Reelin signaling modulates GABA<sub>B</sub> receptor function in the neocortex

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Abstract
Reelin is a protein that is best known for its role in controlling neuronal layer formation in the developing cortex. Here, we studied its role for post-natal cortical network function, which is poorly explored. To preclude early cortical migration defects caused by Reelin deficiency, we used a conditional Reelin knock-out (Reln<sup>cKO</sup>) mouse, and induced Reelin deficiency post-natally. Induced Reelin deficiency caused hyperexcitability of the neocortical network in vitro and ex vivo. Blocking Reelin binding to its receptors ApoER2 and VLDLR resulted in a similar effect. Hyperexcitability in Reln<sup>cKO</sup> organotypic slice cultures could be rescued by co-culture with wild-type organotypic slice cultures. Moreover, the GABA<sub>B</sub> receptor (GABA<sub>B</sub>R) agonist baclofen failed to activate and the antagonist CGP35348 failed to block GABA<sub>B</sub>Rs in Reln<sup>cKO</sup> mice. Immunolabeling of Reln<sup>cKO</sup> cortical slices revealed a reduction in GABA<sub>B</sub>R1 and GABABR2 surface expression at the plasma membrane and western blot of Reln<sup>cKO</sup> cortical tissue revealed decreased phosphorylation of the GABA<sub>B</sub>R2 subunit at serine 892 and increased phosphorylation at serine 783, reflecting receptor deactivation and proteolysis. These data show a role of Reelin in controlling early network activity, by modulating GABA<sub>B</sub>R function.

KEYWORDS
ApoER2 and VLDLR, calcium imaging, GABAB receptors, network activity, Reelin

Abbreviations: 4-OHT, (Z)-4-hydroxytamoxifen; ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; APV, (2R)-amino-5-phosphonopentanoate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; Cux-1, cut-like homeobox 1; DAB1, disabled-1; DIV, days in vitro; F<sub>0</sub>, baseline fluorescence; GABA, gamma-aminobutyric acid; GAD65, glutamate decarboxylase-65; LRPPAP Protein, RAP, LDL receptor-related protein-associated protein 1; OGT, 1-am, oregon green BAPTA-1 acetoxymethyl; OTCs, organotypic cultures; P, post-natal day; Parv, parvalbumin; PFA, paraformaldehyde; PTX, pertussis toxin; Reln<sup>cKO</sup>, Reelin conditional knock-out mice; ROI, region of interest; RRID, research resource identifier; TBS, Tris-buffered saline; WFS, wolframin 1; WT, Reln<sup>flox/flox</sup> wild-type.
Reelin is an extracellular glycoprotein that controls several aspects of mammalian brain development and function. The most prominent role of Reelin is the control of neuronal migration and layer formation in the developing cerebral cortex, as evidenced by numerous histological studies on reeler mutant mice that lack Reelin expression because of a defect of the Reelin gene (Caviness, 1976; Curran & D’Arcangelo, 1998; Lambert de Rouvroit & Goffinet, 1998). Through signaling via its membrane receptors (Bock & May, 2016; Cooper & Howell, 1999), Reelin guides the migration of newborn neurons and orchestrates the development of cortical layers. In the developing and adult brain, the majority of GABAergic interneurons in the neocortex expresses Reelin. The canonical Reelin signaling cascade involves direct binding of Reelin to ApoER2 and VLDLR and subsequent activation of the intracellular adapter protein disabled-1 (DAB1) by tyrosine phosphorylation (Cooper & Howell, 1999; D’Arcangelo et al., 1999; Hiesberger et al., 1999; Howell, Herrick, Hildebrand, Zhang, & Cooper, 2000; Trommsdorff et al., 1999).

In the absence of Reelin or its receptors, the process of neuronal migration is compromised, which causes severe abnormalities in cortical laminar architecture. The resulting phenotype was initially described as an inversion of the layers (Caviness, 1982; Caviness & Sidman, 1973). Although it had already been shown that barrel formation in the somatosensory cortex of the reeler is compromised (Caviness, 1976; Welt & Steindler, 1977), it has been recently demonstrated that the barrel field retains its proper somatotopic organization (Guy, Wagener, Möck, & Staiger, 2015; Wagener, Dávid, Zhao, Haas, & Staiger, 2010). Besides its cortical migration defects, the reeler mutant exhibits cerebellar hypoplasia and a neurological phenotype that is characterized by ataxia (Miyata, Nakajima, Mikoshiba, & Ogawa, 1997). A similar phenotype was observed in human patients carrying homozygous mutations in the Reelin gene, resulting in lissencephaly and cerebellar hypoplasia (Hong et al., 2000). Moreover, in humans, heterozygous Reelin mutations were described that cause autosomal-dominant temporal lobe epilepsy (Dazzo et al., 2015).

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian CNS and plays a key role in modulating neuronal activity. GABA mediates its action via two different classes of receptors, ionotropic receptors (GABA_A, GABA_B) and metabotropic GABA_B receptors. The GABA_A receptors are guanine nucleotide-binding protein (G protein)-coupled metabotropic receptors that modulate Ca^{2+} and potassium (K') channels, and elicit both pre-synaptic and slow post-synaptic inhibition (Benaroch, 2012; Bettler, Kaupmann, Mosbacher, & Gassmann, 2004). In particular, pre-synaptic GABA_ARs are coupled to Ca^{2+} channels, regulating the release of neurotransmitters, while post-synaptic GABA_ARs are coupled to K' inward rectifying (Kir) channels (Kir3), regulating post-synaptic slow inhibition (Pinard, Seddik, & Bettler, 2010). Remarkably, GABA does not mediate hyperpolarization-dependent inhibition during early development, since GABA_AR signaling is mainly depolarizing and excitatory during this period, while GABA_ARs are uncoupled from G-proteins and Kir3 channels until the end of the first post-natal week (Ben-Ari et al., 2012; Fukuda, Mody, & Prince, 1993; Owens & Kriegstein, 2002). Moreover, GABA is abundant in the neonatal nervous system and activates GABA_BRs (Ben-Ari, Gaiarsa, Tyzio, & Khazipov, 2007). In the cortex, GABA_R1 and GABA_R2 were detected by immunocytochemistry as early as E14, and GABA_B2Rs were found to colocalize with GABA_ARs in neurons of the marginal zone and the subplate, indicating that these proteins are coexpressed and could be forming functional GABAergic synapses during perinatal development in vivo (López-Bendito et al., 2002). In the murine neocortex, Reelin haploinsufficiency has been shown to disrupt the developmental trajectory of GABA excitation/inhibition balance (Bouamrane et al., 2016), suggesting a possible interaction between Reelin and the GABA receptors.

Adult conditionally induced Reln^cKO mice exhibited altered hippocampal LTP and a subtle behavioral phenotype while the cortical architecture was shown to be indistinguishable from their wild-type littermates (Lane-Donovan et al., 2015). The question whether Reelin deficiency affects early cortical neuronal activity remains to be solved. To address this question, we investigated the effect of post-natally induced Reelin deficiency on Ca^{2+} signaling and on synaptic function in the neocortex of Reln^cKO mice. Since synaptic release is mainly controlled by GABA_ARs that mediate inhibitory GABA effects around the second post-natal week, we focused here on a potential interaction between Reelin signaling and GABA_AR function.

2 | MATERIAL AND METHODS

2.1 | Reelin conditional knockout mice (Reln^cKO)

Animals were housed in a standard 12-hr light cycle and fed ad libitum with standard mouse chow. All care and use of experimental animals were respected according to the Federal German law with permission Nr. 84-02.04.2016.A383 and the ARRIVE guidelines. The generation of the conditional Reln^cKO line was previously described (Lane-Donovan et al., 2015). To obtain conditional Reelin knockout mice (Reln^lox/lox CAG-CreERT2), we crossed Reln^lox/lox mice with hemizygous tamoxifen-inducible Cre recombinase expressing mice (CAG-CreERT2) (Hayashi & McMahan, 2002). For the experiments, only Reln^lox/lox CAG-CreERT2 male mice were selected and then crossed with Reln^lox/lox female mice to generate Reln^lox/lox wild type (WT) and Reln^lox/lox CAG-CreERT2 (Reln^cKO) siblings as verified by PCR. The cKO mouse line ubiquitously expresses a fusion protein comprising Cre recombinase and a mutated form of the estrogen receptor (Cre-ERT2). Tamoxifen administration induces nuclear Cre activation and knockout of the floxed Reelin gene. 20 μl tamoxifen (Cat# T5648; Sigma) was dissolved in corn oil at a concentration of 20 mg/ml by shaking overnight at 37°C and fed to newly born pups at P1 for five consecutive days. At P14 animals were deep anesthetized with isoflurane CP® (CP-Pharma) to minimize suffering and killed by decapitation. Ex vivo slices from somatosensory cortex were explanted from each animal for acute slice preparation.
at P14 or at P0 for organotypic slice cultures (OTCs) preparation by respecting the coordinates indicated in Allen mouse brain atlas. A graphical flow chart for ex vivo experimental procedures is shown in (Figure 1a). For each outcome measure, we used 5 RelnWT and 5 RelnKO mice (each experiment was repeated three times). In total, 390 animals were used for this study. From each animal, we obtained 3–4 slices. We recorded from each slice three different areas of interests (ROI). In each area of interest, we averaged amplitude, frequency and Ca2+ transient half-width of 6 cells. The average of the three ROI has been plotted in the box plot as single value. This study was not pre-registered. No exclusion criteria were pre-determined and the study was exploratory. No blinding, randomization was performed to allocate subjects in the study. No sample size calculation was performed in this study.

2.2 | PCR and genotyping

All samples were stored at –20°C until PCR analysis. DNA from samples of ear, tail, and brain tissue were isolated with ReliaPrep gDNA kit (Cat# A205; Promega). All procedures were performed according to protocols provided by the manufacturer. The amounts of DNA isolated from the various samples were determined by spectrophotometry with the Genova Nano system (Jenway). DNA was amplified by PCR. PCR reactions were performed in a total volume of 50 μl reaction mixture containing 200 ng of template DNA, Soriano buffer (0.67 M Tris, 0.16 M ammonium sulphate, 67 mM MgCl2, 67 μM EDTA and 50 mM β-Mercaptoethanol), Taq polymerase, 2 μl DMSO, and 10 mM dNTPs. For genotyping we used the following primers: wild-type mice, forward primer 5′-ATAAATCTGGCTGTATGTGACAGG-3′, reverse primer 5′-CTGTATGTGACAGG-3′; flox/flox mice, forward primer 5′-GCTCTGGCCAAGCTTTATC-3′, reverse primer 5′-AGACAATGCTAACAACAGCAAGC-3′ (450 bp). transfection, glutamate receptors were temporarily blocked by Baclofen (10 μM, Cat# 0417; Tocris), CGP35348 (10 μM, Cat# 1245/10; Tocris), PP2 (1 μM, Cat# 1407/10; Tocris), pertussis toxin (PTX, 500 ng/ml, Cat# 3097/50U; Tocris), CNQX (10 μM, Cat# 0190/10; Tocris) and APV (50 μM, Cat# 0190/10; Tocris). As an inhibitor of low-density lipoprotein receptor–related proteins, we used the recombinant Mouse LRAP Protein (LDL receptor-related protein-associated protein 1; also named receptor-associated protein (RAP), 50 ng/ml, Cat# 4480-LR; R&D Systems). RAP serves as a molecular chaperone for LDL receptor family proteins including VLDL and APOER2 and prevents interaction of ligands with these receptors. Therefore, RAP is used to block the canonical Reelin pathway by blocking Reelin from binding to VLDL and APOER2 (Bu & Schwartz, 1998; Gong, Wang, Huang, & Parent, 2007; Herz, Goldstein, Strickland, Ho, & Brown, 1991).

2.4 | Expression plasmids and biolistic transfection

Transfection was performed using a Helios Gene Gun (Bio-Rad) as described previously (Wirth & Wahle, 2003). In brief, cartridges were prepared by coating 10 mg gold particles (Ø = 1µm; Bio-Rad) with genetically encoded Ca2+ indicator pG-P-CMV-GCaMP6s (GCaMP6s). GCaMP6s was a gift from Douglas Kim (RRID: Addgene_40753) (Chen et al., 2013). To prevent excitotoxicity during transfection, glutamate receptors were temporarily blocked with 3 mM kynurenic acid (Cat# K3375; Sigma) and 50 mM APV (Cat# A5282; Sigma) before blasting. The blockers were washed out 3 hr after transfection.

2.5 | Ca2+ imaging using Spinning disc laser microscopy

For studying network activity, Ca2+ imaging was performed in somatosensory cortex of supragranular layers II/III with the Ca2+ indicator Oregon Green BAPTA-1 Acetoxymethyl ester (OGB-1 AM) (Cat# O6807; Molecular Probes). Acute slices (P14) or OTCs DIV14 were loaded according to our previously published protocol (Hamad, Krause, & Wahle, 2015). In brief, a solution of 20% PF127 (Cat# P2443; Sigma) dissolved in DMSO (w/v) (J.T. Baker) was prepared to dissolve OGB-1 AM. The final loading solution concentration was 1 μM OGB-1 AM. After loading, the slices were washed several times to remove excess dye and allowed to recover for an
FIGURE 1  Recording of neuronal activity in Reln\textsuperscript{cKO} mice. (a) Graphical flow chart of experimental procedures. In each experiment, slices were prepared from 5 WT and 5 Reln\textsuperscript{cKO} mice. (b): Confocal image examples of OGB-1 AM loaded P14 acute neocortical WT and Reln\textsuperscript{cKO} slices. Scale bars: 20 µm. (c): Typical example of spike waveform from a recorded cell which shows amplitude and Ca\textsuperscript{2+} transient (spike) half-width. (d): The box plot in the graph represents the values of maximal increase in Ca\textsuperscript{2+} signal amplitude in control WT and Reln\textsuperscript{cKO} acute slices expressed as ΔF/ F\textscript{0}, which is unaltered. (e): The Ca\textsuperscript{2+} frequency is significantly higher in the Reln\textsuperscript{cKO} when compared to WT, and (f) the Ca\textsuperscript{2+} transient half-width is significantly increased in Reln\textsuperscript{cKO} when compared to WT. Mann–Whitney U test; ***p < .001. The number of acute slices analyzed is indicated above the box plots in (d). To assess pyramidal cells and interneurons separately, acute slices were transfected with GCaMP6s plasmid to visualize the cell type (g–j). (g): Confocal images of a GCaMP6s transfected pyramidal cell in a WT P14 acute neocortical slice during Ca\textsuperscript{2+} imaging at resting fluorescence (F\textscript{0}) and at maximal amplitude peak. Scale bars: 20 µm. (h): The box plot in the graph shows no change in maximal Ca\textsuperscript{2+} amplitude signals between control WT and Reln\textsuperscript{cKO} groups for both cell types, but shows (I and J) a significant increase in Ca\textsuperscript{2+} frequency and Ca\textsuperscript{2+} transients half-width in Reln\textsuperscript{cKO} recorded slices in comparison to WT for both pyramidal cells and interneurons. One-way ANOVA on Ranks followed by Dunn’s Multiple Comparison Test, ***p < .001 and **p < .01. The number of single cells in (h–j) is indicated above the box plots in (h). Data are obtained from three independent acute slice preparations.

FIGURE 2  Secreted Reelin rescues impaired Ca\textsuperscript{2+} frequency in Reln\textsuperscript{cKO} OTCs. (a) A graphical flow chart for OTCs experimental procedure. (b) Schematic representation of the co-culture experimental approach. Left: Two co-cultured WT OTCs. Right: Two co-cultured Reln\textsuperscript{cKO} OTCs. (c) Co-culture medium lysates from DIV14 OTCs (40 OTCs obtained from 5 mice for each experimental group) were analyzed by western blot. (d) Quantification of the western blot 180-kDa Reelin signals. An actin antibody was used as loading control. The densitometric quantification showed a decreased Reelin protein level in the WT + Reln\textsuperscript{cKO} co-culture medium and almost complete absence in the Reln\textsuperscript{cKO} + Reln\textsuperscript{cKO} co-culture medium. One-way ANOVA followed by Holm–Sidak Multiple Comparison Test, ***p < .001 WT + Reln\textsuperscript{cKO} compared to WT + WT; ***p < .001 WT + Reln\textsuperscript{cKO} compared to Reln\textsuperscript{cKO} + Reln\textsuperscript{cKO}. At 14 DIV, OTCs were loaded with OGB-1 AM and recorded. (e) The box plot in the graph represents the values of maximal increase in Ca\textsuperscript{2+} signal amplitude, which shows no change in all experimental groups. (f and g) The box plot shows a significant increase in Ca\textsuperscript{2+} frequency and Ca\textsuperscript{2+} transient half-width in Reln\textsuperscript{cKO} co-cultured OTCs. Note that the increased frequency and Ca\textsuperscript{2+} transients half-width observed in the Reln\textsuperscript{cKO} OTC are restored to a normal level when it is co-cultured together with a WT OTC. The number of recorded OTC is indicated above the box plots in (e). One-way ANOVA on Ranks followed by Dunn’s Test, ***p < .001. Data are obtained from three independent OTCs preparations.
the resting fluorescence). Raw data delivered in the form of a linear 16-bit intensity scale were plotted as fluorescence intensity versus time. Pyramidal cell or interneuron somata were chosen as the ROI. The background fluorescence measured near a ROI was then subtracted from these raw data. The baseline fluorescence ($F_0$) was calculated as an average of 20 frames in a time window without neuronal activity (as judged by visual inspection). Subsequently, data were normalized to the mean fluorescence intensities $[\Delta F/F_0 = (F - F_0)/F_0]$, allowing the comparison of data across experiments. Spike (calcium transient) half-width was calculated as the width of the spike at half-maximal amplitude (Weir, Blanquie, Kilb, Luhmann, & Sinning, 2014).
2.6 Immunohistochemistry and surface labeling

At DIV10, OTCs were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 and warmed to 36°C for 1 hr. After washing twice in Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.6) and permeabilization in TBST (TBS, 0.1% Triton X), OTCs were blocked for 1 hr with 1% normal goat serum in TBST. Primary antibodies diluted in TBST were incubated for 24 hr at 23°C. The following antibodies were used: Mouse anti-Reelin G10 (Cat# MAB5364, RRID:AB_2179313, 1:1,000), rabbit anti-Cre recombinase (Cat# 69050, RRID:AB_2314229, 1:2000), rabbit anti-Cux1 (CULT-1, Cat# ab140042; Abcam, 1:500), rabbit anti-Wolframin 1 (Cat# 11558-1-AP, RRID:AB_2216046, 1:2000), mouse anti-Parvalbumin (Parv, Cat# 235, RRID:AB_10000343, 1:3,000), rabbit anti-glutamate decarboxylase-65 (Cat# G5038, RRID:AB_259920, 1:1,000). After washing twice in TBS, the secondary antibodies were added accordingly: Goat anti-mouse or goat anti-rabbit biotinylated (Cat# E043201-8; Dako, 1:300). After several washes in TBS buffer, the slices were subjected to the ABC-horseradish peroxidase method using diaminobenzidine as chromogen. For immunofluorescence, the slices were subsequently washed 3 × 15 min with TBST and incubated with secondary antibodies (anti-mouse IgG coupled to Alexa594 and anti-rabbit IgG coupled to Alexa488) in TBS (ThermoFisher, 1:300) for 30 min at 23°C. After three repetitive washings with TBS-Tween, the OTCs were mounted with sRIMS mounting medium (70% sorbitol w/v in 0.02 M phosphate buffer with 0.01% sodium azide, pH 7.5). Fluorescence was analyzed with the Visiscope spinning-disc confocal system as mentioned above. For GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 surface labeling, P14 WT and Reln<sup>cko</sup> acute slices were fixed with 4% paraformaldehyde. After several washes in TBS, a group of acute slices was permeabilized in TBST to detect total GABA<sub>B</sub>Rs and the other group was not permeabilized to detect only surface expression. The slices were blocked and incubated with the following primary antibodies that recognize only the N-terminus (extracellular) domain of GABA<sub>B</sub>R1 (mouse anti-GABA<sub>B</sub>R1, Cat# ab55051, RRID:AB_941703, 1:500) or the N-terminus (extracellular) domain of GABA<sub>B</sub>R2 (Rabbit anti-GABA<sub>B</sub>R2, Cat# 4819, RRID:AB_2108339, 1:750) for 24 hr at 23°C. The slices were then washed and incubated with appropriate secondary fluorescent antibodies for 30 min. After repetitive washings with TBS, the slices were mounted with sRIMS buffer and mean fluorescence intensity was measured from layers II/III somatosensory cortices with the Visiscope spinning-disc confocal system.

2.7 Western blotting

Equal amounts of cortical tissue samples (22.5 μg) were homogenized in urea assay lysis buffer (100 mM Tris-HCl, pH = 7.5; 12 mM magnesium acetate tetrahydrate and 6M urea) with protease and phosphatase inhibitors and then centrifuged at 10,000 g for 15 min to remove debris and nuclei. The SDS loading buffer was run on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane for 1 hr 40 min in transfer buffer (25 mM Tris/Base, 192 mM glycerine). Membranes were then blocked for 1 hr at 23°C while shaking in blocking buffer with TBS (Cat# P/N 927-50000; Li-Cor). Membranes were incubated overnight (O/N) at 4°C with the primary antibody and for 2 hr at RT with the secondary antibody. After each incubation step, membranes were washed for 3 × 15 min in TBS with 0.1% Tween 20. Membranes were imaged with the Odyssey immunoblot software (Lincoln). The Odyssey software system was also used for densitometric analysis. The following antibodies were used: Rabbit anti-GABA<sub>B</sub>R2 (Cat# 4819, RRID:AB_2108339, 1:500), mouse anti-GABA<sub>B</sub>R1 (Cat# ab55051; Abcam, 1:500), rabbit anti-GABA<sub>B</sub>R2 p-Ser 783 (Cat# TA309142; OriGene Technologies, 1:1,000), p-GABA<sub>B</sub>R2 S892 (Cat# PPS073, RRID:AB_2108325, 1:1,000), mouse anti-Reelin G10 (Cat# MAB5364, RRID:AB_2179313, 1:1,000), and B-Actin (Cat# ab8227, RRID:AB_2305186, 1:10,000).

2.8 Statistical analysis

Statistical analysis was performed with Sigma Stat 12 (SPSS Incorporated). Data are presented as box plots with median (center line), minimum and maximum (whiskers), and 25th–75th percentiles (box). Comparisons between two groups were performed with Student’s unpaired t test when normality test (Shapiro–Wilk) passed, otherwise the Mann–Whitney test. Comparisons between groups larger than two were performed with one-way ANOVA and a Holm–Sidak multiple comparison test for post hoc analysis if normality test passed. If normality failed, we run one-way ANOVA on ranks followed by Tukey’s multiple comparison test for post hoc analysis to isolate the significant groups. If the sample sizes were unequal, the Dunn’s multiple comparison test was used to isolate the significant groups. Results were considered statistically significant at p < .05.

3 RESULTS

3.1 Cortical layering is not altered after post-natal loss of Reelin in Reln<sup>cko</sup> mice

To bypass the cortical layer malformations that are present in the reeler mutant, tamoxifen was fed to newborn pups (~P1) for 5 consecutive days. Around P14, animals were sacrificed, and sections of cortical tissue were subjected to immunohistochemical staining (Figure S1). First, we confirmed that tamoxifen administration induces nuclear Cre activation and knockout of the floxed Reelin gene. We found that tamoxifen administration at P1 induced complete elimination of Reelin immunostaining at P14 (Figure S1a). The pattern of immunostaining against wolframin (Wfs1), a protein expressed by layer II and IV neurons in the mouse somatosensory cortex, was indistinguishable from WT (Figure S1b), while the layer-specific distribution of this protein is disrupted in the reeler cortex (Boyle et al., 2011). Next, we examined the distribution of cut-like
homeobox 1 (Cux-1) protein, a transcription factor expressed in neurons throughout layers II–IV. In the cortex of adult reeler mice, most Cux-1-positive neurons are found in cortical layers V and VI and do not respect the strict boundary that characterizes wild-type cortical layering (Nieto et al., 2004). The distribution of Cux-1 immunostaining in the cortex of Reln<sup>cKO</sup> mice was found in the supragranular layers II–IV and was indistinguishable from WT (Figure S1c). We then examined the distribution of the fast-spiking interneuron marker Parvalbumin (Parv) and found it to be unchanged in the Reln<sup>cKO</sup> when compared to wild-type mice (Figure S1d). These findings confirm that post-natal Reelin elimination did not alter the distribution of layer-specific expression markers in the neocortex.

### 3.2 Excessive Ca<sup>2+</sup> spike frequency in early post-natally Reelin-deficient Reln<sup>cKO</sup> mice

Using Ca<sup>2+</sup> imaging, we compared spontaneous activity in acute slices prepared from WT and Reln<sup>cKO</sup> littermates at P14 (both pup groups were fed with tamoxifen). In our experience (Hamad et al., 2015) as well as in other studies (Yuste, MacLean, Vogelstein, & Paninski, 2011), imaging a large network of neuronal populations loaded with the calcium indicator OGB-1 AM under a spinning-disk confocal microscope, equipped with fast cameras, can be used to image thousands of neurons simultaneously without significant photobleaching, with good signal-to-noise ratio and minimized cellular damage because it does not require electrodes penetrating the tissue. To ensure that tamoxifen per se did not alter basic synaptic transmission, we recorded Ca<sup>2+</sup> amplitude and frequency in DIV14 (days in vitro 14) OTCs loaded with OGB-1 AM from WT mice which were stimulated for five consecutive days either with 4-OHT or vehicle (DMSO) as a control group (Figure S2). Neither 4-OHT nor vehicle groups showed any difference in Ca<sup>2+</sup> amplitude, frequency or Ca<sup>2+</sup> transient half-width when compared to the untreated WT control group (Figure S2). Next, we compared Ca<sup>2+</sup> signal amplitude, frequency and Ca<sup>2+</sup> transient half-width in WT and Reln<sup>cKO</sup> slices using the Ca<sup>2+</sup> indicator OGB-1 AM (Figure 1b–f). Ca<sup>2+</sup> amplitudes were not altered in Reln<sup>cKO</sup> when compared to the WT control group (Figure 1d). Strikingly, the Ca<sup>2+</sup> frequency and Ca<sup>2+</sup> transient half-width were significantly increased in Reln<sup>cKO</sup> (Figure 1e,f). Recordings were performed in acute slices prepared at P14 and transfected with a genetic construct encoding the Ca<sup>2+</sup> indicator GCaMP6s (Chen et al., 2013). GCaMP6s is very sensitive to Ca<sup>2+</sup> changes and we found it to be distributed in soma, dendrites, and axon (Figure 1g). Criteria to distinguish pyramidal cells and interneurons were based on dendritic and axonal patterns (Hamad et al., 2011, 2014; Karube, Kubota, & Kawaguchi, 2004; Kawaguchi, Karube, & Kubota, 2006). To confirm the cell type, after Ca<sup>2+</sup> recording, acute slices were fixed and stained against glutamate decarboxylase-65 (GAD-65) which labels the GABA biosynthesis enzyme in the soma of all GABAergic interneurons but does not label glutamatergic pyramidal cells (Figure S3). Our results confirmed an unchanged amplitude in Reln<sup>WT</sup> compared to Reln<sup>cKO</sup> in both pyramidal cells and interneurons (Figure 1h) and revealed an enhanced frequency and Ca<sup>2+</sup> transient half-width in both pyramidal cells and interneurons in the Reln<sup>cKO</sup> (Figure 1i,j), suggesting that Reln<sup>cKO</sup> mice exhibit defects at the pre-synaptic level. Moreover, Ca<sup>2+</sup> amplitude and Ca<sup>2+</sup> transient half-width did not differ between pyramidal cells and interneurons in both WT and Reln<sup>cKO</sup> slices during GCaMP6s recordings, confirming a previously published study (Weir et al., 2014). Therefore, the subsequent recording experiments were performed with the calcium dye OGB-1 AM.

### 3.3 Wild-type Reelin rescues Ca<sup>2+</sup> frequency in Reln<sup>cKO</sup> neurons

Next, we investigated whether secreted Reelin from WT OTCs might rescue the abnormal neuronal activity observed in the Reln<sup>cKO</sup> OTCs. To address this question, we co-cultured OTCs as follows: WT + WT, or Reln<sup>cKO</sup> + Reln<sup>cKO</sup>, or WT + Reln<sup>cKO</sup> (Figure 2b). To detect the abundance of secreted Reelin in the culture medium under these three conditions, we quantified secreted Reelin protein with western blot from culture medium. As expected, the amount of secreted Reelin was abundant in the WT + WT, a lesser amount was detectable in the WT + Reln<sup>cKO</sup>, whereas Reln<sup>cKO</sup> was almost undetectable in the Reln<sup>cKO</sup> + Reln<sup>cKO</sup> culture medium (Figure 2c,d). Ca<sup>2+</sup> recordings experiments showed that the Ca<sup>2+</sup> amplitude did not change under any of the three experimental conditions (Figure 2e) confirming our interpretation that Reelin does not affect post-synaptic activity. However, in the Reln<sup>cKO</sup> OTCs co-cultured with WT, the Ca<sup>2+</sup> frequency and Ca<sup>2+</sup> transient half-width in the recorded Reln<sup>cKO</sup> OTC was reduced to the same level as in the WT OTC (Figure 2f,g). These results suggest that Reelin secreted from WT OTCs into the incubation medium restored pre-synaptic activity in Reln<sup>cKO</sup> OTCs to a basic level comparable to the WT control group.

### 3.4 GABA<sub>B</sub>R function is impaired in Reln<sup>cKO</sup> mice

GABA<sub>B</sub>Rs are present in GABAergic neuronal terminals (as autoreceptors) and in glutamatergic and other terminals (heteroreceptors) (Benarroch, 2012). Around the second post-natal week, the only source of Reelin in the neocortex are inhibitory interneurons (Pohlkamp et al., 2014). We speculated that the increased Ca<sup>2+</sup> spiking frequency in Reln<sup>cKO</sup> mice in pyramidal cells and interneurons that we observed, was likely due to a defect of GABA<sub>B</sub>R function at the GABAergic or glutamatergic pre-synaptic terminal. If pre-synaptic GABA<sub>B</sub>Rs at the GABAergic neuronal terminals were defective in Reln<sup>cKO</sup> mice, activated GABA<sub>B</sub>Rs would no longer be able to block Ca<sup>2+</sup> channels because pre-synaptic GABA<sub>B</sub> receptors inhibit N type (Cav2.2) or P/Q type (Ca,2.1) Ca<sup>2+</sup> channels, resulting in reduced neurotransmitter release (Benarroch, 2012). In turn, Ca<sup>2+</sup> channels trigger the release of GABA, and thereby inhibit neighboring cells. However, Reln<sup>cKO</sup> mice exhibited a higher Ca<sup>2+</sup> frequency
when compared to WT control (Figure 1), suggesting that pre-synaptic GABA_B Rs at GABAergic neuronal terminals were not defective in Reln^cKO mice. Therefore, we tested a second possibility, i.e. defective pre-synaptic GABA_B Rs at glutamatergic terminals in Reln^cKO mice. To test this possibility, we recorded WT and Reln^cKO OTCs in the presence of the AMPA- and kainate receptor blocker CNQX and the NMDARs antagonist APV at DIV14 (Figure S4). Bath application of 10 µM CNQX immediately decreased the Ca^{2+} amplitude, frequency and Ca^{2+} transient half-width in Reln^cKO to the same extent as in the WT control group (Figure S4a,b). Similarly, APV application elicited the same effect (Figure S4d–f). The observation that the excessive Ca^{2+} frequency in the Reln^cKO can be blocked with CNQX or APV suggests that glutamatergic signaling in the absence of Reelin is still functional. Taken together, we conclude from our findings that most likely GABA_B Rs at glutamatergic pre-synaptic terminals are defective in the Reln^cKO. Moreover, we preclude a possible defect of GABA_B Rs at the post-synaptic site because no significant change in the Ca^{2+} amplitude was observed (Figure 1).

To examine a possible crosstalk between Reelin signaling and GABA_B function, we prepared OTCs from P0 mice. OTCs were treated with 4-OHT at DIV1-5 to induce Reelin deficiency. Subsequent Reelin knockout, Ca^{2+} recordings were performed around DIV14 (Figure 3). First, to confirm conditionally induced Reelin knockout, OTCs were fixed at DIV14 and immunostained against Cre-recombinase and Reelin (Figure S5). While pronounced Reelin staining was seen in WT OTCs, it was absent in Reln^cko OTCs.

Subsequent Reelin knockout, Ca^{2+} recordings were performed around DIV14 (Figure 3). First, to confirm conditionally induced Reelin knockout, OTCs were fixed at DIV14 and immunostained against Cre-recombinase and Reelin (Figure S5). While pronounced Reelin staining was seen in WT OTCs, it was absent in Reln^cko OTCs. Bath application of the GABA_B agonist baclofen (10 µM) immediately activated GABA_B Rs and strongly decreased the Ca^{2+} amplitude by sixfold in WT OTCs, but to a lesser extent (1.6-fold) in Reln^cko OTCs (Figure 3a), suggesting that post-synaptic GABA_B Rs were still functioning in Reln^cko OTCs. However, we do not preclude a post-synaptic functional difference between WT and Reln^cko. Moreover, baclofen induced a profound reduction in the Ca^{2+} frequency and Ca^{2+} transient half-width in WT OTCs (Figure 3b,c and Movie S1), but no effect was observed in Reln^cko OTCs (Figure 3b,c and Movie S2). This suggests an altered GABA_B function at pre-synaptic sites in Reln^cko, probably because of a lack of trafficking and localization of GABA_B Rs at the plasma membrane in Reln^cko mice. Strikingly, the GABA_B antagonist CGP35348 (10 µM) did not influence the Ca^{2+} amplitude, neither in WT nor in Reln^cko OTCs (Figure 3d). However, blockade of GABA_B Rs in WT OTCs dramatically increased the Ca^{2+} signaling frequency and Ca^{2+} transient half-width in WT but not in Reln^cko OTCs (Figure 3e,f). Taken together, these results indicate a pre-synaptic dysfunction of GABA_B Rs in the Reln^cko OTCs.

3.5 | Pre-synaptic GABA_B Rs are modulated by Reelin signaling

Based on our observation that pre-synaptic GABA_B Rs do not properly function in the absence of Reelin, we wondered whether the function of GABA_B Rs might be modulated by canonical Reelin signaling. To address this question, we treated OTCs with RAP, an inhibitor of low-density lipoprotein receptor-related proteins (including VLDL and APOER2; (Herz et al., 1991; Willnow et al., 1996)). Reelin acts via binding to VLDLR and ApoER2 to regulate Dab1 tyrosine phosphorylation (Cooper & Howell, 1999; Trommsdorff et al., 1999). We have found that chronic RAP treatment (50 ng/ml, over 5 days) did not affect the Ca^{2+} amplitude in Reln^cko and WT OTCs (Figure 4a). However, RAP blockade in WT OTCs enhanced the Ca^{2+} frequency and Ca^{2+} transient half-width to a comparable extent as seen in the Reln^cko without RAP treatment (Figure 4b,c). As expected, Reln^cko OTCs treated with RAP did not exhibit any changes in frequency and Ca^{2+} transient half-width (Figure 4b,c). These observations suggest that GABA_B Rs require crosstalk with Reelin signaling for their proper function.

Reelin activates members of Src family tyrosine kinases (SFKs) via ApoER2 and VLDLR and Dab1 (Bock & Herz, 2003; Cooper & Howell, 1999). Therefore, we addressed the question whether inhibition of Src family kinases with the pharmacological blocker PP2 might affect GABA_B function. Our results show that the application of 1 µM PP2 at DIV14 did not alter the Ca^{2+} amplitude neither in WT nor in Reln^cko OTCs (Figure 5a). In turn, the Ca^{2+} frequency and Ca^{2+} transient half-width were increased in WT only but not Reln^cko OTCs (Figure 5b,c). To test whether the inhibition of Src family kinases with the pharmacological blocker PP2 might also affect GABA_B surface expression, OTCs from WT and Reln^cko mice were treated with 1 µM PP2 and immunostained against GABA_B1 and GABA_B2 under non-permeabilized condition to detect their surface expression (Figure S6). The surface expression of GABA_B1 and GABA_B2 was significantly reduced in WT OTC but remained unaltered in the Reln^cko. Together, these results suggest that Reelin signaling through Src is important to maintain GABA_B Rs function and surface expression. In line with these findings, a previous study had shown that the expression of SNAP25, a protein that is involved in the control of transmitter release, was decreased in the hippocampus of reeler mutant mice, but not altered in ApoER2-/-, VLDLR-/- or Dab1-deficient mice (Hellwig et al., 2011). Moreover, GABA_B Rs were found to be down-regulated in the reeler mutant (Cremer, Lübke, Palomero-Gallagher, & Zilles, 2011). Taken together, our findings suggest that pre-synaptic GABA_B function is modulated through the Reelin signaling cascade.

3.6 | Reelin signaling alters GABA_B2 cell surface expression and its phosphorylation at S783 and S892

Previous studies have demonstrated that GABA_B Rs do not undergo agonist-induced internalization (Terunuma, Pangalos, Pangalos, & Moss, 2010), and that GABA_B cytoplasmic domains contain multiple phosphorylation sites (Couve et al., 2002; Fairfax et al., 2004). To find out whether Reelin deficiency might affect GABA_B cell surface expression, we performed immunofluorescence staining of GABA_B1 and GABA_B2 with specific antibodies that recognize only the extracellular N-terminal domain of the
receptor subunits (Figure 6). Mean fluorescence intensity analysis revealed that both GABA_A1 and GABA_A2 surface staining was significantly reduced in non-permeabilized cells of Reln^cKO acute slices in comparison to WT (Figure 6), suggesting a role of Reelin in regulating GABA_A cell surface expression. Next, we investigated whether secreted Reelin from WT OTCs might rescue the reduction of GABA_A1 and GABA_A2 surface expression in the Reln^cKO. To address this question, we co-cultured OTCs from WT + Reln (Figure S7). Our results show that Reelin secreted from WT OTCs into the incubation medium maintained GABA_A1 and GABA_A2 expression in Reln^cKO at a level comparable to the WT control group.

Finally, we performed western blotting, to assess the amount of expressed GABA_A1 and GABA_A2 protein, which however, did not differ significantly between WT and Reln^cKO, though the amount of both receptors showed a tendency to decrease in the Reln^cKO (Figure 7a,b). To find out whether Reelin deficiency might affect GABA_A phosphorylation, we analyzed cortical lysates from WT and Reln^cKO P14 mice by western blotting with antibodies against GABA_A phosphorylation sites. GABA_A2 phosphorylation at Ser892 is mediated by cyclic AMP (cAMP)-dependent protein kinase (PKA) and stabilizes cell surface expression and coupling to G-proteins of the receptors (Couve et al., 2002). Western blotting revealed that in Reelin-deficient tissue, S892 phosphorylation of GABA_A2 was significantly reduced when compared to WT (Figure 7c), suggesting that Reelin signaling is important to stabilize GABA_A2 function at the cell surface. Another GABA_A2 phosphorylation site at S783 is required for degradation, and its phosphorylation is mediated by 5'-AMP-dependent protein kinase (AMPK) (Terunuma, Vargas, et al., 2010). GABA_A2 recycling and degradation is controlled via phosphorylation site S783 (Gassmann & Bettler, 2012). Western blot analysis revealed that GABA_A2 phosphorylation at S783 in Reln^cKO mice was increased when compared to WT, and that phosphorylated GABA_A2s were degraded in Reln^cKO mice only (Figure 7d). Taken together, these findings indicate that Reelin is required to stabilize the

**FIGURE 3** GABA_As are dysfunctional in Reln^cKO. To test the functionality of the GABA_As at DIV14, OTCs were loaded with OGB-1 AM, spontaneous activity was first recorded and then the GABA_A agonist baclofen (10 µm) was added. In each experiment, OTCs were prepared from 5 WT and 5 Reln^cKO mice. (a) The application of baclofen immediately activates GABA_As and thereby strongly decreases Ca^{2+} amplitude in WT OTCs (One-way ANOVA on Ranks followed Tukey’s Test, ***p < .001) but to a lesser extent in Reln^cKO OTCs (*p < .05). Ca^{2+} frequency (b) and Ca^{2+} transient half-width (c) were significantly decreased in the WT but not in Reln^cKO OTCs (One-way ANOVA on Ranks followed Tukey’s Test, ***p < .001). The GABA_A antagonist CGP35348 (10 µM) did not alter Ca^{2+} amplitudes in both WT and Reln^cKO OTCs (d). The application of CGP35348 significantly enhanced Ca^{2+} frequency (e) and Ca^{2+} transient half-width (f) in WT but not Reln^cKO OTCs. One-way ANOVA on Ranks followed by Dunn’s Test, ***p < .001. The number of OTCs analyzed is indicated above the box plot in (d). Data are obtained from three independent OTCs preparations. GABA, gamma-aminobutyric acid
cell surface expression of functional GABA\(_{\text{R}}\)2 receptors, probably by inhibiting receptor degradation.

3.7 | GABA\(_{\text{R}}\) intracellular signaling via G\(_{\text{i/o}}\) proteins is affected in Reln\(^{\text{cKO}}\) mice

To further investigate a potential crosstalk between Reelin, GABA\(_{\text{R}}\) signaling and G-protein coupled receptors, we recorded OTCs in the presence of PTX. Stimulation of GABA\(_{\text{R}}\)s with baclofen inhibits adenylyl cyclase (Xu & Wojcik, 1986), which in turn can be blocked by PTX, known to inhibit G-protein function (Karbon & Enna, 1985; Nishikawa & Kuriyama, 1989). Application of PTX at DIV14 enhanced the Ca\(^{2+}\) amplitude, frequency and Ca\(^{2+}\) transient half-width in WT but not in Reln\(^{\text{cKO}}\) OTCs (Figure S8). These results further support the interpretation that Ca\(^{2+}\) signaling is unaltered by the treatment of Reln\(^{\text{cKO}}\) OTCs because GABA\(_{\text{R}}\) receptors are degraded in the absence of Reelin. It also confirms that Reelin is important to maintain proper GABA\(_{\text{R}}\) signaling.

4 | DISCUSSION

In the present study, we characterized a novel role of Reelin in early post-natal cortical function. We found that post-natally induced Reelin deficiency caused abnormal Ca\(^{2+}\) spike frequency and Ca\(^{2+}\) transient half-width in cortical neurons. RAP blockade of Reelin binding to its receptors ApoER2 and VLDLR mimicked this effect. There are no specific selective antagonists for ApoER2 and VLDLR, therefore we used RAP to broadly block LRP family members. Co-culture of Reln\(^{\text{cKO}}\) OTCs with Reelin secreting WT OTCs rescued deficient network activity in Reln\(^{\text{cKO}}\) OTCs. While the GABA\(_{\text{R}}\) agonist baclofen failed to activate GABA\(_{\text{R}}\)Rs, the GABA\(_{\text{R}}\) antagonist CGP35348 failed to inhibit GABA\(_{\text{R}}\)s in Reln\(^{\text{cKO}}\) OTCs, indicating deactivation and/or degradation of GABA\(_{\text{R}}\)s in Reln\(^{\text{cKO}}\) OTCs. Immunostaining of non-permeabilized cells with antibodies against GABA\(_{\text{R}}\)1 and GABA\(_{\text{R}}\)2 extracellular domains was significantly reduced in Reln\(^{\text{cKO}}\) slices in line with our pharmacological experiments, and confirming a previous study that reported on a down-regulation of GABA\(_{\text{R}}\)s in the reeler

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**FIGURE 4** Effect of chronic blockade with the LDL receptor chaperone RAP. Reln\(^{\text{cKO}}\) or WT OTCs chronically treated with RAP (50 µg) were loaded with OGB-1 AM and recorded. In each experiment, OTCs were prepared from 5 WT and 5 Reln\(^{\text{cKO}}\) mice. Control WT or Reln\(^{\text{cKO}}\) OTCs were treated with H\(_2\)O. (a) RAP blockade did not affect Ca\(^{2+}\) amplitude in WT and Reln\(^{\text{cKO}}\) OTCs. However, RAP significantly increased Ca\(^{2+}\) frequency (b) and Ca\(^{2+}\) transient half-width (c) in WT but not Reln\(^{\text{cKO}}\) OTCs. One-way ANOVA on Ranks followed by Dunn’s Test, ***p < .001. The number of OTCs analyzed is indicated above the box plots in (a). Data are obtained from three independent OTCs preparations

**FIGURE 5** Effect of acute Src kinase inhibition on WT and Reln\(^{\text{cKO}}\) Ca\(^{2+}\) activity. Acute application of PP2 (1 µM) did not affect Ca\(^{2+}\) amplitude in both WT and Reln\(^{\text{cKO}}\) OTCs (a). However, PP2 application enhances Ca\(^{2+}\) frequency (b) and Ca\(^{2+}\) transient half-width (c) in WT but not Reln\(^{\text{cKO}}\) OTCs (b). In each experiment, OTCs were prepared from 5 WT and 5 Reln\(^{\text{cKO}}\) mice. One-way ANOVA on Ranks followed by Dunn’s Test, ***p < .001. The number of OTCs analyzed is indicated above the box plots. Data is obtained from three independent OTCs preparations
mutant (Cremer et al., 2011). When analyzing cortical tissue of conditionally induced Reelin deficient mice by western blotting, we found a decreased GABA\textsubscript{B}R2 phosphorylation at S892 and an increased phosphorylation at S783, both indicative of GABA\textsubscript{B}R2 degradation (Gassmann & Bettler, 2012), and proteolytic processing of GABA\textsubscript{B}R2. Accordingly, in Reln\textsuperscript{cKO} slices we observed a decrease in surface expression of GABA\textsubscript{B}R1 (approx. 35% decrease) and GABA\textsubscript{B}R2 (approx. 39% decrease) respectively. The enhanced Ca\textsuperscript{2+} frequency in the

FIGURE 6 Surface GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 expression are reduced in the Reln\textsuperscript{cKO} slices. Acute slices (P14) from WT and Reln\textsuperscript{cKO} mice were immunostained against total (permeabilized) and surface (non-permeabilized) GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 expression. In each experiment, slices were prepared from 5 WT and 5 RelnKO mice. Confocal image example at 40 x magnification from layers II/III somatosensory cortices of WT (a) and Reln\textsuperscript{cKO} (b) acute slices stained against GABA\textsubscript{B}R1 or GABA\textsubscript{B}R2 (c and d) under permeabilization condition to detect total receptor expression. Confocal image example of WT (e) and Reln\textsuperscript{cKO} (f) acute slices stained against GABA\textsubscript{B}R1 or GABA\textsubscript{B}R2 (G and H) under non-permeabilization condition for receptor surface expression. (i) The box plots represent values of average pixel intensity of total (permeabilized) and surface (non-permeabilized) GABA\textsubscript{B}R1 expression. (j) The box plots represent values of average pixel intensity of total (permeabilized) and surface (non-permeabilized) GABA\textsubscript{B}R2 expression. One-way ANOVA on Ranks followed by Dunn's Test, ***p < .001. The number of acute slices analyzed is indicated above the box plots. Data are obtained from three ex vivo acute slice preparations. Scale bar: 10 µm. GABA, gamma-aminobutyric acid
RelnKO slices can be attributed to the degradation (down-regulation) of the GABA\(_B\)_Rs at the pre-synaptic sites. The main function of pre-synaptic GABA\(_B\)_Rs at the pre-synaptic site is to inhibit calcium channels, which results in inhibition of neurotransmitter release (Benarroch, 2012). Together, our findings suggest a novel role for Reelin in controlling cortical neuronal network maturation, by regulating Ca\(^{2+}\) spiking in glutamatergic neurons via pre-synaptic GABA\(_B\)_Rs.

Prolonged NMDA-R stimulation triggered both the endocytosis of GABA\(_B\)_Rs (Terunuma, Vargas, et al., 2010) and activation of PP2A. PP2A in turn favors dephosphorylation of S783 in GABAB2 and redirection of the endocytosed pool from recycling to degradation. However, surprisingly, despite the observed dephosphorylation of S783 in GABAergic neurons, no increase in the lysosomal degradation of GABA\(_B\)_Rs has been observed by (Padgett et al., 2012). The identification of S783 on the GABA\(_B\)_R2 subunit as an AMPK substrate points to a link between the induction of ischemia and increased phosphorylation of GABA\(_B\)_Rs in the hippocampus (Kuramoto et al., 2007). The increase in GABAB receptor phosphorylation, evident during ischemia, could provide a potentially neuroprotective mechanism that may limit neuronal exposure to excitotoxicity. In absence of Reelin, we also observed enhanced Ca\(^{2+}\) signaling in the RelnKO and increased phosphorylation at Ser783, which might be similarly important to protect neurons from excitotoxicity.

**FIGURE 7** Reelin signaling regulates GABA\(_B\)_ receptor phosphorylation. Cortical lysates from P14 WT and Reln\(^{cKO}\) mice were analyzed by western blot (n = 6 animals from each group; data are obtained from three different tissue explants). Total GABA\(_B\)_R1 (a) and GABA\(_B\)_R2 (b) were unaltered in Reln\(^{cKO}\) in comparison to WT control. (c) Phosphorylation of GABA\(_B\)_R2 at S892 was significantly reduced in Reln\(^{cKO}\) (t-test, **p < .01), whereas phosphorylation at S783 was significantly increased (d) when compared to WT (t-test, **p < .01), and appearance of several bands suggests proteolysis of the receptor (Gassmann & Bettler, 2012; Maier, Marin, Grampp, Sommer, & Benke, 2010). GABA, gamma-aminobutyric acid.
By performing immunohistochemical staining with antibodies against layer-specific markers, we confirm that cortical neurons in post-natal Reln\(^{cKO}\) did not alter their characteristic layer specific marker expression in the absence of Reelin (Boyle et al., 2011). There are two populations of Reelin expressing neurons in the neocortex. At early embryonic stages, Reelin is secreted by Cajal-Retzius (CR) cells. The number of CR cells declines post-natally, and these cells disappear almost completely around P14 by undergoing selective cell death through apoptosis (Anstötz et al., 2014). Moreover, almost all GABAergic interneurons in the neonatal cortex express Reelin, suggesting that in the current study Reelin was mainly expressed by interneurons.

A role of Reelin in modulating glutamatergic activity has been previously reported (Chen et al., 2005). Reelin-haploinsufficiency has been shown to disrupt the developmental course of GABA excitatory/inhibitory balance (Bouamrane et al., 2016). In the current experiments, we studied spontaneous neuronal calcium activity in the Reln\(^{cKO}\) compared to WT littersmates. While the Ca\(^{2+}\) amplitude was unaltered in Reln\(^{cKO}\), the frequency of Ca\(^{2+}\) spiking and Ca\(^{2+}\) transient half-width was dramatically increased in Reln\(^{cKO}\) when compared to WT. The Ca\(^{2+}\) spike half-width narrows around the second post-natal week (14DIV or P14) and this observation has been previously attributed to an increase in cell size and maturation of ion channels (Gold, Henze, & Koch, 2007). Thus, the increased Ca\(^{2+}\) spikes half-width in Reln\(^{cKO}\) may suggest an immature ion channels condition in these mice. Along this line, related observations were reported in the reeler hippocampus. Thus, using electron microscopy, it has been shown that the number of pre-synaptic vesicles was significantly increased in hippocampal CA1 synapses of reeler mutant mice when compared to WT (Hellwig et al., 2011). In contrast, acute Reelin application to dissociated hippocampal neurons enhanced spontaneous neurotransmitter release without affecting properties of evoked neurotransmission (Bal et al., 2013). The discrepancy here may be because of the different models and methodologies used. For instance, acute application of 5 nM Reelin (Bal et al., 2013) versus analysis of the reeler mutant (Hellwig et al., 2011) or the Reln\(^{cKO}\) (present study). In neocortical neurons, neurotransmitter release is controlled by GABAB\(_{R}\) function (see Introduction). Moreover, perturbations of GABAB receptor signaling during development may shift the excitatory/inhibitory balance, culminating in various neurological dysfunctions, including typical and atypical absence seizures (Han, Cortez, & Sneed, 2012). To find out whether Reelin function might interfere with GABAB\(_{R}\) function, we manipulated GABAB\(_{R}\) physiology in Reln\(^{cKO}\) tissue using pharmacological agents. Our observation that a GABAB\(_{R}\) agonist failed to activate GABAB\(_{R}\)S and an antagonist failed to inhibit GABAB\(_{R}\)S in the Reln\(^{cKO}\), points to a novel function of Reelin as a modulator of GABAB\(_{R}\)S during early cortical function. Taken together, our data suggest that in the absence of Reelin, GABAB\(_{R}\)S are down-regulated, as shown by GABAB\(_{R}\) cell surface immunolabeling experiments, and by western blotting that revealed an altered GABAB\(_{R}\) phosphorylation status as well as proteolytic receptor processing.

Next, we investigated possible mechanisms underlying cross-talk of Reelin and GABAB\(_{R}\) signaling. Binding of Reelin to its receptors induces phosphorylation of Dab1 protein by Src kinase (Bock & Herz, 2003). Blocking of WT OTCs with a Src inhibitor increased the Ca\(^{2+}\) signaling frequency to a comparable level as in Reln\(^{cKO}\). Moreover, the Src inhibitor decreased GABAB\(_{R}\) surface expression in WT slices but it did not affect GABAB\(_{R}\) surface expression in the Reln\(^{cKO}\). These observations suggest that Reelin signaling through Src is important to maintain GABAB\(_{R}\)s function. Src may directly interact with the \(\alpha\) subunits of G-proteins (Ma, Huang, Ali, Lowry, & Huang, 2000) and recently it has been shown that Src/Gao interactions provide evidence for a novel type of cross-talk between a non-receptor tyrosine kinase stimulated by Reelin and heterologous G protein-coupled receptors (GPCR) (Cho et al., 2015). Since GABAB\(_{R}\)s are members of the G-protein coupled receptors (GPCRs), it is very well possible that metabotropic GABAB\(_{R}\)s are modulated by Reelin through Src kinase.

Stimulation of GABAB\(_{R}\)s with baclofen can inhibit both basal, and forskolin stimulated adenylyl cyclase (Xu & Wojcik, 1986), which in turn can be blocked by pertussis toxin (PTX), reflecting the involvement of the G\(\alpha\)i/o subunit of the G-protein (Karbon & Enna, 1985; Nishikawa & Kuriyama, 1989). Moreover, immunoprecipitation assays disclosed the possibility that Reelin might increase the active forms of both Src and Gao and promote their direct association (Cho et al., 2015). Thus, our finding that PTX increases Ca\(^{2+}\) frequency in WT OTCs but not in Reln\(^{cKO}\), indicates the possibility of a disrupted Sre\(\alpha\)i/o interaction in the absence of Reelin. For instance, based on our experiments it appears more likely that reduced cell surface expression of GABAB\(_{R}\) is the reason why Ca\(^{2+}\) signaling is unaltered in Reln\(^{cKO}\) OTCs after PTX application.

Functional deficits associated with decreased Reelin expression have often been attributed to the well-known developmental neuronal migration defects in Reelin deficient mice (Eastwood & Harrison, 2003). Moreover, both Reelin deficiency and cortical network dysrhythmias caused by aberrant interneuron activity were also discussed as possible factors contributing to cognitive and behavioral deficits in Alzheimer disease (AD) (Palop & Mucke, 2016; Xiao et al., 2017). Besides Reelin, also different ApoE isoforms act as lipoprotein receptor ligands and are known to influence AD pathogenesis, with ApoE4 being the most important genetic risk factor for AD (Huang, Zhou, Wernig, & Südhof, 2017). GABAB\(_{R}\)s were repeatedly shown to be implicated in synaptic plasticity (Davies, Starkey, Pozza, & Collingridge, 1991; Mott & Lewis, 1991). Until recently, it remained unclear whether GABAB\(_{R}\)s can influence neuronal plasticity through GABAB\(_{R}\) signaling. It has been demonstrated that G-protein mediated signaling through GABAB\(_{R}\)s delays the recruitment of synaptic vesicles during sustained activity and after short-term depression (Sakaba & Neher, 2003). This delay occurs through a reduction of cAMP, which in turn blocks the stimulatory effect of the increased Ca\(^{2+}\) concentration on vesicle recruitment (Sakaba & Neher, 2003). In conclusion, we provide evidence for a novel role of Reelin signaling in controlling early neuronal network activity by regulating neurotransmitter release through GABAB\(_{R}\)s.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT
The Rehnmouse mice require a material transfer agreement from the University of Texas Southwestern Medical Center.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.