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Balance between Neurogenesis and Gliogenesis in the Adult Hippocampus: Role for Reelin

Shanting Zhao[a] Xuejun Chai[a] Michael Frotscher[a, b]

[a]Institut für Anatomie und Zellbiologie und [b]Zentrum für Neurowissenschaften, Albert-Ludwigs-Universität Freiburg, Freiburg, Deutschland

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Reeler mouse · Neurogenesis · Hippocampus · Granule cells · Doublecortin

Abstract
The extracellular matrix protein reelin is essential for the proper radial migration of cortical neurons. In reeler mice lacking reelin, there is a malformation of the radial glial scaffold required for granule cell migration. Immunostaining for glial fibrillary acidic protein (GFAP) reveals abundant radial glial cells with long fibers traversing the granular layer in the wild type, but almost exclusively astrocytes in the reeler mutant. With the concept that radial glial cells are precursors of neurons, we hypothesized that the balance between neurogenesis and gliogenesis is altered in the reeler mutant. To this end, adult reeler mutants and their wild-type littermates were injected with bromodeoxyuridine (BrdU), a marker of newly generated cells. When compared to wild-type animals, we found a reduction in the number of BrdU-labeled cells in the adult reeler dentate gyrus. Moreover, whereas there was a dramatic decrease in the number of newly generated granule cells identified by double labeling for BrdU and NeuN, the number of BrdU-labeled, GFAP-positive astrocytes had increased. Decreased neurogenesis in the adult reeler dentate gyrus was confirmed by immunostaining for doublecortin, a marker of newly generated neurons. These results indicate that adult neurogenesis is altered in the reeler dentate gyrus and that newly generated cells preferentially differentiate into astrocytes.

The subgranular layer of the dentate gyrus is one of the brain regions where substantial postnatal neurogenesis has been shown to occur [Altman and Das, 1965; Kaplan and Hinds, 1977; Kuhn et al., 1996; van Praag et al., 2002]. In the past, most studies have focused on factors stimulating neurogenesis in the dentate gyrus of mature animals. It has been shown, for example, that physical activity in a running wheel, enriched environment, and epileptic activity increase neurogenesis in the dentate gyrus [Kempermann et al., 1997; van Praag et al., 1999a, b; 2002; Parent et al., 1997; Brown et al., 2003a]. So far, little attention has been paid to the molecular signals determining cell fate decisions in the brain regions with ongoing postnatal neurogenesis.

In recent studies, we have shown that the radial glial scaffold in the dentate gyrus is altered in the mouse mutant reeler lacking the extracellular matrix protein reelin. In reeler mice, glial fibrillary acidic protein (GFAP)-positive glial cells do not show the characteristic radial glial

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Michael Frotscher
Institut für Anatomie und Zellbiologie, Albert-Ludwigs-Universität Freiburg
Albertstrasse 17, DE–79104 Freiburg (Germany)
Tel. +49 761 203 5056, Fax +49 761 203 5054
E-Mail Michael.Frotscher@anat.uni-freiburg.de

phenotype with long processes traversing the granular layer but appear as typical astrocytes [Weiss et al., 2003]. We concluded that in the absence of reelin, there is a premature differentiation of radial glial cells into astrocytes [Förster et al., 2002; Frotscher et al., 2003]. Since radial glial cells were found to be precursors of neurons [Malatesta et al., 2000; Noctor et al., 2001], an increase in astrocytes paralleled by a decrease in radial glial cells may indicate an imbalance between neurogenesis and gliogenesis in adult reeler mutants. Thus, reelin may be one of the molecules controlling cell fate decisions in the dentate gyrus.

In the present study, we have analyzed cell proliferation in the dentate gyrus of adult reeler mutants and wild-type animals. Whereas absolute numbers of newly generated cells are difficult to compare between mutants and wild-type mice due to the altered organization of the dentate gyrus in reeler, a study of the relative numbers of newly generated neurons and glial cells appeared feasible. Thus, we combined labeling for bromodeoxyuridine (BrdU), a marker of cell proliferation, with immunostaining for NeuN to determine the number of newly generated neurons in the adult dentate gyrus and double labeling for BrdU and GFAP to monitor gliogenesis. In addition, we employed immunostaining for doublecortin (DCX), a marker of newly generated neurons in the dentate gyrus [des Portes et al., 1998; Gleeson et al., 1998; Brown et al., 2003b; Rao and Shetty, 2004; Couillard-Despres et al., 2005], in order to monitor neurogenesis. Using immunostaining for DCX, we aimed to overcome some of the disadvantages of BrdU labeling such as injection of the tracer into the living animal, dilution with ongoing cell divisions, and toxic effects [see Couillard-Despres et al., 2005].

Our results provide evidence for a decreased cell proliferation in the dentate gyrus of reeler mutants. Moreover, when compared to wild-type animals, reeler mice show a decreased neurogenesis but an increased gliogenesis as revealed in our double-labeling experiments.

Materials and Methods

Animals and BrdU Labeling

Ten adult (5 months old) male reeler mice and 10 wild-type littermates were intraperitoneally injected with BrdU (100 mg/kg body weight) once daily for 10 consecutive days (11:00–12:00 a.m.). A total of 5 reeler mice and 5 wild-type animals were sacrificed 24 h after the last BrdU injection. The remaining animals were allowed to survive until the 35th day after the last BrdU injection. The mutant mice were identified by their well-known morphological malformations in the cortex and hippocampus. In addition, the genotype of reeler mutants was confirmed by PCR analysis of genomic DNAs. The animals were transcardially perfused following anesthesia with an overdose of Narkodorm (500 μl/kg) first with 0.9% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). Brains were removed and postfixed in 4% paraformaldehyde for 24 h. After washing in 0.1 M phosphate buffer (PB, pH 7.4) overnight, brains were cut coronally to contain the entire hippocampus from the rostral to the ventral pole using a Vibratome (50 μm). The sections were then stored in a cryoprotectant solution (25% ethylene, 25% glycerol, 0.05 M PB) at –20°C until they were processed for immunostaining.

Immunostaining

A total of 6 sections containing the dorsal hippocampus were randomly selected from each animal. The free-floating sections were treated with 2 M HCl for 30 min at 37°C. Following neutralization with 0.1 M borate buffer (pH 8.5) and washing in PB, the sections were blocked with 5% normal goat serum containing 0.2% Triton X-100 in 0.1 M PB for 30 min. The sections were then incubated with the first antibodies: rat anti-BrdU (1:500, Serotec, Düsseldorf, Germany), mouse anti-NeuN (1:1,000, Chemicon, Hofheim, Germany), and rabbit anti-GFAP (1:500, DAKO, Hamburg, Germany) in 0.1 M PB for 24 h at 4°C. After washing in 0.1 M PB, the sections were incubated in the secondary antibodies (goat antirat, Alexa 488; goat antimouse IgG, Alexa 555, and goat antirabbit, Alexa 647; dilution 1:300, Molecular Probes, Göttingen, Germany) overnight at 4°C. After rinsing in 0.1 M PB for 2 h, the sections were mounted in Moviol. For DCX immunostaining, 6 sections from the dorsal hippocampus of each animal sacrificed 24 h after the last BrdU injection were used. After blocking in 5% normal donkey serum containing 0.2% Triton X-100 in 0.1 M PB for 30 min, the sections were incubated with goat anti-DCX (1:1,000, Santa Cruz, Heidelberg, Germany) and mouse anti-NeuN (1:1,000) for 24 h at 4°C. This was followed by rinsing the sections in 0.1 M PB for 30 min and incubation in the secondary antibodies (donkey antigoat, Alexa 488, and donkey antimouse IgG, Alexa 555; dilution: 1:300, Molecular Probes) overnight at 4°C. After washing the sections in 0.1 M PB for 2 h, they were mounted in Moviol.

Quantitative Studies

The number of BrdU-positive cells and DCX-immunoreactive neurons, respectively, in the dentate gyrus of each section was counted using an Olympus microscope. Since the dentate gyrus is disorganized in the reeler mutant, we could not restrict our cell counts to the subgranular zone but counted all BrdU-positive cells in the dentate gyrus including the molecular layer, granule cell layer and hilus. A virtual line connecting the free ends of the granule cell layer was used to separate the dentate gyrus from the hippocampus proper. To present the number of immunopositive cells as cells/mm^2 in the dentate gyrus, the area of each dentate gyrus was measured using SIS software. In order to quantify the fate of newly generated cells, the fraction of BrdU-positive cells expressing NeuN or GFAP was quantified by examining individual BrdU-positive cells at a magnification of ×400 using a confocal scanning laser microscope (Zeiss LSM 510). Statistical analysis involved Student's t test and the χ^2 test.

Results

Reduced Cell Proliferation in Reeler Mice

For a study of cell proliferation in wild-type animals and reeler mutants, the number of BrdU-positive cells were quantified in hippocampal sections of animals perfused 24 h after the last BrdU injection. In wild-type animals, BrdU-immunoreactive cells were regularly observed in the subgranular zone of the dentate gyrus (fig. 1A). Only few BrdU-positive cells were found at other locations such as the dentate molecular layer. In reeler mutants, the characteristic lamination of the dentate gyrus is lost, and many granule cells are not densely packed in a granular layer but scattered all over the dentate gyrus. As a result, no clear subgranular layer can be discerned. Only a few BrdU-positive cells can be seen among the many loosely distributed NeuN-positive granule cells (fig. 1B).

For quantification of newly generated cells, the number of BrdU-positive cells/mm^2 dentate gyrus was determined. As shown in figure 1C, the number of BrdU-positive cells 24 h after the last BrdU injection was significantly decreased in the mutant dentate gyrus when compared to the wild type ($p < 0.001$). It appears that the disruption of lamination in the reeler mutant and the loss of a circumscribed proliferative zone, the subgranular layer, interferes with proliferative activity in the adult reeler dentate gyrus. Interestingly enough, this loss of proliferative activity in the dentate gyrus in reeler mice was accompanied by a decrease in the weight of the hippocampus and in overall brain weight (30.7 ± 1.67 mg hippocampal weight in wild-type animals versus 24.6 ± 1.39 mg in reeler mice, and 488.1 ± 21.2 mg total brain weight in wild-type versus 350.5 ± 15.6 mg in reeler mice).

Less New Neurons but More New Glial Cells

Next, in order to study the fate of newly generated cells in the adult reeler dentate gyrus, sections of reeler mutants and wild-type animals perfused 35 days after the last BrdU injection were triple-immunolabeled for BrdU and NeuN, a marker of neurons, and GFAP, a marker of glial cells. In the adult dentate gyrus of wild-type animals, both newly generated neurons (fig. 2A) and glial cells (fig. 2B–D) were mainly observed in the subgranular zone underneath the densely packed granular layer. As described for the BrdU-labeled cells in animals perfused 24 h after the last BrdU injection, BrdU-labeled neurons and glial cells are scattered all over the dentate gyrus in the reeler mutant. A quantitative analysis revealed a significant decrease in the number of newly generated neurons when compared to wild-type animals ($p < 0.001$; fig. 2E). In contrast, the number of newly generated astrocytes/mm^2 dentate gyrus was significantly increased in sections from reeler mice ($p < 0.01$; fig. 2E). These results were confirmed by an estimation of the percentage of NeuN-positive cells and GFAP-positive cells in all BrdU-labeled cells (fig. 2F). While in wild-type animals NeuN-positive cells accounted for 61.2% of all BrdU-labeled cells, the percentage of NeuN-positive cells was reduced to 7.9% in reeler mutants. In contrast, the percentage of double-labeled astrocytes was 4.7% in wild-type animals and 10.7% in reeler mutants. These findings also show that there was a substantial number of BrdU-labeled cells that neither stained for GFAP nor NeuN. In addition to technical problems inherent to double-immunolabeling, proliferation of nonneuronal cells such as microglial cells and endothelial cells may play a role. Studies are in progress to characterize these cells further by using a variety of cell-specific markers. Taken together, this study after a long survival time (35 days) following the last BrdU injection has revealed a clear difference between wild-type animals and reeler mutants with respect to the fate of newly generated cells in the adult dentate gyrus. Loss of lamination in this brain region does not only seem to affect proliferative activity as a whole, but results in significantly decreased neurogenesis in reeler mutants.

DCX-Immunoreactive, Young Neurons Are Decreased in the Reeler Dentate Gyrus

The labeling of newly generated cells with BrdU is dependent on the injection of the tracer and its uptake by progenitor cells. Thus, immunolabeling of proteins exclusively present in newly generated cells may be used to supplement studies with BrdU as a marker. In the present study, we took advantage of DCX, a marker of newly generated granule cells in the dentate gyrus that has recently been found to accurately reflect neurogenesis in the adult brain, even demonstrating modulations of neurogenesis induced by physical activity and epileptic seizures [Couillard-Despres et al., 2005]. In fact, double labeling for NeuN and DCX revealed newly generated neurons in the subgranular zone of wild-type animals, exactly where they were found by using BrdU tracing (fig. 3A). At higher magnification, these newly generated neurons were seen to give rise to long, ascending apical processes traversing the granular layer and invading the molecular layer (fig. 3B). In the reeler mutant lacking a characteristic subgranular zone, DCX-positive cells were scattered

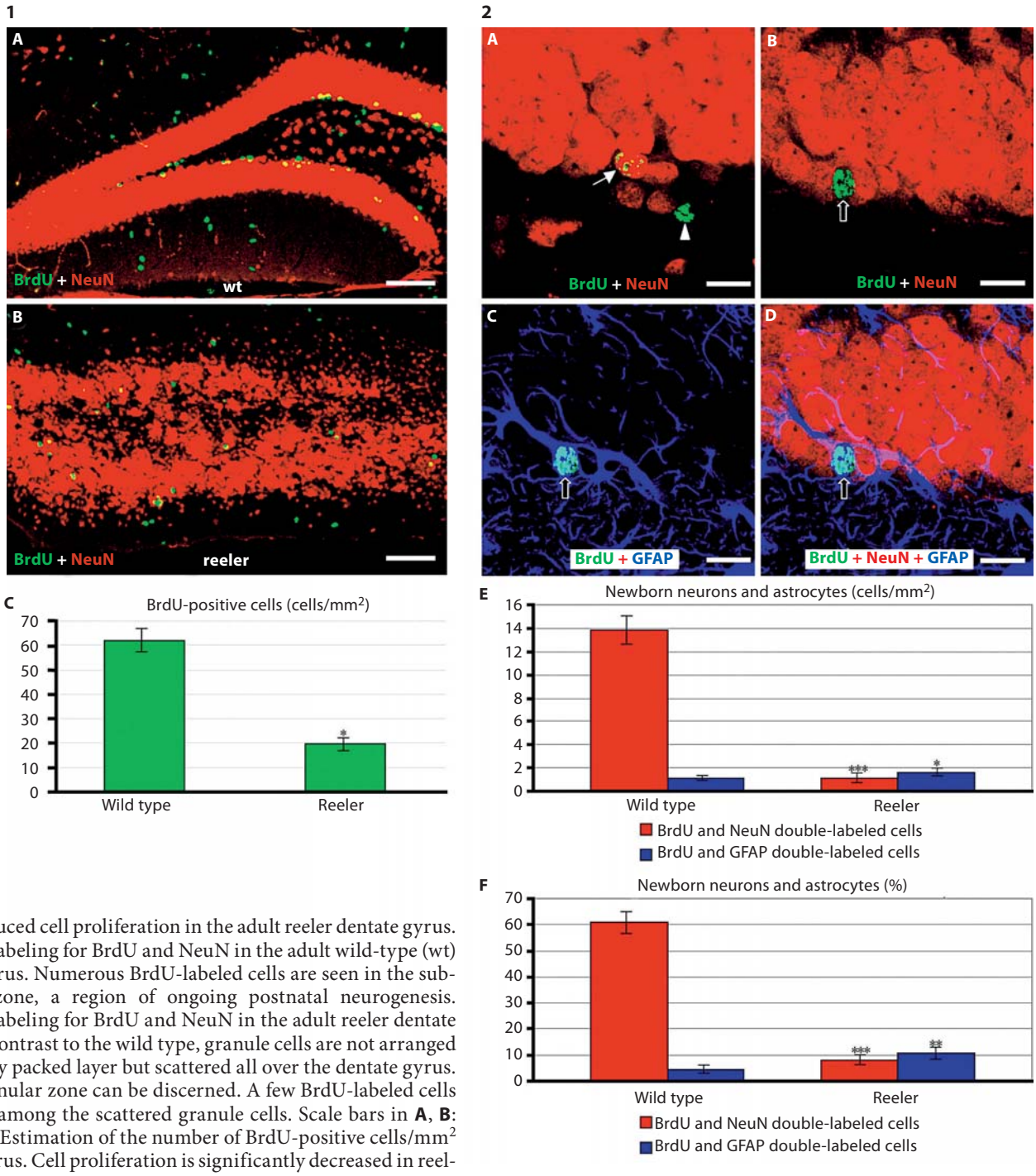

Fig. 1. Reduced cell proliferation in the adult reeler dentate gyrus. **A** Double labeling for BrdU and NeuN in the adult wild-type (wt) dentate gyrus. Numerous BrdU-labeled cells are seen in the subgranular zone, a region of ongoing postnatal neurogenesis. **B** Double labeling for BrdU and NeuN in the adult reeler dentate gyrus. In contrast to the wild type, granule cells are not arranged in a densely packed layer but scattered all over the dentate gyrus. No subgranular zone can be discerned. A few BrdU-labeled cells are found among the scattered granule cells. Scale bars in **A**, **B**: 100 μm. **C** Estimation of the number of BrdU-positive cells/mm^2 dentate gyrus. Cell proliferation is significantly decreased in reeler mutants (mean ± SEM, * $p < 0.001$).

Fig. 2. Reduced neurogenesis but increased gliogenesis in reeler mutants. **A** Double-labeling for BrdU and NeuN. A double-labeled neuron in the subgranular zone of the dentate gyrus is labeled by an arrow. Another cell, only stained for BrdU, is marked by an arrowhead. **B–D** Double labeling for BrdU and NeuN (**B**), BrdU and GFAP (**C**) and triple labeling for BrdU, NeuN and GFAP (**D**). A NeuN-negative, GFAP-positive glial cell in the subgranular zone of the dentate gyrus is labeled by an open arrow. Scale bars (**A–D**): 10 μm. **E** Quantitative estimation of the number of newborn neurons and GFAP-positive astrocytes/mm^2 dentate gyrus. The number of BrdU- and NeuN-double-labeled neurons is significantly decreased in the reeler mutant (mean ± SEM, *** $p < 0.001$). In contrast, the number of GFAP-positive astrocytes is increased (mean ± SEM, * $p < 0.01$). **F** Percentage of newborn neurons and GFAP-positive astrocytes in all BrdU-labeled cells. There are significantly more newly generated neurons in the wild type than in reeler mutants (mean ± SEM, *** $p < 0.001$). The percentage of newly generated astrocytes is significantly increased in the mutant (mean ± SEM, ** $p < 0.005$).

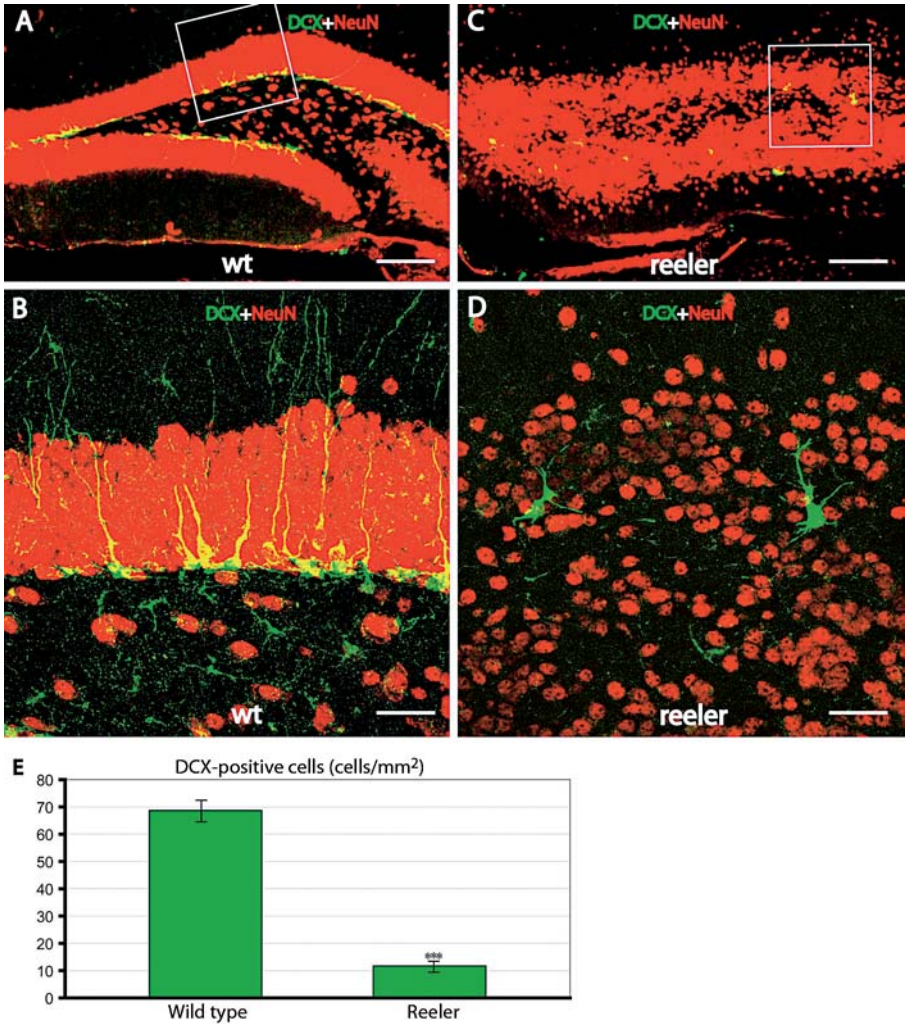

Fig. 3. Double labeling for DCX and NeuN to label newly generated neurons in the wild type (wt) and reeler dentate gyrus. **A**, **B** In the wild type, numerous DCX-positive, newly generated granule cells are observed in the subgranular zone of the dentate gyrus. Boxed area shown at higher magnification in **B** to illustrate DCX-positive dendrites traversing the granular layer. **C**, **D** Double labeling for DCX and NeuN in the reeler dentate gyrus. A few DCX-positive cells are found scattered all over the dentate gyrus. Boxed area shown at higher magnification in **D**. DCX-positive cells give rise to processes oriented into all directions. Scale bars: **A**, **C** 100 μm; **B**, **D** 25 μm. **E** Quantitative estimation of the number of DCX-positive, newly generated neurons/mm^2 dentate gyrus. There are significantly less DCX-positive cells in the reeler dentate gyrus (mean ± SEM, *** $p < 0.001$).

all over the dentate gyrus and appeared to be reduced in number (fig. 3C). At higher magnification, these newly generated neurons in the reeler dentate gyrus were also found to give rise to long processes which, however, did not show a preferential orientation (fig. 3D). A quantitative estimation of the number of DCX-positive cells in both wild-type animals and reeler mutants revealed a significant decrease in DCX-positive cells in the reeler dentate gyrus ($p < 0.001$; fig. 3E). Whereas 68.5 ± 7.6 DCX-positive neurons were counted in the dentate gyrus of wild-type animals, there were only 11.9 ± 2.1 DCX-positive cells in the dentate gyrus of reeler mutants. These studies using a different approach to demonstrate newly generated neurons confirm the reduced neurogenesis in reeler mutants as observed by labeling with BrdU.

Discussion

The present study has provided evidence for a role of reelin in cell proliferation and cell fate decision in the hippocampus. In adult reeler mutants, there was a reduction in cell proliferation in the dentate gyrus, paralleled by a reduction in hippocampal weight. Double labeling with neuronal and glial markers revealed a dramatic reduction in neurogenesis and an increase in gliogenesis. Reduced neurogenesis in the reeler mutant was confirmed by immunostaining for DCX, a marker of newly generated neurons. These findings indicate a hitherto unknown function of reelin, a role in the balance between neurogenesis and gliogenesis.

The mouse mutant reeler has been known for more than half a century [Falconer, 1951] and has been ana-

lyzed in numerous studies. Early reports on changes in the hippocampus noticed a reduced cell number in the dentate gyrus [Stanfield and Cowan, 1979] which could not be confirmed in more recent quantitative studies [Coulin et al., 2001]. Clearly, the reeler mutation is dominated by migration defects mainly in the neocortex, cerebellum and hippocampus, and minor changes in cell number are difficult to document in view of these severe tissue alterations. In the present study, we chose another way by looking at cell proliferation in the adult dentate gyrus, a region known for its persisting postnatal neurogenesis [Altman and Das, 1965; Kaplan and Hinds, 1977; Kuhn et al., 1996; van Praag et al., 2002]. With this approach, we could study the function of reelin in cell proliferation by monitoring the relatively few postnatally generated cells and not by estimating cell numbers. Similar to a recent study by Kim et al. [2001], we found cell proliferation in the adult dentate gyrus to be reduced in the absence of reelin, suggesting a role of this extracellular matrix protein not only in neuronal migration but also cell proliferation. However, postnatal neurogenesis in the dentate gyrus takes place in an anatomically well-defined region, the subgranular zone. The reeler mutant is characterized by a loss of lamination in the dentate gyrus [Stanfield and Cowan, 1979; Drakew et al., 2002] with the granule cells scattered all over the dentate gyrus and no discernible subgranular zone. It remains to be shown whether reelin directly acts on cell proliferation or whether the observed decreased proliferative activity in the adult reeler dentate gyrus is due to the disruption of its normal structure, particularly its subgranular zone.

We regard it as the main finding of the present study that the dramatic decrease in neurogenesis in the adult reeler dentate gyrus is accompanied by an increase in gliogenesis, i.e., an increase in newly generated GFAP-positive astrocytes. We have previously shown that reelin, in addition to directly acting on migrating neurons, also affects glial cells [Förster et al., 2002; Frotscher et al., 2003; Hartfuss et al., 2003; Weiss et al., 2003]. First, we found molecules of the reelin signaling pathway such as the adapter protein disabled 1 expressed by GFAP-positive radial glial cells [Förster et al., 2002]. Second, in a stripe choice assay, GFAP-positive glial cells clearly preferred reelin stripes when compared to control stripes [Förster et al., 2002; Frotscher et al., 2003]. Third, the radial glial scaffold required for proper neuronal migration is severely altered in the reeler dentate gyrus [Weiss et al., 2003], suggesting that the observed migration defect of the granule cells may at least in part be due to a malformation of the radial glial scaffold [Frotscher et al., 2003]. Fourth, in the absence of reelin, no characteristic, long radial glial fibers are formed by GFAP-positive glial cells in the dentate gyrus, suggesting a premature differentiation of radial glial cells into astrocytes [Förster et al., 2002; Frotscher et al., 2003; Weiss et al., 2003; Zhao et al., 2004]. In fact, in the presence of reelin, for instance by incubating slice cultures of reeler hippocampus with recombinant reelin [Zhao et al., 2004], there is a significant increase in the process length of GFAP-positive fibers [Hartfuss et al., 2003; Zhao et al., 2004]. In addition, in coculture experiments, Zhao et al. [2004] showed that reelin is a positional signal for the orientation of radial glial fibers which grow towards the reelin-containing marginal zone, the outer molecular layer of the dentate gyrus. Taken together, reelin seems to be required for the maintenance of radial glial cells in the dentate gyrus. With decreasing reelin expression by Cajal-Retzius cells after birth, the majority of GFAP-positive radial glial cells transforms into astrocytes, a process that seems to be accelerated in reeler mutants. We hypothesize that the increased gliogenesis observed in the reeler mutants of the present study reflects this process. Moreover, early differentiation of radial glial cells into astrocytes may also underlie the observed dramatic decrease in postnatal neurogenesis in the adult dentate gyrus of reeler mutants. It is firmly established that radial glial cells are precursors of both neurons and astrocytes [del Rio et al., 1991; Malatesta et al., 2000; Noctor et al., 2001], and an enhanced differentiation of radial glial cells into astrocytes may exhaust the pool of precursor cells and create an imbalance between neurogenesis and gliogenesis. Studies are in progress that aim to demonstrate whether or not injection of recombinant reelin into the dentate gyrus of reeler mice or treatment of hippocampal slice cultures from reeler mutants with reelin may increase neurogenesis in the dentate gyrus [Zhao et al., in preparation].

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