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Basement membrane attachment is dispensable for radial glial cell fate and for proliferation, but affects positioning of neuronal subtypes

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Radial glial cells have been shown to act as neuronal precursors in the developing cortex and to maintain their radial processes attached to the basement membrane (BM) during cell division. Here, we examined a potential role of direct signalling from the BM to radial glial cells in three mouse mutants where radial glia attachment to the BM is disrupted. This is the case if the nidogen-binding site of the laminin γ1 chain is mutated, in the absence of α6 integrin or of perlecan, an essential BM component. Surprisingly, cortical radial glial cells lacking contact to the BM were not affected in their proliferation, interkinetic nuclear migration, orientation of cell division and neurogenesis. Only a small subset of precursors was located ectopically within the cortical parenchyma. Notably, however, neuronal subtype composition was severely disturbed at late developmental stages (E18) in the cortex of the laminin γ1III4$^{-/-}$ mice. Thus, although BM attachment seems dispensable for precursor cells, an intact BM is required for adequate neuronal composition of the cerebral cortex.

KEY WORDS: Mouse, Basement membrane, Laminin, Cerebral cortex, *Lamc1*, *Itaga6*, *Hspg2*

INTRODUCTION

The morphology of radial glial cells is one of their defining characteristics. Radial glial cells have their somata in the ventricular zone (VZ) and extend long radial processes throughout the wall of the neural tube, attaching via their endfeet to the basement membrane (BM). These radial processes are used as guiding structures by neurons to migrate from their place of birth in the VZ or subventricular zone (SVZ) towards their final position in the cortical plate (CP) (Rakic, 2003). If radial glial processes are altered, as in the *reeler* (Frotscher et al., 2003; Hartfuss et al., 2003; Tissir and Goffinet, 2003) or *Pax6* mutant mice (Caric et al., 1997; Götz et al., 1998), neuronal migration is affected.

However, radial glial cells also act as precursor cells (Malatesta et al., 2003; Malatesta et al., 2000; Noctor et al., 2001), but the role of the radial process in this regard is less clear. Radial glial cells maintain their radial process during cell division (Miyata et al., 2001; Miyata et al., 2004), and the inheritance of the radial process to only one daughter cell may be important in cell fate decisions, as signals from the BM would be perceived only by the cell inheriting the radial process (Fishell and Kriegstein, 2003). While Fishell and Kriegstein (Fishell and Kriegstein, 2003) suggested that the cell inheriting the radial process is and remains a radial glial cell, Miyata and colleagues also observed some cells that maintain the radial process and develop into postmitotic neurons. Thus, the supposed asymmetric inheritance of the radial glia process highlights its potential importance, but the role of BM signalling via the radial glia process for the fate and proliferation of radial glia cells has never been examined.

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The BM is a thin sheet of extracellular matrix (ECM) composed mainly of type IV collagen, nidogen, members of the laminin family and heparan sulphate proteoglycans, such as perlecan and agrin (Erickson and Couchman, 2000; Paulsson, 1992; Timpl, 1996), and is enriched with a variety of growth factors (e.g. Colognato and ffrench-Constant, 2004; Mott and Werb, 2004). Integrins integrate signalling via components of the ECM as well as via growth factors (Colognato and ffrench-Constant, 2004); targeted deletion of either β1 or α6 integrin or the β1 integrin cytoplasmic tail binding protein integrin-linked kinase (ILK) abolishes the attachment of radial glial endfeet to the BM and thereby also disrupts the maintenance of BM integrity (Beggs et al., 2003; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001; Halfter et al., 2002; Mills et al., 2006; Niewmierzycka et al., 2005). Thus, radial glia and later astrocyte endfeet contribute to the formation and maintenance of the BM by integrin-mediated binding. Rupture of the BM then causes type II cobblestone lissencephaly, with cortical neurons protruding into the subarachnoid space (Beggs et al., 2003; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001; Halfter et al., 2002; Niewmierzycka et al., 2005) (see also Blackshear et al., 1997; Costa et al., 2001; Hartmann et al., 1999; Herms et al., 2004). However, it has not been examined to what extent the lack of contact between radial glia endfeet and the BM affects cell proliferation or fate of radial glial cells themselves. Here, we have used several mouse mutants with defects in the glial endfeet attachment to the BM to assess the functional role of BM contact for radial glial cells.

The targeted deletion of the nidogen-binding site within the laminin γ1 chain, γ1III4, results in nidogen depletion from the BM with disintegration and rupture of the BM in the lung, kidney and brain (Halfter et al., 2002; Willem et al., 2002). As previously shown by DiI-tracing of radial glial cells, most of their processes are no longer connected to the BM in the cortex of this mouse mutant (Halfter et al., 2002). A similar phenotype of ruptured BM was also observed in the cortex of α6 integrin$^{-/-}$ mice (Georges-Labouesse et al., 1998). However, as α6β1 integrin binds to laminin that is also present within the intermediate and ventricular zones of the developing cortex (Campos et al., 2004; Liesi, 1985; Sheppard et al.,

1995), defects may also arise within the cortical parenchyma of the α6 integrin$^{-/-}$ mice. We therefore examined a further mouse mutant with deletion of a molecule restricted to the BM, perlecan$^{-/-}$ (Costell et al., 1999) (see also Arikawa-Hirasawa et al., 1999).

MATERIALS AND METHODS

Animals

Laminin γ1III4 (Willem et al., 2002) heterozygous mice were kept on a SV129 background, α6 integrin heterozygous mice (Georges-Labouesse et al., 1998) on C57Bl/6/SV129 background and perlecan heterozygous mice (Costell et al., 1999) on C57Bl/6 background. Crossing of heterozygous mice [the day of the vaginal plug is considered embryonic day (E) 0] allowed to examine wild-type, heterozygous ($^{+/-}$) and homozygous mutant embryo ($^{-/-}$) littermates.

Immunohistochemistry and in-situ hybridization

Embryonic brains were fixed in 4% paraformaldehyde in phosphate-buffered-saline (PBS) and 12 μm frontal sections were cut with a cryostat after cryoprotection. Sections were immunostained using the primary antibodies against the phosphorylated form of Histone H3 (PH3) (rabbit (rbt); Biomol; 1:200), BrdU (mouse IgG1, 1:10, Bioscience Products), calbindin (rbt, 1:2000, SWANT), calretinin (rbt, 1:2000, SWANT), Ki67 (rat Tec-3, 1:50, Dako), pan-Laminin (rbt, 1:50, BD), βIII-Tubulin (mouse IgG2a, 1:100, Sigma), O4 (mouse IgM, 1:1000, kindly provided by Jack Price), GFAP (mouse IgG1, 1:200, Sigma), RC2 (mouse IgM, 1:500, kindly provided by P. Leprince), BLBP (rbt, 1:1500, kindly provided by Nathaniel Heintz), nestin (mouse IgG1, 1:4, Dev. Hybridoma Bank) and reelin (E4, mouse IgG1, 1:500, kindly provided by André Goffinet). The respective secondary antibodies were from Southern Biotechnology Associates and Jackson ImmunoResearch. Specimens were mounted in Aqua Poly/Mount (Polysciences, Northampton, UK) and analysed with a Confocal Microscope (Leica TCS 4NT; Olympus FV 1000) and with the Zeiss Axiophot using the Neurolucida System and the Apotom System. Cell nuclei were visualized by propidium-iodide staining (PI). In situ hybridization was performed as described (Chapouton et al., 2001), and the probes for Cux2, Rorb (RORβ), Math2 and Er81 were obtained from C. Schuurmans (Schuurmans et al., 2004).

Quantification

All quantifications were performed on frontal sections of wild type and the respective mutant littermate telencephali at rostral, intermediate and caudal levels.

Two different approaches were used to quantify the PH3-positive cells: In one set of experiments, we counted the number of PH3-positive cells at the ventricular surface (VS) and at abventricular positions (PH3-positive cells located five or more cell diameters from the VS) (see also Haubst et al., 2004) per section and calculated the percentages of PH3-positive cells in the VZ and at abventricular positions (SVZ) (Fig. 2C). In a second set of experiments, the neocortical area was outlined using the neurolucida system to determine the size of the quantification area. In this area, the numbers of PH3-positive cells at the ventricular surface and at abventricular positions were then quantified and calculated as PH3-positive cells per 100 μm^2 (Fig. 2D) at E14.

For the analysis of neurogenesis the radial width of the entire cortex (Ctx) and the band of the Math2-positive neurons delineating the cortical plate (CP; Fig. 3C) was measured with the neurolucida system and the ratio (CP:Ctx) was calculated.

The angles of cell division in late ana- and telophase were measured at the ventricular surface using Image J as described by Stricker et al. (Stricker et al., 2006). All values are given ±standard deviation (s.d.) and unpaired Student's *t*-test was used to test for significance.

RESULTS

Degree of BM disruption and radial glia endfeet detachment

First, we examined the degree of BM rupture and loss of radial glia endfeet attachment in the cortex of the three different mouse mutants at embryonic day (E) 14, the peak of radial glia-mediated neurogenesis. Immunohistochemistry against laminin, a major component of the BM, revealed the most severe phenotype in BM disruption in the laminin γ1III4$^{-/-}$, with widespread disruptions of the BM and the laminin staining along the blood vessels strongly reduced in the cortex of mutant mice (Fig. 1A,B,a,b). Notably, the partial BM disruptions in the laminin γ1III4$^{-/-}$ cortex analysed in this study appeared slightly more widespread than in the previous analysis, presumably owing to background differences [Halfter et al. (Halfter et al., 2002) performed their analysis with mice of a mixed background, whereas the mice in this analysis were on a pure SV129 background, see Materials and methods]. Among the 20 perlecan$^{-/-}$ embryos, only one did not suffer from exencephali (see also Costell et al., 1999) (see Fig. S2A,B in the supplementary material) and this hemisphere had partial disruptions of the BM (Fig. 1C,c). In the α6 integrin$^{-/-}$ cortex the outer layer of the BM had only small punctuate disruptions, whereas the BM directly overlying the neuroepithelium was largely absent (Fig. 1D,d). These defects of the BM in the perlecan$^{-/-}$ and α6 integrin$^{-/-}$ cortex are consistent with previous electron microscopic observations of disruptions in the BM (Georges-Labouesse et al., 1998; Costell et al., 1999).

We further examined the localization of radial glial processes and endfeet by immunolabelling of the brain-lipid-binding protein (BLBP), a molecule contained in the cytoplasm of radial glial cells (Hartfuss et al., 2001). In wild-type cortex, BLBP immunoreactivity visualizes the radial glia endfeet as a continuous band underlying the pial membranes (Fig. 1E,G), whereas in large regions of the E14 laminin γ1III4$^{-/-}$ cortices, no such band was detectable (Fig. 1F-H). Double-labelling with RC2 (Hartfuss et al., 2001) further revealed large regions with few or no RC2-positive radial glia processes in contact with the pial surface (Fig. 1H), even though mesenchymal cells that are also RC2-immunoreactive were still visible within the pial cell layers (Fig. 1F,H). Only few short disorganized processes immunoreactive for BLBP or RC2 of ectopic clusters of precursors (see below) were detected within the CP of laminin γ1III4$^{-/-}$ cortices (Fig. 1H), while the radial organization of radial glia processes was still maintained within the intermediate and ventricular zones (Fig. 1E-H). These data, together with the previous data (Halfter et al., 2002), clearly demonstrate that the widespread lack of pial endfeet of radial glial cells is due to the broad disruptions of the BM overlying the cerebral cortex by midneurogenesis E14 in the laminin γ1III4$^{-/-}$ cortex (Fig. 1B,b). Similarly, radial glia endfeet were virtually absent in the medial perlecan$^{-/-}$ cortex (Fig. 1I), whereas they appeared less disrupted in the lateral cortex of these mice (data not shown). In the E14 α6 integrin$^{-/-}$ cortex, gaps in radial glial endfeet lining the surface were visible (Fig. 1J).

Ectopic precursor clusters in the cortex of laminin γ1III4$^{-/-}$

Given the severity of the BM phenotype in the cortex of laminin γ1III4$^{-/-}$ we focused our analysis of proliferation and cell fate on this mutant. We first noted BLBP- and RC2-immunopositive cell somata within the cortical plate of the laminin γ1III4$^{-/-}$ (Fig. 1F,H, arrows) that were never observed in the cortex of wild-type littermates where all radial glia somata were located in the VZ (Fig. 1E). These clusters of ectopic BLBP-immunopositive cells continued to divide, as evident from immunostaining against the phosphorylated form of histone H3 (PH3) present in the G2/M-phase of the cell cycle (Hendzel et al., 1997), and against Ki67, an antigen present in all phases of the cell cycle in actively dividing precursors (Gerlach et al., 1997) (Fig. 2A,B). These ectopic

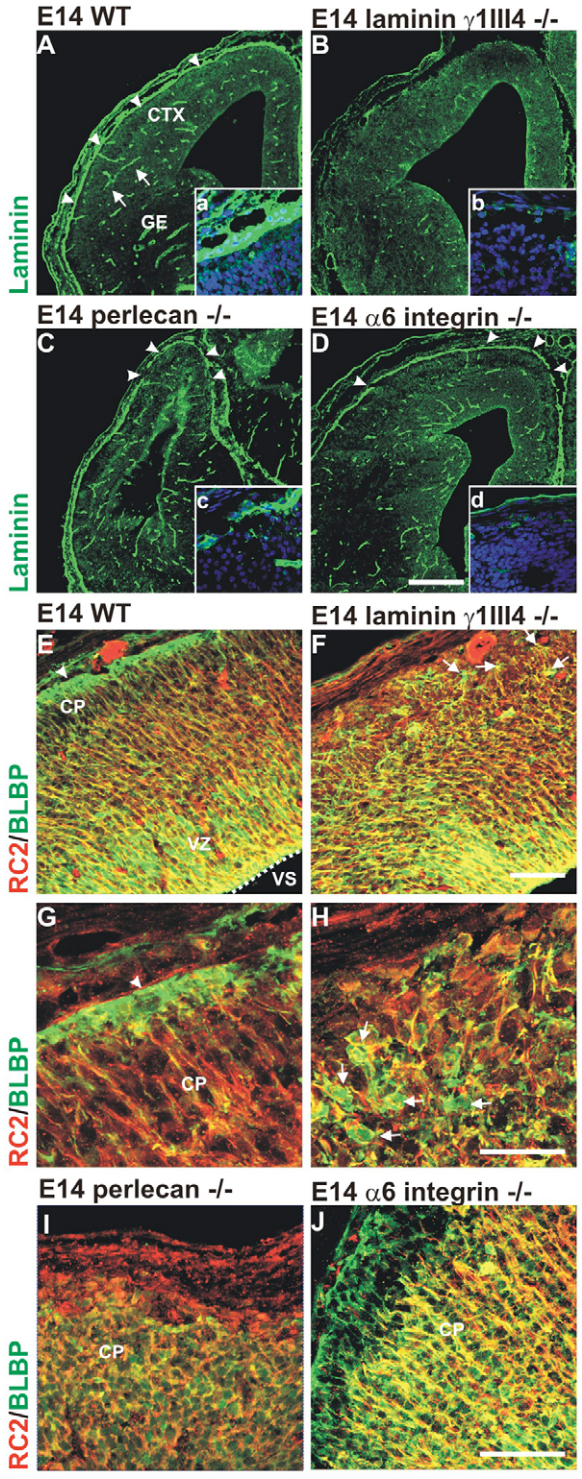

Fig. 1. Basement membrane disruption and radial glial endfeet detachment in laminin γ1III4$^{-/-}$, perlecan$^{-/-}$ and α6-integrin$^{-/-}$ cortex at midneurogenesis. Fluorescent micrographs of immunostained (as indicated in the panels) frontal sections of telencephali of embryonic day (E) 14 wild-type and mutant littermates. (**A-D**) Laminin immunoreactivity underneath the pial surface (arrowheads in A) and surrounding the blood vessels (arrows in A). Insets (a-d) depict representative high-power views of the BM in the respective mouse line. Arrowheads in C,D indicate disruptions of the BM. (**E-J**) The absence of BLBP-immunoreactive radial glia endfeet (arrowhead in E,G) in the laminin γ1III4$^{-/-}$ (F,H), perlecan$^{-/-}$ cortex (I) and α6 integrin$^{-/-}$ cortex (CTX; J) compared with wild type (E,G). Ectopic BLBP-positive cell somata in the laminin γ1III4$^{-/-}$ cortical plate (CP) (arrows in F,H). The broken white line (E) indicates the ventricular surface (VS). CTX, cerebral cortex; GE, ganglionic eminence; VZ, ventricular zones. Scale bars: 200 μm in A-D; 50 μm in E,F; 25 μm in G,H; 100 μm in I,J.

precursor cells in the CP of laminin γ1III4$^{-/-}$ cortices also contained Pax6 (data not shown), which is normally expressed only in neuroepithelial/radial glial precursors in the VZ but not in SVZ precursor cells (Englund et al., 2005; Götz et al., 1998). Consistent with their BLBP and Pax6 immunoreactivity, these ectopically dividing precursors also contained other radial glial markers, such as RC2 or nestin (see Fig. S1 in the supplementary material). They continued to divide until E18 but did not acquire the astroglial or oligodendroglial markers GFAP, O4 or NG2, or neuronal markers (βIII-tubulin, NeuN).

Cell division and interkinetic nuclear migration in the cortex of laminin γ1III4$^{-/-}$

To determine the extent of precursor ectopia in the cortex of the laminin γ1III4$^{-/-}$ mice, we quantified the proportion of precursors dividing at ectopic positions (misplaced proliferating cells in the CP), at the ventricular surface (VZ precursors) or in the SVZ. The SVZ was defined as a band of mitoses not occurring at the ventricular surface (abventricular), but located below the CP and at least five cell diameters distant from the ventricular surface (VS, see Materials and methods) (Haubst et al., 2004). This definition was required to discriminate the abventricular mitoses occurring in the SVZ from those taking place ectopically in the CP. Although in wild-type cortex virtually no dividing cells were observed within the CP (Fig. 2C; 0,6%), they constitute 4.6% of all precursors in the laminin γ1III4$^{-/-}$ cortex (Fig. 2C). Besides this small proportion of ectopically dividing precursors in the laminin γ1III4$^{-/-}$ cortex, the number and proportion of VZ and SVZ precursor cells was not affected (Fig. 2C,D). Because also at later developmental stages (E16) no significant changes in the percentages of cells dividing at the VS or at SVZ positions were observed between wild-type (65.2±8.3% PH3-positive cells dividing at the VS; 34.8±8.3% PH3-positive cells dividing at SVZ position, 22 sections, one animal) and laminin γ1III4$^{-/-}$ cortex (66.9±6.6% PH3-positive cells dividing at the VS; 29.9±7.0% PH3-positive cells dividing at SVZ position, 22 sections, 1 animal), we conclude that proliferation of radial glia cells seems not to be affected by the loss of the BM contact.

A crucial difference between VZ and SVZ precursors is that only the former undergo interkinetic nuclear migration with the nucleus migrating towards basal positions for S phase and then moving back apically to undergo M phase and cytokinesis (Sauer, 1935) [for recent review, see Götz and Huttner (Götz and Huttner, 2005)]. As attachment of the radial glial process to the BM may be crucial as anchoring point to allow interkinetic nuclear migration, we examined interkinetic nuclear migration in the laminin γ1III4$^{-/-}$ cortex by labelling cells in S and M phase of the cell cycle. Injection of the DNA-base analogue 5-bromo-2′-deoxyuridine (BrdU) 0.5 hour prior to sacrifice labels cells in S phase and resulted in a band of BrdU-labelled cells at the basal surface of the VZ, where S phase takes place in both wild-type and laminin γ1III4$^{-/-}$ cortex (Fig. 2E,F). At this time, no cells in S phase (BrdU positive) were co-labelled with PH3, which is

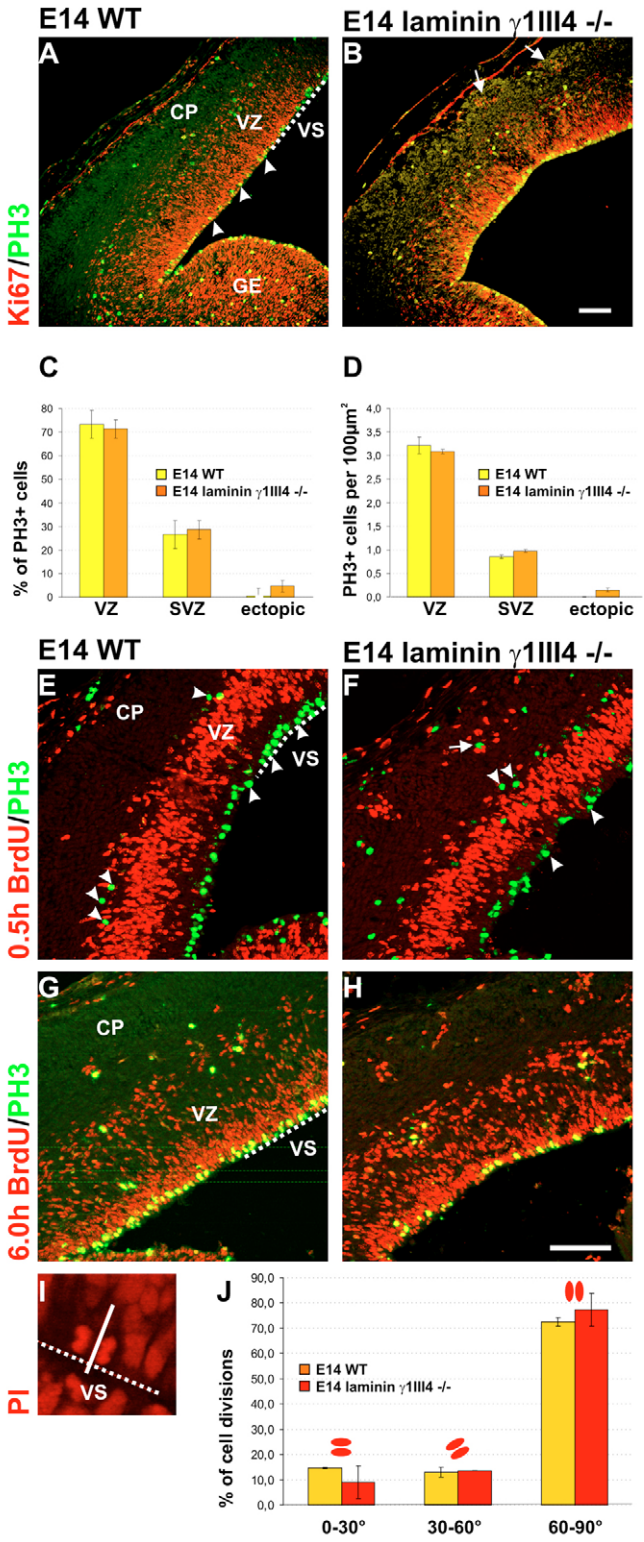

Fig. 2. Proliferation, interkinetic nuclear migration and orientation of cell division in wild-type and laminin γ1III4$^{-/-}$ cortex at midneurogenesis. Fluorescent micrographs of E14 wild-type and laminin γ1III4$^{-/-}$ cortex immunostained as indicated (**A**,**B**,E-H) reveal similar numbers of precursors (Ki67-positive) in mitosis (PH3-positive) at the ventricular surface (VS, arrowheads in A) or at abventricular positions. Ectopic clusters of precursor cells within the laminin γ1III4$^{-/-}$ cortical plate (CP) are indicated by arrows in B. (**C**) Histogram depicting the percentages of precursors dividing at the ventricular surface (VZ in C), subventricular zone (SVZ) or at ectopic positions assessed by the quantification of PH3-positive cells at E14 (wild type: *n*=40 sections, two animals, laminin γ1III4$^{-/-}$: *n*=45, two animals). (**D**) Histogram depicting the number of PH3-positive cells per cortex area (100 μm^2) dividing at VZ or SVZ positions respectively (wild type: *n*=43, two animals; laminin γ1III4$^{-/-}$: *n*=51, two animals). (**E-H**) BrdU-immunostaining (red) reveals cells in S phase (0.5 hours after BrdU-injection; E,F) and 6 hours after S-phase labelling when they have moved towards the ventricular surface (G,H). (**I**) A dividing cell in anaphase labelled with propidium iodide. The angle of cell division was assessed by measuring the angle between a line at the VS and the separating chromatids. (**J**) Histogram depicting the percentages of cells dividing horizontally with respect to the VS (0-30°), obliquely (30-60°) and perpendicularly (60-90°) in E14 cortex (wild type: *n*=103 mitoses, two animals; laminin γ1III4$^{-/-}$: *n*=110, two animals). Scale bars: 100 μm

contained in cells in G2/M phase (Fig. 2E,F, arrowheads). However, most PH3-positive cells in G2/M-phase were also BrdU positive 6 hours after the injection and BrdU-labelled nuclei had progressed towards the apical surface to undergo M phase in both the cortices of wild-type and laminin γ1III4$^{-/-}$ littermates (Fig. 2G,H). Thus, to our surprise, interkinetic nuclear migration occurs normally in the absence of radial glia attachment to the BM. Moreover, this analysis showed that there is no change in the total number of cells in S phase (Fig. 2E,F) nor in the progression from S to M phase in the laminin γ1III4$^{-/-}$ cortex, suggesting that cell cycle progression of radial glia cells occurs normally, despite the absence of BM attachment.

Orientation of cell division in the cortex of laminin γ1III4$^{-/-}$ mice

Next, we examined if the absence of BM attachment may randomize the orientation of cell division at the ventricular surface. The orientation of cell division was determined at the end of M phase in late anaphase and telophase of the cell cycle to avoid further changes in the spindle rotation (Haydar et al., 2003) (see Materials and methods, example in Fig. 2I) (see also Chenn and McConnell, 1995; Estivill-Torrus et al., 2002; Heins et al., 2001; Stricker et al., 2006). As depicted in Fig. 2J, cells dividing at the apical surface were classified in three groups dividing: (1) with an angle of 0-30°, i.e. parallel, with respect to the VS, normally resulting in an asymmetric cell division; (2) with an oblique angle of 30-60° with respect to the VS, still considered as asymmetrically dividing cells; and (3) with an angle vertical to the VS (60-90°), an orientation that may result in symmetric or asymmetric cell division (Chenn and McConnell, 1995; Haydar et al., 2003; Kosodo et al., 2004; Noctor et al., 2002). Notably, no differences were observed in the orientation of apical cell divisions in wild-type and laminin γ1III4$^{-/-}$ cortices at E14 (Fig. 2J), suggesting that anchoring of the basal process at the BM is not required for proper orientation of cell division.

Neurogenesis in the cortex of laminin γ1III4$^{-/-}$ mice

Next, we examined whether the loss of BM attachment may influence the fate of radial glia progeny. To assess the number of neurons, we immunostained for the neuronal antigens βIII-Tubulin and MAP2, but no obvious differences in the thickness of the band of neurons were visible between the cortices of wild-type and mutant

littermates at E14 (Fig. 3A,B), with the exception of some neuron-free areas in the CP of laminin γ1III4$^{-/-}$ mice corresponding to the ectopic clusters of precursors described above (Fig. 3B, arrow). In order to detect subtle changes in neurogenesis, we quantified the thickness of the band of neurons forming the CP (Materials and methods) (see also Haubst et al., 2004) by in situ hybridization for Math2 (Neurod6 – Mouse Genome Informatics) (Schuurmans et al., 2004), a bHLH gene expressed in glutamatergic cortical neurons (Fig. 3C,D). The ratio of the radial thickness of the Math2-positive CP to the total radial width of the cortex (Ctx) (Fig. 3C) did not reveal any significant difference between wild-type and laminin γ1III4$^{-/-}$ cerebral cortices at E14 [wild-type ratio CP:Ctx lateral=0.44±0.24, medial=0.48±0.09, *n*=13 sections, one animal; laminin γ1III4$^{-/-}$ ratio CP:CTX lateral=0.30±0.5; medial=0.41±0.06, *n*=7 sections, one animal; *P*(ratio lateral)=0.17, *P*(ratio medial)=0.06], suggesting that neurogenesis still occurs normally even after loss of radial glia attachment to the BM.

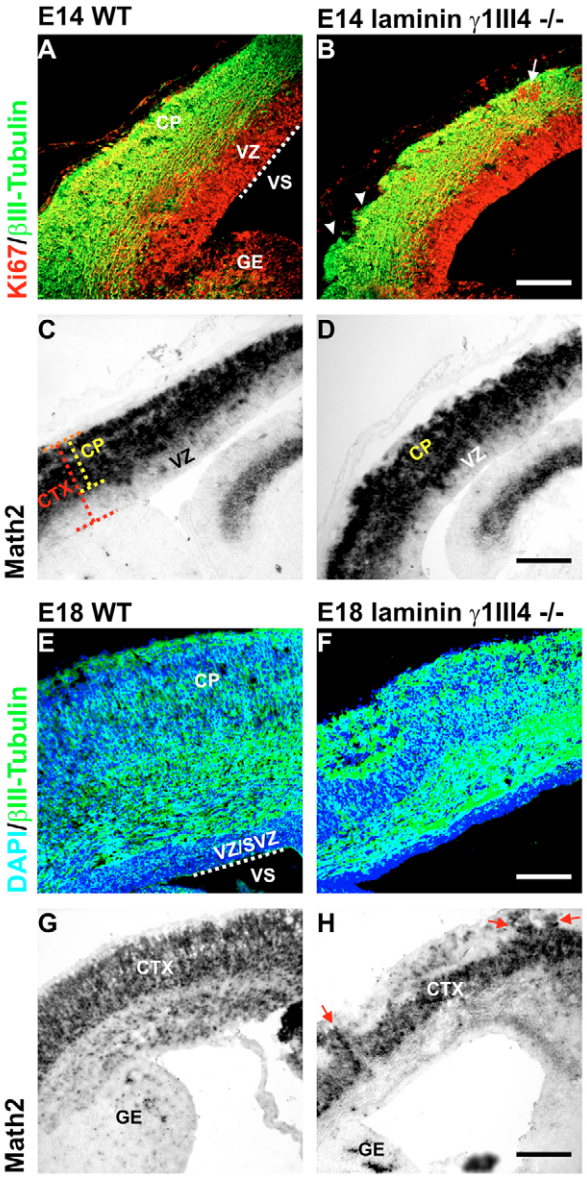

Fig. 3. Neurogenesis in wild-type and laminin γ1III4$^{-/-}$ cortex. (**A-H**) E14 or E18 wild-type and laminin γ1III4$^{-/-}$ cortex sections stained for the neuronal antigen βIII-Tubulin or Math2 mRNA. Arrowheads in B and red arrows in H indicate neuronal ectopias in the subarachnoidal space, arrows in B indicate ectopic clusters of precursors in the laminin γ1III4$^{-/-}$ cortex. The broken yellow and red lines in C delineate the cortical plate thickness in relation to the cortex thickness. Math2 expression is absent in the upper layers of the laminin γ1III4$^{-/-}$ cortex (H), while these neurons still contain βIII-Tubulin (F). Scale bars: 100 μm for A,B,E,F; 200 μm for C,D,G,H.

Neuronal migration and differentiation at late stages in the cortex of laminin γ1III4$^{-/-}$ mice

However, when we examined neuronal markers at E18, we observed a loss of Math2 expression in the outer cortical layers of laminin γ1III4$^{-/-}$ mice (Fig. 3G,H). The width of the Math2-positive CP as ratio of the total cortex width was significantly reduced at this stage [wild-type ratio CP:CTX lateral=0.47±0.09, *n*=40 sections, two animals, medial=0.47±0.06, *n*=27 sections, two animals; E18 laminin γ1III4$^{-/-}$ ratio CP:CTX lateral=0.42±0.09, *n*=53 sections, two animals, medial=0.38±0.12, *n*=43 sections, two animals; *P*(lateral)=0.00046; *P*(medial)=0.0029], while pan-neuronal markers such as βIII-Tubulin were still present (Fig. 3E,F). Thus, some neurons located below the pial surface are not pyramidal neurons that normally express Math2. In order to examine whether they may have other features of cortical pyramidal neurons, we analysed the mRNA for *Cux2*, *Rorb* (which labels upper layer neurons) and *Er81* (Etv1 – Mouse Genome Informatics) (which is expressed in layer V neurons of the cortex) (Schuurmans et al., 2004). Interestingly, *Cux2* and *Rorb* mRNA signal was detected at deeper positions in the cortex of the laminin γ1III4$^{-/-}$ than in wild-type cortex (Fig. 4A-D, red arrow) suggesting that upper layer neurons fail to migrate towards their appropriate layer position. In fact, they seem to locate at the same position as layer V neurons, labelled by Er81 (Fig. 4E,F). Although these data are in agreement with the defects in radial migration in the cortex of mouse mutants with BM disruptions (see Discussion), they still did not reveal the identity of the βIII-tubulin-positive neurons located in the outer part of the cerebral cortex in laminin γ1III4$^{-/-}$ mice.

In a further attempt to identify the subtype of these neurons, we examined *Gad65* mRNA, as well as calbindin- and calretinin-immunolabelling (Fig. 4G-J) to detect GABAergic interneurons. To our surprise, we noted that Gad65- (data not shown), calretinin- and calbindin-positive cells were concentrated in the outer cortical layers of the laminin γ1III4$^{-/-}$ cortex, whereas few of these interneurons were detected at this position in the wild-type cortex (Fig. 4G,I). Calretinin-positive neurons that also contain reelin (Fig. 4G′) are located in layer 1 of the wild-type cortex and are generated at very early stages in cortical development (Stoykova et al., 2003). These neurons were still few in number and scattered in the laminin γ1III4$^{-/-}$ cortex (Fig. 4G,G′,H,H′). These data therefore suggest that neurons in the outer part of the E18 laminin γ1III4$^{-/-}$ cortex are to a large extent GABAergic interneurons containing also calbindin or calretinin.

Notably, at this stage the mutant cortex was also significantly thinner than its wild-type counterpart (11% reduction at lateral cortex; E18 wild-type *n*=40 sections, two animals; E18 laminin γ1III4$^{-/-}$ *n*=53 sections, two animals; $P=1.85\times10^{-5}$). These defects were strongest in the caudal and lateral regions of the cortex, while the medial cortex of laminin γ1III4$^{-/-}$ mice was not significantly thinner than in wild-type mice (*P*=0.21). Although thinner, the E18 laminin γ1III4$^{-/-}$ cortex was longer, as measured by the total length of the ventricular surface from the sulcus delineating cortex and GE to the medial sulcus (60% increase; E18 wild type *n*=21 sections, two animals; E18 laminin γ1III4$^{-/-}$ *n*=32

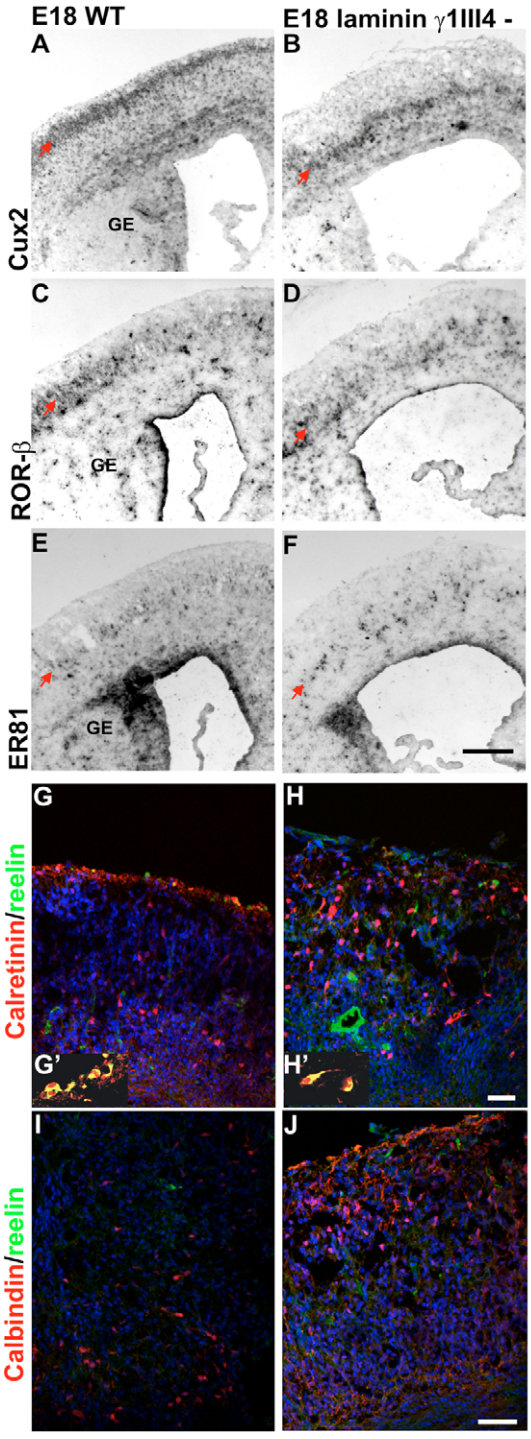

Fig. 4. Neuronal subtypes in the E18 laminin γ1III4$^{-/-}$ cortex. Micrographs of in situ hybridization for the layer-specific mRNAs of Cux2, Rorb (RORβ) and Er81 (**A-F**), and immunostaining for interneurons (**G-J**) in E18 wild-type and laminin γ1III4$^{-/-}$ cortex. *Cux2*- and *Rorb*-expressing layer II-IV neurons are misplaced to the position of Er81-positive deep layer neurons in the laminin γ1III4$^{-/-}$ cortex (E,F). The red arrows indicate the layer specific gene expression. Conversely, GABAergic interneurons containing calretinin (red in G,H) or calbindin (red in I,J) were detected in the outer part of the laminin γ1III4$^{-/-}$ cortex (H,J) in contrast to their scattered position in the wild-type cortex (G,I). Early born reelin-immunoreactive cells (green in G-J) are mostly calretinin positive (G′,H′) and are not increased in the laminin γ1III4$^{-/-}$ cortex (H) compared with wild type (G). Scale bars: 200 μm in A-F; 50 μm in G-J.

sections; two animals; P=0.0002). This thinning and extension of the cortex may be due to a reduced force normally exerted by the pial BM counteracting the pressure of the ventricular fluid or to the failure of radial migration of late generated neurons, or may be related to the overall smaller size of the laminin γ1III4$^{-/-}$ embryos. Indeed, layer II-IV and V neurons colocalize at the same position (Fig. 4B,D,F), although they are normally located on top of each other in wild-type cortex (Fig. 4A,C,E). Taken together, neuronal migration and the overall cortex architecture are severely distorted at the end of neurogenesis after disruption of the BM and radial glia attachment.

At these late embryonic stages, the loss of BM integrity also resulted in severe bleeding (see also Halfter et al., 2002). However, no significant changes in cell death were detectable at E18 in the laminin γ1III4$^{-/-}$ cortex (1.33 activated caspase 3-positive cells per section, n=6, one animal; 1.66 TUNEL-positive cells per section, n=6, one animal) compared with wild type (1.25 activated caspase 3-positive cells per section, n=8, one animal; 0.67 TUNEL-positive cells per section; n=6, one animal; P(activated caspase 3)=0.90; P(TUNEL)=0.12), nor at the earlier stage E14 (laminin γ1III4$^{-/-}$: 0.67 activated caspase 3-positive cells per section, n=18, one animal; wild type: 0.38 activated caspase 3-positive cells per section, n=16, one animal; P=0.26).

Neurogenesis and cell proliferation in the cortex of α6 integrin$^{-/-}$ mice

In order to ensure the general relevance of the above findings, we also examined radial glia cell proliferation and neurogenesis in the α6 integrin$^{-/-}$ cortex as described above. The thickness of the ventricular zones labelled by Ki67 or BrdU immunostaining appeared comparable between wild-type and the α6 integrin$^{-/-}$ littermate cortex (Fig. 5A,B,E,F). This similarity was further substantiated by the equal number of PH3-immunopositive cells at the VS or in the SVZ in wild-type and mutant cortex (Fig. 5A-C), suggesting that proliferation and the proportion of VZ and SVZ precursors are not affected by the absence of α6 integrin. No ectopic clusters of proliferating cells were visible in the CP of this mutant, in contrast to the laminin γ1III4$^{-/-}$. In addition, the orientation of cell division was comparable between wild-type and α6 integrin$^{-/-}$ littermates (Fig. 5D) and the specific loss of α6 integrin did not lead to significant changes in the interkinetic nuclear migration assessed as described above (Fig. 5A,B). Neurogenesis also occurred normally in the absence of α6 integrin, as revealed by immunostaining for βIII-Tubulin (Fig. 5E,F), Map2 (not shown) and Math2 (Fig. 5G,H). These data therefore suggest that α6 integrin-mediated signalling to the radial glial cells does not affect cell division, proliferation or neurogenesis.

Neurogenesis and cell proliferation in the cortex of perlecan$^{-/-}$ mice

As described above, most perlecan$^{-/-}$ embryos (Costell et al., 1999) exhibited exencephali (19 of 20 E14 embryos, consistent with previous observations; see Fig. S2A,B in the supplementary material). As we had to exclude these from our analysis because exencephaly itself may exert many influences on brain development, we could analyse only one cortical hemisphere for proliferation and neurogenesis. Despite severe cobblestone type II neuronal ectopia in this cortex (arrows in see Fig. S2D in the supplementary material), no obvious defects in the band of proliferating cells, in the number of neurons (see Fig. S2C,D in the supplementary material) or in the orientation of cell division (see Fig. S2E in the supplementary material) were detectable in the perlecan$^{-/-}$ cortex.

DISCUSSION

Here, we examined the role of the direct contact of radial glial processes to the BM. Our data show that loss of this contact and/or the lack of the laminin-receptors containing α6 integrin do not affect radial glia proliferation nor their neurogenic progeny in the developing cerebral cortex. Furthermore, radial glial cells without BM anchoring perform normal interkinetic nuclear migration and divide with normal orientations, suggesting that BM attachment of the basal process is not required for any of these processes. However, besides the cobblestone neuronal ectopia below the pial surface in the laminin γ1III4$^{-/-}$ cortex, we also observed some precursors at ectopic positions within the cortical parenchyma and ectopic GABAergic neurons in the outer cortical layers around birth, demonstrating the need of an intact BM for neuronal migration and possibly maturation.

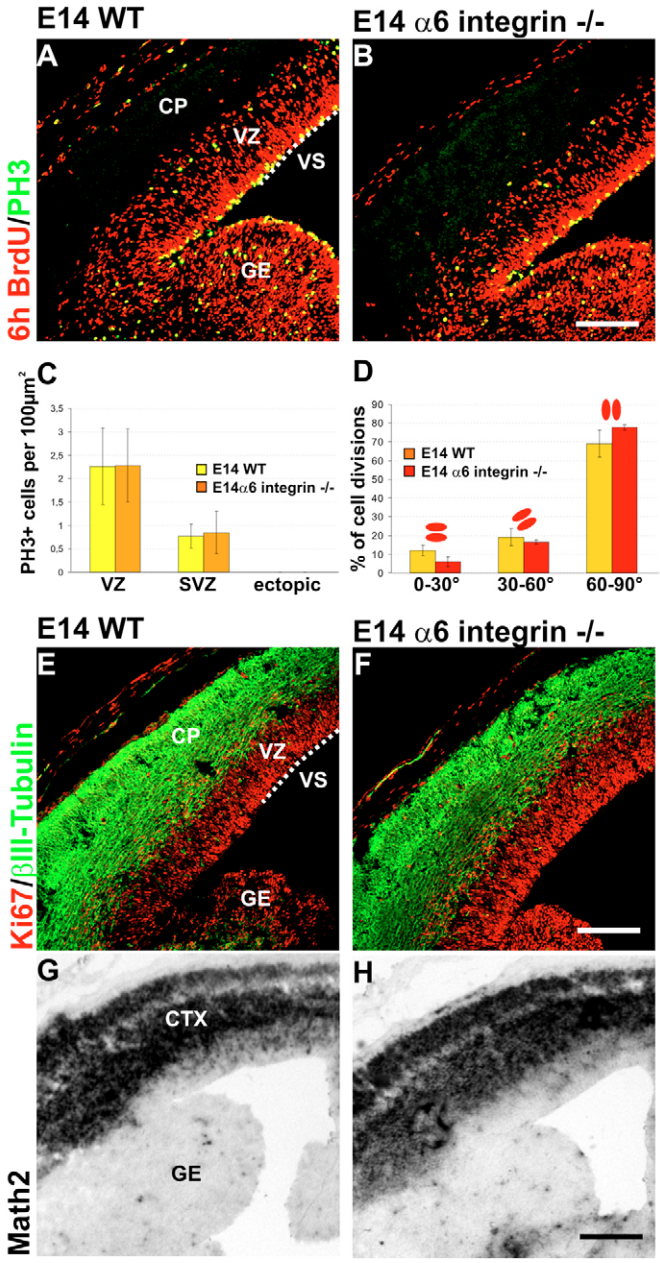

Fig. 5. Proliferation and neurogenesis in α6 integrin$^{-/-}$ cortex. Micrographs of E14 wild-type and α6 integrin$^{-/-}$ cortex sections (**A**,**B**,**E-H**) stained as indicated. (**C**,**D**) Quantification of the number of PH3-positive cells in M phase per cortex area (C; wild type: *n*=39 sections, three animals, α6 integrin$^{-/-}$: *n*=50, three animals) and of the percentages of cells dividing with horizontal, oblique or perpendicular orientation to the ventricular surface (D; wild type: *n*=89 mitoses, one animal, α6 integrin$^{-/-}$: *n*=150, one animal) in wild-type and α6 integrin$^{-/-}$ cerebral cortex. Scale bars: 100 μm.

The role of the BM on radial glial fate and proliferation

Prior to neurogenesis, precursor cells with epithelial properties span the entire thickness of the wall of the neural tube, the neuroepithelial cells (for reviews, see Götz and Huttner, 2005; Fujita, 2003). At the onset of neurogenesis, neuroepithelial cells differentiate into radial glial cells that exhibit reduced tight junctional coupling at the apical side [for junctions between pial endfeet see Balslev et al. (Balslev et al., 1997)], as well as a reduced extent of interkinetic nuclear migration (for a review, see Götz and Huttner, 2005). Owing to the restriction of interkinetic nuclear migration below the cortical plate, previous studies suggested that the radial processes of some precursor cells during neurogenesis would also end there (e.g. Gadisseux et al., 1992). However, 3D reconstruction of precursor cells labelled from the VS revealed that most precursors possess long radial processes reaching above the cortical plate (Hartfuss et al., 2003). Thus, consistent with immunocytochemical evidence (Hartfuss et al., 2001; Noctor et al., 2002) the majority of proliferating precursor cells in the VZ during neurogenesis is radial glial cells that are connected by their radial processes to the BM. Nevertheless, there is some degree of heterogeneity among the precursor cells with subsets of precursors devoid of contact to the pial surface (Hartfuss et al., 2003; Gal et al., 2006). This heterogeneity of precursors during neurogenesis is notably different from the apparent homogeneity at earlier stages, as first described by Fujita (Fujita, 1963).

BM contact has been shown to act as a crucial factor for apicobasal polarity in many cell types (for a review, see Li et al., 2003). Fishell and Kriegstein had suggested that radial glial cells maintaining their contact with the BM may remain precursor cells and proliferate faster than other precursors (Fishell and Kriegstein, 2003; Miyata et al., 2001). This hypothesis would also be consistent with data implicating β1 integrin-mediated signalling in the maintenance of precursor proliferation or even stem cell-like self renewal (Campos et al., 2004). Moreover, growth factor-mediated signalling, e.g. to oligodendrocytes, can be altered upon contact with the BM (Colognato et al., 2002), further supporting the potentially crucial role of such a contact for radial glial cells (Colognato et al., 2005). However, none of these proposed functions of BM contact of radial glial cells was affected in the mouse mutants with severe BM disruptions.

Severe BM disruptions in the laminin γ1III4$^{-/-}$ cortex were evident in large areas of the cortical surface devoid of any laminin immunoreactivity, in the frequent absence of subpial radial glial endfeet with only some ectopic cells positive for radial glial markers scattered in the CP and a severe accumulation of ectopic neurons in the subarachnoid space, the cobblestone-(type II) lissencephaly (Figs 1, 2, 5; see Fig. S1 in the supplementary material). Moreover, two additional mouse lines where the BM disruptions were demonstrated by electron microscopy were included in our analysis (Georges-Labouesse et al., 1998; Costell et al., 1999), thereby ensuring that we did not miss any phenotype caused by BM disruption. None of these mutants exhibited any defects in radial glia cell proliferation, in the generation of basal

progenitors, in the orientation of cell division or in neurogenesis. As BM disruptions occurred already around E12 in the mutants analysed, possible influences on cell proliferation or neurogenesis should manifest by E14 to E18, the stages analysed here. In the absence of anchoring at the BM, radial glia did not transform prematurely into astrocytes or oligodendrocyte precursors, as none of the markers for these cell types was observed until E18 in the BM-deficient cortex (data not shown). Therefore, we conclude that direct signalling from the BM to radial glial cells is not involved in regulating their polarity, proliferation and cell fate.

However, a small proportion of precursors was ectopically located in the cortical parenchyma of the laminin γ1III4$^{-/-}$ mice. As the ectopic precursor cells formed clusters of dividing cells and were still expressing radial glial antigens, we speculate that they result from precursors loosing both basal and apical anchoring at earlier developmental stages. When VZ precursors generate SVZ precursors dividing at abventricular positions, the latter loose their apical contacts but often maintain their anchoring to the basally located BM (Miyata et al., 2004). In the laminin γ1III4$^{-/-}$ cortex, where anchoring of radial processes to the BM is virtually absent, the loss of apical contacts via adherens junctions would result in dispersion of the precursors throughout the parenchyma. In this context, it is interesting to note that, despite the lack of anchoring at the BM a normal band of SVZ precursors was located below the intermediate zone in the laminin γ1III4$^{-/-}$ cortex, suggesting that some other signals may also contribute to localize SVZ cells. In fact, the normal arrangement of the vast majority of precursors in the absence of BM anchoring suggests that this plays only a minor role to position both VZ and SVZ precursors, as fewer than 5% of all precursors were mis-positioned in the laminin γ1III4$^{-/-}$ cortex.

Notably, cell proliferation, neurogenesis or later gliogenesis of radial glial cells were also normal in the cortex of α6 integrin$^{-/-}$ mice. Although these mice had much milder defects in radial glia endfeet attachment to the BM than the laminin γ1III4$^{-/-}$ mice, they lack the laminin receptors containing the α6 integrin subunit [α6β1 and α6β4 (for a review, see Colognato et al., 2005)]. However, the laminin receptors containing α3 and α7 integrins, and dystroglycan may still be present to mediate signalling via parenchymally deposited laminins (De Arcangelis et al., 1999). Thus, signalling via parenchymal ECM components is still present in all the mutant mice analysed here. However, mice with deletions in the components mediating signalling from the ECM, such as the conditional deletion of β1, αv integrin, ILK or the focal adhesion kinase (FAK) in the neuroepithelium (Beggs et al., 2003; Graus-Porta et al., 2001; McCarty et al., 2005; Niewmierzycka et al., 2005), also predominantly exhibit cobblestone ectopia but no obvious defect in cell proliferation or neurogenesis.

The role of the BM for neuronal migration and differentiation

Thus, the attachment of radial glia endfeet to the BM may not be relevant for radial glia proliferation and neurogenesis, but it is functionally relevant for the maintenance of the BM and for neuronal migration. All mutations affecting signalling from the BM (see above) result in BM disruptions similar to the phenotype upon deletion of components integral to the BM, such as in the laminin γ1III4$^{-/-}$ or perlecan$^{-/-}$ mice. BM disruption is in most, but not all (Beggs et al., 2003), cases accompanied by disruption of the layer of reelin-secreting cells (also observed in this study, Fig. 4 and data not shown). Reelin signalling to the radial glial cells promotes their process extension supposedly via the BLBP (Förster et al., 2002; Hartfuss et al., 2003), suggesting that BM disruption affects radial glia process extension directly and indirectly. In the laminin γ1III4$^{-/-}$ cortex, radial glia processes are disorganized within the cortical plate, and hence cannot guide migrating neurons in an organized manner. The lack of BM will also affect the other mode of radial migration of neurons, by somal translocation with neurons pulling the soma towards the pial surface by their apical dendrite anchored at the pial surface (Miyata et al., 2001; Miyata et al., 2004; Morest and Silver, 2003; Nadarajah et al., 2001). Thus, independent of their mode of migration, most neurons will be displaced in a cortex with BM disruption (this work) (Beggs et al., 2003; Chiyonobu et al., 2005; Georges-Labouesse et al., 1998; Halfter et al., 2002; McCarty et al., 2005).

A third mode of neuronal migration is oriented tangentially, in parallel to the ventricular or pial surface. These pathways are mostly pursued by GABAergic interneurons, originating outside the cerebral cortex (for a review, see Marín and Rubenstein, 2003). In this regard, it is of interest that GABAergic neurons settled mostly in the outer part of the laminin γ1III4$^{-/-}$ cortex. Only neurons located in the deeper parts of the cortical grey matter express Math2, a marker for glutamatergic pyramidal neurons (Schuurmans et al., 2004), while neurons located closer to the BM did not express Math2 at E18. Neurons with upper layer marker expression were located deep in the cortex at the same position as deep layer neurons. In the outer cortical regions, only reelin-positive neurons and GABA-, calretinin- or calbindin-positive neurons were detected. Although the number of reelin-positive neurons was not obviously altered, interneurons containing calbindin or calretinin were abnormally concentrated in the outer part of the cortex of laminin γ1III4$^{-/-}$ mice in contrast to their scattered distribution in the wild-type cortex. Thus, tangentially migrating interneurons may lack their normal guidance information in the laminin γ1III4$^{-/-}$ cortex and hence accumulate in the outer part of the cerebral cortex. Indeed, the lower layers of the E18 laminin γ1III4$^{-/-}$ cortex seem to contain fewer GABAergic, calbindin or calretinin-positive neurons than normal, consistent with a misrouting of these neurons.

Alternatively, glutamatergic pyramidal neurons originating within the cortex may change their fate towards GABAergic neurons in the laminin γ1III4$^{-/-}$ mice. However, all neuronal subtypes of pyramidal neurons, including those destined for upper cortical layers were present at lower positions in the mutant cortex, suggesting that these neurons are displaced rather than absent. Moreover, it may also be difficult for mis-specified neurons generated within the cortex to reach the outer layers, while some tangentially migrating neurons anyhow migrate within layer 1 on the outer surface of the cortex (Marín and Rubenstein, 2003). Thus, these data are most consistent with a misrouting of GABAergic interneurons to the outer part of the laminin γ1III4$^{-/-}$ cortex, where glutamatergic neurons fail to migrate to and cortical layers do not develop normally. This is consistent with the mispositioning of GABAergic interneurons in the *reeler* cortex where cortical layering is also disturbed (Yabut et al., 2006). Taken together, our data suggest that contact to an intact BM is important for neuronal migration – both radial and tangential – whereas it is largely dispensable for the precursor roles of radial glial cells.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/16/3245/DC1

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