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Experimental Neurobiology

Reelin and APP Cooperatively Modulate Dendritic Spine Formation *In Vitro* and *In Vivo*

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Amyloid precursor protein (APP) plays an important role in the pathogenesis of Alzheimer's disease (AD), but the normal function of APP at synapses is poorly understood. We and others have found that APP interacts with Reelin and that each protein is individually important for dendritic spine formation, which is associated with learning and memory, *in vitro*. However, whether Reelin acts through APP to modulate dendritic spine formation or synaptic function remains unknown. In the present study, we found that Reelin treatment significantly increased dendritic spine density and PSD-95 puncta number in primary hippocampal neurons. An examination of the molecular mechanisms by which Reelin regulates dendritic spinogenesis revealed that Reelin enhanced hippocampal dendritic spine formation in a Ras/ERK/CREB signaling-dependent manner. Interestingly, Reelin did not increase dendritic spine number in primary hippocampal neurons when APP expression was reduced or *in vivo* in APP knockout (KO) mice. Taken together, our data are the first to demonstrate that Reelin acts cooperatively with APP to modulate dendritic spine formation and suggest that normal APP function is critical for Reelin-mediated dendritic spinogenesis at synapses.

Key words: APP, Reelin, Dendritic spine, Alzheimer's disease, Ras signaling

INTRODUCTION

Many Alzheimer's disease (AD) studies have focused on the synaptotoxic effects of the amyloid β (Aβ) peptide and neglected the possibility that amyloid precursor protein (APP) itself is important for synapse formation and function. We previously demonstrated that APP is highly localized in synapses and is involved in dendritic spine formation. For example, in primary hippocampal neurons

in vitro, overexpression of full-length APP promotes the formation of dendritic spines, while knockdown of APP decreases dendritic spine formation [1]. Consistent with these results, dendritic spine density in cortical layers II/III and in the hippocampal CA1 region is reduced in 1-year-old APP knockout (KO) mice [1], and spine formation and spine plasticity in cortical layer V are impaired in 4-month-old APP KO mice [2]. However, the effects of APP on synaptic/cognitive function have not been studied in detail.

Recruitment of APP to both pre- and post-synaptic sites is required for synapse formation [3]. In addition, APP is involved in presynaptic vesicle release and postsynaptic N-methyl-D-aspartate receptor (NMDAR) trafficking, indicating a role in synaptic connectivity [4]. Furthermore, overexpression of human APP increases synaptic density, while expression of familial Alzheimer's disease (FAD)-mutated APP has no effect [5]. Overexpression of

sAPP, a soluble N-terminal fragment liberated by α-secretases, improves long-term potentiation (LTP) and enhances spatial memory [6]. Thus, the synaptic functions of full-length APP and sAPPα appear to directly oppose the neurotoxic effects of Aβ (and perhaps sAPPβ), the accumulation of which leads to impaired synaptic physiology and synapse loss [7, 8]. Despite the neurotoxic properties of Aβ, enhancement of long-term potentiation (LTP) and spatial memory by APP overexpression requires beta-site APP cleaving enzyme 1 (BACE1), suggesting that the APP intercellular domain (AICD) is also critical for the effect of APP on synaptic function [9].

The extracellular matrix glycoprotein Reelin is required for neuronal migration during embryonic brain development [10] and has been implicated in the pathoprogression of neurodevelopmental diseases such as attention deficit and hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) and in neurodegenerative diseases, including AD. A genome-wide association study (GWAS) revealed that single nucleotide polymorphisms of Reelin are associated with an increased risk of ADHD [11], and patients with ASD have higher plasma levels of Reelin than healthy controls [12]. In addition, Reelin-positive neuronal cells are decreased in a mouse model of AD, whereas Reelin levels in cerebrospinal fluid (CSF) are increased in AD patients [13, 14]. Furthermore, genetic ablation of the *Reelin* gene in ArcAβ mice, a mouse model of AD, exaggerates amyloidogenesis, tauopathy and neuroinflammation compared with single AD mice [15]. These observations indicate that Reelin plays an important role in neurodevelopment and neurodegeneration.

How is Reelin associated with cognitive dysfunction in neurodevelopmental and neurodegenerative diseases? Similar to APP, several studies have reported that Reelin plays a critical role in regulating synaptic function. Specifically, Reelin regulates dendritic spine formation in the early, postnatal hippocampus [16], and intraventricular administration of Reelin enhances dendritic spine density, synaptic plasticity, and spatial learning in the hippocampus in adult wild-type mice [17]. A role for Reelin in regulating synaptic function in the adult brain is further supported by the reduction in LTP in heterozygous *Reeler* mice [18] and the inability of Reelin to enhance LTP in mice deficient in the Reelin receptors apoE receptor 2 (ApoER2) and very low density receptor (VLDLR) [19]. Conversely, mice overexpressing Reelin have enhanced synaptic plasticity and dendritic spine hypertrophy [20].

Ras/ERK signaling upregulates dendritic spine plasticity, and Ras signaling facilitates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) trafficking in cultured hippocampal slices, thereby enhancing LTP [21, 22]. Reelin promotes ERK/p90RSK signaling in mouse cortical neuronal culture [23]. In addition, Reelin regulates Ras-PI3K signaling to suppress cancer cell migration [24]. However, the molecular mechanisms by which Reelin alters dendritic spine formation are unclear. We and others recently reported that Reelin and APP individually alter dendritic spine formation [25], and in this study, we further investigated the physiological function of Reelin and the interaction between Reelin and APP at synapses. We found that Reelin treatment increased dendritic spine formation by regulating Ras/ERK/CREB signaling in mature primary hippocampal neurons. Importantly, Reelin injection did not affect cortical and hippocampal dendritic spine numbers in APP KO mice, suggesting that Reelin is required for the regulation of dendritic spine formation by APP. Taken together, our results indicate that Reelin interacts with APP to facilitate postsynaptic AMPAR trafficking and subsequent activation of Ras signaling to regulate dendritic spine formation.

MATERIALS AND METHODS

Ethics approval

All experiments were approved and performed in accordance with protocols approved by the Animal Welfare and Use Committee of Georgetown University and the Institutional Animal Care and Use Committee of the University of South Florida (approval no. R3336).

Mice

APP KO mice (B6.129S7-APP^tm1DBo/J) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed under a standard 12-h light-dark cycle and fed normal chow *ad libitum*.

Reelin purification

Recombinant Reelin and mock-conditioned medium (Mock medium) were produced using HEK293 cells as previously described [26-28]. Briefly, HEK293 cells were transfected with the plasmid pCrl, which carries the entire open reading frame of mouse Reelin (Addgene plasmid #122443, a gift from Tom Curran), or a control construct. The cells were then incubated in low-glucose DMEM with 0.2% BSA for 2 days, and the medium was collected, filtered, and concentrated by using Centricon® plus-80 centrifugal filter units (Millipore, Bedford, MA, USA). Purified recombinant Reelin was validated via immunoreactivity to an anti-Reelin (G20) antibody (Santa Cruz Biotechnology, Dallas, TX, USA).

Table 1. List of antibodies used for ICC

Immunogen	Host species	Dilution	Manufacturer	Catalog no.
GFP	Mouse	1:200	Novus Biologicals	9F9.F9
GFP	Rabbit	1:200	Invitrogen	A11122
Synaptophysin	Mouse	1:200	Sigma Aldrich	S5768
PSD-95	Mouse	1:200	NeuroMab	07-028
GluA1	Rabbit	1:200	Calbiochem	PC246
GluA2	Mouse	1:100	BD Pharmingen	556341
RasGRF1	Rabbit	1:200	Santacruz Biotechnology	SC-224
pERK1/2	Mouse	1:200	Invitrogen	13-6200
pCREB	Rabbit	1:200	Millipore	06-519
Immunogen	Host species	Dilution	Provider	Epitope
GluN1	Mouse	1:200	Dr. Barry Wolfe	Amino acid 656-811
GluN2A	Rabbit	1:200	Dr. Barry Wolfe	Amino acid 934-1142
GluN2B	Mouse	1:200	Dr. Barry Wolfe	Amino acid 934-1457

Primary hippocampal neuronal culture, transfection, and dendritic spine counting

Primary hippocampal neurons from E18–E19 Sprague Dawley rats were cultured at 150 cells/mm^2 as described previously [29]. Primary hippocampal neurons (DIV 12 or DIV 19) were transfected with GFP plasmid using Lipofectamine 2000 for 24 h and subsequently treated with purified Reelin (0.7 or 1.4 μM) or Mock medium on DIV 13 or DIV 20 for 24 h. Then, immunocytochemistry was conducted on DIV 14 or DIV 21, and secondary dendritic spine density was analyzed to measure the dendritic spine number or puncta number/intensity of synaptic protein levels. In brief, dendritic spines were defined as protrusions ranging in length from 0.2 μm to 2.0 μm with a head and neck (mushroom shaped or thin) or without a neck (stubby) [30]. Immature filopodia were excluded in the analysis of dendritic spine density in primary hippocampal neurons. In addition, we measured the number of dendritic spines located on secondary/tertiary dendritic branches only to minimize the bias/variability caused by the hierarchy of dendrites as previously described [30]. Dendritic spine density was measured by dividing the number of manually quantified dendritic spines by the dendritic segment length, which ranged from 10 to 20 μm.

For *Ras* inhibition, RasN17 (dominant negative for Ras) plasmid or HA plasmid (control for RasN17) was co-transfected with GFP plasmid on DIV 19. For *App* knockdown, APP shRNA (5-GCA CTA ACT TGC ACG ACT A-3, [31]) or PLL (control plasmid for shRNA) was co-transfected with GFP on DIV 19.

Immunocytochemistry

Twenty-four hours after GFP plasmid transfection, primary hippocampal neurons were treated with 1.4 μM Reelin or Mock medium for 24 h. Then, the cells were fixed with 4% paraformaldehyde (to measure the dendritic spine number) or cold methanol (to measure synaptic protein puncta number along the dendritic neuronal processes and dendritic spines) for 10 min, washed 3 times with 1× PBS for 5 min, and incubated with primary antibodies overnight. The next day, the cells were washed 3 times with 1× PBS for 5 min and incubated with secondary antibodies for 1 h at room temperature. Finally, the cells were washed with 1× PBS for 5 min and mounted. Fluorescence intensity and puncta number along the dendritic neuronal processes and dendritic spines were quantified by using the NeuronJ plugin (NeuronJ, NIH, Bethesda, MD, USA) and SynaptoCount. For intensity quantification, regions of interest (ROIs) were acquired by tracing GFP signals; then, the average intensity of the target protein puncta in the dendrites was measured. Table 1 provides the details of the antibodies used in the present study.

Golgi staining and analysis of dendritic morphology in vivo

APP KO mice (3 months old; n=4 mice per genotype per treatment) underwent bilateral intraventricular injections of Reelin (210 nM) or control saline as described previously [17]. Briefly, anesthetized APP KO mice were positioned on a stereotaxic frame (Stoelting, Wood Dale, IL, USA). After making an incision in the scalp and muscle, two holes were drilled in the dura, and Reelin or saline was injected using a Hamilton syringe at coordinates AP -0.35 mm, ML ± 0.75 mm, and DV -2.5 mm from the bregma at a flow rate of 1 μl/min. After the surgery, the mice were allowed to recover and monitored. Five days after the injection, the brains of the mice were perfused with PBS and immersed in the solutions provided in the FD Rapid Golgi Stain kit (FD NeuroTechnologies, Ellicott City, MD) [25]. The dehydrated brains were sliced at a thickness of 150 μm using a vibratome (VT1000S, Leica, Wetzlar, Hessen, Germany) and mounted on glass slides. Images of dendrites in the hippocampal CA1 pyramidal neurons and in cortical

layer II/III were obtained by bright-field microscopy (Axioplan 2. Zeiss, Oberkochen, Baden-Württemberg, Germany) at 63× magnification. Dendritic spines were manually counted using ImageJ software (NIH, Bethesda, MD, USA).

Statistical analyses

GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA) was used for graph generation and statistical analysis. Data are presented as individual data points and the mean±SEM. Student's t test was used for pairwise comparisons, and one-way analysis of variance (ANOVA) with Tukey's multiple-comparisons test was used for multiple comparisons. Asterisks indicate significance: *p<0.05, **p<0.01, and ***p<0.001.

RESULTS

Reelin promotes dendritic spine formation and increases the number of PSD-95 puncta

Dendritic spine density is reduced in organotypic hippocampal cultures from Reeler mice lacking Reelin and can be rescued by the addition of Reelin (Niu et al. 2008). Here, we assessed the ability of Reelin addition to alter dendritic spine density in primary hippocampal neurons. For this experiment, cultured primary hippocampal neurons (DIV 19) were transfected with GFP plasmid and treated with vehicle (Mock medium) or Reelin (0.7 or 1.4 μM) for 24 h, and dendritic spine density was measured in the secondary/tertiary distal dendrites (to reduce variability) [30]. We found that Reelin significantly increased the number of dendritic spines compared with vehicle treatment in a dose-dependent manner in mature primary hippocampal neurons (DIV 21, Fig. 1A). Based on these findings, we chose a Reelin dose of 1.4 μM for subsequent experiments.

We then examined whether Reelin regulates the number of excitatory synapses in vitro. GFP plasmid-transfected primary hippocampal neurons (DIV 19) were treated with 1.4 μM Reelin or vehicle (Mock medium) for 24 h and immunostained with an anti-PSD-95 antibody, and the puncta number in secondary/tertiary dendrites was measured. Reelin significantly increased the number of PSD-95 puncta in mature primary hippocampal neurons, whereas PSD-95 fluorescence intensity did not change (Fig. 1B). These data suggest that Reelin upregulates dendritic spine formation and enhances the number of PSD-95 puncta in mature primary hippocampal neurons.

Reelin selectively alters the levels of NMDAR subunits

A previous study found that Reelin modulates NMDAR subunit expression/trafficking during the peak of synaptogenesis [26];

however, the effects of Reelin on NMDAR expression in mature neurons has not been established. Therefore, we examined whether Reelin regulates NMDAR expression levels in mature neurons. Specifically, GFP plasmid-transfected primary hippocampal neurons (DIV 19) were treated with 1.4 μM Reelin or vehicle (Mock medium) for 24 h and immunostained with antibodies against the NMDAR subunits GluN1, GluN2A, or GluN2B. Interestingly, Reelin treatment did not alter the fluorescence intensity of total GluN1 and total GluN2A but significantly decreased the fluorescence intensity of total GluN2B (Fig. 2A~C). These data suggest that Reelin differentially regulates the total expression levels of GluN1/GluN2A and GluN2B in mature primary hippocampal neurons.

Reelin regulates AMPAR trafficking during the peak of synaptogenesis in vitro

To examine whether Reelin treatment modulates the trafficking of the AMPAR GluA1 and GluA2 subunits during the peak of synaptogenesis in vitro, primary hippocampal neurons were transfected with GFP plasmid (DIV 12) and treated with 1.4 μM Reelin or vehicle (Mock medium) for 24 h. On DIV 14, live cell surface staining was conducted with antibodies recognizing the N-terminus of GluA1 or GluA2, and the puncta number along the dendritic neuronal secondary/tertiary processes was measured. Reelin treatment significantly increased cell surface GluA1 and GluA2 puncta numbers in primary hippocampal neurons at the peak of synaptogenesis (Fig. 3A, B). These data suggest that Reelin promotes AMPAR subunit trafficking during synaptogenesis in primary hippocampal neurons.

Reelin upregulates Ras/ERK/CREB phosphorylation in primary hippocampal neurons

Ras/Rap regulator polo-like kinase 2 (Plk2) suppresses dendritic spine formation and AMPAR trafficking, leading to impaired memory formation [32]. Moreover , we demonstrated that APP increases dendritic spine formation via Ras signaling in vitro and in vivo [1]. These previous findings suggest an essential role of Ras signaling in regulating dendritic spine formation. Therefore, we evaluated whether Reelin upregulates dendritic spinogenesis in a Ras-dependent manner in vitro. For this experiment, GFP plasmid-transfected primary hippocampal neurons (DIV 19) were treated with Reelin (1.4 μM) or vehicle (Mock medium) for 24 h and immunostained with an antibody against RasGRF1, which is upstream of Ras signaling proteins. After immunostaining, the RasGRF1 puncta number was measured in secondary/tertiary dendrites, which revealed a significant increase in RasGRF1 puncta number in Reelin-treated mature primary hippocampal
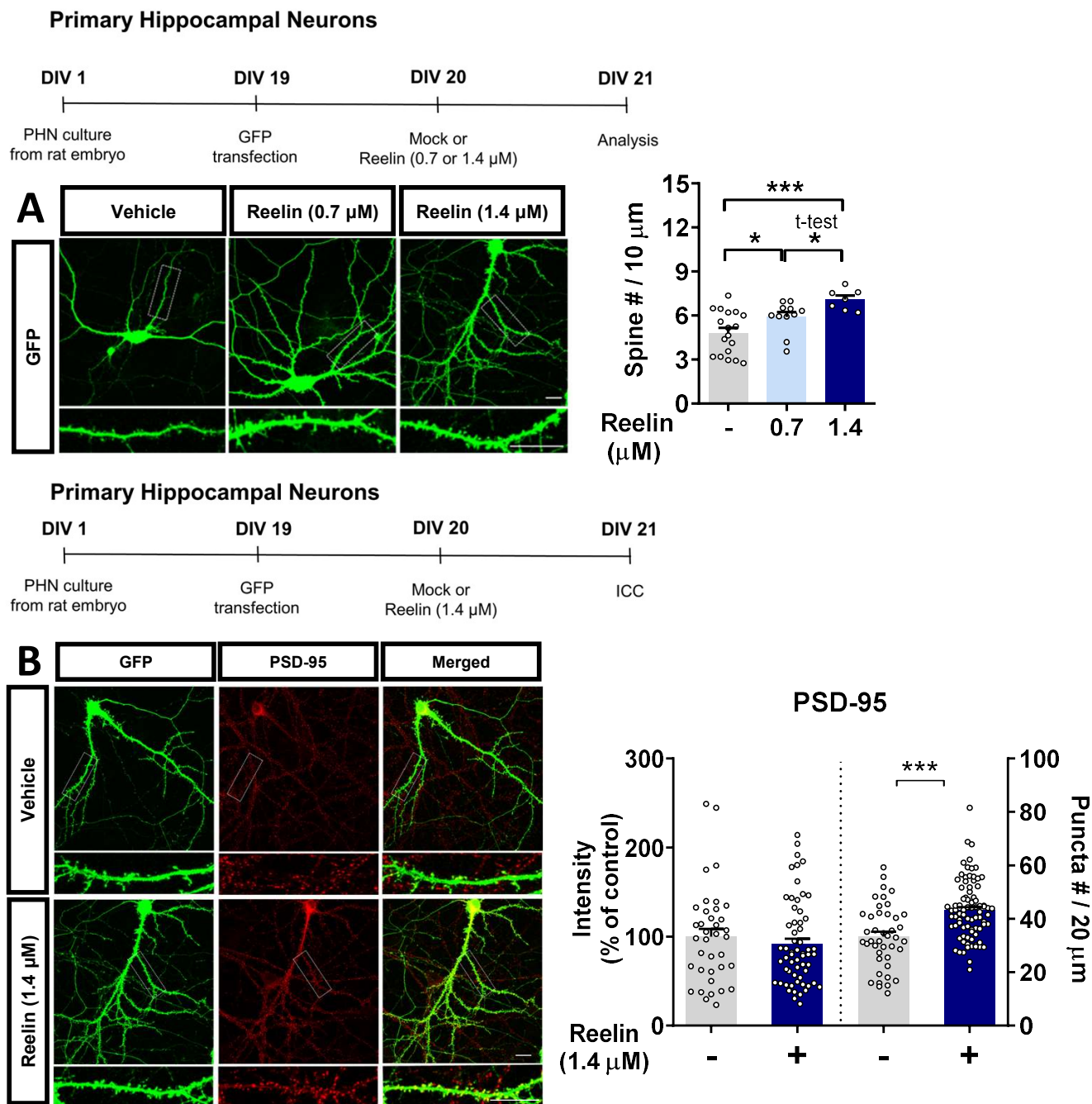
Primary Hippocampal Neurons

Fig. 1. Reelin modulates dendritic spine formation by enhancing the PSD-95 puncta number *in vitro*. (A) Primary hippocampal neurons were transfected with GFP plasmid on DIV 19 and treated with vehicle (Mock medium) or Reelin (0.7 or 1.4 μM) on DIV 20 for 24 h. Then, dendritic spine density was measured (vehicle treated: n=18 dendrites from 9 neurons; 0.7 μM Reelin: n=11 dendrites from 9 neurons; 1.4 μM Reelin: n=7 dendrites from 5 neurons). (B) Primary hippocampal neurons were treated as described above and immunostained with an anti-PSD-95 antibody on DIV 21. The staining intensity and puncta numbers were then quantified (PSD-95 intensity, vehicle: n=37 dendrites from 9 neurons, Reelin: n=61 dendrites from 13 neurons; PSD-95 puncta number, vehicle: n=42 dendrites from 9 neurons, Reelin: n=83 dendrites from 13 neurons). *p<0.05, ***p<0.001. Scale bar=10 μm.

neurons compared with the Mock medium-treated control group (Fig. 4A).

We then examined the effects of Reelin on Ras downstream ERK/CREB signaling *in vitro*. For this experiment, primary hippo-

campal neurons (DIV 19) were transfected with GFP plasmid for 24 h, treated with vehicle (Mock medium) or Reelin (1.4 μM) for 24 h, and immunostained with anti-p-ERK and anti-p-CREB antibodies using methanol fixation for puncta staining in the dendritic
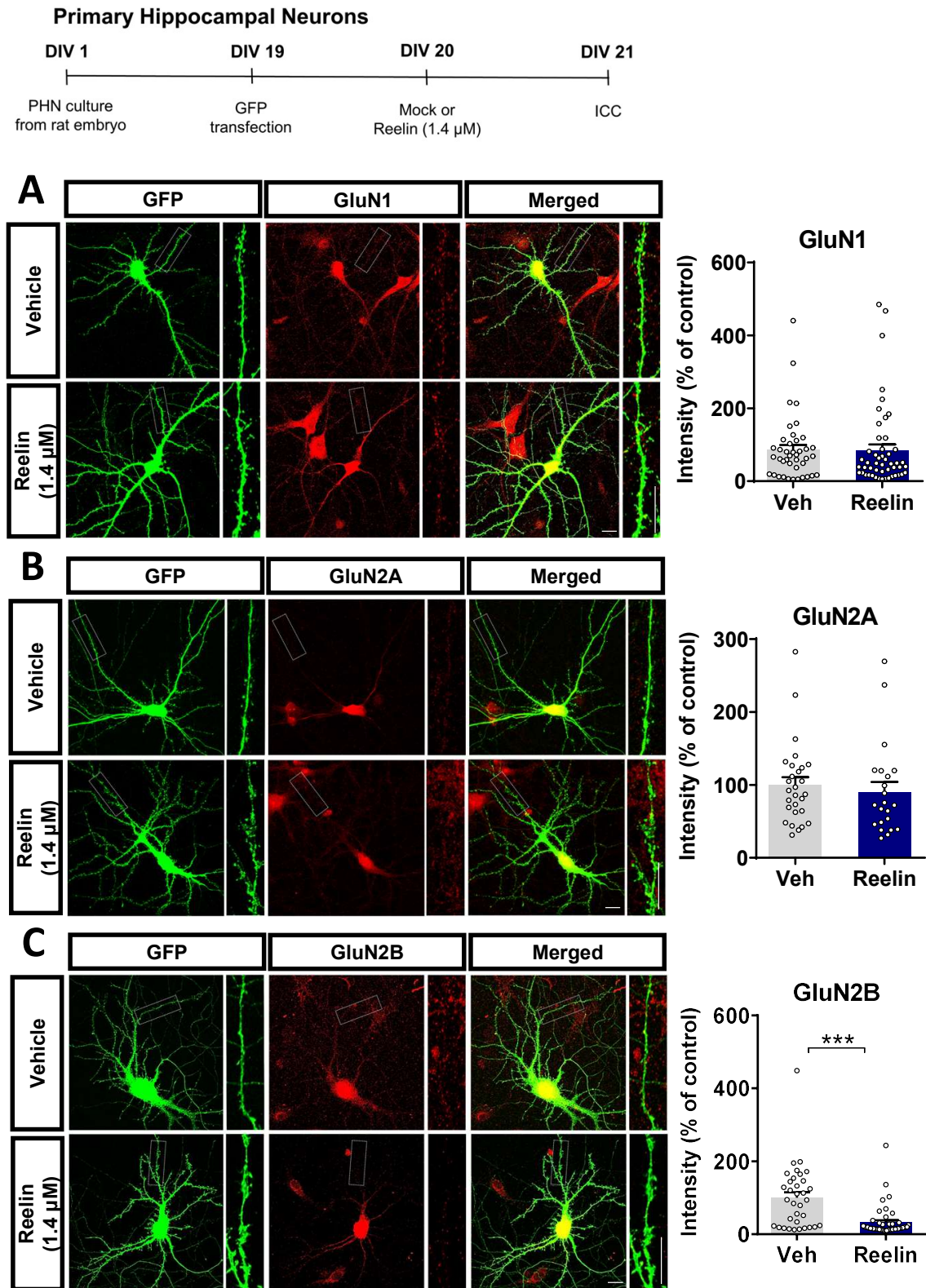
Fig. 2. Reelin selectively alters NMDA receptor subunit levels in primary hippocampal neurons. (A-C) Primary hippocampal neurons were transfected with GFP plasmid on DIV 19, treated with vehicle (Mock medium) or 1.4 μM Reelin on DIV 20 for 24 h, and immunostained with anti-GluN1, anti-GluN2A, or anti-GluN2B antibodies. Then, the intensities of GluN1 (A, vehicle: n=43 dendrites from 7 neurons, Reelin: n=51 dendrites from 6 neurons), GluN2A (B, vehicle: n=28 dendrites from 6 neurons, Reelin: n=22 dendrites from 5 neurons), and GluN2B (C, vehicle: n=34 dendrites from 5 neurons, Reelin: n=46 dendrites from 5 neurons) were measured. ***$p<0.001$. Scale bar=10 μm.
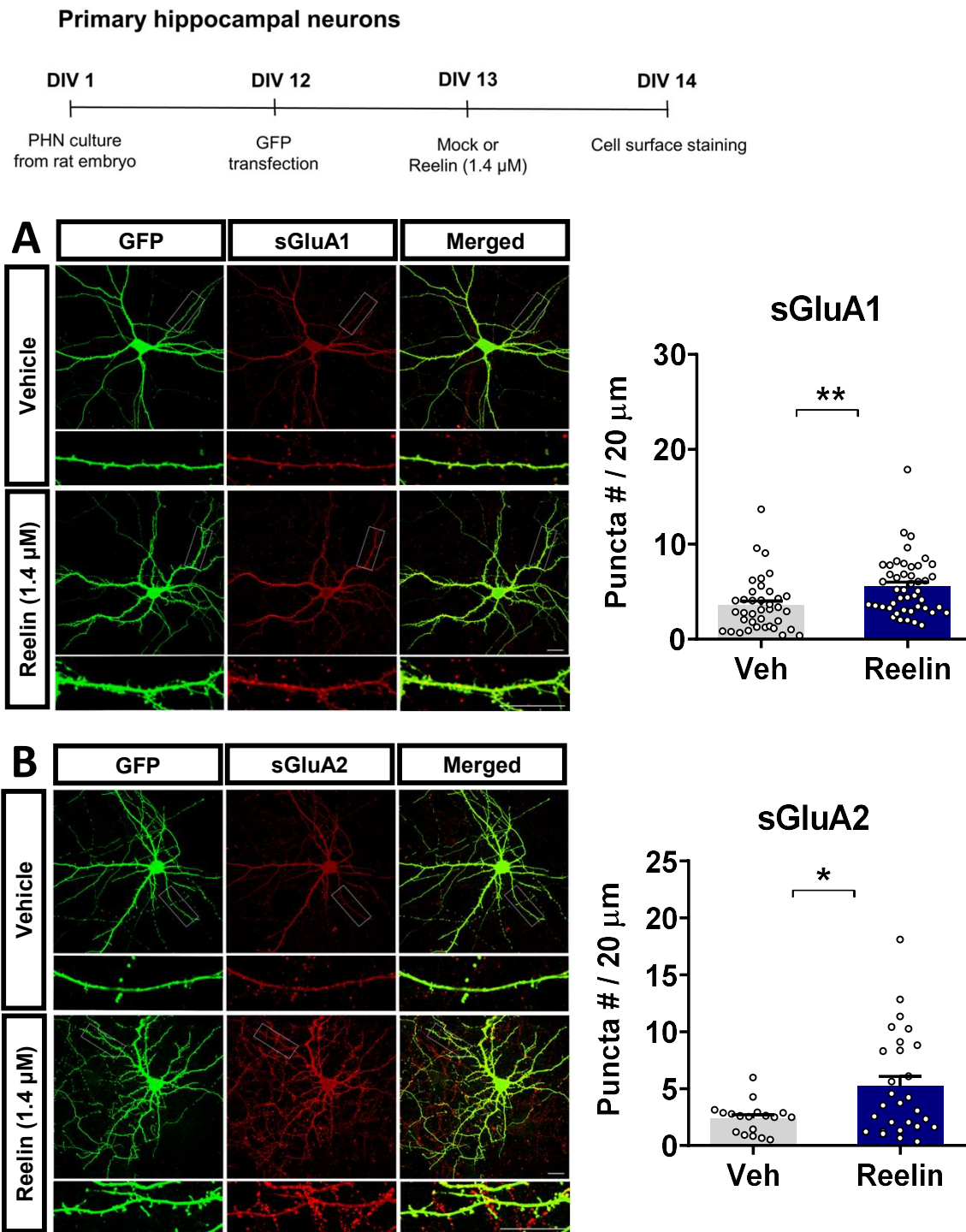
Fig. 3. Reelin increases AMPAR trafficking during synaptogenesis in primary hippocampal neurons. (A, B) Primary hippocampal neurons were transfected with GFP plasmid on DIV 12 and treated with vehicle (Mock medium) or 1.4 μM Reelin on DIV 13 for 24 h. On DIV 14, live cell surface staining was conducted with antibodies recognizing the N-terminus of GluA1 or GluA2. Then, puncta numbers of GluA1(A, vehicle: n=38 dendrites from 6 neurons, Reelin: n=46 dendrites from 8 neurons) and GluA2 (B, vehicle: n=18 dendrites from 4 neurons, Reelin: n=27 dendrites from 6 neurons) were measured. *p<0.05, **p<0.01. Scale bar=10 μm.

neuronal processes. Reelin treatment markedly enhanced p-ERK and p-CREB puncta numbers in secondary/tertiary dendrites in mature primary hippocampal neurons (Fig. 4B, C).

Next, we tested whether Ras activity is required for the effects of Reelin on dendritic spinogenesis. Primary hippocampal neurons (DIV 19) were transfected with GFP plasmid and HA plasmid (control for RasN17) or GFP plasmid and RasN17 plasmid (Ras inactivator) and treated with Reelin (1.4 μM) or vehicle (Mock medium) for 24 h. Dendritic spine number was then measured in secondary/tertiary distal dendrites. Consistent with the findings in Fig. 1A, Reelin treatment significantly increased dendritic spine density in mature primary hippocampal neurons compared with Mock treatment (Fig. 4D). However, RasN17 treatment blocked the Reelin-mediated increase in dendritic spine number in mature primary hippocampal neurons (Fig. 4D). These data suggest that Reelin promotes dendritic spine formation via Ras signaling in mature primary hippocampal neurons.

Reelin works with APP to promote dendritic spine formation in vitro and in vivo

Previously, we found that APP enhances dendritic spine formation in vitro and in vivo [1]. Since APP and Reelin individually modulate dendritic spine formation, we examined whether Reelin and APP act cooperatively or independently to affect dendritic spinogenesis. For this experiment, primary hippocampal neurons (DIV 19) were transfected with GFP plasmid and PLL (control) or with GFP plasmid and APP shRNA and then treated with Reelin (1.4 μM) or vehicle (Mock medium) for 24 h. After Reelin treatment, we measured the dendritic spine number in secondary/tertiary distal dendrites. Compared with the corresponding controls, Reelin increased dendritic spine density, whereas APP knockdown decreased dendritic spine density in mature primary hippocampal neurons (Fig. 5A). Importantly, Reelin treatment did not enhance dendritic spine density when APP was knocked down, indicating that Reelin is necessary for APP to promote dendritic spinogenesis in mature primary hippocampal neurons (Fig. 5A).

Next, we investigated whether APP is required for Reelin-mediated dendritic spine formation in vivo. We chose a Reelin dose of 210 nM because we previously found that this dose significantly increased hippocampal spine density in wild-type and ApoER2 KO mice [17]. Three-month-old APP KO mice were bilaterally intraventricularly injected with Reelin (210 nM) or saline (control). Since Reelin was previously shown to enhance dendritic spine density in cortical layers II/III and hippocampal area CA1 in wild-type mice 5 days after injection [17], the Reelin- or saline-injected APP KO mice were sacrificed for Golgi staining on day 5, and the dendritic spine number was measured in secondary/tertiary distal

dendrites. In both the hippocampal CA1 region and cortical layers II/III, Reelin-injected APP KO mice did not increase the dendritic spine density of AO and BS dendrites compared with control-treated APP KO mice (Fig. 5B, C). This result further demonstrates that the interaction of Reelin and APP is essential to regulate dendritic spine formation in vitro and in vivo.

DISCUSSION

Previous studies have separately examined the roles of Reelin and APP in dendritic spine formation and synaptic plasticity in vitro and in vivo [17, 20, 33]. However, the mechanism by which Reelin modulates dendritic spinogenesis has not been clearly established, and whether APP and Reelin work together or independently to alter dendritic spine formation is unknown. This study is the first to demonstrate that Reelin regulates dendritic spinogenesis through Ras/ERK/CREB signaling and acts cooperatively with APP to promote dendritic spine formation in vitro and in vivo. Our findings indicate that abnormal APP function (including amyloidogenic processing of APP) may not only contribute to AD pathology (via Aβ accumulation) but also impair normal APP/Reelin signaling at synapses.

In a previous study, we investigated the effects of Reelin and APP on dendritic neurite outgrowth and dendritic branching in developing hippocampal neurons (DIV 14, the peak of synaptogenesis). Given that Reelin has a pivotal role in embryonic neuronal translocation/migration and postnatal dendritic growth/synaptic development [34], our previous findings implicated an interaction between Reelin and APP in the regulation of dendritic outgrowth during neuronal development [25]. Interestingly, a recent study demonstrated that organotypic hippocampal cultures from *Reeler* mice (disruption of the *reelin* gene) exhibit decreased dendritic spine density that is rescued by exogenous Reelin treatment [16]. We previously reported that Reelin-treated wild-type mice exhibit significantly increased dendritic spine density and synaptic plasticity in the hippocampus [17]. In the present study, Reelin treatment increased the dendritic spine number and PSD-95 puncta number in primary hippocampal neurons (Fig. 1). Consistent with our findings, another study found that inhibition of Reelin with the neutralizing antibody CR50 suppresses PSD-95 immunoreactivity without affecting synaptophysin levels in rat primary hippocampal neuronal culture [35]. Overall, previous studies and our findings suggest that Reelin enhances dendritic spinogenesis by modulating postsynaptic function in mature primary hippocampal neurons.

Trafficking of NMDARs and AMPARs to the surface of excitatory postsynaptic neurons plays a critical role in LTP induction and
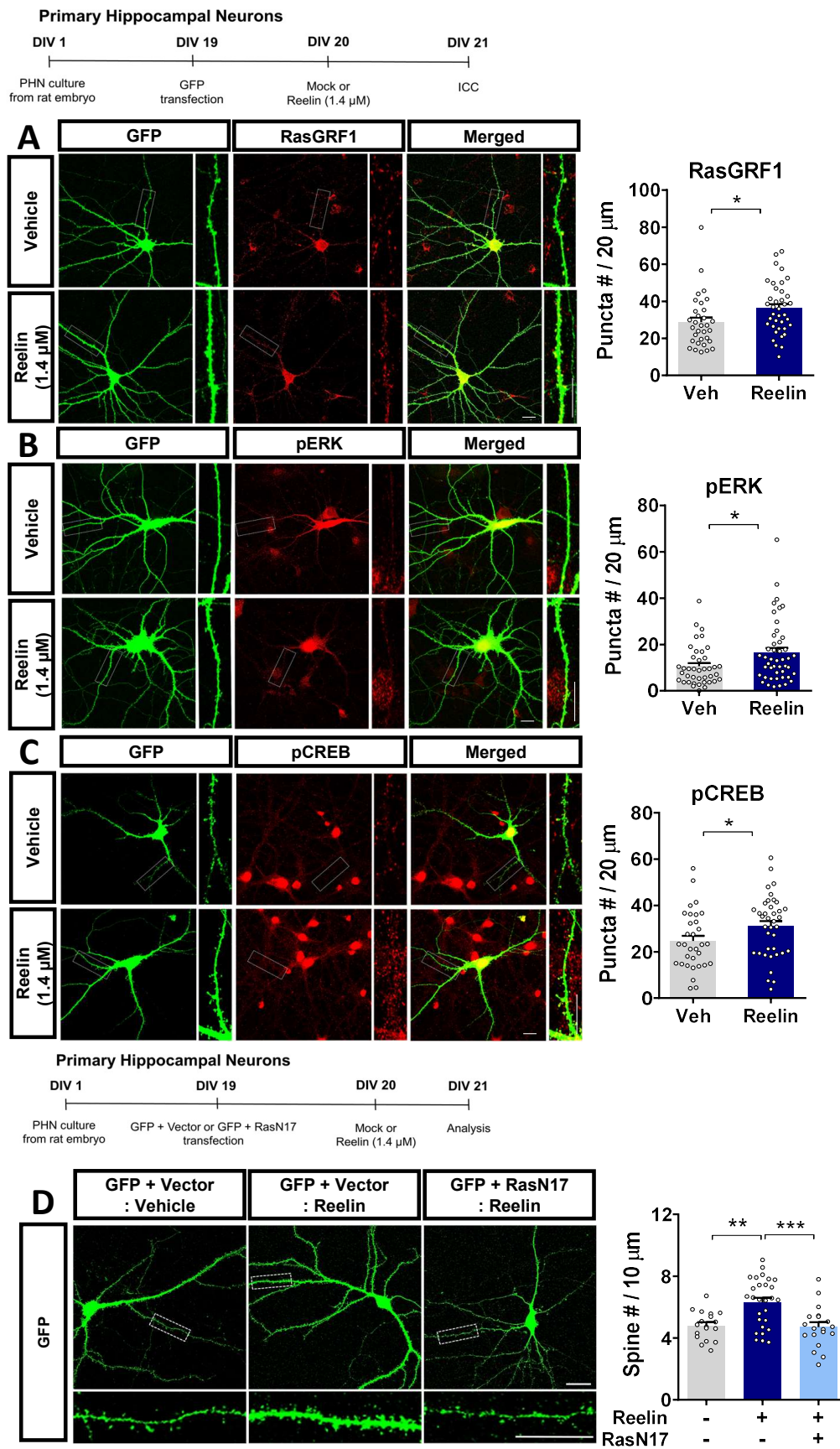
Fig. 4. Reelin promotes dendritic spine formation through Ras signaling in primary hippocampal neurons. (A) Primary hippocampal neurons were transfected with GFP plasmid on DIV 19 and treated with vehicle (Mock medium) or 1.4 μM Reelin on DIV 20 for 24 h. On DIV 21, the neurons were immunostained with an anti-RasGRF1 antibody, and the puncta numbers were measured (vehicle: n=32 dendrites from 6 neurons, Reelin: n=38 dendrites from 7 neurons). (B, C) Primary hippocampal neurons were treated as described above and immunostained with anti-p-ERK and anti-p-CREB antibodies on DIV 21, and the puncta numbers were measured (p-ERK, vehicle: n=40 dendrites from 6 neurons, Reelin: n=46 dendrites from 9 neurons, p-CREB, vehicle: n=32 dendrites from 8 neurons, Reelin: n=38 dendrites from 8 neurons). (D) Primary hippocampal neurons were transfected with GFP plasmid and HA plasmid (control plasmid for RasN17) or GFP plasmid and RasN17 (Ras inactivator) on DIV 19 and treated with vehicle (Mock medium) or 1.4 μM Reelin on DIV 20 for 24 h. Then, dendritic spine density was measured (GFP plasmid+HA plasmid+vehicle: n=18 dendrites from 9 neurons, GFP plasmid+HA plasmid+Reelin: n=28 dendrites from 12 neurons, GFP plasmid+RasN17+Reelin: n=19 dendrites from 7 neurons) *p<0.05, **p<0.01, ***p<0.001. Scale bar=10 μm.
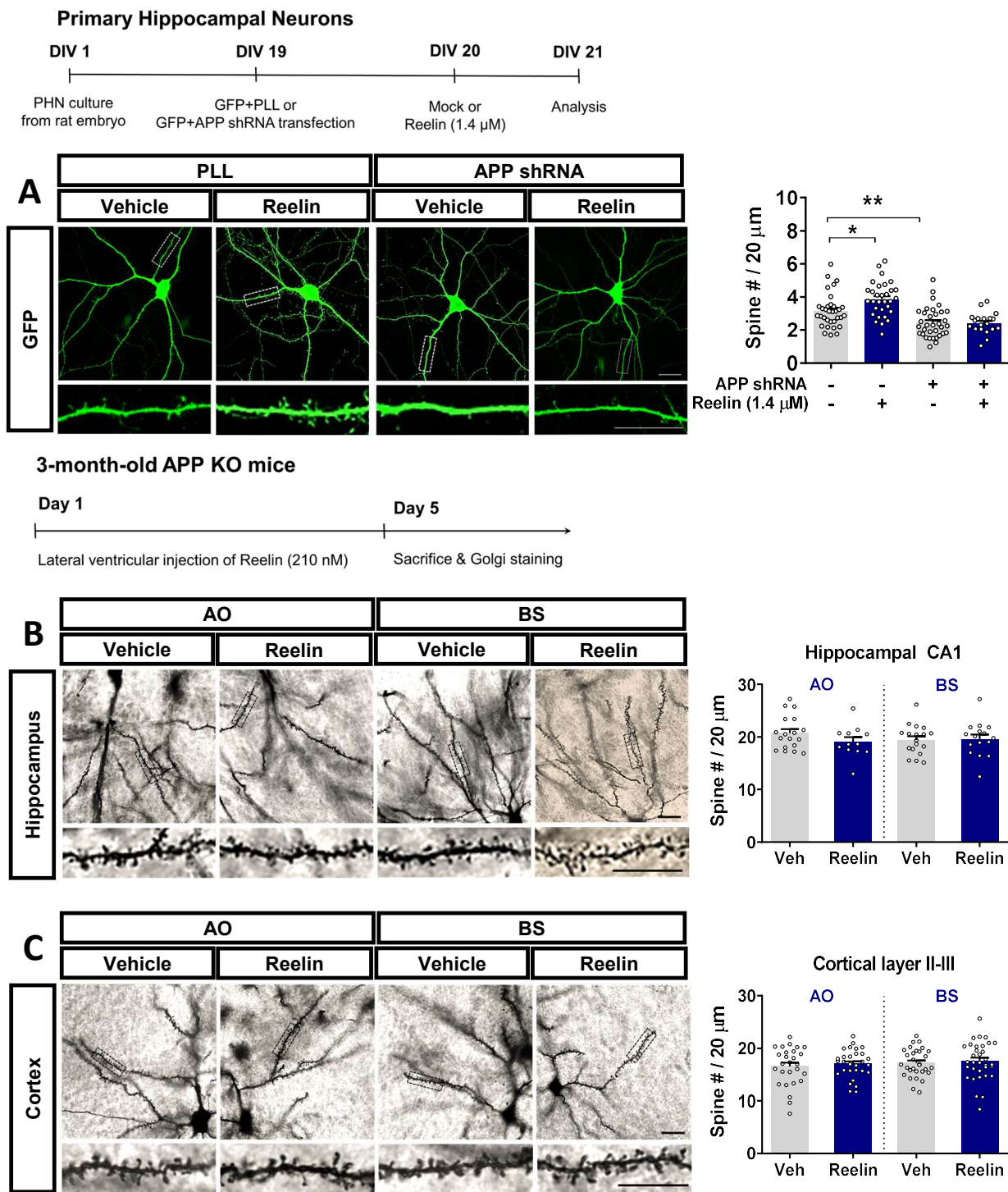
Fig. 5. Reelin and APP cooperatively regulate dendritic spine density *in vitro* and *in vivo*. (A) Primary hippocampal neurons were transfected with GFP plasmid and PLL (control) or GFP plasmid and APP shRNA on DIV 19 and treated with 1.4 μM Reelin or vehicle (Mock medium) on DIV 20. Then, dendritic spine density was measured (GFP plasmid+PLL+vehicle: n=33 dendrites from 13 neurons, GFP plasmid+PLL+Reelin: n=31 dendrites from 14 neurons, GFP plasmid+APP shRNA+Vehicle: n=34 dendrites from 14 neurons, GFP plasmid+APP shRNA+Reelin: n=19 dendrites from 9 neurons). Scale bar=10 μm. (B, C) Three-month-old APP KO mice received bilateral intraventricular injections of 210 nM Reelin or saline. After 5 days, Golgi staining was performed, and dendritic spine density was measured in the AO and BS regions of hippocampal area CA1 (for AO, vehicle: n=18 dendrites from 18 neurons, Reelin: n=12 dendrites from 12 neurons; for BS, vehicle: n=18 dendrites from 18 neurons, Reelin: n=16 dendrites from 16 neurons, n=4 mice/group) and cortical layer II/III (for AO, vehicle: n=27 dendrites from 27 neurons, Reelin: n=29 dendrites from 29 neurons; for BS, vehicle: n=30 dendrites from 30 neurons, Reelin: n=33 dendrites from 33 neurons, n=4 mice/group). *p<0.05, **p<0.01. Scale bar=20 μm.

memory consolidation [36]. In primary hippocampal neurons, blocking Reelin activity upregulates GluN2B-expressing dendritic spines [35]. However, Reelin treatment downregulates surface and total GluN2B levels but upregulates surface and total GluN2A levels in hippocampal slice cultures [26]. This NMDAR subunit switch from GluN2B to GluN2A and subsequent increase in GluA1 are accompanied by downregulation of silent synapses and synaptic transmission failure, implying that Reelin plays a critical role in synaptic plasticity by modulating synaptic NDMAR/AMPAR subunit composition [26]. Interestingly, a previous study demonstrated that NMDAR GluN1 subunit levels are reduced in primary hippocampal neurons from *Reeler* mice compared with those in primary hippocampal neurons from wild-type mice during the peak of synaptogenesis [26]. However, whether Reelin modulates NMDAR expression in mature primary hippocampal neurons (DIV 21) has not been examined. Thus, we investigated the effects of Reelin on NMDAR expression in mature primary hippocampal neurons and found that Reelin decreased NMDAR GluN2B subunit expression, whereas the expression of GluN1 and GluN2A was not altered (Fig. 2). GluN2B expression is associated with AKT dephosphorylation, which exacerbates excitotoxicity, and the absence of GluA2-containing AMPARs accelerates calcium permeability and consequently reduces cell viability [37, 38]. Importantly, suppression of GluN2B levels in hippocampal CA1 synapses attenuates memory decline in aged mice and blocking GluN2B-containing NMDARs ameliorates the Aβ1-42- or TNF-α-mediated LTP reduction in rat CA1 pyramidal neurons [39, 40]. Overall, these data suggest that Reelin may differentially regulate NMDAR subunit expression between the peak of synaptogenesis and mature neurons to alter cognitive and synaptic function.

Regarding the effects of Reelin on AMPAR trafficking and expression, several studies have confirmed that Reelin treatment enhances surface GluA1 expression and GluA2 phosphorylation in primary hippocampal neuronal culture [26, 41]. In addition, we previously reported that primary hippocampal neurons cultured from *Reeler* mice exhibit decreased GluA1 puncta numbers compared with primary hippocampal neurons cultured from wild-type mice during the peak of synaptogenesis [9]. Moreover, another study demonstrated that Reelin affects cell surface AMPAR GluA1 and GluA2 subunit levels in mature neurons [42]. Thus, in this study, we further examined whether Reelin treatment modulates AMPAR GluA2 subunit trafficking in primary hippocampal neurons during the peak of synaptogenesis. We found that Reelin treatment enhanced surface GluA1 and GluA2 puncta numbers compared with Mock treatment (Fig. 2, 3). However, we have not yet examined whether Reelin modulates AMPAR GluA1 and GluA2 subunit trafficking in mature primary hippocampal

neurons, which will be addressed in a future study. Taken together with the literature, our data indicate that Reelin regulates AMPAR GluA1 and GluA2 subunit trafficking to affect dendritic spine formation and synaptic function.

NMDARs trigger Ras signaling via RasGRF, and the subsequent activation of the Ras-ERK cascade leads to CREB phosphorylation, which facilitates synaptic plasticity and memory formation [43, 44]. The Ras-ERK pathway has been implicated in dendritic complexity in hippocampal neuronal culture [45]. In addition, we and others have found that Reelin regulates NMDAR/AMPAR expression/trafficking to alter dendritic spine formation, but the underlying molecular mechanism remains to be elaborated. To address this gap, we examined the effects of Reelin on Ras signaling for the first time in the present study and found that Reelin treatment activated the Ras-ERK-CREB pathway and promoted dendritic spinogenesis in a Ras signaling-dependent manner (Fig. 4). Interestingly, an association of Reelin with neuronal maturation via enhancement of ERK signaling in a VLDLR/ApoER2-independent manner in cortical neuronal culture has been reported [23]. Given that Reelin binds to APP [25], we assume that Reelin interacts with APP and/or other receptors (e.g., VLDLR and ApoER2) to activate the Ras-ERK-CREB cascade and thereby promote dendritic spinogenesis *in vitro*.

We and others have reported that APP also plays an important role in dendritic spinogenesis and synapse formation [1]. We previously demonstrated that APP interacts with Reelin extracellularly and that this interaction has a synergistic effect on neurite outgrowth [25]. The present study is the first to examine whether Reelin regulates dendritic spine formation, which is involved in cognitive function, by cooperating with APP *in vitro* and *in vivo*. We found that Reelin promoted dendritic spine formation in the presence of APP, whereas silencing APP expression prevented Reelin-mediated enhancement of dendritic spine density in mature primary hippocampal neurons (Fig. 5). Further supporting our *in vitro* findings, Reelin did not rescue the deficits in dendritic spine density in the cortex and hippocampus in APP KO mice (Fig. 5). It is possible that APP and ApoE receptors (e.g., ApoER2 and VLDLR) compete for Reelin to alter dendritic spinogenesis. Another possibility is that adaptor proteins such as Dab1, JIP1 and FE65 bind to APP/ApoER and that the receptor complex works in concert with Reelin to modulate dendritic spinogenesis. Future studies will address this issue. Overall, our findings strongly demonstrate that Reelin acts cooperatively with APP to modulate dendritic spine formation *in vitro* and *in vivo*.

How do Reelin and APP alter dendritic spinogenesis? The mechanisms that Reelin utilizes to regulate synapse formation may not be mutually exclusive. Dendritic spines have high con-

centrations of actin fibers, and actin likely mediates the fluctuating sizes of dendritic spines over time. Interestingly, APP is highly enriched in the motile regions of neuronal growth cones [46] and affects cell movement *in vitro* [47], suggesting that APP signaling modulates actin dynamics. Recent studies have also established a role of Reelin in regulating actin cytoskeleton dynamics through the actin-depolymerizing protein n-cofilin [48, 49] and the Rho GTPase Cdc42 [50]. Since n-cofilin and Cdc42 are important for regulating dendritic spine stability in adults [51, 52], it is tempting to speculate that Reelin and APP utilize these pathways to regulate dendritic spine formation and/or stabilization. The extent to which APP contributes to known mechanisms of Reelin signaling is currently being explored.

In conclusion, this study is the first to demonstrate that Reelin regulates the formation of dendritic spines in an APP-dependent manner. These data are further evidence of the important function of APP in dendritic spinogenesis and its distinction from the function of Aβ, which can result in synaptic dysfunction in AD. In particular, the present data suggest that APP is a downstream target of the Reelin signaling pathway in normal brain function and that alterations in this relationship may be relevant to the cognitive disturbances associated with AD.

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