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Role of Laminin gamma 1 in Cortical Development

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ROLE OF LAMININ $\gamma 1$ IN CORTICAL DEVELOPMENT

A Thesis Presented to the Faculty of
The Rockefeller University
in Partial Fulfillment of the Requirements for
the degree of Doctor of Philosophy

by

Véronique Haegeli

June 2006

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ROLE OF LAMININ $\gamma 1$ IN CORTICAL DEVELOPMENT

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The Rockefeller University 2006

Laminins are a major component of basement membranes, including those in the central nervous system (CNS). To explore the role of laminin $\gamma 1$, a subunit common to all laminins found within the CNS, we developed a murine model in which laminin $\gamma 1$ is conditionally disrupted in the nervous system. These mice show cortical developmental abnormalities including undulations in the embryonic cortical plate and resultant adult cortical layers and abnormal positioning of pyramidal neurons and interneurons. We have investigated the mechanisms underlying these abnormalities. Neuronal migration via the establishment of the radial glial scaffold and the Cajal-Retzius cell layer was affected in the mutants. Furthermore, axon pathfinding was impaired with developing axons projecting along the abnormally structured cortical plate. Laminin $\gamma 1$ also plays a role at the cellular level, as disturbances in normal neuronal morphology were observed in mutant animals, but laminin was not required for cell survival or cell proliferation. These data indicate a fundamental role for laminin $\gamma 1$ in both the cellular and structural development of the cerebral cortex.

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CHAPTER 1: INTRODUCTION

1.1 Laminins in the CNS

1.1.1 Definition of laminin

Laminins are heterotrimeric proteins that are critical components of the extracellular matrix (ECM) (Timpl, 1996). At present, five α chains, four β chains and three γ chains have been identified, and of the possible 60 heterotrimers, 15 have been observed.

Laminins have multiple roles in mammalian tissues: they compose a major structural element of the basement membrane and provide attachment sites for cells through cell surface proteins. They also act as ligands for receptors on cells and thereby initiate signals that influence cell behavior and survival.

Laminins can be found in a compact form in basal laminae or in a punctate pattern throughout a tissue (Libby et al., 2000).

Furthermore, although laminin is stably expressed in basement membranes, the punctate form of laminin is often transiently expressed, for example along the path of the developing optic nerve in chicks (Dong et al., 2002). This raises the possibility that laminin in basement membranes may provide a survival signal for cells whereas punctate laminin expressed throughout a tissue may have a role in differentiation.

1.1.2 Role of laminin in the CNS

Although the presence of laminins in the CNS was initially uncertain, it is now clear from the work of numerous laboratories that this protein is present (Hagg et al., 1989; Zhou, 1990; Jucker et al., 1996; Chen and Strickland, 1997; Hagg et al., 1997; Tian et al., 1997; Nakagami et al., 2000; Grimpe et al., 2002; Indyk et al., 2003; Yin et al., 2003). Most notably, gene trapping in transgenic mice whereby a LacZ gene is inserted under the control of the laminin $\gamma 1$ promoter leads to β gal expression within laminin $\gamma 1$ -expressing cells (Yin et al., 2003; Indyk et al., 2003). This system has allowed detection of laminin $\gamma 1$ promoter activation within the CNS both during embryogenesis and adulthood (Skarnes et al., 1995; Mitchell et al., 2001; Yin et al., 2003). Laminins are expressed in the cortex and in the pial basement membrane (Grimpe et al., 2002; Indyk et al., 2003; Yin et al., 2003).

Since homozygous mutant mice die at embryonic day (E) 5.5 (Smyth et al., 1999) it is not possible to use these mutants to investigate the role of laminin $\gamma 1$ in CNS development. Similar results were obtained with transgenic mice where the laminin $\gamma 1$ gene has been replaced with LacZ (Skarnes et al., 1995; Mitchell et al., 2001). ES cells prepared from laminin $\gamma 1$ mutant blastocysts can form embryoid bodies but not basement membranes (Murray

and Edgar, 2000). *In vitro* systems have been useful in deciphering the role of laminin at later time points.

1.1.3 Function of laminins in the CNS

There is substantial evidence that laminins play an important role in neurite outgrowth and axon pathfinding *in vitro* (Rogers et al., 1986; Letourneau et al., 1988; McLoon et al., 1988; Timpl, 1996); for review see (Luckenbill-Edds, 1997). The rate of neurite extension is increased for hippocampal neurons growing on a laminin substrate versus a poly-lysine substrate. Laminin also enhances the polarity of the newly-formed neurites by promoting growth of the major neurite while preventing further extension of minor neurites (Lein et al., 1992; Lochter and Schachner, 1993). Neurons grown on alternating stripes a few hundred μm wide of laminin and poly-lysine will extend neurites toward the laminin stripe and the first neurite to reach a laminin substrate will develop preferentially to become the axon (Esch et al., 1999). It has also been shown that laminin is the permissive cue to induce growth cone extension (Gomez and Letourneau, 1994; Bonner and O'Connor, 2001). Therefore, in cell culture systems, laminin can promote the formation of neurites, and differentiation of neurites into axons as well as serve as a chemoattractant for developing neurites.

Laminin also has a role in axon regeneration. For example, regeneration of severed axons in rat hippocampal slices is prevented by a decrease of laminin $\gamma 1$ expression by RNAi (Grimpe et al., 2002). Function-blocking laminin antibodies prevent regrowth of severed mouse axons in the absence of GFAP expression *in vitro* (Costa et al., 2002). Laminin's role in regeneration extends to the PNS, as laminin $\gamma 1$ is required for regeneration in the mouse sciatic nerve (Chen and Strickland, 2003).

1.2 Cortical development

The mammalian adult cerebral cortex exhibits a highly organized laminar structure with six neuronal layers. During development, waves of neuroblasts generated from the ventricular neuroepithelium migrate towards the pia to form successive cortical layers (Rakic, 1990). Starting at E11, neurons generated from the proliferative ventricular zone migrate to form the preplate. This coincides with the migration of Cajal-Retzius cells from the medial ganglionic eminences. From E14 to E18, successive waves of newly generated neurons migrate radially towards the pial surface. These neurons form the cortical plate by splitting the preplate into the marginal zone (above) and the subplate (below).

The six adult cortical layers in emanate from the embryonic cortical plate. Therefore, proper migration of cortical plate neurons is essential for correct formation of the neuronal layers. After E18, neuron maturation occurs, with extension of processes and the formation of synaptic connections (Rakic, 1990).

The migration of cortical plate neurons has been extensively studied. One of the most distinctive features of the cortex is the “inside out” position of the cortical neurons: earlier-born neurons are present in deeper layers whereas later-born neurons are found in superficial layers. Therefore, the later-born neurons migrate past earlier-born neurons. The earlier-born neurons travel short distances, possibly by somal translocation (from E10-13)(Nadarajah et al., 2001; Nadarajah and Parnavelas, 2002) whereas later-born neurons (from E14-18), which travel further, migrate along radial glial fibers (Angevine and Sidman, 1961; Sidman and Rakic, 1973). The radial glial network is formed by glial processes extending from the ventricle toward the surface of the brain with endfeet attaching to the pia basement membrane (Marin and Rubenstein, 2003) One proposed function of the laminin-containing pial basement membrane is to provide an attachment site for radial glial endfeet. Interestingly, radial glial

cell proliferation produces neurons which migrate on the glial cell which gave rise to them (Noctor et al., 2001).

By contrast, interneurons are generated in the ganglionic eminence and migrate tangentially to the cortex (Marin and Rubenstein, 2003). This tangential migration occurs within several different zones of the developing cortex. It has been suggested that interneurons migrate along axons. The neuronal adhesion molecule TAG1 has a role in promoting migration to the cortex although migrating cells do not overlap with TAG1-expressing axons (Denaxa et al., 2001; Tanaka et al., 2003).

The pathways that regulate cortical layering are complex. Cajal-Retzius cells, which form cortical layer I, have a role in the inside-out positioning of cortical neurons. Cajal-Retzius cells secrete proteins such as Calbindin, Calretinin and Reelin, a large extracellular glycoprotein and a predicted serine protease (D'Arcangelo et al., 1995; Ogawa et al., 1995). A mutation in the reelin gene results in an inversion of cortical layers in *reeler* mice (Frotscher, 1998). It has been suggested that Reelin cleaves laminin (Quattrocchi et al., 2002). Reelin binds to a known laminin receptor, $\alpha 3\beta 1$ integrin (Dulabon et al., 2000); however the significance of these findings is not clear. Reelin can bind to two

receptors: the very low density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor (ApoER) (D'Arcangelo et al., 1999; Hiesberger et al., 1999). The intracellular molecule Dab1, which also binds VLDLR and ApoER, is phosphorylated in the presence of Reelin. Mutations in both VLDLR and ApoE or in Dab1 result in inversion of the cortical layers, similar to the *reeler* mutant (Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997; Trommsdorff et al., 1999). Therefore, signaling by Cajal-Retzius cells in the marginal zone has a role in the formation of the cortical layers.

1.3 Role of the pia and laminin in cortical development

Since the direct analysis of whole body laminin $\gamma 1$ knock-out mice is not possible, other investigators have turned to genes which have a role in the formation and assembly of the laminin-containing pia basement membrane. Numerous mouse mutants have been generated containing a similar CNS phenotype of neurons migrating through breaches in the basement membrane. Selective ablation of meningeal cells, which secrete the majority of ECM proteins, by 6-hydroxydopamine leads to extrusions of neurons through the discontinuous basement membrane (Sievers et al., 1994). Mutations in ECM components such as perlecan lead to discontinuous expression of laminin in the pia basement membrane and cortical dysplasia (Costell et al., 1999). Deletion of

the nidogen-binding site of the laminin $\gamma 1$ gene leads to the same phenotype and furthermore results in a disorganized radial glial scaffold (Halfter et al., 2002). Mutations in receptors for laminins, such as dystroglycan and $\alpha 6$ and $\beta 1$ integrins have the same effects (Beggs et al., 2003; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001). Lack of cortical expression of the intracellular molecules focal adhesion kinase (FAK) and integrin-linked kinase (ILK) also lead to similar phenotypes, placing them in the same genetic pathway (Beggs et al., 2003; Niewmierzycka et al., 2005). Therefore, the assembly of laminin into a functional basal lamina is required for normal neuronal migration. However, it has been suggested that laminin deposited along radial glial cells also has a role in neuronal radial migration (Liesi, 1985a, b, 1992).

1.4 Axon guidance

Laminin has a role in axonal guidance (Garcia-Alonso et al., 1996; Tessier-Lavigne and Goodman, 1996). Interaction of laminin with other chemo-attractive or -repulsive molecules can alter their properties (Hopker 1999; Suh 2004). For example, netrin has chemo-attractive properties (Culotti and Kolodkin, 1996) which can be converted to chemo-repulsive in the presence of laminin (Hopker et al., 1999). There is some evidence that defective neuronal migration leads to aberrant axon pathfinding, but not

always as some axons from misplaced neurons do reach their target. Thus the axon pathfinding defect may be secondary to neuronal mispositioning (Bielas et al., 2004).

1.5 Human diseases with similar defects

Similar neuronal migration defects with neuronal extrusions are observed in human diseases such as type II (cobblestone) lissencephaly (Olson and Walsh, 2002; Ross, 2002). These diseases are caused by hypoglycosylation of ECM components, most notably α -dystroglycan. In Muscle-Eye-Brain (MEB) disease and Walker-Warburg syndrome (WWS), mutations in the POMGnT1 and POMT1 genes respectively, lead to hypoglycosylation of α -dystroglycan (Kim et al., 2004). Fukuyama-type muscular dystrophy (FCMD) is caused by a mutation in the fukutin gene, which is a glycolipid modifying enzyme (Yoshida et al., 2001; Takeda et al., 2003).

Mouse models for some of these genes have been constructed. For example, a mutation in the LARGE gene, coding for a putative glycosyl transferase, leads to abnormal glycosylation of dystroglycan in mice and humans (Longman et al., 2003).

Hypoglycosylation of dystroglycan has also been observed in muscular dystrophy (*myd*) mice (Michele et al., 2002). Fukutin null mice are embryonic lethal and exhibit a decrease in

glycosylation of α -dystroglycan and defective basement membranes (Kurahashi et al., 2005).

1.6 Disruption of the laminin γ 1 gene in mice

In order to investigate the role of laminin γ 1 both in the pia basement membrane and throughout the cortex, we analyzed the brains of mice with the laminin γ 1 gene disrupted in the CNS (Chen and Strickland, 2003). The effect of laminin in the CNS has been indirectly studied in mice mutant for perlecan, dystroglycan, integrins and FAK which all exhibit cortical layering defects associated with depletion of laminin in the pia basal lamina. Therefore, laminin γ 1 conditional mutants will allow us to study the direct role of laminin γ 1 in the CNS.

Mutants show abnormal cortical development with layering defects both in the embryonic cortical plate and in adult cortical layers. In mutant embryos, areas of disruption of laminin in the pia basement membrane correlate with neuronal extrusions. Gaps in the pial basal lamina are accompanied by a disorganized radial glial scaffold and impaired neuronal migration. Furthermore, axon guidance is impaired in mutants with embryonic thalamo-cortical axons and adult corpus callosum and external capsule axons straying toward the surface of the brain. Cell death and

proliferation were not affected in mutants. These results demonstrate that laminin $\gamma 1$ in the CNS is an essential component of the pial basal lamina which is required for the formation of the radial glial scaffold, neuronal migration, and axon pathfinding.

CHAPTER 2: MATERIALS AND METHODS

2.1 Mouse lines and analysis of Cre activity

Exon 2 of the laminin $\gamma 1$ gene was flanked by LoxP sites (Chen and Strickland, 2003). The mutant mice used in this study were homozygous for the floxed laminin $\gamma 1$ allele and carried a Cre recombinase transgene under the Ca-Calmodulin Kinase II α promoter (R1ag5 line) (Dragatsis and Zeitlin, 2000) (CaMKII/Cre:flAMy1 mice). Although mutant mice were smaller than control littermates, they survived to adulthood. Cre recombinase activity was monitored by using the LacZ/EGFP double reporter mouse line (Novak et al., 2000). Recombination of the laminin $\gamma 1$ gene in the forebrain was monitored by PCR using the following primers: 5'-GGG TGG GAT TAG ATA AAT GCC TGC TCT TTA CTG-3' and 5'-GAT TTT CAA AGA AGC AGA GTG TG 3'. The PCR conditions were 95°C for 5 minutes, 57°C for 1 minute, 72°C for 3 minutes, then 35 cycles (95°C for 30 seconds, 57°C for 1 minute, 72°C for 2 minutes), then a 7 minute extension at 72°C. The PCR products were the following: unrecombined allele 1kb, recombined allele -500 bp. Protocols for genotyping mouse tail genomic DNA for the floxed allele, Cre and EGFP have been described (Chen and Strickland, 2003).

2.2 Histology and immunohistochemistry

All animals were maintained according to Animal Welfare guidelines at the Rockefeller University. Animals were anesthetized deeply with an injection of 2.5% avertin (0.02 ml/gm of body weight), then perfused with cold 1x PBS, pH 7.4 followed by 4% paraformaldehyde. Adult brains or embryonic heads were dissected; tissues were fixed for 48 hours in 4% paraformaldehyde and cryoprotected in 30% sucrose for 48 hours at 4°C. Specimens were embedded in OCT (Miles) and 30 µm coronal sections were collected on SuperFrost Plus slides (Fisher Scientific). Sections were blocked in 5% normal goat or donkey serum (Vector Laboratories and Jackson ImmunoResearch Laboratories Inc) and 0.3% Triton in 1x PBS, pH 7.4 for one hour. Primary antibodies were incubated overnight at 4°C. Primary antibodies used were:

- β-gal (1:1000, MP Biomedicals)
- BLBP (gift from Dr. Nathaniel Heintz, the Rockefeller University)
- calbindin (1:1000, Swant CB-38a, Bellinzona, Switzerland)
- dystroglycan (1:50, Chemicon, MAB 371)
- integrin β1 (1:300, Chemicon)
- laminin I (1:2000, Sigma L-9393)
- laminin γ1 (1:500, Chemicon MAB1914)
- MAP1 (1:100, Sigma, M-4278)
- MAP2 (1:500, Sigma, M-4403)

Netrin (1:50, Calbiochem, PC364T)

NeuN (1:100, Chemicon, MAB377)

neurofilament (1:2000, Chemicon AB5539)

PECAM (1:30, BD Pharmingen, 550274)

perlecan (1:1000, Chemicon, MAB 1948)

reelin (1:500, Chemicon, MAB5364)

SMI32-neurofilament (1:2500, Sternberger Monoclonals Inc)

TuJ1 (1:500, Covance, MMS-435P).

Sections were rinsed in 1x PBS, pH 7.4 , incubated for one hour at room temperature in 1:500 fluorescent-conjugated secondary antibody (FITC or rhodamine, Jackson ImmunoResearch Laboratories Inc) in 3% serum, 0.3% Triton in 1x PBS, pH 7.4 , rinsed again and mounted in Vectashield mounting medium with DAPI (Vector Laboratories Inc). Sections stained with the SMI-32-NF antibody were incubated with 1:200 biotinylated secondary antibody (Vector Laboratories Inc), rinsed, incubated in ABC kit solution (Vectastain Elite Standard ABC kit, Vector Laboratories Inc) for 1 hour, then rinsed again, and DAB was used for visualization.

Luxol Fast Blue staining was performed overnight at 60°C with 0.1% Luxol Fast Blue in 95% ethanol and 0.05% acetic acid. Color was developed by alternate rinses in 0.05% Li carbonate and 70% ethanol.

Cresyl violet staining was done on fixed sections in 0.018% cresyl violet for 15-30 minutes, dehydrated then mounted in DPX (Sigma-Aldrich).

The stained sections were examined with a Zeiss microscope.

2.3 BrdU Pulse and birthdating

For BrdU labeling of embryos, pregnant mice were injected. The day of detection of the vaginal plug was counted as embryonic day (E) 0.5 and pregnant mice were injected at E12.5, E14.5 or E16.5 with 100 µg/gm of BrdU. Detection of BrdU (Kuhn et al., 1997), was done either 2 hours after injection (pulse labeling) or once the mice were 14 days old (migration/birthdating studies). Briefly, DNA denaturation was performed on fixed sections by incubating in 50% formamide in 2x SSC at 65°C for 2 hours, then in 2N HCl at 37°C for 45 minutes, followed by blocking in 5% goat serum 0.3% Triton for one hour and overnight incubation with monoclonal anti-BrdU antibody (1:400, Boehringer Mannheim-Roche). Sections were incubated in 1:500 rhodamine-conjugated secondary antibody for pulse labeling. For migration/birthdating studies, sections were incubated in 1:200 biotinylated secondary antibody (Vector Laboratories Inc.), rinsed, then incubated in ABC kit solution (Vectastain Elite Standard ABC kit, Vector

Laboratories Inc) for 1 hour, rinsed again, and DAB was used for visualization.

2.4 Mixed cortical cell culture

The cortices of E13.5 embryos were dissected in Hank's Buffered Saline Solution (HBSS, Gibco InVitrogen), then dissociated in 0.25% trypsin in HBSS for 15 minutes at 37C. Once trypsin was inactivated by addition of fetal bovine serum (FBS, Sigma), cells were triturated using a fire-polished pipette, spun at 700g for 5 minutes and resuspended in NeuroBasal media (Gibco InVitrogen) supplemented with 2 mM glutamine (Gibco InVitrogen), B27 (Gibco InVitrogen) and 10% FBS (plating media). After four hours, media was replaced with plating media without FBS. Cells were grown for 5 days, fixed with 4% paraformaldehyde and immunostained as above.

2.5 Timm staining

Adult mice were perfused with 0.4% sodium sulfide in PB, then 1% paraformaldehyde, 1.25% glutaraldehyde (fixative). Brains were dissected and fixed overnight in fixative at 4C, then cryoprotected in 30% sucrose in PB for 48 hours. Thirty μ m sections were collected with a microtome, then processed according to Cavazos (Sutula et al., 1989). Briefly, sections developed in the dark for 40

minutes in a mixture 12:6:2 of gum Arabic (20% W/V), hydroquinone (5.6% W/V), citric acid/sodium citrate buffer with 17% silver nitrate solution. Sections were then rinsed, dehydrated and mounted in Permount.

2.6 Golgi staining

Adult brains were processed using a Rapid Golgi staining kit (FD Neurotechnologies). Briefly, brains were dissected and immersed in solution A/B in the dark and at room temperature for two weeks. Brains were then incubated in solution C for 24 hours at 4°C, frozen with dry ice and sectioned in the cryostat at 100 µm thickness. Color was developed in 1 part solution D, one part solution E, 2 parts water. Sections were dehydrated and mounted in Permount.

CHAPTER 3: RESULTS

3.1 Disruption of laminin γ 1 expression the CNS

We have generated conditional knock mice for the laminin γ 1 gene by using loxP sites. In these mice, the floxed exon 2 of the laminin γ 1 gene can be excised by Cre recombinase. We have crossed these floxed mice to mice expressing Cre recombinase under the CaMKII promoter, ensuring excision of laminin γ 1 in the CNS. We have demonstrated previously that significant recombination of the laminin γ 1 gene occurs in CaMKII/Cre:flAMy1 mice (hereafter termed mutant mice) in the hippocampus, spinal cord and peripheral nerves, but not in muscle or heart (Chen and Strickland, 2003). Therefore, the expression of Cre recombinase from line R1ag5 was not as hippocampal-specific as in other CaMKII-Cre lines (Tsien et al., 1996). To monitor the excision by CaMKII-Cre recombinase within the cortex, we used the double reporter mouse strain LacZ/EGFP (Z/EG) (Novak et al., 2000). In this mouse strain, in the absence of Cre activity, only LacZ is expressed, and after Cre excision of the LacZ gene, only EGFP is expressed. We generated mice containing both the CaMKII-Cre and the Z/EG transgenes. In these mice, EGFP expression, indicating Cre activity, was observed in some, but not all cells

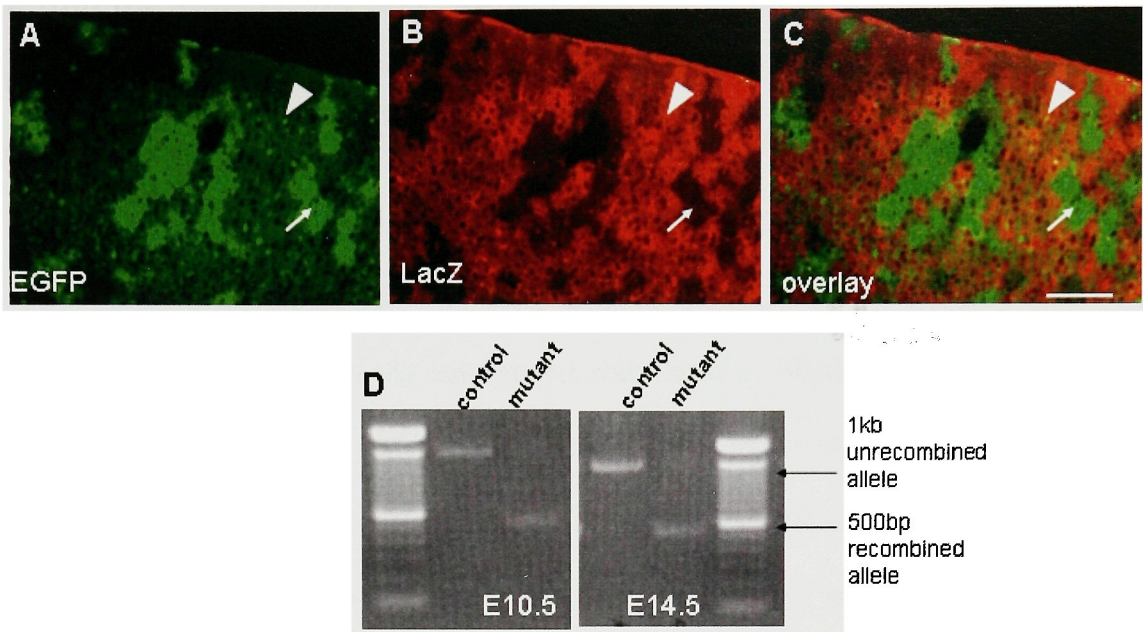


Figure 1. Recombination of the laminin $\gamma 1$ gene in the cortex.

A-C. Analysis of CaMKII-Cre recombinase activity in adult CaMKII-Cre; Z/EG reporter double transgenic mice. A. EGFP fluorescence (green), indicating Cre recombinase activity, was present both in individual cells (arrowhead) and in clusters of cells (arrow). B. LacZ immunostaining allowed detection of areas where recombination did not occur. C. Overlay of A and B shows that EGFP-expressing regions did not overlap with LacZ stained areas, demonstrating that cells either underwent recombination or not. Scale bar 100 μ m.

D. PCR monitoring recombination of the laminin $\gamma 1$ gene. DNA from forebrains of control (fLAM $\gamma 1$) E10.5 and E14.5 embryos led to amplification of a 1kb product (upper arrow) demonstrating lack of recombination. DNA from cortices of mutant (CaMKII/Cre: fLAM $\gamma 1$) E10.5 and E14.5 embryos led to amplification of a 500 bp product (lower arrow), showing that recombination had occurred. As PCR is biased towards the formation of the smallest product, no 1kb band is detectable in mutants even though there may be cells present which did not undergo recombination. Outer lanes show DNA ladder.

Left panel: E10.5 embryos. Right panel: E14.5 embryos.

within the adult cortex (Fig. 1A-C), including some neurons (data not shown). β -galactosidase immunostaining allowed detection of areas where recombination did not take place. There was no overlap of EGFP fluorescence and β -galactosidase expression. EGFP fluorescence was found both in deeper layers of the cortex and in proximity to the pia basement membrane. Within the cortex both individual neurons and clusters of neurons, possibly originating from one proliferating cell, showed EGFP expression. Therefore in mutant mice, excision of the laminin γ 1 gene by CaMKII-Cre recombinase occurred both near the pia basement membrane and within the cortex, allowing us to investigate the role of laminin throughout the cortex.

We also investigated at what time point Cre-mediated recombination was initiated. It has been shown, in a mouse line with the huntingtin gene homozygously floxed and containing the same CaMKII-Cre transgene, that a decrease in the level of the Huntingtin protein occurs at E15.5 (Dragatsis et al., 2000). Using PCR to monitor recombination, we have found that excision of the laminin γ 1 gene is detectable in the forebrain of conditional mutants as early as E10.5 (Fig. 1D).

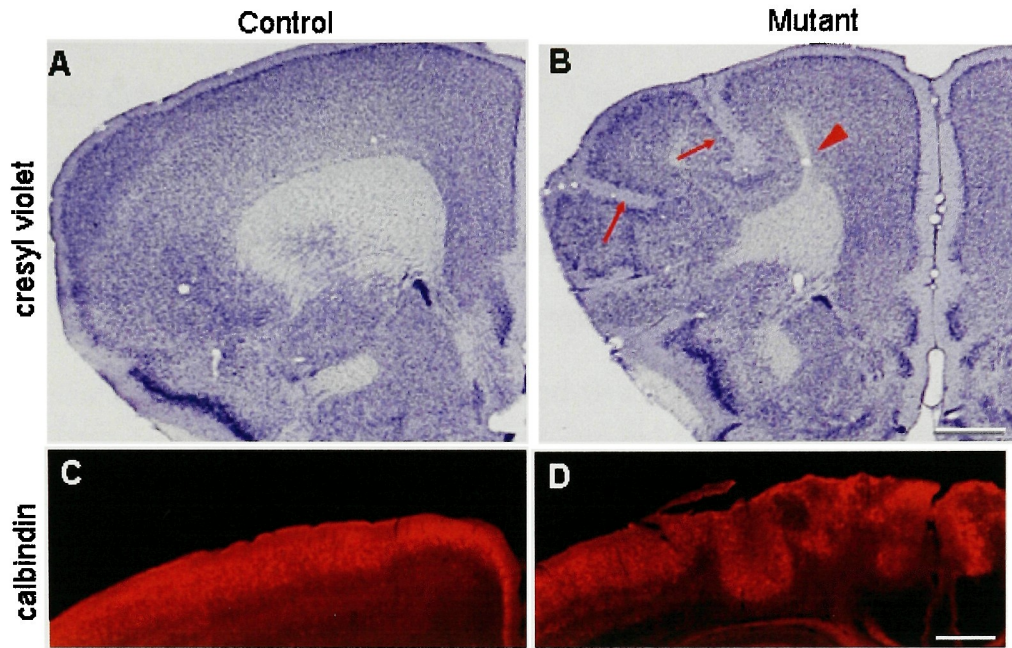


Figure 2. Lack of laminin γ 1 leads to cortical dysplasia.

Coronal sections through the cortex were analyzed by Cresyl Violet staining. A and B. In control frontal cortices (A), neurons were organized in structured layers, whereas in mutant frontal cortices (B), defects in cortical layering were apparent; the marginal zone invaded deeper layers of the cortex (arrows in B) and the forceps minor callosus had an abnormal morphology (arrowhead in B). C-D. Interneuron positioning is affected in mutants. Interneurons were visualized in adult coronal sections by Calbindin immunostaining. C. In control brains, interneurons were found in a superficial layer of the cortex. D. In mutant brains, interneurons formed undulating layers. Scale bars 500 μ m.

3.2 Cortical dysplasia in mutant adults

The majority of mutant adult mice (81%) showed severe defects in cortical cytoarchitecture. The laminar structure of the cortex was affected with cells having both over-migrated and under-migrated, resulting in an overall undulating appearance of the cortical layers (Fig. 2). In the frontal cortex, the marginal zone deeply invaded into the cortex (arrows in Fig. 2B) and the morphology of the forceps minor corpus callosum was also changed (arrowhead in Fig. 2B). In some areas, the hemispheres were not separated by meninges (data not shown). Since ~75% of cortical neurons are pyramidal neurons and 25% are interneurons, we investigated whether interneuron migration was also affected. Interneurons, as visualized by calbindin immunostaining, were aberrantly positioned (Fig. 2C-D), with undulations being formed. We also examined the cerebellum, where recombination does not occur, but no obvious malformations were observed (data not shown).

In order to analyze the formation of specific cortical layers, we performed an immunostaining using a monoclonal antibody against a non-phosphorylated epitope of the neurofilament heavy chain, SMI-32, (Fig. 3A-D). This antibody labels dendrites of pyramidal neurons that are specifically found in layers III and V (Campbell and Morrison, 1989). In mutant adult brains, layers III

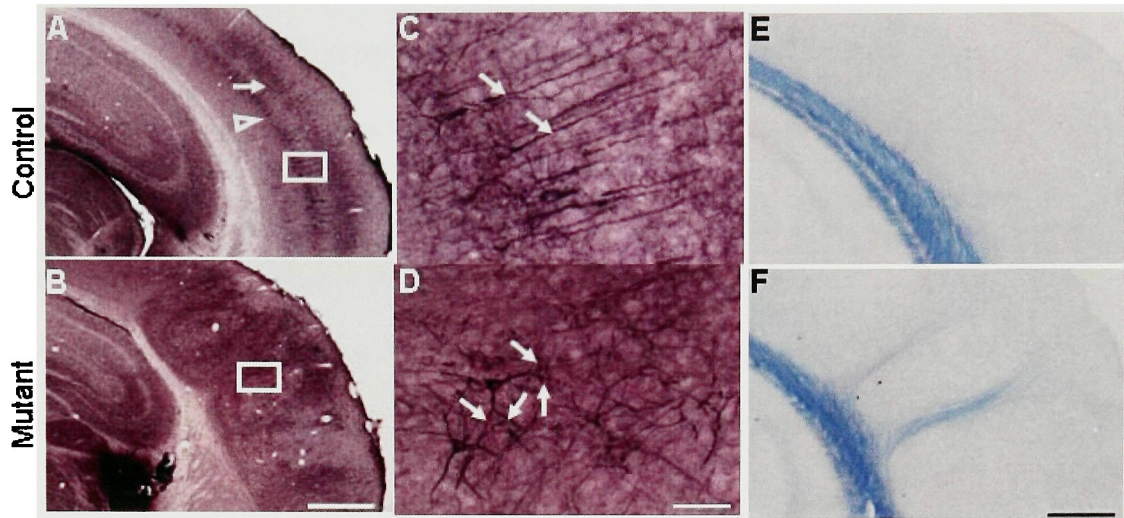


Figure 3. Lack of laminin $\gamma 1$ leads to cortical layering defects

Morphology of layer III and V pyramidal neurons (labeled for SMI-32). A. Layers III and V appeared linear and compact in controls (III: arrow and V: arrowhead). B. In mutants, layers III and V appeared ruffled. Boxed areas in A and B are magnified and shown in C and D. C. In control animals, neurons extended processes in the same orientation (arrows in C), whereas in mutants (D) neuronal polarity was lost (arrows in D). E-F. Luxol Fast Blue labeling of axons. E. Control cortices showed compact and linear labeled axons within the external capsule whereas mutants had straying axons (F). Scale bars: A-B: 500 μm , C-D: 50 μm , E-F: 200 μm ,

and V were disorganized and had a ruffled appearance (Fig. 3B). Furthermore, the polarity of labeled dendrites was severely affected: instead of projecting parallel extensions towards the surface of the brain, layer III and V neurons formed a disorganized dendritic arbor with no preferential polarity (Fig. 3D).

Cortical lamination was further disrupted with cortical axons in the external capsule and the corpus callosum abnormally migrating toward the pial surface (Fig. 3F, see also Chapter 4, figures 20, 22, 23, 24, 25, 26 and 27). Neuronal cell bodies were pushed aside by these aberrantly positioned axon bundles (data shown in chapter 4 and figures 21 and 22).

Cortical layering is established by waves of migrating neurons between embryonic day (E)12 and E18 from the ventricular proliferating zone to the cortical plate. Therefore, the defects in cortical layering observed in adults could arise from impaired neuronal migration during development. We investigated whether neuronal migration was impaired in embryos, and at what time point. At E14.5, extrusions of cells occurred through the gaps in pial laminin (open arrowheads in Fig. 4B) and, in severely affected mutants, the laminar structure of the cortical plate was entirely abolished (Fig. 4C). Therefore laminin $\gamma 1$ has a role in cortical layer formation during development.

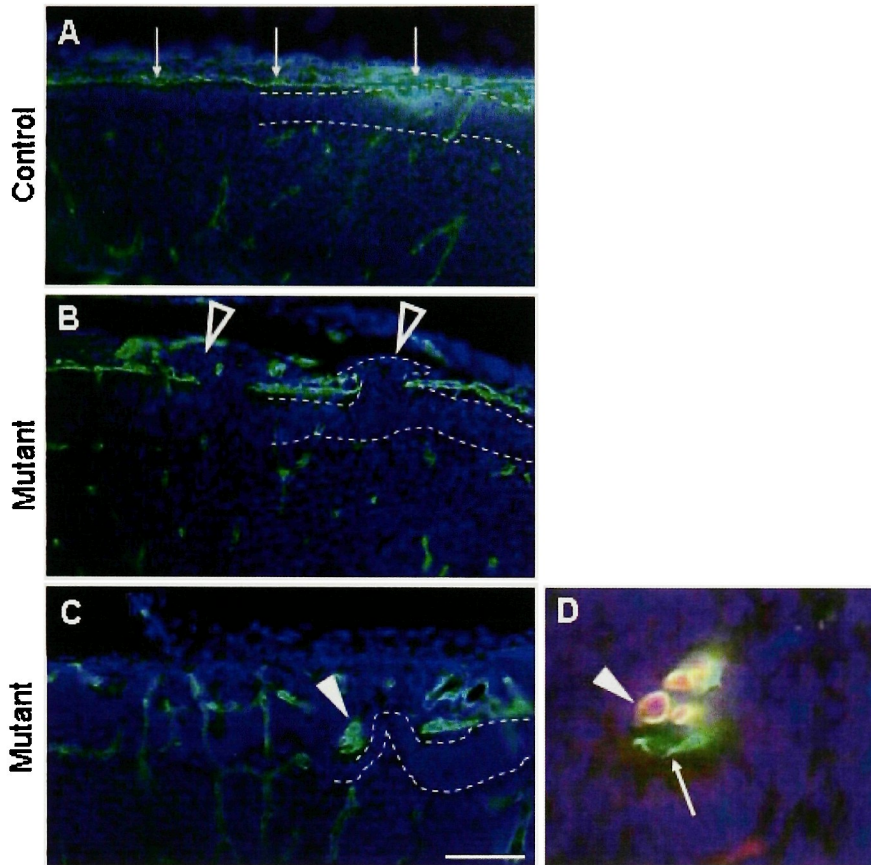


Figure 4. Pial laminin expression is disrupted or absent in mutants.

A-C. Laminin $\gamma 1$ immunohistochemistry (green) and DAPI counterstaining (blue) in the pial basal lamina and the cortical plate (dashed white lines on the right side of each panel) in E14.5 embryos. A. In control cortices, pial laminin I expression (green) was continuous (arrows). B. In mutants, cortical extrusions occurred in areas where pial laminin expression was disrupted (open arrowheads). C. In severely affected mutants, pial laminin was absent, and the cortical plate was arranged in undulating structures (outlined in dashed white lines) around laminin-secreting blood vessels (arrowhead). D. Analysis of vascular and pial laminin expression patterns in E14.5 mutants. Boxed area in C is represented in D. Blood vessels were identified by PECAM immunohistochemistry (red). Laminin I staining demonstrated that laminin is present both in the vasculature (arrowhead) and in remaining fragments of the pial basal lamina (arrow). Scale bars: A-C: 200 μm .

3.3 Discontinuous cortical laminin expression during development

To confirm that Cre recombinase expression leads to disruption of laminin $\gamma 1$ expression, we stained for laminin in mutant brains. In E14.5 mutant embryos, cortical laminin expression was discontinuous in the pia basement membrane of mutant mice as opposed to control mice where laminin expression was uninterrupted (Fig. 4). There were degrees of severity in the perturbation of pial laminin expression, ranging from localized disruptions to complete lack of a basal lamina (compare Fig. 4B,C) which correlated to the severity of the cortical plate defect. In mutants with discrete breaches in pial laminin, the cortical plate was disrupted only directly below the laminin gap, whereas in mutants with undetectable levels of pial laminin, the cortical plate was severely disorganized. The vascular laminin expression pattern was not affected. Interestingly, adult pia membranes did not exhibit defects and expressed wild-type levels of laminin (data shown in Chapter 4 and figure 9). Therefore a decrease in CNS-produced pial laminin during development led to defects in the formation of the cortical plate, and there was a correlation between the amount of pial laminin and the extent of the cortical plate malformation.

We observed that in severely affected mutant cortices, instead of forming a linear structure, the cortical plate had a ruffled appearance (outlined by white dashed lines in Fig. 4B-C) and occasionally formed semi-circular structures around remaining fragments of laminin (arrowhead in Fig. 4C-D, see also Figs 5, 6, 7). Since laminin is expressed both in blood vessels and in the pia, this raised the possibility that in the absence of pial laminin, vascular laminin may influence cortical plate formation. A double immunostaining for blood vessels and laminin revealed that the semi-circular cortical plate structures surrounded laminin originating from two sources: small remaining fragments of the pial basal lamina (arrow in Fig. 4D) and vascular laminin (arrowhead in Fig. 4D). Therefore, small remaining fragments of laminin retain the ability to induce proper neuronal migration in localized areas, surrounded by disorganized areas that lack laminin, thereby causing the cortical plate to form undulations.

3.4 Pial laminin is required for the formation of the radial glial scaffold

During development, neurons migrate along radial glial fibers. If the radial glial scaffold is impaired, then neuronal migration is affected. In wild-type mice, radial glial cells extended fibers from

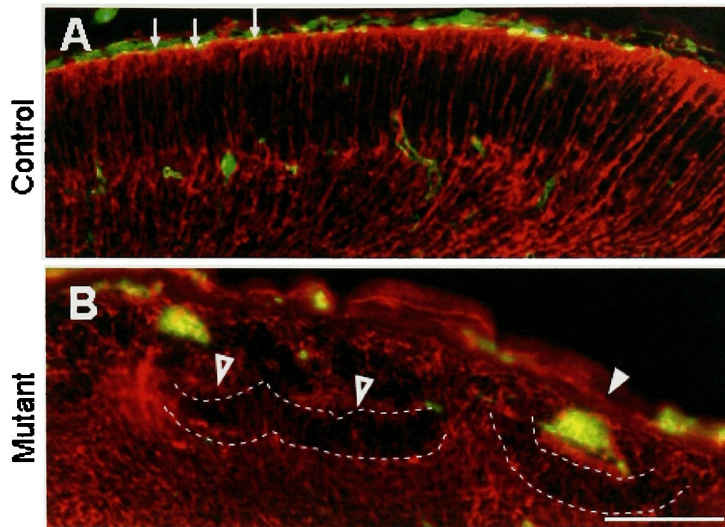


Figure 5. Pial laminin is required for orientation of the radial glial scaffold

The laminin $\gamma 1$ expression pattern (green) was compared to the structure of the radial glial scaffold, visualized by BLBP immunostaining (red). A. In control cortices, laminin was present throughout the pia membrane (arrows) and in blood vessels. The radial glial scaffold formed a parallel structure. B. In mutants, pial laminin was discontinuous (open arrowheads show gaps in laminin expression, arrowhead shows remaining laminin). The radial glial scaffold was severely disrupted. There were areas of localized organization into a scaffold in the presence (arrowhead) and absence (open arrowhead) of laminin, interrupted by areas with no discernable organization. White dashed lines outline the cortical plate. Scale bar 100 μm .

the ventricle to the pial surface, where they formed endfeet attaching to the basal lamina. The ensuing radial glial network was a parallel arrangement of fibers which enables the formation of organized cortical layers. We therefore examined the radial glial scaffold and compared it to the pial laminin expression pattern in mutant mice. The radial glial scaffold was impaired to varying degrees. In areas where laminin $\gamma 1$ was still expressed, a localized glial network formed (arrowhead in Fig. 5). In areas where laminin $\gamma 1$ expression was absent, either a short, localized radial glial scaffold formed (open arrowheads Fig. 5) or the radial glial cells extended processes without any specific polarity. Therefore, pial laminin $\gamma 1$ was required for the formation of an ordered radial glial network and could lead to the observed neuronal migration defects.

3.5 Cajal-Retzius cells are aberrantly positioned in mutants

Although the pia basement membrane has an important role in the formation of a layered cortex, other molecules are involved. Cajal-Retzius cells, which form layer I of the cortex, secrete Reelin and calbindin and have an important role in cortical neuron migration. We analyzed whether Cajal-Retzius cells were still present in mutant brains, whether they were properly positioned, and

whether they still secreted Reelin and Calbindin. In control mice, Calbindin expression at E16.5 was confined to a tightly regulated layer corresponding to cortical layer I (Fig. 6A). However, in mutant mice, Calbindin expression, although it was maintained, was severely affected. Calbindin expression was expanded to a wider area, was discontinuous and was less compacted (Fig. 6D). The expression of Reelin in mutant brains was similarly affected (data shown in Chapter 4, figure 15). Therefore, although Cajal-Retzius cells were present and maintained a proper expression profile, they were not positioned properly. However, it is unclear whether the aberrant positioning of Cajal-Retzius cells was a consequence of defects in the pia basement membrane and/or the radial glial scaffold or whether the Cajal-Retzius cells were an additional cause of defective neuronal migration.

3.6 The pathfinding of thalamo-cortical axons (TCA) is affected in mutants

Calbindin is also expressed in one subset of axons called the thalamo-cortical axons (TCAs). In control animals, the TCAs project from the thalamus to the cortex, where they form a compact linear structure abutting the cortical plate (Fig. 6A-B). In mutant embryonic cortices, the TCAs appropriately developed directly below the cortical plate and extended to their proper

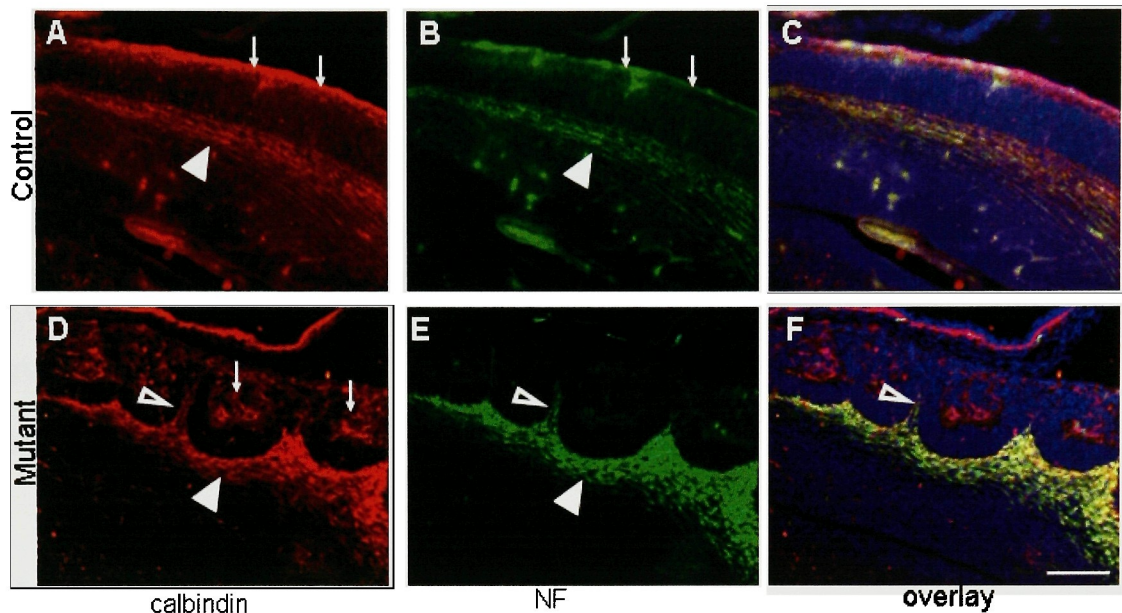


Figure 6: Pial laminin is required for a proper Calbindin expression pattern

The formation of the Cajal-Retzius cell layer and pathfinding of the TCAs were analyzed in E16.5 embryos and correlated with defects in cortical plate structure. Calbindin immunostaining (A, D) labeled both Cajal-Retzius cells and TCAs, whereas neurofilament immunostaining (B, E) labeled the TCAs exclusively. A. In control embryos, Calbindin expression was found in cortical layer I (arrows) and in the TCAs. B. Neurofilament immunostaining labeled the TCAs (arrowhead). C. DAPI staining overlayed with A and B revealed the linear structure of the cortical plate and of cortical layer I. D. In mutant embryos, cortical layer I was wider, more scattered and discontinuous (arrows) and the TCAs had a wavy appearance (arrowhead) as evidenced by Calbindin immunostaining. Some straying axons migrated aberrantly towards the pia (open arrowheads). E. Neurofilament staining in mutants showed abnormal pathfinding of TCAs (arrowhead) and some axons migrating towards the pia (open arrowhead). F. In mutants, DAPI staining overlayed with D and E revealed the aberrant structure of the cortical plate and of cortical layer I. Scale bar 100 μ m.

length, but since the cortical plate forms an aberrantly wavy structure, the TCAs also appeared wavy (Fig.6D-E). Therefore, the defect in axon pathfinding of the TCAs mirrored the undulation of the mutant cortical plate. Furthermore, some axons aberrantly migrated radially through gaps in the cortical plate, towards the pia (Fig. 6E-F). These results suggest that the defect in axon guidance of the TCAs was a secondary defect, due to the aberrant structuring of the cortical plate.

3.7 Proliferation and cell death

ECM proteins have been shown to have a role in proliferation and in survival. We investigated whether the lack of cortical laminin had an impact on proliferation and death of neurons.

Furthermore, both the overall size and the size of the brain were smaller in mutant mice than in control littermates. We investigated whether the discrepancy in brain size was due to increased cell death or decreased cell proliferation. Cell death was assessed by TUNEL assay at E16.5 (Fig 7A-D). Cell death was not increased compared to control; therefore the decrease in brain size was not due to cell death. To analyze cell proliferation at different time points during development, we performed a BrdU pulse assay whereby we measured BrdU incorporation in E12.5, E14.5 and

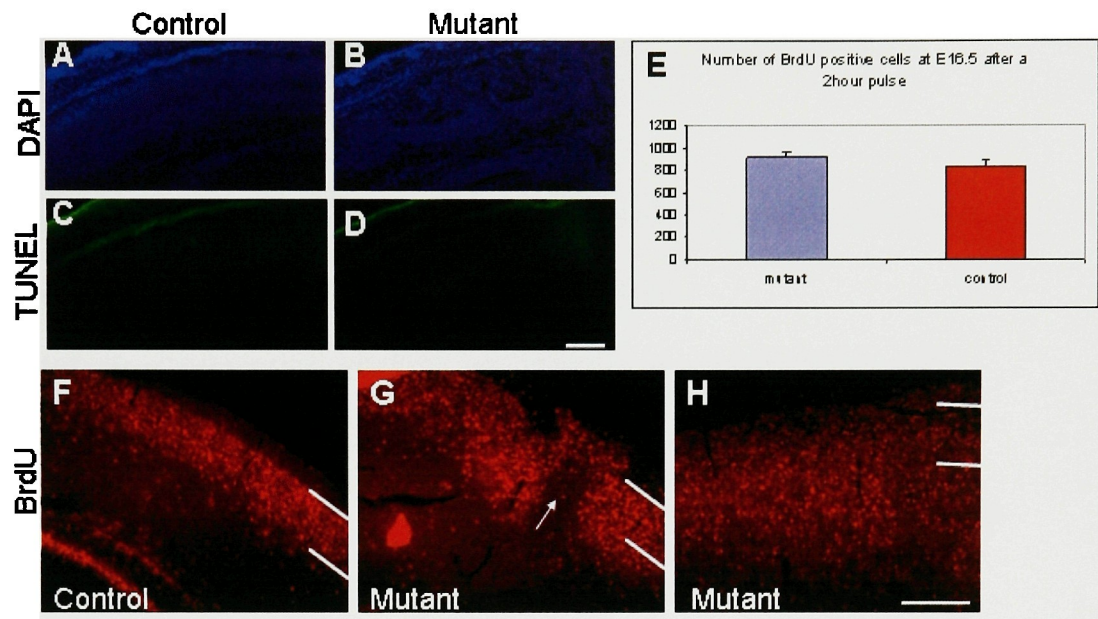


Figure 7: Lack of laminin does not induce changes in cell death or proliferation

A-D. Coronal sections of control and mutant cortices were stained for DAPI (A, B) and TUNEL (C, D). Cell death was not increased in mutants (B, D) compared to control (A, C). E. BrdU pulse assay. The number of BrdU-positive cells in the ventricular zone were counted and compared in mutants and controls at E16.5 after a 2 hour pulse of BrdU. The number of proliferating cells was not significantly altered in mutants. F-H. Birthdating and migration of layer III-IV cells was determined by in utero BrdU injection at E14.5 and the distribution of BrdU-labeled neurons was determined at P14 by immunohistochemistry. F. In controls, BrdU-labeled cells were present in layers III-IV, delineated by white lines. G. In mutant brains, BrdU-labeled cells formed undulations. H. In severely affected mutants, BrdU-labeled cells were evenly dispersed throughout the cortex and were not restricted to layers III-IV (delineated by white lines). The number of dividing neurons did not appear to change in mutants compared to controls (compare F with G and H). Scale bars 200 μ m.

allows detection of cells generated in a short time frame, in this case, two hours. Cell proliferation was not significantly altered in mutant embryos.

We also investigated whether the formation of specific cell layers were affected in mutant cortices. To perform birthdating/migration assays, we injected pregnant dams with BrdU at E14.5, then analyzed the progeny at post-natal day 14 (P14). In control animals, the neuroblasts generated at E14.5 are destined to form adult cortical layers III-IV. In mutant animals, the cells generated at E14.5 did not migrate properly although their number is normal. In some mutants, there was a ruffling in the cortical layers containing cells generated at E14.5 (Fig. 7F) whereas in other, more affected mutants, labeled cells are found scattered throughout the cortex (Fig. 7G). In mutant cortices, labeling with the monoclonal antibody SMI-32 and BrdU showed that BrdU-positive adult cells in layer III expressed the SMI-32 antigen (data not shown). Therefore, neuroblasts generated at E14.5 maintained their proper expression profile as adults even while they were abnormally positioned.

CHAPTER 4. ADDITIONAL RESULTS

4.1 ADDITIONAL PHENOTYPES

4.1.1 Abnormal cortical plate structure in mutant embryos

In order to investigate when the defect in migration of cortical plate cells occurred, we performed a Cresyl Violet staining. The migration of cortical plate cells begins at E14.5, and their migration was aberrant at that time point in mutant embryos (Fig 8, see also figures 4, 5 and 6). In control embryos, the cortical plate cells were regularly aligned directly below the surface of the brain. In mutant embryos, the cortical plate cells did not form a linear structure, but a wavy structure, where some cells appeared to have over-migrated and others under-migrated. Furthermore, some pockets of cells were detected above the cortical plate-like structures (arrows in Fig 8).

4.1.2 Laminin in the pia of adult mice

We have shown that laminin expression is discontinuous in the pia basement membrane of mutant embryos starting at E14.5 (Fig 4). In order to investigate whether this discontinuity persisted into adulthood, we performed laminin immunostaining of adult mutant brains (Fig 9). Surprisingly, laminin expression is restored by

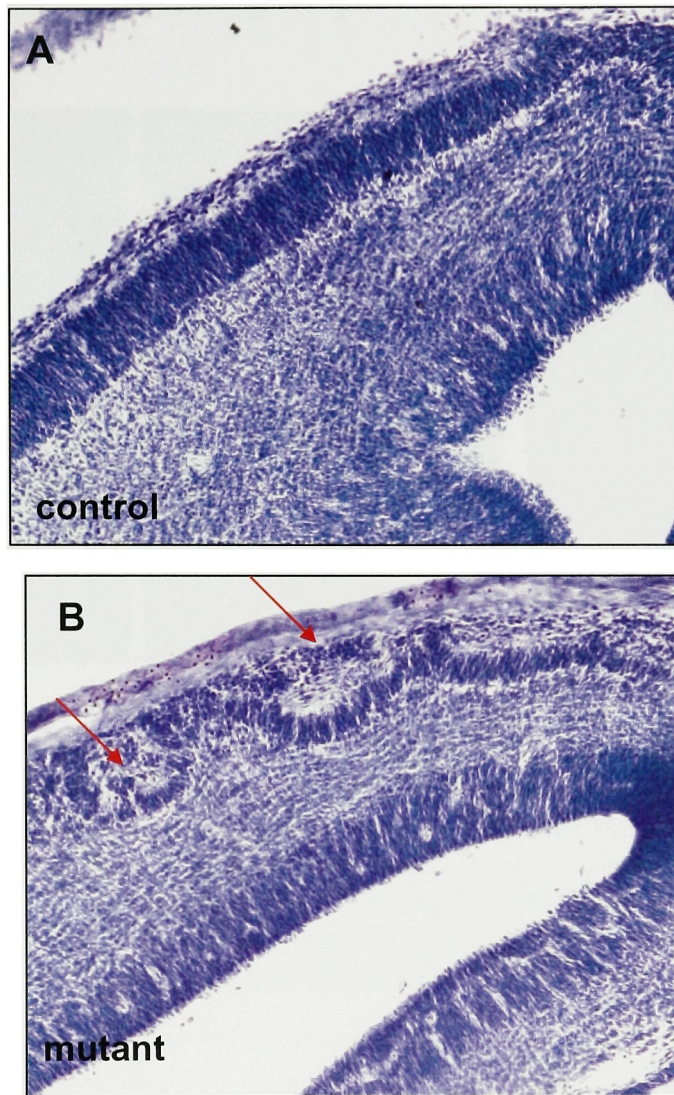


Figure 8: Defects in cortical lamination emerge at E14.5.

Defects in the formation of the cortical plate were detected by Cresyl Violet staining. The cortical plate was aberrant in mutant embryos, with malformations in the cortical plate. Cortical plate cells were not aligned as in control embryos (A), but had a wavy appearance (B). Some cells were found above the putative cortical plate (arrows in B).

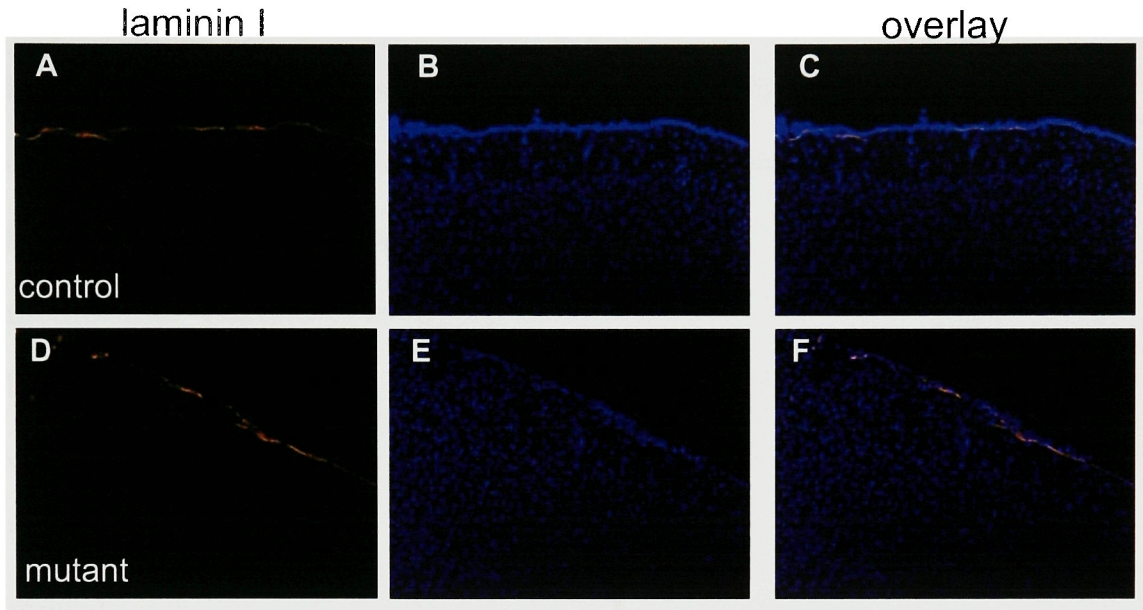


Figure 9: Mutant adults have intact pial laminin.

A. Control adult mice express laminin I in their pial basement membranes. B. DAPI staining shows the different layers in adult control mice. C. is an overlay of A and B. D. In mutant adult mice, laminin I expression persists. E. DAPI staining shows the perturbation of the cortical layers in mutant mice. F. is an overlay of D and E.

adulthood in mutants. However, several cell types secrete ECM proteins contributing to the pia basement membrane, and it is possible that not all these cell types undergo excision of the laminin $\gamma 1$ gene in mutants. The massive expansion of brain vesicles during development is probably correlated with a high-level expression of ECM proteins comprising the basement membrane. Therefore, reducing the number of cells expressing laminin $\gamma 1$ would have a drastic effect during embryogenesis, whereas, in adults, only a slower turnover of ECM proteins would be required. Therefore, expression of laminin $\gamma 1$ after neuronal migration is complete would not rescue the phenotype.

4.1.3 Genetic pathway

It is well known that laminins can bind to multiple receptors, including integrin $\beta 1$ and dystroglycan. We investigated whether the expression of these receptors were affected in mutant embryos. Immunostaining for both integrin $\beta 1$ and dystroglycan has revealed that their expression pattern is perturbed (Fig 10 and 11). In area of intact cortical plate formation, both receptors were present near the pial basement membrane directly above cortical plate cells. In areas of neuronal extrusion through breaches in the basement membrane, receptor expression, although maintained directly above the cortical plate cells, was displaced to outside the

brain. Therefore, the expression of these laminin receptors was not decreased in the absence of laminin $\gamma 1$, but rather seemed to be displaced by the aberrantly migrating neurons.

The ECM is composed of several proteins crosslinked together. We analyzed the expression pattern of another ECM component, perlecan (Fig 12). Mutations in perlecan in mice generate a phenotype similar to laminin $\gamma 1$ conditional mutants (Costell et al., 1999). Immunostaining for perlecan revealed a correlation between laminin and perlecan expression; in areas lacking laminin, perlecan is absent as well. Therefore, laminin is required for the assembly of other components into a functional basement membrane.

4.2 NEURONAL MIGRATION

4.2.1 Radial glial cells

The role of radial glial cells in cortical plate neuron migration has been well established. We have shown that the radial glial scaffold is severely disorganized in mutant embryos (see Fig 5). In some severely affected mutants, the radial glial projections extended in scattered directions and are shortened. However, we have observed that in mildly affected mutants, the radial glial scaffold extended beyond the brain. At E16.5 we have observed mutant

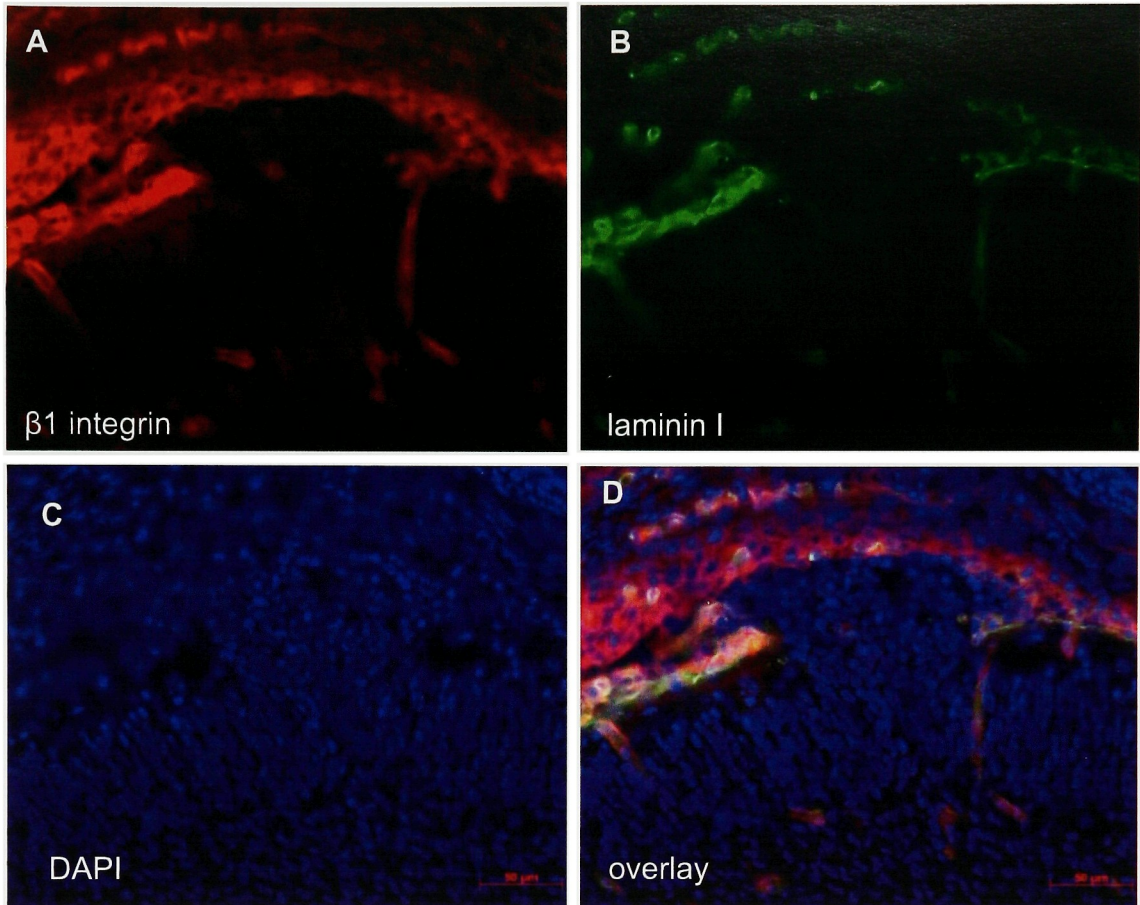


Figure 10: Laminin disruption in the pia was correlated with disruption in the expression of the laminin receptor $\beta 1$ integrin. Immunostaining for the laminin receptor $\beta 1$ integrin and for laminin I shows aberrant expression patterns. A. Integrin $\beta 1$ expression persisted above the cortical plate neurons. In areas where neurons over-migrated, integrin $\beta 1$ expression was displaced. B. Laminin I staining shows that areas lacking laminin expression correlate with extrusions of neurons. C. DAPI staining allows visualization of all cell nuclei. D. is an overlay of A, B and C.

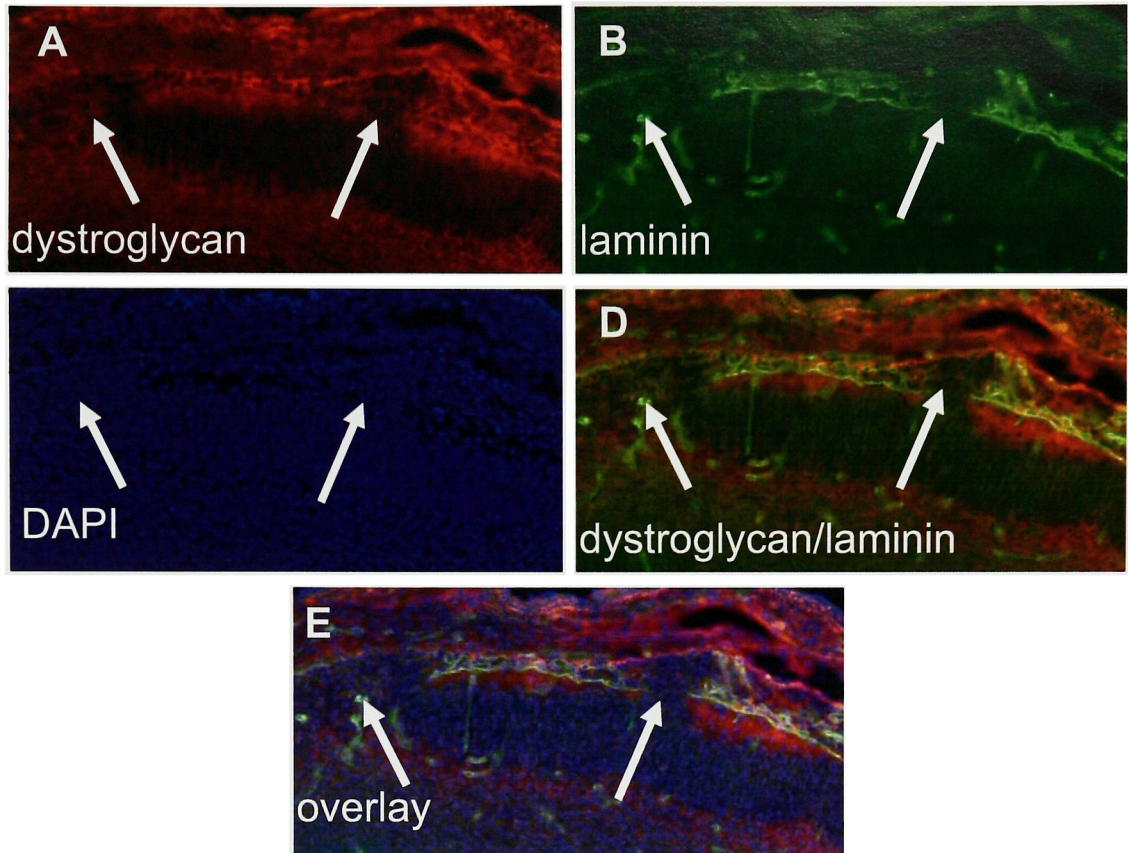


Figure 11: Laminin disruption in the pia is correlated with disruption in the expression of the laminin receptor dystroglycan.

Immunostaining for the laminin receptor dystroglycan and for laminin I shows aberrant expression patterns. A. Dystroglycan expression persisted but was pushed by over-migrating cortical plate neurons (arrows). B. Laminin I staining was discontinuous in the pia. Areas lacking laminin are shown by arrows. C. Overlay of laminin and dystroglycan shows that areas lacking laminin have abnormal expression of dystroglycan. E. DAPI staining allows visualization of all cell nuclei. Arrows show areas of over-migration of neurons. D. is an overlay of A and B. E. is an overlay of A, B and C.

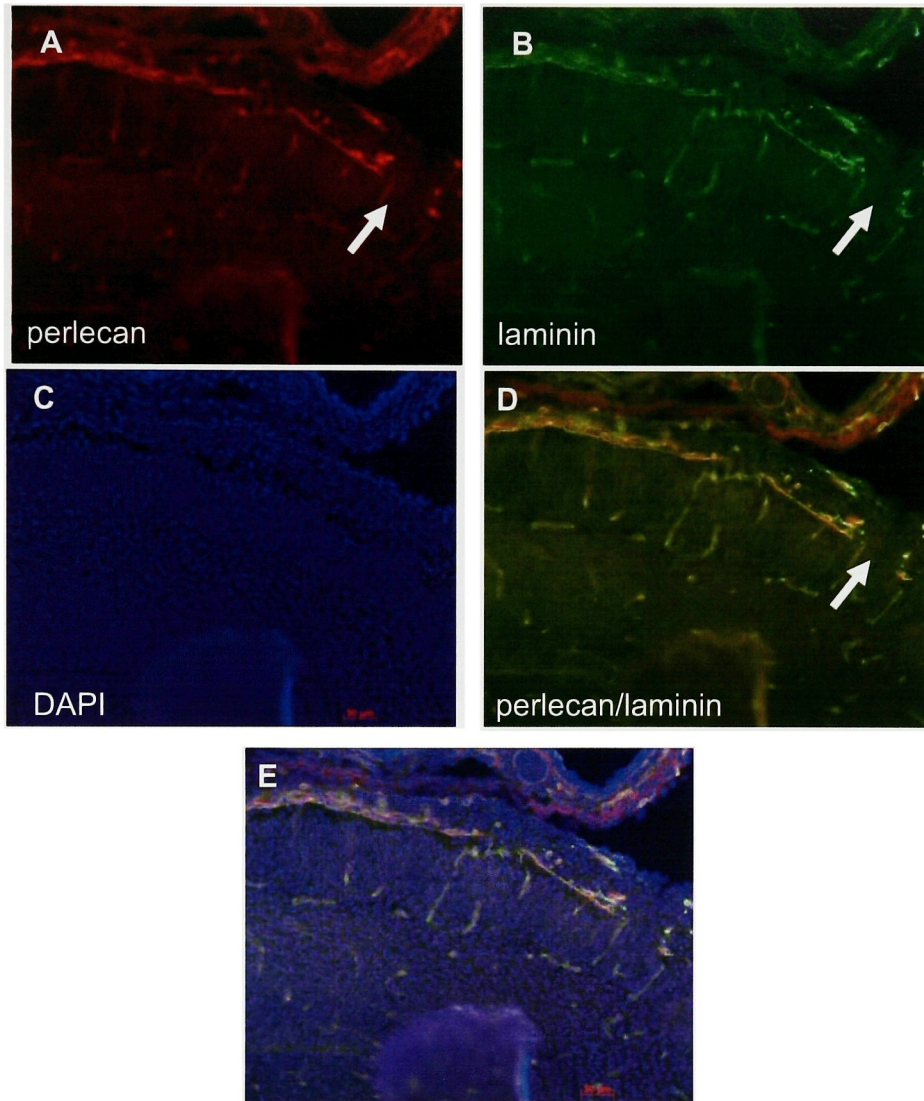


Figure 12: Laminin disruption in the pia is correlated with disruption of the ECM protein perlecan.

Immunostaining for perlecan and laminin I revealed aberrant pattern in mutant embryos. A. Perlecan expression was discontinuous in the pia. An area lacking perlecan expression is shown by the arrow. B. Laminin I expression is also discontinuous. The area shown by the arrow also lacks laminin expression. C. DAPI allowed visualization of all nuclei. D. is an overlay of A and B showing that the expression pattern of laminin I and perlecan overlapped and that areas lacking laminin also lack perlecan expression. E. is an overlay of A, B and C. Scale bar shows 50 μm .

cortices with localized areas showing an over-migration of the radial glial extensions, as identified by BLBP immunostaining (Fig 13). We hypothesize that in severely affected mutants, the radial glial scaffold loses its radial structure entirely whereas in less affected mutants, the scaffold is affected only locally. These data demonstrate the role of laminin $\gamma 1$ in the outgrowth of radial glial cells extension. Pial laminin may act both as a chemoattractant for developing glial extensions and serve to anchor glial endfeet, thereby terminating migration. Therefore, in the complete absence of laminin $\gamma 1$, glial extensions would not have any chemoattractive signal to entice growth towards the pia, whereas if laminin $\gamma 1$ levels are decreased, glial extension would proceed normally. In localized areas lacking laminin in the pia, radial glial extension may continue migrating outside of the brain, as there is no laminin to anchor it.

4.2.2 Cajal-Retzius cells

Cajal-Retzius cells have a role in cortical neuron migration (Frotscher, 1998; Tissir and Goffinet, 2003). Cajal-Retzius cells secrete various proteins, including Calbindin, Calretinin and Reelin, the most extensively studied molecule. We have analyzed the expression pattern of Reelin in mutant E14.5 embryos (Fig 14). In control embryos, Reelin staining is present at cortical layer I in

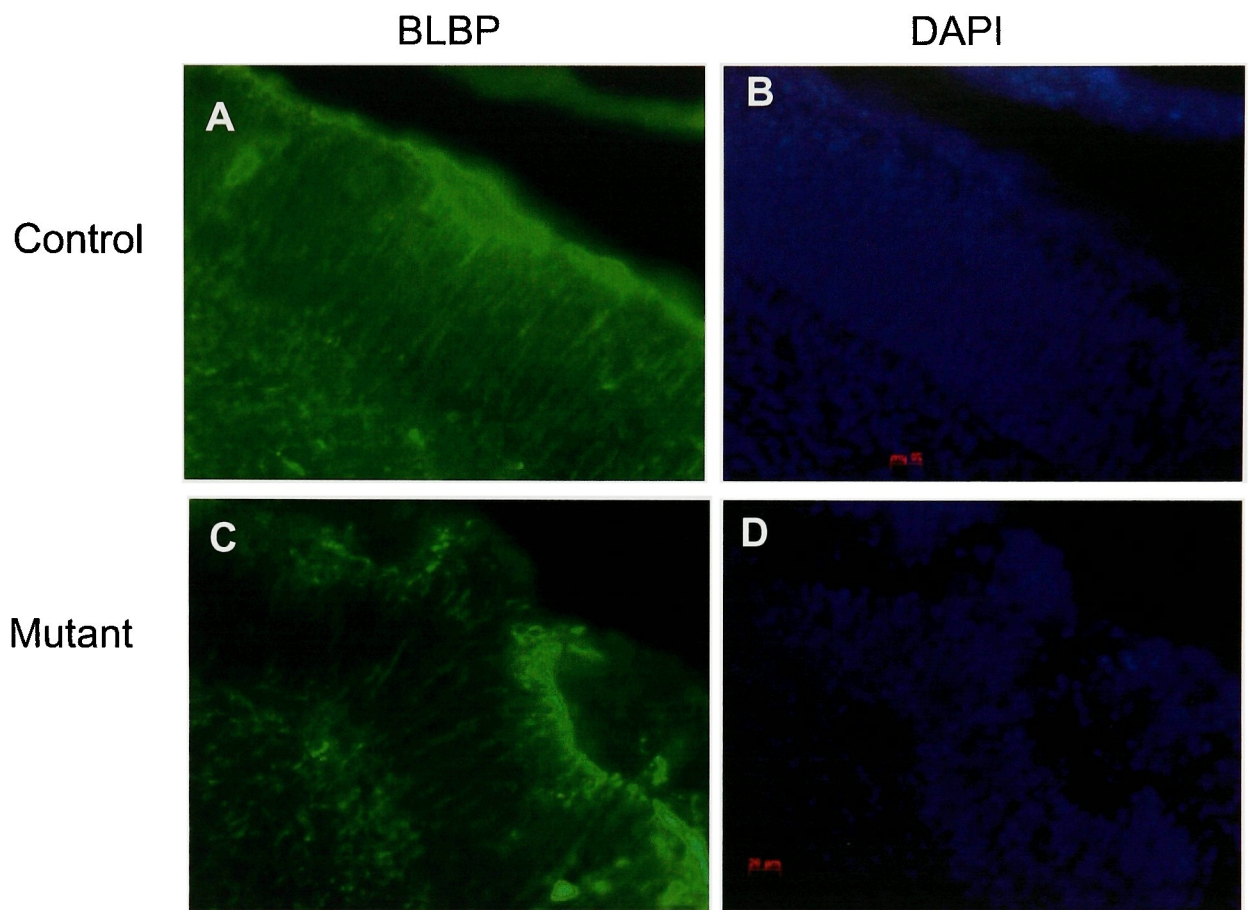


Figure 13: The radial glial scaffold is impaired in mutant embryos.

Immunostaining of the radial glial scaffold using the BLBP antibody revealed defects in mutant E16.5 embryos. A. BLBP expression in control brains shows the alignment of the radial glial extensions. B. DAPI staining shows the linear cortical plate. C. In mutant brains, BLBP expression shows that the radial glial extensions are not aligned properly. In some areas, they over-migrate. D. DAPI staining shows the over-migration of cortical plate neurons in the same area.

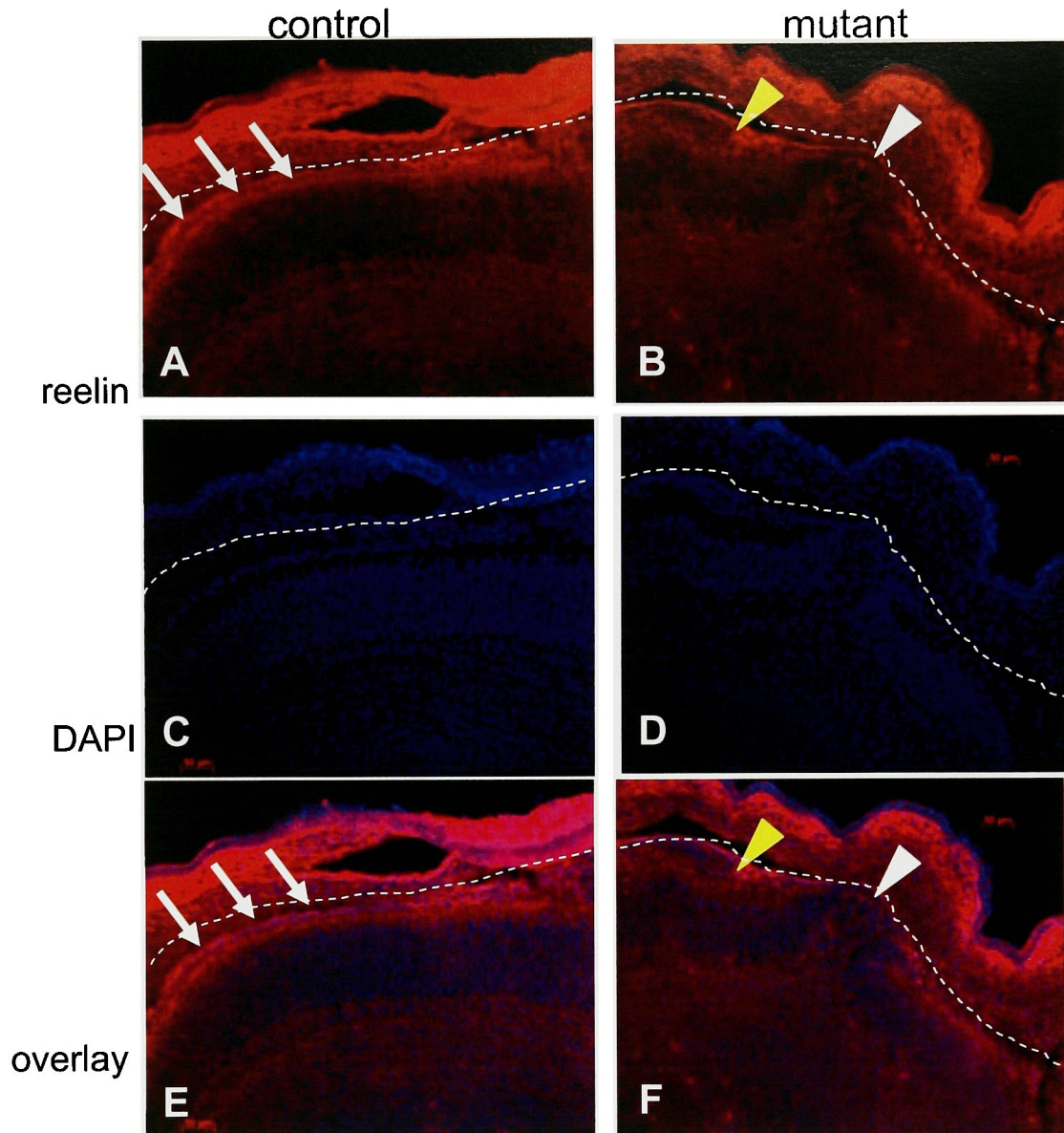


Figure 14: Reelin expression pattern is perturbed in mutant embryos.

Reelin expression was monitored by immunostaining at E16.5. High background staining was observed outside of the CNS (above dashed lines). A. Reelin expression in control embryos was restricted to cortical layer (arrows). B. In mutant embryos, reelin expression was discontinuous; in areas where over-migration had occurred there was less reelin staining (white arrowhead) whereas in areas where neurons had under-migrated there was more reelin staining (yellow arrowhead). C and D. DAPI staining allows detection of the cortical plate structure. E and F. Overlays of A/C and B/D.

a linear pattern. In mutant embryos, the expression pattern of Reelin is perturbed. In some areas, Reelin expression is absent whereas in others, Reelin is expressed in a broader pattern. We have shown that the expression pattern of two Cajal-Retzius proteins, Calbindin and Reelin is abnormal, demonstrating that the positioning of Cajal-Retzius cells is abnormal.

4.3 NEURONAL MORPHOLOGY

We have observed, by SMI 32 immunostaining, that the morphology of cortical neurons is abnormal (see Fig 3). In order to further investigate this defect, we have performed immunostaining for other markers. Immunostaining by MAP1 was used to analyze axonal extensions (Fig 15) and MAP2 for dendritic and axonal morphology (Fig 16). MAP1 immunostaining revealed that axons in the CA1 region of the hippocampus from mutants were shorter than in controls. MAP2 immunostaining in the cortex of mutant adult mice showed that dendrites are shorter and more disorganized. Specifically, neuronal extensions labeled by MAP2 projected in random directions instead of extending towards the pial surface.

We performed Golgi staining in adult mutant brains to analyze the morphology of cortical cells (Fig 17). Some cortical neurons

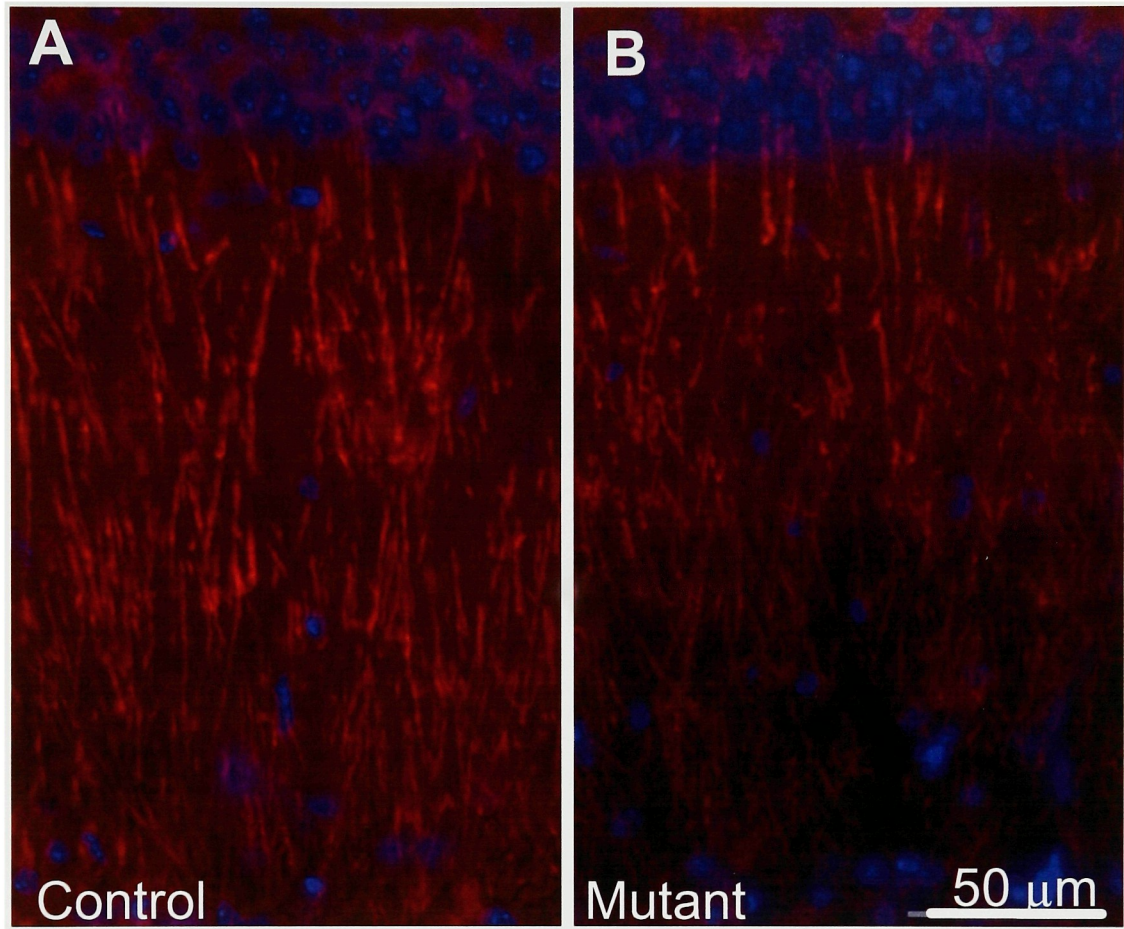


Figure 15: Abnormal dendritic extension in the hippocampal CA1 region of mutant mice.

The dendritic morphology of hippocampal neurons was analyzed by MAP1 immunostaining. A. In control adult brains parallel processes extended from the CA1 region of the hippocampus. B. In mutants, these processes were shorter.

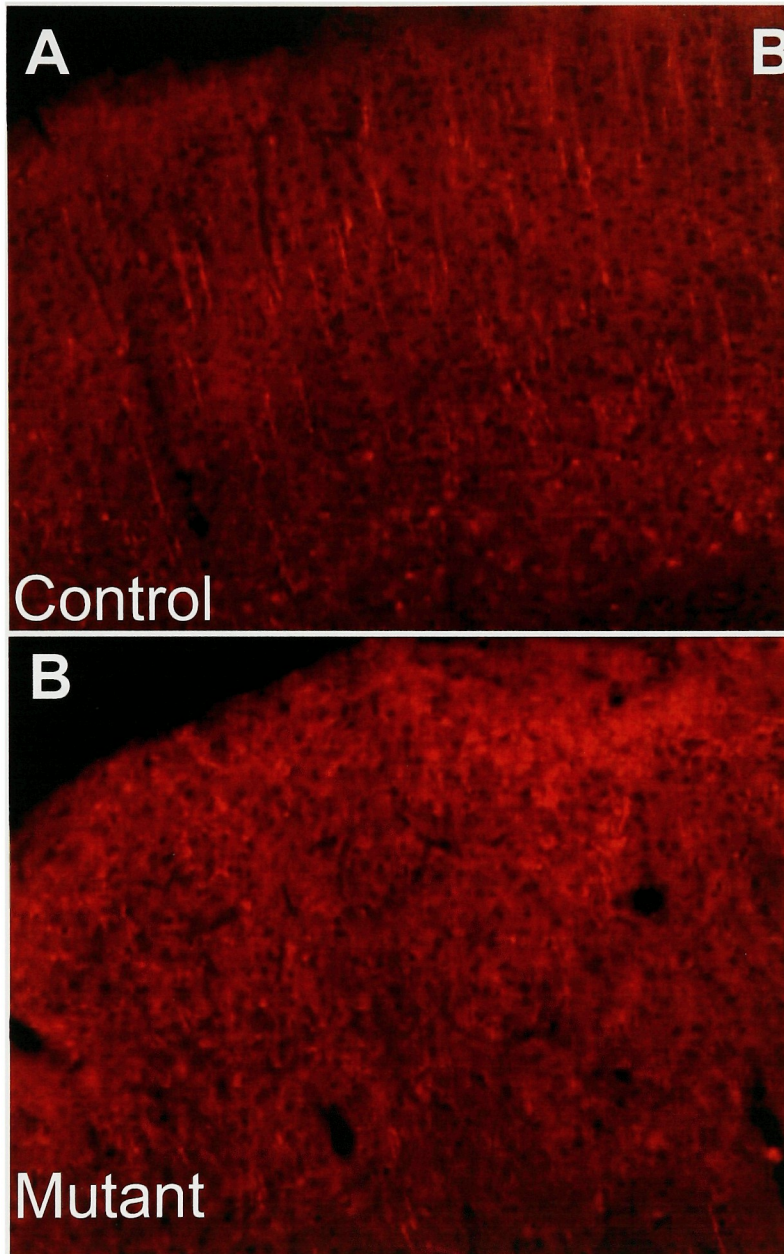


Figure 16: Abnormal orientation of axons and dendrites in the cortex of mutant mice.

The orientation of axons in adult mice was determined by immunostaining for MAP2.

A. In control brains, MAP2 staining showed axons extending towards the pial surface. B. In mutant brains, MAP2 staining revealed that axons are shorter and disorganized.

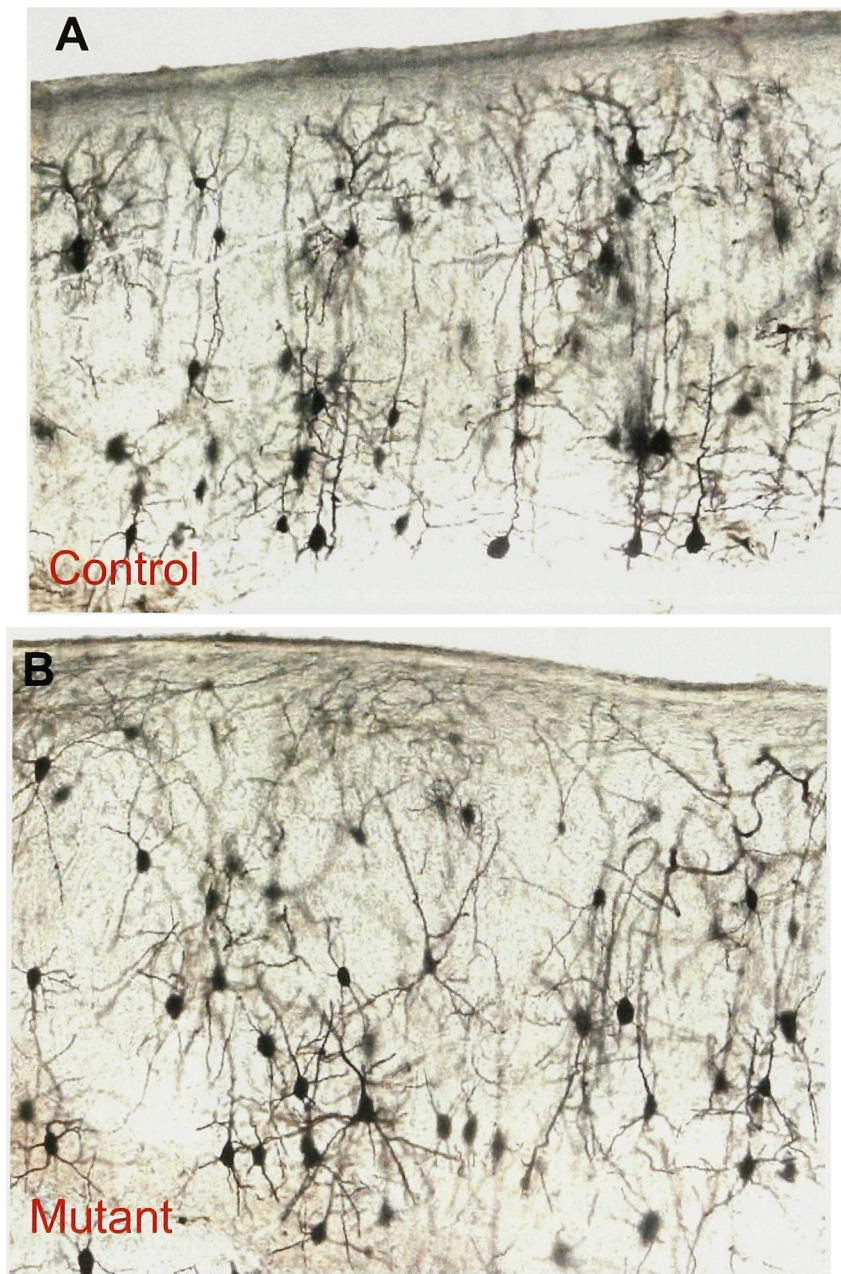


Figure 17: Golgi staining reveals defects in the positioning of the cortical layers and in the orientation of dendrites.

Golgi staining was performed in adult mice. A. In control brains, the neuronal processes were oriented towards the pia. B. In mutant embryos, the processes extend in random directions.

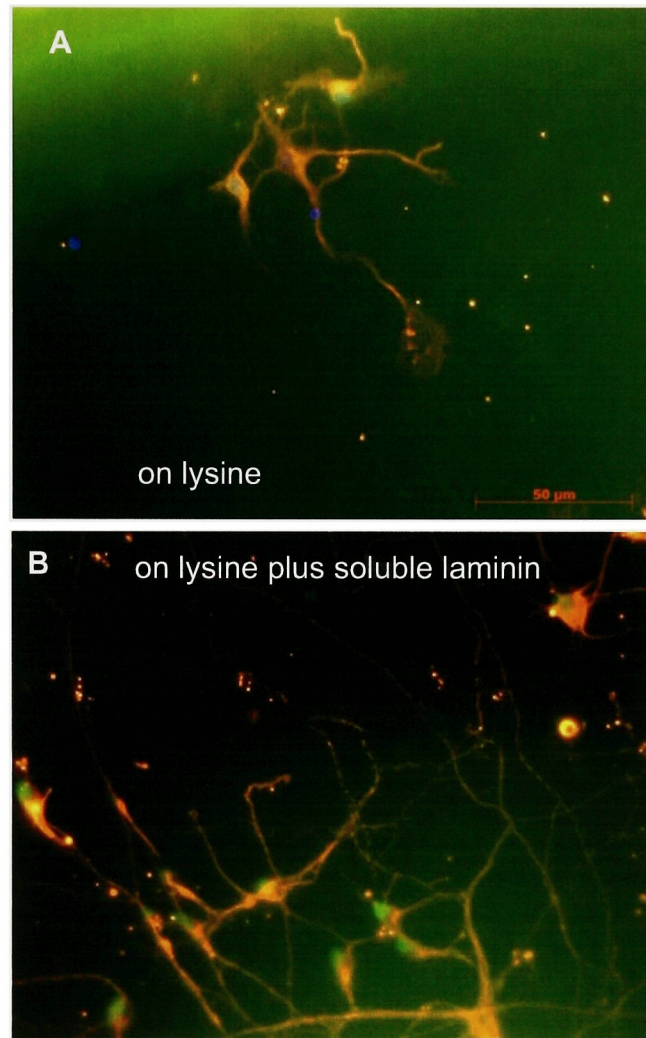


Figure 18: Mutant neurons project shorter neurites in culture. Mixed cortical cultures from mutant E13.5 embryos were identified by EGFP fluorescence. A. Mutant neurons (expressing EGFP) grown on lysine extended a few short neurites, whereas control neurons from the same brain (not expressing EGFP) extended longer neurites. B. Cultures grown on lysine with added soluble laminin in the media showed robust extension of neurites.

projected in random directions instead of towards the pial surface. Therefore, laminin is required for the extension of axons and dendrites in the proper orientation. In order to analyze the role of laminin in neurite extension during development, we turned to *in vitro* systems. Mixed cortical cell cultures from mutant E13.5 embryos containing the Z/EG reporter were cultured for 5 days (Fig 18).

In this system, only cells which have undergone recombination express EGFP. This allowed us to analyze both mutant and control neurons from the same animal. Mutant cells grown on a lysine substrate show limited neurite outgrowth, whereas control cells from the same plate exhibit extensive neurite outgrowth (Fig 18A). Therefore, laminin expression by a neuron is required for the induction of neurite growth. The addition of soluble laminin in the media rescues this phenotype, with all neurons showing extensive neurite outgrowth (Fig 18B).

4.4 AXON GUIDANCE

We have observed widespread defects in axon guidance in mutants. Axons were labeled using various methods, such as Luxol Fast Blue, neurofilament antibody and Timm staining.

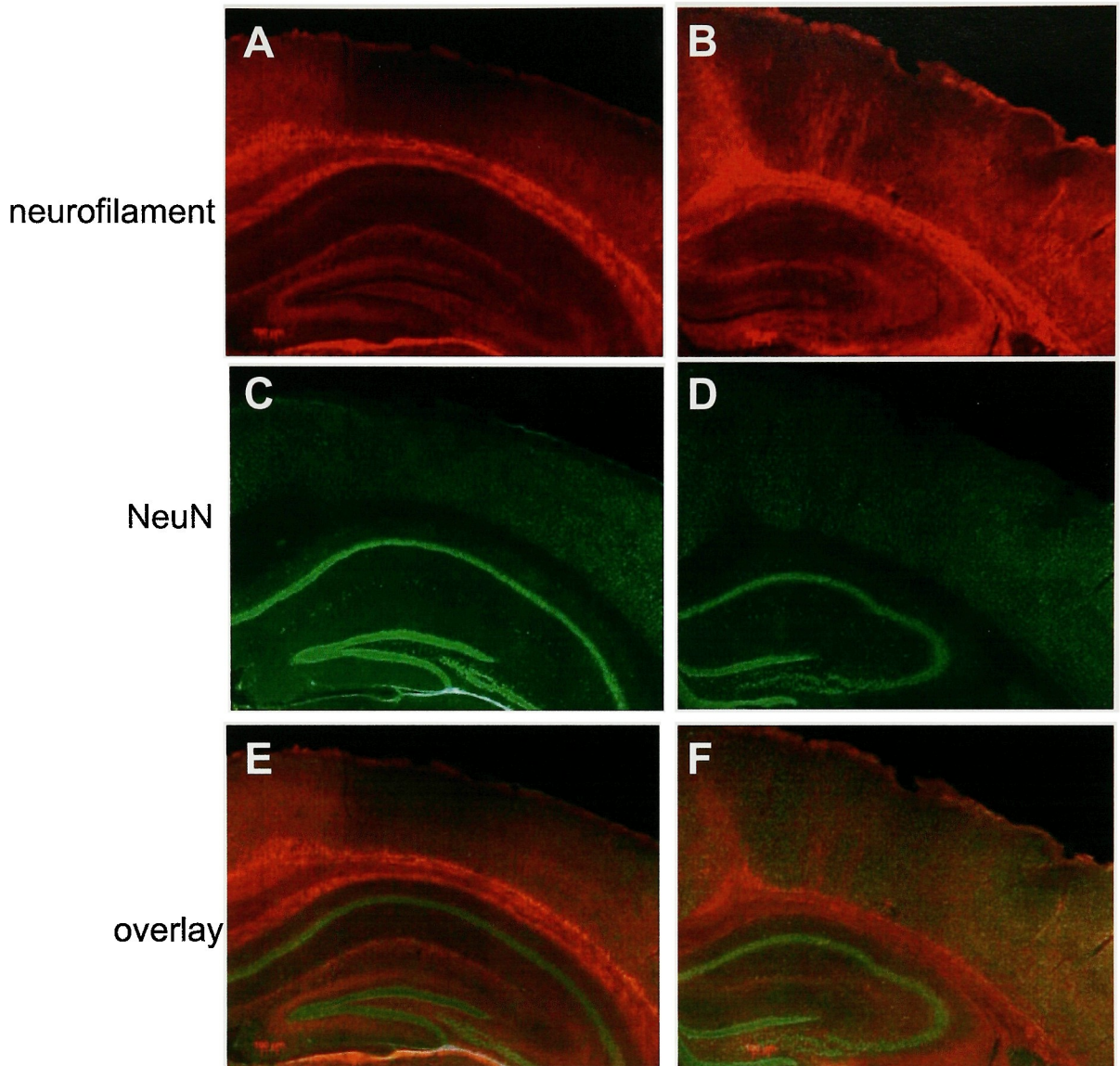


Figure 19: Misguided axonal bundles displace neuronal cell bodies in the cerebral cortex.

Immunostainings for neurofilament and NeuN (for neuronal cell bodies) were performed in adult brains. A. In control brains, axons were present in the corpus callosum. B. In mutant brains, some axonal bundles strayed from the corpus callosum towards the pial surface. C. In control brains, neuronal cell bodies were distributed evenly. D. In mutant brains, neuronal cell bodies were pushed aside by misguided axons. E and F. Overlays of A/C and B/D.

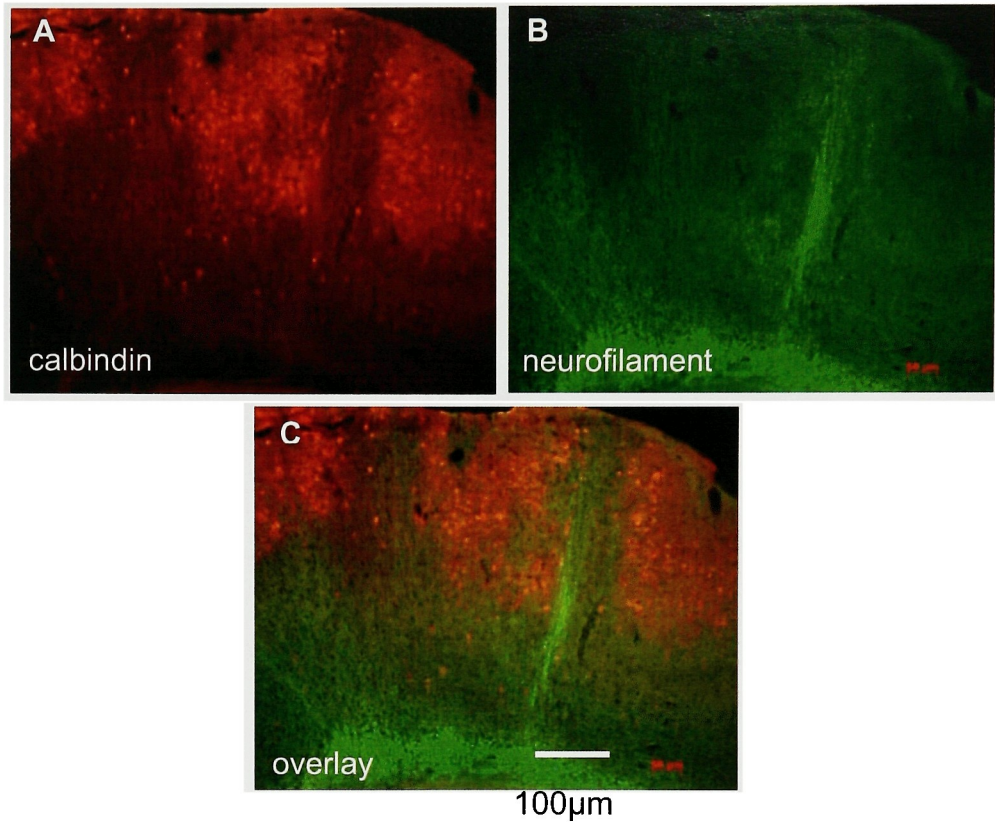


Figure 20: Interneuron positioning defect correlates with straying axons.

Immunostaining for interneurons (Calbindin) and axons (neurofilament) was performed in adult mutant brain. A. Calbindin staining shows the aberrant migration pattern of interneurons in the cortex. B. The neurofilament staining shows straying bundles of axons. C. The overlay of A and B demonstrated that straying axons and interneurons never overlap, suggesting that developing aberrant axons may push interneurons aside.

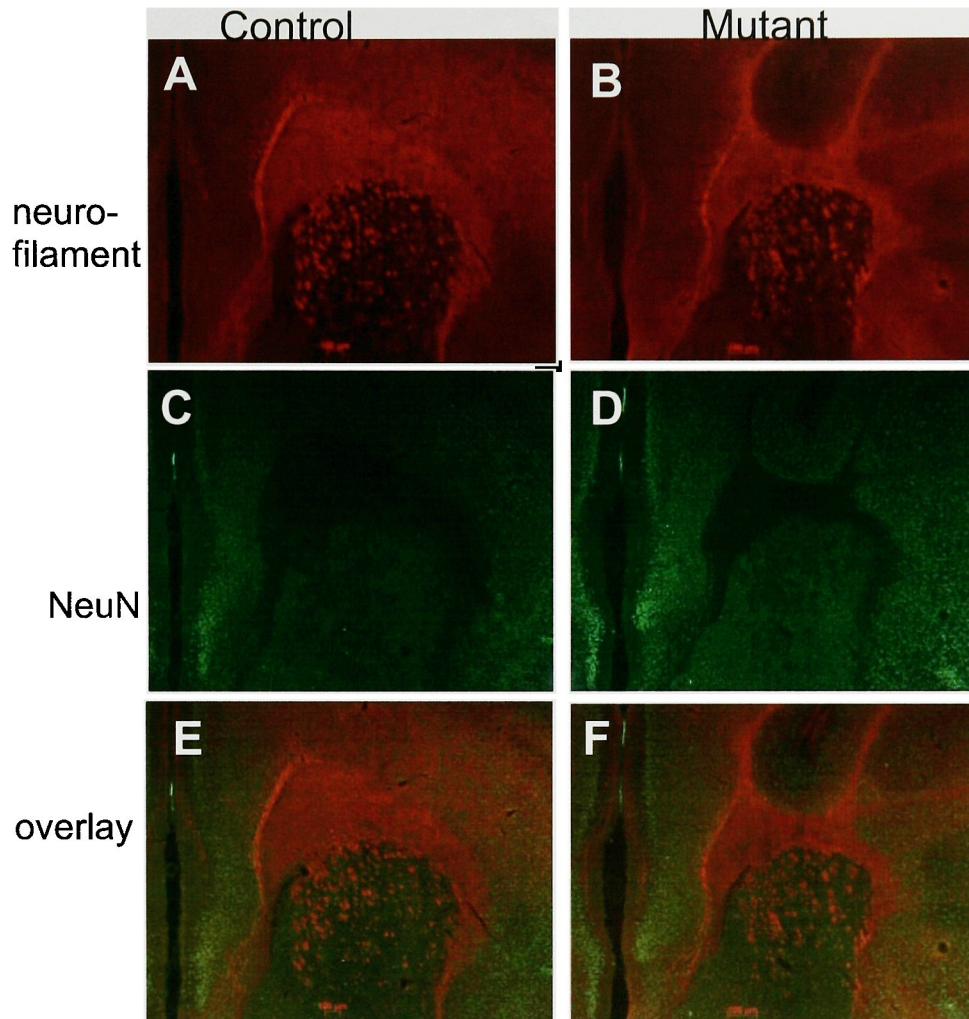


Figure 21: Misguided axonal bundles displace neuronal cell bodies in the external capsule of adult mutants.

Immunostainings for neurofilament and NeuN (for neuronal cell bodies) were performed in adult brains. A. In control brains, axons were present in the external capsule. B. In mutant brains, some axonal bundles strayed from the external capsule towards the pial surface. C. In control brains, neuronal cell bodies were distributed evenly, except in areas where axons were present. D. In mutant brains, neuronal cell bodies were pushed aside by misguided axons. E and F. Overlays of A/C and B/D.

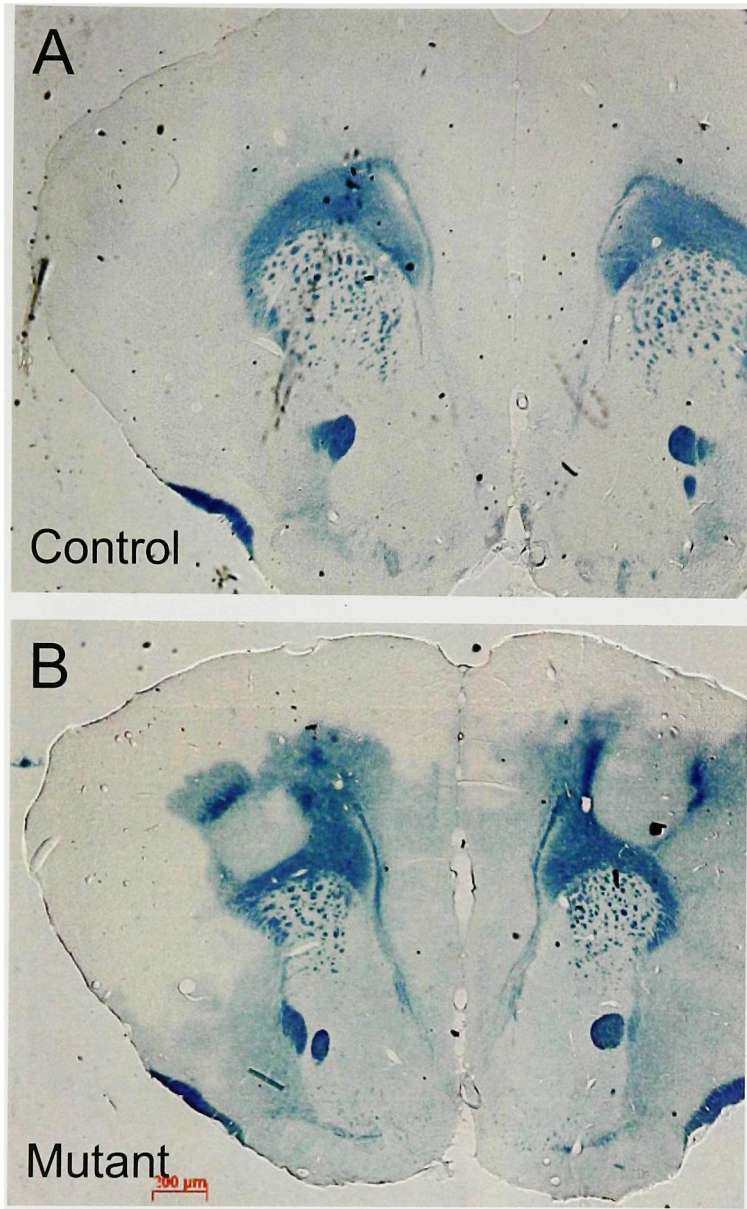


Figure 22: Defect in the formation of the forceps minor corpus callosum in mutant mice.

Axon guidance was analyzed by Luxol Fast Blue (LFB) staining in the anterior cortex of adult brains.

A. In control brains, LFB is present in the minor corpus callosum.

B. In mutant embryos, axons, stained by LFB, stray towards the pial surface.

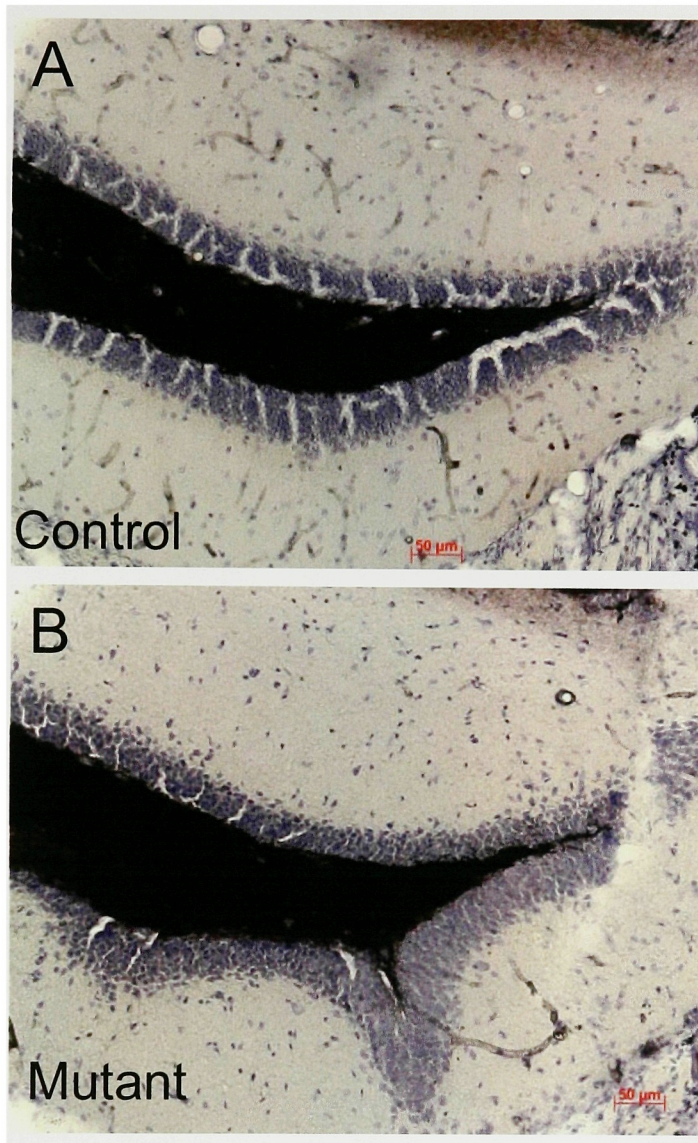


Figure 23: Abnormal positioning of neurons in the dentate gyrus (DG) of mutant mice.

Axons were stained using Timm staining and cell bodies with Cresyl Violet. A. In control mice, axons were restricted to the DG. B. In mutant mice, some neurons were abnormally positioned and some axons strayed outside of the DG.

Straying axons were observed in mutant adults in various regions of the brain. Neurofilament immunostaining in the adult cortex at the level of the hippocampus revealed axonal bundles straying from the corpus callosum to the pial surface (Fig 19). This result was similar to the Luxol Fast Blue staining in adults (Fig 3), confirming the observed defect. Furthermore, NeuN immunostaining to label neuronal cell bodies showed that the straying axonal bundles had displaced neurons (Fig 19 C and D). In order to investigate the identity of these misplaced neurons, we labeled interneurons by immunostaining for Calbindin. We observed that interneurons were also displaced by misguided axons (Fig 20).

Bundles of axons straying to the pial surface were also observed in the external capsule by immunostaining with neurofilament (Fig 21) and by Luxol Fast Blue staining (Fig 22). In the adult hippocampus, mossy fibers, labeled by Timm staining, were observed to extend beyond the dentate gyrus (Fig 23).

In order to investigate at what time point these axonal guidance defect occurred, we performed immunostainings in mutant embryos. At E18.5, axonal guidance defects were also observed in various areas of the brain. Straying axons were observed in other regions of the brain in E18.5 embryos. In the hippocampus, axon

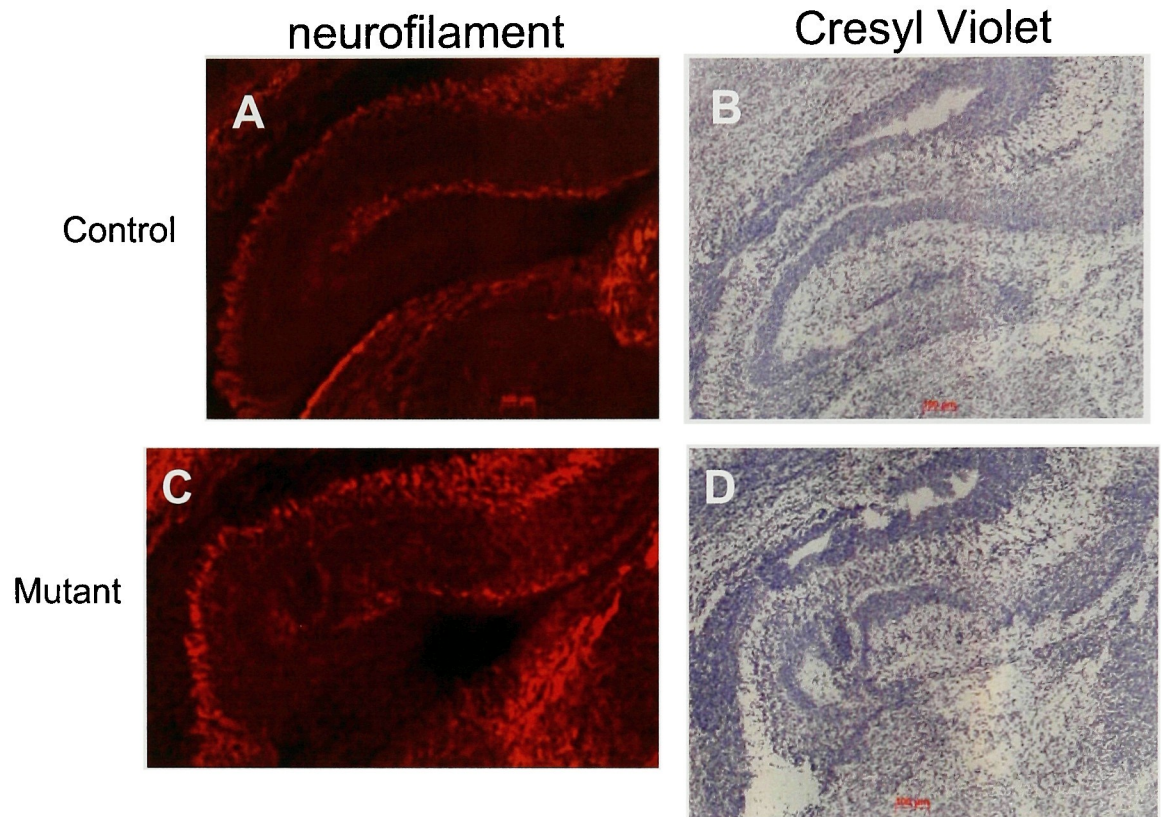


Figure 24: Hippocampal defects in E18.5 mutant mice.

Axon guidance was analyzed by neurofilament immunostaining and hippocampus structure was analyzed by Cresyl Violet (CV) staining at E14.5.

A. Neurofilament staining shows axons guidance in the hippocampus. B. CV staining shows the position of cells in the hippocampus. C. Mutant embryos have slightly disorganized axon guidance. D. Mutant embryos show impaired migration of cells composing the hippocampus.

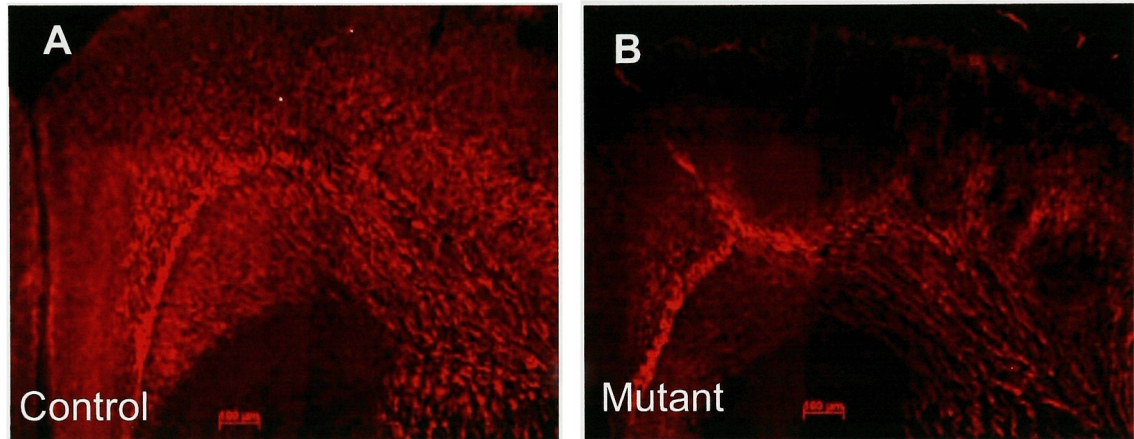


Figure 25: Axonal guidance defects in anterior portions of the brain.

Neurofilament immunostaining shows thalamocortical axons (TCA) and the corpus callosum at E14.5. A. In control embryos, the TCAs form an arc. B. In mutant embryos, the TCAs wander towards the pial surface.

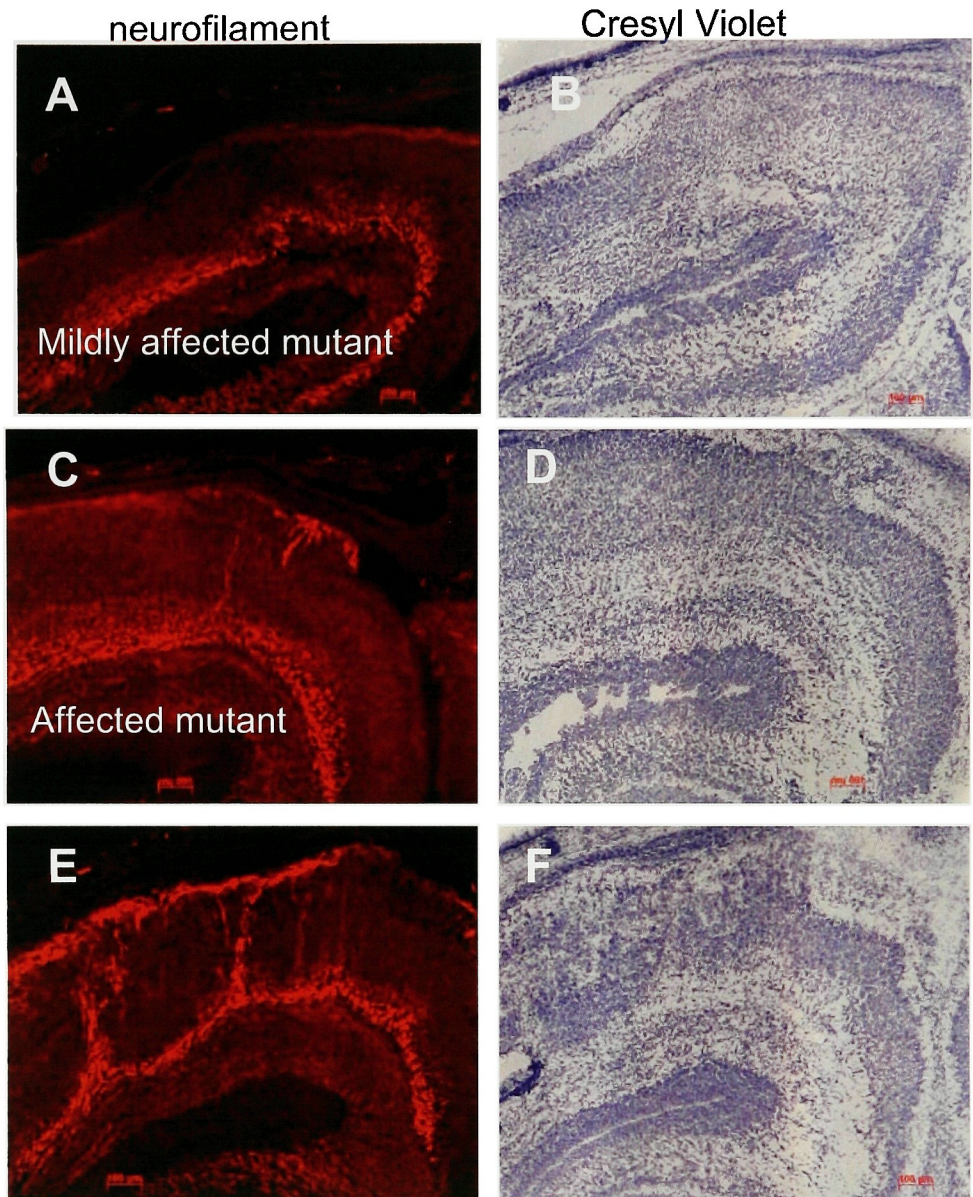


Figure 26: Axonal migration defects in the cortex are present at E18.5.

Adjacent sections from E18.5 mutants were immunostained for neurofilament and stained with Cresyl Violet (CV). A. Mildly affected mutants showed a slight waviness in TCA pathfinding. B. CV staining showed only mild cortical defects. C. Affected mutants showed one or more straying axons. D. CV staining showed that the straying axons did not push any cells aside. E. Strongly affected mutants exhibit several bundles of straying axons. F. CV staining does not show any cells being displaced by the aberrantly migrating axons.

migration was slightly perturbed (Fig 24), and in the anterior cortex, axons strayed towards the pial surface (Fig25). In the cortex, axons from the thalamo-cortical tract, immunolabelled by a neurofilament antibody, extended towards the pial surface (Fig 26). The severity of the mutant, as determined by Cresyl Violet staining of adjacent sections, correlated with the axonal guidance defect. Therefore, in mutants with mild defects in the structure of the cortical plate, axons appeared somewhat perturbed; whereas mutants with increasing cortical plate waviness exhibited increasing numbers of straying axons. However, it is not clear whether malpositioned cortical plate neurons had an impact on subsequent axon pathfinding, or whether these two processes are both dependent on laminin.

Many molecules have been identified which play a role in axon pathfinding. Laminin has been shown to interact with netrin and change its axon-attractive property to axon-repulsive (Hopker et al., 1999). Therefore, decreased levels of cortical laminin could alter the environment encountered by axons. For example, if netrin and laminin are both present throughout the cortex, they would present a chemo-repulsive signal and ensure that axons do not migrate to the cortex. In the absence of laminin expression, this chemo-repulsive signal may be converted to a chemo-

attractive signal and induce axons to traverse the cortex. In order to investigate the role of netrin in axon guidance within the cortex, we performed immunostainings in E14.5 embryos (Fig 28). Netrin expression was not observed throughout the cortex but was confined above the cortical plate in control embryos (Fig 28A). In mutant embryos, netrin expression was disrupted. Netrin expression was present above clusters of cortical plate fragments (Fig 28B, arrows). Therefore, instead of presenting a solid linear structure which may prevent axons from straying towards the pial surface, the netrin expression pattern in mutants is discontinuous. It is possible that axons may extend through the breaches in netrin expression.

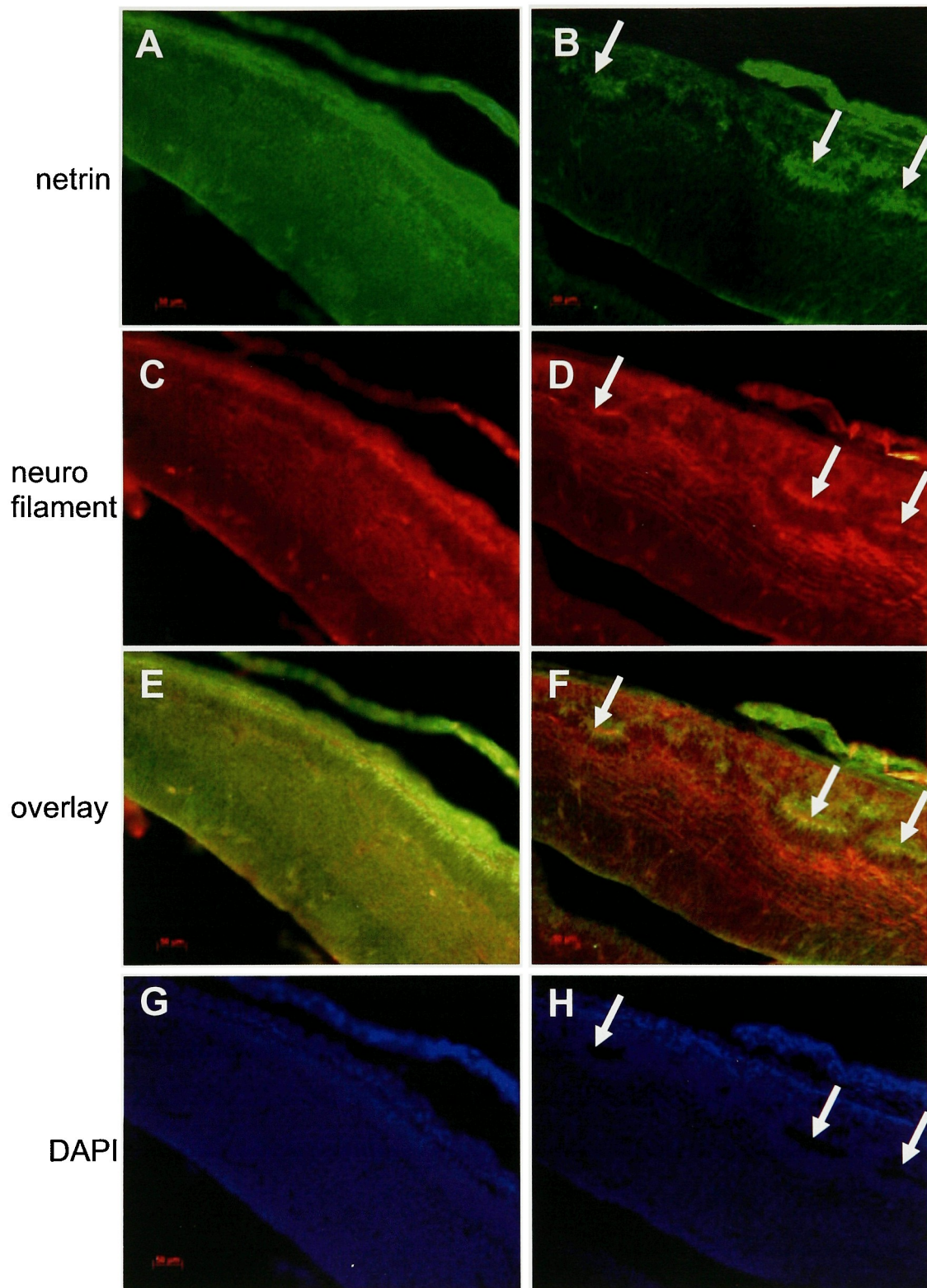


Figure 27: Netrin expression is perturbed in mutant embryos.

Netrin and neurofilament expression were determined by immunostaining at E14.5. A. In control embryos, netrin expression was confined to an area above the cortical plate. B. In mutant embryos, netrin expression was found in patches (arrows). C. In control embryos axons migrated below the cortical plate. D. In mutant embryos, axon guidance is impaired, but the axons remained below the cortical plate. E and F. is an overlay of A/C and B/D demonstrating that axons migrate along the cortical plate, and that netrin expression is above the cortical plate. G and H. show a DAPI staining to visualize the cortical plate neurons.

CHAPTER 5: DISCUSSION

Selective excision of the laminin $\gamma 1$ gene in the cerebral cortex using the CaMKII promoter-driven Cre recombinase results in mutant mice which develop severe brain abnormalities, including abnormal cortical layering and aberrant axonal positioning. We propose that laminin deficiency both in the pial basal lamina and within the cortex leads to a cascade of events with multiple consequences. Lack of pial laminin results in a discontinuous basal lamina. Since the developing radial glial fibers normally extend and attach to the pial basal lamina, breaches within the pial basement membrane lead to disorganizations of the radial glial scaffold. Neuroblast migration along the radial glial scaffold is therefore affected, leading to a malformation of the embryonic cortical plate and abnormal adult cortical layers. Furthermore, thalamocortical axons which migrate along the cortical plate exhibit aberrant pathfinding. Finally, laminin is not required for proliferation or survival.

5.1 Laminin as a structural component of the ECM

In the Cre mouse line we used in this study, excision of the laminin $\gamma 1$ gene occurred in cortical neurons at E10.5 and led to a discontinuous or completely absent pia basal lamina during

embryogenesis; however, laminin expression and the basal lamina were restored by adulthood. Although the Cre recombinase was only active in a limited subset of cells, the phenotype appeared robust throughout the cerebral cortex. We hypothesize that after excision of the laminin $\gamma 1$ gene and during the rapid expansion of the cortex, the basal lamina is stretched and then ruptured, resulting in disruptions or a complete absence of the pia. In our mutants, we have also observed a decrease in the expression of other ECM proteins, such as perlecan, correlating with disruptions of pial laminin, indicating a requirement for laminin in the assembly of other ECM components into a functional basal lamina. Surprisingly, laminin $\gamma 1$ is also required for expression of integrin $\beta 1$, a laminin receptor. Therefore, depletion of laminin $\gamma 1$ impacts other cortical molecules.

Mutations in components of the basement membrane (such as perlecan) or laminin receptors (such as integrin $\alpha 6$, integrin $\beta 1$ and dystroglycan) or the intracellular protein FAK all exhibit similar defects such as defective assembly of laminin into a functional basal lamina, radial glial scaffold disorganization and neuronal migration defects (Georges-Labouesse et al., 1998; Graus-Porta et al., 2001; Moore et al., 2002; Beggs et al., 2003). However, not only do these mutants affect laminin indirectly, but

they also exhibit other phenotypes, rendering it difficult to discern whether the pial basement membrane defect is an effect of the mutation or the cause of the other phenotypes. Furthermore, these mutants exhibit extensive defects outside of the CNS. For example, perlecan mutants have severe myocardial defects and die at E10 (Costell et al., 1999) and deletion of the nidogen1-binding site of laminin γ 1 results in abnormalities in the kidneys, lungs and CNS vasculature, leading to lethality at birth (Halfter et al., 2002; Willem et al., 2002). In mutants with deletion of the nidogen1 binding site of laminin γ 1 both cortical and vascular basal laminae are disrupted and cerebral hemorrhaging is observed. Therefore, analysis of laminin γ 1 conditional mutants allowed us to conclude that laminin is directly responsible for the formation of the radial glial scaffold and neuronal migration.

5.2 Laminin is critical for normal development at the cellular level

Laminin is known to have a role in neuronal polarity (Rogers et al., 1986; Letourneau et al., 1988; McLoon et al., 1988; Gomez and Letourneau, 1994; Kuhn et al., 1995; Luckenbill-Edds, 1997; Bonner and O'Connor, 2001) and in neurite extension (Costa et al., 2002; Grimpe et al., 2002). In our mutants, cortical neurons extended neurites in a disorganized array, suggesting that the

defects in neuronal morphology induced by laminin deficiency may be due to impaired laminin signaling.

ECM components are required for cell survival (Meredith et al., 1993; Chen and Strickland, 1997; Boudreau and Jones, 1999) and their absence can induce cell death (Frisch and Francis, 1994; Frisch and Screaton, 2001). Our present investigation demonstrates that cell death of cortical neurons is not increased in our mutants. Therefore, laminin is not important for neuronal survival during development.

5.3 The role of laminin in the formation of cortical layers

We propose two possible mechanisms for the cortical layering defects observed in our laminin $\gamma 1$ conditional mutant mice. First, the radial glial scaffold is known to support migration of neuroblast from the ventricle to the surface of the brain. Radial glial cells form a network by extending parallel processes from the ventricle to the surface of the brain, then attaching to the basement membrane via endfeet. In our mutants, the radial glial scaffold was severely disorganized with radial glial cells and neurons clustering around remaining fragments of laminin.

Second, Cajal-Retzius cells are also known to have a role in neuronal migration. In our mutants, Cajal-Retzius cells are dispersed and form clusters instead of a compact and linear cell layer. The exact mechanism of migration of Cajal-Retzius cells is not well characterized. It is not understood whether Cajal-Retzius cells migrate by gliophilic migration which is dependent on laminin or by somal translocation. Since Cajal-Retzius cells are abnormally positioned in laminin $\gamma 1$ conditional mutants, then laminin is required for the proper positioning of Cajal-Retzius cells. Cajal-Retzius cells secrete several molecules which have a role in cortical lamination, including Reelin. Reelin was originally thought to act as a stop signal to terminate neuroblast migration by promoting detachment from radial glial cells (Frotscher, 1998; Pearlman et al., 1998). Reelin and laminin are known to bind to the integrin $\alpha 3 \beta 1$ integrin receptor (Dulabon et al., 2000), leading to the hypothesis that a switch of substrates for this integrin receptor could lead to detachment from radial glial cells. However, it has been shown that ectopic expression of Reelin can restore partial migration and therefore Reelin does not function simply as a positional signal (Magdaleno et al., 2002). In our mutants, although the aberrantly positioned Cajal-Retzius cells still secrete both Reelin and Calbindin, the expression patterns of Reelin and Calbindin are discontinuous and could impact migration of cortical

neurons. However, it is unclear whether lack of laminin $\gamma 1$ indirectly impacts neuronal migration due to the aberrant positioning of Cajal-Retzius cells or whether laminin has a direct role in neuronal migration.

5.4 The role of laminin in interneuron migration

Interneurons, generated in the lateral and medial ganglionic eminences, must migrate much farther distances than neuroblasts generated at the ventricular zone. Axons have been proposed as the substrate for the migration of interneurons (Tanaka et al., 2003; McManus et al., 2004). The observed axon pathfinding defects in laminin $\gamma 1$ conditional mutants are a possible cause of impaired interneuron migration. However, the defect in interneuron positioning could also be due to defects in the cortical plate.

Analysis of laminin $\gamma 1$ conditional mutants showed a requirement for laminin during cortical development. We have demonstrated that the lack of laminin $\gamma 1$ alone is sufficient to cause disruptions in the pia basal lamina leading to defects in cortical layering and disorganizations in the radial glial scaffold. The decrease in cortical laminin also led to a defect in the morphology of neurons, but had no impact on survival or proliferation.

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