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Divergent roles of ApoER2 and Vldlr in the migration of cortical neurons

Iris Hack[1,*], Sabine Hellwig[1,2,*], Dirk Junghans[3], Bianka Brunne[4], Hans H. Bock[4], Shanting Zhao[1] and Michael Frotscher[1,4,†]

Reelin, its lipoprotein receptors [very low density lipoprotein receptor (Vldlr) and apolipoprotein E receptor 2 (ApoER2; also known as Lrp8)], and the cytoplasmic adaptor protein disabled 1 (Dab1) are important for the correct formation of layers in the cerebral cortex. Reeler mice lacking the reelin protein show altered radial neuronal migration resulting in an inversion of cortical layers. ApoER2 Vldlr double-knockout mutants and Dab1 mutants show a reeler-like phenotype, whereas milder phenotypes are found if only one of the two lipoprotein receptors for reelin is absent. However, the precise role of the individual reelin receptors in neuronal migration remained unclear. In the study reported here, we performed fate mapping of newly generated cortical neurons in single and double receptor mutants using bromodeoxyuridine-labeling and layer-specific markers. We present evidence for divergent roles of the two reelin receptors Vldlr and ApoER2, with Vldlr mediating a stop signal for migrating neurons and ApoER2 being essential for the migration of late generated neocortical neurons.

KEY WORDS: Lipoprotein receptors, disabled 1, Reelin, Layer formation, Neuronal migration

INTRODUCTION

The mammalian neocortex consists of six layers of neurons with distinct functional and morphological identities. These layers are generated in an inside-out sequence, with early born neurons located in the deep layers and late born cells in superficial layers (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic and Caviness, 1995; Takahashi et al., 1999). The formation of individual neuronal layers involves migration of neurons in radial and tangential directions to their final laminar positions. To generate an inside-out pattern, cells migrating to the cortical plate must be able to pass earlier generated neurons and stop at their appropriate destinations. The nature of interaction of migrating cells with their environment and the molecular signals that regulate this process and subsequent differentiation are complex, and as yet poorly understood (reviewed by Marin and Rubenstein, 2003).

One pathway regulating the migration of neurons involves the extracellular matrix protein reelin (reviewed by Rakic and Caviness, 1995; Curran and d'Arcangelo, 1998; Frotscher, 1998; Tissir and Goffinet, 2003; Soriano and Del Rio, 2005; Förster et al., 2006a; Förster et al., 2006b). Reelin is a glycoprotein secreted by Cajal-Retzius cells in the developing marginal zone of the cortex and other laminated brain regions (D'Arcangelo et al., 1995; D'Arcangelo et al., 1997; Lambert de Rouvroit and Goffinet, 1998; Meyer and Goffinet, 1998; Rice et al., 2001). Different models of reelin action have been proposed. Reelin may act as a stop signal (Dulabon et al., 2000), a chemoattractant (Gilmore and Herrup, 2000) and a detachment signal for migrating neurons (Hack et al., 2002; Sanada et al., 2004).

Neurons migrating radially towards the cortical plate express at least three essential elements of the reelin signaling cascade: the two reelin receptors Vldlr (very low density lipoprotein receptor) and ApoER2 (apolipoprotein E receptor 2; also known as Lrp8 – Mouse Genome Informatics) (D'Arcangelo et al., 1999; Trommsdorff et al., 1999; Hiesberger et al., 1999), and the adapter molecule disabled 1 (Dab1) that binds to the intracellular domains of these receptors (Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997; Rice et al., 1998; Trommsdorff et al., 1999). Binding of reelin to its membrane receptors VLDLR or ApoER2 has been shown to induce phosphorylation of Dab1 (Hiesberger et al., 1999; Howell et al., 1999; Trommsdorff et al., 1999). Mutations in the reelin gene, the Dab1 gene and in both the Vldlr and ApoER2 gene result in a reeler-like phenotype characterized by a severely altered cortical layering (Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997; Lambert de Rouvroit and Goffinet, 1998; Trommsdorff et al., 1999). The analysis of single receptor mutants, however, revealed a minor phenotype with Vldlr being more important for the development of the cerebellum and ApoER2 for cortical lamination (Trommsdorff et al., 1999; Benhayon et al., 2003). To further characterize possible individual roles of these two reelin receptor molecules for the development of cortical layers, we have examined cortical lamination in Vldlr and ApoER2 single and double-knockout mutants, reeler mice and Dab1 mutants using layer-specific markers and bromodeoxyuridine (BrdU) labeling. With this combined approach we provide new evidence for divergent roles of ApoER2 and Vldlr in neuronal migration and cortical lamination. Whereas ApoER2 is important for the proper migration of late generated neurons, Vldlr mediates a stop signal for reelin, preventing migrating neurons from entering the marginal zone.

MATERIALS AND METHODS

Animals

Experiments were performed in agreement with the German law on the use of laboratory animals. ApoER2 and Vldlr single and double-mutant mice were maintained on a mixed 129SvEv×C57BL/6J strain background. The

[1]Institut für Anatomie und Zellbiologie and [2]Neurologische Universitätsklinik, Neurozentrum, Albert-Ludwigs-Universität Freiburg, D-79104 Freiburg, Germany. [3]Max-Planck-Institut für Immunbiologie, D-79108 Freiburg, Germany. [4]Zentrum für Neurowissenschaften, Albert-Ludwigs-Universität Freiburg, D-79104 Freiburg, Germany.

*These authors contributed equally to this work
†Author for correspondence (e-mail: Michael.Frotscher@anat.uni-freiburg.de)

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day of appearance of the vaginal plug was considered embryonic day (E) 0.5 and the day of birth postnatal day (P) 0. Mice were anesthetized with CO_2 and killed by cervical dislocation.

BrdU injection and staining

Pregnant mice (n=2 for each developmental time point) were injected intraperitoneally with a sterile solution of BrdU (Roche Diagnostics, Mannheim, Germany) in 0.9% NaCl (50 mg/kg) at E12.5, E13.5, E14.5, E15.5 and E16.5. Newborn mice were killed on P0. Brains were fixed in freshly prepared 4% paraformaldehyde (PFA) in phosphate-buffered saline (1×PBS) and cut into 30 μm serial sagittal sections on a vibratome. For BrdU detection, sections were washed in distilled water for 30 minutes at room temperature. They were then incubated in 2 M HCl for 30 minutes at 37°C and washed in borate buffer (120 mM NaCl, 17 mM sodium tetraborate, 100 mM boric acid in distilled water, pH 8.4) three times for 10 minutes at room temperature. Slides were then rinsed for 15 minutes in 0.1 M phosphate buffer (PB) and incubated in blocking solution [5% normal goat serum (NGS), containing 0.2% Triton X-100 in PB] for 60 minutes at room temperature. This was followed by incubation in a monoclonal mouse anti-BrdU antibody (1:1000; Roche Diagnostics) in PB containing 4% bovine serum albumin (BSA), 1% NGS, and 0.2% Triton X-100 overnight at 4°C. After three washes for 15 minutes each in PB at room temperature, sections were exposed to the secondary antibody (goat anti-mouse IgG, Alexa Fluor 488; Invitrogen, Karlsruhe, Germany), 1:300 in PB containing 4% BSA overnight at 4°C. After three washes for 15 minutes in PB, the sections were embedded in fluorescent mounting medium (S3023 DAKO, Glostrup, Denmark).

In situ hybridization

Probe generation

Total RNA from P0 wild-type brains was reverse-transcribed to cDNA with reverse transcriptase (Supercript II, Invitrogen) and used as a template for the PCR reaction (Eppendorf, Hamburg, Germany). The following oligonucleotide primers were used (MWG-Biotech AG, Ebersberg, Germany): *ApoER2* forward 5′-GCTGTCATTGGGGTCATCG-3′, reverse: 5′-GCTTGCACTTGACGACAGGC-3′. The primers also included T7 polymerase binding sites (not listed). The combination of primers led to the amplification of the expected single band of 398 bp. The PCR template was purified (Qiaquick purification kit, Qiagen, Hilden, Germany), sequenced (GATC, Konstanz, Germany), and used for in vitro transcription.

In vitro transcription

In vitro transcription was performed with 1 μg linearized plasmid DNA or PCR template, in the presence of ATP, GTP, CTP and digoxigenin-11-UTP (DIG), RNasin, transcription buffer and T3, SP6 or T7 RNA polymerase (Roche Diagnostics), for 2 hours at 37°C following the manufacturer's recommendations.

RNA detection by in situ hybridization

For in situ hybridization the following DIG-labeled riboprobes were used: *Cux2* (Zimmer et al., 2004) (also known as *Cutl2* – Mouse Genome Informatics), *ER81* (Lin et al., 1998) (also known as *Etv1* – Mouse Genome Informatics), *RORbeta* (Schaeren-Wiemers et al., 1997) (also known as *Rorb* – Mouse Genome Informatics), *Tle4* (Beffert et al., 2006) and *ApoER2*. Brains (n=5-10 for each marker and developmental time point) were fixed in freshly prepared 4% PFA in 1× PBS, cryoprotected in 30% sucrose, and frozen in isopentane at –60°C. In situ hybridization was performed on 50 μm free-floating cryostat sections as described previously (Haas et al., 2000). Briefly, free-floating sections were prehybridized in hybridization buffer (50% formamide, 4× SSC, 50 mM NaH_2PO_4, 250 μg/ml heat-denatured salmon sperm DNA, 100 μg/ml tRNA, 5% dextransulfate and 1% Denhard's solution) for 60 minutes at 45°C or 65°C. Hybridization was performed in the same buffer including 700 ng/ml of the riboprobes at 45°C or 65°C overnight. After hybridization, the sections were washed in 2× SSC (twice for 15 minutes each) at room temperature, 2× SSC and 50% formamide, 0.1× SSC and 50% formamide for 15 minutes each and finally in 0.1× SSC (twice for 15 minutes each) at 55°C or 64°C. Immunological detection with anti-DIG-AP was performed as recommended by the manufacturer (Roche Diagnostics). Colorimetric detection was accomplished using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

For double fluorescent in situ hybridization (FISH), sections were co-hybridized with a digoxigenin (DIG)-labeled *ApoER2* or *RORbeta* probe and a FITC-labeled *Cux2* probe and processed as described by Dufour et al. (Dufour et al., 2006). To allow for a comparison of the layer-specific markers with Nissl-stained sections, part of the sections were stained with Cresyl Violet, dehydrated in ethanol and xylene, and coverslips applied with Histokit (Shandon, Pittsburgh, USA).

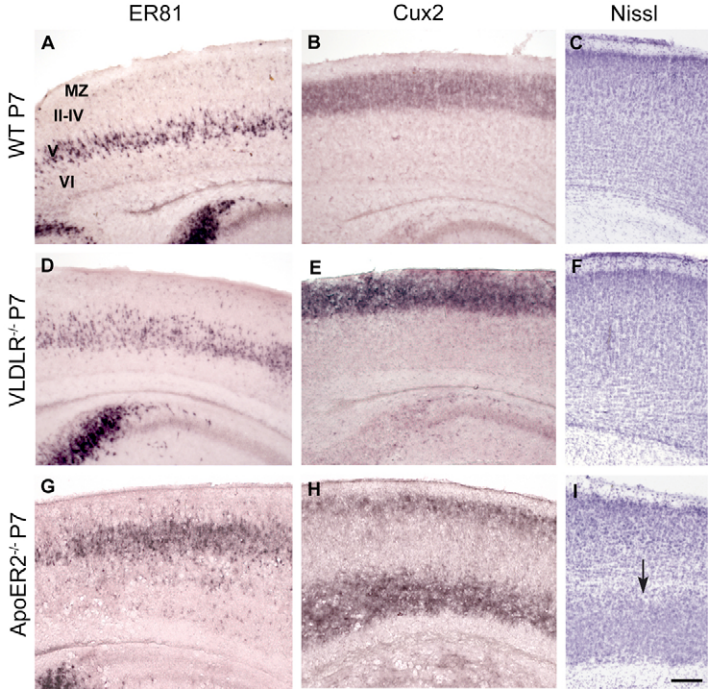

Fig. 1. Labeling of early and late born cortical neurons in wild-type, *Vldlr*$^{-/-}$ and *ApoER2*$^{-/-}$ mice at P7 using *ER81* and *Cux2*. (**A**,**D**,**G**) In situ hybridization for *ER81*, a marker of early generated neurons, showed strongest expression in cortical layer V neurons on sagittal brain sections of wild-type animals (A) and *Vldlr*$^{-/-}$ mice (D). In *ApoER2* mutants (G), labeling in the outer portion of the cortex, normally occupied by layer II-IV neurons, was found, indicating a more superficially located layer V. (**B**,**E**,**H**) In situ hybridization for *Cux2*, a marker of late generated neurons, revealed expression in the upper cortical layers (II-IV) in wild-type animals (B). In *Vldlr* mutants (E), the *Cux2*-positive population showed a similar distribution, but the marginal zone was invaded by many *Cux2*-positive cells. In *ApoER2* mutants (H), in situ hybridization for *Cux2* showed two bands, a superficial one underneath layer I, and a deep one, located in the innermost portion of the cerebral wall. A comparison of the expression patterns of *ER81* (G) and Cux2 (H) in this mutant suggests that the upper *Cux2*-positive cells represent layer IV neurons. (**C**,**F**,**I**) The prominent phenotype of *ApoER2* mutants when compared to *Vldlr*$^{-/-}$ mice is also seen in Nissl-stained sections. Arrow in I indicates cell accumulation in deep cortical layers. Scale bar: 200 μm.

Immunohistochemistry

Staining procedure

Brains (n=4-10 for each genotype and developmental time point) were fixed in 4% PFA in 1× PBS and sliced sagittally on a vibratome (50 μm). Sections were pre-incubated for 60 minutes in blocking solution [10% fetal calf serum (FCS) in 1× PBS] at room temperature. Subsequently, sections were incubated with the primary antibodies in 3% FCS containing 0.1% Triton-X 100 in 1× PBS overnight at 4°C. After washing three times for 10 minutes each in 1× PBS at room temperature, sections were incubated in secondary antibodies for 2 hours at room temperature. After rinsing in 1× PBS (three times for 30 minutes), sections were mounted in fluorescent mounting medium (DAKO).

Dehydrated, PFA-fixed brains were embedded in paraffin wax and sectioned at 12 μm. Immunohistochemistry for Tbr1 and Foxp2 was performed on paraffin sections as described previously (Batlle et al., 2002).

Visualization of antibody binding by diaminobenzidine (DAB) staining was performed using the ABC Standard Kit (Vector Laboratories, Burlingame, USA) with DAB and H_2O_2 as substrates in accordance with the manufacturer's suggestions. Finally, sections were mounted on gelatin-coated slides, dehydrated and coverslips applied using Histokit.

Antibodies

The following primary antibodies were used: mouse anti-reelin (mAb5364, 1:250, Chemicon, Hofheim, Germany), mouse anti-NeuN (also known as Neuna60 – Mouse Genome Informatics; mAb377, neuron-specific nuclear protein, 1:1000; Chemicon), rabbit anti-calbindin (CB38, 1:1000; Swant, Bellinzona, Switzerland) and rabbit anti-calretinin (769914, 1:1000; Swant), mouse anti-GAD67 (also known as Gad1 – Mouse Genome Informatics; mAb5406, 1:1000; Chemicon), rabbit anti-parvalbumin (PV28, 1:1000; Swant), guinea pig anti-GLAST (also known as Slc1a3 – Mouse Genome Informatics; mAb1782, 1:500; Chemicon), rabbit anti-brain lipid binding protein (BLBP; (also known as Fabp7 – Mouse Genome Informatics; AB9558, 1:500; Chemicon), mouse anti-nestin (Rat401, 1:50; Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA), rabbit anti-Tbr1 (AB9616, 1:10,000; Chemicon), goat anti-Foxp2 (N16) (sc-21069, 1:500; Santa Cruz Biotechnology, Heidelberg, Germany), and mouse anti-SMI32 (1:100; Sternberger Monoclonals, Lutherville, Maryland, USA). Secondary antibodies: goat anti-mouse Alexa Fluor 568 (A-11004, 1:1000; Invitrogen), goat anti-rabbit Alexa Fluor 488 (A-11008, 1:1000; Invitrogen), rabbit anti-goat Alexa Fluor 488 (A-11078, 1:300; Invitrogen) and biotinylated horse anti-mouse (BA-2000, 1:250; Vector Laboratories).

Quantitative stereology

The number of NeuN-positive neurons was obtained by the optical dissector/fractionator method (West et al., 1991). For quantitative stereology, frontal sections of adult brains (n=4 for each group) were visualized on a computer screen attached to an Olympus BX60 microscope F5 (Olympus Optical, Düsseldorf, Germany). A computer-controlled stepper motor stage and focus assembly allowed movement in the x-, y- and z-axes. Cell counts were performed using Stereo Investigator software (version 3.0; MicroBrightField, Inc., Colchester, VT). The marginal zone, as the region of interest, was first marked for every single section using low-power magnification (4×/0.10 objective). For subsequent cell counts, the following parameters were added to the program: counting frame, 50×30 μm; guard zone, 2 μm, and counting depth, 8 μm. Thereafter, using high-power magnification (oil objective lens, 100×/1.35), NeuN-positive cells that fulfilled the criteria of the unbiased counting rules were marked and added to the probe run list. The total cell numbers estimated by the optical dissector were subsequently analyzed by Wilcoxon's exact rank sum test.

RESULTS

Development of cortical layers in *ApoER2* and *Vldlr* mutants

It has been suggested that for the proper formation of cortical layers either ApoER2 or Vldlr is required, with ApoER2 playing a more significant role than Vldlr at later stages of cortical migration (Benhayon et al., 2003). To investigate the role of these two reelin receptors in more detail, we first used layer-specific markers to examine the laminar structure of the cerebral cortex in *ApoER2*$^{-/-}$ and *Vldlr*$^{-/-}$ mice at different developmental stages. The transcription factor ER81 is strongly expressed by cells of cortical layer V (Hevner et al., 2003). Accordingly, we used ER81 as a marker for cortical cells committed to a deep layer fate. These cells are born at early stages of cortical development, around E12-E13 (Takahashi et al., 1999). *ER81*-positive neurons are positioned in the inner part of the cortex of wild-type mice 1 week after birth (Fig. 1A). In general, the pattern of *ER81* staining was similar when we analyzed *Vldlr* mutant mice: *ER81*-positive neurons were localized in an organized layer in the inner part of the cortex (Fig. 1D). By contrast, in situ hybridization for *ER81* in *ApoER2*$^{-/-}$ mice showed positive cells in an organized layer located ectopically in more superficial positions of the cortex (Fig. 1G). This part of the cortex is usually occupied by layer II-IV neurons. Since *ER81* expression may not be restricted to layer V pyramidal cells (Wonders and Anderson, 2005), we studied additional markers of deep layer neurons, such as *Tbr1*, *Foxp2* and *Tle4*. Similar observations as with *ER81* were made when these markers were used (Fig. 2A-D).

The homeodomain transcription factor Cux2 is specifically expressed within cortical layers II-IV (Zimmer et al., 2004). Accordingly, the *Cux2*-positive population in wild-type animals was positioned in the outer portion of the cortical plate, which gives rise to the upper cortical layers II-IV (Fig. 1B). In *Vldlr* mutant mice, the *Cux2*-positive cells were also localized in the upper part of the cortex (Fig. 1E) but, unlike in wild-type mice, we observed numerous *Cux2*-positive cells in layer I of the cortex (see also Fig. 7). Furthermore, a sharply separated marginal zone could not be discerned in *Vldlr* mutant mice (compare Fig. 1B and E, Fig. 7A and

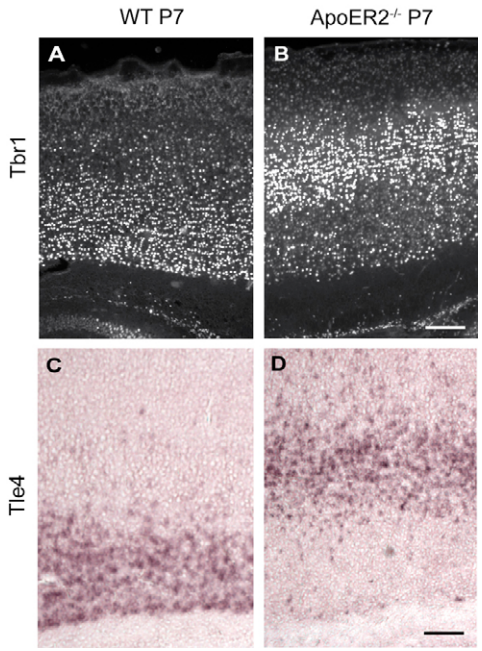

Fig. 2. Labeling of early born cortical neurons in wild-type and *ApoER2*$^{-/-}$ mice at P7 using Tbr1 and Tle4. Immunostaining for Tbr1 (**A**,**B**) and in situ hybridization for *Tle4* (**C**,**D**) on sagittal brain sections revealed the strongest signal in deep cortical layers of wild-type animals (A,C). Similar to the staining for *ER81*, both markers show a shift in early born neurons towards more superficial regions in the *ApoER2* mutant cortex (B,D). Scale bars: 200 μm in A,B; 100 μm in C,D.

B). These findings suggest an ectopic invasion of *Cux2*-positive neurons into the cortical marginal zone. The invasion of layer I first became obvious at P7, when this layer was large enough to be clearly defined.

A completely different pattern was observed when we analyzed *Cux2* expression in *ApoER2* mutants. In sections of these animals, two prominent *Cux2*-positive bands were visible in the cortex (Fig. 1H). One band was localized in the upper part of the cortex, right underneath the marginal zone. The second band was localized close to the ventricular zone. Thus, labeling with markers of early generated and late generated cortical neurons revealed significant differences between *Vldlr*$^{-/-}$ mice and *ApoER2* mutants. The more prominent phenotype of *ApoER2* mutants is similarly seen in Nissl-stained sections (Fig. 1C,F,I).

In order to compare the expression patterns of *Cux2* and *ER81* in *ApoER2*$^{-/-}$ mice and *Vldlr*$^{-/-}$ mice with those in other mutants lacking various components of the reelin signaling pathway, we performed in situ hybridization studies in reeler mice, *Dab1*$^{-/-}$ mice and *ApoER2 Vldlr* double-knockout mice using *ER81* and *Cux2* riboprobes. As expected, no distinct layer formation was visible in the three mutants, reflecting the more severe phenotype in these animals (see Fig. S1 in the supplementary material).

ApoER2 is important during late stages of cortical layer formation

In situ hybridization for *Cux2* on sections of *ApoER2* mutant mice revealed two bands of expression in the cortex, one in normal superficial position, and a second one in an abnormal position deep in the cortex (compare Fig. 1B with H). Comparing the expression patterns of *ER81* and *Cux2*, the *Cux2*-positive upper band was localized directly above the *ER81*-positive layer V (Fig. 1G,H), suggesting that these *Cux2*-labeled cells mainly belonged to layer IV. If this were true, the deep *Cux2*-positive cells would mainly include neurons normally destined to layers II-III. To confirm that the *Cux2*-positive cells in the superficial band of the cortex of *ApoER2* mutants were relatively early generated neurons, we used BrdU labeling at different developmental time points.

Following BrdU injections at E12.5 and E13.5, wild-type animals exhibited the most heavily BrdU-labeled cells in inner and middle locations of the cortex when analyzed at P0 (Fig. 3A,B). BrdU injections at E14.5-E16.5 mainly labeled cells destined to outer portions of the cortex (Fig. 3C-E). An altered pattern was observed in *ApoER2* mutants (Fig. 3F-J). Here, BrdU-labeling at E13.5, and more clearly at E14.5 and E15.5, led to the formation of two separate BrdU-positive bands. Injection of BrdU at E16.5 labeled a distinct band of deeply located neurons in *ApoER2* mutants (Fig. 3J). Together, these findings are in line with the observation of two separate layers of late born, *Cux2*-positive cells. They suggest that in *ApoER2* mutants, a large fraction of late born neurons fail to migrate to their destinations in outer cortical layers.

To substantiate further that the upper BrdU-positive band in the *ApoER2* mutants represents neurons that are normally destined to layer IV, we performed in situ hybridization for *RORbeta*, a marker for layer IV neurons (Nakagawa and O'Leary, 2003). In adult wild-type animals, *RORbeta* mRNA expression is, in fact, mainly seen in layer IV cells (Fig. 4A). By contrast, in *ApoER2*

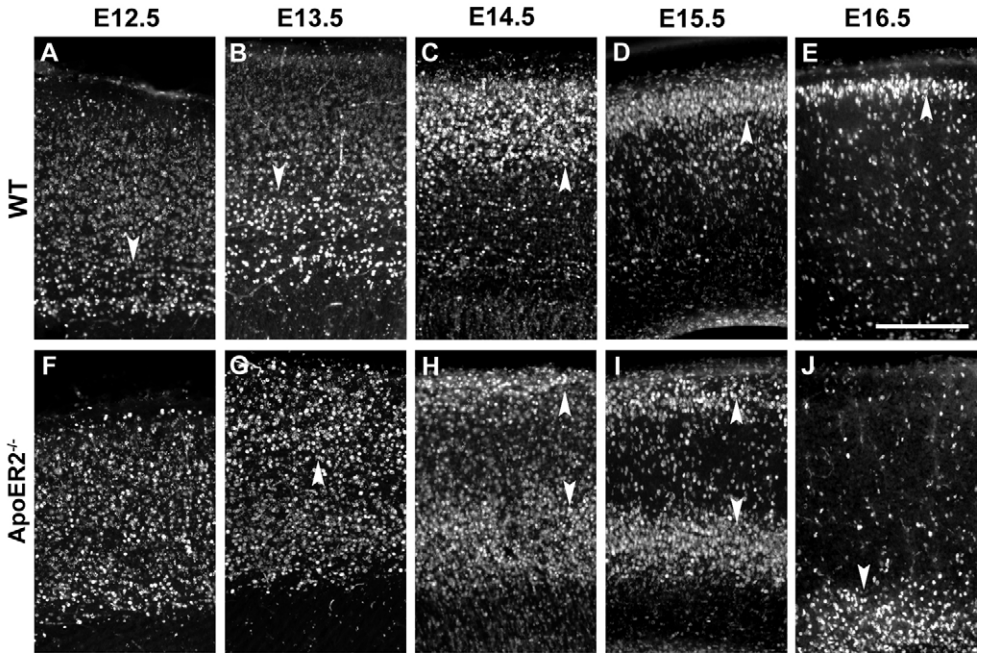

Fig. 3. ApoER2 controls late stages of cortical layer formation. (**A**,**F**) BrdU-labeling at E12.5 and staining of sagittal brain sections through the cerebral cortex at P0 showed the strongest signal close to the ventricle in wild-type mice (A, arrowhead). By contrast, in *ApoER2* mutants (F), many heavily BrdU-labeled cells were also found in superficial portions of the cortex. (**B**,**G**) Labeling at E13.5 revealed many BrdU-stained cells in the inner to middle portion of the cortex in wild-type animals (B, arrowhead), whereas in *ApoER2* mutants (G) an altered pattern was observed (arrowhead), and the formation of two separate bands became apparent. When labeling for BrdU was carried out at E14.5 (**C**,**H**) and E15.5 (**D**,**I**), the separation of these two layers was more clearly visible (H,I, arrowheads), whereas in wild-type (C,D) mice, only the upper cortical layers were strongly labeled (arrowheads). (**E**,**J**) Labeling at E16.5, a late stage of corticogenesis, resulted in a heavily stained band in the outermost portion of the cortex in wild-type animals (E, arrowhead), whereas in *ApoER2* mutants (J) strongly labeled cells were located close to the ventricle (arrowhead). Scale bar: 200 μm.

mutants a dispersion of *RORbeta*-positive cells, with strongest expression levels underneath the marginal zone and close to the ventricle, is observed (Fig. 4E). Double in situ hybridization for *RORbeta* and *Cux2* in wild-type animals (Fig. 4B-D) revealed a *Cux2*-positive band just above *RORbeta*-stained cells, but also double-labeled neurons (Fig. 4D). By contrast, in *ApoER2* mutants *Cux2*-positive and *RORbeta*-positive neurons were scattered, forming cell accumulations underneath the marginal zone and near the ventricular zone (Fig. 4F-H). We conclude that early born layer IV neurons migrate properly, consistent with the superficial *RORbeta*-positive population, whereas later generated layer IV and layer II-III cells remain close to the proliferative zone. These data together with the BrdU studies suggest that ApoER2-mediated signaling is important for late phases of neuronal migration in the cortex.

ApoER2 is expressed by cells in upper cortical layers during development

Our findings imply that during cortical development late born neurons need ApoER2 signaling for proper migration. Thus, we next studied the expression of *ApoER2* by neurons in wild-type animals performing in situ hybridization for *ApoER2* at different developmental time points (Fig. 5A-F). At E16.5 and E18.5, when late-generated neurons reach their destinations in upper layers, *ApoER2* is expressed throughout the cortical wall with the strongest expression in upper cortical layers (Fig. 5A-D). This staining pattern is still seen 1 week after birth (Fig. 5E,F). These observations indicate that mainly late born neurons destined to superficial layers strongly express the receptor. Double labeling for *ApoER2* and *Cux2* supports this conclusion (Fig. 5G-I).

Recent studies indicated that correct layer destination of cortical GABAergic interneurons does not directly depend on reelin signaling (Pla et al., 2006). Interneurons seem to invade their target layers well after synchronously generated projection neurons reach their final destinations, so that projection neurons guide cortical interneurons to their appropriate layer. Since we found a prominent migration defect in *ApoER2* mutants, we expected also interneurons to be misplaced in these animals. Immunostaining for the interneuron marker calretinin revealed positive neurons mainly in superficial cortical layers in wild-type animals (see Fig. S2A in the supplementary material). As expected, in *ApoER2* mutants calretinin-positive cells were found in superficial and deep cortical layers (see Fig. S2B in the supplementary material). Immunostaining for parvalbumin, another marker of GABAergic interneurons, shows a scattered distribution of labeled neurons in sections of wild-type animals and *ApoER2* mutants (see Fig. S2C,D in the supplementary material).

The radial glial scaffold is not altered in *ApoER2* mutants

Our results indicate that ApoER2 is involved in the migration of late generated neurons that mainly use radial glia-guided migration to reach their destinations in superficial cortical layers (Nadarajah and Parnavelas, 2002). Could the migration defect seen in *ApoER2* mutants be due to an altered radial glial scaffold? We studied the organization of the radial glial scaffold at E16.5 by using antibodies against BLBP, nestin and GLAST, but were unable to find obvious differences in morphology and arrangement of radial glial cells when comparing sections of wild-type and mutant cortex (Fig. 6A-F). As the glial scaffold does not seem to be altered in the mutant cortex, the observed neuronal phenotype in *ApoER2*$^{-/-}$ mice could be due to a failure of the neurons to attach properly to the radial glial fiber. This attractive hypothesis needs to be tested in future real-time microscopy studies.

Cortical neurons invade the marginal zone in *Vldlr* mutants

In situ hybridization for *Cux2* showed an invasion of cells into the marginal zone of *Vldlr* mutants (Fig. 7A,B), but not of *ApoER2* mutants, suggesting different roles of these two lipoprotein receptors. As *Cux2* expression only indicates a neuronal subpopulation within layers II-IV, we performed immunohistochemistry for the neuronal marker NeuN. In fact, staining for NeuN revealed more prominent differences when comparing the presence of neurons in the marginal zone of wild-type mice (Fig. 7C), *ApoER2*$^{-/-}$ (Fig. 7D) and *Vldlr*$^{-/-}$ (Fig. 7E) mice. Stereological estimation of the number of NeuN-positive cells in the marginal zone and subsequent statistical analysis revealed a significant increase in NeuN-positive neurons in *Vldlr* mutant mice

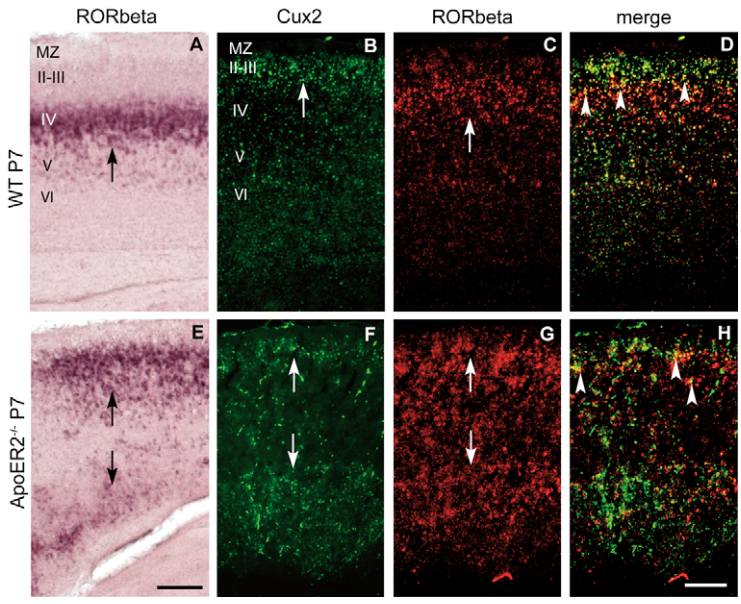

Fig. 4. *Cux2* and *RORbeta* staining in wild-type animals and *ApoER2* mutants at P7. (**A**,**E**) In situ hybridization for *RORbeta*, a marker of layer IV neurons, showed strong expression in layer IV in sections of wild-type animals (arrow in A). In *ApoER2* mutants (E), strongest *RORbeta* expression was found in superficial portions of the cortex underneath the marginal zone and close to the ventricle (arrows). (**B-D**,**F-H**) Double fluorescence in situ hybridization for *Cux2* (green) and *RORbeta* (red) revealed that *Cux2* labels mainly layer II-III neurons and only a superficially located subpopulation of layer IV in wild-type animals (B-D). Arrowheads in D point to double-labeled cells. In *ApoER2* mutants (F-H), *Cux2*- and *RORbeta*-labeled neurons were scattered throughout the cortex with cell accumulations underneath the marginal zone and close to the ventricle (arrows). Arrowheads in H point to double-labeled cells. Scale bars: 200 μm.

compared to both wild-type animals and *ApoER2* mutants. By contrast, no significant difference was found when wild-type animals and *ApoER2* mutants were compared (Fig. 7F).

It has been shown that in the telencephalon *Cux2* is expressed by two subpopulations of neurons which are of different regional origin and show different migratory behaviors and phenotypic characteristics (Zimmer et al., 2004). One subpopulation consists of cortical interneurons born in the subpallium, migrating tangentially to the cortex. The other population comprises cortical projection neurons born in the dorsal telencephalon. These cells migrate radially from the subventricular zone (SVZ) to superficial positions before they differentiate into upper cortical layer neurons (Tan and Breen, 1993; Tan et al., 1998; Nadarajah and Parnavelas, 2002). To determine the types of cells in the marginal zone of *Vldlr*$^{-/-}$ mice, we used antibodies against different interneuron markers such as GAD67, parvalbumin and calretinin, and antibodies against calbindin and reelin and the pyramidal neuron markers SMI32 and Tbr1 (Fig. 8). Immunostaining for calbindin, a marker of both interneurons and pyramidal cells, revealed many cells in the marginal zones of *Vldlr* mutants when compared with wild-type animals (Fig. 8A,B). Similarly, we found SMI32-positive pyramidal cells and Tbr1-positive neurons only in the marginal zones of *Vldlr* mutant mice (Fig. 8C-G). As known from studies in the reeler mutant, some of these displaced pyramidal cells were inverted with the apical dendrite directed towards the white matter (Fig. 8E-G). We were unable to find clear-cut differences in the labeling of interneurons in layer I between *Vldlr* mutants and wild-type animals using antibodies against calretinin, parvalbumin, GAD67 and reelin (see Fig. S2E-L in the supplementary material).

The different roles of ApoER2 and Vldlr do not seem to be restricted to the migration of neocortical neurons. Thus, we observed a neuronal migration defect in the olfactory bulb of *ApoER2* mutants but not *Vldlr* mutants when staining sections of adult animals for calbindin (see Fig. S3A-C in the supplementary material). As in the neocortex, no alterations of the radial glial scaffold were observed (see Fig. S3D,E in the supplementary material).

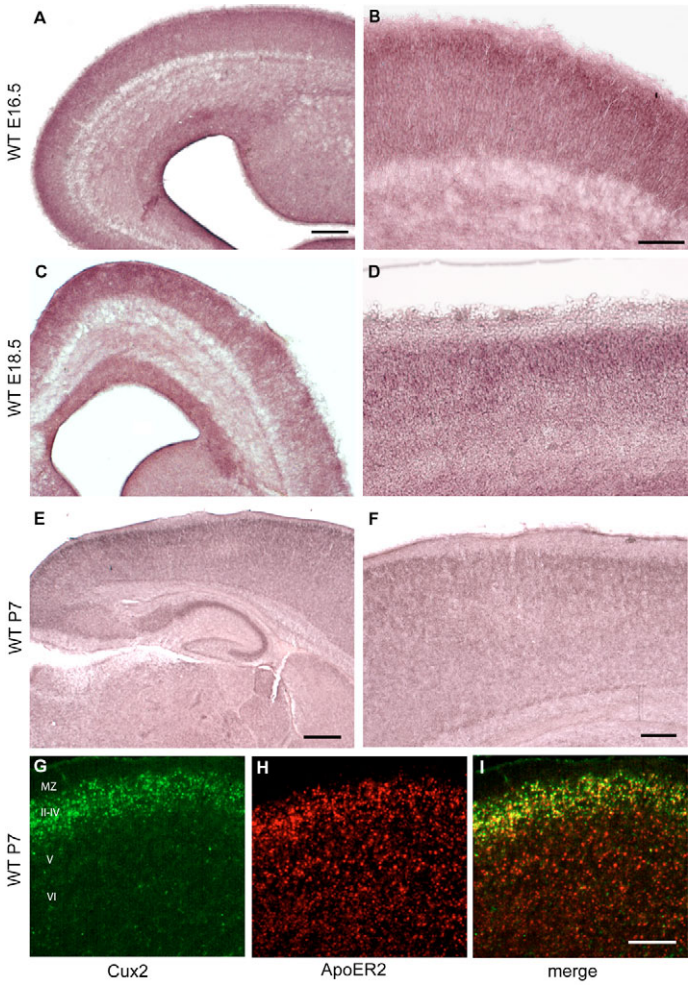

Fig. 5. Expression pattern of *ApoER2* transcripts at different developmental time points. (**A-F**) In situ hybridization for *ApoER2* on sagittal sections of E16.5 (A), E18.5 (C) and P7 (E) wild-type cortex showed expression throughout the whole cortical wall. Higher magnification of the neocortex revealed the strongest signal in upper cortical layers (B,D,F). (**G-I**) Double fluorescence in situ hybridization for *Cux2* (G, green) and *ApoER2* (H, red) shows colocalization (I, yellow) of the two markers, indicating that late generated cortical neurons express *ApoER2*. Scale bars: 200 μm in A,C,F-I; 100 μm in B,D; 500 μm in E.

DISCUSSION

The results of the present study indicate that the two reelin receptors Vldlr and ApoER2 exert different functions during layer formation of the cerebral cortex. In *Vldlr* mutants, but not in *ApoER2*$^{-/-}$ mice, numerous neurons invade the marginal zone in addition to the few interneurons and Cajal-Retzius cells normally present in this layer. Vldlr thus seems to mediate a 'stop signal' function of reelin

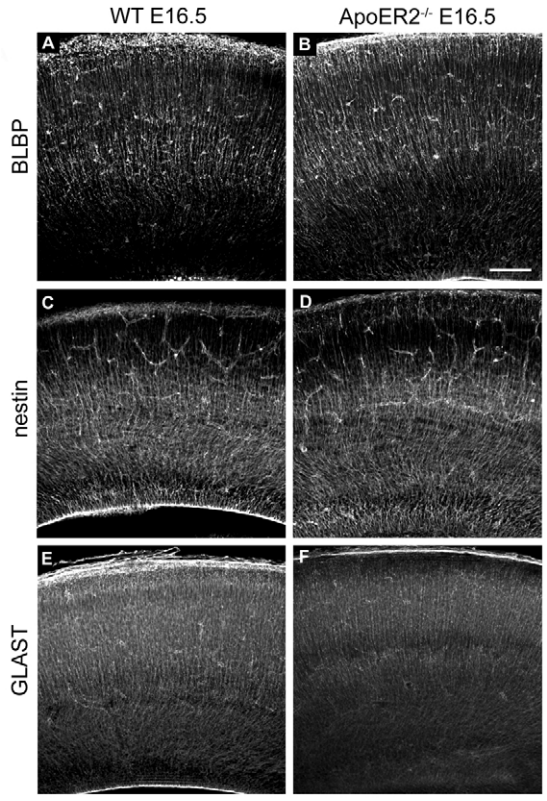

Fig. 6. Cortical radial glial scaffold in wild-type and *ApoER2*$^{-/-}$ mice. Staining for BLBP, nestin and GLAST of frontal sections through the telencephalon of *ApoER2*$^{-/-}$ (**B**,**D**,**F**) mice at E16.5 showed no alteration of the radial glial scaffold when compared to wild-type animals (**A**,**C**,**E**). Scale bar: 100 μm.

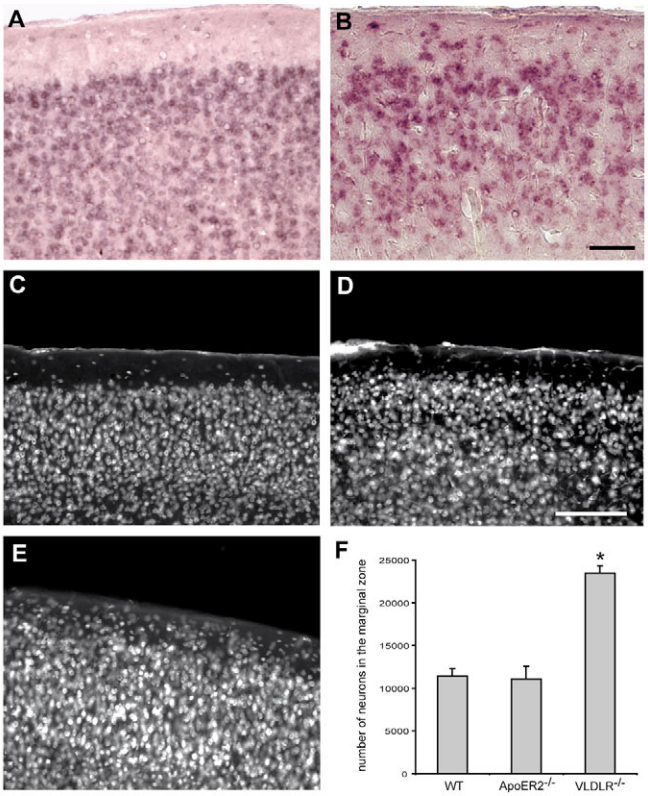

Fig. 7. Invasion of the marginal zone in *Vldlr* mutants. (**A**,**B**) In situ hybridization for *Cux2* on sagittal brain sections through the neocortex of adult wild-type mice (A) showed only few neurons in layer I, whereas many *Cux2*-positive cells were found in the marginal zone of *Vldlr*$^{-/-}$ mice (B). (**C-E**) Sagittal brain sections through adult neocortex stained for NeuN similarly showed few neurons in wild-type animals (C) and *ApoER2* mutants (D), but numerous cells in *Vldlr* mutants (E). Scale bars: 100 μm in A,B; 200 μm in C-E. (**F**) Stereological estimation of cell numbers in the marginal zone was performed in four animals of each group. *Vldlr*$^{-/-}$ mice showed significantly more neurons (mean±s.e.m.) in the marginal zone compared with wild-type animals and *ApoER2* mutant mice (P=0.029). No significant difference was found between wild-type animals and *ApoER2* mutants.

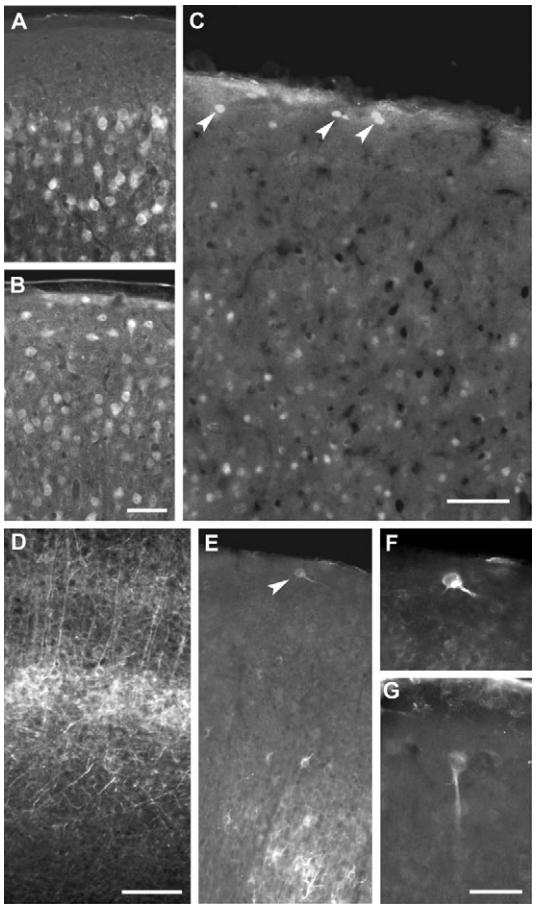

Fig. 8. Ectopic pyramidal cells in the marginal zone of *Vldlr* mutants. (**A**,**B**) Calbindin-immunoreactive neurons were almost absent from the marginal zone in wild-type animals (A), but were numerous in *Vldlr* mutants (B). (**C**) Immunostaining for Tbr1, a deep layer marker, revealed labeled cells in the marginal zone of *Vldlr* mutant mice. (**D-G**) Immunolabeling for SMI32, a pyramidal cell marker, revealed strongest expression in layer V on sagittal sections through adult neocortex of wild-type mice (D). In *Vldlr* mutants (E), but not in wild-type animals and ApoER2 mutants, a few SMI32-positive cells were observed in the marginal zone (arrowhead; higher magnification in F). Reminiscent of the reeler phenotype, inverted pyramidal cells were occasionally observed (G). Scale bars: 50 μm in A,B,F,G; 100 μm in C,D,E.

(Dulabon et al., 2000; Frotscher, 1998). *ApoER2* mutants, but not *Vldlr*$^{-/-}$ mice, show migration defects of late generated neurons destined for superficial layers.

ApoER2 signaling controls the formation of late generated cortical layers

Studies in recent years have provided evidence for different modes of migration of various neuronal cell types in the developing cortex (Nadarajah et al., 2001; Nadarajah and Parnavelas, 2002). Early generated neurons, giving rise to the formation of deep cortical layers and the establishment of corticofugal connections, are likely to use somal translocation to move from the germinal ventricular zone to their definitive positions in the cortical plate. This mode of migration may be necessary in the absence of a glial scaffold early in the development of the cortex. Later on in development, when a radial glial scaffold has formed (Kriegstein and Götz, 2003), neurons destined to superficial cortical layers use radial glial processes as a template to move away from the proliferative zone. A glia-guided migration appears necessary to govern the increasingly longer migration of late generated neurons. This way, the layers of the cortex are formed in an inside-out manner (Caviness and Rakic, 1978; Rakic and Caviness, 1995).

The cortex of the reeler mutant is characterized by an abnormal cortical lamination despite a virtually normal development of the preplate (Caviness, 1982; Sheppard and Pearlman, 1997). A plausible explanation may be that early generated cortical neurons adopt a mode of migration that is unaffected by the cascade of signaling mechanisms that regulate the glia-guided migration of late generated cortical neurons. The data of the present study are consistent with this hypothesis. In the *ApoER2* mutant, early generated layers are formed almost normally, with one subpopulation of layer IV neurons lying above layer V. These cells are labeled with the layer IV-specific marker *RORbeta*. Thus, the formation of these early layers is largely independent of ApoER2

signaling. By contrast, the formation of superficial, late generated layers is severely altered. Late born cells are unable to bypass earlier ones and remain close to the ventricular zone. It is well-known that the cells destined to layers IV and V are among early generated neurons that start to migrate before E14.5, likely using somal translocation as their mode of migration. By contrast, later generated neurons follow thereafter and may increasingly use radial glia-guided migration (Nadarajah and Parnavelas, 2002; Sanada et al., 2004). As these late forming layers are affected in the *ApoER2* mutant, we suggest that late glia-guided migration is controlled by ApoER2 signaling. This is consistent with the finding that in the wild-type cortex strongest *ApoER2* mRNA expression was found in upper cortical layers. Since we were unable to find obvious changes in the radial glial scaffold, we assume that the interaction of the migrating neuron with the radial fiber is altered in a yet unknown way. Studies are in progress analyzing glia-guided migration of late generated cortical neurons in wild-type mice and *ApoER2* mutants by means of real-time microscopy.

Vldlr – a stop signal for migrating cortical neurons?

In situ hybridization for *Cux2* and immunostaining for NeuN and subsequent stereological quantification revealed that significantly more neurons were present in the marginal zone of *Vldlr* mutants than in wild-type animals or *ApoER2* mutant mice. In the marginal zone of adult wild-type animals some GABAergic interneurons but no pyramidal cells are present. By contrast, staining with SMI32 and Tbr1 showed a small population of pyramidal cells in the marginal zone of *Vldlr* mutant mice that was absent in wild-type animals and *ApoER2* mutants. The presence of these neurons in the marginal zone of *Vldlr* mutants indicates that at least some of these cells are early-generated pyramidal cells that were not prevented from invading the marginal zone.

During development, Vldlr is expressed in radially migrating neurons that are about to signal reelin in the marginal zone (Trommsdorff et al., 1999; Perez-Garcia et al., 2004). This is consistent with a role of Vldlr as a receptor terminating the migratory process. Signaling via Vldlr results in the phosphorylation of Dab1 at tyrosine 220 and 232, which in turn leads to the detachment of the migrating neuron from the radial glial fiber (Sanada et al., 2004). Thus, it appears that Vldlr is the receptor mediating the 'stop signal' function of reelin in the marginal zone.

Another scenario may hold true for GABAergic interneurons known to migrate from the lateral ganglionic eminence in a tangential direction to settle in the various layers of the cerebral cortex. In late stages of corticogenesis (E14-E16), GABAergic interneurons are first destined to the ventricular zone of the cortex where they stay for a short while before they migrate radially towards the pial surface to their final destinations in the cortical plate (Nadarajah et al., 2002). Recently it has been shown that tangential migration and layer acquisition of cortical GABAergic interneurons are independent of reelin signaling (Pla et al., 2006); however, it has been postulated that interneurons require a normal distribution of projection neurons to adopt their correct laminar positions (Pla et al., 2006).

In conclusion, consistent with the present findings, reelin, mediated via Vldlr is likely to provide a stop signal for radially migrating cells of the cortical plate, preventing them from entering the marginal zone. Together with the aberrant migration of late generated cortical neurons in *ApoER2* mutants, the results of the present study point to specific functions of the two lipoprotein receptors for reelin.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/21/3883/DC1

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