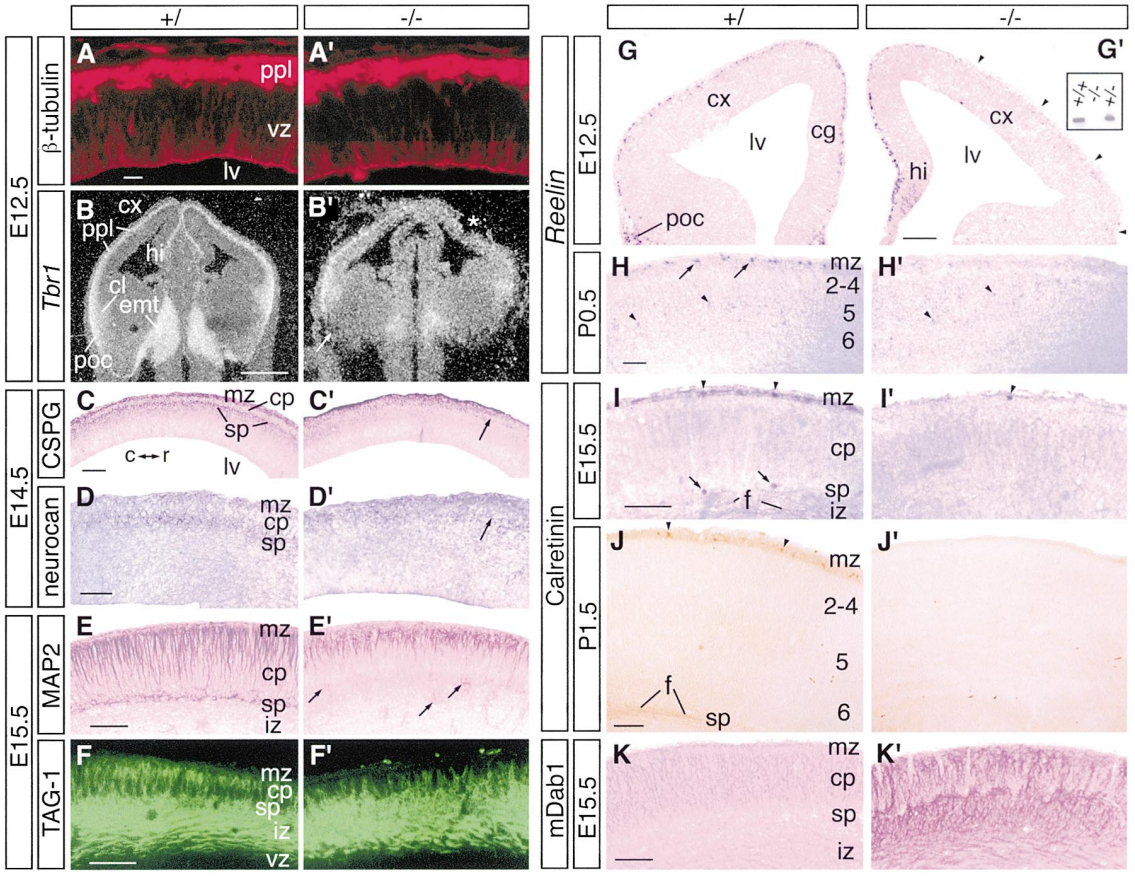

Figure 4. Defective Preplate Splitting and *Reelin* Downregulation in *Tbr1*$^{-/-}$ Mutants

Controls (A, B, C, D, E, F, G, H, I, J, and K), mutants (A′, B′, C′, D′, E′, F′, G′, H′, I′, J′, and K′).

(A and A′) Immunofluorescence for βIII-tubulin, a neuronal marker, E12.5. lv, lateral ventricle; vz, ventricular zone. Scale bar: 20 μm.

(B and B′) *Tbr1* in situ hybridization, E12.5. *Tbr1*-expressing cells were present in the mutant preplate, but not in the primary olfactory cortex (poc) (arrow). *, dissection artifact; cl, claustrum; cx, neocortex; emt, eminentia thalami; hi, hippocampus. Scale bar: 0.5 mm.

(C and C′) CSPG immunohistochemistry (sagittal sections), E14.5. Preplate splitting was disturbed in mutants. Partial splitting occurred focally (arrow). cp, cortical plate; mz, marginal zone; sp, subplate. Axes: c, caudal; r, rostral. Scale bar: 100 μm.

(D and D′) Neurocan immunohistochemistry (sagittal sections), E14.5. The neurocan^{+} subplate was abnormally superficial. Partial splitting was evident (arrow). Scale bar: 50 μm.

(E and E′) MAP2 immunohistochemistry, E15.5. MAP2^{+} subplate neurons were shifted to superficial positions, with only a few deeper cells (arrows). Scale bar: 100 μm.

(F and F′) TAG-1 immunohistochemistry, E15.5. TAG-1^{+} cortical plate neurons shifted to deeper positions, below the subplate. Scale bar: 100 μm.

(G and G′) *Reelin* in situ hybridization, E12.5. *Reelin* was downregulated in the dorsolateral neocortex and primary olfactory cortex (arrowheads), but not in the cingulate (cg) cortex or hippocampus. Inset: Western blot showing decreased Reelin full-length protein in E12.5 neocortex of *Tbr1* homozygous mutants. Scale bar: 100 μm.

(H and H′) *Reelin* in situ hybridization, P0.5. High-level expression, characteristic of Cajal-Retzius cells (arrows), was lacking in mutant neocortex. Low-level expression, typical of GABAergic interneurons (Alcántara et al., 1998), was present (arrowheads). Scale bar: 100 μm.

(I and I′) Calretinin immunohistochemistry, E15.5. Cajal-Retzius cells (arrowheads), subplate cells (arrows), and fibers (f) were labeled in controls, but mostly missing in mutants. Scale bar: 100 μm.

(J and J′) Calretinin immunohistochemistry, P1.5. Cajal-Retzius cells (arrowheads) and subplate fibers were labeled at this age in controls, but were mostly absent in mutants. Scale bar: 100 μm.

(K and K′) mDab1 immunohistochemistry, E15.5. Expression was increased in cortical plate cells. Scale bar: 100 μm.

Retzius neurons was unaffected by *Tbr1* deficiency (data not shown). Thus, CR and *Reelin* expression showed similar regional effects, suggesting that Tbr1 regulates the molecular properties of neocortical Cajal-Retzius neurons.

Gene Expression in the Cortical Plate

As Tbr1 is expressed in migratory and postmigratory cortical neurons (Figures 1A–1C), we assayed the molecular properties of *Tbr1* mutants to test two questions: (1) does the migration defect have a cell autonomous basis, in addition to the defect in Reelin expression, and (2) since Tbr1 is expressed at high levels in cortical layer 6 (Figure 1C), is this layer preferentially affected by the mutation?

We found that mRNA levels were normal in the *Tbr1* mutants for several genes known to be required for radial migration of cortical neurons (reviewed by Rice and Curran, 1999; Gleeson and Walsh, 2000). These included Reelin receptors (*apoER2* and *VLDR*), and cyto-

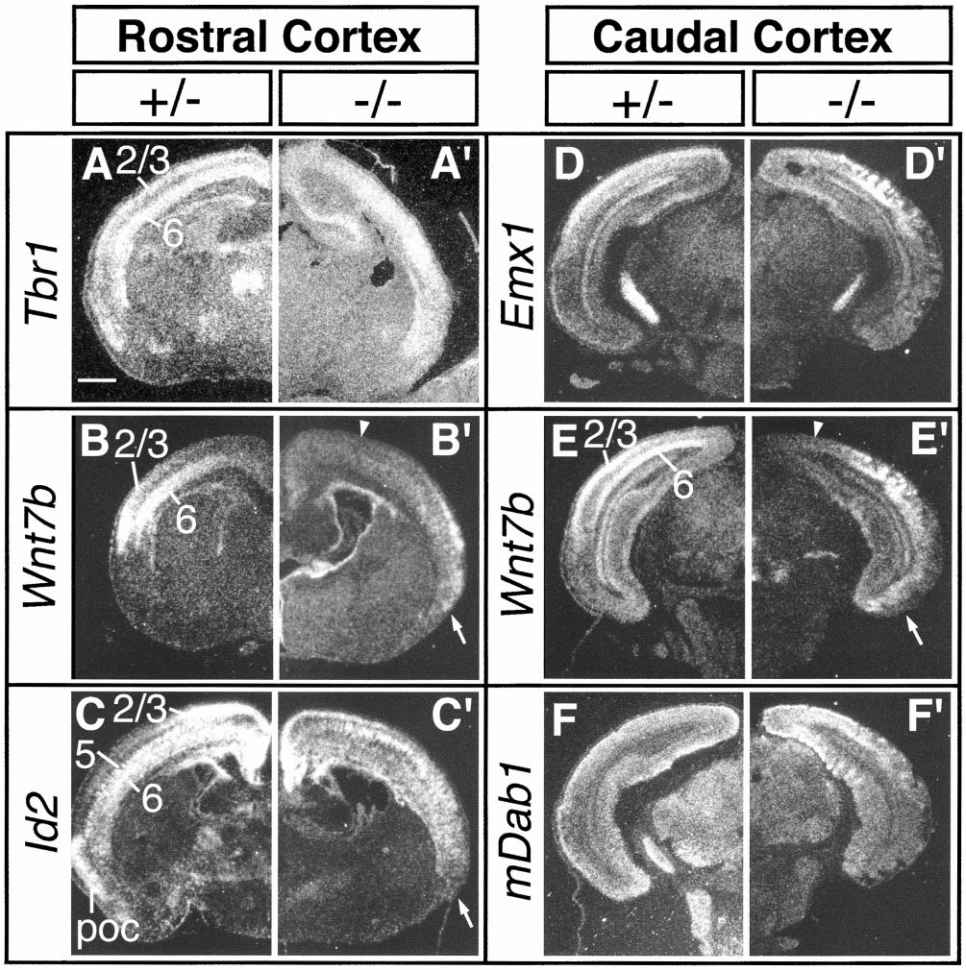

Figure 5. In Situ Hybridization for Cortical Plate Gene Expression, P0.5

Controls (A, B, C, D, E, and F), mutants (A′, B′, C′, D′, E′, and F′).

(A–C′) Rostral cortex.

(A and A′) *Tbr1* expression was maintained.

(B and B′) *Wnt7b* was downregulated in layer 6 (arrowhead), and ectopically expressed in paleocortex (arrow).

(C and C′) *Id2* expression was reduced in the primary olfactory cortex (poc) (arrow).

(D–F′) Caudal cortex.

(D and D′) *Emx1* expression was patchy.

(E and E′) *Wnt7b* was downregulated in layer 6 (arrowhead), had patchy expression in lateral cortex, and was ectopically expressed in entorhinal cortex (arrow).

(F and F′) *mDab1* had patchy expression.

Scale bar: 0.5 mm.

plasmic components in Reelin signal transduction (*mDab1*, *p35*, *p39*, and *cdk5*) (Figures 5F and 5F′ and data not shown). *Doublecortin* (a microtubule-associated protein) expression was also normal (data not shown). However, like in *reeler* mutants, mDab1 protein levels were markedly increased (Figure 4K′).

We also examined the expression of genes specific for different layers of the neocortex. Markers for most neocortical layers were not reduced (data not shown), but a marker of layer 6 in the dorsomedial cortex, *Wnt7b*, was downregulated (arrowheads, Figures 5B′ and 5E′). Although layer 5 projections to the cerebral peduncle were abnormal (see below), genes expressed in layer 5 such as *ER81* and *Otx1* (Weimann et al., 1999), continued to be expressed in laminar patterns (data not shown). In general, genes expressed in multiple cortical layers, such as *Id2*, *Emx1*, *apoER2*, *VLDR*, *mDab1*, *p35*, *p39*, and *cdk5* were not downregulated, but their laminar distributions were perturbed (Figures 5C–5D′, 5F, and 5F′ and data not shown). In caudal areas, many genes had patchy expression, corresponding to cell clusters (Figures 5D′, 5E′, and 5F′). Defective development of the primary olfactory cortex was evident from reduced expression of *Id2* (arrow, Figure 5C′) and from ectopic expression of *Wnt7b* (arrows, Figures 5B′ and 5E′). Defects in olfactory bulb projection neurons were reported previously (Bulfone et al., 1998). Thus, those cells that expressed the highest levels of Tbr1—i.e., Cajal-Retzius cells (Figures 4G′, 4H′, 4I′, and 4J′), subplate cells (see below), layer 6 neurons (Figures 5B′ and 5E′), olfactory

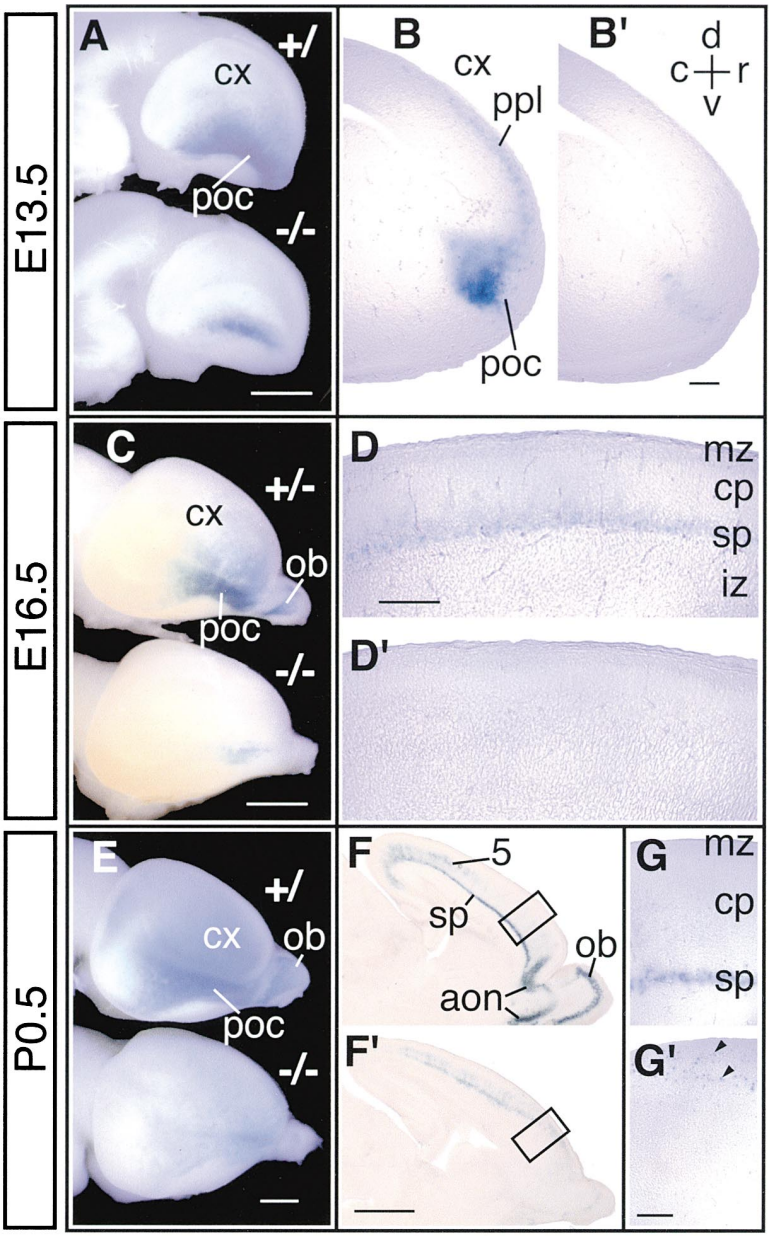

Figure 6. Downregulation of Subplate Marker *golli-lacZ* in *Tbr1*$^{-/-}$ Mutants

Controls (B, D, F, and G), mutants (B′, D′, F′, and G′). Whole mounts (left), sagittal sections (right).

(A–B′) E13.5. Expression was reduced in the primary olfactory cortex (poc) and absent from neocortex (cx). ppl, preplate. Axes: c, caudal; d, dorsal; r, rostral; v, ventral. Scale bars: 1 mm (A), 200 μm (B and B′).

(C–D′) E16.5. Expression was weak in the primary olfactory cortex and almost nil in neocortex and the olfactory bulb (ob). cp, cortical plate; iz, intermediate zone; mz, marginal zone; sp, subplate. Scale bars: 1 mm (C), 200 μm (D and D′).

(E–G′) P0.5. In controls, expression was specific for subplate cells in rostral cortex, but labeled layer 5 and subplate cells in caudal cortex. In mutants, *golli-lacZ*$^{+}$ cells were superficial in rostral cortex (arrowheads), but multilaminar in caudal cortex. Expression was generally weak. aon, anterior olfactory nuclei. Scale bars: 1 mm (E, F, and F′), 200 μm (G and G′).

cortex neurons (Figures 5B′, 5C′, and 5E′), and olfactory bulb projection neurons—had molecular expression defects.

Tbr1 Is Required for the Development of Subplate Neurons

Tbr1 is highly expressed in subplate cells (Figure 1G). To test whether subplate cell properties were affected by *Tbr1* deficiency, we studied a subplate-specific marker, *golli-lacZ* (Landry et al., 1998). We bred *golli-lacZ* transgenic mice with *Tbr1* mutant mice and assayed *golli-lacZ* expression by β-galactosidase histochemistry.

In controls, neocortical expression of *golli-lacZ* was first detected at E13.5 in the preplate (Figure 6B) (Landry et al., 1998). In E13.5 *Tbr1* mutants, preplate expression of *golli-lacZ* was absent, indicating that subplate differentiation was impaired (Figures 6A–6B′). *Golli-lacZ* expression was also severely reduced in the primary olfactory cortex.

At E16.5, *golli-lacZ* was strongly expressed by many cells in the subplate (and a few cells in the marginal zone) of controls, but remained almost undetectable in the mutant neocortex (Figures 6C–6D′). A few *golli-lacZ*$^{+}$ cells were present, but were barely visible due to low expression levels (data not shown). Calretinin, another marker of subplate cells in the embryonic neocortex (Fonseca et al., 1995), was also downregulated in embryonic *Tbr1* mutants (Figure 4I′). The olfactory cortex and bulb likewise showed reduced expression of *golli-lacZ* at E16.5.

At P0.5, *golli-lacZ* in controls was expressed not only in the subplate and olfactory areas, but also in layer 5 of the caudal cortex (Figures 6E and 6F). In P0.5 mutants, *golli-lacZ* expression remained low in the neocortex and olfactory areas (Figures 6E and 6F′). In the rostral cortex,

where *golli-lacZ* expression was subplate specific (Figure 6G), expression in mutants was weak and superficial (arrowheads, Figure 6G′). This suggested that subplate cells were present in the *Tbr1* mutant cortex, but their differentiation was delayed. The ectopic locations of the cells were consistent with BrdU birthdating studies indicating that early-born cells occupied abnormally superficial positions (Figures 3A1′ and 3A2′). In more caudal regions, where *golli-lacZ* was expressed in layer 5 as well as the subplate (Figure 6F), the expression in *Tbr1* mutants was difficult to interpret (Figure 6F′). Some *golli-lacZ*-expressing cells were superficial, some were deep, and it was unclear which cells corresponded to layer 5 or subplate cells.

The altered molecular properties of subplate cells suggested that their functions might be disrupted. To study the functions of subplate (and layer 6) cells, we examined the development of major axon tracts entering, leaving and interconnecting the neocortex.

Neocortical Connections Develop Abnormally in *Tbr1* Mutants

Subplate and layer 6 cells have been implicated in the development of cortical afferent and efferent connections, including the corticothalamic (CT), cerebral peduncle (CP), corpus callosum (CC), and thalamocortical (TC) pathways (McConnell et al., 1989, 1994; Ghosh et al., 1990; De Carlos and O'Leary, 1992; Ghosh and Shatz, 1993; Koester and O'Leary, 1994). To study these pathways, we used DiI tracing and immunohistochemistry.

Cortical DiI injections at P1.5 were used to label efferent pathways anterogradely, and afferent pathways retrogradely. In *Tbr1* mutants, major defects in the CT, CC, and TC pathways were revealed (Figures 7A1–7A2′). CT axons grew only as far as the internal capsule, where they were tipped by growth cones (inset, Figure 7A2′). CC fibers mostly terminated in the Probst bundle (pb, Figure 7A1′) without crossing the midline, though some crossed through the hippocampal commissure, or through a rudimentary CC (data not shown). Retrograde labeling of TC neurons (arrowheads, Figure 7A2) was abolished in *Tbr1* mutants (Figure 7A2′), indicating that cortical areas did not receive thalamic innervation. However, DiI injections of frontal and cingulate cortical areas revealed that some CT and TC connections were present in these areas, albeit reduced (data not shown).

The CP appeared to contain fewer cortical efferent axons in mutants than in normals (data not shown). To further investigate this, DiI injections in the ventrolateral midbrain were used to label CP neurons retrogradely (Figures 7B1–7B2′). In controls, CP neurons were found in all neocortical areas, but in mutants, CP neurons were found only in lateral areas (Figures 7B1 and 7B1′), indicating that dorsal and medial areas did not project into the midbrain CP. The morphology and laminar position of the retrogradely labeled *Tbr1*$^{-/-}$ CP neurons were mostly normal (Figure 7B2′).

Cortical efferent pathways were further assessed by immunohistochemistry for TAG-1, a marker of cortical, but not thalamic axons (Wolfer et al., 1994; Fukuda et al., 1997). In controls, TAG-1^{+} CT axons entered the thalamus (Figure 7C), but in *Tbr1* mutants, CT projections did not reach the thalamus; instead, they ended in the internal capsule, without crossing the boundary into diencephalon (dashed line, Figure 7C′). This pattern replicated the DiI results (Figure 7A2′). Also, the callosal defect in *Tbr1* mutants was confirmed by TAG-1 labeling of the Probst bundle, and the CP defect was confirmed as a reduction of TAG-1 labeling in the midbrain CP (data not shown).

Thalamic DiI injections at P1.5 were used to label TC projections anterogradely, and CT projections retrogradely. In mutants, TC innervation of the cortex was absent (Figure 7D′). TC axons grew into the internal capsule, but then turned basolaterally away from the cortex, and into the external capsule and amygdala. Also, the mutant cortex lacked retrogradely labeled CT neurons, which were present in layer 6 and the subplate of controls (Figures 7D and 7D′). (However, thalamic DiI injections did reveal some CT and TC connections with frontal and cingulate cortex, as mentioned above.) These results showed that TC axons became misrouted while in the internal capsule and remained in the basal telencephalon rather than entering the cortex.

TC projections were also studied using serotonin immunohistochemistry, which labels thalamic sensory relay nuclei and axons (Lebrand et al., 1996). In neonatal controls, serotonin^{+} axons innervated sensory cortical areas (Figure 7E). In mutants, serotonin^{+} fibers did not enter the cortex, but instead grew into the internal capsule and basal telencephalon (Figure 7E′), similar to the pattern seen by DiI tracing (Figure 7D′). On the other hand, serotonin-containing fibers from the brainstem, identified by their thick, varicose morphology (Lebrand et al., 1996), were present throughout the cortex of mutants as well as controls (data not shown), indicating that brainstem and thalamic projections utilized different guidance mechanisms.

To investigate whether archicortical connections were affected by Tbr1 deficiency, we used DiI tracing to label projections from the entorhinal cortex to the hippocampus. In P1.5 controls, hippocampal DiI injections retrogradely labeled neurons in entorhinal layers 2 and 3 (Figure 7F). In *Tbr1* mutants, retrogradely labeled entorhinal neurons were grouped in clusters rather than layers (Figure 7F′). DiI injections into the entorhinal cortex labeled the entorhino-hippocampal projections anterogradely. The alveus and fimbria were appropriately targeted in mutants, though the stratum lacunosum-moleculare appeared thickened (Figure 7G′). Thus, in contrast to neocortical connections, a major archicortical connection was intact.

In sum, major efferent (CT, CP, and CC) and afferent (TC) connections of the neocortex developed abnormally in *Tbr1* mutants. Analysis of the reciprocal CT and TC defects over developmental time showed that the lack of connectivity was due to a defect in the guidance and/or growth of TC and CT axons beyond the internal capsule (data not shown).

Discussion

Cortical Expression of Tbr1 Is Restricted to Glutamatergic Neurons

Previous studies of the forebrain distribution of *Tbr1* mRNA demonstrated that its expression is largely restricted to postmitotic cells in pallial structures, including all of the neocortex (Bulfone et al., 1995; Puelles et

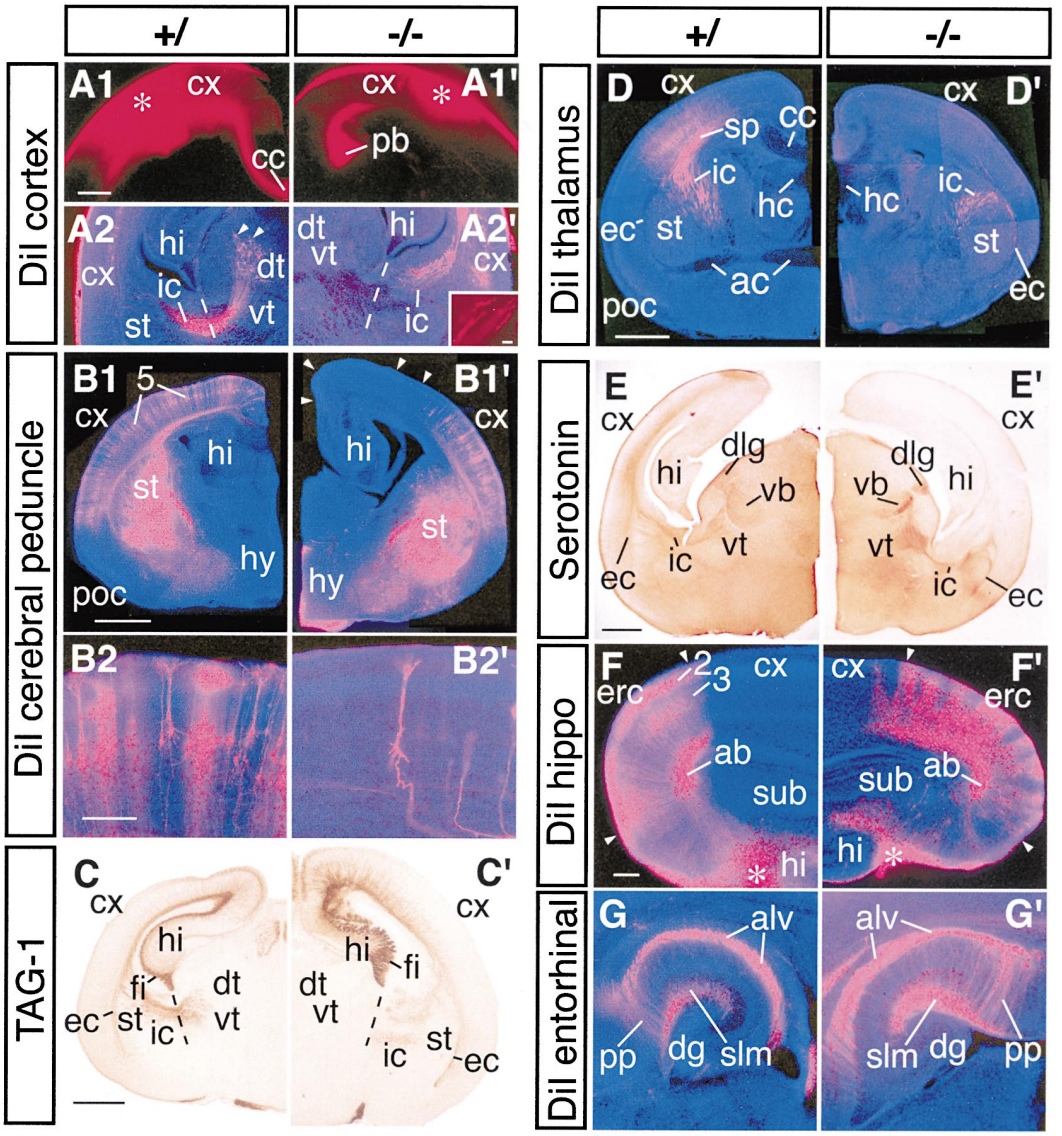

Figure 7. Axon Guidance Errors in *Tbr1*$^{-/-}$ Mutants

Controls (A1, A2, B1, B2, C, D, E, F, and G), mutants (A1′, A2′, B1′, B2′, C′, D′, E′, F′, and G′).

(A1–A2′) DiI labeling (red or pink) from parietal cortex, E18.5. Some sections counterstained with DAPI (blue). Callosal fibers formed a Probst bundle (pb). Retrograde labeling of thalamic neurons (arrowheads) was absent. Efferent fibers grew into the internal capsule (ic) and were tipped by growth cones (inset, [A2′]). Dashed lines, diencephalon/telencephalon boundary. *, DiI injection site; cx, neocortex; cc, corpus callosum; dt, dorsal thalamus; hi, hippocampus; st, striatum; vt, ventral thalamus. Scale bars: 0.5 mm (A1–A2′), 20 μm (inset, [A2′]).

(B1–B2′) DiI labeling from cerebral peduncle, P0.5. In controls all neocortical areas contained retrogradely labeled layer 5 neurons, but in mutants retrograde labeling was absent from dorsal and medial areas (arrowheads). Cellular morphology was not affected. hy, hypothalamus; poc, primary olfactory cortex. Scale bars: 1 mm (B1 and B1′), 200 μm (B2 and B2′).

(C and C′) TAG-1 immunohistochemistry, P0.5. TAG-1^{+} corticothalamic axons ended in the internal capsule. External capsule (ec) labeling was increased, and the fimbria (fi) thickened. Dashed lines, diencephalon/telencephalon boundary. Scale bar: 0.5 mm.

(D and D′) DiI labeling from ventrobasal nucleus of dorsal thalamus, P1.5. Thalamocortical axons entered the subplate (sp) and cortical plate of controls, but not *Tbr1* mutants. Some thalamic fibers entered the external capsule. ac, anterior commissure; hc, hippocampal commissure. Scale bar: 1 mm.

(E and E′) Serotonin immunohistochemistry, P1.5. Sensory relay thalamic axons were misrouted into the external capsule. dlg, dorsal lateral geniculate nucleus; vb, ventrobasal complex. Scale bar: 0.5 mm.

(F and F′) DiI labeling from hippocampus (horizontal sections), P0.5. Retrogradely labeled cells formed clusters in mutants. *, DiI injection site; ab, angular bundle; sub, subiculum. Scale bar: 200 μm (F–G′).

(G and G′) DiI labeling from entorhinal cortex (horizontal sections), P0.5. Routes to hippocampus through the alvear (alv) and perforant (pp) pathways were intact, but the stratum lacunosum-moleculare (slm) was thickened. dg, dentate gyrus.

al., 2000). Here, we have shown that Tbr1 protein is expressed in the same distribution and, moreover, is restricted to glutamatergic neurons (Figure 1J). Its exclusion from GABAergic cortical neurons (Figure 1I) is consistent with recent evidence that these cells are derived from the basal telencephalon and reach the pallium

through a tangential migration (reviewed by Anderson et al., 1999). The few Tbr1$^+$ cells in the basal telencephalon also appear to express glutamate and not GABA. Thus, we hypothesize that Tbr1 expression marks glutamatergic neurons, regardless of location. We do not know whether Tbr1$^+$ cells in the basal telencephalon arise from local subpallial progenitors or from distant pallial progenitors.

In the cortex, we propose that Tbr1$^+$ cells arise from the pallial neuroepithelium. At E12.5, most or all preplate neurons are Tbr1$^+$ (Figure 1D). Since cells in the preplate at E12.5–E13.5 differentiate as Cajal-Retzius and subplate neurons, which are likewise Tbr1$^+$ (Figures 1F and 1G), it is probable that these neurons are derived mostly or entirely from cortical neuroepithelium. Conversely, it seems unlikely that many Cajal-Retzius neurons are derived from subcortical sources, though a few could be (Lavdas et al., 1999). The idea that many preplate cells originate from a source at the base of the olfactory bulb—the retrobulbar region (Meyer et al., 1998, 1999)—is not ruled out by our results, as this region is Tbr1$^+$.

Tbr1 Function Is Essential for the Differentiation of Preplate and Layer 6 (Early-Born) Cortical Neurons

The laminar pattern of Tbr1 expression showed that it was highest in early-born cortical cells (Figures 1A–1E). *Tbr1*-deficient animals displayed defects in most cortical areas; only medial structures (cingulate cortex and hippocampus) were relatively spared (Figure 2D′ and data not shown). Olfactory structures were severely affected, as most projection neurons failed to differentiate in the olfactory cortex (Figures 2C′ and 2D′ and data not shown) and olfactory bulb (Bulfone et al., 1998). In the neocortex, lamination and connections were abnormal, and changes in molecular expression pointed to defects in the differentiation of early-born neurons, i.e., preplate derivatives (Cajal-Retzius and subplate cells) and layer 6 neurons (Figures 4 and 5). Despite their impaired differentiation, early-born neurons apparently survived at least to P0.5, but occupied ectopic locations, as indicated by apoptosis studies (data not shown) and BrdU birthdating (Figure 3).

Cajal-Retzius cells expressed reduced levels of *Reelin* and calretinin (Figures 4G–4J′). The loss of *Reelin* expression may have been directly due to the *Tbr1* deficiency, as Tbr1 activates transcription of an artificial enhancer element derived from the *Reelin* gene (Hsueh et al., 2000). The only cortical region where Reelin expression was not reduced in *Tbr1* mutants was the medial cortex, including the hippocampus (Figure 4G′). Presumably, Reelin expression in the medial cortex can be activated by other transcription factors in the absence of Tbr1. Excellent candidates are *Tbr2* (*eomesodermin*), another T box gene expressed in the developing cortex (Bulfone et al., 1999), and *Emx1* and *Emx2* (Cecchi and Boncinelli, 2000). *Emx1* and *Tbr2* are expressed in the preplate (Briata et al., 1996; Bulfone et al., 1999), and Emx2 has been detected in Cajal-Retzius cells (Cecchi and Boncinelli, 2000). Furthermore, *Emx2* mutants show reduced expression of Reelin and calretinin, and a migrational defect (Mallamaci et al., 2000). In *Tbr1* mutants, the expression of *Tbr2* and *Emx1* was maintained (Figure 5D′ and data not shown).

Impaired differentiation of subplate and layer 6 neurons was indicated by molecular and axonal defects. Molecularly, the expression of subplate markers *golli-lacZ* (Figure 6) and calretinin (Figure 4I′) was severely reduced, and the expression of a layer 6 marker (*Wnt7b*) was decreased (Figures 5B′ and 5E′). Axonal guidance errors were observed in major efferent and afferent pathways that depend on the subplate and layer 6 for their development (Figure 7).

Unlike the defects found in early-born cortical neurons, later-born neurons appeared to differentiate more normally, based on molecular and axonal criteria. Neonatal *Tbr1* mutants expressed markers of postmitotic cortical neurons including glutamate (Figures 2H′ and 2I′), *Tbr1* (Figure 5A′), mDab1 (Figures 4K′ and 5F′), and *doublecortin* (data not shown). Furthermore, the expression of markers specific for layers 2–5 was maintained (data not shown). Axonal projections to the cerebral peduncle, characteristic of layer 5 neurons, were reduced in dorsomedial areas (Figure 7B1′), but development of this pathway depends on the integrity of subplate as well as layer 5 neurons (McConnell et al., 1994). Thus, although layers 2–5 may not develop normally in *Tbr1* mutants, the evidence suggests that Tbr1 primarily regulates the differentiation of Cajal-Retzius, subplate, and layer 6 neurons.

Reelin Downregulation and the Cortical Malformation in *Tbr1* Mutants

The reduction of *Reelin* expression in *Tbr1* mutants produced a migration defect that, in the rostral neocortex, was a phenocopy of that seen in *reeler* mice which lack a functional *Reelin* gene (reviewed in Rice and Curran, 1999). The *reeler*-like features included defective splitting of the preplate (Figures 4C–4D′); superficial positioning of early-born neurons (Figures 3A1–3A2′); increased dispersion and approximate inversion of cell positions in the cortical plate (Figures 3B1–3C2′); and upregulation of mDab1, an intracellular adaptor protein that mediates the response of cortical plate cells to Reelin (Figure 4K′). Expression levels of the Reelin receptor genes *VLDLR* and *apoER2*, and of other molecules implicated in cortical cell migrations (*p35*, *p39*, *cdk5*, and *doublecortin*), appeared normal in *Tbr1* mutants (data not shown). Radial glia appeared morphologically normal by RC2 immunohistochemistry in *Tbr1* mutants (data not shown). Thus, downregulation of *Reelin* appeared to fully account for the *reeler*-like phenotype in the rostral cortex of *Tbr1* mutants.

On the other hand, the organization of caudal cortex in *Tbr1* mutants was significantly different from that in *reeler* mutants. *Tbr1* mutants formed neuronal clusters, which have not been reported in *reeler* mice. The clusters tended to consist of cells with similar properties, e.g., birth date (Figures 3B2′ and 3C2′), neurotransmitter phenotype (Figures 2I′ and 2K′), gene expression (Figures 5D′, 5E′, and 5F′), and axonal connections (Figure 7F′). Since these properties are normally associated with cortical layers, the clusters may be conceptualized as malformed layers. It is still unclear why the clusters form in *Tbr1* and not *reeler* mutants, and why they are restricted to the caudal cortex. One explanation could be the patchy expression of *Reelin* in the cortex of *Tbr1* mutants, which was most apparent in caudal areas (Figure 4G′ and data not shown).

It is intriguing that the neuronal clusters in the *Tbr1*$^{-/-}$ caudal neocortex resemble clusters that normally form in the entorhinal cortex (Blackstad, 1956). Furthermore, in the entorhinal cortex of *Tbr1* mutants, neuronal clustering was exaggerated (Figures 2E′ and 7F′). Perhaps, Tbr1 normally suppresses the development of entorhinal-like features in the caudal cortex.

Impaired Subplate Differentiation and Defective Thalamocortical Axon Guidance

Previous studies have shown that subplate cells are necessary for the development of many efferent and afferent cortical connections. The majority of early or "pioneer" corticofugal axons originate from cells in the subplate (McConnell et al., 1989; De Carlos and O'Leary, 1992), though some come from cells in the marginal zone as well (Meyer et al., 1998; Molnár et al., 1998a). Excitotoxic ablation of subplate neurons reduces the formation of cortical descending projections (McConnell et al., 1994) and prevents thalamocortical innervation of the lesioned area (Ghosh et al., 1990; Ghosh and Shatz, 1993). Despite this knowledge, the mechanisms of how subplate cells promote afferent and efferent innervation are unknown.

Using calretinin and *golli-lacZ* as subplate markers (Fonseca et al., 1995; Landry et al., 1998), we found that subplate differentiation was deficient throughout the neocortex of *Tbr1* mutants at embryonic ages (Figures 4I′ and 6). Consistent with the defect of subplate differentiation, thalamocortical and corticothalamic projections were lacking in most cortical areas (Figures 7A1–7A2′ and 7C–7E′). These changes were distinct from the phenotype of *reeler* mice, which retain thalamocortical and corticothalamic projections (Molnár et al., 1998b). *Reeler* mice also form barrel–like structures in the somatosensory cortex (Polleux et al., 1998), which were absent from the *Tbr1* mutant cortex (data not shown). On the other hand, the thalamocortical phenotype in *Tbr1* mutants did resemble that of COUP-TF1 knockout mice, in which impaired subplate development was associated with failure of thalamocortical axons to exit the internal capsule (Zhou et al., 1999).

How does the defect of subplate differentiation in *Tbr1* mutants explain the misrouting of thalamocortical axons? Intriguingly, thalamocortical misrouting began in the striatal portion of the internal capsule, before thalamic axons entered the cortex (Figures 7D′ and 7E′). The internal capsule is also the site where subplate and thalamic axons first meet (De Carlos and O'Leary, 1992; Molnár and Blakemore, 1995). It has been proposed that the subplate axons act as guide wires and lead the thalamic axons from the internal capsule to the cortex (Molnár et al., 1998a). A disrupted interaction or "handshake" (Molnár and Blakemore, 1995) between subplate and thalamic axons could explain the thalamocortical phenotype in the internal capsule. Alternatively, changes in the cortical production of diffusible factors could alter thalamocortical axon guidance. However, in vitro evidence suggests that cortical diffusible factors have only trophic effects, and no tropic effects on thalamic axons (Molnár and Blakemore, 1999).

Impaired Callosal, Corticothalamic, and Cerebral Peduncle Projections

The callosal pathway is pioneered by early-born neurons in the medial cortical plate, and not subplate neurons (Koester and O'Leary, 1994). In *Tbr1* mutants, callosal fibers did not cross the midline (Figure 7A1′), and early-born neurons in the medial cortical plate developed abnormally as evidenced by reduced expression of *Wnt7b* (Figures 5B′ and 5E′). Thus, molecular defects in the early–born cells may have caused errors of callosal pioneer axon guidance. Alternatively, cell-autonomous defects in later–born callosal projection neurons, or defects of putative glial structures implicated in callosal axon guidance (Silver et al., 1993), could have played a role as well.

The causes of the decreased corticothalamic and cerebral peduncle projections in *Tbr1* mutants (Figures 7A–7C′) are likewise unresolved and may have included cell-autonomous defects in layer 5 and 6 projection neurons and/or errors of subplate pioneer axon guidance (McConnell et al., 1994). In *Tbr1* mutants, we found clear evidence of gene expression defects in cells of the subplate (Figures 4I′ and 6) and layer 6 (Figures 5B′ and 5E′), but markers of layer 5 (e.g., *Otx1* and *ER81*) continued to be expressed (data not shown). The nearly normal laminar position of cortical cells projecting into the cerebral peduncle (Figure 7B2′), in view of the marked migrational defects (Figure 3), suggests that axonal projection targets may not be specified by cellular birth date alone, but also by other factors such as the position of the cells.

Conclusion

The most prominent phenotype of *Tbr1* mutants is the abnormal differentiation of early-born cortical neurons. In the neocortex, these neurons regulate later steps of development. While progress has been made in identifying how Cajal-Retzius cells regulate radial migration (Rice and Curran, 1999; Dulabon et al., 2000; Gleeson and Walsh, 2000), much less is known about how subplate and layer 6 neurons regulate the growth of thalamocortical, corticothalamic, and corpus callosum axons. Thus, future studies of the *Tbr1* mutants should be aimed at identifying the molecules produced by subplate and layer 6 neurons that regulate the patterned growth of afferent and efferent axons.

Experimental Procedures

Mice

Mice were cared for according to animal protocols approved by the Committee on Animal Research at UCSF. Generation of *Tbr1* mutant mice (Bulfone et al., 1998) and transgenic *golli-lacZ* mice (line 1.3E) (Landry et al., 1998) were described previously. Genotypes were determined by PCR (Bulfone et al., 1998).

Immunohistochemistry and Immunoblotting

Immunohistochemistry was performed as described previously (Anderson et al., 1997), with the following additions. For fluorescence microscopy, fluorescent secondary antibodies were used (Alexa series, Molecular Probes). For confocal microscopy, paraformaldehyde-fixed brains were sectioned at 50 μm on a VT1000S vibrating microtome (Leica), processed free-floating for immunofluorescence, and mounted. Triton X-100 was omitted from anti-O4 reactions. Antibodies were used as follows: monoclonal anti-βIII-tubulin (clone TuJ1; Babco), 1:500; monoclonal anti-BrdU (clone BU1/75; Harlan),

1:15; polyclonal anti-calretinin (Chemicon), 1:1000; monoclonal anti-CaMKII (clone 6G9; Boehringer), 1:100; monoclonal anti-chondroitin sulfate proteoglycan (clone CS56; Sigma), 1:200; monoclonal anti-GABA (clone GB-69; Sigma), 1:2000; polyclonal anti-GABA (Sigma), 1:10,000; monoclonal anti-GFAP (clone 6F2; Dako), 1:500; monoclonal anti-glutamate (clone GLU-4; Sigma), 1:10,000; polyclonal anti-glutamate (Sigma), 1:10,000; monoclonal anti-MAP2 (clone AP20; Boehringer), 1:500; polyclonal anti-mDab1 (gift of B. W. Howell), 1:500; polyclonal anti-neurocan (gift of A. Oohira), 1:1000; monoclonal anti-O4 (clone 81; Boehringer), 1:25; monoclonal anti-Reelin (clone G10; Lambert de Rouvroit et al., 1999), 1:1000; polyclonal anti-serotonin (Incstar), 1:5000; polyclonal anti-TAG-1 (Fukuda et al., 1997), 1:1000; polyclonal anti-Tbr1 (Tbr-C; Hsueh et al., 2000), 1:100.

Reelin immunoblotting was performed using the G10 monoclonal antibody (Lambert de Rouvroit et al., 1999).

In Situ Hybridization

In situ hybridization was performed using ^{35}S- or digoxigenin-labeled riboprobes, as described (Bulfone et al., 1998). The following riboprobes were synthesized from cDNA templates: *ApoER2* (Trommsdorff et al., 1999), *cdk5* (gift of L.-H. Tsai), *doublecortin* (gift of F. Francis), *Emx1* (gift of A. Simeone), *Id2* (Bulfone et al., 1998), *mDab1* (gift of T. Curran), *Reelin* (gift of T. Curran), *p35* (gift of L.-H. Tsai), *p39* (gift of L.-H. Tsai), *Tbr1* (Bulfone et al., 1995), *VLDLR* (Trommsdorff et al., 1999), and *Wnt7b* (gift of A. McMahon).

BrdU Labeling and Analysis

Single injections of BrdU (40 mg/kg i.p.) were done as described previously (Anderson et al., 1997). Pregnant dams were injected at E10.5 (n = 3), E11.5 (n = 2), E13.5 (n = 5), or E16.5 (n = 2). To determine the distribution of cells born on different birth dates in normal and *Tbr1* mutant cortex, sections through the rostral cortex (level of anterior commissure) and caudal neocortex (occipital lobe) were divided into ten horizontal bins from superficial to deep. Two sections from each of two littermates were used for counting, and the percentage of cells in each bin was calculated.

Apoptosis Detection

The In Situ Cell Death Detection Kit, POD (Boehringer) was used, per the manufacturer's instructions.

β-Galactosidase Histochemistry to Detect *golli-lacZ* Expression

Brains were processed for β-galactosidase histochemistry in whole mounts as described (Landry et al., 1998), then either cryosectioned at 10 μm or sectioned without freezing on a Leica VT1000S vibrating microtome at 40–50 μm.

DiI Tract Tracing

DiI experiments were done using standard methods (Bulfone et al., 1998). Single crystals of DiI were placed in the cerebral cortex or dorsal thalamus; three to five crystals were injected in the cerebral peduncle, hippocampus, or entorhinal cortex. 3–8 weeks were allowed for tracer diffusion.

Acknowledgments

We thank T. Curran for *mDab1* and *Reelin* cDNAs, F. Francis for *doublecortin* cDNA, Brian Howell for anti-mDab1, M. Israel for Id2 cDNA, T. Jessell for ER81 cDNA, H. Kawano for anti-TAG-1, A. McMahon for *Wnt7b* cDNA, A. Oohira for anti-neurocan, A. Simeone for *Emx1* and *Otx1* cDNAs, M. Trommsdorff for *ApoER2* and *VLDLR* cDNAs, and L.-H. Tsai for *cdk5*, *p35*, and *p39* cDNAs. This research was supported by the following grants: NIH grant NS01973 to R. F. H., as well as funding to J. L. R. R. from Nina Ireland, NARSAD, NINDS R01 #NS34661, and K02 #MH01046.

Received September 21, 2000; revised November 30, 2000.

References

Alcántara, S., Ruiz, M., D'Arcangelo, G., Ezan, F., de Lecea, L., Curran, T., Sotelo, C., and Soriano, E. (1998). Regional and cellular patterns of *reelin* mRNA expression in the forebrain of the developing and adult mouse. J. Neurosci. *18*, 7779–7799.

Allendoerfer, K.L., and Shatz, C.J. (1994). The subplate, a transient neocortical structure: its role in the development of connections between thalamus and cortex. Annu. Rev. Neurosci. *17*, 185–218.

Anderson, S.A., Qiu, M., Bulfone, A., Eisenstat, D.D., Meneses, J., Pedersen, R., and Rubenstein, J.L.R. (1997). Mutations of the homeobox genes *Dlx-1* and *Dlx-2* disrupt the striatal subventricular zone and differentiation of late born striatal neurons. Neuron *19*, 27–37.

Anderson, S., Mione, M., Yun, K., and Rubenstein, J.L.R. (1999). Differential origins of neocortical projection and local circuit neurons: role of *Dlx* genes in neocortical interneuronogenesis. Cereb. Cortex *9*, 646–654.

Angevine, J.B., and Sidman, R.L. (1961). Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. Nature *192*, 766–768.

Bicknese, A.R., Sheppard, A.M., O'Leary, D.D.M., and Pearlman, A.L. (1994). Thalamocortical axons extend along a chondroitin sulfate proteoglycan-enriched pathway coincident with the neocortical subplate and distinct from the efferent path. J. Neurosci. *14*, 3500–3510.

Blackstad, T.W. (1956). Commissural connections of the hippocampal region in the rat, with special reference to their mode of termination. J. Comp. Neurol. *105*, 417–537.

Briata, P., Di Blas, E., Gulisano, M., Mallamaci, A., Iannone, R., Boncinelli, E., and Corte, G. (1996). EMX1 homeoprotein is expressed in cell nuclei of the developing cerebral cortex and in the axons of the olfactory sensory neurons. Mech. Dev. *57*, 169–180.

Bulfone, A., Smiga, S.M., Shimamura, K., Peterson, A., Puelles, L., and Rubenstein, J.L.R. (1995). *T-brain-1*: a homolog of *Brachyury* whose expression defines molecularly distinct domains within the cerebral cortex. Neuron *15*, 63–78.

Bulfone, A., Wang, F., Hevner, R., Anderson, S., Cutforth, T., Chen, S., Meneses, J., Pedersen, R., Axel, R., and Rubenstein, J.L.R. (1998). An olfactory sensory map develops in the absence of normal projection neurons or GABAergic interneurons. Neuron *21*, 1–20.

Bulfone, A., Martinez, S., Marigo, V., Campanella, M., Basile, A., Quaderi, N., Gattuso, C., Rubenstein, J.L.R., and Ballabio, A. (1999). Expression pattern of the *Tbr2* (*eomesodermin*) gene during mouse and chick brain development. Mech. Dev. *84*, 133–138.

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

Cecchi, C., and Boncinelli, E. (2000). *Emx* homeogenes and mouse brain development. Trends Neurosci. *23*, 347–352.

De Carlos, J.A., and O'Leary, D.D.M. (1992). Growth and targeting of subplate axons and establishment of major cortical pathways. J. Neurosci. *12*, 1194–1211.

del Río, J.A., Martínez, 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 *1*, 13–21.

Dulabon, L., Olson, E.C., Taglienti, M.G., Eisenhuth, S., McGrath, B., Walsh, C.A., Kreidberg, J.A., and Anton, E.S. (2000). Reelin binds α3β1 integrin and inhibits neuronal migration. Neuron *27*, 33–44.

Fonseca, M., Del Río, J.A., Martínez, A., Gómez, S., and Soriano, E. (1995). Development of calretinin immunoreactivity in the neocortex of the rat. J. Comp. Neurol. *361*, 177–192.

Fukuda, T., Kawano, H., Ohyama, K., Li, H.-P., Takeda, Y., Oohira, A., and Kawamura, K. (1997). Immunohistochemical localization of neurocan and L1 in the formation of thalamocortical pathway of developing rats. J. Comp. Neurol. *382*, 141–152.

Ghosh, A., and Shatz, C.J. (1993). A role for subplate neurons in the

patterning of connections from thalamus to neocortex. Development *117*, 1031–1047.

Ghosh, A., Antonini, A., McConnell, S.K., and Shatz, C.J. (1990). Requirement for subplate neurons in the formation of thalamocortical connections. Nature *347*, 179–181.

Gleeson, J.G., and Walsh, C.A. (2000). Neuronal migration disorders: from genetic diseases to developmental mechanisms. Trends Neurosci. *23*, 352–359.

Hsueh, Y.-P., Wang, T.-F., Yang, F.-C., and Sheng, M. (2000). Nuclear translocation and transcription regulation by the membrane-associated guanylate kinase CASK/LIN-2. Nature *404*, 298–302.

Koester, S.E., and O'Leary, D.D.M. (1994). Axons of early generated neurons in cingulate cortex pioneer the corpus callosum. J. Neurosci. *14*, 6608–6620.

Lambert de Rouvroit, C., de Bergeyck, V., Cortvrindt, C., Bar, I., Eeckhout, Y., and Goffinet, A.M. (1999). Reelin, the extracellular matrix protein deficient in reeler mutant mice, is processed by a metalloproteinase. Exp. Neurol. *156*, 214–217.

Landry, C.F., Pribyl, T.M., Ellison, J.A., Givogri, M.I., Kampf, K., Campagnoni, C.W., and Campagnoni, A.T. (1998). Embryonic expression of the myelin basic protein gene: identification of a promoter region that targets transgene expression to pioneer neurons. J. Neurosci. *18*, 7315–7327.

Lavdas, A.A., Grigoriou, M., Pachnis, V., and Parnavelas, J.G. (1999). The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J. Neurosci. *19*, 7881–7888.

Lebrand, C., Cases, O., Adelbrecht, C., Doye, A., Alvarez, C., El Mestikawy, S., Seif, I., and Gaspar, P. (1996). Transient uptake and storage of serotonin in developing thalamic neurons. Neuron *17*, 823–835.

Liu, X.-B., and Jones, E.G. (1996). Localization of alpha type II calcium calmodulin-dependent protein kinase at glutamatergic but not γ-aminobutyric acid (GABAergic) synapes in thalamus and cerebral cortex. Proc. Natl. Acad. Sci. USA *93*, 7332–7336.

McConnell, S.K., Ghosh, A., and Shatz, C.J. (1989). Subplate neurons pioneer the first axon pathway from the cerebral cortex. Science *245*, 978–982.

McConnell, S.K., Ghosh, A., and Shatz, C.J. (1994). Subplate pioneers and the formation of descending connections from the cerebral cortex. J. Neurosci. *14*, 1892–1907.

Mallamaci, A., Mercurio, S., Muzio, L., Cecchi, C., Pardini, C.L., Gruss, P., and Boncinelli, E. (2000). The lack of *Emx2* causes impairment of *Reelin* signaling and defects of neuronal migration in the developing cerebral cortex. J. Neurosci. *20*, 1109–1118.

Meyer, G., Soria, J.M., Martínez-Galán, J.R., Martín-Clemente, B., and Fairén, A. (1998). Different origins and developmental histories of transient neurons in the marginal zone of the fetal and neonatal rat cortex. J. Comp. Neurol. *397*, 493–518.

Meyer, G., Goffinet, A.M., and Fairén, A. (1999). What is a Cajal-Retzius cell? A reassessment of a classical cell type based on recent observations in the developing neocortex. Cereb. Cortex *9*, 765–775.

Miller, B., Sheppard, A.M., Bicknese, A.R., and Pearlman, A.L. (1995). Chondroitin sulfate proteoglycans in the developing cerebral cortex: the distribution of neurocan distinguishes forming afferent and efferent axonal pathways. J. Comp. Neurol. *355*, 615–628.

Molnár, Z., and Blakemore, C. (1995). How do thalamic axons find their way to the cortex? Trends Neurosci. *18*, 389–397.

Molnár, Z., and Blakemore, C. (1999). Development of signals influencing the growth and termination of thalamocortical axons in organotypic culture. Exp. Neurol. *156*, 363–393.

Molnár, Z., Adams, R., and Blakemore, C. (1998a). Mechanisms underlying the early establishment of thalamocortical connections in the rat. J. Neurosci. *18*, 5723–5745.

Molnár, Z., Adams, R., Goffinet, A.M., and Blakemore, C. (1998b). The role of the first postmitotic cortical cells in the development of thalamocortical innervation in the *reeler* mouse. J. Neurosci. *18*, 5746–5765.

Polleux, F., Dehay, C., and Kennedy, H. (1998). Neurogenesis and commitment of corticospinal neurons in *reeler*. J. Neurosci. *18*, 9910–9923.

Probst, M. (1901). Über den Bau des vollständig balkenlosen Großhirns, sowie über Mikrogirie und Heterotopie der grauen Substanz. Arch. Psychiatr. Nervenkr. *34*, 709–786.

Puelles, L., Kuwana, E., Puelles, E., Bulfone, A., Shimamura, K., Keleher, J., Smiga, S., and Rubenstein, J.L.R. (2000). Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. J. Comp. Neurol. *424*, 409–438.

Rice, D.S., and Curran, T. (1999). Mutant mice with scrambled brains: understanding the signaling pathways that control cell positioning in the CNS. Genes Dev. *13*, 2758–2773.

Sheppard, A.M., and Pearlman, A.L. (1997). Abnormal reorganization of preplate neurons and their associated extracellular matrix: an early manifestation of altered neocortical development in the reeler mutant mouse. J. Comp. Neurol. *378*, 173–179.

Silver, J., Edwards, M.A., and Levitt, P. (1993). Immunocytochemical demonstration of early appearing astroglial structures that form boundaries and pathways along axon tracts in the fetal brain. J. Comp. Neurol. *328*, 415–436.

Smart, I.H.M., and Smart, M. (1977). The location of nuclei of different labelling intensities in autoradiographs of the anterior forebrain of postnatal mice injected with [^{3}H]thymidine on the eleventh and twelfth days post-conception. J. Anat. *123*, 515–525.

Supèr, H., del Río, J.A., Martínez, A., Pérez-Sust, P., and Soriano, E. (2000). Disruption of neuronal migration and radial glia in the developing cerebral cortex following ablation of Cajal-Retzius cells. Cereb. Cortex *10*, 602–613.

Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R.E., Richardson, J.A., and Herz, J. (1999). Reeler/disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell *97*, 689–701.

Weimann, J.M., Zhang, Y.A., Levin, M.E., Devine, W.P., Brûlet, P., and McConnell, S.K. (1999). Cortical neurons require Otx1 for the refinement of exuberant axonal projections to subcortical targets. Neuron *24*, 819–831.

Wolfer, D.P., Henehan-Beatty, A., Stoeckli, E.T., Sonderegger, P., and Lipp, H.-P. (1994). Distribution of TAG-1/axonin-1 in fibre tracts and migratory streams of the developing mouse nervous system. J. Comp. Neurol. *345*, 1–32.

Wood, J.G., Martin, S., and Price, D.J. (1992). Evidence that the earliest generated cells of the murine cerebral cortex form a transient population in the subplate and marginal zone. Dev. Brain Res. *66*, 137–140.

Zhou, C., Qiu, Y., Pereira, F.A., Crair, M.C., Tsai, S.Y., and Tsai, M.-J. (1999). The nuclear orphan receptor COUP-TFI is required for differentiation of subplate neurons and guidance of thalamocortical axons. Neuron *24*, 847–859.