

β 1-Class Integrins Regulate the Development of Laminae and Folia in the Cerebral and Cerebellar Cortex

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Summary

Mice that lack all β 1-class integrins in neurons and glia die prematurely after birth with severe brain malformations. Cortical hemispheres and cerebellar folia fuse, and cortical laminae are perturbed. These defects result from disorganization of the cortical marginal zone, where β 1-class integrins regulate glial endfeet anchorage, meningeal basement membrane remodeling, and formation of the Cajal-Retzius cell layer. Surprisingly, β 1-class integrins are not essential for neuron-glia interactions and neuronal migration during corticogenesis. The phenotype of the β 1-deficient mice resembles pathological changes observed in human cortical dysplasias, suggesting that defective integrin-mediated signal transduction contributes to the development of some of these diseases.

Introduction

Cortical structures in the mature mammalian central nervous system exhibit an ordered laminar organization. Defined cell layers form by a series of well-orchestrated cell migration events. In the cerebral cortex, large numbers of neurons are generated in the ventricular neuroepithelium and migrate radially along glial fibers until they reach the cortical marginal zone, where they coalesce to form distinct cell layers. The birthdate of a neuron defines its position within the cortex. Earlier born neu-

rons migrate first and occupy deeper layers than later born neurons that occupy more exterior positions. In the cerebellar cortex, several cell types such as Purkinje cells and interneurons migrate radially from the primary germinal zone toward the cerebellar surface. Cerebellar granule cell precursors first move tangentially from the rhombic lip across the surface of the developing cerebellum. They form a secondary germinal zone, the external granule cell layer (EGL), which lies between the meningeal and Purkinje cell layers (PCL). Granule cells that are generated in the EGL migrate subsequently radially along Bergman glial fibers through the PCL to form the internal granule cell layer (IGL) (reviewed in Herrup and Kuemerle, 1997; Hatten, 1999).

The molecular mechanisms that govern the formation of cortical structures are not well understood. The development of cortical cell layers requires that neurons exit the cell cycle at specific times, adhere to and move along glial fibers, detach from these fibers, and assemble into defined layers. Cell surface receptors that regulate adhesive interactions are likely to play important roles in these events. Cortical neurons express cadherins and cadherin-related neuronal receptors (CNRs), which may regulate cell-cell and cell-extracellular matrix (ECM) interactions (Kohmura et al., 1998; Senzaki et al., 1999; reviewed in Redies, 2000). Cortical neurons and glial cells also express several members of the β 1-class integrin family (Pinkstaff et al., 1999), a family of at least 12 different receptors formed by heterodimerization of a common β 1 subunit with different α subunits. β 1-class integrins interact with ECM molecules and cell surface counterreceptors (reviewed in Hemler, 1999). They have been implicated in the regulation of corticogenesis by several lines of evidence. First, the migration of tectal neurons is perturbed in chickens upon infection with retroviruses expressing antisense mRNAs of the integrin β 1 or α 6 subunits (Galileo et al., 1992; Zhang and Galileo, 1998). Second, neurons invade the cortical marginal zone in mice that carry a targeted mutation inactivating the integrin α 6 subunit (Georges-Labouesse et al., 1998). Third, the α 3 β 1 integrin has been reported to regulate the rate of neuronal migration along glial fibers (Anton et al., 1999) and to bind to reelin (Dulabon et al., 2000), an ECM component that is essential for the formation of cortical cell layers (reviewed in Cooper and Howell, 1999; Rice and Curran, 1999). Fourth, integrins containing the α v subunit are expressed in glial fibers and may regulate migratory events (Hirsch et al., 1994; Anton et al., 1999).

The expression pattern of several integrin ligands in the nervous system further implicates integrins in the regulation of cortical development. For example, laminin and collagen IV are components of the basement membrane underlying the meningeal cells and could serve as guidance cues for granule cell precursors during their tangential movement from the rhombic lip (Rakic and Sidman, 1970; Hausmann and Sievers, 1985; Altman and Bayer, 1997). It has also been reported that laminin, thrombospondin, and tenascin show expression patterns in the cerebellum consistent with a role in radial

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migration and that antibodies against these molecules perturb granule cell migration in organotypic slice cultures (Liesi, 1985; O'Shea et al., 1990; Bartsch et al., 1992; Husmann et al., 1992). In vitro, purified granule cells attach to and migrate on ECM components such as laminin in an integrin-dependent manner (Nagata and Nakatsuji, 1990; Husmann et al., 1992; Fishman and Hatten, 1993).

We provide here genetic evidence that $\beta 1$ -class integrins have an unexpected function in cortical development. We have used Cre/Lox-mediated recombination to generate mice with a $\beta 1$ null allele in the precursors of neurons and glia, thereby inactivating all $\beta 1$ -class integrin receptors in the nervous system. The formation of cortical laminae and cerebellar folia is severely perturbed in the mutants. Surprisingly, $\beta 1$ -class integrins are neither essential for neuron-glia interactions nor for neuronal migration during corticogenesis. The defects are rather in large part a consequence of defects in the cortical marginal zone, where integrins are required for the anchorage of glial endfeet, for basement membrane assembly and/or remodeling, and for the formation of the Cajal-Retzius (CR) cell layer. We conclude that $\beta 1$ -class integrins regulate the formation of cortical layers by affecting cells and signals in the cortical marginal zone that are essential for the assembly of neurons into defined layers.

Results

Generation of Mice that Carry a $\beta 1$ -flox Allele

To analyze the function of $\beta 1$ -class integrins in nervous system development, we have generated a $\beta 1$ allele suitable for Cre/Lox-mediated gene inactivation (Figure 1). A gene-targeting vector was constructed where two loxP sites flank the first coding exon of the integrin $\beta 1$ subunit gene. A selection cassette containing a *neomycin* gene, a *thymidine kinase* gene, and a third loxP site followed the second loxP site (Figure 1A). ES cells were electroporated with the linearized vector, and neomycin resistant targeted ES cell clones were identified by Southern blot (Figure 1B). To remove the selection cassette, cells were retransfected with a Cre-expressing plasmid, and clones that lost the selection cassette but retained two loxP sites flanking the first coding exon were identified (Figure 1B). We will refer to this allele as the $\beta 1$ -flox allele. Germline-transmitting chimeric mice were generated (Figure 1C). Mice that carried either the $\beta 1$ -flox allele in the homozygous configuration or one $\beta 1$ -flox and one $\beta 1$ null allele (Stephens et al., 1995) showed no overt abnormal phenotype (data not shown). Thus, the presence of two loxP sites does not affect integrin $\beta 1$ function in vivo.

Characterization of the Nestin-Cre Mice

To inactivate $\beta 1$ -class integrins in the nervous system, we used a transgenic mouse line that expresses Cre under the control of the neuron-specific enhancer of the nestin promoter (Tronche et al., 1999). First, we characterized the nestin-Cre mice by crossing them with a reporter mouse line that carries a *Rosa26lacZ-loxP* gene. In this mouse line, lacZ expression is induced by Cre-mediated recombination (Mao et al., 1999). In whole-mount staining, lacZ expression was detected

throughout the embryonic neural tube as early as embryonic day (E) 10.5 (Figure 1D). Staining of sagittal sections of E12.5 animals for lacZ revealed widespread Cre activity within the developing cortical wall, with less intense but detectable expression in the ventricular neuroepithelium (Figure 1E). LacZ staining was evident in all cortical layers at postnatal ages (Figure 1E). The blood vessels and meninges were lacZ negative (Figures 1E and 1F and data not shown). Similar widespread Cre-mediated recombination was evident in cerebellar sections from E15.5 and P7 animals (data not shown), consistent with the lacZ staining patterns seen in whole mounts (Figure 1D). We also stained dissociated cerebellar cultures and observed lacZ expression in essentially all neurons and glial cells (Figure 1G). We conclude that the *nestin-Cre* allele induces efficient and widespread recombination in precursors of neurons and glia starting around embryonic day E10.5, consistent with the known properties of the neuron-specific enhancer of the nestin promoter (Zimmerman et al., 1994).

Mice that Lack $\beta 1$ -Class Integrins in the Nervous System Show Severe Defects in Brain Development

We inactivated the integrin $\beta 1$ subunit gene by crossing the $\beta 1$ -flox mice with the *nestin-Cre* mice. The *nestin-Cre* allele was maintained as a heterozygous locus on a background heterozygous for a null allele of the integrin $\beta 1$ subunit gene. These mice were bred with mice homozygous for the $\beta 1$ -flox allele. The mutant offspring, carrying a $\beta 1$ -flox allele, a $\beta 1$ null allele, and the *nestin-Cre* transgene were born with the expected Mendelian frequency. A small fraction of the mutants died shortly after birth, but the vast majority survived and died prematurely at varying ages during adulthood. The survivors grew more slowly than wild-type littermates, were ataxic, and had partially closed eyes (data not shown). The dissected mutant brains showed several irregularities (Figure 2A). While the olfactory bulbs appeared normal, the cerebral cortical hemispheres and the cerebellum were reduced in size. Furthermore, while the vermis and hemispheres had formed in the cerebellum, they lacked fissures (Figure 2B).

In situ hybridization confirmed that the integrin $\beta 1$ subunit was expressed in wild-type mice throughout the developing cerebral cortex and cerebellum (Figure 2C and data not shown). $\beta 1$ protein was also detected by Western blots in extracts from neural tissue of wild-type animals at E12.5, E15.5, and P1 (Figure 2E). To confirm that the $\beta 1$ gene was inactivated in the nervous system of the mutant mice, we monitored recombination of the $\beta 1$ allele at the DNA level and $\beta 1$ expression at the protein level. Recombination in neural tissue was readily detectable at the DNA level (Figure 2D). The $\beta 1$ protein was present in low amounts in extracts from dissected neural tubes from mutant animals at E12.5. $\beta 1$ protein was essentially absent at later time points (Figure 2E), and only very low levels could be revealed upon long exposure of the films (data not shown). This was expected, since $\beta 1$ is expressed in meningeal cells and blood vessels, where Cre was not active (Figure 1), but that contaminated the dissected neural tissue. We conclude that expression of $\beta 1$ and, thus, the activity of all $\beta 1$ -class integrins is effectively abolished in neural tissue at E12.5 but likely earlier, since Cre is already active at E10.5 (Figure 1D).

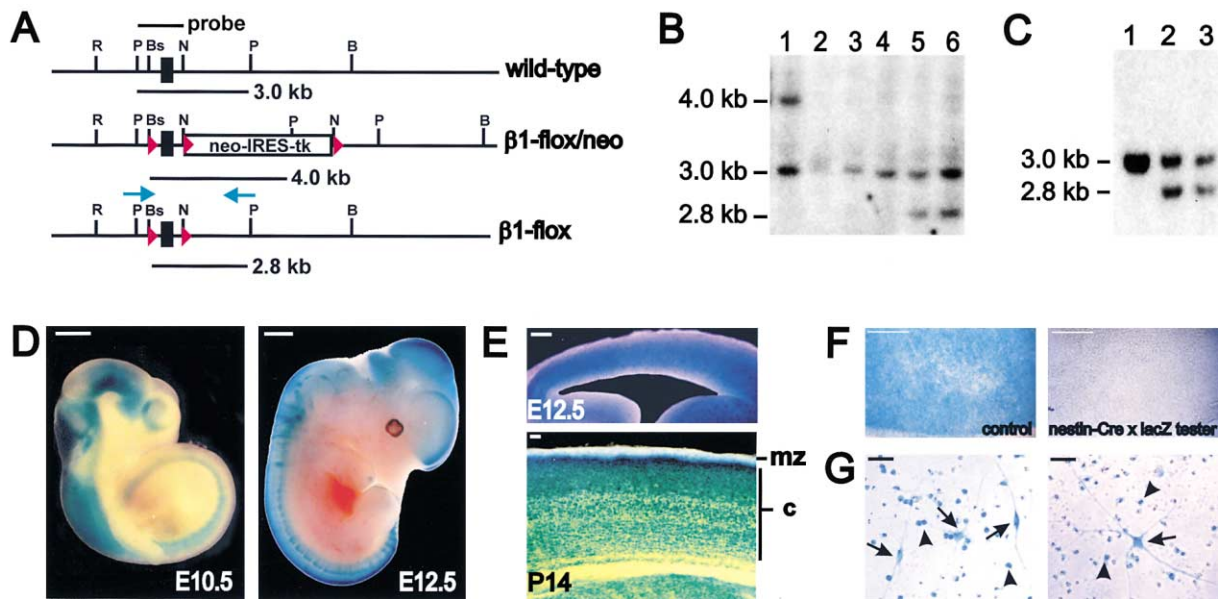


Figure 1. Generation of the β 1-*flox* Allele and Analysis of the Nestin-Cre Mouse

(A) Diagram of the integrin β 1 wild-type and β 1-*flox* allele. The first coding exon (black rectangle), the loxP sites (red triangles), the Southern blot probe, and the expected Southern blot fragments are indicated. Restriction endonuclease sites are marked (R, EcoRI; P, PvuII; N, NheI; B, BamHI; and Bs, BstEII), and the position of primers for PCR analysis to monitor Cre recombination is indicated (blue arrows). (B) DNA was analyzed by Southern blot from an ES clone carrying the β 1-*flox/neo* allele in the heterozygous configuration (lane 1) and from subclones obtained after transfection with a Cre-expressing plasmid (lanes 2 through 6). The DNA was digested with PvuII, and the probe indicated in (A) was used to identify clones that had lost the neo-IRES-tk cassette but retained the first β 1 coding exon flanked by two loxP sites. Two clones (lanes 5 and 6) had the expected pattern, while three (lanes 2, 3, and 4) had also lost the β 1 coding exon. (C) Southern blot analysis of DNA from a wild-type mouse (lane 1) and offspring from a germline-transmitting chimera (lanes 2 and 3). (D) A *Rosa26lacZ-loxP* reporter mouse was crossed with a nestin-Cre mouse, and embryos were analyzed by whole-mount X-gal staining. Widespread lacZ expression was evident throughout neural tissue. (E) Histological sections revealed Cre recombination in the cerebral cortex (c) at E12.5 and P14. Meningeal cells in the marginal zone (mz) did not recombine. (F) The meningeal cell layer was dissected from P7 control mice that contained a *Rosa26lacZ-loxP* transgene that was recombined in all tissues (control) or from mice obtained from intercrosses of nestin-Cre mice with *Rosa26lacZ-loxP* tester mice. The meningeal tissue was spread out on a coverslip and photographed from the top. Nestin-Cre did not induce recombination in meninges. (G) Cell cultures derived from P7 cerebella of intercrosses between *Rosa26lacZ-loxP* and *nestin-Cre* mice were stained for lacZ. Essentially all neurons (arrowheads) and glial cells (arrows) were lacZ positive. Size bars: (D), (F), and (G), 50 μ m; (E), 40 μ m.

Defects in the Laminar Structure of the Cerebral and Cerebellar Cortex

To investigate the effects of loss of β 1-class integrins on the development of the central nervous system, we prepared histological sections from brains at varying ages starting at E15.5 (Figure 3 and data not shown). The overall organization of the β 1-deficient brain appeared normal, but the cerebral and cerebellar cortex showed striking alterations. In the cerebral cortex, the cortical wall had formed, but the layers had a wavy appearance at E15.5, indicative of abnormal positioning of cortical neurons (Figures 3A and 3B). In several areas, neurons invaded the marginal zone. In adjacent areas, cortical neurons accumulated deep in the cortical wall (arrows in Figure 3B). Defects in the cortex were also evident at all subsequent ages. At P2, layers I through IV and in some cases layer V were disrupted (Figures 3C and 3D). In addition, coronal sections revealed that the cortical hemispheres were fused at the midline (Figures 3E and 3F).

Changes in the cytoarchitecture of the cerebellum became apparent at postnatal ages. At P0, the cerebellar anlage was indistinguishable between wild-type and mutant mice. The EGL was normal in size and thickness, and Purkinje cells were aligned below the EGL (Figures 3G and 3H). Several abnormalities became obvious at

P4 and were pronounced by P7 and P14 (Figures 3I–3L). First, the development of cerebellar folia was defective. The position and number of folia along the axis of the cerebellum was not altered (data not shown), but fusion between adjacent folia was evident from the onset of their development (Figures 3J and 3L). With age, the folia became progressively more distorted, and the cerebellum was reduced in size as prominently evident from the decreased depth of the folia (compare Figures 3I to 3J and 3K to 3L). Second, while a rudimentary IGL formed, a large number of granule cells formed ectopia along the fusion lines of adjacent folia (arrows in Figure 3L) and at the cerebellar surface underlying the meninges (arrowheads in Figure 3L). In conclusion, the development of both the cerebral and cerebellar cortex is severely impaired in the absence of β 1-class integrins, leading to fusion between folia and hemispheres and to defects in the organized laminar cytoarchitecture of cortical structures.

Defective Remodeling of the Meningeal Basement Membrane

Meningeal cells secrete ECM components that are assembled into a basement membrane between the meningeal cells and the neural tissue (Sievers et al., 1994). Defects in the expansion of the meningeal cell layer

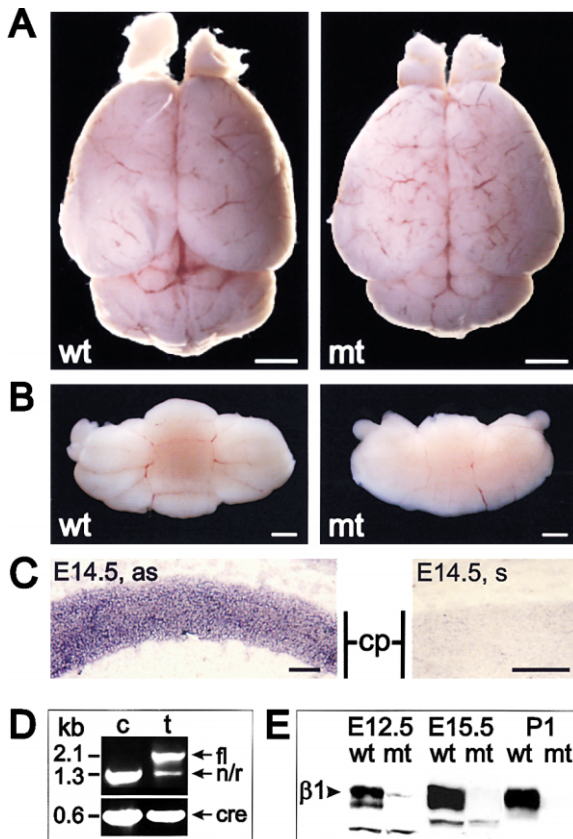


Figure 2. Mice that Lack β 1-Class Integrins in the Nervous System Show Severe Brain Abnormalities

(A) The brain was dissected from a 3-week-old wild-type and mutant mouse. The cortical hemispheres and the cerebellum were reduced in size in the mutants, and the cerebellum lacked external foliation. (B) Higher magnification view of the cerebellum from 3-week-old mice. The mutant cerebellum was devoid of fissures. (C) In situ hybridization revealed that at E14.5 the integrin β 1 subunit mRNA is expressed throughout the developing cortical plate (cp) of wild-type mice (antisense probe [as]; control sense probe [s]). (D) Cre-mediated recombination was analyzed by PCR with DNA obtained from the cerebral cortex (c) or tail (t) from P0 animals. A 2.1 kb band indicative of the β 1-*flox* allele (fl) and a 1.3 kb band indicating the β 1 *null* allele (n) were visible in tail DNA. The 2.1 kb DNA band was absent in DNA from the cerebral cortex. Instead, a 1.3 kb band indicative of both the β 1 *null* and the recombined β 1-*flox* (r) allele was evident. The presence of the *nestin-Cre* transgene was confirmed by PCR. (E) Western blot analysis for the β 1 protein. Extracts were prepared from neural tissue dissected at E12.5, E15.5, and P1. The β 1 protein was readily detected in wild-type mice but not in the mutants. Size bars: (A), 2 mm; (B), 1 mm; (C), 40 μ m.

and in basement membranes during growth of cortical structures could lead to abnormal lamination and foliation. We therefore analyzed the integrity of the meningeal cell layer and the distribution of ECM components in the developing brain (Figure 4). Meninges covered the outer surface of the mutant and wild-type brain at all ages analyzed and could be removed as a single uninterrupted sheet (Figure 1F). However, the meningeal cell layer in mutants did not extend into the developing cerebellar folia (Figures 4A and 4B) and between the cortical hemispheres (Figure 3F).

In wild-type mice, both the cerebral and cerebellar

cortices were covered with a basement membrane containing laminin (Figures 4C, 4E, 4G, and 4I), collagen IV, and nidogen/entactin (data not shown), and this basement membrane penetrated into and separated the cerebellar folia (Figures 4C and 4E). In contrast, a relatively intact ECM layer was visible in the mutants only at early stages of cortical development, i.e., at E15.5 in the cerebral cortex (Figure 4H) and by P2 covering the cerebellar cortex (Figure 4D). At E15.5, only small areas of the surface of the cerebral cortex were devoid of ECM, and they coincided with areas where cortical neurons invaded the marginal zone (Figure 4H, arrowhead). At subsequent ages, basement membrane defects became severe, with areas devoid of ECM and widespread depositions of granules, indicating active ECM degradation (Figures 4H and 4J, arrowheads). In the cerebellum, ECM molecules were never observed within the developing folia (Figures 4D and 4F) and were absent by P7 from areas of the brain surface underlying the meninges (Figure 4F). The basement membranes around blood vessels were unaffected (Figures 4C–4J, arrows).

Previous data suggested that meningeal cells are essential for the formation of the basement membranes at the brain surface (Sievers et al., 1994). Since the *nestin-Cre* mice did not induce recombination in meningeal cells, our data suggest that expression of β 1-class integrins in neurons and/or glia is required to assemble and/or remodel the meningeal basement membranes during the growth phase of cortical structures. Fusion of cerebellar folia and cortical hemispheres are likely caused by defects in the basement membranes, which results in lack of expansion of the overlying meningeal cell layer into the folia and between the cortical hemispheres.

Defective Development of the Glial Endfeet

Glial endfeet formation has been proposed to be dependent on interactions of glial fibers with basement membranes (Sievers et al., 1994), suggesting a role for integrins in this process. Abnormal glia-basement membrane interactions in the β 1-deficient mice could cause defects in basement membranes and glial fibers. This in turn could lead to the development of distorted cortical layers. We therefore visualized glial fibers in cortical sections between E18.5 and P14 by immunohistochemistry (Figure 5). At E18.5, radial glial fibers spanned the cerebral cortical wall in wild-type and mutant mice, with no apparent difference in alignment and density (Figures 5A and 5B). In contrast to wild-type animals, glial fibers in the mutants did not develop glial endfeet but terminated at varying positions within the marginal zone close to the meningeal cell layer (compare Figures 5C and 5D). Glial fibers that extended toward the cerebellar surface were also readily detectable in wild-type and mutant mice at E18.5 (Figures 5E and 5F). At later stages, a regular glial network was visible in wild-type mice, and the glial endfeet formed a continuous layer (Figures 5G and 5I). In contrast, the glial fibers in mutant mice were highly irregular. Within the folia, they occasionally invaded the granule cell layer but did not form expanded endfeet at any age (Figure 5J). Glial endfeet were also absent at the surface of the cerebellum (Figure 5H), even though ECM components were initially present (Figure 4D).

The data provide strong evidence that β 1-class integ-

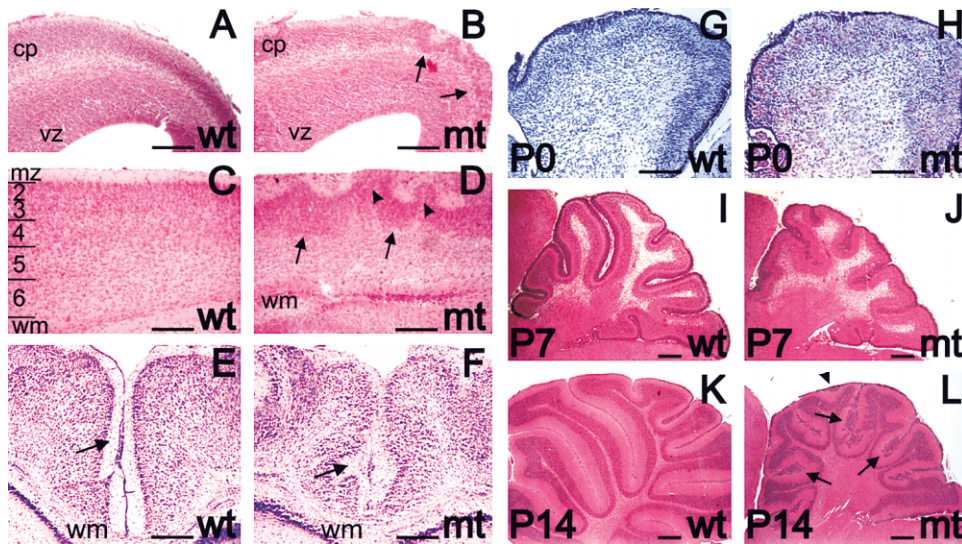


Figure 3. Developmental Defects in the Cerebral and Cerebellar Cortex

(A–D) Sagittal sections through the cerebral cortex were analyzed by hematoxylin and eosin staining. (A and B) Sections of E15.5 embryos. The cortical plate (cp) in mutants was disorganized (arrows in [B] (vz, ventricular zone)). (C and D) Sections of P2 animals. The marginal zone (mz), cortical layers 2 through 6, and the white matter tract (wm) were visible in wild-type mice. In the mutants, neurons accumulated in the cortical wall (arrows in [D]), and ectopia formed in the cortical marginal zone (arrowheads in [D]). (E and F) Coronal sections through the cerebral cortex at P14 were stained with cresyl violet. The hemispheres were separated by meninges in wild-type animals (arrow in [E]) but not in the mutants. Cells that are likely remnants of a disrupted meningeal cell layer were occasionally visible in mutants (arrow in [F]). (G–L) Midsagittal sections through the cerebellum were prepared and stained with Nissel (G and H) or hematoxylin and eosin (I–L). The cerebellar cortex in mutant mice was normal at birth. Folia started to develop at subsequent ages. At ages after P7, the cerebellar folia in mutants were significantly smaller when compared to wild-type animals, and the laminar organization of the mutant cerebellum became progressively more distorted. (L) Granule cell ectopia formed along fusion lines between folia (arrows) and below the pial surface (arrowheads). Size bars: (A), (B), and (G–L), 200 μ m; (C) and (D), 50 μ m; (E) and (F), 250 μ m.

rins in glial cells are required for the anchorage of glial endfeet to the meningeal basement membrane. Basement membrane defects are likely caused at least in part by defects in these interactions. In contrast, β 1-class integrins are not essential for the extension of glial fibers per se, as prominently visible in the cerebral cortex where glial fibers extend throughout the cortical wall (Figures 5A and 5B). This suggests that defects in cortical lamination do not develop as a direct consequence of a perturbed glial network. We therefore analyzed whether β 1-class integrins have a direct role in regulating neuron-glia interactions and neuronal migration that could lead to the lamination defects.

Neuronal Migration in the Cerebral Cortex

We performed several experiments to analyze the extent of neuronal migration in the absence of β 1-class integrins. First, we determined with molecular markers whether neurons within the cerebral cortex had migrated into appropriate layers. Immunohistochemical analysis with an antibody that recognizes specifically the non-phosphorylated form of neurofilament, a marker for a subset of pyramidal neurons in layers III and V (Campbell and Morrison, 1989), revealed that these layers had formed in wild-type and mutant animals (Figures 6A and 6B), even though the layers in the mutants had the same wave-like appearance as observed in histological stains (Figure 3).

To detect potential defects in the migration of cortical neurons directly, we labeled proliferating neurons with

bromodeoxyuridine (BrdU) at E13.5, E14.5, and E15.5 and analyzed the extent of their migration at P4 (Figures 6C–6F and data not shown). In wild-type and mutant mice, BrdU-positive neurons had assumed appropriate positions at P4. For example, most cells labeled at E15.5 were found in layers II/III, with some scattered neurons located in intermediary migratory zones in wild-type and mutant mice. Strings of BrdU-labeled cells reminiscent of neurons attached to glial fibers were readily visible (Figures 6C and 6D, arrowheads). In fact, double-immunofluorescence staining with BrdU and GFAP showed that these cells were in close proximity to glial fibers (Figures 6I and 6J), and we confirmed by electron microscopy that neurons were attached to glial fibers (Figures 6G and 6H). However, while most neurons reached the appropriate layers in the cortical wall in the mutant mice, some abnormalities were evident. First, and in accordance with our histological data (Figure 3), the BrdU-labeled cortical layers were undulating (Figure 6D). Second, defects in cell migration occurred close to the pial surface of the cortex where neurons invaded the marginal zone (Figure 6F, arrows). Third, the cell bodies of cortical neurons were less tightly packed within cortical layers (compare Figures 6E to 6F). These data provide strong evidence that defects in neuronal migration arise close to the cortical marginal zone but that β 1-class integrins are not essential for neuron-glia interactions and neuronal migration per se. We can at present not exclude that integrins have some regulatory function during cell migration or are redundant with other receptor systems (see Discussion).

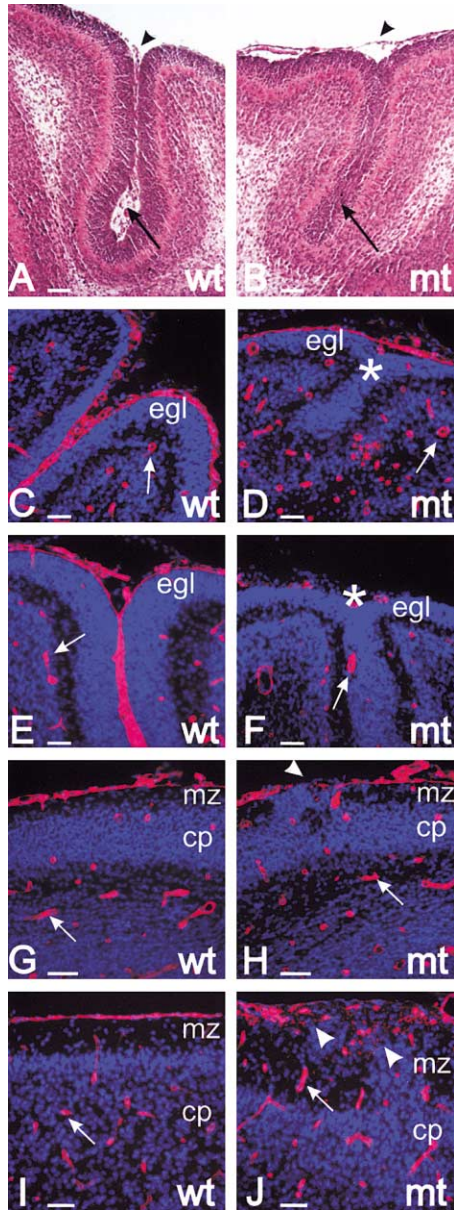


Figure 4. Defective ECM Deposition into Meningeal Basement Membranes

(A and B) Sagittal sections through the cerebellar cortex were stained with hematoxylin and eosin. The meninges (arrowheads) covered the outer surface of the cerebellum but did not penetrate into the folia in mutant mice (arrow). (C–J) Sagittal sections of the cerebellar and cerebral cortex of wild-type and mutant animals were stained with antibodies to laminin (red) and with DAPI (blue). Note that laminin was deposited around blood vessels in wild-type and mutant mice at all ages (arrows in [C]–[J]). (C–F) Sagittal sections of the cerebellar cortex. Laminin was incorporated at P2 in the meningeal basement membranes overlying the EGL in wild-type and mutant mice (C and D). At P7 (E and F), laminin in mutant mice was severely reduced at the cerebellar surface and absent in the folia (asterisk in [D] and [F]). (G–J) Sagittal sections of the cerebral cortex. At E15.5 (G and H), the meningeal basement membrane in mutants showed interruptions (arrowhead in [H]). At P2 (I and J), granular laminin deposits were evident in the cerebral cortex of mutants (arrowheads in [J]). Cortical neurons invaded the marginal zone in areas where basement membrane defects were apparent. Size bars: 40 μ m.

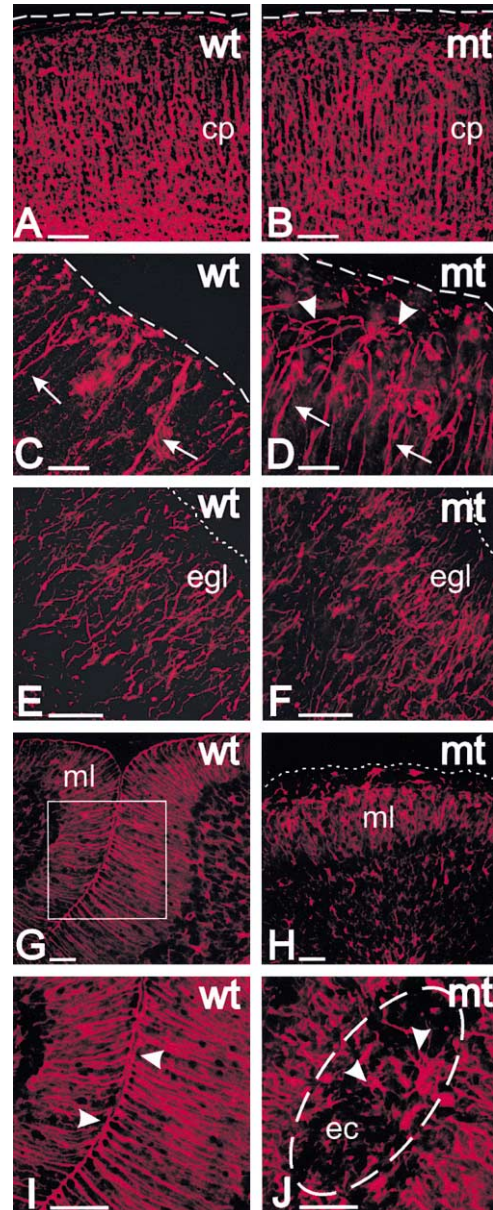


Figure 5. Defects in the Development of the Glial Network

(A and B) Radial glial fibers in the cerebral cortex of E18.5 (stained for GFAP) were extending through the cortical plate (cp) in wild-type and mutant mice. (C and D) Glial fibers (arrows) in the marginal zone of the cerebral cortex at P0 (stained for RC2). In wild-type mice, the glial fibers extended to the meningeal cell layer (dashed line). In the mutants, the glial fibers terminated at varying positions within the marginal zone (arrowheads). (E and F) Sagittal sections of the cerebellar cortex at E18.5, stained for RC2. Glial fibers in wild-type and mutant mice extended through the external granule cell layer (egl) toward the meninges (dashed line). (G and H) Sagittal cerebellar sections at P14 stained for GFAP. (G) Glial fibers in wild-type animals formed a regular network and were anchored with glial endfeet. (I) Enlargement of the square in (G). The arrowheads point to endfeet. (H and J) Glial fibers in mutant animals were irregular and meandered through the molecular layer (ml). They lacked endfeet at the cerebellar outer surface (dashed line in [H]) and within the fusion lines between folia (J). The glial fibers (arrowheads in [J]) occasionally invaded granule cell ectopia (ec; approximately outlined with a white dashed line) at fusion lines between folia. Size bars: 40 μ m.

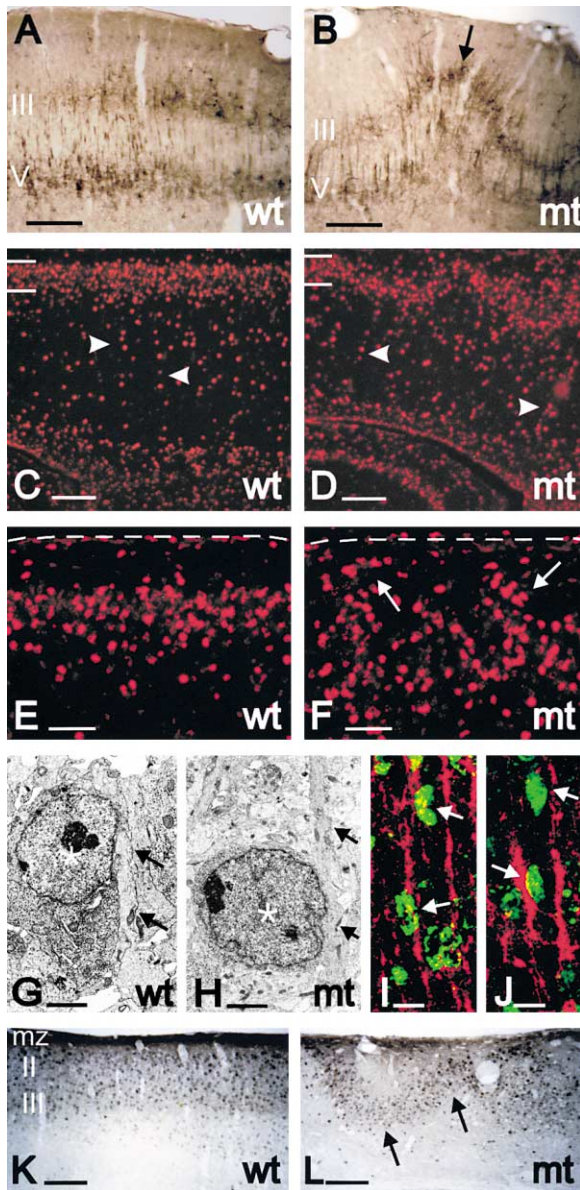


Figure 6. Cell Migration and Layer Formation in the Cerebral Cortex (A and B) Sagittal sections through the cerebral cortex (P28) were stained with an antibody specific for a nonphosphorylated form of neurofilament that labels a subset of neurons in layers III and V. Both layers had formed in wild-type and mutant mice but were undulating in the mutants (arrow). (C–F) Cell migration was analyzed by BrdU pulse labeling. Pregnant mice were injected at E15.5, and the distribution of BrdU-labeled neurons was determined in offspring at P4. The majority of neurons in wild-type and mutant mice reached layer II/III (indicated by horizontal lines). Strings of cells reminiscent of neurons attached to glial fibers were detected in wild-type and mutant mice (arrowheads in [C] and [D]). (E–F) Higher magnification views. The dashed line indicates the meningeal cell layer. The width of layer II/III in the mutants was increased (compare [E] to [F]). Neurons in the mutants invaded the cortical marginal zone (arrows in [F]). (G and H) Interactions between neurons (the star marks the nucleus of a neuron) and glial fibers (arrows) were visualized by electron microscopy of cortical sections at P0. (I and J) Sagittal sections of the cortex of wild-type (I) and mutant (J) mice were analyzed by double immunofluorescence. Migrating neurons were labeled with BrdU at E15.5 and visualized at E18.5 (green, arrows). Glial fibers were stained with antibodies to GFAP (red). (K

Cortical interneurons are generated in the ganglionic eminence and migrate tangentially into the cerebral cortex (e.g., Anderson et al., 1997). In wild-type and mutant brains, interneurons invaded the cortex and assumed their appropriate positions in layers II/III (Figures 6K and 6L). In the mutant mice, the tangentially migrating interneurons were organized in undulating layers. These data provide further evidence that the cortical marginal zone is perturbed and that neuronal layers, although wave-like in appearance, form and contain positional cues that are recognized by the tangentially migrating interneurons.

Defective Positioning of Cajal-Retzius Cells and Reelin in the Cortical Marginal Zone

In the β 1-deficient cerebral cortex, migration of neurons progressed relatively normal and was mostly perturbed close to the marginal zone. This zone is an organizing center for the developing cerebral cortex. In particular, it contains CR neurons that secrete reelin, an ECM molecule that regulates the formation of cortical layers (reviewed in Cooper and Howell, 1999; Rice and Curran, 1999). Defects in the organization of the marginal zone and in CR cells could account for the abnormalities detected in the lamination in the β 1-deficient cortex. Therefore, we examined the presence of CR cells and reelin in wild-type and mutant mice. In wild-type mice, CR cells formed a well-organized layer at all ages analyzed (Figures 7A and 7C). In the mutants, the CR cell layer was perturbed. Defects were evident at E15.5, such as small gaps in the CR cell layer (Figure 7B), which coincided with the gaps in the meningeal basement membrane (data not shown). The defect worsened at later ages, with CR neurons forming ectopia within the cortical wall (Figure 7D). The orientation of the cell bodies and processes of CR cells were also abnormal. While the cell bodies and processes of wild-type cells were aligned parallel to the meningeal cell layer (Figure 7C), those of mutant cells were more randomly oriented (Figures 7D–7F). The CR neurons of mutant mice still expressed reelin, indicating that loss of β 1-class integrins did not inhibit reelin synthesis (Figures 7G–7L). Importantly, the waves in the cortical layers followed the territories delineated by CR cells. Neurons invaded areas of the cortical marginal zone that lacked CR cells (Figure 7B, arrows) and accumulated below CR cell ectopia within the cortical wall (Figure 7D, arrowheads).

Taken together, our data demonstrate that migrating neurons are confronted at the cortical marginal zone with a drastically perturbed environment. CR cells and signaling molecules such as reelin are absent in some places and deposited ectopically in others. Undulating cortical waves form likely in part as a consequence of these perturbations. The accumulation of cortical neurons underneath the CR cell ectopia also suggest that

and L) Interneurons were identified at P28 by staining for calbindin. They were appropriately positioned in layer II/III. The layers were meandering in the mutants (arrows in [L]). Size bars: (A), (B), (K), and (L), 250 μ m; (C) and (D), 150 μ m; (E) and (F), 40 μ m; (G) and (H), 2 μ m; (I) and (J), 10 μ m.

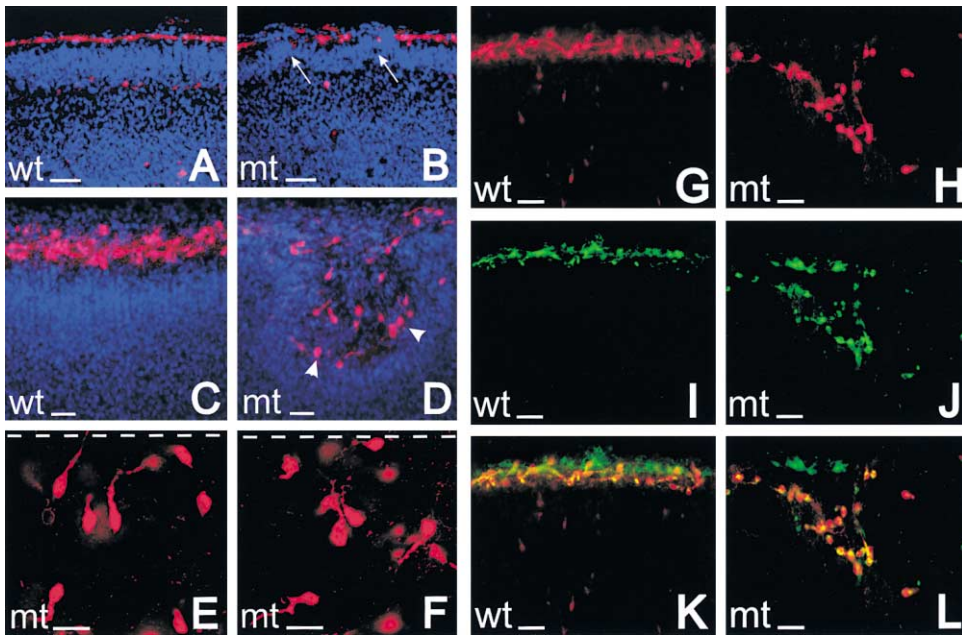


Figure 7. Defects in the Cajal-Retzius Cell Layer

(A–D) CR cells were stained with antibodies to calretinin (red) and all cell nuclei with DAPI (blue). In wild-type mice, the CR cells formed a layer at E15.5 (A) and P0 (C). The CR cell layer in mutants showed interruptions at E15.5 (B) and invasions of neurons into the gaps (arrows in [B]). The interruptions became worse by P0, with CR cells forming ectopia in the cortical wall (arrowheads in [D]) and cortical neurons accumulating below the ectopia. (E and F) Higher magnification view of CR ectopia in mutants at P0. The CR cell bodies and processes were oriented randomly relative to the cortical surface (dashed line). (G–L) Double immunofluorescence at P0 for calretinin (red in [G] and [H]) to reveal CR cells and reelin (green in [I] and [J]). CR cells in wild-type and mutant mice expressed reelin. A double exposure is shown (K and L). Size bars: (A)–(D) and (G)–(L), 40 μ m; (E) and (F), 20 μ m.

these neurons, despite their lack of β 1-class integrins, still respond to guidance cues emitted from CR cells.

Neuronal Migration in the Cerebellar Cortex

The analysis of the cerebral cortex suggests that β 1-class integrins are not essential for neuronal migration during corticogenesis. This raises the possibility that the formation of granule cell ectopia (Figure 3) in the cerebellum is not directly caused by defects in migration due to lack of β 1-class integrins in cerebellar granule cells. In fact, the IGL in wild-type and mutant animals was similar in size around P4, at a time when the glial network was relatively intact (Figures 8A and 8B). To compare cell migration quantitatively, we labeled proliferating granule cells with BrdU at P3 and analyzed their extent of migration by P5. Short-term BrdU labeling experiments revealed that fewer cells were labeled in the EGL of mutant mice when compared to wild-type animals, indicative of proliferation defects in granule cell precursors (data not shown). To quantify the rate of migration, we had to compensate for this defect. Accordingly, we determined the number of BrdU-positive cells generated at P3 in the EGL during a 2 hr pulse labeling. Next, we determined the number of BrdU-labeled neurons that had reached the IGL 2 days later. We then established the ratio between the two cell numbers (Figure 8C). The ratio was essentially identical in wild-type and mutant animals, providing strong evidence that cell migration progresses initially normally. The later accumulation of granule cells in ectopic sites, i.e., along

the cerebellar surface and along fusion lines between folia, is probably a secondary consequence of perturbances in the glial network. In agreement with this hypothesis, ectopia start to form at the same time when perturbances in the glial network are detectable, and ectopia are most prominent in areas where the glial network is most defective, i.e., along the fusion lines of adjacent folia (Figure 5).

To investigate this further, we analyzed neuron-glia interactions directly in vitro (Figures 8D–8G). Cells attached to cell processes were readily detected in cultures derived from both wild-type and mutant animals (Figures 8D and 8E). We confirmed by double immunofluorescence with specific markers that these were neurons attached to glial fibers (Figures 8F and 8G). The elongated shape of many of the granule cell bodies suggested that they were migrating. Similar observations were also made in vivo (data not shown), in agreement with the results shown for neuron-glia interactions in the cerebral cortex (Figures 6G–6J). These data provide further evidence that β 1-class integrins are not essential in either neurons or glia for their interaction. We cannot exclude, however, that β 1-class integrins modulate these interactions or have redundant functions with other receptor systems (see Discussion).

Discussion

We have inactivated the integrin β 1 gene selectively in the precursors of neurons and glia by Cre/Lox-mediated

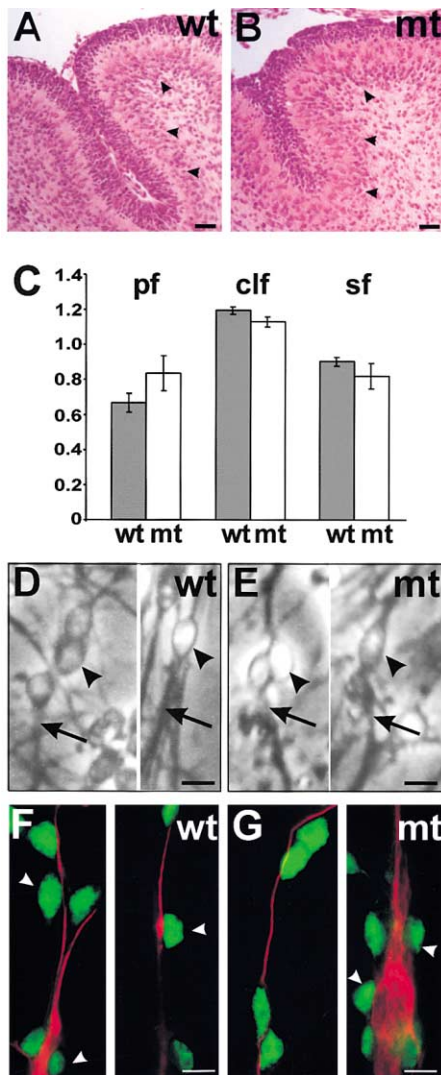


Figure 8. Cell Migration in the Cerebellum
(A and B) Hematoxylin and eosin staining of P4 sagittal sections reveals that the thickness of the IGL (arrowheads) was similar in wild-type and mutant animals. (C) Mice were labeled at P3 with BrdU. The number of replicating BrdU-positive cells in the EGL was determined in sections prepared 2 hr postlabeling, and the number of BrdU-positive cells that had migrated into the IGL in sections prepared 2 days later. Three different folia were analyzed in two mice each (primary fissure [pf], secondary fissure [sf], central folium [clf]). The ratio of BrdU-labeled cells in the EGL and IGL was determined and was essentially identical in wild-type (dark bars) and mutant (white bars) mice. (D and E) Dissociated mixed cerebellar cultures were prepared from P7 animals and plated on Poly-D-Lysine-coated coverslips. Wild-type and β 1-deficient neurons (arrowheads) attached to cell processes (arrows). (F and G) To determine the identity of cells in culture, the cells were stained with antibodies to GFAP (red) and NeuN (green). Neurons (arrowheads) attached to glial fibers were readily visible. There was no difference between cultures from wild-type and mutant animals. Size bars: (A and B), 40 μ m; (D)–(G), 10 μ m.

gene inactivation and show here that the mutant mice develop severe brain abnormalities. The defects include fusion of the cortical hemispheres and cerebellar folia and abnormal laminar organization of cortical structures. These are characteristic features of inherited cor-

tical malformation diseases (reviewed in Walsh, 1999), suggesting that integrin-activated signal transduction pathways may be affected in some of the diseases. Our data suggest that cortical foliation and lamination defects arise in large part as a consequence of perturbations in the cortical marginal zone, where the anchorage of glial endfeet, the remodeling of basement membranes, and the extension of the meningeal cell layer are perturbed in the absence of β 1-class integrins. Unexpectedly, β 1-class integrins are not essential for neuron-glia interactions and for neuronal migration during corticogenesis. In the cerebral cortex, neurons migrate toward the marginal zone; however, they invade CR/reelin-free areas or accumulate below CR/reelin ectopia. In the cerebellum, granule cells accumulate along fusion lines of folia and at the pial surface, at a time when the glial fiber network is most dramatically perturbed. The granule cell ectopia appear to form as a result of perturbations in the glial network that prevent physical contact between some granule cells and glial fibers and consequently the initiation of cell migration by some neurons.

β 1-Class Integrins and the Assembly and Remodeling of the Meningeal Basement Membrane

The deposition of ECM components into the meningeal basement membrane is defective in the absence of β 1-class integrins. Deposition is initiated early in development, but defects become pronounced during the rapid growth phase of cortical structures. Previous studies have shown that meningeal cells express ECM components and that they are important for the assembly of the meningeal basement membrane (Hausmann and Sievers, 1985; Sievers et al., 1994). In the current study, integrins were only inactivated in neurons and glia. This suggests that the initial deposition of ECM components is dependent on meningeal cells, but proper basement membrane maintenance and/or remodeling is also dependent on β 1-class integrins in neurons and/or glia. Strikingly, expanded glial endfeet do not form in the mutants, suggesting that interactions between β 1-class integrins in glial cells and ECM components are important to anchor the endfeet at the basement membrane and to maintain and/or remodel the basement membranes.

Glial cells express numerous integrin heterodimers, containing different β subunits, including β 1 (Milner and Ffrench-Constant, 1994). Our data suggest that β 1-class integrins have a predominant role in mediating glial endfeet anchorage. It is at present not clear which integrin α subunit(s) heterodimerize(s) with β 1 to mediate anchorage, but α 6 is one candidate. Invasions of neurons into the marginal zone and punctuate laminin deposits form in mice carrying a targeted mutation in the integrin α 6 subunit gene. However, the mechanism leading to the defects is unclear. In these mice, basement membranes are only slightly affected, CR cell ectopia do not form, and neurons do not accumulate within the cortical wall (Georges-Labouesse et al., 1998). Thus, inactivation of α 6 alone leads to a much less severe defect than inactivation of all β 1-class integrins. Also, the α 6 subunit forms heterodimers with the integrin β 1 and β 4 subunits. It is not clear whether the defects described previously are caused by inactivation of the

integrin $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ (Georges-Labouesse et al., 1998). Likewise, it is not known whether $\alpha 6$ is required in neurons, glia, or meningeal cells. Further studies will be necessary to address these points and to identify additional integrin α subunits that are important during cortical development.

Cortical defects are observed in humans and mice carrying mutations in the laminin $\alpha 2$ chain gene (reviewed in Miyagoe-Suzuki et al., 2000) and in mice with a *perlecan* mutation (Costell et al., 1999). Laminin is a ligand for several $\beta 1$ -class integrins, including $\alpha 6\beta 1$. Laminin and perlecan are also components of the meningeal basement membranes. The data suggest that a common molecular pathway involving $\beta 1$ -class integrin-dependent basement membrane assembly is affected in the different mutants.

Formation of Cajal-Retzius Cell Ectopia

In the cerebral cortex of the $\beta 1$ -deficient mice, neurons invaded the marginal zone, while others accumulated within the cortical wall. Our data suggest that the abnormal layering is in large part a consequence of defects in the cortical marginal zone, including perturbations of the CR cell layer. Defects in the CR cell layer arise after E15.5 in parallel to perturbances in the meningeal basement membranes. Proliferation of CR cells is probably not strongly affected, since these cells are generated well before E15.5. The subsequent expansion of the CR cell layer during the growth phase of the cortex has been attributed to morphological changes in CR cells (reviewed in Marin-Padilla, 1998). Our data are consistent with a model in which these morphological changes are dependent on reciprocal interactions between CR cells and ECM components and/or glia, interactions that are perturbed in the absence of $\beta 1$ -class integrins. As a consequence, the CR cell layer is not maintained, leading to CR-free areas and the ectopic accumulation of CR cells in the cortical wall. However, further experiments are necessary to analyze directly $\beta 1$ -dependent interactions of CR cells with ECM components, including reelin.

Similar CR cell ectopia develop in cortical slice cultures upon application of neurotrophin-4 (NT-4) and in transgenic mice that overexpress brain-derived neurotrophic factor (BDNF) (Brunstrom et al., 1997; Ringstedt et al., 1998). The mechanism by which these ectopia form is unclear. BDNF may mediate its effects by affecting reelin expression, since reelin levels are reduced in the nestin-BDNF transgenic mice (Ringstedt et al., 1998). In the mutant described here, reelin expression was maintained, although we cannot exclude that its levels were altered. It will be interesting to address whether NT-4, BDNF, integrins, and reelin are part of a molecular network that regulates CR cell differentiation.

Granule cell ectopia are also found in the $\beta 1$ -deficient cerebellum. They form likely as a secondary consequence to perturbances in the glial network. Some granule cells that are too far away from the malformed glial fibers never contact glial processes and fail to initiate migration. This model is consistent with the observation that migration progresses normally at early stages of cerebellar development when the glial network is relatively intact. Furthermore, formation of ectopia is most

prominent in areas where the glial network is strongly perturbed, such as along the fusion lines of cerebellar folia.

$\beta 1$ -Class Integrins and Cell Migration

Our data provide evidence that $\beta 1$ -class integrins are not essential for neuron-glia interactions and for neuronal migration during cortical development. Importantly, BrdU pulse-labeling experiments and staining of neurons with layer-specific markers show that cortical neurons migrate into appropriate cortical layers. Migratory defects are mostly pronounced close to the marginal zone, where neurons invade areas devoid of CR cells or accumulate below CR cell ectopia. This suggests that migratory defects are in large part a consequence of a perturbed marginal zone. We also demonstrate by immunohistochemistry and electron microscopy that neurons and glial cells establish direct contact in vivo and in vitro, even in the absence of $\beta 1$ -class integrins in both cell types.

Previous studies have provided evidence that integrins contribute to the regulation of migration of cortical neurons. Infection of tectal neurons with a retrovirus expressing an integrin $\beta 1$ antisense mRNA leads to the accumulation of infected cells in the ventricular zone and to their death. However, a direct effect on migration has not been demonstrated, and it is not clear whether migratory defects are caused secondarily to defects in proliferation (Galileo et al., 1992). These defects would likely not occur in the mice studied here, since the nestin promoter used to activate Cre expression is not or only weakly active in early neural progenitors (Zimmerman et al., 1994). In contrast, the retrovirus vectors used in the previous study infect these cells (Galileo et al., 1992). It has also been reported that antibodies against the integrin $\alpha 3$ subunit reduce the rate of neuronal migration along glial fibers in vitro and that some neurons in integrin $\alpha 3$ -deficient mice do not reach appropriate layers (Anton et al., 1999). The only known heterodimer partner for $\alpha 3$ is $\beta 1$ (reviewed in Hemler, 1999), predicting that defects in cell migration should be apparent in the $\beta 1$ -deficient mice. We do not detect such defects. However, we cannot exclude that $\beta 1$ -class integrins have a modulatory role and that the rate of migration is affected. A quantitative study of neuronal migration in the cerebral cortex was unfortunately not possible, since layer formation is affected to a varying degree in different parts of the cortex. Further studies will be necessary to address this point.

Once neurons reach the cortical marginal zone, they terminate migration and form distinct layers. The ECM component reelin has been suggested to act as a stop signal for migrating cortical neurons. Integrin $\alpha 3\beta 1$, CNR proteins, and members of the low-density lipoprotein receptor family have been reported to bind to reelin (reviewed in Rice and Curran, 1999; Cooper and Howell, 1999). Mice carrying targeted mutations inactivating two members of the low-density lipoprotein receptor family, VLDLR and ApoER2, show the same phenotype as mice lacking reelin, providing genetic evidence that these receptors are essential to transmit the reelin signal (Trommsdorff et al., 1999). It has also been reported that the integrin $\alpha 3\beta 1$ is essential to transmit a reelin stop

signal (Dulabon et al., 2000), but our data do not support the hypothesis that β 1-class integrins are involved. Accordingly, β 1-deficient migrating cortical neurons accumulate within the cortical wall below CR cell/reelin ectopia. Invasions of neurons into the cortical marginal zone only occur in those areas where CR cells and reelin are absent. We can at present not explain the difference between the two studies, and further in vitro experiments will be important to define the role of β 1-class integrins in reelin signaling. It will also be important to address whether integrins, CNRs, and VLDLR/II/ApoERII may have redundant functions in this process.

In vitro culture experiments suggest that integrins in glia regulate neuron-glia interactions. In particular, the integrin α _V subunit is expressed in glia (Hirsch et al., 1994; Milner and Ffrench-Constant, 1994; Anton et al., 1999). Antibodies against α _V cause detachment of neurons from glial fibers in vitro (Anton et al., 1999). Glial cells express multiple integrin β subunits besides β 1 (Milner and Ffrench-Constant, 1994), and α _V can heterodimerize with several β subunits (reviewed in Hemler, 1999). We did not observe strong defects in neuron-glia interactions, suggesting that other β subunits are important to mediate these interactions or that several β subunits have redundant functions.

β 1-Class Integrins and Genetic Diseases that Lead to Cortical Malformations

Cortical defects similar to the ones described here have been observed in patients suffering from Lissencephaly type II, Muscle Eye Brain disease, Walker-Warburg syndrome, and Fukuyama Congenital Muscular Dystrophy. The pathology includes fusion of cortical hemispheres, lack of folia, defects in the meningeal basal lamina, and disrupted cortical layers. Some forms of cortical dysplasia can be accompanied by lamination defects in the eye and by muscular dystrophy (reviewed in Walsh, 1999). Lamination defects in the eye have been observed in mice carrying a targeted mutation in the integrin α 6 subunit gene and in double knockouts for α 3 and α 6 (Georges-Labouesse et al., 1998; De Arcangelis et al., 1999). Dystrophic symptoms are observed in muscle fibers lacking the integrin α 5 and α 7 subunits or the laminin α 2 chain (Mayer et al., 1997; Taverna et al., 1998; reviewed in Miyagoe-Suzuki et al., 2000). These findings suggest that perturbations in cell-ECM interactions are a common cause for the development of these diseases.

Experimental Procedures

Generation of β 1-flox Mice

An EcoRI/BamHI fragment containing the first coding exon of the integrin β 1 subunit gene was isolated from a YAC-vector (Genome Systems; Figure 1) and subcloned into a pGEM5 vector (Promega). A double-stranded oligonucleotide containing a PvuII site and a loxP site was inserted into the BstEII site 5' to the first coding exon, and a loxP-neo-IRES-tk-loxP cassette was inserted into the NheI site 3' of the same exon. ES cell transfection, selection, and screening was carried out as described (Müller et al., 1997), using the probe indicated in Figure 1. One positive clone was retransfected with a Cre-expressing plasmid (Gu et al., 1993), counterselected in ganciclovir, and clones that had retained two loxP sites flanking the first coding exon were identified by Southern blot. Germline-transmitting chimeras were generated as described (Müller et al., 1997).

Analysis of Cre-Mediated Recombination

DNA was isolated as described (Müller et al., 1997) and analyzed by PCR. The position of the PCR primers for the β 1 gene locus are indicated in Figure 1 (primers: 5'-GCCGCCACAGCTTTCTGCTGTAGG-3' and 5'-GGCCCTGCTGTATACATTCTCCGC-3'). The *nes-tin-Cre* transgene was identified by PCR (primers: 5'-GACATGTT CAGGGATCGCCAGGCG-3' and 5'-GCCGGTCGTGGGCGGATGG TGC-3'). PCR conditions were as follows: 5 min at 94°C, followed by 30 cycles (1 min, 94°C; 1 min, 60°C; 1 min, 72°C), and 10 min extension at 72°C. PCR reaction products were 2.1 kb (β 1-flox), 1.3 kb (β 1-flox recombined and β 1 null), and 0.6 kb (Cre).

Detection of the Integrin β 1 Subunit

In situ hybridization was carried out as described (Littlewood Evans and Müller, 2000). For detection of β 1 protein, the neural tubes of E12.5 and E15.5 embryos and the brains of P1 mice were dissected, proteins extracted and analyzed by Western blots (Müller et al., 1995), with a polyclonal antibody against β 1 (Tomaselli et al., 1990), and a secondary anti-rabbit antibody coupled to peroxidase (Jackson ImmunoResearch).

Histology

For histology, tissues were dissected, fixed overnight at 4°C in 4% paraformaldehyde in PBS or in Carnoy (60% ethanol, 30% chloroform, 10% acetic acid), embedded in paraffin, and sectioned at 8 μ m. Sections were stained with hematoxylin and eosin or with cresyl violet as described (Müller et al., 1997).

X-gal staining was performed as described (Farinas et al., 1996), either on whole-mount embryos or on 30–50 μ m cryosections after fixation in 2% paraformaldehyde. Meninges were dissected and fixed in 2% paraformaldehyde prior to staining. For staining of cells in culture, cerebellar cells were isolated (see below) and cultured for 4 days prior to fixation.

For BrdU labeling, pregnant mothers were injected intraperitoneally with 100 μ g BrdU/g body weight. Offspring were sacrificed at P4, their brains dissected, fixed in Carnoy, and processed for paraffin sectioning. Deparaffinized and rehydrated sections were treated with 4 N HCl for 10 min to denature DNA, neutralized for 5 min with 0.1 M sodium borate buffer (pH 8.5), followed by the addition of primary antibody (see below).

Cerebellar Cell Culture

Cerebellar cells were purified using a modification of the procedure described by Hatten et al. (1998). Cerebella were dissected, cut into small pieces, and incubated at 37°C for 5–10 min in digestion buffer consisting of EBSS-CMF (GIBCO-BRL), supplemented with 0.1% glucose, 1% trypsin, 0.1% DNase (Worthington), 1 mM MgSO₄, and 6 mM NaOH. The digestion buffer was replaced with BME (GIBCO-BRL) containing 0.05% DNase and 0.25% glucose, and the tissue was triturated using pipettes of decreasing bore size to obtain a single-cell suspension. Cells were harvested by centrifugation at room temperature, resuspended in EBSS containing 0.1% glucose, the cell suspension was passed through a cell strainer (Becton Dickinson) to remove debris, recentrifuged and resuspended in BME supplemented with glutamine, penicillin/streptomycin, 0.35% glucose, 10% horse serum, and 5% fetal calf serum. Cells were plated onto plastic dishes coated with 100 μ g/ml Poly-D-Lysine (Sigma) or Matrigel (Sigma) and were cultured in a humidified atmosphere at 37°C/5% CO₂. Cell numbers were determined by counting in a haemocytometer.

Immunohistochemistry and Electron Microscopy

Immunohistochemistry of dissociated neuronal cultures and histological sections was carried out as described (Müller et al., 1995; Littlewood Evans and Müller, 2000). For immunohistochemistry using the RC2 antibody (Misson et al., 1988), brains were freshly embedded in OCT compound (Tissue Tek), cryosectioned at 50 μ m, and treated for 10 min with Methanol at –20°C. Sections were blocked for 30 min in PBS⁺ (10% normal goat serum [NGS], 0.1% Triton X-100 in PBS), and incubated overnight at 4°C with RC2 antibody in PBS²⁺ (3% NGS, 0.1% Triton X-100 in PBS). For staining with anti-neurofilament (Campbell and Morrison, 1989) and anti-calbindin (Anderson et al., 1997; Chemicon) antibodies, mice were

perfused with 4% paraformaldehyde, the brains dissected and treated for cryosectioning as described above. For staining with antibodies to laminin (kindly provided by L. Sorokin), calretinin (Weisenhorn et al., 1994; Chemicon), reelin (G4; de Bergueyck et al., 1999), NeuN (Mullen et al., 1992; Chemicon), GFAP (Bignami et al., 1972; DAKO), and BrdU (PharMingen), brains were fixed in Carnoy and processed for paraffin sectioning. Deparaffinized and rehydrated sections were blocked for 30 min at room temperature with PBS⁺ and incubated overnight at 4°C or for 2 hr at room temperature with primary antibodies in PBS²⁺. For immunofluorescence, sections were washed in PBS and incubated for 1 hr at room temperature with FITC-, or TRITC-labeled secondary antibodies (Jackson ImmunoResearch) and DAPI (Hoechst No. 33342; Sigma). Sections were washed in PBS and mounted with Vectashield (Vector Labs) or Mowiol (Calbiochem). Images were collected on a Deltavision System. In experiments with anti-neurofilament or anti-calbindin antibodies, detection was performed with peroxidase-coupled secondary antibodies and the Vectastain ABC kit (Vector Laboratories).

For electron microscopy, tissues were fixed with 2.5% glutaraldehyde in 0.03 M potassium-phosphate buffer (pH 7.4, 370 mOsm) by perfusion of the blood vessels (Burri, 1974). Samples were postfixed in 0.1 M sodium cacodylate (pH 7.4, 340 mOsm), containing 1% OsO₄, dehydrated in a graded series of ethanol, and embedded in Epon 812. Sections of ~80 nm thickness were cut, picked up on Formvar-coated copper grids, double stained with lead citrate (Reynolds, 1963) and uranyl acetate (Frasca and Parks, 1965), and viewed in a Philips 400 electron microscope.

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