

Reeler/Disabled-like Disruption of Neuronal Migration in Knockout Mice Lacking the VLDL Receptor and ApoE Receptor 2

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Summary

Layering of neurons in the cerebral cortex and cerebellum requires Reelin, an extracellular matrix protein, and mammalian Disabled (mDab1), a cytosolic protein that activates tyrosine kinases. Here, we report the requirement for two other proteins, cell surface receptors termed very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2). Both receptors can bind mDab1 on their cytoplasmic tails and are expressed in cortical and cerebellar layers adjacent to layers that express Reelin. mDab1 expression is upregulated in knockout mice that lack both VLDLR and ApoER2. Inversion of cortical layers and absence of cerebellar foliation in these animals precisely mimic the phenotype of mice lacking Reelin or mDab1. These findings suggest that VLDLR and ApoER2 participate in transmitting the extracellular Reelin signal to intracellular signaling processes initiated by mDab1.

Introduction

The formation of the central nervous system is marked by a series of migratory waves during which postmitotic neurons leave their birthplace in the ventricular zone and move along glial guidance fibers to their proper places (e.g., in the cortex or in the cerebellum). Neuronal migration entails both radial and tangential components (Walsh and Cepko, 1992; Kornack and Rakic, 1995; O'Rourke et al., 1995; Komuro and Rakic, 1998; Pearlman et al., 1998). The molecular events that regulate the interaction of the migrating cells with their environment and the cues they receive to inform them of their spatial position during the course of development are complex and, as a whole, poorly understood. They involve various classes of adhesion or signaling molecules that either

mediate direct cellular interactions or generate attractant or repulsive signals.

A molecular pathway that regulates the migration of neurons along the radial glial fiber network involves the large extracellular protein Reelin (reviewed in Curran and D'Arcangelo, 1998). In the cortex, this modular protein is either associated with the extracellular matrix or with the surface of a special class of neurons that produce it in the outermost layer just beneath the pial surface. These so-called Cajal-Retzius neurons are formed during the early stages of neural development. In the *reeler* strain of mice (Falconer, 1951), the gene encoding Reelin is defective (D'Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995). As a result, in these mice migratory neurons apparently do not receive a critical cue that informs them of their position, leading to an inversion of the cortical layers. These layers normally form from the inside out, with later-born neurons migrating past older ones to form progressively more superficial, and thus younger, layers of the neocortex. In the cerebellum, Reelin is required for the Purkinje cells to migrate outward, where they form a well-defined cortical plate through which postmitotic granule cells migrate inward to form the internal granular layer (Miyata et al., 1997; Komuro and Rakic, 1998; Wechsler-Reya and Scott, 1999). Both of these laminated structures do not form in the *reeler* mouse.

The cytoplasmic adaptor protein mDab1 is related to the *Drosophila disabled* gene product. It is predominantly expressed in neurons and has been shown to function downstream of Reelin. mDab1-deficient mice (identified as a naturally occurring strain called *scrambler* and also generated by gene knockout) develop a phenotype indistinguishable from *reeler* (Sweet et al., 1996; Howell et al., 1997b; Sheldon et al., 1997; Ware et al., 1997). Furthermore, in *reeler* mice, mDab1 protein expression is greatly increased even after the end of the migratory period, indicating a failure of neurons to adjust its expression due to lack of Reelin signal input (Rice et al., 1998; Howell et al., 1999a). mDab1 contains a protein interaction domain that binds to NPxY motifs in the cytoplasmic tails of receptors (Pawson and Scott, 1997). It can undergo tyrosine phosphorylation and subsequently interact with nonreceptor tyrosine kinases of the Abl and Src family (Howell et al., 1997a), suggesting that the Reelin signaling pathway involves coupling of the signal, via mDab1, to intracellular kinase pathways. However, the cell surface receptors that mediate transmission of the signal across the neuronal plasma membrane are unknown.

We and others have recently described the interaction of mDab1 with several membrane proteins that are expressed by neurons (Trommsdorff et al., 1998; Howell et al., 1999b). They include two members of the low-density lipoprotein (LDL) receptor gene family (i.e., the LDL receptor and the LDL receptor-related protein [LRP]), as well as the amyloid precursor protein (APP). These recycling plasma membrane proteins bind mDab1, presumably through interaction with NPxY motifs that are present in their cytoplasmic tails.

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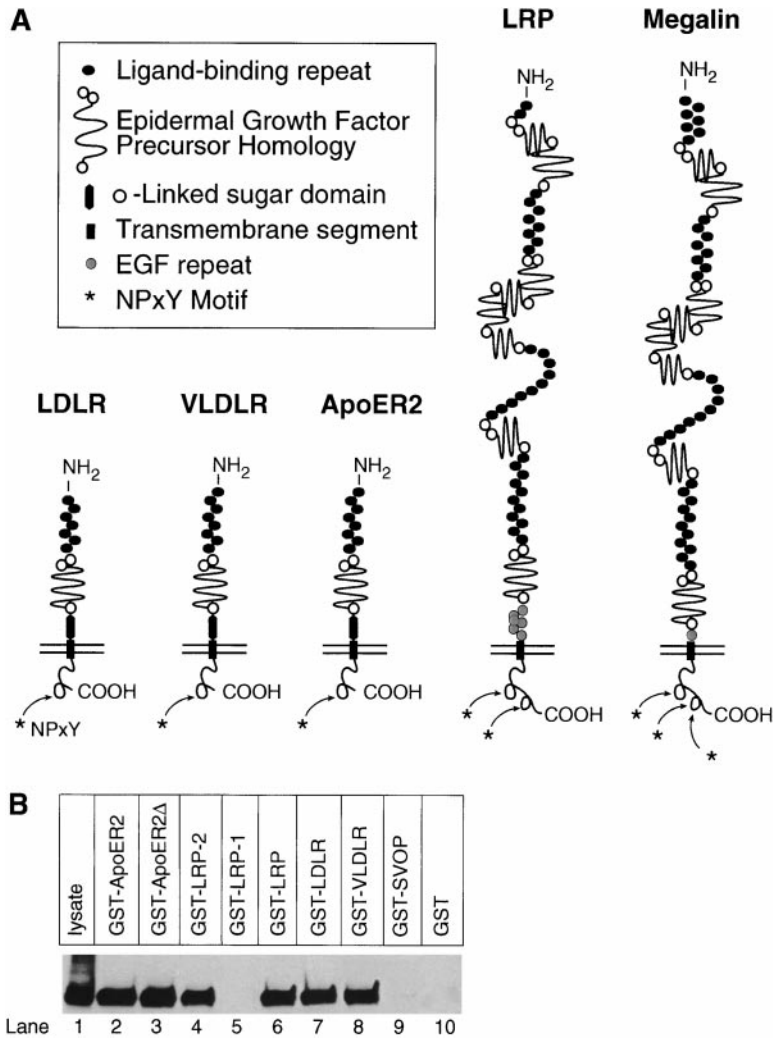


Figure 1. mDab1 Binds to the Cytoplasmic Tails of the Members of the LDL Receptor Gene Family

(A) Structural organization of LDL receptor (LDLR), VLDL receptor (VLDLR), ApoE receptor 2 (ApoER2), the LDL receptor-related protein (LRP), and Megalin. Cytoplasmic NPxY motifs that serve as endocytosis signals and interaction sites for protein interaction domains are indicated by asterisks.

(B) Interaction of mDab1 with recombinant GST-fusion proteins containing the complete cytoplasmic tails of ApoER2 (lane 2), LRP (lane 6), LDL receptor (LDLR, lane 7), VLDLR (lane 8), or SVOP (lane 9) (Janz et al., 1998), as well as fusion proteins containing either the first (lane 5) or the second (lane 4) of the NPxY motifs present in the LRP tails (GST-LRP-1 and -2). GST-ApoER2Δ (lane 3) contains a cytoplasmic tail of ApoER2 that lacks an alternatively spliced 59 amino acid insert. GST-SVOP (lane 9) and GST (lane 10) were used as controls. Cell lysate from 293 cells transfected with the mDab1 expression plasmid (lane 1) was loaded for comparison.

The LDL receptor gene family comprises a group of structurally related multifunctional cell surface receptors that mediate endocytosis of extracellular ligands (Krieger and Herz, 1994). The five known mammalian members of the family are the LDL receptor, LRP, Megalin, VLDLR, and ApoER2 (Figure 1A). The role of the LDL receptor in the regulation of cholesterol homeostasis is well understood. LRP and Megalin are both multifunctional and bind a diverse spectrum of ligands, including lipoproteins, proteases and their inhibitors, peptide hormones, and carrier proteins for vitamins (Krieger and Herz, 1994). The VLDLR and ApoER2, like all members of the family, can bind ApoE (Takahashi et al., 1992; Kim et al., 1996; Novak et al., 1996; Brandes et al., 1997), but the relevance of this interaction is unclear and their true physiological functions are unknown. Although the amino acid sequence of the VLDLR is highly conserved between species (Takahashi et al., 1992), VLDLR knock-out mice appear grossly normal and have a normal life span (Frykman et al., 1995).

Here, we demonstrate that mDab1 specifically interacts with the cytoplasmic tails of the VLDLR and ApoER2. Using gene targeting in mice we show that both of these receptors are required for cortical layering, cerebellar foliation, and the migration of Purkinje cells. mDab1

protein expression is upregulated in brains of mice lacking both receptors. These findings establish novel signaling functions for the LDL receptor gene family and implicate the VLDLR and ApoER2 as essential components of the Reelin pathway that either directly or indirectly interact with extracellular Reelin and couple the signal to mDab1 in the cytosol.

Results

Interaction of mDab1 with VLDLR and ApoER2

We have recently reported the binding of mDab1 to the cytoplasmic tails of the LDL receptor and the LRP (Trommsdorff et al., 1998). This finding has raised the possibility that the LDL receptor gene family could be involved in the development of the central nervous system. Within the gene family, LDLR, VLDLR, and ApoER2 are structurally most closely related to each other (Figure 1A), suggesting that they share similar functions. In addition to the close structural conservation of the extracellular domains, all three receptors also share a high degree of homology in a region within their cytoplasmic tails containing the NPxY endocytosis signal to which mDab1 likely binds. To explore whether mDab1 also interacts with the VLDLR and ApoER2, we tested

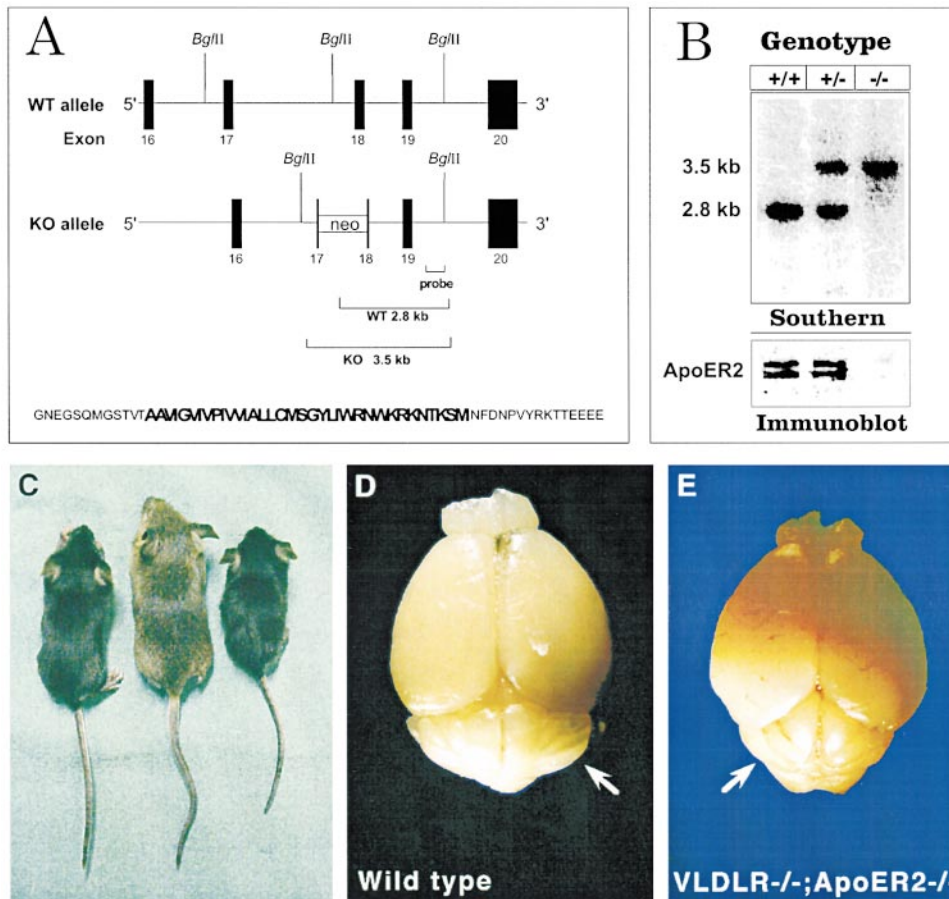


Figure 2. Targeting of the *apoER2* Gene and Macroscopic Phenotype of *vldlr/apoER2* Double Knockout Mice

(A) Targeting strategy. Sequences from exons 17 and 18 containing the transmembrane segment and part of the cytoplasmic tail, and intron 17 were replaced by a *pol2neo* cassette (Soriano et al., 1991). Deleted amino acids are shown in bold letters. Location of the Southern probe fragment and diagnostic restriction sites are indicated.

(B) Genotype analysis by Southern blotting and immunoblotting of ApoER2 in mouse brain extracts. The 3.5 kb band represents the knockout allele, and the 2.8 kb band represents the wild-type allele. ApoER2 protein is detected as a doublet representing the mature (upper) and precursor (lower band) form of the protein. No protein is present in the knockout.

(C) Twenty-day-old double knockout mice (black mice) are smaller than normal littermates (agouti animal in center). Double knockouts frequently show muscle wasting (right animal) and progressive hind limb paralysis.

(D and E) Wild-type and double knockout brain (P20). Arrows point to normal and rudimentary cerebellum in (D) and (E), respectively.

the ability of mDab1 to bind to bacterially expressed GST-fusion proteins containing the cytoplasmic tails of these receptors. As shown in Figure 1B, mDab1 expressed in transfected 293 cells (lane 1) strongly interacted with the complete cytoplasmic tails of the ApoER2 (lane 2), LRP (lane 6), LDL receptor (lane 7), and VLDLR (lane 8), but not with GST alone (lane 10) or with a GST-SVOP control fusion protein (lane 9). The latter contains the cytoplasmic tail of SVOP, a synaptic vesicle protein, which lacks an NPXY motif and is thus not expected to interact with mDab1. mDab1 interacted with specific sites in the cytoplasmic tails. In the case of LRP, which contains two NPXY motifs, mDab1 interacted only with the cytoplasmic tail segment containing the second motif (GST-LRP-2, lane 4).

Generation of Receptor-Deficient Mutant Mice

All members of the LDL receptor gene family are expressed in neurons of adult brain. To test whether the closely related LDL receptor, VLDLR, and ApoER2 might

be involved in the transmission of the Reelin signal to mDab1, we decided to determine the effect of gene inactivation of these receptors on the development of the central nervous system in mice. Consistent with the findings in human LDL receptor-deficient patients, LDL receptor knockout mice did not show any overt neurological abnormalities (Ishibashi et al., 1993). Similarly, VLDLR-deficient mice appeared neurologically normal (Frykman et al., 1995). To inactivate the *apoER2* gene in mice, we used the targeting strategy shown in Figure 2A. Exons 17 and 18, which encode most of the membrane-spanning segment and part of the cytoplasmic tail, were replaced by a *pol2neo* cassette. Southern blotting was used to determine the genotype of heterozygous and homozygous knockout mice, and no protein could be detected in knockout brains with an antibody directed against the cytoplasmic tail of ApoER2 (Figure 2B). Homozygous mice were born at the expected Mendelian ratio and were grossly normal. Specifically, there was no ataxia, a hallmark of *reeler* and mDab1-deficient

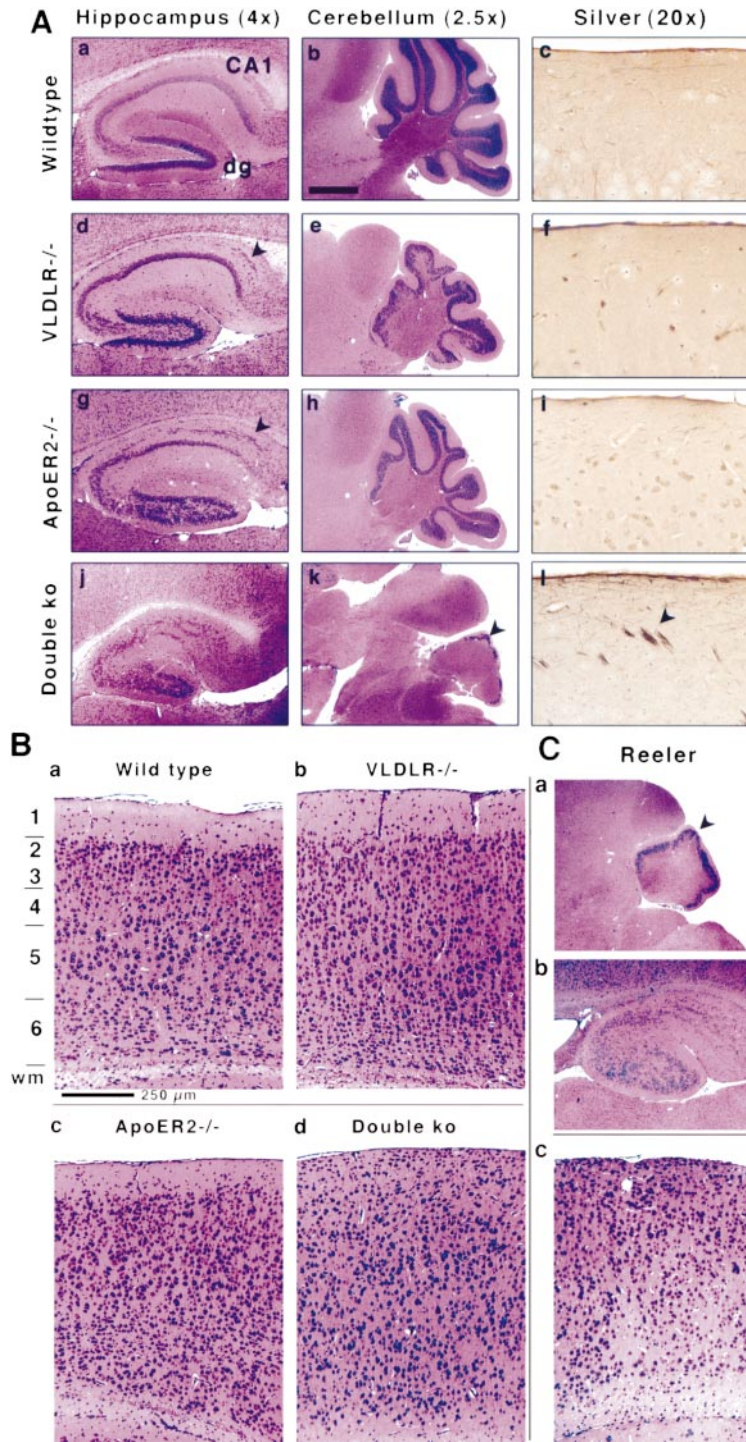


Figure 3. Histologic Analysis of Wild-Type and Mutant Mice

(A) Histoanatomy of hippocampus and cerebellum in wild-type and mutant mice. Hematoxylin/eosin or silver-stained sagittal sections of hippocampus at 4× (a, d, g, and j), cerebellum at 2.5× (b, e, h, and k), and cortex (c, f, i, and l) at 20× relative magnification of P20 wild type (a–c), *vldlr*^{-/-} (d–f), *apoER2*^{-/-} (g–i), and double knockout (j–l) are shown. Arrows in (d) and (g) point at the split in the CA1 region, which is also a characteristic feature in *reeler* and *mDab1*-deficient mice. The arrow in (k) points to the small rim of dysplastic granule cells in the double knockout. The arrow in (l) points to aggregated silver-positive neurofilaments in double knockout cortex (revealed by the Siever-Munger method). Scale bar in (b), 1 mm (2.5×), 625 μm (4×), 125 μm (20×).

(B) Cortical layering in wild-type and mutant mice. Sagittal sections of P13 wild-type (a), *vldlr*^{-/-} (b), *apoER2*^{-/-} (c), and double knockout (d) mouse brains were stained with H&E. Normal cortical layers are indicated by numbers 1–6 next to (a).

(C) Analogous sections of a *reeler* brain at the same magnifications.

scrambler mice. Male *apoER2*^{-/-} mice showed reduced fertility. A detailed analysis of this phenotype will be reported elsewhere.

To explore whether LDL receptor, VLDLR, and ApoER2 share overlapping functions, we generated lines of double knockout mice lacking combinations of these receptors. Of these three combinations, only mice lacking both the VLDLR and the ApoER2 presented with a neurological phenotype. Around day 10 after birth (P10), failure to thrive became apparent in *vldlr*^{-/-};*apoER2*^{-/-} mice

(in the following also referred to as “double knockout”). Between P13 and P15, neurological symptoms developed, characterized by progressive ataxia, wide gait, and tremor. When they tried to walk, double knockout animals frequently flipped on their backs. By P20, double knockout mice were clearly smaller than their unaffected littermates (Figure 2C). Progressive hind limb paralysis also developed between P16 and P20, and most double knockout animals died shortly afterward. On autopsy, their brains (Figure 2E) were smaller than those

of wild-type animals of the same age (Figure 2D). This difference was most apparent for the cerebellum (indicated by the arrow), which in the double knockout was present only in a rudimentary form.

***reeler*- and *scrambler*-like Neuropathology in Receptor Double Knockout Mice**

To investigate the relative contributions of the VLDLR and ApoER2 gene defects to this striking neuroanatomical phenotype, we prepared histological sections from the brains of P20 wild-type, *vldlr*, *apoER2*, and double knockout animals. Characteristic differences between the individual genotypes are shown in Figure 3A in the hippocampus (a, d, g, and j) and the cerebellum (b, e, h, and k). While in wild-type mice neurons in the hippocampus and the dentate gyrus were tightly packed, they were more loosely associated in the single knockouts, particularly in the CA1 region and the dentate (dg). In *apoER2*^{-/-} and double knockout mice, the CA1 region was clearly split into two layers (indicated by the arrow in [g]). In the double knockout, hippocampal neurons were scattered in a barely recognizable pattern. Discernible differences between the single gene defects and the wild type were also present in the cerebellum. In both the *vldlr* and the *apoER2* knockout, the cerebellum was smaller and less foliated than in wild-type littermates (Figure 3A, middle panel). In the double knockout, only a rudiment was present that completely lacked foliation (k). In the *vldlr* knockout (e), the granule cell layer appeared less compact with fewer cells than in the wild type (b). In the *apoER2* knockout (h), structural abnormalities were less pronounced.

The striking disorganization of the neurons in the hippocampus and the Purkinje cells in the cerebellum of *vldlr/apoER2* double knockout mice suggests that these cells are unable to properly respond to migratory cues from their environment. Cortical neurons as well as Purkinje cells follow radial migration paths along glial fibers that guide them to their proper layers. If the neurons do not receive the proper migratory signals, their extensions frequently form abnormal aggregates, a hallmark of *reeler* and *scrambler* mice, that can be detected by silver staining methods. No aggregates were seen in wild-type or *vldlr*^{-/-} brains (Figure 3A, c and f). In *apoER2*^{-/-} brains, minor silver-positive aggregates were often present in the cell-free layer below the brain surface (i). In the double knockout, prominent and easily recognizable aggregates (indicated by the arrow in [l]) were present at irregular angles, and neuronal cell bodies containing Nissl granules were abundant throughout the superficial layers.

To determine whether in mice lacking VLDLR, ApoER2, or both proteins cortical lamination is affected in a way similar to that seen in *reeler* and *mDab1*-deficient mice, we prepared histological sections of their neocortex (Figure 3B). In the wild type, the six neuronal layers were easily distinguishable by H&E (a). Layer 1 is normally free of cell bodies. Layer 5 contained easily recognizable, large pyramidal neurons. In *vldlr*^{-/-} mice, layer 1 was also cell free; however, the remaining layers were no longer clearly separated, and the neurons were arranged in a striking radial pattern (b). This lamination defect was more pronounced in *apoER2*^{-/-} mice, where

it was difficult to distinguish the individual layers 2–6 (c). In contrast to the *vldlr* knockout, however, the neurons appeared to be packed into tight horizontal layers. Finally, in double knockout brains (d), individual cortical layers were no longer distinguishable, and a large number of cell bodies were present in layer 1. Figure 3C shows analogous sections through the cerebellum (a), hippocampus (b), and cortex (c) of a *reeler* mouse. *vldlr*^{-/-};*apoER2*^{+/-} brains were not readily distinguishable from *vldlr*^{-/-};*apoER2*^{+/+} brains (data not shown). Any residual protein that may be derived from the *apoER2* knockout allele is thus unlikely to exert a significant dominant-negative effect.

Abnormal Distribution of Purkinje Cells and Dendrites in Receptor-Deficient Mice

To investigate the cerebellar and cortical malformations in single and double knockout animals in more detail, we used *in situ* hybridization (Figures 4a–4d) and immunohistochemistry (Figures 4e–4p) to detect calbindin (a–h) and microtubule-associated protein 2 (MAP-2, [i–p]). Calbindin is a marker for Purkinje cells. Immunohistochemical staining for this abundant cytoplasmic protein allows visualization of the cell bodies and of the intricate dendritic arborization pattern that is typical for Purkinje cells. MAP-2 specifically stains dendritic extensions of postmitotic cortical neurons. At low magnification, the normal foliation of the wild-type cerebellum (a) was revealed by the single layer of Purkinje cell bodies. The arrow in (e) points to the intricate network of normal dendritic arbors. In the *vldlr*^{-/-} cerebellum, Purkinje cells have failed to assemble in a tight layer (arrow in [b]) and dendritic arbors were abnormal (f). In contrast, in the *apoER2*^{-/-} cerebellum (c and g) the Purkinje cells have formed a single layer, although their arbors were less complex than in the wild type. In the double knockout, calbindin-positive cells lacked all signs of organized arborization (h). Purkinje cell migration did not occur, and the cells remained scattered among the neurons of the deep cerebellar nuclei (d). As a consequence, the granule cells, which depend on proliferative signals from Purkinje cells, failed to proliferate (arrow in Figure 3A, k).

MAP-2 staining of cortical neuronal projections in the CA1 region of the hippocampus (Figure 4i–4l) and the overlying neocortex (m–p) also revealed characteristic differences between wild type, single, and double knockouts. Arrows point to neuronal cell bodies in (i) and (k) (enhanced by pseudocoloring in [j]–[l]). The CA1 region was split in the ApoER2 knockout and the double knockout (k and l). A dense network of radial projections emanated from the hippocampal neurons in wild-type (i) and single knockouts (j and k), but not in the double knockout (l), emphasizing the disarray of this neuronal cell population. In the overlying cortex, MAP-2-positive neuronal processes were arranged in a radial pattern that spanned layers 2–6 in the wild-type (m), but was absent from layer 1 (arrow). In *vldlr*^{-/-} cortex, similar radial projections were present, but more diffuse staining was also seen in layer 1 (n). In the *apoER2* knockout, this radial pattern was poorly developed (o), while it was completely absent in the disorganized double knockout cortex (p).

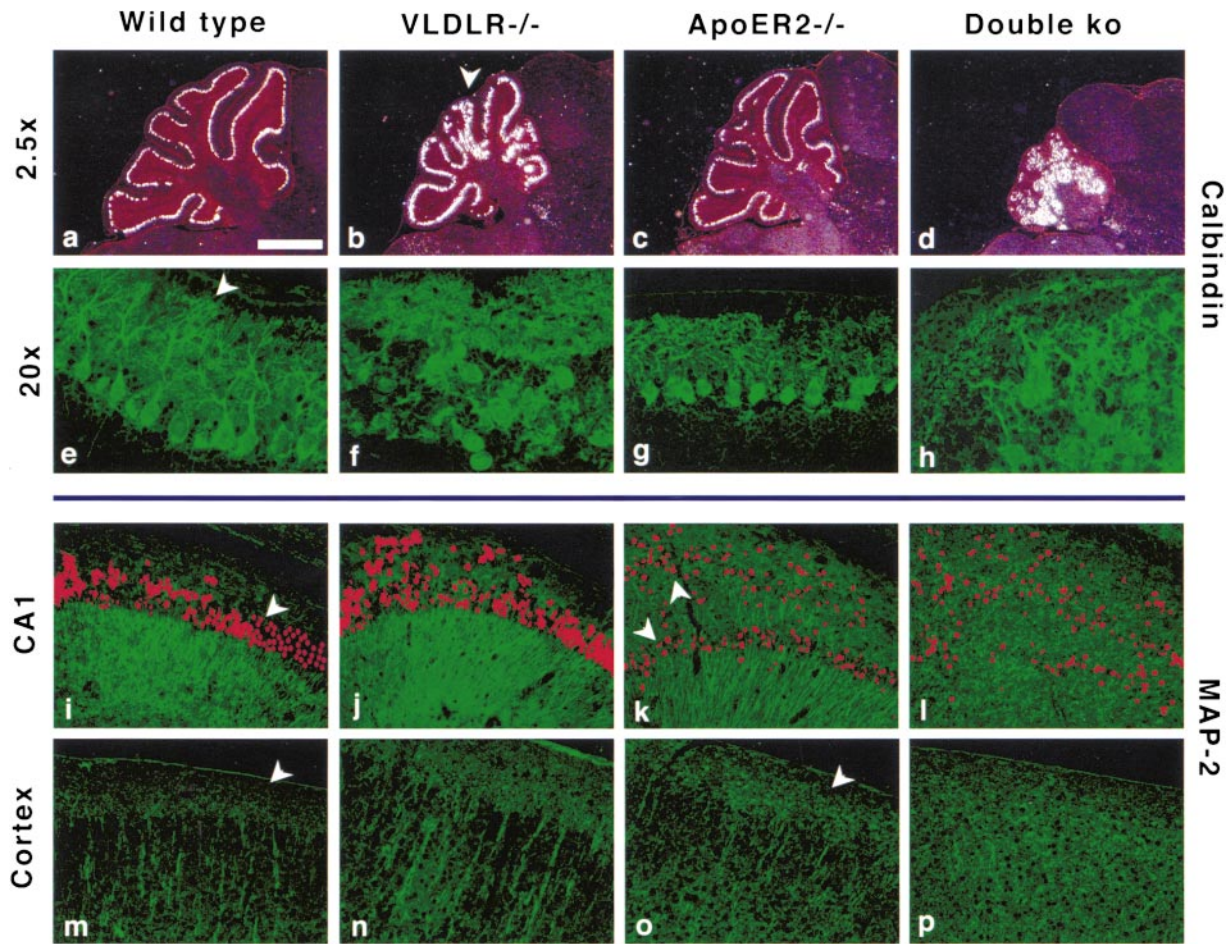


Figure 4. In Situ Hybridization and Immunohistochemical Detection of Calbindin and MAP-2 in Sagittal Sections of Wild-Type and Mutant 20-Day-Old Mice

(a–d) In situ hybridizations for Calbindin (dark field image). (e–h) Immunohistochemical staining for Calbindin. (i–p) Immunohistochemical staining for MAP-2. Arrow in (b) points at folia where Purkinje cells have not completed migration. Cell bodies of hippocampal neurons are identified by arrows and by pseudocoloring (i–l). Arrows in (m) and (o) indicate cortical layer 1, which contains few MAP-2-expressing cells in the wild type (m). MAP-2 expression is disordered and increased in (o). Scale bar in (a): 1 mm (a–d), 125 μ m (e–h), 250 μ m (i–p).

Expression of VLDLR, ApoER2, and mDab1 in Cortical Layers Adjacent to Reelin

The neuroanatomical abnormalities of *vldlr*^{-/-}; *apoER2*^{-/-} mice described in Figures 2–4 are indistinguishable from those seen in *reeler* and mDab1-deficient mice. Taken together with the interaction of mDab1 with the cytoplasmic tails of VLDLR and ApoER2 (Figure 1B), these findings are consistent with a model in which VLDLR and ApoER2 function as obligate components of a pathway that transmits the Reelin signal to mDab1. If this hypothesis is correct, then VLDLR and ApoER2 should be expressed in or near layers of the cortex that express Reelin during the neuronal migration period. Figure 5 shows in situ hybridizations of wild-type mouse brains at E13.5 (left and middle panels) and E15.5 (right panels) using a control (sense) probe (a–c) and antisense probes for *reelin* (d–f), *vldlr* (g–i), *apoER2* (j–l), and *mDab1* (m–o). The developing neocortex is indicated by the arrow in (a), and the hippocampal formation is indicated by the arrow in (b). Reelin was abundantly expressed by Cajal-Retzius neurons in the marginal zone just underneath

the cortical surface and in the hippocampus (d and e). Reelin-expressing granule cells were present on the surface of the cerebellum at this time of development (arrow in [f]). Both the VLDL receptor (g and h) and mDab1 (m and n) were predominantly expressed in the intermediate zone and the cortical plate immediately adjacent to the Reelin-expressing cells. In the cerebellum, both genes were expressed in the deep layers where Purkinje cells reside at this time (i and o). ApoER2 was abundantly expressed by postmitotic neurons that had left the ventricular zone and were en route to or had arrived at their final layer (j and k), consistent with our hypothesis which requires coexpression of mDab1, VLDLR, and ApoER2. In the cerebellum, ApoER2 was also ubiquitously expressed (l).

Inverted Cortical Layers in Receptor-Deficient Mice

Disruption of the Reelin signaling pathway results in the inversion of cortical layers. To investigate whether this characteristic inversion of the layers is present in *vldlr*^{-/-};

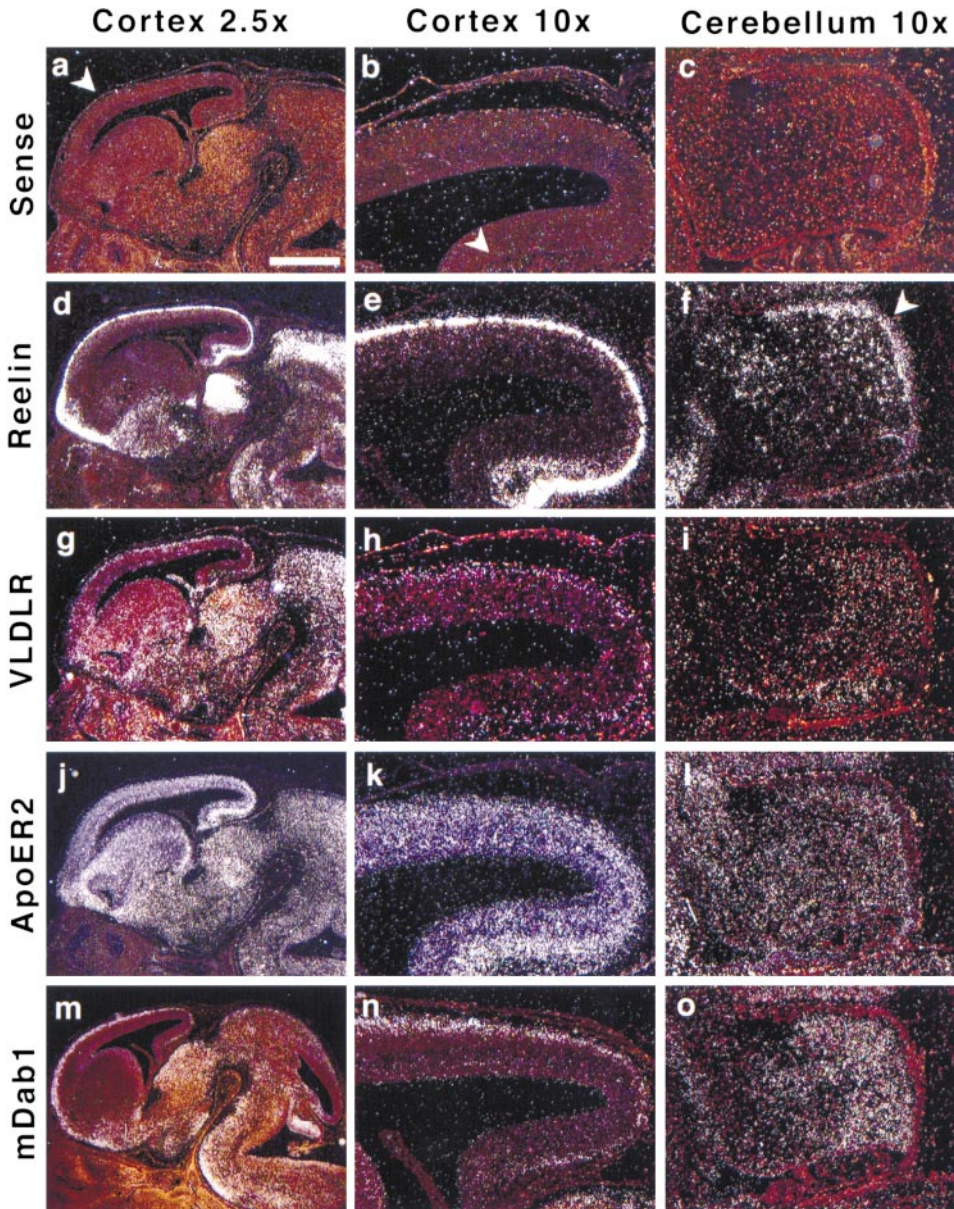


Figure 5. In Situ Hybridization of Wild-Type Embryonic Mouse Brain at E13.5 and E15.5

Probes used were as follows: sense control (a–c), and *reelin* (d–f), *vldlr* (g–i), *apoER2* (j–l), and *mDab1* (m–o) antisense. (a), (d), (g), (j), and (m) show an overview at 2.5× magnification. (b), (e), (h), (k), and (n) show a section of the cortex including the developing hippocampal formation at E13.5. The developing cerebellum (E15.5) is seen in (c), (f), (i), (l), and (o). Arrows in (a) and (b) indicate neocortex and hippocampal formation, respectively. Arrow in (f) indicates superficial Reelin-expressing granule cells. Dark field images of sagittal sections are shown. Scale bar in (a): 1 mm (2.5×), 250 μm (10×).

apoER2^{-/-} mice, we performed neuronal birth date analysis in E12 and E15 mouse embryos. Pregnant double heterozygous female mice that had been mated to male animals of the same genotype were injected with a single dose of [³H]thymidine or bromodeoxyuridine. The offspring were sacrificed 16 days after birth and perfused with fixative. Brains obtained from animals that were either wild type or lacking either VLDLR, ApoER2, or both proteins were sectioned and processed for autoradiography to visualize nuclei of neurons that had incorporated the label while undergoing their final cell division

at the time of injection. Sagittal sections in Figure 6 show the final positions of neurons born at E12 and E15 in wild-type (a and b), *vldlr*^{-/-} (c and d), *apoER2*^{-/-} (e and f), and double knockout (g and h) mouse brains. In the wild-type and the *vldlr*^{-/-} animals, the neurons born at E12 and E15 have taken up their proper positions in the deepest layers (E12) and in the most superficial layers (E15). In contrast, in the *apoER2* knockout, E12 neurons are scattered throughout the cortex. Many E15 neurons have failed to migrate to the surface and have remained in the periventricular zone. Some neurons,

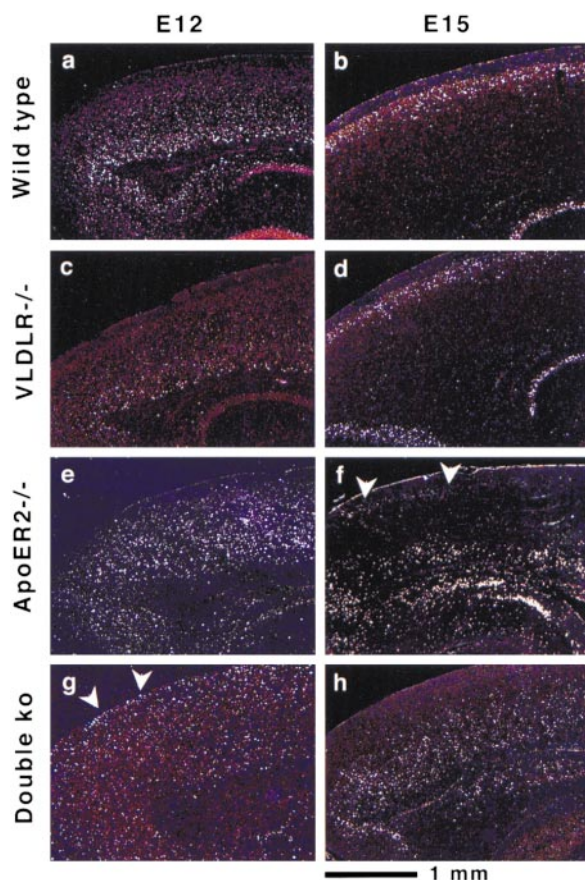


Figure 6. Neuronal Birth Date Analysis in Wild-Type and Mutant Mouse Cortex at E12 and E15

Pregnant double heterozygous female mice were injected with [³H]thymidine (a–e, g, and h) or BrdU (f) at E12 and E15. Pups were perfused with 4% paraformaldehyde 16 days after birth, and brains were removed, embedded in paraffin, and sectioned sagittally. After deparaffinization, sections were either coated with photographic emulsion and exposed for 2–3 weeks at 4°C or processed for immunohistochemical detection of incorporated BrdU as described in Experimental Procedures. Arrows in (f) point to labeled neurons that have arrived at their proper layer. Arrows in (g) point at misplaced superficial neurons.

however, have migrated to more superficial layers (arrows in [f]). As expected, this migration defect and the general disorganization of the neuronal distribution pattern were even more prominent in the double knockout brains (g and h), where E12 neurons are crowded in the normally cell-free layer 1 immediately beneath the pial surface (arrows in [g]). In contrast, all E15 neurons have failed to migrate to superficial layers.

Upregulation of mDab1 Protein Expression

In the absence of Reelin, mDab1 protein expression is increased severalfold. If VLDLR and ApoER2 mediate the transmission of the Reelin signal across the plasma membrane, mDab1 should also be upregulated in *vldlr/apoER2* double mutant mice. To investigate this, we performed Western blot analysis of brain extracts from newborn mice that were either wild type (Figure 7, lane 1), heterozygous for *vldlr* and *apoER2* (lane 2), *vldlr*^{-/-}

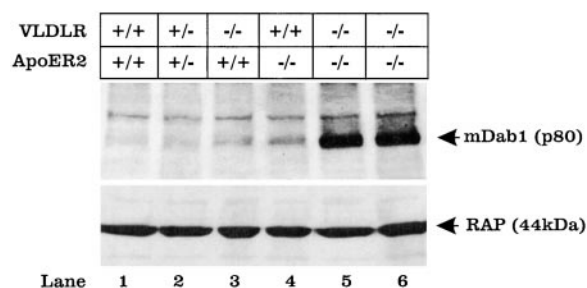


Figure 7. Expression of mDab1 and RAP in Wild-Type and Mutant Newborn Mouse Brain

Lysates were prepared from wild type (lane 1), *vldlr*^{+/-};*apoER2*^{+/-} (lane 2), *vldlr*^{-/-} (lane 3), *apoER2*^{-/-} (lane 4), and *vldlr*^{-/-};*apoER2*^{-/-} (lanes 5 and 6) newborn mouse brains. Proteins were separated by 4%–15% SDS gel electrophoresis, transferred to nitrocellulose, and the 80 kDa isoform of mDab1 was detected by immunoblotting with an antibody raised against the carboxy-terminal 15 amino acids of full-length murine mDab1. mDab1 expression is only slightly elevated in *vldlr*^{-/-} and *apoER2*^{-/-} brains, but dramatically increased in the double knockout. Expression of RAP, an ER chaperone that controls expression of the LDL receptor gene family, is unchanged.

(lane 3), or *apoER2*^{-/-} (lane 4), or homozygous deficient for both genes (lanes 5 and 6). The p80 isoform of mDab1 was detected with an anti-peptide antibody raised against the carboxyl terminus of the full-length mouse protein (Rice et al., 1998). Weak mDab1 expression was detected in wild-type (lane 1) and double heterozygous brain extracts (lane 2). mDab1 protein levels were increased 2- to 3-fold in each of the single knockouts (lanes 3 and 4) and approximately 13-fold in the brain extracts from double knockouts (lanes 5 and 6). The same result was obtained with an independently derived antibody directed against the protein interaction domain of mDab1 (data not shown). A nonspecific band that cross-reacted with the antibody is seen above mDab1. Neither the expression of this protein nor that of RAP, a specialized ER chaperone that controls the biosynthesis of the LDL receptor gene family, was changed in any of the samples, indicating that equal amounts of protein were loaded in each lane.

Discussion

We have used gene targeting in mice to show that two cell surface receptors, the VLDL receptor and ApoER2, are required for radial neuronal migration in the brain. In the absence of both receptors, the cortical neuronal layers are inverted and cerebellar development is arrested, due to failure of Purkinje cell migration. The phenotype of the double knockout is indistinguishable from *reeler* and *scrambler* (Sweet et al., 1996; Gonzalez et al., 1997; Howell et al., 1997b; Sheldon et al., 1997; Ware et al., 1997; Curran and D'Arcangelo, 1998; Gallagher et al., 1998), naturally occurring mouse mutants in which the Reelin and mDab1 gene, respectively, are defective. Both receptors can bind the cytosolic adaptor protein mDab1 on their cytoplasmic tails. In *reeler* (Rice et al., 1998; Howell et al., 1999a) and in *vldlr*^{-/-};*apoER2*^{-/-} newborn mice, mDab1 expression is greatly increased, suggesting that mDab1 functions in the same pathway. Taken together, these findings suggest that the VLDL

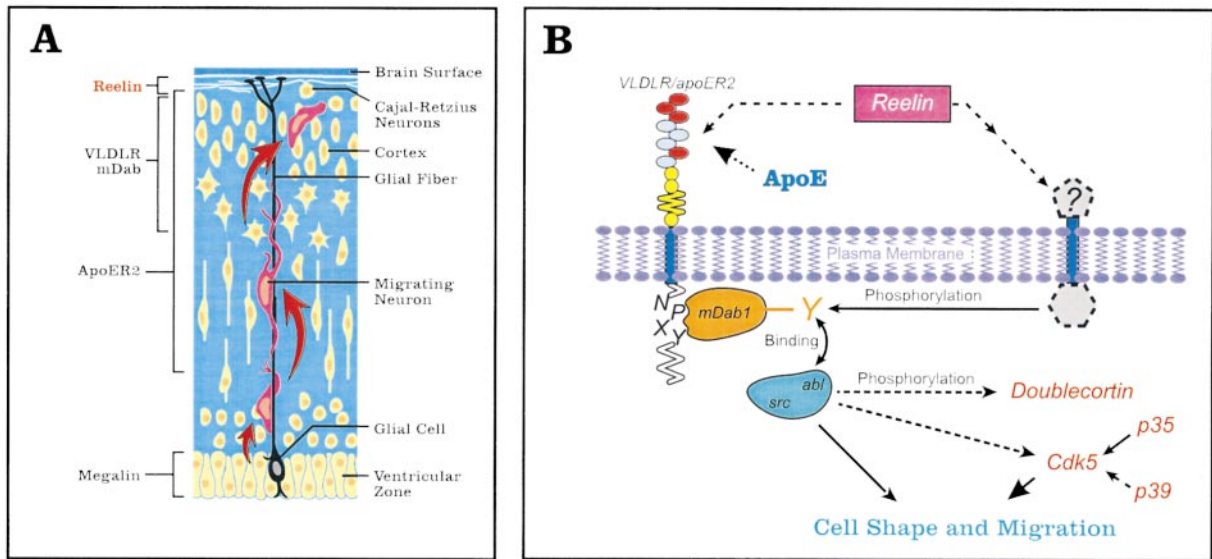


Figure 8. Role of Reelin, Receptors, and mDab1 in Neuronal Migration

(A) A schematic section through the cortex is shown. A radial glial cell is anchored in the ventricular zone and sends its guidance fiber to the surface of the brain, where it is anchored through several foot processes. After their birth in the ventricular zone, postmitotic neurons migrate along the guidance fiber to the surface, where they encounter Reelin, expressed by Cajal-Retzius neurons, detach from the fiber, and migrate horizontally to their final position. VLDLR and mDab1 are expressed in the intermediate zone immediately adjacent to the Reelin-containing marginal zone. ApoER2 is expressed throughout the developing neocortex with the exception of the ventricular zone, where Megalin is present on the apical surface of premigratory neuroepithelial stem cells.

(B) Hypothetical pathway of Reelin signaling. Reelin may directly or indirectly interact with the VLDLR and ApoER2 on the neuronal surface. mDab1 is recruited to the cell surface by binding to the cytoplasmic NPXY motif in both receptors. Tyrosine phosphorylation of mDab1 is thought to be induced by receptor ligation, through interaction of VLDLR and ApoER2 with extracellular components, possibly Reelin itself, or alternatively indirectly by a membrane tyrosine kinase (?) that is activated by Reelin. Tyrosine phosphorylated mDab1 recruits and activates nonreceptor tyrosine kinases of the Src and Abl family that act on downstream components of the signaling pathway. Cdk5, p35, and p39 (activators of Cdk5) may lie downstream of mDab1. VLDLR and ApoER2 also bind ApoE, which could potentially modulate this signaling pathway. ApoER2 and VLDLR share the same basic structure. The main structural difference is in the ligand-binding domain (cluster of oval symbols), where blue repeats are alternatively spliced in *apoER2*.

receptor and ApoER2 are obligate components of a signaling pathway that mediates the transmission of the Reelin signal across the plasma membrane to intracellular kinases that regulate neuronal migration during development.

Distinct Roles of VLDLR and ApoER2 in Neuronal Migration

Three models have been proposed to explain the phenotype of Reelin- and mDab1-deficient mice (Curran and D'Arcangelo, 1998). One model suggests that Reelin acts as a repellent that induces the cortical preplate to split into marginal zone and subplate. The failure of the preplate to separate in *reeler* causes the cortical plate to develop ectopically underneath the subplate neurons. In another model, a Reelin gradient may selectively induce cortical plate neurons to migrate past the subplate neurons. In a third model, Reelin is thought to act as a stop signal that instructs migrating neurons to detach from their glial guidance fiber.

The present findings are consistent with all these models, but they also indicate that neuronal migration along radial glia is more complex than these current models suggest. We have shown that VLDLR and ApoER2 are both required for normal neuronal migration. Both receptors coordinate the development of the cortex and of the cerebellum; however, the phenotype

of the VLDLR defect manifests itself mainly in the cerebellum, whereas deficiency in ApoER2 predominantly affects the development of the neocortex. Only the absence of both receptors causes an almost exact neuro-anatomical phenocopy of the *reeler* and *scrambler* (mDab1-deficient) mutation. In the cortex, VLDLR and mDab1 are selectively expressed in the migrating neurons that are about to make contact with Reelin in the marginal zone (Figure 8A), consistent with a role of VLDLR as a receptor for a migratory stop signal. Also consistent with such a model is the finding that in VLDLR-deficient cerebellum, Purkinje cells are ectopically located, apparently due to their inability to properly respond to the Reelin signal that emanates from the granule cells in the external granular layer. In contrast, ApoER2 is more ubiquitously expressed throughout the developing brain. Thus, other non-cell autonomous functions for this receptor cannot be ruled out. Absence of ApoER2 alone prevents a large portion of neurons from completing their migration and causes a partial inversion of the layers in the neocortex. As a result, the large and easily identifiable pyramidal neurons end up in a relatively ordered, but more superficial layer than the layer 5 in which they normally reside.

Furthermore, in VLDLR-deficient cortex, neurons are strictly radially aligned, and although they apparently have reached their normal assigned layer, they have

failed to distribute within that layer. In contrast, in ApoER2-deficient cortex, there is no recognizable radial pattern, and neurons instead are packed into tight consecutive horizontal layers. In the absence of either VLDLR or ApoER2, neurons do not invade layer 1, in contrast to the double knockout and to *reeler* and mDab1 mutants (Sweet et al., 1996; Goldowitz et al., 1997; Gonzalez et al., 1997; Howell et al., 1997b; Sheldon et al., 1997; Ware et al., 1997).

Taken together, these findings suggest that VLDLR and ApoER2 function in a coordinated and partially overlapping fashion. Either receptor is apparently capable of interpreting the stop signal conferred by Reelin, thus allowing migrating neurons to detach from the radial guidance fiber and preventing them from invading layer 1. Consistent with this interpretation is our finding that mDab1 expression is only slightly increased in either of the single knockouts. The distinct neuroanatomical phenotypes of *vldlr* and *apoER2* knockouts may indicate that both receptors further interact with specific sets of other matrix or glial surface components. Other adaptor proteins besides mDab1 may also be involved and control different stages of neuronal migration.

Roles in the Organization of the Cytoskeleton

Both receptors function not only during the migratory phase of the neurons, but also affect the organization of their cytoskeleton. This is revealed by staining for MAP-2 in cortical neurons and for calbindin in Purkinje cells. In the latter, absence of either receptor severely affects the intricate pattern of the dendritic arbors and spines. Although in the absence of ApoER2 most Purkinje cells migrate to a single, well-defined cortical layer, their cell bodies are smaller and their dendritic processes are disorganized. This finding further supports a model in which VLDLR and ApoER2 receive distinct signals from the extracellular environment that are translated into cellular shape changes. Also consistent with this hypothesis is the finding that two nonreceptor tyrosine kinases, Abl and Src, which interact with mDab1 (Howell et al., 1997a) and are thus likely mediators of the Reelin signal, are involved in the organization of the cytoskeleton (Insogna et al., 1997; Koleske et al., 1998; Ishida et al., 1999).

How Are the Migratory Signals Transmitted?

Neither the VLDLR nor ApoER2 contains a kinase domain in its cytoplasmic tail, suggesting that signaling depends on receptor ligation and recruitment of adaptor proteins and kinases into the complex, a mechanism similar to those described for other signaling pathways (reviewed in Pawson and Scott, 1997; Schillace and Scott, 1999). Our findings suggest that VLDLR and ApoER2 function as obligate components in the Reelin/Disabled-mediated neuronal migration pathway. So far, we have not been able to determine conclusively whether Reelin interacts directly with the extracellular domains of VLDLR and ApoER2. The reason for this may lie in the inherent technical difficulties of expressing sufficient amounts of this large (>300 kDa) protein, or alternatively accessory factors may be necessary for binding. Alternatively, VLDLR and ApoER2 may interact with a different set of extracellular components as part of a larger

complex in which another Reelin-specific receptor induces tyrosine phosphorylation of mDab1 bound to their cytoplasmic tails (Figure 8B). In the simplest model, Reelin and other extracellular matrix or glial surface components could induce the receptors to cluster into a complex. This would allow for transphosphorylation of mDab1 and result in the amplification of the basal activity of the tyrosine kinases.

Possible Functions beyond Neuronal Migration

VLDLR, ApoER2, and mDab1 continue to be coexpressed in adult brain (our own unpublished observations), although tyrosine phosphorylation of mDab1 is greatly reduced after the end of the neuronal migration period (Howell et al., 1997a, 1999a). It is nevertheless possible that this pathway remains active at a low level to direct neurons that continue to be born even in adult brains (Gage, 1998) to their proper place. Alternatively, this pathway may participate in the reorganization of dendritic and axonal connections that continue in adult brain at a constant rate. This hypothesis is underscored by the requirement of VLDLR and ApoER2 for Purkinje cell arborization, a process that in the mouse takes place within the first few postnatal weeks after the cells have migrated to their layer (Hatten and Heintz, 1995).

A role of this pathway in the reorganization of the cytoskeleton is also supported by two other mouse models in which the kinase Cdk5, which phosphorylates neurofilaments, or its activating subunit, p35, have been destroyed (Ohshima et al., 1996; Chae et al., 1997). In these animals, cortical layers are also inverted. This raises the possibility that Cdk5 functions downstream of the VLDLR and ApoER2.

In conclusion, the present findings have established a novel role for the LDL receptor gene family in cellular signaling events that goes beyond the endocytosis of ligands. The genetic identification of two receptors that are required for normal neuronal migration and that may function in the transmission of the Reelin signal will facilitate the further dissection of the biochemical processes that govern the migration of neurons to their final position in the cortex.

Experimental Procedures

GST Pull-Down Experiments

Interactions between mDab1 and recombinant GST fusion proteins containing cytoplasmic tails of LDL receptor gene family members were performed as described (Trommsdorff et al., 1998). Briefly, murine full-length mDab1 was expressed in HEK293 cells by transfection, and cell lysates were prepared. The entire cytoplasmic tails of the receptors of the LDL receptor family and segments containing only one NPxY in the case of the LRP tail were amplified and cloned into pGEX-KG. Primers used for amplification of LDLR, LRP, and mDab1 sequences have been previously described (Trommsdorff et al., 1998). The following primer pairs were used for amplification of other tail sequences. Murine ApoER2: 5'-TTTTGAATTCGGAGAACTGGAAGCGGAAGAACA3' and 5'-AAAAAAGCTTCAGGGTAGTCCATCATCTTCAAG3', which amplified the ApoER2 tail with or without an alternatively spliced insert (Kim et al., 1996). Murine VLDLR: 5'-TTTTGAATTCGGAGGAATTGGCAACATAAAAACATG-3' and 5'-TTTAAGCTTTCAGCCAGATCATCATCTGTGC-3'.

All fragments were verified by sequencing. GST pull-downs were performed by incubating the fusion proteins with mDab1-containing lysates, followed by addition of glutathione agarose. Beads were

washed briefly, bound proteins were separated by SDS gel electrophoresis, and mDab1 was detected by immunoblotting.

In Situ Hybridization

Templates used for in situ hybridization were cloned into PCRII-TOPO (Invitrogen) and sequenced. The different murine cDNA fragments were nt 1844–2403 for *vldlr*, nt 5062–5702 for *reelin*, nt 2164–stop (no insert) for *apoER2*, nt 1–543 for *mDab555*, and nt 458–878 for *calbindin*. For each labeling reaction, 0.5 µg of linearized template was transcribed using T7 RNA polymerase or Sp6 polymerase (Ambion, TX) and 100 µCi of ³³P-UTP (Amersham) producing sense and antisense probes for each of the fragments.

Time-mated wild-type females at 13.5 or 15.5 day postcoitum were anaesthetized with metofane and perfused via the left ventricle with PBS followed by 4% paraformaldehyde. Whole embryos were immersed in 4% paraformaldehyde overnight at 4°C, then changed to PBS. The tissue was placed in 70% ethanol, dehydrated through graded ethanol solutions, cleared in xylene, and infused with paraffin. Sagittal sections were cut at 5 µm intervals and mounted on Vectabond-treated slides (Vector laboratories). Contiguous sections were probed with sense or antisense transcripts of the different probes.

In situ hybridization was performed to determine the expression pattern of ApoER2, VLDLR, Reelin, and mDab1 in wild-type mice during neuronal development. Paraffin was removed from the sections with xylene, followed by graded ethanol hydration, postfixation in 4% paraformaldehyde, Pronase digestion (20 µg/ml Pronase for 7.5 min), and acetylation (0.1 M triethanolamine-HCl [pH 7.5]/0.25% acetic anhydride for 5 min). Hybridization was conducted for 12 hr at 55°C in a solution containing 50% formamide, 0.3% dextrane sulfate, 1× Denhardt's solution, 0.5 mg/ml tRNA, and 7.5 × 10⁶ cpm/ml riboprobe. After hybridization, the slides were washed in 5× SSC/100 mM DTT at 65°C for 30 min. K.5 nuclear emulsion (Ilford) was applied to the slides before exposure at 4°C for 21 to 28 days, depending on the probes.

Neuronal Birth Date Analysis

These experiments were performed essentially as described (Angevine, 1965) with the following modifications. Female mice, heterozygote for *apoER2* and *vldlr*, were mated with double heterozygous males and injected at E15.5 with 500 µCi [³H]thymidine (American Radiolabeled Chemicals, Inc., MO) or 500 µg/g of body weight BrdU (Sigma). Pups were sacrificed at day 16 after birth and perfused with 4% paraformaldehyde. Brains were dissected and embedded in paraffin. For autoradiography, sections were cut at 7 µm at the level of the cerebellar peduncle. Slides were deparaffinized, dipped in K.5 nuclear emulsion, and exposed at 4°C for 14 to 28 days.

Immunohistochemistry

BrdU Staining

Slides were cut at 5 µm thickness, deparaffinized by several incubations in xylene, and hydrated through a gradient of ethanol to PBS. DNA was denatured by incubating the slides in 2N HCl for 60 min at 37°C. Slides were neutralized in 0.1 M borate buffer (pH 8.5) two times for 5 min each, washed three times for 5 min each in PBS, and permeabilized in 0.3% Triton/PBS two times for 5 min each. Slides were then washed in PBS three times for 5 min each and blocked in 1.5% normal horse serum/0.1% BSA/PBS for 30 min at RT. Slides were then incubated with anti-BrdU antibody (Boehringer-Mannheim-Roche) diluted 1/25 in the blocking buffer overnight at 4°C. The next day, slides were washed three times for 5 min in PBS, incubated with biotinylated horse anti-mouse (Vector laboratories) diluted 1/200 in 1% horse serum/0.1% BSA/PBS for 30 min at RT. Slides were washed three times for 5 min each in PBS.

For immunofluorescence slides were incubated with FITC-streptavidin (Vector laboratories) diluted 1/50 in PBS (pH 8.2) at RT in a dark place. Slides were washed in PBS, three times for 5 min each, and mounted using Vectashield (Vector laboratories).

For Dab staining, slides were quenched in 0.3% H₂O₂/CH₃OH for 30 min at 22°C, then washed in PBS three times for 5 min each. Slides were incubated with peroxidase-streptavidin (Vector laboratories), diluted 1/500 in PBS for 30 min at 22°C, and washed three times for 5 min each in PBS. Diaminobenzidine chromagen (DAKO, CA)

was applied, and development was monitored under the microscope and stopped by incubating the slides in H₂O. Slides were counterstained with hematoxylin, washed in H₂O, dehydrated in 100% EtOH, three times for 1 min each, xylene three times for 1 min each, then mounted with permount.

Calbindin and MAP-2 Staining

Slides were essentially treated the same way as described, except that there was no denaturation step in HCl. Rabbit anti-calbindin antibody was used at a 1:1000 dilution (Swant, Switzerland), and mouse anti-MAP-2 antibody was diluted 1:20 (Sigma). As secondary antibody, a goat anti-rabbit (Vector laboratories) was used at 1:200 dilution.

Immunoblotting

Brains of newborn pups were dissected and weighed, and proteins were extracted in 5 vol of lysis buffer (0.5% NP40, 150 mM NaCl, 50 mM Tris-HCl [pH 7.4] with an EDTA protease inhibitor cocktail (Boehringer-Mannheim-Roche). After lysis on ice for 10 min, nuclei were centrifuged for 30 min (14,000 rpm, 4°C). One hundred micrograms of protein was loaded per lane and separated by 4%–15% SDS polyacrylamide gel electrophoresis. After transfer to nitrocellulose, mDab1 and ApoER2 were detected using a rabbit antibody directed against the carboxy-terminal 15 amino acids of the proteins. Relative protein concentrations were quantitated using NIH Image software.

Generation of *apoER2* Knockout Mice

Two fragments derived from isogenic murine DNA were cloned into the KO vector. An 11 kb fragment containing the EGF precursor and O-linked sugar domains was amplified by long-range PCR (Takara), using the primers SN11 5'-TTCTGGACAGACCTGGAAAATGAGG-3' and MT11 5'-TGACTGTTGAGCCCATCTGGCTGCC-3'. A 1 kb fragment containing most of the cytoplasmic tail was amplified by PCR using primers MT1 5'-TGAATTTTCGACAACCCAGTGACAG-3' and MT-3 5'-TGCCACAGTGGGCGATCATAGTT-3'.

Homologous recombination resulted in the replacement of approximately 5 kb of genomic sequences containing the transmembrane domain by a *pol2neo* cassette (Soriano et al., 1991). Two copies of the herpes simplex virus thymidine kinase gene were inserted in tandem at the 3' end of the short homology region. Electroporation of linearized replacement vector into murine embryonic stem cells SM1 and derivation of germline chimeras from two independent stem cell clones were performed according to standard protocols (Willnow and Herz, 1994). The presence of the disrupted allele was confirmed by hybridizing BgIII-digested genomic DNA with a 0.5 kb fragment located 3' of the short arm.

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Note Added in Proof

vdLr and *apoER2* knockout mice have been submitted to the Induced Mutant Resource at the Jackson Laboratories, Bar Harbor, ME.