Reelin Signaling Facilitates Maturation of CA1 Glutamatergic Synapses

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Qiu S, Weeber EJ. Reelin signaling facilitates maturation of CA1 glutamatergic synapses. J Neurophysiol 97: 2312–2321, 2007. First published January 17, 2007; doi:10.1152/jn.00869.2006. Reelin signaling through the low-density lipoprotein receptor family members, apolipoprotein E receptor 2 (apoER2) and very-low-density lipoprotein receptor (VLDLR), plays a pivotal role in dictating neuronal laminating during embryonic brain development. Recent evidence suggests that this signaling system also plays a role in the postnatal brain to modulate synaptic transmission, plasticity, and cognitive behavior, mostly likely due to a functional coupling with N-methyl-D-aspartate receptor (NMDAR) subunit NR1 and AMPAR subunit GluR1 clustering were increased surface expression of AMPARs in CA1 tissue. In cultured hippocampal slices, reelin treatment increased the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents and the evoked AMPA/NMDA receptor current ratios. In addition, reelin treatment also reduced the number of silent synapses, facilitated a developmental switch from NR2B to NR2A of NMDARs, and increased surface expression of AMPARs in CA1 tissue. In cultured hippocampal neurons from reeler embryos, reduced numbers of AMPAR subunit GluR1 and NMDAR subunit NR1 clustering were observed compared with those obtained from wild-type embryos. Supplemeting reelin in the reeler culture obliterated these genotypic differences. These results demonstrate that reelin- and lipoprotein receptor-mediated signaling may operate during developmental maturation of hippocampal glutamatergic function and thus represent a potential important mechanism for controlling synaptic strength and plasticity in the postnatal hippocampus.

INTRODUCTION

Reelin is a large extracellular matrix protein that is secreted by Cajal-Retzius (CR) cells located in the marginal zone and serves as a molecular guiding cue for the subventricular migrating neurons during development (D’Arcangelo et al. 1995; Del Rio et al. 1997; Frotscher et al. 2003). Reelin binds to two members of the low-density lipoprotein receptor (LDLR) family, apolipoprotein E receptor 2 (apoER2), and very-low-density lipoprotein receptor (VLDLR) and results in phosphorylation of disabled 1 (db1) and activation of Src family protein tyrosine kinases (D’Arcangelo et al. 1999; Hiesberger et al. 1999; Howell et al. 1997, 1999). Recent studies have shown that reelin signaling may also play an important role in the adult brain function. For example, bath perfusion of recombinant reelin onto hippocampal slices enhances tyrosine phosphorylation of N-methyl-D-aspartate receptor (NMDAR) subunits, increases NMDAR-mediated whole cell currents, and elevates the magnitude of long-term potentiation (Beffert et al. 2005; Chen et al. 2005; Weeber et al. 2002). On the other hand, genetic ablation of either apoER2 or VLDLR leads to impaired memory function in mice (Weeber et al. 2002).

It has been recently shown that reelin is required for normal development of dendritic structures (Niu et al. 2004), maturation of somatic NMDARs (Sinagra et al. 2005), and modulation of glutamate-induced NMDAR activities (Chen et al. 2005). These findings also raise an interesting question of whether reelin signaling affects maturation of hippocampal glutamatergic function in vivo. It is well established that NMDAR function is dependent on its subunit composition. Two major forms of NR2 subunits in adult hippocampus, NR2A and NR2B, are developmentally regulated (Chavis and Westbrook 2001; Monyer et al. 1994; Sheng et al. 1994), subjected to activity- and behavior-induced changes (Barria and Malinow 2005; Carmignoto and Vicini 1992; Quinlan et al. 2004), and contribute to CA1 synaptic plasticity (Malenka and Nicoll 1993; Nicoll and Malenka 1995). NR2A has faster kinetics and gradually increases during the first several postnatal weeks in mice (Monyer et al. 1994). Moreover, both NR2A and NR2B can be tyrosine phosphorylated in responses to synaptic activity (Lau and Huganir 1995; Lu et al. 1998; Saltar and Kalia 2004; Yu et al. 1997) and reelin signaling (Beffert et al. 2005; Chen et al. 2005). Therefore changes of NMDAR activity through developmental alteration of subunit composition and/or tyrosine phosphorylation by reelin could have a dramatic impact on synaptic strength and plasticity.

The maturation of CA1 synapses is also reflected by an increased AMPAR response at synapses that initially contain only NMDARs (silent synapses) (Isaac et al. 1995; Kullmann 1994; Liao et al. 1995). In addition, CA1 synaptic strength and plasticity could be accomplished by altered AMPAR subunits phosphorylation (Barria et al. 1997; Lee et al. 2000) and/or trafficking to postysaptic sites following synaptic activity (Hayashi et al. 2000; Shi et al. 1999; Song and Huganir 2002). In this study, we provide evidence that reelin-induced signaling facilitates the developmental maturation of hippocampal glutamatergic function in the postnatal hippocampus.

METHODS

Experimental animals

The B6C3Fe strain mice that carry either a single or both alleles of RELN gene were obtained from the Jackson Laboratory. These mice were mated to produce progenies of various genotypes that were determined by PCR, as previously described (Qiu et al. 2005). Mice at postnatal days 6–7 or embryonic days 16–17 were used for slice
cultures and hippocampal neuronal cultures, respectively. Animal care and use protocol was according to National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

**Hippocampal slice cultures and drug treatment**

Hippocampal slice cultures were prepared using the protocol described by Stoppini et al. (1991). Briefly, horizontal slices were made from 6- to 7-day-old litters and were cultured on Millicell culture plate inserts (Millipore, Billerica, MA) in DMEM/F12 medium supplemented with 10% FBS. Drugs were applied after 3 days in culture and replenished every 3 days thereafter. Recombinant reelin was produced by using HEK293 cells that were stably transfected with the full-length reelin construct pCrl vector (D’Arcangelo et al. 1997). The expression of GST-RAP fusion protein was obtained by using DH5α cells carrying the GST-RAP vector construct [provided by Dr. G. Bu (Zhuo et al. 2000)]. The preparation and purification of reelin and GST-RAP; preparation of mock conditioned medium was essentially as previously described (Sinagra et al. 2005). The chemicals including TTX, 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX), D-AP5, and bicuculline were purchased from Tocris-Cookson (Ellisville, MO).

Electrophysiology

The cultured slices were transferred to the recording chamber and perfused with artificial cerebrospinal fluid (ACSF; containing in mM: 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 1.2 MgCl₂, 2.0 CaCl₂, and 10 glucose, pH 7.3–7.4, and bubbled with 95% O₂-5% CO₂) perfused with artificial cerebrospinal fluid (ACSF; containing in mM: 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 1.2 MgCl₂, 2.0 CaCl₂, and 10 glucose, pH 7.3–7.4, and bubbled with 95% O₂-5% CO₂). 20 μM bicuculline was included in the perfusate. Slices were incubated in the recording chamber at room temperature for ≥1.5 h before recording.

Spontaneous or synaptically evoked whole cell responses of CA1 pyramidal neurons were recorded at room temperature (22–24°C) using an EPC10 double amplifier (Heka Elektronik, Germany) with the aid of DIC microscopy (Leica DM-LSFA). The patch electrodes (4–7 MΩ) were filled with a solution containing (in mM) 147 Cs gluconate, 10 tetraethylammonium-Cl, 0.3 NaGTP, and 0.2 EGTA, and adjusted to pH 7.2, 280–290 mOsm. CA1 pyramidal neurons were voltage clamped at −65 mV in whole cell configuration for ≥10 min to achieve stable holding currents. Series resistance (8–15 MΩ) was monitored by applying 5-ms hyperpolarizing voltage steps. Experiments were terminated if serial resistance or input resistance changed by >15%. To measure AMPAR-mediated spontaneous miniature EPSCs (mEPSCs), slices were preincubated with 20 μM bicuculline and 1 μM TTX for 30 min, and recordings were carried out in the continued presence of these chemicals.

Monosynaptically evoked whole cell responses, as verified by their high synchrony across varying stimulus intensities, were recorded at either −65 or +40 mV. Electrical stimulus was driven by a Master-8-cp stimulator (AMPI) and delivered through a bipolar tungsten electrode (Frederick Haer, Bowdoinham, ME) to Schaffer collateral fibers. To obtain NMDAR-mediated whole cell currents, neurons were clamped at +40 mV in the presence of 10 μM CNQX, and the response was confirmed by washing in 100 μM d-AP5 on termination of experiments. Electric signals were digitized either by the EPC10 or Digidata 1322A at 10 kHz and filtered at 2 kHz, stored on a PC computer, and analyzed off-line.

To determine the proportion of silent synapse and synaptic failures of CA1 pyramidal neurons in cultured hippocampal slices, a minimum stimulation protocol was adopted (Liao et al. 1995). CA1 pyramidal neurons were voltage clamped at −65 mV, and stimulus intensity was gradually increased from zero to obtain a minimum stimulus intensity that elicits a mixture of responses and failures. This stimulus intensity was kept constant and synaptic responses were recorded for ≥15 min at 0.2 Hz. Cells were then clamped at +40 mV to record the mixed AMPAR- and NMDAR-mediated EPSCs. d-AP5 (100 μM) was washed in at the end of the experiment to ensure the failure rates of AMPAR-mediated EPSCs at −65 and +40 mV were the same, only those cells showed same failure rates were included in analysis.

**Cell surface protein biotinylation and Western blotting**

Cultured slices treated with reelin or mock for 6–7 days or non-treated controls were biotinylated in ACSF with 1 mg/ml sulfo-NHS-S-S-biotin for 30 min at 4°C, using a Pinpoint cell surface protein isolation kit (Pierce, Rockford, IL), similar to that described previously for biotinylation in slices (Broutman and Baudry 2001). CA1 regions were dissected out and sonicated in RIPA buffer [contains 50 mM Tris–HCl, pH 7.4, 10 mM EDTA, 100 μM leupeptin, 1 mM pepstatin, 10 μg/ml aprotinin, 10 μg/ml bacitracin, 100 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate and 1% Nonidet P40]. Half volume of the tissue lysate was used for probing total proteins and the other half was mixed with immobilized neutravidin beads (Pierce) and rotated for 2 h at 4°C. The beads were then washed three times in RIPA buffer and, along with the total protein fraction, treated with 2X Laemmli buffer supplemented with 50 mM DTT. Both the total protein and biotinylated proteins were separated by SDS-PAGE by running through 9% SDS-polyacrylamide gels. The proteins were then transferred to PVDF membranes (Immobilon-P, Millipore) and incubated with antibodies against Glur1, NR1, NR2A or NR2B diluted in 5% nonfat dry milk and 1% BSA for 2 h at RT. The membranes were then washed extensively in 0.1M PBS-Tween 20 and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies (Promega, Madison, WI) and developed using an enhanced chemiluminescence method. Rabbit anti-NR2A and anti-NR2B antibodies were purchased from Upstate Biotechnology. Rabbit anti-GluR1, anti-NR1 and monoclonal NR1 antibodies were obtained from Chemicon (Temecula, CA). The final dilution of antibodies was between 1:1,000 and 1:5,000. The optical density of immunoreactive bands was quantified by densitometry using Quantity One software (Bio-Rad, Hercules, CA).

**Hippocampal neuronal culture, immunocytochemistry, and image analysis**

Hippocampal neuronal cultures were prepared from 16- to 17-day embryonic mice. Briefly, hippocampi from fetal brains were collected and digested in Hank’s balance salt solution containing 0.5 mg/ml papain at 37°C for 20 min. In the meanwhile, the tails of the embryos were collected for genotype analysis. The hippocampi were then separated into individual cells using a glass micropipette. The cells were seeded on poly-D-lysine-coated 12-mm glass coverslips at a density of 5,000 cells/cm² and grown in Neurobasal medium supplemented with 2% B27. Every 5 days after plating, half of the medium was replaced with fresh plating medium containing 10 μM cytosine arabinoside. At day 14–20, neurons were fixed in 4% paraformaldehyde in PBS for 20 min followed by prechilled (−20°C) methanol for 10 min. The neurons were permeabilized for 20 min in 0.2% Triton-X 100, and were blocked with 0.01M PBS containing 10% normal serum and 1% BSA, incubated overnight with primary antibodies (rabbit anti-GluR1 and mouse anti-NR1, both 1:500 dilutions), followed by a 2-h incubation with Alexa Fluor 488-conjugated donkey anti-rabbit secondary IgG (Invitrogen, Carlsbad, CA) and CY3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA).

Digital images were taken with a Zeiss LSM510 confocal microscope using a 40× oil-immersion lens (numerical aperture: 1.3). All images from the same experiment were acquired with identical settings for laser power, photomultiplier gain, offset and a fixed pinhole size of one Airy unit. Images were encoded to analyze blindly using...
MetaMorph software (Molecular Devices, Sunnyvale, CA). Thresholds for GluR1 and NR1 immunostaining were chosen such that all visually recognizable punctate labeling were included for analysis. For colocalization analysis, regions were defined around the thresholded puncta in one channel (NR1, red), and these regions were used to create a mask and then overlaid on the second channel (GluR1, green). Thresholded puncta defined by at least partially overlapping regions were considered colocalizing. ≥10 neurons were analyzed per group and the sections of 50–100 μm of all the primary dendrites were counted and averaged to determine the number of GluR1, NR1, or colocalized puncta. Each experiment was repeated in four cultures.

**Data analysis**

The amplitude of whole cell EPSCs were defined as the averaged 2–4 ms value around the peak minus the stable holding current immediately preceding the stimulus artifact and was quantified by averaging 12 consecutive traces during a 1-min bin. To determine the time constant of EPSC_{NMDA} decay, the fallen phase of averaged EPSC_{NMDA} was fitted with a single exponential decay using Igor Pro software. Although NMDAR whole cell current can be best fitted by multi-exponential kinetics (Kampa et al. 2004), a single-exponential function nevertheless provides an accurate estimation on the decay time constant, especially in early developmental stages (Barth and Malenka 2001). The peak current amplitude was used to quantify AMPA/NMDA ratio and ifenprodil sensitivity. Detection and analysis of mEPSCs were done using Clampfit 9.0, and each detected event was confirmed by visual inspection.

To estimate the proportion of silent synapses and quantify synaptic failure rates, the amplitude distribution histograms of minimum stimulus-evoked EPSC at either −65 or +40 mV were plotted and a smooth curve was drawn using the Spline function provided in GraphPad Prism 4.0 software. The integral of the peak centered at 0 pA represents failures of transmission and matches the noise amplitude distribution. The failure rates were measured by using the fraction of responses with amplitude less than zero and then timed by two based on the symmetry of noise (Liao et al. 1995). Failure rates were also visually inspected in a subset of cells to ensure that this approach provided an accurate estimation. The decrease of failure rate at +40 mV (mixed AMPAR + NMDAR response) compared with that in −65 mV (AMPAR response) indicate the presence of silent synapses, whose numbers are proportional to the differences of failure rates (Liao et al. 1995).

For statistical analysis, data were represented by means ± SE. An unpaired Student’s t-test, one-way ANOVA (with post hoc Student Neuman-Keuls test when overall significance was achieved) or two-way ANOVA analysis were performed when data passed normality test. For the comparison of cumulative histogram of mEPSC amplitudes, Kolmogorov-Smirnov test was used. Significance was assigned for all tests at *P < 0.05.

**RESULTS**

**Chronic reelin treatment enhances AMPA receptor function and facilitates the developmental switch from NR2B to NR2A of synaptic NMDA receptors**

Reelin binds to its lipoprotein receptors, apoER2 and VLDLR, and initiates a signaling cascade that is transmitted by Dab1 and Src (Bock and Herz 2003; Hiesberger et al. 1999; Howell et al. 1999; Trommsdorff et al. 1999). In adult hippocampal slices, this signaling leads to increased NMDAR tyrosine phosphorylation, increased NMDAR-mediated currents, and elevated magnitude of LTP (Befert et al. 2005; Chen et al. 2005; Weeber et al. 2002). It is not known whether reelin signaling affects glutamatergic transmission during postnatal hippocampal development. We therefore tested this using hippocampal slice cultures prepared from 6- to 7-day-old mice. Either 5 nM reelin or reelin in combination with the functional lipoprotein receptors antagonist GST-RAP (20 μM) (Herz et al. 1991) or Src inhibitor PP1 (10 μM) (Hanke et al. 1996) were applied to slice cultures at day 3. CA1 pyramidal neurons were then recorded for spontaneous miniature activities or synaptically evoked whole cell responses at 6–7 days after drug application. To quantify reelin effects on spontaneous mEPSCs, ~2,000 consecutive events pooled from 8 to 10 CA1 pyramidal neurons derived from six to eight slices were analyzed for each group. It was found that reelin treatment markedly increased spontaneous mEPSC amplitude, an effect that was blocked by either GST-RAP or PP1 (Fig. 1, A–C compared with mock treated group, *P < 0.01 for reelin; *P > 0.05 for reelin + GST-RAP and reelin + PP1. Kolmogorov-Smirnov test). No significant change in mEPSC frequency was seen after reelin treatment [Fig. 1D, *F(3,30) = 2.43, *P > 0.05]. Therefore chronic reelin treatment significantly increased spontaneous AMPAR-mediated responses in CA1 pyramidal neurons.

It has been well established that the maturation of CNS synapses is mirrored by an increased recruitment of AMPARs to the synapses containing only NMDARs, rendering it functional at hyperpolarized potentials (Isaac et al. 1995; Kullmann 1994; Liao et al. 1995). In addition, the subunit composition of NMDARs at murine CA1 synapses normally changes over the two to three postnatal weeks (Monyer et al. 1994) with a developmental switch from NR2B to NR2A subunits. We first compared the relative current ratio of AMPAR and NMDAR under mock- and reelin-treated conditions. The AMPA/NMDA current ratio of mock-treated slices was significantly lower than that from slices treated with reelin [Fig. 2B, mock treated, 0.84 ± 0.07; reelin treated, 1.73 ± 0.12. *F(3,35) = 10.4, *P < 0.01], an effect that was not observed when reelin was co-applied with GST-RAP or PP1. These results indicate that chronic reelin treatment enhances AMPAR function and facilitates glutamatergic transmission in the developing hippocampus.

Next we tested whether reelin facilitates the switch from NR2B to NR2A during hippocampal in vitro development. CA1 cells were tested for their sensitivity to the NR2B specific antagonist, ifenprodil (3 μM). In reelin-treated slices (Fig. 2C), perfusion of ifenprodil resulted in an averaged 18.8 ± 1.6% reduction of peak EPSC_{NMDA}, which was significantly lower than that derived from mock-treated slices [38.3 ± 2.2%, *F(4,36) = 14.7, *P < 0.001]. Mock treatment had no effect on ifenprodil sensitivity compared with nontreated control group. In addition, the decay time constant (τ) of EPSC_{NMDA} was also significantly smaller after reelin treatment [Fig. 2D, reelin treated, 93.6 ± 10.5 ms; mock treated, 185.8 ± 17.2 ms, *F(3,25) = 8.7, *P < 0.01]. Pharmacological blockade of reelin signaling with GST-RAP or PP1 abolished reelin effects on both ifenprodil sensitivity and EPSC_{NMDA} decay τ, suggesting an accelerated switch to faster NMDAR kinetics occurred in the developing hippocampus as a result of reelin signaling.

**Differential effects of reelin treatment on surface levels of AMPAR and NMDAR subunits**

Because of the increased AMPA component of synaptic response, altered of EPSC_{NMDA} kinetics and ifenprodil sensitivity after chronic reelin treatment, we asked whether the...
amounts of AMPAR and NMDAR subunits were changed by reelin in CA1. Both total and surface levels of GluR1, NR1, NR2A, and NR2B were probed by Western blotting. We first examined whether GluR1, an AMPAR subunit that is increasingly expressed during developmental maturation (Petràlia et al. 1999; Wu et al. 1996) and subjected to regulated trafficking during synaptic plasticity (Hayashi et al. 2000; Shi et al. 1999), was increased on CA1 cells surfaces. Figure 3 shows, as expected, that reelin treatment significantly increased levels of surface GluR1 [F(2,11) = 15.6, P < 0.001] as well as total GluR1 [F(2,11) = 11.2, P < 0.01] compared with mock-treated groups, indicating regulated expression and surface insertion of AMPARs at least partly account for the increased mEPSC<sub>AMPA</sub> and AMPA/NMDA current ratio after chronic reelin treatment. No changes of either surface or total NR1 levels were observed. In comparison, both total and surface NR2A showed significant increase after reelin treatment compared with mock treatment [total protein, F(2,14) = 9.75, P < 0.01; surface protein, F(2,11) = 44.9, P < 0.001]. Moreover, both total and surface NR2B showed significant decrease [total protein, F(2,11) = 4.1, P < 0.05; surface protein, F(2,11) = 22.6, P < 0.001] after reelin treatment.

**Chronic reelin treatment reduces number of CA1 silent synapses and decreases synaptic failures**

If synapses acquire more AMPARs at postsynaptic sites as a result of reelin treatment, then the number of silent synapses, which contain only NMDARs and therefore are quiescent at normal resting membrane potentials, should decrease. This scenario could also explain increased AMPA/NMDA ratio and mEPSC<sub>AMPA</sub> amplitudes after reelin treatment. We further tested this hypothesis by comparing the proportion of silent synapses in CA1 neurons in mock- and reelin-treated slices by using a minimum stimulation protocol (see METHODS). In both mock- and reelin-treated slices, neurons clamped at −65 mV displayed a consistently higher failure rate than that obtained when clamped at +40 mV, indicating the existence of silent synapses (Fig. 4, A and E). The failure rates were further quantified and frequency distributions of synaptic EPSC amplitudes were analyzed. Histogram of EPSCs revealed that reelin treatment increased clustering of EPSCs at larger amplitudes at both −65 and +40 mV holding potentials (Fig. 4, D and H). When the failure rates obtained at −65 and +40 mV holding potentials were compared (Fig. 4, I–K), mock- and reelin-treated cells showed a 41.4 ± 5.4 and 5.8 ± 1.9% reduction in failure rates at +40 mV, respectively (n = 7–8, P < 0.001, t-test), indicating reelin treatment significantly reduced the proportion of silent synapses compared with mock treatment. In addition, at −65 mV, reelin-treated cells showed 39.2% ± 4.4% less failures compared with mock-treated cells (reelin, 59.5 ± 5.3%; mock treated, 20.3 ± 4.7%, n = 6, P < 0.01, t-test); this suggests facilitated synaptic transmission by AMPAR at normal resting membrane potentials after reelin treatment.

If a physiological level of reelin is required for normal maturation processes in CA1 through reelin receptors- and Src-mediated signaling, then blocking reelin receptors or Src activity would delay the normal maturation of glutamatergic function. We tested this hypothesis by applying GST-RAP or PP1 to the cultured wild-type slices and compared synaptic failures and ifenprodil sensitivity under these conditions. Chronic treatment with either GST-RAP or PP1 for 6–7 days significantly increased CA1 failure rates at −65 mV compared with control slices receiving no treatment [Fig. 5A, control, 54.1 ± 3.8%; GST-RAP, 74.6 ± 5.3%; PP1, 78.4 ± 6.1%. F(3,20) = 6.4, P < 0.01)]. Treatment with GST was without effect (P = 0.22). In addition, both GST-RAP and PP1 signifi-
FIG. 2. Chronic treatment of reelin facilitates CA1 glutamatergic maturation. A: representative traces showing increased AMPA/N-methyl-D-aspartate (NMDA) current ratio of synaptic response after prolonged reelin treatment. Reelin-treated traces are scaled so that its peak EPSC$_{\text{NMDA}}$ overlaps that of mock treated EPSC$_{\text{NMDA}}$. Note the faster decay kinetics of EPSC$_{\text{NMDA}}$ at +40-mV holding potential after reelin application compared with mock-treated conditions. B: reelin treatment significantly increased AMPA/NMDA current ratio compared with mock treatment (n = 7–12, **p < 0.01), an effect that was blocked by co-application with GST-RAP or PP1 (ns, P > 0.05). C: reelin treatment significantly reduced ifenprodil sensitivity in CA1 neurons (n = 8, ***p < 0.001; ns, P > 0.05, compared with mock treatment). Inset denotes representative traces of NMDAR-mediated whole cell currents before and after ifenprodil. D: NMDAR current decay constant was significantly reduced in reelin-treated cells (n = 8, **p < 0.01; ns, P > 0.05, compared with mock treatment). Scale bar in C, 400 pA, 50 ms.

significantly increased ifenprodil sensitivity of CA1 neurons [Fig. 5B, nontreated control, 38.4 ± 5.3%; GST-RAP, 64.5 ± 7.3%; PP1, 55.4 ± 4.6%. F(3,27) = 9.1, P < 0.01]. These results suggest that a constitutive level reelin signaling is required for physiological maturation of glutamatergic synapses in CA1.

Reelin rescues the reeler phenotypes on glutamatergic synapse formation and function in cultured primary hippocampal neurons

Recent findings have indicated that reelin is required for normal development of dendritic structure and maturation of somatic NMDARs in cultured hippocampal neurons (Niu et al. 2004; Sinagra et al. 2005). We hypothesized that reelin signaling also facilitates the formation and function of glutamatergic synapses through cell autonomous mechanisms and tested this hypothesis in cultured low-density embryonic hippocampal neurons. In agreement with the previous findings (Niu et al. 2004), we observed that in cultures obtained from reeler embryos, neurons generally exhibited stunted neurite growth and less number of ramifications at 14 days after plating, which could be corrected by supplying 5 nM reelin in the culture medium from day 3 after plating (Fig. 6, A–C). To quantify the potentially functional glutamatergic synapses, we labeled both NR1 and GluR1 proteins using immunofluorescence techniques. In wild-type neurons, primary dendrites showed extensive punctate labeling of NR1 and GluR1 (Fig. 6A), which represented potential sites for synapses. In addition, a considerable proportion of NR1 positive puncta also contained GluR1. In contrast to those puncta that show only NR1 immunoreactivity (therefore are silent at resting membrane potentials), acquisition of GluR1 in these silent synapses is believed to render them capable of fast synaptic transmission. In neurons obtained from reeler embryos, less number of NR1 and GluR1 clustering was observed (Fig. 6B), and this deficit was corrected by supplying reelin in the culture medium (Fig. 6C).

We next quantified the number of potential glutamatergic synapses formed under different culture conditions and examined whether it is correlated with a functional phenotype. In reeler culture, a significantly reduced number of NR1 and GluR1 puncta was observed, which can be reversed by supplementing 5 nM reelin in the culture [Fig. 6D, number of NR1 puncta per 50 μm primary dendrites; wild type (WT), 61.6 ± 10.3; reeler, 27.1 ± 9.5; reeler with reelin, 54.9 ± 11.2. F(2,17) = 4.7, P = 0.04, one-way ANOVA]. Similar results were obtained for GluR1 puncta [number per 50 μm primary dendrites; WT, 42.4 ± 6.5; reeler, 13.3 ± 4.8; reeler with reelin, 35.6 ± 8.3. F(2,17) = 5.18, P = 0.019, one-way ANOVA]. In addition, the proportion of NR1 positive puncta that also contained GluR1 was 57.3 ± 6.2% in WT neurons (Fig. 6E), which was significantly higher than that from reeler cultures [26.1 ± 4.8%, F(2,26) = 6.65, P = 0.005, one-way ANOVA] but not different from reeler cultures supplemented with reelin (42.5 ± 7.6%, P > 0.05). To test whether these structural distinctions were correlated with functional alterations, we recorded low-density neurons under above culturing conditions. It was observed that in the absence of reelin, neurons had significantly reduced amplitude of AMPAR-mediated mEPSCs [Fig. 6F, Z = 2.28, P = 0.02, compared with WT neurons, Kolmogorov-Smirnov test; −2,000 mEPSCs pooled from 8 to 10 neurons for each group]. This difference was eliminated when 5 nM reelin was supplemented in the culture medium in reeler cultures (Z = 0.26, P > 0.05, compared with WT neurons, Kolmogorov-Smirnov test). In addition, mEPSC frequency was significantly lower in cultured reeler neurons compared with that from WT neurons. Supplementation of reelin in the reeler culture fully restores mEPSC frequency to the WT levels (number of mEPSCs, WT, 2.35 ± 0.24; reeler, 0.72 ± 0.33; reeler + reelin, 2.11 ± 0.37; F(2,19) = 4.9, P = 0.026, data not shown). Therefore these results suggest that reelin signaling facilitates glutamatergic synapse formation and function through cell autonomous mechanisms that do not require neuronal activities under in vivo conditions.

**DISCUSSION**

Reelin-mediated signaling components involving apoER2 and VLDLR, the intracellular adaptor protein dab1 and Src family protein tyrosine kinases, are critical in embryonic brain development (Bock and Herz 2003; D’Arcangelo et al. 1995;
circuitries of the hippocampal formation are known to mature when slice cultures are prepared from young rodents, neural hippocampal slice cultures and embryonic neuronal cultures. These potential questions, we examined effects of reelin on the activity (Lu and Constantine-Paton 2004; Monyer et al. 1994; Nicoll 1990). Moreover, maturation and subunit switch of NMDARs in vivo is critically dependent on neuronal circuitry required for normal development of several structural and functional aspects of hippocampal neurons. For example, hippocampal neurons derived from reeler embryos showed stunted neurite growth due to mechanisms involving apoER2/VLDLR and dabl, a deficit that can be rescued by adding reelin to the culture medium (Niu et al. 2004). However, from this study it is not clear whether a more elaborated neurite network is correlated with a more mature and functional glutamatergic synapse. A recent functional study by Sinagra et al. (2005) has shown that reelin signaling is required for normal developmental switch of somatic NMDAR subunits from NR2B to NR2A in cultured hippocampal neurons. However, this finding may not necessarily reflect the in vivo conditions and offers limited knowledge on reelin effects on synaptic NMDARs, which play a determinant role in CA1 synaptic plasticity (Zalutsky and Gahwiler 1984).

We have first examined reelin effects on spontaneous synaptic transmission by analyzing AMPAR-mediated mEPSCs, which represent postsynaptic responses to single quanta of neurotransmitter releases. Reelin application for 6–7 days in cultured slices significantly increases amplitude of mEPSCs, an effect that is dependent on apoER2/VLDLR, suggesting reelin signaling leads to enhanced AMPAR function in CA1. We next examined effects of reelin signaling on synaptic responses mediated by NMDARs and AMPARs. In agreement with previous findings (Sinagra et al. 2005), reelin facilitated a functional switch from NR2B to NR2A, as shown by increased surface expression of NR2A and decreased surface expression of NR2B, reduced ifenprodil blockade of synaptic EPSC_NMDA and decreased τ of EPSC_NMDA decay current. Importantly, these effects were dependent on apoER2/VLDLR and Src, as they were blocked by either GST-RAP or PP1 (Fig. 2). Decreased τ of NMDAR decay through NR2A acquisition during maturation has been described in many CNS regions (Carmignoto and Vicini 1992; Flint et al. 1997; Hestrin 1992). Reduced ifenprodil sensitivity and faster τ of EPSC_NMDA indicate more NR2A subunits are present at synaptic sites in reelin-treated slice cultures. Because NMDAR subunit composition determines surface delivery of AMPAR subunits (Kim et al. 2005), differentially couples with intracellular biochemical events (Barria and Malinow 2005; Kim et al. 2005), and may differentially affect CA1 synaptic plasticity (i.e., LTP or LTD) (Bartlett et al. 2006; Morishita et al. 2006), an accelerated switch to NR2A by reelin could have a profound impact on synaptic strength and plasticity.

We next examined whether reelin signaling leads to increased efficacy of synaptic transmission by comparing spontaneous miniature events, synaptically evoked AMPA/NMDA current ratios, synaptic failures and estimated relative abundance of silent synapses. As CNS synapses mature, those containing only NMDARs acquire AMPARs in an activity-dependent manner (Isaac et al. 1995; Kullmann 1994; Liao et al. 1995). We have found that both mEPSC\textsubscript{AMPA} amplitude and to maintain a surprising three-dimensional, organotypic organization for many weeks in vitro (Gahwiler et al. 1997; Zimmer and Gahwiler 1984).

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AMPA/NMDA current ratio were significantly increased after chronic reelin treatment. These effects were also dependent on apoER2/VLDLR and Src. The increased AMPAR responses might be due to increased levels of surface expression GluR1 subunit (Fig. 3). However, it is not clear whether this increased GluR1 expression is mainly synaptic and whether other AMPAR subunits, such as GluR2 and GluR4 (Kolleker et al. 2003; Shi et al. 2001; Zhu et al. 2000), also contribute to enhanced AMPAR function. Moreover, in the presence of GST-RAP or PP1, there was a significant reduction in NMDAR subunit switch and an increased synaptic failure rate compared with untreated slice cultures undergoing normal in vitro development (Fig. 5). These results indicate that a constitutive level of reelin signaling is required to maintain the physiological maturation process in CA1. Taken together, our data show that prolonged reelin treatment during development facilitates maturation of CA1 glutamatergic function through an enhanced AMPAR-mediated synaptic transmission and a functional switch from NR2B to NR2A. It is interesting to note that reduced silent synapses should result in increased mEPSCAMPA frequency, which was not observed in our cultured slices. This may be due to decreased presynaptic release probability, structural changes of synapse and/or reduced number of total synapse formed in CA1 neurons in the presence of reelin. Moreover, reduced NR2B contents and enhanced AMPAR currents as a result of reelin signaling should not favor LTP induction. This apparent discrepancy with our

FIG. 4. Chronic treatment reduces number of silent synapses and failure rates of synaptic transmission. A: superimposed consecutive 15 trials elicited by minimum stimulation at −65 mV (bottom) and +40 mV (top) in a CA1 pyramidal neuron receiving mock treatment. The averaged traces of these 15 responses are shown in B. C: plotting of the amplitudes of 400 EPSCs evoked by minimum stimulation at either holding potentials of this mock-treated neuron. The amplitude frequency distribution of EPSCs (from 7 mock-treated cells obtained from 7 slices) is shown in D. Note the large fraction of failures of response at −65 mV compared with that at +40 mV. E and F: superimposed consecutive 15 trials (E) and their averages (F) elicited by minimum stimulation at −65 mV (bottom) and +40 mV (top) in a CA1 pyramidal neuron receiving reelin treatment. G: representative EPSC responses of minimum stimulation from a reelin-treated CA1 pyramidal neuron at either holding potentials. The histogram of amplitude distribution of EPSCs (8 cells from 8 reelin treated slices) is shown in H. I: summary of failure rate changes at −65 and +40 mV from 7 mock-treated and 8 reelin-treated cells. J: reelin treatment resulted in significantly reduced difference in failure rate at two holding potentials (failure rate from mock-treated cells, 41.4 ± 5.4%; failure rate from reelin-treated cells, 5.8 ± 1.9%, n = 7–8, ***P < 0.001, t-test). K: reelin treatment significantly reduced failure rate at −65 mV compared with that at +40 mV (***P < 0.01), indicating facilitated synaptic transmission at hyperpolarized potentials after reelin treatment.

FIG. 5. Chronic blockade of reelin receptors or inhibition of Src significantly delayed normal maturation of glutamatergic function. A: treatment of either GST-RAP or PP1, but not GST, in cultured slices increased failure rates of synaptic transmission at −65 mV (n = 5–6, *P < 0.05, **P < 0.01, compared with nontreated control group). B: chronic treatment of either GST-RAP or PP1 significantly increased ifenprodil sensitivity compared with the control group receiving no treatment (n = 5–8, *P < 0.05, **P < 0.01). The representative traces before and after ifenprodil in each group were shown above the plot. Scale bar, 400 pA, 50 ms.
previous report that reelin enhances LTP in acute hippocampal slices (Weeber et al. 2002) may be due to different tissue preparations and the timing of reelin signaling under these two distinct conditions.

Our immunocytochemistry data using cultured low-density hippocampal neurons provide strong connection between reelin signaling with glutamatergic synapse formation and support a cell autonomous rather than activity-dependent mechanism for reelin signaling. During development, newly formed synapses contain exclusively NMDARs (silent) and progressively acquire AMPARs in an activity-dependent manner (Liao et al. 1999; Wu et al. 1996). In the absence of reelin, impaired neurite outgrowth occurs (Niu et al. 2004). We have further shown that neurons from reeler cultures exhibit less glutamate receptor-mediated activity, significantly reduced numbers of GluR1 and NR1 clusters and colocalized puncta that contains both GluR1 and NR1. Moreover, in the absence of reelin, more NR1 puncta did not show GluR1 immunoreactivity. In comparison, neurons derived from wild-type embryos or reeler cultures supplemented with recombinant reelin showed increased number of NR1 and GluR1 clustering and a larger proportion of double immunoreactivity for GluR1 and NR1 (therefore reduced number of silent synapses). By employing this morphological correlate of silent synapses, we provide direct evidence that reelin signaling through cell autonomous mechanisms facilitates maturation of glutamatergic function through increased clustering of GluR1 and NR1 and reduces the number of silent synapses by promoting AMPAR synaptic insertion. However, the molecular mechanisms for increased AMPAR insertion remain unclear. One possible mechanism may be that enhanced tonic NMDAR activity after reelin signaling and Src activation leads to increased acquisition of AMPARs and spontaneous activity at NMDAR-containing synapses over a prolonged developmental process similar to that described previously (Zhu and Malinow 2002). This potential mechanism may function in the maturation process of cultured hippocampal slices as well.

In light of our observation that increased reelin signaling accelerates maturation of CA1 glutamatergic synapses, it would be intriguing to know whether a decreased level reelin signaling may manifest the opposite effects. Complete loss of reelin in the reeler mouse disrupts many laminated brain...
structures and precludes a meaningful electrophysiological or behavioral testing in these animals. A partial loss (~50%) of reelin in heterozygote mice results in a significant impairment of memory performance and long-term plasticity (Qi et al. 2005; Tueting et al. 1999; Yabut et al. 2005). It is not clear whether the phenotypes found in heterozygote reeler mouse are primarily due to deficits in network connections or due to insufficient signaling caused by reelin haploinsufficiency. Our data using slice and neuronal cultures support the latter in that chronic reelin application facilitates maturation of glutamatergic responses that is dependent on reelin receptors and the intracellular signaling component Src.

Based on the findings of this study, it would be tempting to speculate that a reduced level of reelin signaling may have broader implications in distinct brain regions from development to senescence. It is especially intriguing considering some recent interesting findings that aberrant reelin signaling may play an etiological role in some neurodegenerative or neuropsychiatric diseases such as Alzheimer’s disease (Botella-Lopez et al. 2006), schizophrenia, lissencephaly, bipolar disease, and autism (for a review, see Qi et al. 2006). The novel reelin signaling events uncovered by this study could modify glutamatergic transmission, plasticity, and, potentially, memory and cognition in a profound manner as to affect the pathogenesis and outcome of these neurological disorders.

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REFERENCES


REELIN FACILITATES MATURATION OF GLUTAMATERGIC SYNAPSES


