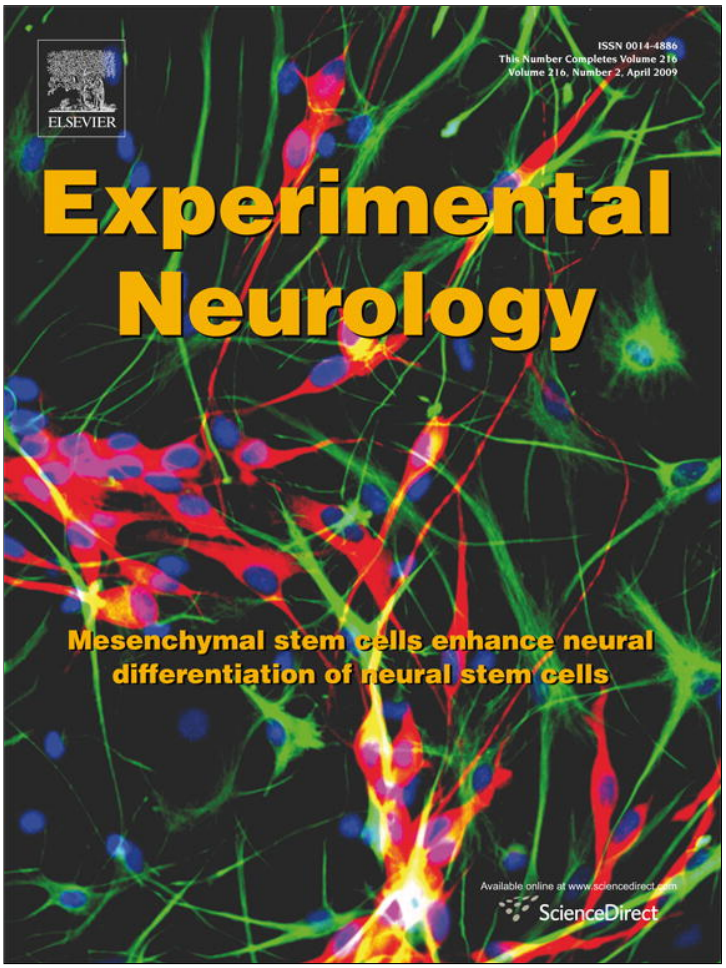

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Exogenous reelin prevents granule cell dispersion in experimental epilepsy

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ABSTRACT

Temporal lobe epilepsy (TLE) is often accompanied by granule cell dispersion (GCD), a migration defect of granule cells in the dentate gyrus. We have previously shown that a decrease in the expression of reelin, an extracellular matrix protein important for neuronal positioning, is associated with the development of GCD in TLE patients. Here, we used unilateral intrahippocampal injection of kainate (KA) in adult mice which is also associated with GCD formation and a decrease of reelin expression. In this mouse epilepsy model we aimed to prevent GCD development by the application of exogenous reelin. As a prerequisite we analyzed whether the reelin signaling transduction cascade was preserved in the KA-injected hippocampus. Using *in situ* hybridization and Western blot analysis we found that the expression of the reelin signaling components, apolipoprotein E receptor 2, the very-low-density lipoprotein receptor and the intracellular adaptor protein disabled 1, was maintained in dentate granule cells after KA injection. Next, recombinant reelin was infused into the KA-injected hippocampus by osmotic minipumps over a period of 2 weeks. Quantitative analysis of granule cell layer width revealed a significant reduction of GCD in reelin-treated, but not in saline-infused animals when compared to KA injection alone. Our findings highlight the crucial role of reelin for the maintenance of granule cell lamination in the dentate gyrus of adult mice and show that a reelin deficiency is causally involved in GCD development.

Introduction

Cortical neuronal migration defects are often associated with the occurrence of epilepsy (Palmini et al., 1991; Gleeson and Walsh, 2000). Also in temporal lobe epilepsy (TLE), a migration defect of dentate granule cells, termed granule cell dispersion (GCD), is frequently observed (Houser, 1990). The extracellular matrix protein reelin has been suggested as one candidate molecule critically involved in the development of GCD, since the reelin-deficient *reeler* mouse (D'Arcangelo et al., 1995; Hirotsune et al., 1995) shows a similar granule cell migration defect (Rakic and Caviness, 1995; Frotscher et al., 2003). In fact, a reelin deficiency was found in resected hippocampi from TLE patients where a decreased expression of reelin mRNA by hippocampal Cajal–Retzius cells correlated with the extent of GCD (Haas et al., 2002). Also after unilateral intrahippocampal kainate (KA) injection in adult mice, an animal epilepsy model, GCD develops and correlates temporally with a decrease of reelin expression (Heinrich et al., 2006).

During normal brain development reelin is synthesized and secreted by Cajal–Retzius cells in the marginal zones of the neocortex and hippocampus where it provides a positional cue for migrating neurons, thus regulating layer formation (for review see Tissir and Goffinet 2003; Förster et al., 2006). The reelin signaling pathway involves two reelin receptors, apolipoprotein E receptor 2 (ApoER2), the very-low-density lipoprotein receptor (VLDLR), and the intracellular adaptor protein disabled 1 (dab1; Howell et al., 1997; Sheldon et al., 1997; Trommsdorff et al., 1999; D'Arcangelo et al., 1999). Mouse mutants deficient in these molecules also show granule cell migration defects. Recent evidence suggests that reelin is not only important during development, but also in the adult hippocampus for the maintenance of lamination. *In vivo* neutralization of endogenous reelin by infusion of the function-blocking CR-50 antibody resulted in a loss of granule cell lamination in naïve mice (Heinrich et al., 2006).

Based on these findings we aimed to rescue GCD formation by chronic infusion of recombinant reelin into the hippocampi of KA-injected mice. As a first step we investigated the mRNA and protein expression of the reelin signaling components after KA injection to ensure their existence during the infusion of reelin. Second, we induced GCD experimentally by intrahippocampal KA injection in adult mice and infused recombinant reelin in the same animals over a period of 14 days. We present evidence that the reelin signaling pathway is preserved in the epileptic mouse hippocampus and that exogenous application of reelin prevents GCD formation.

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Materials and methods

Animals

Experiments were performed on adult C57Bl/6NCrl male mice (8–10 weeks of age; animal facility of the University Hospital Freiburg). After surgery (see below), mice were housed in individual cages with food and water *ad libitum* and kept in a 12 h light/dark cycle (room temperature 22±1 °C). All animal procedures were carried out in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Animal surgery

Unilateral, intrahippocampal KA injections were performed as described elsewhere (Heinrich et al., 2006). In brief, mice were anaesthetized with a mixture of ketamine (100 mg/kg body weight, i. p.), xylazine (5 mg/kg body weight, i. p.) and atropine (0.1 mg/kg) in 0.9% NaCl and placed in a stereotaxic frame in flat skull position. 50 nL (1 nmol) kainic acid solution (KA; Sigma) in 0.9% sterile NaCl (saline) were stereotaxically injected into the right dorsal hippocampus (AP=−1.9, ML=−1.5, DV=−1.9 mm, with bregma as reference) over a period of 60 s using a micro-pump (CMA/100, Carnegie Medicine, Stockholm, Sweden) operating a 0.5 μL micro-syringe (Hamilton, Bonaduz, Switzerland). After recovery from anesthesia the animals were kept under observation for 8–10 h after KA injection. During this period the animals experienced a status epilepticus characterized by mild clonic movements of the forelimbs, rotations and immobility as previously described (Riban et al., 2002). This characteristic behavior has been shown to be associated with seizure activity consisting of bursts of spikes and discharges of spikes, poly-spikes and spike-and-waves recordable in both cortices and hippocampi that lasted at most for 24 h. After this period, the animals had recovered and no behavioral impairment was observed. Only mice showing this characteristic behavioral pattern after KA injection were kept for further analysis.

One day after the initial status epilepticus, KA-injected mice were divided into three experimental groups (group 1: KA − only, $n=4$; group 2: KA+ saline, $n=4$; group 3: KA+ reelin; $n=5$). Mice of groups 2 and 3 were anaesthetized again for the implantation of minipumps. A brain infusion cannula (ALZET Brain Infusion Kit 3, Durect Corp., Cupertino, CA) was stereotaxically placed into the right hippocampus (AP=−1.9, ML=−1.5, DV=−1.5 mm) and fixed to the skull with dental cement. The osmotic pump (Alzet, volume 200 μl, flow rate 0.5 μl/h, duration of infusion: 2 weeks) was implanted subcutaneously on the back of the animal and was connected via catheter tubing with the infusion cannula. The pumps contained either purified recombinant reelin (5 μg/ml in 0.9% saline) obtained from HEK-293 cells (for details see below) or saline as a negative control. The animals were allowed to survive for 17 days after pump implantation (see Fig. 1).

Another group of animals was subjected to KA injection alone and was allowed to survive for 2 days, 7 days or 14 days. These animals were either killed by decapitation for Western blot analysis ($n=2$, each time point) or they were transcardially perfused for *in situ* hybridization (7 days and 14 days, $n=2$ each; see below).

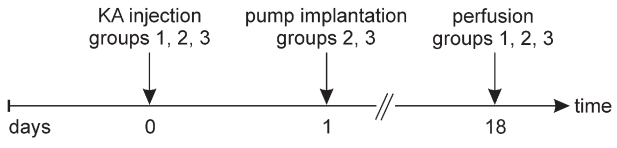

Fig. 1. Schedule for KA injection, pump implantations and experimental groups used in this study.

Perfusion and tissue preparation

Mice were deeply anaesthetized with a mixture of ketamine (200 mg/kg), xylazine (10 mg/kg) and acetylpromazine (0.2 mg/kg) followed by transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 10 min. The brains were post-fixed for 5 h at 4 °C, rinsed in PB overnight, and cut in coronal sections (50 μm) on a vibratome (Leica, VT100S) for immunocytochemistry, or subjected to cryoprotection (20% sucrose overnight at 4 °C) followed by cryostat sectioning (50 μm, coronal plane) for *in situ* hybridization.

In situ hybridization histochemistry

Murine cDNA fragments specific for VLDLR, ApoER2 (Trommsdorff et al., 1999) and dab1 mRNAs (Carroll et al., 2001) cloned either into pCRII-TOPO (Invitrogen, Karlsruhe, Germany) or pGEM (Promega, Mannheim, Germany) vectors were transcribed *in vitro* in the presence of digoxigenin (DIG)-labeled nucleotides. cRNA probes were used for *in situ* hybridization as described by Heinrich et al. (2006).

Western blot analysis

After sacrifice, brains were removed from the skull, the hippocampi were resected and divided in a dorsal and ventral portion. Only the dorsal parts of both hippocampi were homogenized in RIPA-lysis-buffer in the presence of protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA), since mainly the dorsal hippocampus is affected by the kainate injection (Heinrich et al., 2006). Homogenates were centrifuged at 15,000 *g* for 10 min at 4 °C and the supernatants were collected. Total protein content was determined in each supernatant using the Bicinchoninic Acid (BCA) protein assay kit (Pierce, Rockford, USA) following the manufacturer's instructions. Briefly, samples were mixed with the BCA working reagent and incubated for 30 min at 37 °C. Absorbance was measured at 562 nm, and in each sample the protein concentration was determined by comparison with a bovine serum albumin standard curve.

Prior to electrophoresis samples containing equal amounts of total protein (22.5 μg), 1% lithium dodecyl sulfate and 1% dithiothreitol were denatured at 70 °C for 10 min, cooled on ice, and loaded on 4–12% Bis–Tris polyacrylamide gels (NuPAGE, Invitrogen). Electrophoresis was performed in the presence of 50 mM, pH 7.7, SDS-MOPS (3-morpholinopropane-1-sulfonic acid) running buffer (according to the manufacturer's recommendations) for 60 min at 200 V. After size-fractionation gels were blotted by electrophoretic transfer on polyvinylidene difluoride (PVDF) membranes (Roche, Mannheim Germany).

For immunodetection PVDF membranes were pre-treated with I-Block buffer (1 h at room temperature; Tropix, Bedford, USA) followed by incubation with rabbit polyclonal anti-ApoER2 (1:1000; Trommsdorff et al., 1999) or mouse monoclonal anti-VLDLR (1:200) and goat polyclonal anti-actin (1:500, both from Santa Cruz). Signals were detected by incubation with respective alkaline phosphatase-conjugated secondary antibodies (1:10000, 1 h at room temperature, Tropix) and by enhanced chemiluminescence using CDP Star as a substrate according to the manufacturer's instructions (Tropix). Actin served as control for equal loading. Chemiluminescence signals were captured using the *Chemismart* System (Peqlab Biotechnologies, Erlangen, Germany) equipped with a CCD camera.

Purification and stability of recombinant reelin

In order to compensate the endogenous reelin loss after KA injection, biologically active, recombinant reelin, which had been successfully used previously in *in vitro* assays (Förster et al., 2002; Bock et al., 2003; Frotscher et al., 2003), was obtained as follows: reelin-containing supernatants of reelin-transfected HEK-293 cells

were 10× concentrated using the Stirred Ultrafiltration Cell (Millipore, Schwalbach, Germany). For further purification, these concentrated supernatants were centrifuged at 20,000 *g* for 2 h. The resulting pellet was washed and re-suspended. The concentration of purified reelin was estimated by the BCA method (500 μg/ml).

In order to test the thermal stability of recombinant reelin, a pre-requisite for its structural integrity during the 2 week infusion time via osmotic minipumps, we incubated reelin samples at 37 °C for the same period of time. Subsequently, Western blot analysis was performed of recombinant reelin stored at −70 °C or incubated at 37 °C for 2 weeks. Reelin samples (400 ng protein sample; concentration: 22 μg/mL) were subjected to SDS-PAGE (3–8% Tris/Acetate gels) and Western blot analysis as described above, except that mouse monoclonal anti-reelin (1:1000; Chemicon Int., Temecula, CA, USA) was used for detection. Reelin signals were digitalized as described above.

Immunocytochemistry

Immunocytochemistry was performed on tissue sections using a free-floating standard protocol. Primary antibodies were as follows: rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, 1:500, DAKO, Glostrup, Denmark), mouse monoclonal anti-NeuN (1:100,

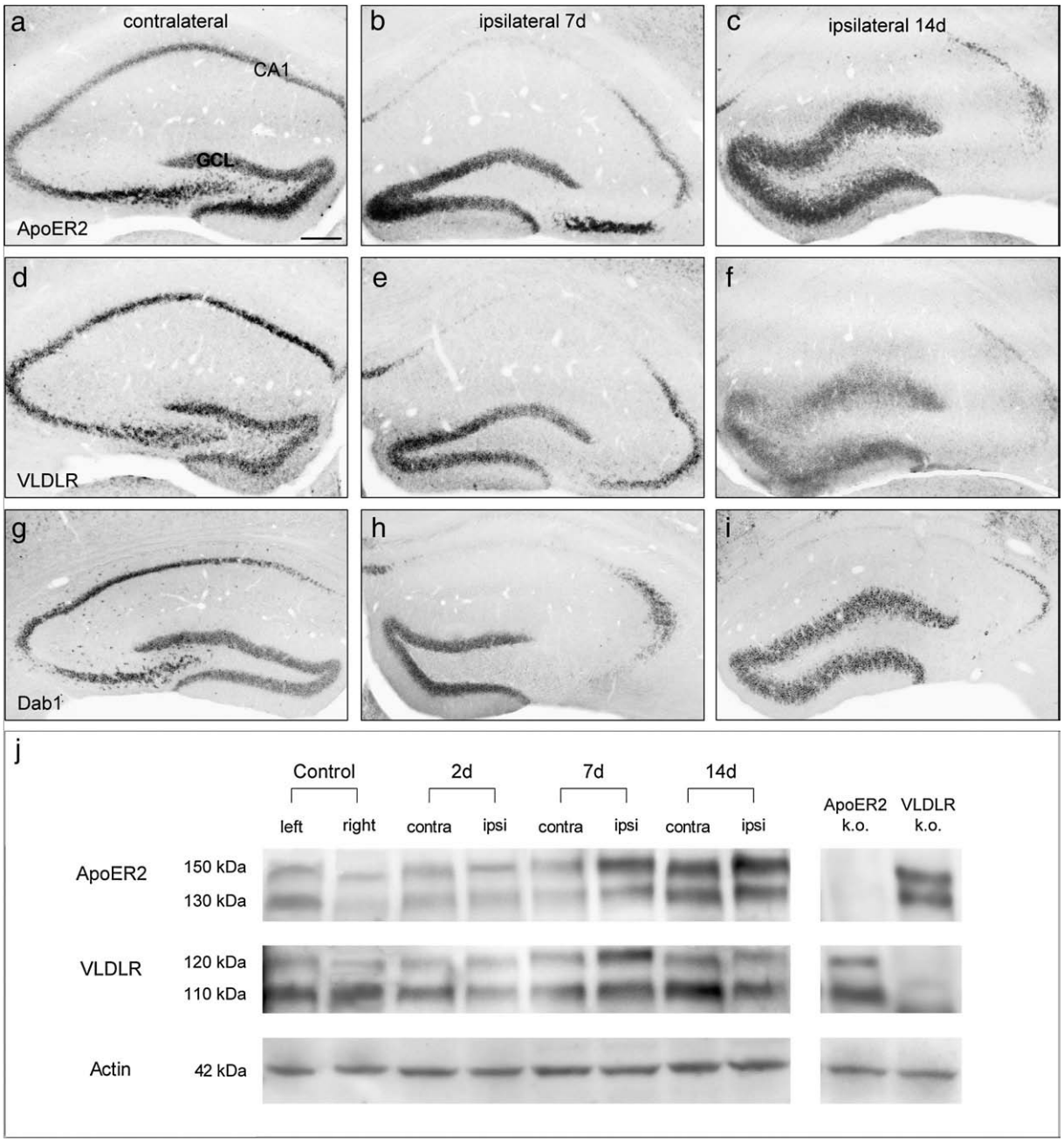

Fig. 2. Expression of reelin signaling components in the KA-injected hippocampus. *In situ* hybridization for ApoER2 (a–c), VLDLR (d–f) and dab1 (g–i) mRNAs in the contralateral (a, d, g) and ipsilateral hippocampus at seven (b, e, h) and 14 days (c, f, i) post injection. In the contralateral hippocampus all three mRNA species are expressed in pyramidal cells of the cornu ammonis, in granule cells of the dentate gyrus and in the hilus (a, d, g). The expression of ApoER2, VLDLR and dab1 mRNAs is maintained in granule cells of the ipsilateral dentate gyrus and in CA2 at both time points after KA injection. Note the loss of labeling in CA1 and hilus due to selective cell death in these regions (b, c, e, f, h, i). GCD becomes apparent at 2 weeks after KA injection as seen in c, f, i. j. Western blot analysis of ApoER2 and VLDLR protein expression in hippocampal extracts at 2 days, 7 days, and 14 days after KA injection. The same membrane was sequentially incubated with ApoER2, VLDLR and Actin (as loading control) antibodies. Similar levels of VLDLR and ApoER2 can be observed in control hippocampi and at all postlesional time points with slight differences at 2 days and 7 days post KA. Molecular weight is shown in kilodalton. Extracts from ApoER2 or VLDLR knockout mice show no signal at the respective molecular weight of the deleted receptor. GCL, granule cell layer; CA1, cornu ammonis. Scale bar: 200 μm.

Chemicon Int.), goat polyclonal anti-doublecortin (DCX, 1:500, sc-8066, Santa Cruz), and mouse monoclonal anti-reelin (G10, 1:1000, a gift of Dr. A. Goffinet, Center for Neurosciences, Brussels, Belgium). After washing in PB, sections were incubated with appropriate secondary antibodies conjugated with Cy™3 or Cy™2 (1:200, 3 h at room temperature; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or Alexa Fluor-488 (Molecular Probes, Eugene, OR, USA) followed by washing in PBS for 1.5 h, mounting on gelatin-coated slides and coverslipping.

Cresyl violet staining

In order to analyze the width of the granule cell layer serial coronal sections of the dorsal hippocampus were subjected to Nissl staining. Tissue sections were immersed in 0.1% cresyl violet solution for 10 min, dehydrated in increasing concentrations of ethanol and coverslipped in Hypermount (Thermo Fisher Scientific, Dreieich, Germany).

Measurement of granule cell layer width

To assess the effect of the chronic reelin infusion, width of the granule cell layer was determined at 18 days after KA injection in all three experimental groups. We chose the area of maximal GCD in the dorsal part of the injected hippocampus and measured the width of the granule cell layer in 5 consecutive sections/animal in this region. In each section, measurements were taken in 100 μm intervals along the entire superior and inferior blade of the dentate gyrus using AxioVision software (Carl Zeiss). Measurements were performed blinded and by two observers independently.

Statistical analysis

Averaged values of GCL width per animal were subjected to a nonparametric Kruskal–Wallis one-way analysis of variance. Differences in the average GCL width were then assessed by a post hoc analysis using a Dunnett's multiple comparison test between the reelin-infused and the saline-infused mice. Statistical analysis was performed with GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when the p-value was <0.05.

Results

Expression of reelin signaling components in the KA-injected hippocampus

Since an effect of exogenously applied reelin on dentate granule cells requires an intact signaling machinery we performed *in situ* hybridization for ApoER2, VLDLR, and dab1 mRNAs on hippocampal tissue sections at 7 days and 14 days after KA injection, the time period of GCD formation.

In the contralateral, non-injected hippocampus all three reelin signaling components were expressed in pyramidal cells of the cornu ammonis, in granule cells of the dentate gyrus and in hilar neurons (Figs. 2a, d, g). On the ipsilateral side, ApoER2, VLDLR, and dab1 mRNA expression was maintained in granule cells and surviving pyramidal cells in CA2 at 7 days (Figs. 2b, e, h) and 14 days (Figs. 2c, f, i) after KA injection. After 7 days KA-induced cell death in CA1 and hilus was apparent, but GCD had not yet fully developed. At 14 days after KA injection GCD was obvious with dispersed granule cells expressing all three reelin signaling components.

To study reelin receptor expression on the protein level we performed Western blot analysis of extracts from micro-dissected hippocampi at several time points (2 days, 7 days, 14 days) after KA injection. During the whole time period investigated, VLDLR and ApoER2 receptors were expressed in the contra- and ipsilateral hippocampi (Fig. 2j). At 2 days after KA injection, VLDLR expression appeared slightly reduced on the ipsilateral side when compared to the contralateral side. In contrast, expression levels of both receptors were elevated on the KA-injected side at 7 days post KA. Western blot analysis of forebrain extracts obtained from adult VLDLR or ApoER2 knockout mice were devoid of the corresponding VLDLR or ApoER2 signal, thus proofing the specificity of both antibodies (Fig. 2j).

Taken together, we show on the mRNA and protein level that expression of the reelin signaling components was maintained in the dentate gyrus after KA injection, an important prerequisite for reelin infusion.

Control experiments

Recombinant reelin synthesized by *reelin*-transfected HEK 293 cells is well characterized and has been shown to be an effective inducer of reelin signaling in a number of studies (Förster et al., 2002; Bock et al., 2003; Frotscher et al., 2003). For our *in vivo* infusion experiments we tested whether recombinant reelin was stable under the actual experimental conditions (exposure to 37 °C body temperature inside the minipump over 2 weeks). To this end we performed Western blot analysis of recombinant reelin stored either at −70 °C or at 37 °C for 2 weeks using the monoclonal G10 reelin antibody for detection. Reelin secreted by HEK 293 cells consists of several isoforms (400, 320 and 180 kDa) (Frotscher et al., 2003). We found the same isoform pattern of the three reelin fragments under both experimental conditions (Fig. 3) indicating that there was no obvious degradation of reelin during a long-term incubation at 37 °C.

Next, we wanted to verify that the recombinant reelin infused by osmotic pumps indeed reached the KA-injected hippocampus. To this end, we performed intrahippocampal KA injection combined with reelin infusion for 2 weeks and performed immunolabeling for reelin at 18 days after KA. As shown in Fig. 4a, strong immunofluorescence was observed in the region of the infusion tract extending from the cortex down to the hippocampus, showing the presence of infused reelin in the brain parenchyma. In addition, many reelin-immunolabeled neurons were detected in the contralateral cortex and hippocampus (Figs. 4a, b) showing the specificity of the reelin-immunolabeling. In the ipsilateral hippocampus, there was a diffuse immunolabeling for reelin (Fig. 4a), but only a few reelin-positive cells (Fig. 4c) showing the lack of endogenous reelin on the lesioned side.

As a further control experiment, we wanted to exclude potential effects of the continuous reelin infusion on changes known to be

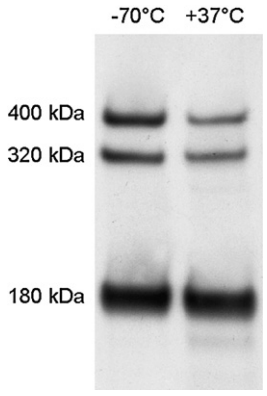

Fig. 3. Thermal stability of reelin. Western blot analysis of recombinant reelin stored either at −70 °C or at 37 °C for 2 weeks. All three characteristic reelin fragments (400, 320 and 180 kDa) were detected under both experimental conditions indicating that recombinant reelin was stable after incubation at 37 °C for 2 weeks.

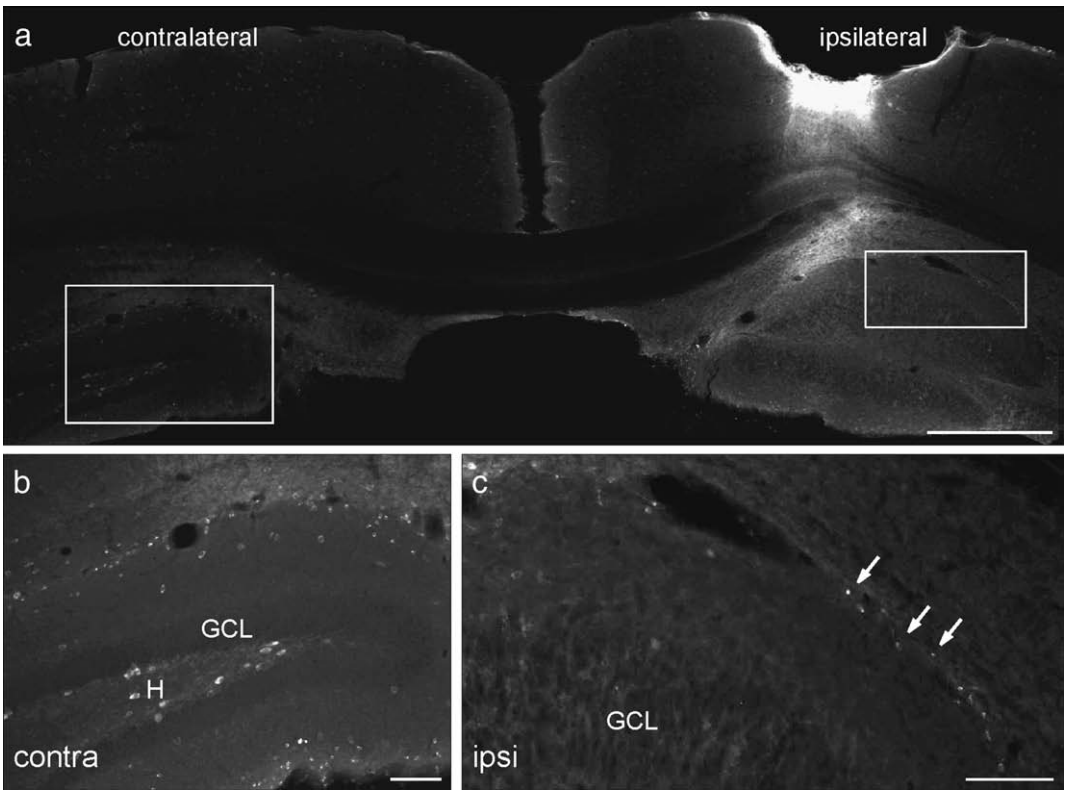

Fig. 4. Presence of recombinant reelin at the infusion site. (a) Coronal section of a reelin-infused mouse brain at the level of the infusion site. Reelin was detected by immunocytochemistry with the G10 antibody. The ipsilateral side shows a cortical injury in the area where the cannula was located. In the cortical region directly beneath the injection site and in the hippocampus strong immunofluorescence shows the diffusion gradient of the delivered reelin. In addition, many reelin-immunopositive cells can be detected in the contralateral cortex and hippocampus. (b) Higher magnification of the left framed hippocampal portion in a. Reelin is mainly expressed in the hilus and at the hippocampal fissure. (c) Higher magnification of the framed area in the ipsilateral, reelin-infused side in a. Reelin-immunopositive cells are maintained only at the hippocampal fissure (arrows). GCL, granule cell layer; H, hilus. Scale bars: a, 500 μm; b–c, 100 μm.

caused by intrahippocampal KA injection such as selective cell death in CA1, CA3 and hilus, loss of neurogenesis and gliosis (Bouilleret et al., 1999; 2000; Kralic et al., 2005; Heinrich et al., 2006). To address this question we performed intrahippocampal KA injection combined with reelin or saline infusion for 2 weeks and subsequently analyzed tissue sections of these animals by cresyl violet staining and immunolabeling for DCX or GFAP, markers for newly generated neurons and astrocytes, respectively (Fig. 5). In both groups, we observed severe cell death in CA1, CA3 and hilus (Figs. 5b, c) and a complete loss of DCX-immunolabeling in the subgranular zone of the dentate gyrus (Figs. 5e, f). In addition, a GFAP-positive glial scaffold was detected in the region of GCD in both experimental groups (Figs. 5h, i). The described changes occurred only in the ipsilateral, but not in the contralateral hippocampus (Figs. 5a, d, g). These results show that reelin and saline infusions do not interfere with KA-induced cell death, loss of neurogenesis or the development of a glial scaffold previously reported in this model.

Significant reduction of GCD by chronic reelin infusion

Intrahippocampal KA injection leads to a progressive development of GCD within 2 weeks (see also Fig. 2) accompanied by a strong reduction of the number of reelin mRNA-expressing cells (Heinrich et al., 2006). With the aim to compensate the endogenous reelin deficiency we infused recombinant reelin into the KA-injected hippocampus for a period of 2 weeks during the time of GCD formation. We analyzed the effect of exogenous reelin infusion on GCD development by measuring the width of the granule cell layer in cresyl violet-stained hippocampal sections of KA+ reelin-infused mice and compared the results to two control groups (KA+ saline, KA − only) at 18 days after KA injection. Immunolabeling for reelin confirmed that endogenous reelin synthesis was decreased in the ipsilateral hippocampus of these animals (see Fig. 4).

Infusion of reelin over a period of 2 weeks resulted in a significant reduction of granule cell layer width (140 ± 3.0 μm, Figs. 6a, b, f) when compared to the saline-infused control group (157 ± 3.7 μm; Figs. 6c, f). Reduction of GCD in KA+ reelin-animals was most pronounced in the proximity of the infusion tract (Fig. 6 b) indicating that the effect of reelin was limited by diffusion (see also Fig. 4).

In accordance with the reduction of GCD, we observed a higher density of granule cells in reelin-infused hippocampi when compared to saline-infused animals where granule cells were separated by enlarged intercellular spaces (Figs. 6d, e). The mean width of the granule cell layer in saline-infused animals was almost identical to that of KA − only mice (159 ± 2.5 μm) indicating that the inhibitory effect of reelin on GCD formation was specific (Fig. 6f).

Discussion

In this study we show for the first time, that *in vivo* application of exogenous reelin significantly attenuates GCD formation in the adult, epileptic mouse hippocampus. This result demonstrates that a reelin loss is causally involved in the TLE-associated widening of the granule cell layer and that reelin is important for the maintenance of granule cell positioning in the adult brain.

We have previously shown that the development of GCD is correlated with a loss of reelin synthesis in human TLE patients (Haas et al., 2002) and that a decrease of reelin expression coincides with

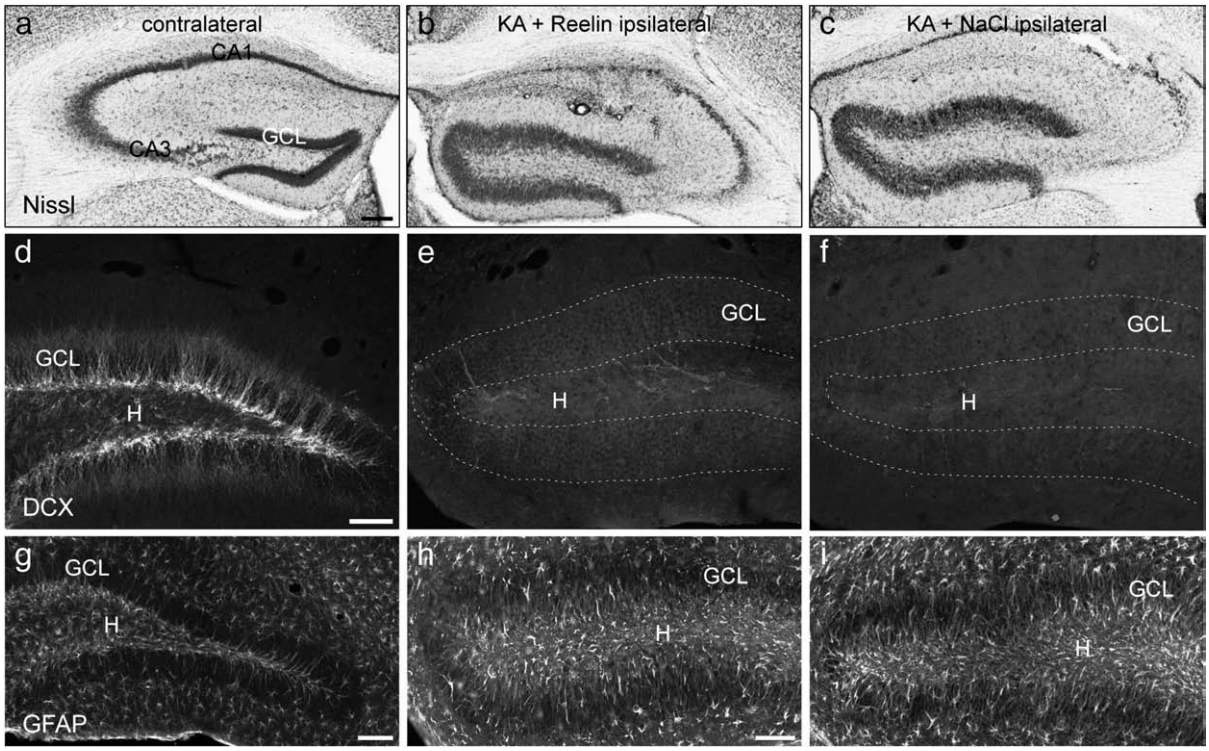

Fig. 5. KA-induced cell death, loss of neurogenesis and gliosis are untouched by reelin or saline infusion. Animals were injected with KA followed by a 2 week reelin infusion (contralateral side: a, d, g; ipsilateral side: b, e, h) or saline infusion (c, f, i) and were sacrificed 18 days postlesion. (a–c) Cresyl violet staining. The characteristic KA-induced cell death pattern is detectable in the reelin- (b) and saline- (c) infused hippocampi. d–f. Immunolabeling for DCX. Many DCX-immunopositive cells are present in the subgranular layer of the contralateral dentate gyrus (d). No DCX-immunoreactivity can be observed in the ipsilateral dentate after KA+ reelin (e) or KA+ saline (f). g–i. Immunolabeling for GFAP. On the contralateral side, GFAP-immunopositive astrocytes are evenly distributed in the dentate gyrus (g). Portion of the ipsilateral dentate gyrus after KA+ reelin (h) or KA+ saline (i). Note the increase of GFAP immunoreactivity and radially orientated GFAP-positive fibers in the granule cell layer under both experimental conditions. GCL, granule cell layer; H, hilus. Scale bars: a–c, 200 μm; d–i, 100 μm.

GCD development after intrahippocampal KA injection (Heinrich et al., 2006). In addition, a local GCD-like phenotype occurs after the infusion of the reelin-neutralizing antibodies into the hippocampus of naïve mice (Heinrich et al., 2006). Hence, we hypothesized that decreased reelin levels in the epileptic hippocampus may be causally involved in the malpositioning of dentate granule cells resulting in GCD. Here we provide strong evidence that this is really the case, while the prerequisites for the action of reelin (i.e. the components of the signaling pathway) are not affected by KA injection. We infused recombinant reelin into the hippocampus during the first 2 weeks after KA injection, which is the time window of GCD formation. We found a significant reduction of granule cell layer width in mice that had received recombinant reelin, but not in saline-infused mice. Thus, we could compensate for the endogenous reelin loss and significantly inhibit GCD formation.

We performed a number of control experiments to ensure specificity. (1) Western blot analysis demonstrated the stability of recombinant reelin incubated at 37 °C for 2 weeks, since the reelin isoforms (400, 320 and 180 kDa) were preserved under these conditions. Thus, we are confident that reelin, contained in osmotic minipumps at body temperature for 2 weeks, was thermally stable and not degraded. (2) We proved by immunolabeling for reelin that recombinant, infused reelin had indeed reached the hippocampus and was still present 3 days after the end of the infusion period. These results further indicate that exogenously applied reelin is rather stable. (3) We showed that the pump implantation following KA injection did not influence major neuropathological changes known to reliably occur after intrahippocampal KA injection: in the ipsilateral hippocampi of reelin- and saline-infused animals segmental cell death in CA1, CA3 and hilus was present, DCX-immunolabeled neurons disappeared and a glial scaffold developed in the area of GCD as described previously (Bouilleret et al., 1999, 2000; Kralic et al., 2005; Heinrich et al., 2006). Thus, the effects of KA injection were not attenuated by pump implantations following the initial status epilepticus. The only difference was the reduction of GCD in the KA+ reelin animals ensuring the specificity of the reelin effect.

Expression of reelin receptors on dentate granule cells is an important prerequisite for rescue of granule cell lamination. Binding to VLDLR and ApoER2 and activation of an intracellular signaling cascade has been shown to be necessary for correct positioning of neurons during the development of laminated structures such as the neocortex, hippocampus and cerebellum (for review see Herz and Bock, 2002; Tissir and Goffinet, 2003; Förster et al., 2006). Dentate granule cells have been shown to express reelin signaling components (Haas et al., 2002; Niu et al., 2004). Here we show that dentate granule cells express VLDLR, ApoER2 and dab1 mRNAs and that the protein levels of VLDLR and ApoER2 are maintained after KA injection. Thus, although endogenous reelin levels drop after KA injection, dispersed granule cells appear to maintain their ability to respond to the application of exogenous reelin.

Despite the presence of reelin receptors on dentate granule cells during the infusion period, only a partial, but significant rescue of GCD was achieved by infusion of exogenous reelin. One reason could be that infused reelin reached not all granule cells due to limited diffusion. The infusion cannula was placed close to the hippocampal fissure implying that the infused reelin had to diffuse through the molecular layer to reach the granule cells and their dendrites, respectively. Indeed, the rescue effect was mainly restricted to the outer portion of both granule cell layers beneath the infusion site indicating that reelin diffusion was limited (see also Fig. 4a). It is not known how far the different reelin isoforms can diffuse in intact brain tissue. Jossin et al. (2007) proposed that the 400 kDa full-length reelin

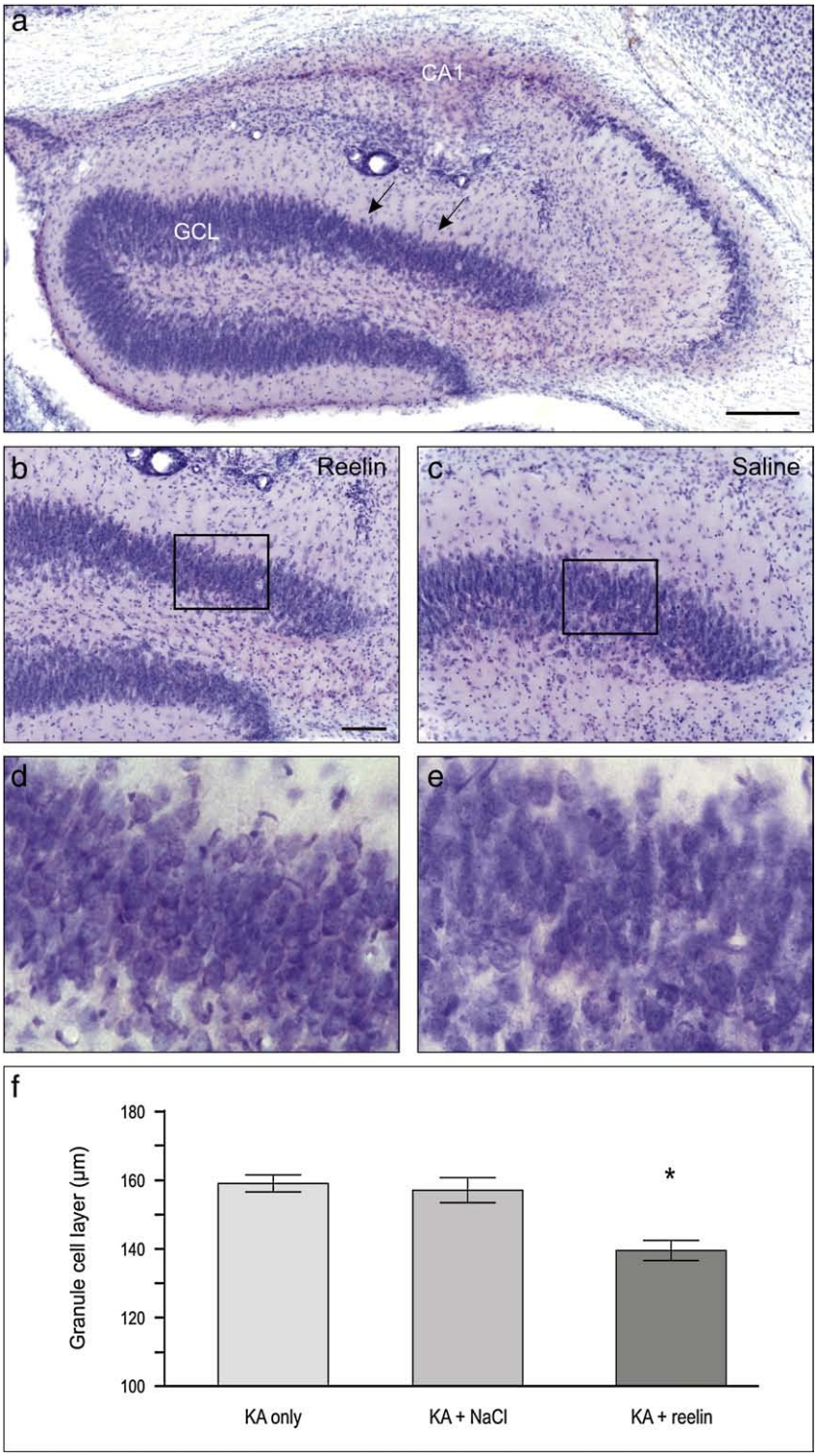

Fig. 6. Chronic infusion of recombinant reelin reduces GCD in the KA-injected hippocampus. GCL width was measured in cresyl violet-stained sections of KA+ reelin, KA+ saline and KA − only animals as described in Materials and methods. (a) Representative section of a KA+ reelin-infused hippocampus. Note reduced width of GCL at the outer portion of the suprapyramidal blade of the dentate granule cell layer (arrows). (b) Higher magnification of the outer portion of the GCL shown in a. Note reduction of GCL width in the region below the infusion cannula tract. (c) Corresponding region below the infusion cannula tract in a saline-infused hippocampus. The GCL is strongly dispersed. (d) High magnification of the framed area in b. Cellular density appears higher in reelin-infused animals compared to saline-infused mice (e). (e) High magnification of the framed area in c. Granule cells are loosely distributed and separated by intercellular spaces. (f) Histograms showing mean GCL width in KA-injected hippocampi of KA − only ($n=4$), KA+ saline ($n=4$) and KA+ reelin ($n=5$) mice. Mean values (±SEM) are shown. GCD is significantly reduced by reelin infusion ($p<0.05$). GCL, granule cell layer; H, hilus. Scale bars: a, 200 µm; b, c, 100 µm; d, e, 25 µm.

cannot diffuse freely, it rather remains anchored to the extracellular matrix until cleavage by metalloproteases secreted by migrating neurons. Only after proteolytic processing the central reelin fragment is released and can bind to reelin receptors (Jossin et al., 2007). We can only speculate, whether this mechanism described for the development of cortical layers holds also true for the adult KA-injected hippocampus. In case diffusion and processing of the 400 kDa reelin was impaired in our study, one would assume that the 320 kDa reelin fragment was still available and obviously potent enough to prevent the formation of GCD in a discrete area.

GCD is a process affecting differentiated granule cells, since neurogenesis is lost and replaced by gliogenesis in the dentate gyrus after intrahippocampal KA injection (Kralic et al., 2005; Heinrich et al., 2006). Hence in our study exogenously applied reelin conveys positional information to adult dentate granule cells which is an interesting new finding. Former studies which reported a role for reelin in neuronal positioning concerned young migrating neurons during the process of layer formation (Zhao et al., 2004; Gong et al., 2007). Also in our previous study, in which infusion of reelin-neutralizing antibodies into the hippocampus of naïve mice produced a GCD-like phenotype (Heinrich et al., 2006), migration of newly-generated granule cells probably contributed to the expansion of the granule cell layer. Since ongoing dentate neurogenesis takes place in the subgranular zone of adult mice and newly-born neurons integrate in the granule cell layer (for review see Kempermann et al., 2004), the local reelin neutralization most likely led to an uncontrolled migration of newly formed granule cells.

In view of the present results, we suggest that reelin is not only important for the formation but also for the maintenance of granule cell lamination in the adult hippocampus. During development reelin controls granule cell lamination by acting as a positional signal for migrating neurons (e.g. Zhao et al., 2004). The present study provides evidence that reelin also acts as a positional cue for adult granule cells since they disperse after KA-induced loss of reelin, but stay in position when reelin is supplied.

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