Reelin Regulates Neuronal Excitability through Striatal-Enriched Protein Tyrosine Phosphatase (STEP\textsubscript{61}) and Calcium Permeable AMPARs in an NMDAR-Dependent Manner

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Alzheimer’s disease (AD) is a progressive neurodegenerative disease marked by the accumulation of amyloid-\(\beta\) (A\(\beta\)) plaques and neurofibrillary tangles. A\(\beta\) oligomers cause synaptic dysfunction early in AD by enhancing long-term depression (LTD; a paradigm for forgetfulness) via metabotropic glutamate receptor (mGluR)-dependent regulation of striatal-enriched tyrosine phosphatase (STEP\textsubscript{61}).

Reelin is a neuromodulator that signals through ApoE (apolipoprotein E) receptors to protect the synapse against A\(\beta\) toxicity (Durakoglugil et al., 2009). Reelin signaling is impaired by ApoE4, the most important genetic risk factor for AD, and A\(\beta\)-oligomers activate metabotropic glutamate receptors (Renner et al., 2010). We therefore asked whether Reelin might also affect mGluR-LTD. To this end, we induced chemical mGluR-LTD using DHPG (Dihydroxyphenylglycine), a selective mGluR5 agonist. We found that exogenous Reelin reduces the DHPG-induced increase in STEP\textsubscript{61}, prevents the dephosphorylation of GluA2, and concomitantly blocks mGluR-mediated LTD. By contrast, Reelin deficiency increased expression of Ca\(^{2+}\)-permeable GluA2-lacking AMPA receptors along with higher STEP\textsubscript{61} levels, resulting in occlusion of DHPG-induced LTD in hippocampal CA1 neurons. We propose a model in which Reelin modulates local protein synthesis as well as AMPA receptor subunit composition through modulation of mGluR-mediated signaling with implications for memory consolidation or neurodegeneration.

**Key words:** AMPA receptor; ApoE; NMDA receptor; Reelin; STEP; synaptic plasticity

**Significance Statement**

Reelin is an important neuromodulator, which in the adult brain controls synaptic plasticity and protects against neurodegeneration. Amyloid-\(\beta\) has been shown to use mGluRs to induce synaptic depression through endocytosis of NMDA and AMPA receptors, a mechanism referred to as LTD, a paradigm of forgetfulness. Our results show that Reelin regulates the phosphatase STEP, which plays an important role in neurodegeneration, as well as the expression of calcium-permeable AMPA receptors, which play a role in memory formation. These data suggest that Reelin uses mGluR LTD pathways to regulate memory formation as well as neurodegeneration.
Introduction

Alzheimer’s disease (AD) is a socioeconomic burden of sweeping proportions. Dysregulation of synaptic function occurs in AD before overt clinical manifestation. One of the main characteristics of AD are amyloid-β (Aβ) plaques. Aβ oligomers cripple the synapses through multiple modes of action and hijack the core neuronal machinery leading to inhibition of long-term potentiation (LTP) and enhancement of long-term depression (LTD), paradigms for learning and memory or forgetfulness, respectively.

At excitatory synapses, glutamate binds to the ionotropic AMPA and NMDA receptors (AMPA and NMDAR, respectively) as well as metabotropic glutamate receptors (mGluRs). AMPARs regulate fast synaptic transmission under resting conditions. NMDARs are coincidence detectors that flux Ca2+ on activation, which in turn regulates LTP through transcriptional mechanisms. In contrast, mGluR activation regulates LTD by modulating local synaptic mRNA translation. (Ebert and Greenberg, 2013).

AMPARs are tetramers consisting of combinations of four subunits: GluA1-4. GluA2 is critical for determining AMPAR function, and GluA2 depletion ablates mGlur-LTD starting at the age of 3 months in mice (Zhou et al., 2011; Cao et al., 2017; 2018). During LTD, one of the main translated proteins is striatal-enriched tyrosine phosphatase (STEP61). Phosphorylation of STEP61 regulates synaptic plasticity by dephosphorylation of the NMDAR subunit GluN2B and AMPAR subunit GluA2, which lead to the endocytosis of the receptors (Pelkey et al., 2002; Snyder et al., 2005; Zhang et al., 2008). Importantly, Aβ induces endocytosis of GluA2-containing AMPARs, mimicking and occluding mGluR-dependent LTD (Hsieh et al., 2006). In AD patients and mouse models, STEP61 levels are increased (Kurup et al., 2010). Blocking mGluR5 rescues cognitive impairment in an AD mouse model (Renner et al., 2010; Rammes et al., 2011; Chen et al., 2013). Importantly, the inhibition (Xu et al., 2014) or genetic knockdown (Zhang et al., 2011) of STEP61 ameliorates the behavioral and cognitive impairments seen in AD mouse models, implicating STEP61 as a downstream target of Aβ and mGluR5.

We have shown previously that the extracellular signaling protein Reelin, by binding to ApoE (apolipoprotein E) receptors, can antagonize Aβ at the synapse (Durakoglugil et al., 2009) and protect neurons against Aβ toxicity in vivo (Lane-Donovan et al., 2015). This Reelin protective effect is inhibited by ApoE4, the most important genetic risk factor for late-onset AD, as it impairs the recycling of Apoer2 and glutamate receptors (Chen et al., 2010; Xian et al., 2018). Reelin regulates the surface expression of both AMPARs (Qiu et al., 2006a; Qiu and Weeber, 2007) and NMDAR (Weeber et al., 2002; Chen et al., 2005; Durakoglugil et al., 2009) and is essential for the NMDAR subunit switch during development (Groc et al., 2007).

In this current study, we investigated whether Reelin can modulate mGlur-LTD by either applying exogenous Reelin on wild-type hippocampal slices or by using Reelin-deficient slices and determining STEP61 and AMPAR composition. We used a mouse model with induced genetic depletion of Reelin [conditional KO (cKO)], which has an architecturally normal adult mouse model with induced genetic depletion of Reelin [conditional KO (cKO)], which has an architecturally normal adult mouse model with induced genetic depletion of Reelin [conditional KO (cKO)], which has an architecturally normal adult mouse model with induced genetic depletion of Reelin [conditional KO (cKO)], which has an architecturally normal adult mouse model with induced genetic depletion of Reelin [conditional KO (cKO)]. Here, we now show that acute Reelin application reduces both mGlur-LTD and STEP61 levels. Reelin cKO mice, on the other hand, display increased STEP61 levels and increased expression of surface GluA2-lacking AMPARs, which results in the occlusion of mGlur-LTD. Overall, our data demonstrate that Reelin, through regulation of local protein synthesis, controls the assembly of AMPAR subunits on the synaptic surface, with implications for memory consolidation and neurodegeneration.

Materials and Methods

Animals. All mice were housed under a 12:12 light/dark cycle and fed a normal chow diet. All animals were killed by inhalation of isoflurane followed by decapitation according to strict regulations set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the University of Texas Southwest Animal Care and Use Committee. Pregnant Sprague Dawley rats were purchased from Charles River Laboratories. The Reelin conditional knock-out animals have been described previously (Lane-Donovan et al., 2015). Briefly, Eox1 of the Reln gene was flanked by loxP sites. Homozygous Reelin-floxed (Relnfl/fl) mice were then crossed with transgenic CAG-CreERT2 mice (Hayashi and McMahon, 2002). To induce conditional Reelin knockout, Cre-positive Relnfl/fl mice were injected with tamoxifen (Reelin cKO) as described (Lane-Donovan et al., 2015). As a control, Cre-negative Relnfl/fl mice were also injected with tamoxifen. For electrophysiological recordings, tamoxifen injections were performed at 4 weeks of age. For biochemical analysis, tamoxifen injections were performed at 8 weeks of age by daily intraperitoneal injections for 5 d with 135 mg/kg tamoxifen (Sigma) dissolved in sunflower oil and 10% ethanol. Electrophysiological recordings and biochemical analysis were performed at 3 or 6 months, as indicated. This gave us the advantage of testing the in vivo function of endogenous Reelin in adult mice after the end of the neuronal migration period for which Reelin is required (Herz and Chen, 2006).

Recombinant protein. Recombinant Reelin was secreted by 293HEK cells stably transfected with the mouse Reelin cDNA plasmid (pGri). Reelin was purified from the media as described (Weeber et al., 2002).

In vitro electrophysiology (whole-cell patch-clamp recordings). Cells were recorded from the CA1 region of 2-3-month-old mice. The extracellular solution contained the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 28 NaHCO3, 0.5 CaCl2, 1 MgSO4. The intracellular solution contained the following (in mM): 130 CsMeSO4, 5 NaCl, 10 HEPES, 1 CaCl2, 4 MgCl2, 0.5 EGTA, four MgATP, 5 QX314, 0.3 GTP and 0.1 spermine (pH adjusted with CsOH). Spontaneous recordings of AMPAR currents were performed at room temperature (26°C) in the presence of the NMDAR antagonist N-AP5 (D-(-)-2-Amino-5-phosphono pentanoic acid; 50 μM) and GABA receptor antagonist picrotoxin (50 μM). Stimulus intensity was adjusted to evoke currents approximately one-third of the maximal current response, typically resulting in currents between 100 and 200 pA. Current voltage curves were obtained by plotting the current response at different holding potentials (−70 to +50 mV with 10 mV increments). The rectification index (RI) was calculated as the ratio of the average peak current responses at −70 mV to those at 50 mV. NASPM [1-Naphthylacetyl spermine trihydrochloride; philanthotoxins (PhTX), 100 μM] was applied after a 20 min stable baseline for about 20 min and washed out for at least 20 min.

Extracellular field recordings. Hippocampal slices were prepared from 3-month-old Reelin cKO mice and Cre-negative, tamoxifen-injected littermate controls. The brains were quickly removed and placed in cold high sucrose cutting solution containing the following (in mM): 110 sucrose, 6 NaCl, 3 KCl, 1.25 NaH2PO4, 28 NaHCO3, 0.5 CaCl2, 5 glucose, 0.6 Ascorbic acid, 1 MgSO4. The transverse sections of 400 mμ were cut using a vibratome. Slices were then transferred into an incubation chamber containing 50% artificial CSF (aCSF) containing the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, 10 D-glucose, 2 CaCl2, 1 MgSO4 and 50% sucrose cutting solution. In the recording chamber, slices were perfused with aCSF.
only. For stimulation, concentric bipolar electrodes were used (catalog #CBBRC75, FHC) and placed into the stratum radiatum. Experiments were performed in the presence of n-AP5 (50 μM) and picrotoxin (10 μM). Twenty minutes after stable baseline, DHPG (100 μM) was applied for 10 minutes. Stimulus intensity was set at 40 of maximum response and delivered through an Isolated Pulse Stimulator (Model 2100, A-M. Systems). A custom-written program in LabVIEW 7.0 was used for recording and analysis of LTD experiments.

Pharmacological treatment of slices. Experiments were performed in six-well plates (see above, Extracellular field recordings). Briefly, 300 μM WT slices were continuously bubbled with 95% O2 and 5% CO2 in aCSF containing AP-5 and picrotoxin (Fig. 1C). Four of the slices obtained were incubated with 50 μM AP-5 and 10 μM picrotoxin; the other four were incubated with 5 μM Reelin with AP-5 and picrotoxin. Twenty minutes later, two of the slices from both the control group and the Reelin-treated group were incubated with 100 μM DHPG (AP-5+picrotoxin± Reelin). Ten minutes later, slices were washed in either AP-5- and picrotoxin-containing aCSF or in AP-5-, picrotoxin-, and Reelin-containing aCSF for 30 min, and then collected for biochemical analysis.

For the experiments on Reelin cKO (Cre-positive mice injected with tamoxifen) and Control (Cre-negative littermates injected with tamoxifen; see Fig. 5) slices were bubbled with 95% O2 and 5% CO2 in acSF for 10 min followed by a washout (post-DHPG). Slices were oxygenated in aCSF containing picrotoxin and AP-5 in a six-well plate. Twenty minutes after pretreatment with or without Reelin, slices were moved to DHPG-containing wells for 10 min. DHPG was washed out, and slices were incubated for an additional 30 min with or without Reelin. Total STEPs levels were analyzed by immunoblotting and quantified by averaging seven independent experiments from seven different mice (bar graph). ANOVA test was performed on the average of control slices or Reelin-treated slices 50 min after DHPG treatment (Control n = 14, Reelin n = 11, p = 0.0311). Exogenous Reelin decreases STEPs1 in wild-type slice from 3-month-old mice treated with or without DHPG for 10 min followed by a washout (post-DHPG). Slices were oxygenated in aCSF containing picrotoxin and AP-5 in a six-well plate. Twenty minutes after pretreatment with or without Reelin, slices were moved to DHPG-containing wells for 10 min. DHPG was washed out, and slices were incubated for an additional 30 min with or without Reelin. Total STEPs1 levels were analyzed by immunoblotting and quantified by averaging seven independent experiments from seven different mice (bar graph). ANOVA F(1,23) = 5.591 p = 0.0347. Holm–Sidak multiple comparisons test shows a significant difference between Control versus Reelin (p = 0.0096) and Control versus post-DHPG with Reelin (p = 0.0482). All data are plotted as mean ± SEM. B, Exogenous Reelin reverses DHPG-induced reduction of phospho-GluA2 (pGluA2) in rat primary neurons. Neurons were pretreated for 30 min in aCSF containing picrotoxin and AP-5. Reelin and/or DHPG was added for an additional hour before neurons were harvested for Western blot. The quantification of pGluA2/GluA2 is shown in the bar graph (bottom; n = 4 independent cultures, p = 0.03, unpaired t test). All data are plotted as mean ± SEM. E, Reelin activates SFK and stabilizes GluA2 on the cell surface, whereas STEP61 dephosphorylates and removes GluA2 from the surface. We propose a model in which Reelin blocks mGluR-dependent protein synthesis and LTD through STEP61. Error bars represent the standard error of the mean (SEM). (*p < 0.05, **p < 0.01).

Figure 1. Reelin blocks DHPG-induced LTD through regulating STEP and phospho-GluA2 levels. A, Field EPSPs were recorded from stratum radiatum in the presence of AP-5 (50 μM) and picrotoxin (10 μM). Mock or Reelin was added 10 min before recordings and was present throughout the experiment (—30 to +60). Stable baseline EPSP was recorded for 20 min (—20 to 0 min) before DHPG application and normalized to 1. DHPG was applied for 10 min (0–10 min). Sample traces before and after (—10, +40 min) DHPG in the presence or absence of Reelin are shown (bottom right inset; 3-month-old mice; Control, n = 14 slices, a = 8 animals; Reelin, n = 11, a = 6). Two-way RM ANOVA: Reelin-int: F(1,23) = 9.68, p = 0.0049. Sidak’s post hoc: Control versus Reelin, 50–60 min: p = 0.0493-0.0016, time-int: F(6,62) = 55.8, p < 0.0001; Sidak’s post hoc: Control: —20 versus 60, p < 0.0001; Reelin: —20 versus 60 min, p = 0.3385. -int, -interaction.

B, Bar graph shows the fEPSP slope average of the last 10 min of recording from A (50–60 min). Unpaired Student’s t test was performed on the average of control slices or Reelin-treated slices 50 min after DHPG treatment (Control n = 14, Reelin n = 11, p = 0.0031). C, Exogenous Reelin decreases STEPs1 in wild-type slice from 3-month-old mice treated with or without DHPG for 10 min followed by a washout (post-DHPG). Slices were oxygenated in aCSF containing picrotoxin and AP-5 in a six-well plate. Twenty minutes after pretreatment with or without Reelin, slices were moved to DHPG-containing wells for 10 min. DHPG was washed out, and slices were incubated for an additional 30 min with or without Reelin. Total STEPs1 levels were analyzed by immunoblotting and quantified by averaging seven independent experiments from seven different mice (bar graph). ANOVA F(1,23) = 5.591 p = 0.0347. Holm–Sidak multiple comparisons test shows a significant difference between Control versus Reelin (p = 0.0096) and Control versus post-DHPG with Reelin (p = 0.0482). All data are plotted as mean ± SEM. D, Exogenous Reelin reverses DHPG-induced reduction of phospho-GluA2 (pGluA2) in rat primary neurons. Neurons were pretreated for 30 min in aCSF containing picrotoxin and AP-5. Reelin and/or DHPG was added for an additional hour before neurons were harvested for Western blot. The quantification of pGluA2/GluA2 is shown in the bar graph (bottom; n = 4 independent cultures, p = 0.03, unpaired t test). All data are plotted as mean ± SEM. E, Reelin activates SFK and stabilizes GluA2 on the cell surface, whereas STEP61 dephosphorylates and removes GluA2 from the surface. We propose a model in which Reelin blocks mGluR-dependent protein synthesis and LTD through STEP61. Error bars represent the standard error of the mean (SEM). (*p < 0.05, **p < 0.01).
indicate an increase in STEPs in Reelin cKO mice. Top, Confirmation of Reelin conditional knockout efficiency after the treatment in 3-month-old Reelin cKO and Control slices in CA1 region in the presence of AP-5 (50 μM) and picrotoxin (10 μM). Sample fEPSP traces before and after DHPG (inset, bottom right). (Control, n = 7; Reelin cKO, n = 13; a = 3, α = 0.0055). STEP61 levels were measured by immunoblotting hippocampal lysates from 6-month-old Reelin cKO and tamoxifen-injected Control mice (Control, n = 13, a = 11, Reelin cKO, n = 10, a = 10; unpaired t test, p = 0.0280). Error bars represent the SEM. (*p < 0.05, **p < 0.01).

**Figure 2.** mGluR-LTD is occluded when Reelin is conditionally knocked out. A, Normalized fEPSP slopes before and after DHPG treatment in 5-month-old Reelin cKO and Control slices in CA1 region in the presence of AP-5 (50 μM) and picrotoxin (10 μM). Sample fEPSP traces before and after DHPG (inset, bottom right). (Control, n = 11 slices, a = 3 animals; cKO, n = 13, a = 3; two-way RM ANOVA: Reelin-int: F(1,22) = 10.68, p = 0.0035; Holm–Sidák post hoc: Control cKO: 38–60 min, p = 0.0441–0.001; time-int: F(5,110) = 20.39; p < 0.0001; Holm–Sidák post hoc: Control: –20 vs 60 min, p < 0.0001; cKO: –20 vs 60 min, p > 0.9999). B, Reelin knockdown completely blocked DHPG-induced LTD. Quantification of average fEPSP in the last 5 min is shown. Unpaired Student’s t test was performed on the average of Control slices or Reelin cKO-treated slices 50 min after DHPG treatment (Control n = 11, Reelin cKO n = 13, p = 0.0055). B, Input-output analysis shows increased excitability in the Reelin cKO mice in the presence of AP-5 + picrotoxin (filled square). D, Hippocampal protein analysis indicates an increase in STEPs levels in Reelin cKO mice. Top, Confirmation of Reelin conditional knockout efficiency after the injections. (Control, n = 7, a = 7; Reelin cKO, n = 5, a = 5; unpaired t test, p = 0.0002). STEP3 levels were measured by immunoblotting hippocampal lysates from 6-month-old Reelin cKO and tamoxifen-injected Control mice (Control, n = 13, a = 13; Reelin cKO, n = 10, a = 10; unpaired t test, p = 0.0280). Error bars represent the SEM. (*p < 0.05, **p < 0.01).

**aCSF.** Both Control and Reelin cKO slices were incubated in wells containing AP-5- and picrotoxin-containing aCSF for 1 h. Slices were collected for biochemical analysis (see below, Surface biotinylation and Western blotting).

**Surface biotinylation.** Biotinylation experiments were performed as described previously (Nosyreva and Huber, 2005). Slices were biotinylated on ice for 30 min using PBS buffer containing a nonpermeable biotin solution, 1.0 mg/ml sulfo-NHS-biotin (Pierce). Slices were homogenized by forcing the suspension 15–20 times through a 23-gauge needle in 300 μl of ice-cold lysis buffer containing the following (in mM): 65 Tris-HCl pH 7.4, 150 NaCl, 1 EDTA, 0.5% deoxycholic acid, 1% Triton X-100, 0.1% SDS. Homogenate was spun down at 14,000 rpm for 15 min at 4°C. Protein concentration of the supernatant was determined using the Bio-Rad DC protein assay. Surface proteins were pulled down using neutravidin beads (Thermo Scientific). Beads were boiled for 20 min in 5 × SDS sample buffer (250 mM Tris-HCl, pH 6.8), 500 mM DTT, 10% SDS, 0.5% bromphenol blue, 50% glycerol) at 90°C. For total protein, SDS sample buffer (1 × final) was added to 10 μg of hippocampal lysate and incubated for 10 min at 90°C. The surface and total protein samples were separated on an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane.

**Western blotting.** Six-month-old Reelin cKO mice were perfused, and brains were quickly isolated and flash frozen. Hippocampi were dissected in PBS with protease inhibitors and then lysed with lysis buffer containing the following (in mM): 0.1 ammonium molybdate, 2 EDTA, 2 sodium pervanadate, 50 sodium fluoride, 10 sodium pyrophosphate, 320 sucrose; 5× sample buffer was added to hippocampal lysate. Samples were boiled at 80°C for 10 min and then loaded on a Tris/Glycine gradient gel and transferred onto a PVDF membrane.

The surface and total protein samples were separated on an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane. Surface and total levels of GluA subunits were determined with antibodies directed against GluA2/3 (catalog #07-598, Millipore), GluA1 (catalog #ab1504, Abcam), or GluA4 (catalog #3824S, Cell Signaling Technology). STEP61 of total fractions was visualized with an antibody directed against STEP (Cell Signaling Technology, 23e5) and GAPDH with anti-GAPDH antibody (catalog #ab9485, Abcam). Protein levels were calculated by using the LI-COR Biosciences system. The ratio of surface to total protein levels was calculated after normalizing to GAPDH. Reelin knockdown was confirmed by probing with anti-Reelin antibody (G10; de Bergeyck et al., 1998).

**Primary cortical neuronal cultures.** Primary cortical neuronal cultures were prepared from rat (SD, Charles River Laboratories) at E18 as described previously (Chen et al., 2005). Neurons were cultured in Poly-D-Lysine-coated six-well plates (1 million/9 cm²) in the presence of neurobasal medium supplemented with B27, glutamine, and penicillin-streptomycin and incubated at 37°C with 5% CO2. Primary neurons were used at DIV 13–15 for experiments. Media was changed to aCSF 2 h before DHPG (100 μM) was applied with or without Reelin (5 nM) for 1 h in the presence of AP-5 (50 μM) and picrotoxin (10 μM).

**Drugs.** (RS)-3,5-DHPG, t-A5P, and spermine tetrahydrochloride were purchased from R&D Systems. NASPM and picrotoxin were purchased from Sigma-Aldrich.

**Statistical analysis.** Data were expressed as the mean ± SEM and evaluated using two-tailed Student’s t test for two groups with one variable tested and equal variances or one-way ANOVA with Dunnett’s post hoc or Tukey’s post hoc for multiple groups with only variable tested. The differences were considered significant at p < 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001). Software used for data analysis was ImageJ (National Institutes of Health), LabVIEW 7.0 (National Instruments), Odyssey Imaging Systems (LI-COR Biosciences), Prism 7.0 (GraphPad Software).

**Results.** Reelin blocks DHPG-Induced LTD in hippocampal slices from wild-type mice

Synaptic plasticity is mediated by both LTD and LTP. Previously, we found that Reelin enhances LTP in the adult brain through enhancing NMDAR currents (Weeber et al., 2002; Durakoglugil et al., 2009). Along this line, Reelin treatment for 1 h increases surface expression of GluA1 and GluA2 in the hippocampal CA1 in a PI3K-dependent manner (Qi et al., 2006a). In this study, we investigated whether Reelin treatment influences mGluR5 agonist DHPG-induced LTD. Extracellular field
potentials were recorded from the stratum radiatum. During the entire experiment, hippocampal slices from WT mice were maintained in oxygenated aCSF containing picrotoxin (GABA receptor antagonist, 10 μM) and d-AP5 (NMDAR antagonist, 50 μM). Slices were pretreated with Reelin for 30 min followed by a 10 min treatment with DHPG. After the washout of DHPG, slices were incubated with or without Reelin for an additional 50 min. In 3-month-old mice, Reelin (5 nm) application reduced the long-term LTD response after DHPG (100 μM) treatment (Control 58.16 ± 4.61%, n = 14 slices, animals (a) = 8 mice vs Reelin-treated slices 84.62 ± 6.86%, n = 11 slices, a = 6 mice) of baseline (n indicates the slice numbers and a denotes animal numbers; Fig. 1A,B), suggesting that Reelin regulates mGluR-LTD.

Exogenous Reelin reduces STEP₆₁ levels and increases GluA2 phosphorylation

Dephosphorylation of the AMPAR subunit GluA2 by tyrosine phosphatases results in AMPAR endocytosis and is essential for DHPG-induced LTD (Moult et al., 2002). DHPG increases the abundance of STEP₆₁, a brain-specific phosphatase of GluA2 (Zhang et al., 2008). Thus, to understand how Reelin reduces mGluR-LTD, we examined STEP₆₁ levels on Reelin treatment. STEP₆₁ protein levels were analyzed in wild-type hippocampal slices after AP-5, picrotoxin, Reelin, and/or DHPG treatment. Mouse hippocampal slices were treated in six-well plates with AP-5, picrotoxin, Reelin, and/or DHPG as described extensively (see above, Materials and Methods). STEP₆₁ levels compared with control levels 50 min after DHPG washout were not significantly different (Fig. 1C; Control 102.7 ± 3.53% vs post-DHPG: 94.59 ± 12.05%, n = 7 experiments, a = 7, n.s.). By contrast, in slices pretreated with AP-5, picrotoxin, and Reelin, basal STEP₆₁ levels were significantly decreased (Reelin 65.32 ± 5.72%, n = 7 experiments, a = 7 animals, p = 0.0096). Moreover, we saw a decrease in STEP₆₁ in samples cotreated with AP-5, picrotoxin, Reelin, and DHPG (post-DHPG with Reelin: 73.20 ± 5.51%, n = 7, a = 7, p = 0.0482; Fig. 1C, 3-month-old mice). These data support the conclusion that Reelin blocks DHPG-induced LTD through STEP₆₁.

Activated STEP₆₁ dephosphorylates GluA2. Given the observed Reelin-mediated block of DHPG-induced LTD and decreased STEP₆₁ levels (Fig. 1A–C), we quantified tyrosine phosphorylation (Y869/873/876) of GluA2 after applying DHPG to primary rat cortical neurons (DIV 14) in the absence and presence of AP-5, picrotoxin, and Reelin (Fig. 1D). Coapplication of AP-5, picrotoxin, Reelin, and DHPG increased GluA2 phosphorylation (DHPG 100 ± 7.49% vs DHPG + Reelin 124.34 ± 7.16%, n = 4 different cultures from four separate rats). Whether Reelin directly increases phosphorylation of GluA2 or indirectly through STEP₆₁ still needs to be determined. We propose a model where Reelin blocks mGluR-dependent LTD through increasing the phosphorylation of GluA2 along with a reduction in STEP₆₁ levels (Fig. 1E).

Reelin cKO mice have elevated STEP₆₁ levels

LTD induction depends on both the phosphorylation and surface expression of GluA2, which is regulated by STEP₆₁ levels (Won et al., 2019). We postulated that initial loss of Reelin by tamoxifen-driven Cre-recombination would remove the brake on the LTD suppression, resulting in elevated LTD and increased levels of STEP₆₁. To test this hypothesis, we measured hippocampal STEP₆₁ levels in 6-month-old Reelin cKO mice. Ablation of Reelin significantly increased STEP₆₁ levels (Control 100.0 ± 5.1% vs Reelin cKO 174.5 ± 35.6%; Control n = 13, a = 13; Reelin cKO n = 10, a = 10, unpaired t test, p = 0.0280; Fig. 2D). These data, along with the phosphorylation of GluA2 containing...
AMPAR currents using whole-cell recordings in the Reelin cKO mice. We found a significantly greater magnitude of average AMPAR current in CA1 neurons from Reelin cKO mice compared with those from control mice at all stimulus intensities, as seen with a leftward shift of the input-output (IO) curve in the Reelin cKO mice compared with the Controls (Fig. 3A; Control, n = 18 cells, a = 5; Reelin cKO, n = 10 cells, a = 3). This leftward shift in the IO curve indicates an increase in conductance of AMPARs. Lack of the GluA2 subunit increases the conductance of AMPARs and the ability of polyamines (spermine) to block the receptor at positive holding potentials (Swanson et al., 1997). Thus, we can use the ratio of the AMPAR peak current evoked at −70mV over those evoked at +50mV to calculate the rectification index (RI). An increase in RI would indicate the amount of inwardly rectifying receptors or, as in our case, GluA2-lacking AMPARs. Therefore, we measured the RI in 2- to 3-month-old Reelin cKO mice. Indeed, loss of Reelin increased the RI, suggesting the presence of inwardly rectifying GluA2-lacking AMPARs (Control 1.168 ± 0.14, n = 15 cells, a = 11 vs Reelin cKO 2.507 ± 0.4, n = 8 cells, a = 5; Fig. 3B). Reelin deficiency increases excitability and RI, which is consistent with a relative lack of GluA2 on the surface.

**Evoked AMPAR currents from Reelin cKO mice have a higher proportion of GluA2-lacking receptors**

PhTXs are polyamines that specifically block GluA2-lacking AMPARs. To determine the relative amount of GluA2-lacking AMPARs in the cKO mice, we compared the PhTX sensitivity of AMPAR components of stimulus-evoked excitatory postsynaptic currents (EPSCs) in the CA1 region of 2- to 3-month-old Reelin cKO to the Control mice. The exogenous PhTX (NASPM) induced a robust reduction in AMPAR currents in Reelin cKO (from 140.49 to 85.20 ± 8.66 pA; n = 7 cells, a = 5), whereas it had no effect on control hippocampi (from 138.99 ± 18.55 to 145.51 ± 17.70 pA; n = 6 cells, a = 4; Fig. 3C), confirming that Reelin cKO mice have an increase in GluA2-lacking AMPARs.

**Postdevelopmental loss of Reelin increases rectification index in CA1 neurons**

During development, lack of Reelin disrupts normal neuronal migration leading to perturbations in lamination of the hippocampus and neocortex. In the adult mice, Reelin can modulate NMDARs and synaptic plasticity (Lane-Donovan et al., 2014, 2015), yet it is not clear how Reelin can regulate glutamatergic receptor surface expression under stress conditions. Because deletion of Reelin after 6 months of age increases STEP61 levels, we suspect that downstream targets of STEP61 are altered as well. As STEP61 regulates surface expression of GluA2-containing AMPARs, we first evaluated the functional properties of evoked AMPARs (Fig. 1D), further support a role for Reelin in the regulation of STEP61.

**Spontaneous AMPAR minis have higher amplitude and frequencies in Reelin cKO mice**

To further validate if the increased excitability in Reelin cKO mice is mediated by an increase in GluA2-lacking AMPARs, we analyzed spontaneous AMPAR minis, which represent the AMPAR current responses to glutamate release at individual synapses. Reelin cKO neurons display increased amplitude (Control 10.15 ± 0.30 pA, n = 10 cells, a = 7 vs Reelin cKO 14.84 ± 1.23 pA; n = 9 cells, a = 5) and frequency (Control 0.61 ± 0.08 Hz vs Reelin cKO 1.128 ± 0.06 Hz) of spontaneous AMPAR minis.
currents compared with control neurons (Fig. 4A–C). However, both were reduced in Reelin-cKO compared with Control and Reelin-cKO neurons not treated with the inhibitor. The reduced amplitude after NASPM is consistent with an increased number of GluA2-lacking receptors at Reelin-cKO synapses.

**Surface GluA2 expression is significantly reduced in Reelin cKO mice**

The electrophysiology data suggests an increase in GluA2-lacking AMPARs; thus, we next performed surface biotinylation experiments to determine the AMPAR subunits residing on the surface of Reelin cKO neurons. The slices were treated with D-AP5 (50 μM) and picrotoxin (10 μM) to mimic the conditions of the electrophysiological recording experiments. Our results show a significant reduction in surface GluA2 levels (Control 100.0 ± 21.4%, n = 6, a = 6; Reelin cKO 23.9 ± 6.4%, n = 6, a = 6), along with a trend to a reduction in surface GluA1 (Control 100.0 ± 31.2%; Reelin cKO 76.7 ± 15.3%) and no significant change in surface GluA4 (Control 100.0 ± 23.9%; Reelin cKO 118.0 ± 20.5), providing further evidence that Reelin depletion in adult mice results in an increase in calcium-permeable GluA2-lacking AMPARs (Fig. 5A–C).

**Discussion**

We have shown that Reelin inhibits mGluR-LTD, a type of synaptic plasticity that is exacerbated by Aβ in AD mouse models. Specifically, we show that exogenous Reelin reduces STEP61 levels. Thus, in the presence of DHPG, Reelin suppresses the dephosphorylation of GluA2 receptors leading to the inhibition of mGluR-LTD (Fig. 6A). Conversely, Reelin depletion in the adult mouse brain leads to an increase of STEP61, while causing a reduction in surface expression of GluA2-receptors after NMDAR blockade. Although this finding seems counterintuitive at first, our data suggest that in the Reelin cKO mice, STEP levels are already saturated and GluA2-containing AMPARs are already internalized. Therefore, application of DHPG does not cause further internalization and thus no longer induces LTD (Fig. 6B), an effect known as occlusion, which also occurs in amyloid precursor protein and presenilin-1 mice, which can be rescued by repression of the elf2α kinase PERK (Yang et al., 2016). Importantly there is evidence that ApoE4 treatment in neurons increases excitotoxicity (Buttini et al., 2010). Indeed, our preliminary unpublished data suggest that slices from ApoE4 mice show increased expression of CPARs in an age-dependent manner. This would be consistent with Reelin resistance developing during aging in ApoE4 mice in parallel with ApoE4 mediated risk for AD.

We propose two mechanisms for how Reelin regulates mGluR-LTD: (1) either through directly enhancing GluA2 surface expression and phosphorylation and/or (2) by regulating translation of proteins required for LTD. The first is supported by a study indicating that Reelin enhances the surface expression of AMPARs-containing GluA1 and/or GluA2 (Qiu et al., 2006b). Reelin binding to Apoer2 activates Src family kinases (SFK), which phosphorylate Disabled 1 (Dab1), the first step of Reelin pathway activation. SFKs also phosphorylate GluA2 at residue Y876 (Kohda et al., 2013). Thus, Reelin might have a direct effect on GluA2 phosphorylation through SFKs. Supporting the second mechanism, our current data show that Reelin regulates the protein level of STEP51, an important LTD protein. STEP51 dephosphorylates tyrosines on GluA2 to induce its endocytosis (Won et al., 2019). Reelin reduces STEP51 levels and therefore prevents GluA2 dephosphorylation and endocytosis.

We have shown that exogenous Reelin treatment increases tyrosine (Y869/Y873/876) phosphorylation of GluA2 (Fig. 1D), and others showed that Reelin increases GluA2 surface expression (Qiu et al., 2006a; Qiu and Weeber, 2007). The role of Reelin in increasing surface GluA2 and phospho-GluA2 is further supported by decreased surface GluA2 levels we found in Reelin cKO brain slices. It is likely that Reelin controls surface...
GluA2 through both mechanisms mentioned above. Future studies need to delineate how Reelin regulates STEP$_{61}$ levels. It is possible that Reelin modulates STEP$_{61}$ levels through translational regulation of LTD genes. This model is supported by the finding that Reelin regulates the mTOR and ribosomal protein S6 Kinase (S6K) pathway (Bock et al., 2003; Jossin et al., 2003). S6K regulates translation of LTD proteins like FMRP and STEP$_{61}$. Additionally, our complementary study in Reelin cKO mice shows that STEP$_{61}$ levels are elevated along with a reduction in surface GluA2 receptors (Fig. 5). Consequently, Reelin cKO neurons are unable to respond to DHPG, and LTD is occluded.

The subunit composition of synaptic AMPARs can undergo dynamic changes during physiological as well as pathologic conditions (Weiss, 2011). Physiologically, GluA2-lacking AMPARs are transiently incorporated into the synapse during LTP, which is followed by insertion of GluA2-containing AMPARs that are required for LTD consolidation (Terashima et al., 2008). Pathologically, however, the absence or insufficient expression of GluA2 subunits renders neurons more susceptible to excitotoxicity and neuronal cell death (Liu and Ziskin, 2007). Since their discovery, Ca$^{2+}$-permeable AMPARs (CPARs) have been implicated in the pathogenesis of various neurologic conditions, including epilepsy (Egbenya et al., 2018), amyotrophic lateral sclerosis, ischemic insult (Weiss, 2011), and AD (Gaissler-Salomon et al., 2014). Our data support the findings that in the adult mouse brain, Reelin regulates AMPAR subunit composition during neurophysiological and neurodegenerative conditions. In an autosomal dominant form of temporal lobe epilepsy (TLE) patients have increased GluA2-lacking AMPARs (Egbenya et al., 2018). Interestingly, TLE causes neuronal positioning defects in the dentate gyrus of patients because of deficient Reelin expression (Haas et al., 2002; Haas and Frotscher, 2010; Tinnes et al., 2011). Thus, the link between Reelin deficiency and GluA2-lacking AMPARs might be relevant for TLE as well.

GluA2 mRNA is a substrate of adenosine deaminase acting on RNA (ADAR2) and is normally edited by this enzyme to cause a Q to R change at amino acid 607. AMPARs containing the unedited GluA2(Q) subunit are Ca$^{2+}$ permeable, whereas the presence of edited GluA2(R) renders the receptor Ca$^{2+}$ impermeable (Swanson et al., 1997). AMPAR-containing GluA2(Q) and GluA2-lacking AMPAR are collectively known as CPARs, and play an important role in synaptic and homeostatic plasticity and in the pathophysiology of various neurologic disorders (Henley and Wilkinson, 2016). Recently, it was found that Reelin-expressing stellate cells of the medial entorhinal cortex are subject to age-dependent GABA receptor subunit α3 editing (Berggaard et al., 2019). Whether Reelin can regulate GluA2 editing remains to be determined.

The N terminus of GluA2 is indispensable for hippocampal mGluR-LTD (Zhou et al., 2011) and depletion of GluA2 ablates mGluR-LTD in adult mice (3 months), but not in younger mice (<1 month), suggesting that GluA2 is involved in mGluR-LTD in an age-dependent manner (Cao et al., 2017, 2018). GluA2-dependent mGluR-LTD in mature mice is mediated through Rho GTPase Rac1 and its downstream actin-Regulator cofilin (Zhou et al., 2011). Cofilin phosphorylation is selectively reduced in the mature mouse brain. Conversely, Reelin reduces cofilin activity via phosphorylation through LIMK1 (LIM Domain Kinase 1), a downstream effector of Rac1 (Chai et al., 2009), suggesting that the Reelin/cofilin axis provides another potential LTD-regulating pathway. Thus, it is possible that Reelin is a key regulator in the transition from the juvenile to the mature brain. Future studies are required to dissect these interactions to understand their contributions to overall plasticity and neurodegeneration. Whereas Reelin seems to regulate synaptic plasticity in mature brains dependent on GluA2, other plasticity regulators such as PICK1 (protein interacting with C-Kinase 1) are effective in the juvenile brain independent of GluA2 (Cao et al., 2018).

LTD is induced through activation of Group1 mGluRs (mGluR1 and mGluR5), which induces a cell signaling cascade mediated by the PI3 kinase (PI3K)/Akt and mTOR pathways. This cascade controls the phosphorylation of proteins, for example Fragile X mental retardation protein (FMRP) a critical regulator for the translation of LTD proteins. Phosphorylated FMRP controls protein synthesis by stalling ribosomes on target LTD mRNAs and suppresses the translation of proteins such as STEP$_{61}$, Arc, and MAP1b (Richter and Klann, 2009; Waung and Huber, 2009; Gontopoulos-Pournazis et al., 2020). Importantly, and comparable to AD mouse models, FMRP-KO mice have higher STEP$_{61}$ levels (Chatterjee et al., 2018). In this current study, we have shown that Reelin controls STEP$_{61}$ levels, suggesting a role in protein synthesis.

Dysregulation of the mTOR pathway occurs in numerous neurodegenerative and neurodevelopmental diseases including AD and autism (Hoeffe and Klann, 2010; Sharma et al., 2010). Reelin and mTOR signaling pathways share pivotal hub molecules, such as PI3K and Akt. Whereas Reelin acts upstream of PI3K/Akt (Bock et al., 2003), mTOR acts downstream (Khlebodarova et al., 2018). Heterozygous Reelin-KO mice, a genetic model for schizophrenia, have reduced spine density and abnormal fear memory, which is alleviated by a single in vivo injection of ketamine or a GluN2B antagonist. The rescue effect by ketamine can be prevented by rapamycin, an inhibitor of the mTOR pathway (Iafriati et al., 2014), further bolstering the role of mTOR in Reelin-related psychiatric and neurodegenerative disorders.

Brain-derived neurotrophic factor (BDNF) is an upstream activator of mTOR during memory formation (Slipczik et al., 2009). There is evidence for cross-talk between Reelin and BDNF (Ringstedt et al., 1998); BDNF, by binding to its receptors on the Cajal-Retzius cells of the cerebral cortex, downregulates Reelin expression during early postnatal development. In dendrites of cultured neurons, BDNF activates signaling of both 4E-BP and S6K, translational regulators downstream of mTOR (Takei et al., 2001). BDNF promotes proteolysis of PTEN (phosphatase and tensin homolog deleted on chromosome 10), which is a phosphatase that inhibits Akt activity and signaling to mTORC1 and degradation of tuberous sclerosis complex proteins TSC1 and TSC2, both of which are negative regulators of mTORC1 (Ebert and Greenberg, 2013). Along this line, in heterozygous Reeler mice, decreased levels of PTEN were found in the postsynaptic density fractions (Ventruti et al., 2011).

Local protein synthesis and actin remodeling in neurons involves activation of TrkB receptors by the neurotrophin BDNF and Group1 mGluRs by DHPG. As previously described, DHPG increases local translation and tyrosine phosphatase activity of STEP$_{61}$ to dephosphorylate and induce endocytosis of the GluA2-containing AMPARs. Intriguingly, BDNF increases STEP$_{61}$ degradation through PLCγ-dependent proteosomal activation, which requires tyrosine kinase activity (Saavedra et al., 2016). Activation of mGluRs enhances dendritic protein synthesis through mTOR- and ERK-dependent mechanisms. In summary, our data show that Reelin can reduce STEP$_{61}$ translation induced by DHPG application, suggesting a regulatory function at the level of protein synthesis.
References
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