Rescue of the Reeler Phenotype in the Dentate Gyrus by Wild-Type Coculture Is Mediated by Lipoprotein Receptors for Reelin and Disabled 1

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ABSTRACT

Reelin is a positional signal for the lamination of the dentate gyrus. In the reeler mutant lacking Reelin, granule cells are scattered all over the dentate gyrus. We have recently shown that the reeler phenotype of the dentate gyrus can be rescued in vitro by coculturing reeler hippocampal slices with slices from wild-type hippocampus. Here we studied whether Reelin from other brain regions can similarly induce this rescue effect and whether it is mediated via the Reelin receptors apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR). We found that coculturing reeler hippocampal slices with slices from wild-type olfactory bulb, cerebellum, and neocortex rescued the reeler phenotype as seen before with hippocampal slices, provided that the Reelin-synthesizing cells of these regions were placed near the marginal zone of the reeler hippocampal slice. However, coculturing wild-type hippocampal slices with hippocampal slices from mutants deficient in ApoER2 and VLDLR did not rescue the reeler-like phenotype in these cultures. Similarly, no rescue of the reeler-like phenotype was observed in slices from mutants lacking Disabled 1 (Dab1), an adapter protein downstream of Reelin receptors. Conversely, reeler hippocampal slices were rescued by coculturing them with slices from Dab1−/− mutants or ApoER2−/−/VLDLR−/− mice. These findings show that Reelin from other brain regions can substitute for the loss of hippocampal Reelin and that rescue of the reeler phenotype observed in our coculture studies is mediated via lipoprotein receptors for Reelin and Dab1. J. Comp. Neurol. 495:1–9, 2006. © 2006 Wiley-Liss, Inc.

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Little is known about the signals that control the characteristic formation of cell and fiber layers in the dentate gyrus and the integration of postnatally generated granule cells into the hippocampal network. The extracellular matrix protein Reelin is known to play an important role, because the characteristic lamination of the dentate gyrus is lost in the reeler mutant lacking Reelin (Stanfield and Cowan, 1979; Drakew et al., 2002). Reelin is synthesized and secreted by Cajal-Retzius cells located in the outer molecular layer close to the hippocampal fissure (D’Arcangelo et al., 1995, 1997; del Rio et al., 1997; Frotscher, 1998), the marginal zone of the dentate gyrus.

We have recently shown that the reeler phenotype of the dentate gyrus can be rescued in slice cultures of reeler hippocampus by coculturing them with wild-type hippocampal slices providing a Reelin-containing marginal...
zone (Zhao et al., 2004). After an incubation period of about 24 hours, the scattered granule cells of the reeler dentate gyrus formed a compact cell band reminiscent of a granular layer. In contrast, no granular layer formed when recombinant Reelin was added to the culture medium (Zhao et al., 2004). We concluded from these studies that Reelin has to be in a specific location to exert its effects on granule cell laminarization.

These experiments did not clarify the signaling cascade involved. Several Reelin receptors have been described. The lipoprotein receptors apolipoprotein E receptor 2 (ApoER2) and the very-low-density lipoprotein receptor (VLDLR) are known to be Reelin receptors (Trommsdorff et al., 1999). Alpha3beta1 integrins (Dulabon et al., 2000) have been reported to function as Reelin receptors as well, although binding of Reelin to them has recently been phosphorylated upon Reelin binding (Hiesberger et al., 1999; Howell et al., 1999).

In the present study, we used our coculture assay to determine the signaling molecules involved in the rescue of the reeler phenotype in the dentate gyrus. First, we show that wild-type hippocampal slices can be replaced by slices of neocortex, olfactory bulb, and cerebellum, which all contain different neuronal types synthesizing Reelin, suggesting that the rescue effect is brought about by Reelin and does not depend on tissue-inherent properties. We then provide evidence that Reelin signaling via ApoER2 and VLDLR and the adapter protein Dab1 underlies the observed rescue of granule cell laminarization in slices of reeler hippocampus.

**MATERIALS AND METHODS**

**Preparation of slice cultures**

For the preparation of slice cultures, newborn (P0) homozygous reeler mice, newborn (P0) and young postnatal (P3–5) ApoER2/VLDLR double knockout mice, Dab1 knockout mice, and wild-type mice or rats were used. The mutant mice were identified by their well-known morphological malformations in the cortex and hippocampus; the genotype of reeler mice, ApoER2/VLDLR double knockout mice, and Dab1 mutants was confirmed by PCR analysis of genomic DNAs as described elsewhere (Deller et al., 1999; Deffert et al., 2002). Brains were removed following decapitation and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) overnight. Then, the brains were cut on a vibratome (50 μm).

Cultures were fixed after 7–10 DIV with 4% paraformaldehyde for 2 hours and then washed several times in 0.1 M PB. Thereafter, the cultures were resliced on a vibratome (50 μm). Tissue sections and sections of cultures were preincubated in a blocking solution (5% normal goat serum, 0.2% Triton-X 100 in 0.1 M PB) for 30 minutes at room temperature. After being rinsed in 0.1 M PB, sections were incubated with the following primary antibodies overnight at 4°C: monoclonal mouse anti-Reelin G10 (1:1,000; Chemicon, Hofheim, Germany; MAB 5364, immunogen: a fusion protein containing amino acids 164–496 of mouse Reelin) or polyclonal rabbit anti-Prox-1 (1:1,000; Chemicon, AB 5475, immunogen: last 15 amino acids of the C-terminus of mouse Prox-1; see Bagri et al., 2002). After being washed in 0.1 M PB, sections were incubated in secondary antibodies (Alexa 488 goat anti-rabbit IgG, A11008, and/or Alexa 568 goat anti-mouse IgG, A11004; 1:600; Molecular Probes, Göttingen, Germany) at 4°C overnight. The monoclonal antibody G10 against Reelin specifically stained Reelin-synthesizing neurons as originally described by de Bergeyck et al. (1998; see Fig. 1). It stained the 400-kD Reelin protein and the 300-kD and 180-kD Reelin fragments in Western blots (see Fig. 2). Identical immunolabeling was observed with the original G10 antibody (de Bergeyck et al., 1998; kindly provided by Dr. A. Goëffin) and the commercial G10 antibody from Chemicon. No immunostaining was observed with tissue from reeler mutants lacking Reelin. The Prox-1 antibody specifically stained the granule cells of the dentate gyrus as previously described (Bagri et al., 2002). No double labeling was observed with markers for GABAergic interneurons and mossy cells (data not shown). In the present experiment, we used this characteristic staining pattern of Prox-1 to monitor the rescue of granule cell laminarization by wild-type coculture (see, e.g., Fig. 3).

Sections of immersion-fixed brains were incubated with neurotrace (1:500; Molecular Probes; N21480) for 30 minutes at room temperature. After being rinsed in 0.1 M PB for 2 hours, they were mounted in Moviol.

Sections were photographed with a Zeiss LSM-510 confocal microscope. Confocal images were exported from the Zeiss LSM image browser and stored as TIFF files. Figures were prepared in Adobe PhotoShop 6.0 and Adobe
ILLUSTRATOR 10 (Adobe, San Jose, CA). Image brightness, contrast, and sharpness were adjusted.

Western blot analysis for Reelin

Western blot analysis included lysates of hippocampal tissues from P0 reeler mice and P0 rats, lysates of neocortex from P0 wild-type mice, and olfactory bulb and cerebellum from P3 wild-type mice. Brains were removed following decapitation under hypothermic anesthesia. The hippocampus, neocortex, olfactory bulb, and cerebellum were dissected and collected in 1.5-mL tubes on ice. The samples were weighed and immediately frozen in liquid nitrogen. Six volumes (v/v) of hypotonic lysis buffer (50 mM Tris buffer, 150 mM NaCl, 5 mM EDTA, and 1% protease inhibitor cocktail; Sigma, Munich, Germany; pH 7.6) were added to each sample, and the tissue was lysed by repeated thawing at 37°C and freezing in liquid nitrogen (five times). After sonication for 5 minutes and trituration with a pipette tip to homogenize larger tissue pieces, the suspension was centrifuged at 20,000g for 20 minutes (0°C). The resulting crude supernatants were stored at −80°C or directly used. Supernatants (20 μg) from freshly prepared tissues were diluted with sample buffer (Invitrogen, Karlsruhe, Germany) and boiled for 5 minutes. Proteins were separated by 3–8% gradient Tris-acetate gel electrophoresis (SDS-PAGE; Invitrogen) and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes. A monoclonal mouse antibody against Reelin G10 (Chemicon; MAB 5364, immunogen: recombinant Reelin amino acids 164–496) was used as primary antibody at a dilution of 1:3,000, followed by an alkaline phosphatase-conjugated secondary antibody (goat anti-mouse IgG, 1:10,000; Invitrogen; G21060). The immunoreaction was visualized by a chemiluminescence reaction (Invitrogen).

RESULTS

Reelin expressed by different cell types in different brain regions is qualitatively similar

Reelin is synthesized in different brain regions and by different types of cells. In the hippocampus and neocortex, Cajal-Retzius (CR) cells in the marginal zone synthesize Reelin. Thus, CR cells of the dentate gyrus are concentrated in the outer molecular layer, the marginal zone of the dentate gyrus (Fig. 1A). The granule cells, nonimmunoreactive to Reelin, accumulate underneath, thereby forming a second c-shaped band beneath the larger, similarly c-shaped layer of CR cells. Neocortical sections from P0 mice show a distinct labeling of CR cells in the marginal zone, future layer I, when immunostained for Reelin (Fig. 1B). Nonimmunoreactive neurons seem to arrest underneath, suggesting that they were prevented from invading the marginal zone. In the olfactory bulb, Reelin is synthesized by the mitral cells, which form a ring under which nonimmunoreactive neurons accumulate (Fig. 1C). In the cerebellum, Reelin is known to be synthesized by granule cell precursors in the external granular layer (Fig. 1D). The size and ratio of Reelin and its fragments from these different cell types and brain regions were similar when analyzed by Western blotting (Fig. 2).

Rescue of the reeler phenotype by Reelin from different sources

As described previously, coculturing of a reeler slice next to a wild-type slice with the Reelin-containing marginal zone of the wild-type dentate gyrus attached to the reeler dentate gyrus induced formation of a compact granular layer in the reeler slice (Zhao et al., 2004; Fig. 3A). Because Reelin from different brain regions and cell types turned out to be similar (Fig. 2), we reasoned that it might be possible to rescue the reeler phenotype of the dentate gyrus by coculturing reeler slices with wild-type tissue from these other regions. In fact, when the marginal zone of a neocortical slice was placed next to the dentate gyrus of a reeler slice, we again observed the formation of a compact granular layer (Fig. 3B). Similarly, a dense band of accumulating granule cells was observed when a reeler slice was placed next to Reelin-synthesizing mitral cells of the olfactory bulb (Fig. 3C). Finally, a granular layer also formed when the reeler slice was cocultured with a cerebellar slice containing Reelin-synthesizing cerebellar granule cell precursors (Fig. 3D). These findings indicate that Reelin from different cell types and brain regions can rescue the reeler phenotype of the dentate gyrus. Moreover, these experiments show that tissue-specific properties or factors depending on the developmental stage of the secreting cells are not critical for the observed Reelin effect.

The Reelin-synthesizing cells of the various brain regions had to be in close apposition to the reeler dentate gyrus to provide a Reelin-containing marginal zone. Thus, no rescue was seen when the ventricular zone of a neocortical slice was cocultured next to the reeler dentate gyrus. Accordingly, when a neocortical slice was cocultured with two reeler slices, one adjacent to the marginal zone and the other one next to the ventricular zone (Fig. 4A), only the reeler slice opposed to the marginal zone showed the formation of a compact granular layer (Fig. 4B,C).

Lipoprotein receptors for Reelin and Dab1 are required for granule cell lamination

Double-knockout mice deficient in ApoER2 and VLDLR show a phenotype indistinguishable from that of reeler (Trommsdorff et al., 1999; Drakew et al., 2002). We accordingly hypothesized that the rescue of granule cell lamination in slices from postnatal reeler mutants observed after coculturing with wild-type is mediated by lipoprotein receptors for Reelin. In fact, coculturing of wild-type tissue with ApoER2⁻/⁻/VLDLR⁻/⁻ mutant slices, which lack wild-type slices expressed Reelin but otherwise showed the scattered distribution of granule cells characteristic of the reeler phenotype, did not induce the formation of a compact granular cell layer in the receptor mutant slices (Fig. 5A). Similarly, no rescue of granule cell lamination was observed when wild-type slices were cocultured with slices from Dab1 knockout mice (Fig. 5B). Dab1 mutants, as with ApoER2/VLDLR double-knockout mice, exhibit a reeler-like phenotype. Conversely, both slices from ApoER2⁻/⁻/VLDLR⁻/⁻ mutants and slices from Dab1-deficient mice rescued the reeler phenotype in cocultured reeler slices, likely because of their Reelin expression (Fig. 6A,B). Together, these findings show that lipoprotein receptors for Reelin and the adapter protein Dab1 are required for the rescue of granule cell lamination in slices of reeler hippocampus. Because we used slices
from postnatal hippocampus, our results also suggest that these molecules control the integration of postnatally generated granule cells into the granular layer in vivo.

**DISCUSSION**

By coculturing reeler slices and wild-type slices with the marginal zone of the wild-type slice opposing the reeler dentate gyrus, we could rescue the reeler phenotype, thus confirming the results of our previous studies (Zhao et al., 2004). Here we provide evidence that Reelin from different brain regions and different cell types can exert this effect. Moreover, by coculturing wild-type tissue to slices from Reelin receptor mutants and mutants lacking the adapter protein Disabled-1, we provide evidence that the rescue of granule cell lamination is mediated via these molecules of the Reelin signaling cascade. We conclude that the formation of a laminated dentate gyrus and the integration of granular layer (EGL) of the P3 mouse cerebellum. In A–C, nonimmunoreactive neurons are counterstained with Neurotrace (green). CP, cortical plate; DG, dentate gyrus; EGL, external granular layer; g, granular layer; h, hilus; iml, inner molecular layer; IZ, intermediate zone; MC, mitral cells; MZ, marginal zone; oml, outer molecular layer; SP, subplate. Scale bars = 80 μm in A,B; 250 μm in C,D.
postnatally generated granule cells require Reelin signaling via ApoER2, VLDLR, and Dab1.

**Reelin from different sources is equivalent in rescuing granule cell lamination**

Developmental processes in the various brain regions containing Reelin-synthesizing cells are quite different, the only common trait being the formation of laminated structures. In the neocortex, Reelin controls the formation of cortical layers in an inside-out manner, with late-generated superficial neurons migrating past the early-generated neurons of the deep layers. In contrast, in the dentate gyrus, Reelin is required for the development of a tightly packed granular layer, which develops in an
Fig. 4. Positioning of the marginal zone containing Reelin-immunoreactive Cajal-Retzius cells is crucial for the rescue of granule cell lamination. A: DAPI-stained triplet culture with a wild-type neocortical slice (P0) sandwiched between two reeler cultures (P0). A tightly packed granule cell layer (g) has formed only in the reeler culture (DG1) facing the marginal zone (MZ) of the neocortical culture. In the reeler culture (DG2) facing the ventricular zone (VZ), neurons remain loosely distributed all over the dentate gyrus. Dotted lines indicate borders between cultures; boxed areas are shown in B and C, respectively. B: Reeler culture (DG1) placed next to the marginal zone of wild-type neocortex shown at higher magnification. Double labeling for Prox-1 to label granule cells (green) and Reelin (red) to label CR cells. Note the formation of a dense layer of granule cells (g) near Reelin-immunoreactive CR cells. C: Reeler culture (DG2) placed next to the ventricular zone (VZ) of the wild-type culture, remote from Reelin-synthesizing CR cells. No compact granule cell layer has formed. Scale bars = 200 μm in A; 60 μm in B,C.
outside-in fashion. In the olfactory bulb, Reelin was found to act as a detachment signal for chain-migrating precursors of olfactory bulb interneurons (Hack et al., 2002). In the cerebellum, Reelin synthesized by granule cell precursors in the external granular layer controls the proper positioning of Purkinje cells (Miyata et al., 1997). Despite these various functions in brain development, Reelin from these different brain regions invariably rescued the formation of a compact granule cell layer in our coculture experiments. In line with this uniform effect of Reelin from different sources, Western blots for Reelin from these regions gave very similar results. We conclude that developmental differences among these brain regions arise from different Reelin receptors of the target cells, differences in the Reelin signaling pathway, and/or additional molecular signals.

ApoER2, VLDLR, and Dab1 control development of the dentate gyrus

Several Reelin receptors have been described. There is reason to assume that, for development of the dentate gyrus and for integration of postnatally generated granule cells, the lipoprotein receptors ApoER2 and VLDLR as well as the adapter protein Disabled-1 play an important role. In the absence of lipoprotein receptors or Dab1, no compact granule cell layer formed in coculture experiments. These findings do not exclude a role for other Reelin receptors, such as alpha3beta1 integrins. In fact, localized migration defects of the granule cells, likely caused by a maldifferentiation of the radial glial scaffold, have been described for alpha3beta1-deficient mutants ( Förster et al., 2002). In comparing ApoER2 mutant mice and mice lacking VLDLR, the former were found to show a more severe migration defect in the dentate gyrus (D r akew et al., 2002). Absence of ApoER2 plus VLDLR results in a phenotype indistinguishable from that of reeler mutants ( T rommsdorff et al., 1999; D r akew et al., 2002). Reelin binding to both types of lipoprotein receptors involves Dab1, and scrambler mutants lacking Dab1 exhibit a reeler phenotype ( Howell et al., 1997; S heldon et al., 1997; W are et al., 1997; H iesberger et al., 1999). The results of the present study add to the observations made in these mutants that Reelin, its lipoprotein receptors, and Dab1 have to be present as late as in the early postnatal period to allow for the formation of a compact granule cell layer in the dentate gyrus.

Reelin binding to lipoprotein receptors induces Dab1 phosphorylation by the nonreceptor tyrosine kinase fyn ( Arnaud et al., 2003; B eck and H erz, 2003) and modulates tau phosphorylation (H iesberger et al., 1998; B effert et al., 2002). Phosphorylation of Dab1 at tyrosines 220 and 232 was recently found to be involved in the disconnection of the migrating neuron from the radial glial fiber, which terminates the migration process (S anada et al., 2004). Thus, phosphorylation of Dab1 may underlie the presumed stop signal function of Reelin ( C urran and D’ A rcangelo, 1998; F rotscher, 1998). In the rescue experiments of the present study, we regularly observed an accumulation of granule cells at some distance from the Reelin-synthesizing Cajal-Retzius cells of the cocultured wild-type tissue. Similarly, in the wild-type dentate gyrus, granule cells form a densely packed layer at some distance...
from the CR cells in the marginal zone and do not invade the molecular layer.

**Role of Reelin in laminating the dentate gyrus**

Whereas various molecules of the Reelin signaling cascade have been identified in recent years, we still know very little about the role of Reelin signaling in the various steps leading to the formation of the granular layer. As mentioned, Reelin has been regarded as a stop signal for migrating neurons brought about by Dab1 phosphorylation resulting in the disconnection of the migrating neuron from the radial glial fiber (Sanada et al., 2004). In the reeler mutant, many granule cells remain in the hilus and do not migrate toward the granular layer, suggesting that Reelin in the marginal zone could also act as an attractive signal. It should be noted, however, that Reelin, in addition to its direct effects on neurons, also acts on glial fibrillary acidic protein (GFAP)-positive radial glial cells by increasing their process length (Förster et al., 2002; Frotscher et al., 2003; Weiss et al., 2003). Moreover, Reelin was found to be a positional signal for outgrowing radial glial fibers (Zhao et al., 2004). We hypothesize, based on these data, a dual function of Reelin in the development of the dentate gyrus. As a first step, Reelin induces the formation of a regular radial glial scaffold with the outgrowing radial fibers heading toward the Reelin-containing marginal zone. Granule cells then migrate along these radial glial fibers to reach their final destination in the granular layer. This, in turn, is achieved by Reelin’s second function, its direct effect on the migrating neuron. After Reelin binding to neuronal lipoprotein receptors, Dab1 is phosphorylated, which results in the disconnection of the neuron from the radial glial fiber. This terminates neuronal migration and leads to an accumulation of granule cells in the granular layer (Zhao et al., 2004).

Alternatively, radial glial fibers extending toward the Reelin-containing marginal zone may be inherited by newly generated granule cells and become their apical dendrites (Miyata et al., 2001), insofar as radial glial cells are precursors of neurons (Malatesta et al., 2000; Noctor et al., 2001). Migration of the granule cell from the proliferation zone in the hilus may take place by nuclear translocation (Kriegstein and Noctor, 2004), a process that may be terminated by Reelin signaling. Future live microscopy studies will be needed to determine which of the two scenarios predominates during the long-lasting period of granule cell neurogenesis or holds true at all for the development of the dentate gyrus.

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**LITERATURE CITED**


