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Very low density lipoprotein receptor regulates dendritic spine formation in a RasGRF1/CaMKII dependent manner

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Abstract

Very Low Density Lipoprotein Receptor (VLDLR) is an apolipoprotein E receptor involved in synaptic plasticity, learning, and memory. However, it is unknown how VLDLR can regulate synaptic and cognitive function. In the present study, we found that VLDLR is present at the synapse both pre- and post-synaptically. Overexpression of VLDLR significantly increases, while knockdown of VLDLR decreases, dendritic spine number in primary hippocampal cultures. Additionally, knockdown of VLDLR significantly decreases synaptophysin puncta number while differentially regulating cell surface and total levels of glutamate receptor subunits. To identify the mechanism by which VLDLR induces these synaptic effects, we investigated whether VLDLR affects dendritic spine formation through the Ras signaling pathway, which is involved in spinogenesis and neurodegeneration. Interestingly, we found that VLDLR interacts with RasGRF1, a Ras effector, and knockdown of RasGRF1 blocks the effect of VLDLR on spinogenesis. Moreover, we found that VLDLR did not rescue the deficits induced by the absence of Ras signaling proteins CaMKIIα or CaMKIIβ. Taken together, our results suggest that VLDLR requires RasGRF1/CaMKII to alter dendritic spine formation.

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Authorship

Experiments and analysis were conducted by AMD, SBD, and JMS. GB, EW, HSH and GWR provided reagents. All contributed to the writing of the manuscript.

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Keywords

VLDLR; Dendritic Spine; Ras; CaMKII; Alzheimer's disease; apoE receptor

1. Introduction

In addition to participating in cholesterol transport, apolipoprotein E (apoE) receptors have been implicated in neuronal development, synaptic function, and Alzheimer´s disease (AD). Two of these receptors with 50% homology to one another, ApoE Receptor 2 (ApoER2) and Very Low Density Lipoprotein Receptor (VLDLR), are important for nervous system development [1–4]. Specifically, the double ApoER2 and VLDLR knockout caused deficits in neuronal migration in the developing brain [5]. Polymorphisms in VLDLR have also been associated with Alzheimer's disease risk later in life [6]. Moreover, the gene encoding for the ligand of this receptor, APOE, is the strongest known genetic risk factor for Alzheimer's disease [7].

In addition to their roles in neuronal development and disease, apoE receptors have also been implicated in synaptic function. In particular, ApoER2 knockout mice demonstrate severe defects in LTP and dentate granule cell organization while the VLDLR knockout mice have moderate defects in the hippocampus [8, 9]. Moreover, we have previously shown that ApoER2 can alter dendritic spine formation with different cytoplasmic adaptor proteins moderating this effect [10]. Blocking the function of another apoE receptor, low-density lipoprotein receptor-related protein-1 (LRP1), can also induce deficits in the late phase of LTP [11] and decrease dendritic spine density [12]. Taken together, this research suggests that apoE receptors are important in synapse function and dendritic spine formation, and therefore, in learning and memory. However, it is unknown whether VLDLR alters dendritic spines. Moreover, the molecular mechanisms by which apoE receptors could alter spinogenesis are still unknown.

In the present study, we are the first to demonstrate the effect of VLDLR on dendritic spine number and its expression at pre- and post- synaptic densities. Additionally, we discovered that VLDLR selectively affects the puncta number of synaptophysin as well as the expression of glutamatergic receptors at the synapse. Moreover, we report that the knockdown of Ras signaling proteins RasGRF1, CaMKIIα or CaMKIIβ block the effect of VLDLR on mediating spine density. Taken together, our results suggest that VLDLR increases dendritic spine density through a RasGRF1/ CaMKII-dependent signaling mechanism.

2. Materials and Methods

2.1. Mus musculus

VLDLR knockout mice were raised from stocks originally created through targeted gene deletion [13]. All experiments used wild-type littermates as controls. The animals were provided a standard rodent chow diet (Diet 7001, Harlan Teklad, Madison, WI) and water *ad libitum*. All procedures were performed in accordance with the protocols approved by the

Institutional Committee for Use and Care of Laboratory Animals of the University of South Florida and the Animal Care and Use Committee of Georgetown University.

2.2. Cell lines and culture conditions

COS7 cells (Lombardi Co-Resources Cancer Center, Georgetown University, Washington DC, USA) were maintained in Opti-MEM (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA.) in a 5% CO_2 incubator. To examine cell surface levels of VLDLR by RasGRF1, COS7 cells were transiently transfected with VLDLR + Vector or VLDLR + RasGRF1, with using FuGENE 6 (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol followed by cell surface biotinlyation. After 24 hours, cell surface biotinlyation was conducted as previously described ([10]).

2.3. Antibodies

We used the following antibodies: anti-PSD-95 (Chemicon Billerica, MA), anti-GFP (Invitrogen Carlsbad, CA, USA), β-actin (Chemicon, Temecula, CA, USA), anti-GluA1 (Calbiochem. Darmstadt, Germany), anti-GluA2 (Chemicon, Billerica, MA), anti-c-myc (Abcam, San Francisco, CA, USA), anti-synaptophysin (Sigma, St. Louis, MO), anti-VLDLR (5F3, generous donation from Dr. Dudley Strickland; IIII, generated in the laboratory of Dr. Guojun Bu; 74, generous gift from Dr. Joachim Herz), anti-GluN1 (Neuromab, US Davis, Davis, CA, ISA), anti-GluN2A (Chemicon Temecula, CA, USA), anti-GluN2B (Neuromab, UC Davis, Davis, CA, USA), anti-Ras (BD Biosciences, San Jose, Ca, USA), anti-RasGRF1 (BD Biosciences, San Jose, CA, USA, Santa Cruz Biotechnology, Santa Cruz, CA), anti-CaMKIIα (Abcam, San Francisco, CA, USA), anti-p-CaMKIIα (Abcam, San Francisco, CA, USA), CaMKIIβ (Abcam, San Francisco, CA, USA).

2.4. Primary neuronal culture and transfection

Hippocampal neurons from embryonic day 18–19 Sprague–Dawley rats (Rattus Norvegicus) were cultured at 150 cells/mm^2 as described [14]. These cells are mixed cultures, as no glial inhibitor is added to the media. To test the effect of VLDLR on spinogenesis, primary hippocampal cultures were transfected with GFP + vector, GFP + VLDLR, or GFP + VLDLR shRNA using lipofectamine 2000 (Invitrogen). After 72 hours, dendritic spine density was measured using Scion ImageJ. To examine which domain of VLDLR is responsible for its effect on spinogenesis, primary hippocampal neurons were transfected with GFP + vector, GFP + VLDLR deletion #1 (without VLDLR ligand binding domain), GFP + VLDLR deletion #2 (without VLDLR extracellular domain), GFP + full length VLDLR for 72 hours, and spine density was measured. To measure the number of excitatory synapses, primary hippocampal neurons were transfected with GFP + vector, GFP + VLDLR, or GFP + VLDLR shRNA for 72 hours. After 72 hours, the puncta number of synaptophysin and PSD-95 was measured, as well as spinogenesis related proteins. To examine whether VLDLR promotes spinogenesis in a RasGRF1-dependent manner, primary hippocampal neurons were transfected with GFP + Vector + PLL, GFP + VLDLR + Vector, GFP + RasGRF1 shRNA + Vector, GFP + RasGRF1 shRNA + VLDLR for 72 hours, and spine density was measured. To test the effect of CaMKIIα and CaMKIIβ on VLDLR mediated spinogenesis, primary hippocampal neurons were transfected with GFP + PLL + Vector, GFP + VLDLR+ Vector, GFP + CaMKIIα or CaMKIIβ shRNA + Vector, or GFP +

VLDLR + CaMKIIα or CaMKIIβ shRNA for 72 hours, and dendritic spine density was measured.

2.5. Spine Density Analysis

For quantification of spine number, images of the dendritic shaft were collected at 63× magnification in the GFP channel. Groups were coded to prevent bias, and dendritic spines were counted on 100 μm dendritic segments (one to three dendritic shafts per neuron). The numbers of spines were divided by the length measured to measure the density along the dendritic shaft. The spine measurements were collected from at least 8 neurons per group. For each construct, individual spine measurements were first grouped and averaged per neuron; means from several neurons were then averaged to obtain a population mean (presented as mean ± SEM).

2.6. Plasmids

The VLDLR shRNA constructs were a gift from Dr. Tracy Young-Pearse. The following was the target sequence for VLDLR shRNA #1: 5' – CCGGCCAGCAATGAACCTTGTGCTACTCGAGTAGCACAAGGTTCATTGCTGGTTTTTG-3', for VLDLR #3: 5'-CGGGCCGAGTCTGATCTTCACTAACTCGAGTTAGTGAAGATCAGACTCGGCTTTTTG-3' and the shRNAs were expressed in a pLKO.1 plasmid vector. The CaMKIIα and β RNAi constructs were gifts from Dr. Dan Pak at Georgetown University.

The following sequences were targeted: for CaMKIIα; 5′-CCACTACCTTATCTTCGAT-3′; for CaMKIIβ; 5′-GAGTATGCAGCTAAGATCA-3′ and the RNAi were expressed in the pSuper plasmid vector. The VLDLR-myc constructs were expressed in a pSEC, tag2 hygro plasmid under the CMV promoter. The GFP construct was expressed in the pEGF1 N1 plasmid under the CMV promoter.

2.7. Immunocytochemistry

To test the co-localization between VLDLR and various synaptic proteins related to spinogenesis for Figure 1, primary hippocampal neurons (DIV 21) were fixed with 4% paraformaldehyde for 5 minutes, and with methanol for 5 minutes. After fixation, cells were washed with 1× PBS three times, and then incubated with primary antibody overnight (VLDLR (IIII) and one of the following antibodies: synaptophysin, PSD-95). Images were taken on a single plane using a confocal microscope (Zeiss LSM 510). To measure synaptic protein intensity, primary hippocampal neurons were fixed in either 4% paraformaldehyde at room temperature (for morphological analysis) or in methanol at −20°C (for immunostaining of endogenous synaptic markers) for 10 min. After fixation, cells were incubated with primary antibodies in GDB buffer (0.1% gelatin, 0.3% Triton X-100, 16 mM sodium phosphate pH 7.4, 450 mM NaCl) overnight. The next day, cells were washed with 1× PBS three times, and cells were incubated in secondary antibody with AlexaFluor 488 or AlexaFluor 555. To measure the cell surface levels of AMPA receptor subunits, live neuronal cultures were incubated with antibodies directed against extracellular N-termini of AMPA receptor subunits (GluA1, GluA2, 10 mg/mL in conditioned medium, non-permeabilizing conditions) for 10 min. Then, cells were fixed with 4% paraformaldehyde for

5 minutes and washed with 1× PBS three times. We then incubated with Alexa Fluor 555–linked anti-mouse secondary antibodies for 1 hr. Z-stacked images from a confocal microscope (Zeiss LSM510) were imaged and analyzed for the number of puncta and total intensity using Metamorph and ImageJ software. Dendrites were straightened using a customized macro, and puncta number was measured in the 0–50 micron dendritic region from the soma. Puncta density was calculated by dividing the puncta number by the area of the dendritic branch.

2.8. Co-immunoprecipitation

Primary cortical and hippocampal lysates or whole brain lysate were incubated with anti-synaptophysin, PSD-95, GluA1, GluA2, GluN1, GluN2A, GluN2B, CamKIIα or CaMKIIβ and protein G- or protein A-Sepharose beads overnight at 4°C (Amersham Biosciences, Pittsburgh, PA, USA). The next day, cell or brain lysate was washed with IP buffer 3 times, and re-suspended in SDS sample buffer, and Western blot was conducted.

2.9. Synaptosomal fractionation

Synaptosomal fractionation was performed as described [10]. Mouse brains were homogenized in Sucrose HEPES buffer (0.32 M sucrose, 4 mM HEPES-NaOH, pH 7.3 with protease inhibitors), and centrifuged at 10,000g for 10 min to recover the S1 supernatant and the P1 pellet. The S1 supernatant was subsequently spun at 12,0000g for 15 min to obtain the pellet P2 (Crude synaptosome) and the supernatant S1. The P2 fraction was lysed via hypo-osmotic shock and centrifuged at 25,0000g for 20 min to generate the pellet LP1 and the supernatant LS1. The LS1 fraction was ultracentrifuged to obtain the synaptic vesicle fraction (LP2 or SV). The LP1 pellet was applied to a discontinuous sucrose gradient consisting of 1.0, 1.5, and 2.0 M sucrose layers. After a 200,000g ultracentrifugation for 2 hours, the PSD fraction was recovered at the 1.5/2.0 M sucrose interface and solubilized in detergent (0.16 M sucrose, 5 mM Tris- HCl, pH 8.0, 0.5% Triton X-100, 0.5 mM β-ME, 1 mM EDTA, and protease inhibitors). This fraction was ultracentrifuged to obtain the final PSD2 fraction.

2.10. Statistical analyses

All data were analyzed using either a two-Tailed T-test or ANOVA with Graphpad Prism 4 software. Post-hoc analyses were completed with Tukey's Multiple Comparison test with $p<0.05$ significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

3. Results

3.1. VLDLR expression in synapses

To determine whether VLDLR is expressed at the synapse, we immunostained primary hippocampal neurons with VLDLR (green, IIII antibody) and synaptophysin (red, pre-synaptic marker) antibodies. We found that VLDLR is expressed along the dendrites and spines with a punctate staining that partially co-localizes with synaptophysin (Fig. 1A, upper panels). We also conducted a parallel experiment by staining primary hippocampal cultures with VLDLR (green, IIII antibody) and PSD-95 (red, post-synaptic marker) antibodies.

VLDLR also co-localized with PSD-95 (Fig. 1A, lower panels), suggesting that VLDLR could be present in both pre- and post-synaptic compartments.

As an alternative approach to defining VLDLR subcellular expression, we conducted synaptosomal fractionations from wild-type mouse brain lysate and probed for VLDLR as well as various synaptic proteins related to spinogenesis. Consistent with our immunostainings, we found that VLDLR was present in both the pre- and post- synaptic fractionations. However, VLDLR was either more pre- or post-synaptic depending upon the antibody used. Interestingly, we found that VLDLR was present in both the pre- and post-synaptic fractionations when we used an antibody recognizing the ligand binding domain (LBD) of VLDLR (5F3, monoclonal) (Fig. 1B, upper left panel). However, another polyclonal VLDLR antibody raised against the LBD of VLDLR (IIII) predominantly localized to the post-synaptic fractionation (Fig. 1B, second left panel). Another N-terminal antibody, recognizing the LBD for VLDLR (α-74, polyclonal), suggested that VLDLR is mostly localized to the pre-synaptic fractionation (Fig. 1B, third left panel). To test for nonspecific binding of these VLDLR antibodies, we probed the immunoblots of wild-type and VLDLR knockout tissue with these three VLDLR antibodies. We were unable to see any immunoreactivity in the VLDLR deficient tissue at 120 kDa, the molecular weight for VLDLR (Fig. 1C). These data suggest that the antibody used for VLDLR is important to consider when examining VLDLR expression at the synapse.

To screen for spinogenesis-related synaptic proteins, we examined the distribution of CaMKIIα, CaMKIIβ, and RasGRF1 (Fig. 1B, right panel). Consistent with the literature, we found that CaMKIIα is predominantly post-synaptic, but can also be at the pre-synaptic compartment [15]. Likewise, CaMKIIβ and RasGRF1 are mostly expressed at the post-synaptic density (Fig. 1B, right panel). To monitor the purity of the synaptosomal fractionations, we probed for synaptophysin and PSD-95 and found them only in the expected compartments (Fig. 1B, lower right panel). Overall, these data suggest that VLDLR can be present in both pre- and post-synaptic compartments of synaptosomal fractionations depending on which region in the LBD of VLDLR is targeted.

3.2. VLDLR promotes dendritic spine formation in vitro

We and others have found that APOE receptors, LRP1 and ApoER2, can affect dendritic spine formation [10, 12]. Here, we investigated whether another ApoE receptor involved in learning and memory, VLDLR, could also alter dendritic spine formation. To test this hypothesis, primary hippocampal neurons (at DIV14, highest peak of *in vitro* synaptogenesis) were transfected with GFP + vector, GFP + VLDLR, GFP + PLL (control vector for shRNA) or GFP + VLDLR shRNA for 72 hours and dendritic spine density was measured. Overexpression of VLDLR caused a trend toward increased spine density (Fig. 2A–B, $p<0.06$), while knockdown of VLDLR did not significantly alter spine density (Fig. 2C–D) compared to controls.

We then examined whether VLDLR regulated dendritic spine number in mature neurons. For this experiment, primary hippocampal neurons (DIV21) were transfected with GFP + Vector, GFP + VLDLR, GFP + PLL, or GFP + VLDLR shRNA for 72 hours and dendritic spine density was measured. Interestingly, we found that overexpression of VLDLR

significantly increased spine density by 42% (Fig. 2E–F, *p<0.05), while knockdown of VLDLR significantly decreased dendritic spine density by 33% (Fig. 2G–H, ***p<0.001). These data suggest that VLDLR may have differential effects on dendritic spine density during stages of spine formation and stability. To determine the effectiveness of VLDLR shRNA knockdown, we transfected COS7 cells with rodent VLDLR and shRNA constructs. Two VLDLR shRNA constructs (#1 and #3) were tested, but only #3 was effective in reducing VLDLR expression by > 90% (Fig. 2I). As an independent assay, we also stained primary hippocampal cultures that had been transfected with empty vector or with shVLDLR (Fig. 2J). After shRNA exposure, we observed a large reduction in VLDLR staining compared to control, validating the specific pattern of VLDLR staining (Fig 1A) and the efficacy of VLDLR knockdown. For the experiments in Figure 2, as well as the rest of the experiments, we use this VLDLR shRNA #3 construct (please see the Materials and Methods for more detail).

We then examined which domain of VLDLR was responsible for its effect on dendritic spine formation. Primary hippocampal neurons (DIV18) were transfected with GFP + VLDLR deletion #1 (without the ligand binding domain of VLDLR), GFP + VLDLR deletion #2 (without the extracellular domain of VLDLR; VLDLR c-terminal fragment), or GFP + full length VLDLR for 72 hours followed by quantification of dendritic spine density (Fig. 2K–L). Deletion of the ligand binding domain of VLDLR eliminated the effect of VLDLR on dendritic spine density (Fig. 2L). These data suggest that VLDLR may play an important role in spinogenesis, and specifically, that the extracellular domain of VLDLR is required for this effect.

3.3. Knockdown of VLDLR decreases the puncta number of synaptophysin

Since we observed that VLDLR can enhance dendritic spine density, we investigated whether VLDLR could affect functional synapses. To initially test this hypothesis, we examined whether the VLDLR IIII antibody would be effective in co-immunoprecipitation assays. Therefore, wild-type and VLDLR KO brain lysates were homogenized in immunoprecipitation (IP) buffer, immunoprecipitated with α-IIII (for VLDLR) or IgG (negative control), and immunoblotted for α-5F3 (another VLDLR antibody) or α-APP (an interacting protein) as a positive control (Fig S1A). Once we confirmed that the VLDLR α-IIII antibody was successful in immunoprecipitating VLDLR and detecting its interaction partners, we conducted a parallel experiment to test whether VLDLR could interact with synaptophysin or PDS-95 (Fig. 3A–B). We found that VLDLR did not co-precipitate with either of these synaptic proteins. Although these proteins do not interact with VLDLR, we next investigated whether VLDLR expression levels could alter the puncta number of synaptophysin and PSD-95. To test this hypothesis, we transfected primary hippocampal neurons at DIV14 or DIV21 with GFP + PLL or GFP + VLDLR shRNA for 72 hours and immunostained with synaptophysin or PSD-95 antibodies (Fig. 3C, 3F). Interestingly, knockdown of VLDLR significantly decreased synaptophysin puncta number at DIV14 and DIV21 by approximately 30% (Fig. 3D, 3G, *p<0.05). However, knockdown of VLDLR did not alter PSD-95 puncta number at DIV14 and DIV21 (Fig. 3E, 3H). These data suggest that VLDLR may alter presynaptic composition prior to postsynaptic alterations such as dendritic spine formation.

3.4. VLDLR alters levels of NMDA receptor subunit GluN1

We then investigated whether VLDLR could alter other postsynaptic markers beyond PSD-95 at the synapse. Specifically, we examined whether VLDLR can interact with NMDA receptor subunits, which are involved in spinogenesis and synaptic plasticity (for review, see [16, 17]). For this experiment, wild-type brain lysates were homogenized in IP buffer, immunoprecipitated with α-IIII (for VLDLR) or IgG (negative control), and immunoblotted with α-GluN1, α-GluN2A, or α-GluN2B. We found that VLDLR did not co-precipitate with these NMDA receptor subunits (Fig. 4A–C). Next, we tested whether VLDLR altered NMDA receptor subunit levels. Primary hippocampal neurons (DIV18) were transfected with GFP + Vector, GFP + VLDLR, GFP + PLL, or GFP + VLDLR shRNA for 72 hours and immunostained with α-GluN1. Overexpression of VLDLR did not alter total GluN1 levels. However, knockdown of VLDLR significantly decreased GluN1 levels by approximately 33% (Fig. 4D–G, $^{**}p<0.01$). We also conducted a parallel experiment with GluN2A and GluN2B, and found that knockdown of VLDLR trended toward decreasing GluN2A levels (Fig. 4H–I, $p<0.07$), but did not alter GluN2B levels (Fig. 4J–K).

3.5. VLDLR affects cell surface levels of AMPA receptor subunit GluA1

Since we observed that VLDLR knockdown decreases the levels of the GluN1 NMDA receptor subunit, we next investigated whether VLDLR could alter the levels of AMPA receptor subunits. Specifically, we examined whether VLDLR interacted with AMPA receptor subunits GluA1 or GluA2. For this experiment, we immunoprecipitated wild-type mouse brain lysates with anti-VLDLR (IIII) or IgG, and probed for anti-GluA1 or GluA2. We found that VLDLR co-immunoprecipitated with GluA1, suggesting that VLDLR may directly or indirectly interact with GluA1 (Fig. 5A, S1B). However, VLDLR did not co-precipitate GluA2 (Fig. 5B). We then conducted double immunostainings in primary hippocampal cultures for VLDLR and GluA1, or VLDLR and GluA2, and found that VLDLR partially co-localized with GluA1 and GluA2 in puncta along neuronal processes (data not shown).

To examine the effect of VLDLR on AMPA receptor subunit trafficking, we transfected primary hippocampal neurons with GFP + PLL or GFP + VLDLR shRNA for 72 hours and conducted live cell surface staining (non-permeabilizing conditions) or total immunostaining (permeabilizing conditions) with an N-terminal recognizing GluA1 or GluA2 antibody. We found that knockdown of VLDLR significantly decreased the cell surface levels of GluA1 by 19%, without changing total levels, compared to control vector (Fig. 5C–F, $^{*}p<0.05$). Interestingly, knockdown of VLDLR did not significantly alter cell surface or total levels of GluA2 (Fig. 5G–J). These data suggest that VLDLR differentially interacts with glutamate receptor subunits in addition to altering glutamate receptor subunit trafficking.

3.6. VLDLR requires Ras activation for altering dendritic spine density

Ras signaling is implicated in dendritic spine formation as well as neurodegeneration such as Alzheimer's disease (AD). Specifically, protein-specific guanine nucleotide releasing factor 1, RasGRF1, has been linked to Ras activation, synaptic delivery of AMPA receptors, and dendritic spine formation [18]. In a recent study, we found that APP promotes dendritic

spine formation in a Ras-dependent manner [19], APP interacts with VLDLR, and APP affects the trafficking of VLDLR [20]. Based on the literature and our findings, we hypothesized that VLDLR may affect dendritic spine density by regulating the levels or activity of Ras signaling proteins.

To test this hypothesis, we first examined whether VLDLR physically associated with RasGRF1 *in vivo*. Wild-type brain lysates were immunoprecipitated with IIII (for VLDLR) or IgG, and immunoblotted for RasGRF1. We found that VLDLR co-immunoprecipitated with RasGRF1 (Fig. 6A, S1B). We then examined whether RasGRF1 altered the cell surface levels of VLDLR. COS7 cells were transfected with VLDLR + Vector or VLDLR + RasGRF1 for 24 hours and cell surface biotinylation was conducted. Overexpression with RasGRF1 did not alter cell surface or total levels of VLDLR (Fig. 6B).

We then examined whether VLDLR could alter the levels of RasGRF1. To test this hypothesis, we transfected primary hippocampal neurons with GFP + Vector, GFP + VLDLR, GFP + PLL, or GFP + VLDLR shRNA for 72 hours. We then immunostained with RasGRF1 and found that altering VLDLR levels did not significantly change the levels of RasGRF1 (Fig. 6C–F). Next, we investigated whether VLDLR required RasGRF1 to alter dendritic spine number. Primary hippocampal neurons were transfected with GFP + PLL + Vector, GFP + VLDLR + Vector, GFP + RasGRF1 shRNA + Vector, or GFP + RasGRF1 shRNA + VLDLR for 72 hours followed by quantification of spine density. Consistent with our previous findings, overexpression of VLDLR significantly increased dendritic spine density by 40% (Fig 2, Fig. 6G–H, *$p<0.05$). Interestingly, knockdown of RasGRF1 prevented the effect of VLDLR on spinogenesis, suggesting that VLDLR-mediated spinogenesis requires RasGRF1 (Fig. 6G–H).

To examine whether VLDLR alters Ras activity, we conducted a GST-pull down assay from wild-type and VLDLR knockout brain lysate by incubating it with GST-Raf-RBD purified protein (the active form of Ras) and immunoblotted for anti-Ras. We found that VLDLR knockout brain lysates had decreased levels of the active form of Ras compared to wild-type brain lysates (Fig. 6I). These data suggest that the expression levels of VLDLR can affect Ras activity.

3.7. Interaction between VLDLR and CaMKIIα alters dendritic spine formation

Ras signaling is downstream of CaMKII [20]. CaMKII induces Ras signaling, which promotes dendritic spine growth and LTP [20]. Thus, we examined whether VLDLR can interact with CaMKIIα using co-immunoprecipitation from primary cortical neurons and wild-type mouse brain lysate. We found that VLDLR interacted with CaMKIIα as well as with the p-CaMKIIα (active form) in primary cortical neurons and brain lysates (Fig. 7A (left panel), Fig.S1B). We then examined whether VLDLR can interact with another CaMKII isoform, CaMKIIβ, and found that VLDLR did not immunoprecipitate with CaMKIIβ (Fig. 7A, right panel).

We examined whether CaMKIIα affected cell surface levels of VLDLR. For this experiment, COS7 cells were transfected with VLDLR + Vector, VLDLR + CaMKIIα WT, VLDLR + CaMKIIα T286D (non-autophosphorylatable form) or VLDLR + CaMKIIα

K42R (kinase dead form) for 24 hours and cell surface biotinylation was conducted. Overexpression of CaMKIIα WT increased cell surface and total levels of VLDLR, but not CaMKIIα T286D or K42R, suggesting that both kinase activity and phosphorylation of CaMKIIα are required for this effect (Fig. 7B).

Next, we examined whether the expression levels of VLDLR can alter the levels of CaMKIIα. To test this, we transfected primary hippocampal neurons with GFP + Vector, GFP + VLDLR, GFP + PLL, or GFP + VLDLR shRNA for 72 hours and immunostained with CaMKIIα. Knockdown of VLDLR significantly decreased the levels of CaMKIIα by about 40% (Fig. 7E–F, *$p<0.05$), while overexpression of VLDLR trended toward increasing CaMKIIα (Fig. 7C–D, $p<0.06$). Based on these findings, we then examined whether VLDLR mediated dendritic spine density through CaMKIIα. Primary hippocampal neurons were transfected with GFP + Vector + PLL, GFP + VLDLR + Vector, GFP + CaMKIIα shRNA + Vector, or GFP + VLDLR + CaMKIIα shRNA for 72 hours, and dendritic spine density was measured. Again, we found that overexpression of VLDLR increased dendritic spine density (Fig. 7G–H, *$p<0.05$). Interestingly, knockdown of CaMKIIα prevented the effect of VLDLR on spinogenesis, suggesting that VLDLR-mediated spinogenesis is dependent on CaMKIIα (Fig. 7G–H).

To test whether VLDLR altered levels of CaMKIIβ, we transfected primary hippocampal neurons with GFP + PLL or GFP + VLDLR shRNA for 72 hours and immunostained with CaMKIIβ. Knockdown of VLDLR significantly decreased the levels of CaMKIIβ by around 25% in primary hippocampal neurons (Fig. 7I, *$p<0.05$). We next investigated whether CaMKIIβ could affect VLDLR enhancement of dendritic spine density. Surprisingly, depletion of CaMKIIβ also inhibited the VLDLR-mediated effect on dendritic spine density (Fig. 7J). Taken together, these data indicate that VLDLR requires CaMKIIα and CaMKIIβ to exert its effects on dendritic spine formation.

Reelin, an extracellular ligand of VLDLR, can also impact dendritic spine formation, and requires either VLDLR or ApoEr2 for this effect [21, 22]. Therefore, we asked whether Reelin could modulate the interaction of VLDLR with GluA1, RasGRF1, and/or CamKIIα. To test this, we treated primary hippocampal neurons (DIV 21) with 50 ng/ml of Reelin or vehicle. Following a 1 hour treatment, we immunoprecipitated the neuronal lysates with IIII or IgG and probed for the different VLDLR interacting partners (Fig. 8A). We observed that Reelin treatment decreased the immunoprecipitation of VLDLR with each of the binding partners identified here, GluA1, RasGRF1, and CamKIIα. These data indicate that extracellular ligands, such as Reelin, impact the intracellular interactions of VLDLR to the RasGRF1 signaling cascade.

4. Discussion

In the present study, we identify a novel role for VLDLR on dendritic spine formation and its mechanism of action. We found that VLDLR is expressed at synapses both pre- and post-synaptically (Fig. 1) and that VLDLR overexpression promotes, while VLDLR knockdown decreases, dendritic spine density in primary hippocampal cultures (Fig. 2). Interestingly, we observed that VLDLR alters presynaptic puncta number without affecting the postsynaptic

puncta number (Fig. 3). Additionally, we found that VLDLR expression selectively affects NMDA/AMPA receptor subunit expression (Fig. 4 and 5). Moreover, VLDLR regulates spine density, potentially by regulating Ras signaling molecules, including RasGRF1 and CaMKIIα/CaMKIIβ (Fig. 6 and 7). Taken together, our results strongly suggest that VLDLR regulates dendritic spinogenesis in a RasGRF1/CaMKII dependent manner.

We and others have previously reported that apoE receptors, LRP1 and ApoER2, are expressed at the synapse and regulate spinogenesis [1, 10–12, 22–24]. ApoER2 and VLDLR are 50% homologous, and both receptors are involved in the neural development of the neocortex through Reelin signaling [25]. These two receptors are the exclusive Reelin signaling receptors, and bind to Reelin with similar affinities [26]. Interaction with Reelin induces both receptors to bind to the adaptor protein Dab1, which subsequently leads to the activation of Src family tyrosine kinases [27]. Although ApoER2 and VLDLR share many similarities, they can also interact with different ligands suggesting that they may also have non-overlapping functions. For example, VLDLR interacts with the Pafah1b complex, which is involved in neuronal migration, while ApoER2 does not. Moreover, ApoER2 has a PDZ domain in Exon 19, which is absent in VLDLR. This PDZ domain can interact with Jip1 and PSD95. Further, the cellular distribution in membrane compartments seems to be different between the two receptors, with ApoER2 found in lipid rafts while VLDLR is usually not [28]. For a more extensive review about the similarities and differences of these two receptors, please see [29]. Here, we asked whether VLDLR, like ApoER2, would also be involved in synaptic plasticity.

We are the first to identify that VLDLR, similar to ApoER2, is expressed at synapses and regulates dendritic spine formation *in vitro*. Interestingly, we found that VLDLR more effectively alters spinogenesis at DIV21 compared to DIV14, suggesting that VLDLR may have a more profound impact on the later stages of spine formation or stability. We also examined whether reducing VLDLR protein levels affects functional synapses in particular by immunostaining with synaptophysin and PSD-95. Surprisingly, we found that VLDLR affects synaptophysin puncta number without altering PSD-95 puncta number. Because VLDLR induces a pre-synaptic effect prior to post-synaptic alterations (synaptophysin, DIV14; dendritic spine density, DIV21), it is possible that the VLDLR-mediated impact on post-synaptic spine density is an indirect effect of VLDLR influence on pre-synaptic sites. Another possibility is that other apoE receptors known to be expressed post-synaptically (such as ApoER2) may be altered as a compensatory mechanism when VLDLR expression is reduced. We had also conducted a parallel experiment to test whether overexpression of VLDLR could enhance synaptic protein expression. Interestingly, we did not observe any changes in these synaptic markers following overexpression (data not shown). These data suggest that perhaps the overexpression of VLDLR enhances actin motility to increase dendritic spine number, but that these dendritic spines may be silent and non-functional. This idea will be further explored with functional assays such as electrophysiology in future experiments.

Unlike other apoE receptors known to impact dendritic spine density, we also observed VLDLR in the pre-synaptic compartment (Fig. 1A, B). Moreover, depending on the antibody used, VLDLR was observed more in the pre- or post-synaptic compartment. We

tested whether there was differential VLDLR antibody detection for various splice variants of VLDLR (such as full length VLDLR compared to VLDLR lacking exon 16 (the O-linked sugar domain)) (data not shown). We have not observed that any of these antibodies differentially recognize VLDLR splice variants. This observation does not exclude the possibility that other splice variants or dimerization of the receptor could be the mechanistic explanation for the variation in pre-synaptic and post-synaptic VLDLR identification by different antibodies. Because we observed that 2 out of 3 different N-terminal antibodies for VLDLR detected VLDLR predominantly in the pre-synaptic compartment and it is known that VLDLR can be cleaved by γ-secretases, it would be interesting to investigate whether this cleavage results in enhanced pre-synaptic signaling. Future studies will elucidate these findings by measuring whether secretase activity can alter VLDLR localization at the synapse, subsequently altering synapse formation *in vitro* and *in vivo*.

Much of the research regarding APOE receptor ligands (i.e., ApoE, F-spondin, Reelin) at the synapse has focused on modulations of post-synaptic signaling, highlighting that post-synaptic alterations can indirectly impact pre-synaptic ones (and vice versa). Pre-synaptic localization of VLDLR may hint at a new role for APOE receptors. Synaptophysin levels are affected by APOE genotype following lipopolysaccharide injection [30], increased by F-spondin overexpression in an AD model [31], and decreased with Reelin inhibition [32]. VLDLR may be involved in the mechanism for these observations.

To further investigate the effect of VLDLR at the synapse, we also examined the effects of VLDLR on NMDA and AMPA receptor levels. Our results demonstrate that VLDLR knockdown decreased the total levels of GluN1 and the surface levels of GluA1 in primary hippocampal neurons. These results suggest that although PSD-95 is unaffected, VLDLR can still impact the composition of postsynaptic densities. To support our findings, VLDLR knockout mice were observed to have moderate decreases in synaptic potentiation [8]. It is possible that the shift of AMPA and NMDA receptor subunit composition contributes to this effect. Indeed, it is well known that GluA1/A2 heteromers comprise the predominant AMPA receptor population in the hippocampus [33, 34]. The decrease in surface expression of the GluA1 subunit may reflect a shift to a GluA2/A3 subunit composition, subsequently altering the kinetics of the AMPA receptors. Moreover, studies have shown that minor alterations in GluN1 levels can cause changes in synaptic signaling such as LTP [35, 36].

Intriguingly, we also observed that VLDLR co-precipitated with the GluA1 receptor subunit, but not with GluA2 or the NMDA receptor subunits (Fig. 4, 5). This suggests that VLDLR may directly or indirectly complex with GluA1 to alter both its trafficking to the cell surface and its signaling. Reelin, a ligand for VLDLR, is known to enhance GluA1 composition through VLDLR and ApoER2 receptor signaling [37]. However, how Reelin enhances surface GluA1 expression is unknown. These new findings shed light onto a potential mechanism for this effect.

To elucidate the molecular mechanism by which VLDLR could promote synapse formation, we examined Ras signaling as a potential downstream target. The Ras family of signaling molecules is important for spine dynamics and synaptic plasticity, with Ras promoting surface delivery of AMPA receptors and stimulating dendritic spine formation [18, 38, 39].

Our group has recently shown that APP, a receptor that interacts with VLDLR [40], co-precipitates with RasGRF1, a downstream guanine exchange factor, which activates Ras signaling [19]. Moreover, RasGRF1 is required for APP to modulate dendritic spine formation [19]. In the present study, we found that VLDLR can also co-precipitate with RasGRF1 (Fig. 6A), and that RasGRF1 is required for the effects of VLDLR on dendritic spine formation. These results indicate that RasGRF1 is a potential downstream signaling molecule of VLDLR. Furthermore, we also observed that VLDLR knockout animals have significantly reduced levels of the activated form of Ras, although total Ras levels are unchanged (Fig. 6I). These findings further contribute to the idea that Ras activity is downstream of VLDLR signaling.

CaMKII is another molecule implicated in synaptic efficacy and morphogenesis [41–45]. The CaMKIIα isoform is usually associated with synaptic efficiency and the CaMKIIα and β isoforms are associated with dendritic spine morphogenesis [41–43, 46]. Interestingly, overexpressing CaMKII also caused an increase in VLDLR expression levels; however, it is not clear whether this is a direct or indirect effect. For example, it is known that CAMKIIα overexpression can cause increases in synaptic strength and size [47]. Therefore, it is possible that the increase in spine size can in turn lead to an additional increase in VLDLR. We report here that CaMKIIα, but not CaMKIIβ, interacts with VLDLR in brain lysates. However, both CaMKII isoforms are required for the VLDLR-mediated increase in dendritic spine density *in vitro* (Fig 7). Because VLDLR and APP can form a complex, our future studies will investigate whether VLDLR and APP cooperate or compete to alter spinogenesis through these signaling pathways.

A recent study showed that ligands to apoE such as F-spondin or Reelin could cause ApoER2 to homo-cluster with itself or hetero-cluster with VLDLR [48]. Therefore, it is possible that the ligand-binding domain that is required to observe the VLDLR-mediated effect on dendritic spines (Fig 2) could be through this same mechanism. In our working model (Fig. 8 B,C), VLDLR may be clustering trans- or cis- synaptically through the ligand binding domain, providing structural support for dendritic spine growth. Moreover, this interaction of VLDLR with another VLDLR receptor, or with known interaction binding partners (such as APP or ApoER2), has already been implicated in synaptic development. Transient extracellular ligand treatment also altered intracellular signaling cascades by decreasing the interaction of VLDLR with RasGRF1 signaling proteins in addition to altering dendritic spine formation.

What is the biological significance of our findings? VLDLR has been identified as an AD risk gene in Japanese cohorts [6, 49], and European Caucasian cohorts [50–52]. Moreover, VLDLR deletions have been associated with a non-progressive cerebellar ataxia with intellectual disability in the human population [53]. There has been no thorough investigation of the impact of this VLDLR homozygous deletion on dendritic spines in the hippocampal regions. Therefore, it is possible that the signaling pathways identified here may contribute to both of these diseases. Moreover, these studies begin to clarify the molecular mechanism by which the apoE receptor family may participate in the development and functional maintenance of the nervous system. In particular, these studies highlight a novel role for VLDLR at the synapse and its mechanism of action.

5. Conclusions

In summary, our results demonstrate that VLDLR alters dendritic spine formation through a RasGRF1/CaMKII dependent mechanism. This discovery and characterization of a novel role for VLDLR at the synapse is critical to furthering our understanding of central nervous system development and functional maintenance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Very Low Density Lipoprotein Receptor (VLDLR) is present in neuronal synapses
- VLDLR overexpression increases, while knockdown decreases, dendritic spine number
- VLDLR knockdown decreases surface levels of GLUA1
- The VLDLR-mediated effect on dendritic spine number requires RASGRF1 expression
- The VLDLR-mediated effect on dendritic spine number requires CAMKIIa expression

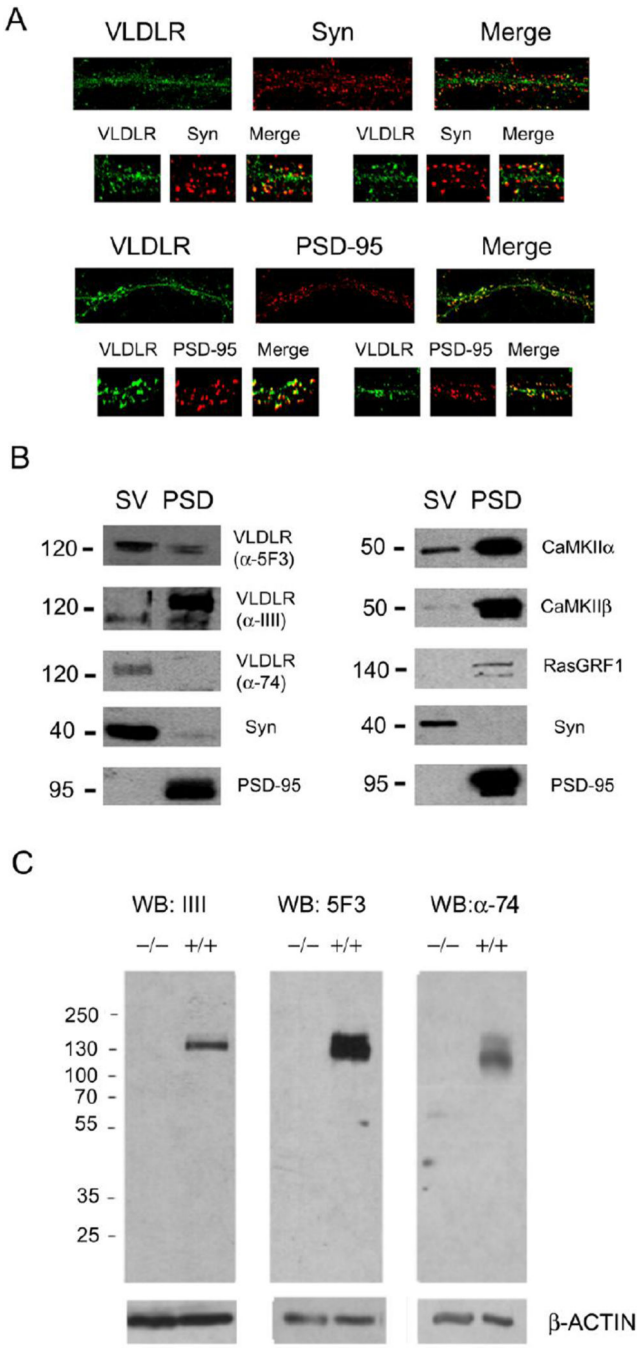

Fig. 1.

VLDLR is expressed at synapses. (A) Primary hippocampal neurons (DIV 21) were co-immunostained with Synaptophysin (red) and VLDLR (green, IIII antibody, upper panels) or PSD-95 (red) and VLDLR (green, IIII antibody, lower panels). (B) Immunoblot following synaptosomal fractionation for VLDLR, CAMKIIα, CAMKIIβ, RasGRF1, Synaptophysin and PSD-95 in pre-synaptic vesicles (SV) and post-synaptic density (PSD) fractions. (D) Immunoblot comparing VLDLR levels in wild-type (WT) and VLDLR knockout (KO)

tissue using VLDLR antibodies α-IIII (left), α-5F3 (middle), α-74 (right), and β-Actin as a loading control.

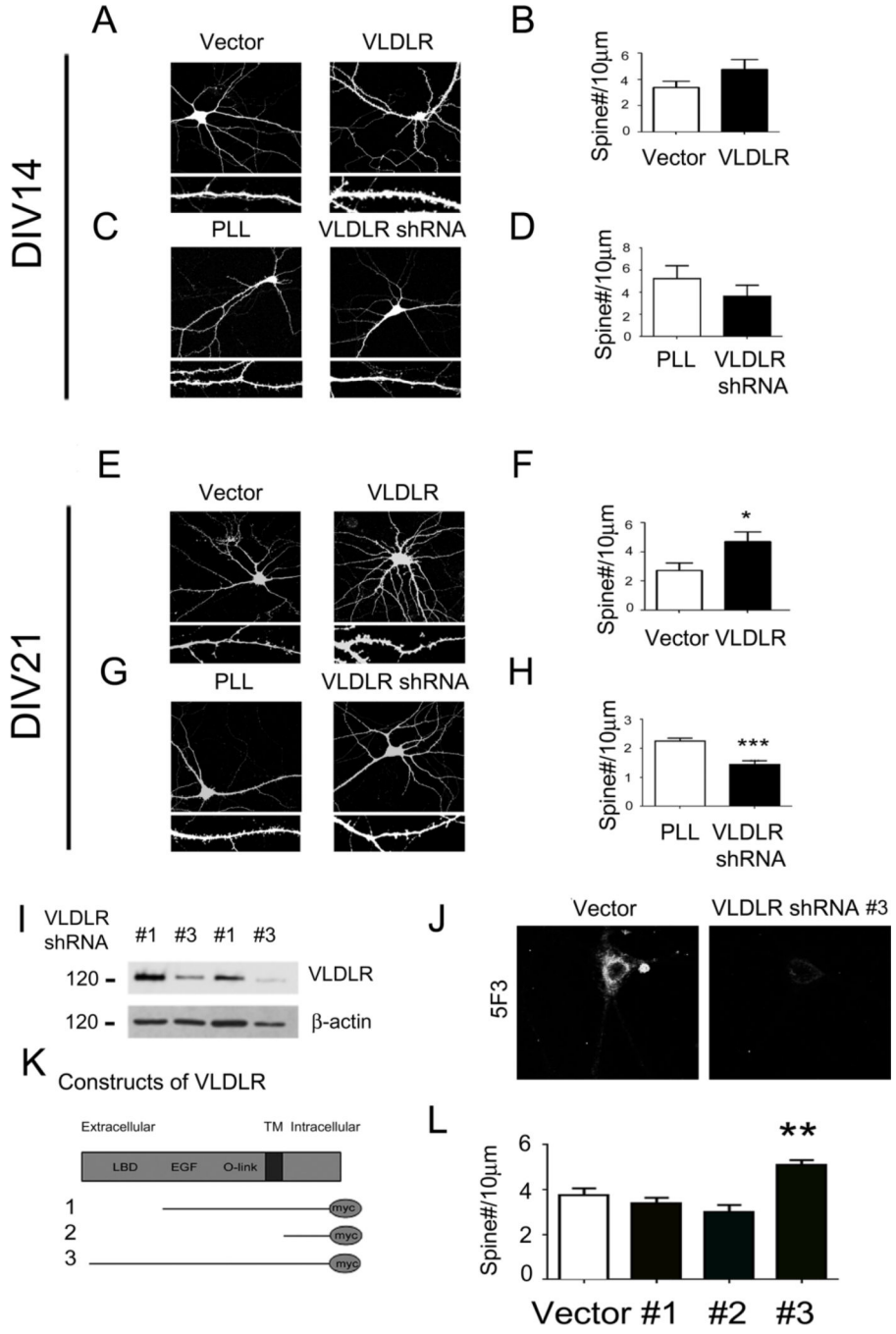

Fig. 2.

VLDLR promotes dendritic spine density in primary hippocampal neurons. (A, C) Primary hippocampal neurons were transfected with GFP + Vector (n=12), GFP + VLDLR (n=10), GFP + PLL (control vector for shRNA, n=5), or GFP + VLDLR shRNA (n=4), for 3 days. Cells (DIV 14) were then fixed, immunostained for GFP, and dendritic spines were counted on primary dendrites. (B, D) Quantification of A and C. (E, G) Hippocampal neurons (DIV18) were transfected with GFP + Vector (n=10), GFP + VLDLR (n= 8), GFP + PLL (n=8), or GFP + VLDLR shRNA (n=8) for 3 days. Cells (DIV 21) were then fixed,

immunostained with GFP, and dendritic spines were counted on primary dendrites. (F, H) Quantification of E and G (*p<0.05, ***p<0.001). (I) COS7 cells were co-transfected with rodent VLDLR and VLDLR shRNA #1 or VLDLR shRNA #3 or control PLL vector. VLDLR in cell lysates was measured with antibody IIII. (J) A representative image of hippocampal neurons (DIV 21) transfected with empty vector or VLDLR shRNA(#3) immunostained for VLDLR. (K) Schematic of the different deletion constructs for VLDLR. (L) Primary hippocampal neurons (DIV18) were transfected with GFP + Vector (n=8), GFP + VLDLR construct #1 (lacking the ligand binding domain of VLDLR, n=8), GFP + VLDLR construct #2 (lacking the extracellular domain of VLDLR, n=5), or GFP + VLDLR construct #3 (full length VLDLR, n=9). Dendritic spines were analyzed and quantified (**p <0.01). Error bars represented as S.E.M.

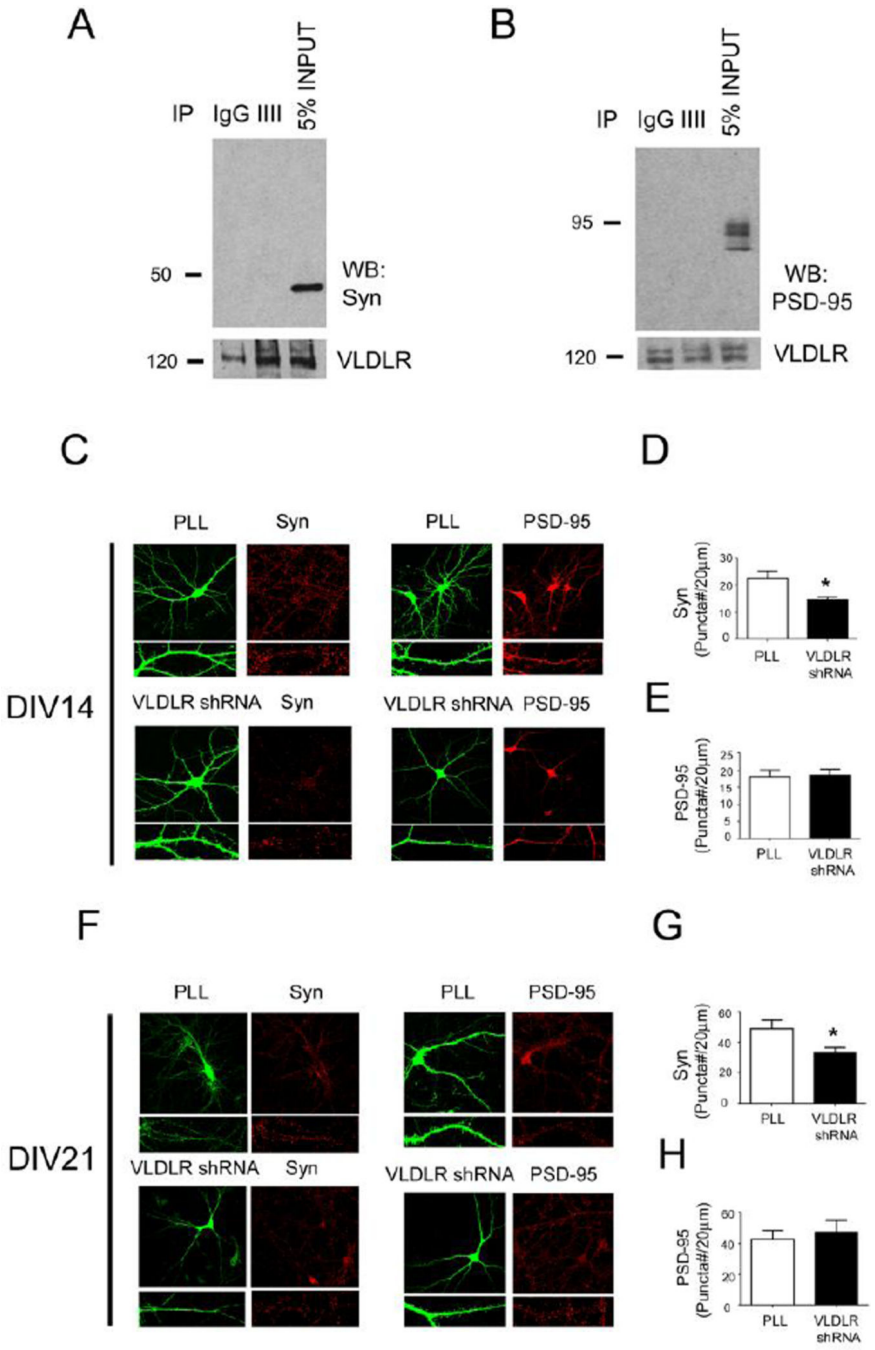

Fig. 3.

Knockdown of VLDLR significantly decreases synaptophysin puncta number. (A–B) Mouse brain lysates were immunoprecipitated with IgG or VLDLR (IIII) followed by an immunoblot with synaptophysin (A) and PSD-95 (B). (C) Primary hippocampal neurons (DIV12) were transfected with GFP + PLL or GFP + VLDLR shRNA for 3 days. Cells were then fixed and immunostained with Synaptophysin (left) or PSD-95 (right). (D) Quantification of synaptophysin puncta number in C (PLL n=6 neurons, VLDLR shRNA n=7 neurons, *p<0.05). (E) Quantification of PSD-95 puncta number in C (PLL n=6

neurons, VLDLR shRNA n=8 neurons) (F) Primary hippocampal neurons (DIV18) were transfected with GFP + PLL or GFP + VLDLR shRNA for 3 days. Cells were then fixed and immunostained with Synaptophysin (left) or PSD-95 (right). (G) Quantification of synaptophysin puncta number in F (PLL n=5 neurons, VLDLR shRNA n=7 neurons, *$p<0.05$). (H) Quantification of PSD-95 puncta number in F (PLL n=8 neurons, VLDLR shRNA n=8 neurons).

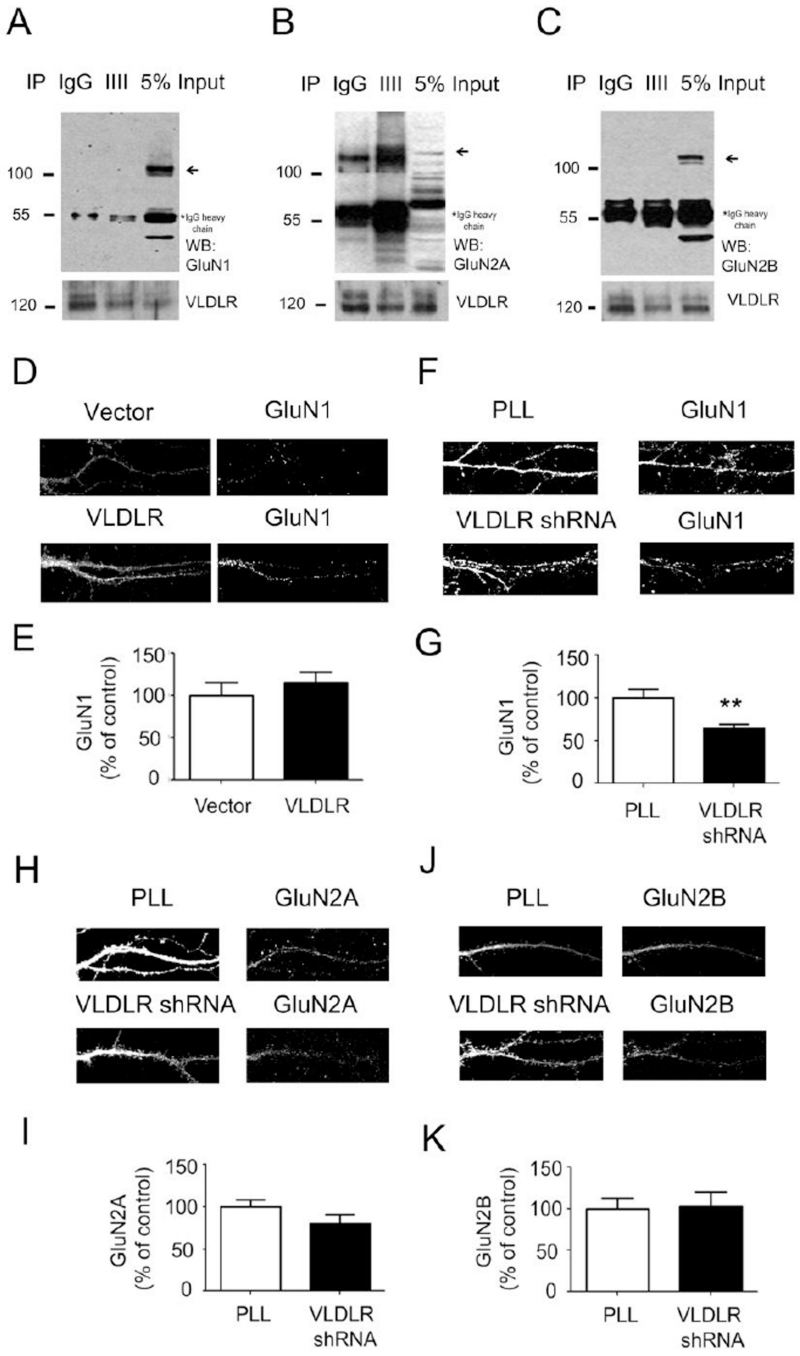

Fig. 4.

Knockdown of VLDLR decreases GluN1 levels. (A–C) Mouse brain lysates were immunoprecipitated with IgG or VLDLR (IIII) followed by immunoblot with GluN1 (A), GluN2A (B), and GluN2B (C). Arrows indicate specific bands at the expected molecular weight. (D) Hippocampal neurons (DIV18) were transfected with GFP + Vector (n= 11 neurons) or GFP + VLDLR (n=11 neurons) for 3 days. Cells were then fixed and immunostained for GluN1. Representative images for each condition are shown. (E) Quantification of GluN1 integrated intensity in D. (F) Hippocampal neurons (DIV18) were

transfected with GFP + PLL (n=7 neurons) or GFP+VLDLR shRNA (n=8 neurons) for 3 days. Cells were then fixed and immunostained for GluN1. Representative images for each condition are shown. (G) Quantification of GluN1 integrated intensity in F (**$p<0.01$). (H–K) Hippocampal neurons (DIV18) were transfected with GFP + PLL or GFP + VLDLR shRNA for 3 days. Cells were fixed and immunostained for GluN2A or GluN2B. (I) Quantification of GluN2A integrated intensity in H (n=7–16 neurons/group). (K) Quantification of GluN2B integrated intensity in J (n=8–18 neurons/group).

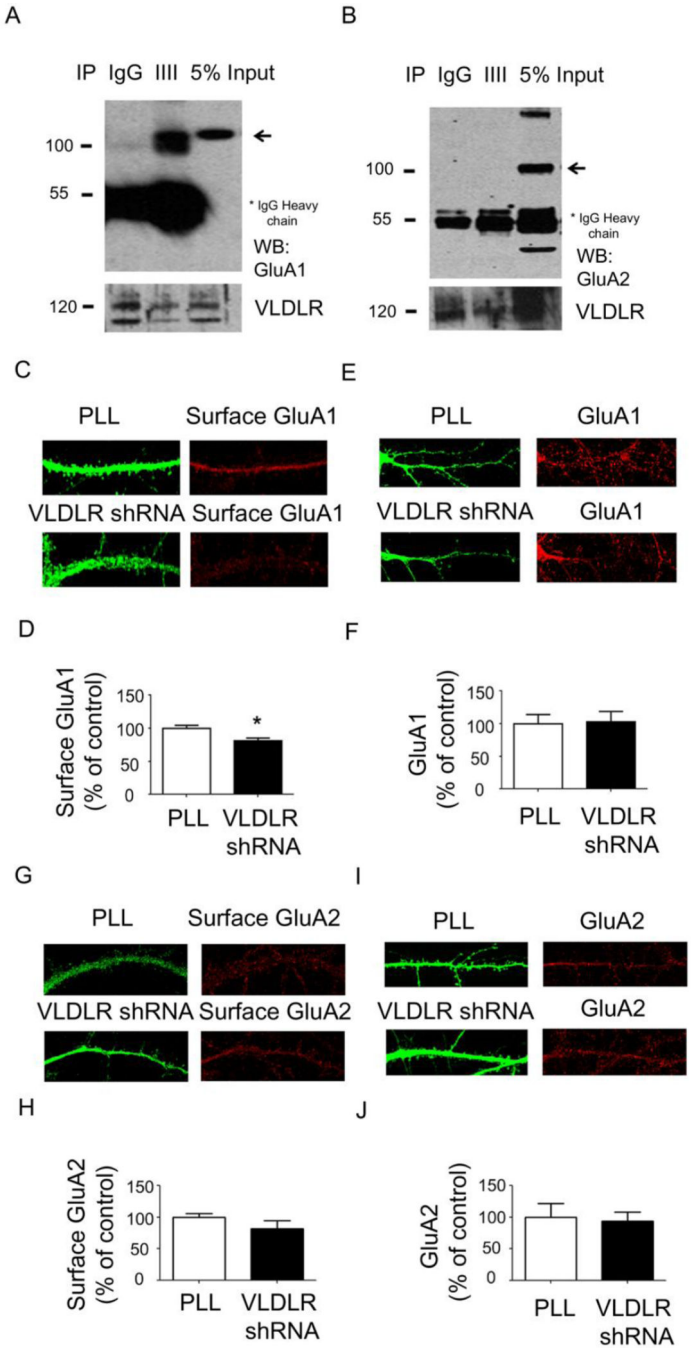

Fig. 5.

Knockdown of VLDLR decreases cell surface GluA1 levels. (A, B) Mouse brain lysates were immunoprecipitated with IgG or VLDLR (IIII) followed by immunoblot with GluA1 (A) and GluA2 (B). Arrows indicate specific bands at the expected molecular weight. (C–F) Primary hippocampal neurons (DIV18) were transfected with GFP + PLL or GFP + VLDLR shRNA for 3 days. Cells were then fixed in a non-permeabilizing and permeabilizing condition to detect for cell surface (C–D, PLL n=10 neurons, VLDLR shRNA n=8 neurons) and total levels (E–F, PLL n=9 neurons, VLDLR shRNA n=9 neurons) of GluA1. (*$p<0.05$)

(G–J) Primary hippocampal neurons (DIV18) were transfected with GFP + PLL or GFP + VLDLR shRNA for 3 days. Cells were then fixed in a non-permeabilizing and permeabilizing condition to detect for cell surface levels of GluA2 (G–H, PLL n=10 neurons, VLDLR shRNA n=12 neurons) and total levels of GluA2 (I–J, PLL n=9 neurons, VLDLR shRNA n=9 neurons).

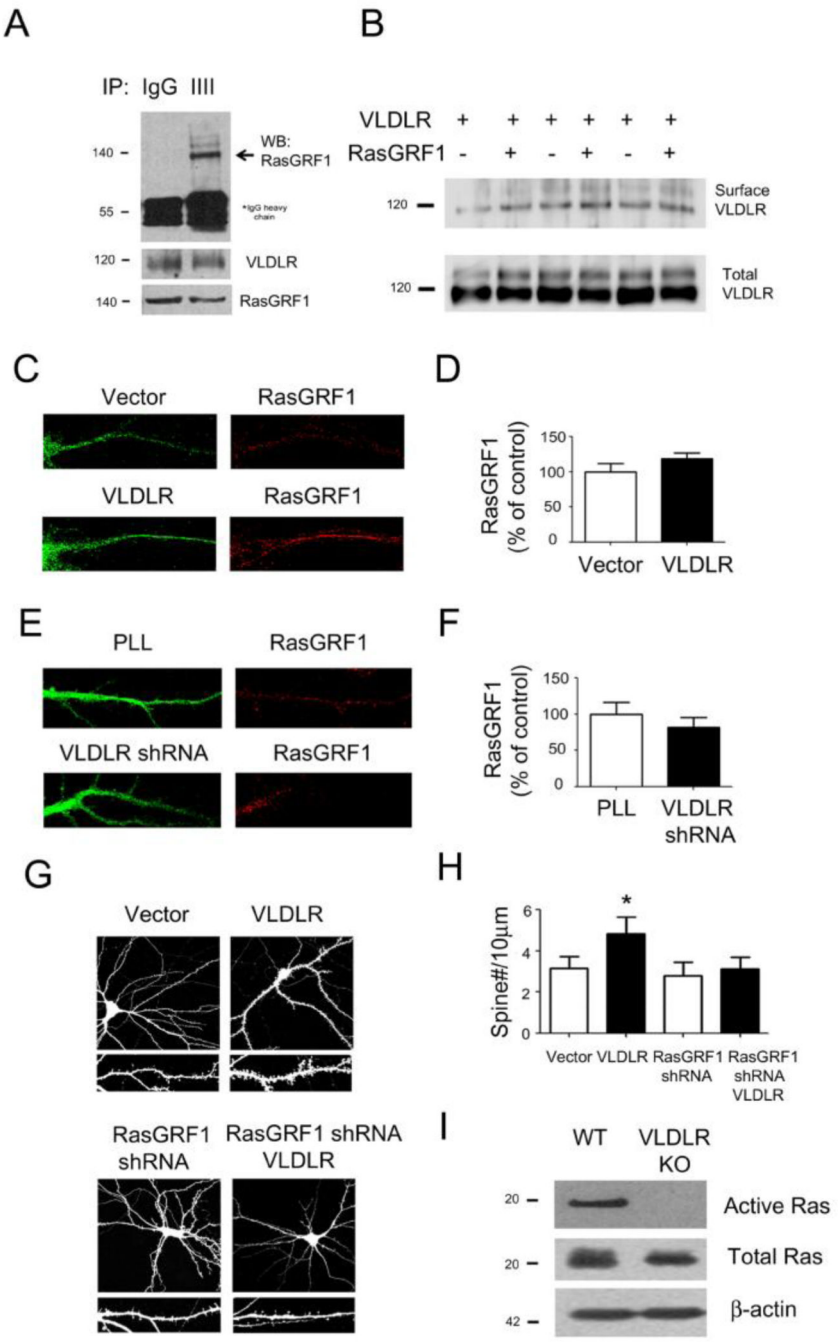

Fig. 6.

VLDLR requires RasGRF1 to mediate dendritic spine density (A) Mouse brain lysates were immunoprecipitated with IgG or VLDLR (IIII) followed by immunoblot with RasGRF1. Arrow indicates specific band at the expected molecular weight. (B) COS7 cells were transfected with VLDLR + Vector or VLDLR + RasGRF1, and cell surface biotinylation was performed to measure cell surface levels of VLDLR (n=3/group). (C) Primary hippocampal neurons (DIV18) were transfected with GFP + Vector or GFP+ VLDLR for 72 hours. Cells were then fixed and immunostained for RasGRF1. Representative images from

each condition are shown. (D) Quantification of RasGRF1 integrated intensity from C (n=7–10 neurons/group). (E) Primary hippocampal neurons (DIV18) were transfected with GFP + PLL or GFP + VLDLR shRNA for 72 hours and cells were then fixed and immunostained for RasGRF1. (F) Quantification of RasGRF1 integrated intensity from E (n=5–11 neurons/group). (G) Primary hippocampal neurons (DIV18) were transfected with GFP + PLL+ empty vector (n=9 neurons), GFP + VLDLR + vector (n=10 neurons), GFP + RasGRF1 shRNA + vector (n=11 neurons), or GFP + RasGRF1 shRNA + VLDLR (n=10 neurons) for 72 hours. After 72 hours, cells were fixed and immunostained for GFP and dendritic spine density was measured. (H) Dendritic spines visualized by GFP immunostaining under the conditions in G were quantified (*$p<0.05$). (I) A GST-pulldown assay was conducted to measure the levels of active Ras from wild-type and VLDLR knockout brains (n=3/group).

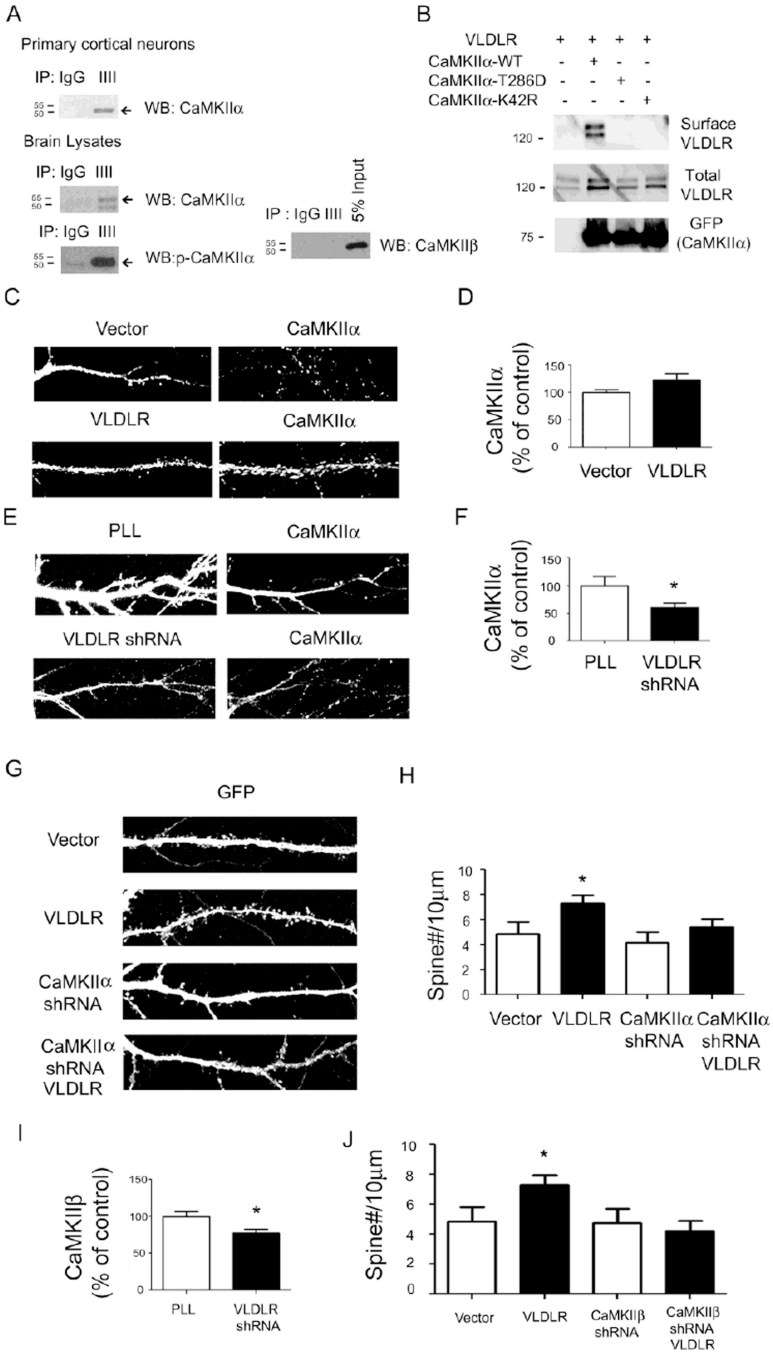

Fig. 7.

VLDLR interacts with CaMKIIα and requires both CaMKIIα and CaMKIIβ to alter dendritic spine formation (A) Primary cortical neurons and mouse brain lysates were immunoprecipitated with IgG or VLDLR (IIII) followed by immunoblot with CaMKIIα, p-CaMKIIα, or CaMKIIβ. Arrows indicate specific bands at the expected molecular weights. (B) COS7 cells were transfected with VLDLR + Vector, VLDLR + CaMKIIα WT, VLDLR + CaMKIIα T286D (non-autophosphorylatable form), or VLDLR + CaMKIIα K42R (kinase dead form), and cell surface biotinylation was performed to measure surface levels of

VLDLR (n=2/group). (C) Primary hippocampal neurons (DIV18) were transfected with GFP + Vector or GFP + VLDLR for 72 hours. Cells were then immunostained for CaMKIIα. (D) Quantification of CaMKIIα levels in C (n=7–13 neurons/group). (E) Primary hippocampal neurons (DIV18) were transfected with GFP+PLL or GFP+VLDLR shRNA for 72 hours. Cells were then immunostained for CaMKIIα. (F) Quantification of CaMKIIα levels in E (n=7 neurons/group, *p<0.05). (G) Primary hippocampal neurons (DIV18) were transfected with GFP + Vector +PLL (n=12 neurons), GFP + VLDLR + Vector (n=11 neurons), GFP + CaMKIIα shRNA +Vector (n=7 neurons), or GFP + VLDLR + CaMKIIα shRNA (n=11 neurons) for 72 hours. Cells were then immunostained for GFP to visualize dendritic spines. Representative images are shown. (H) Quantification of dendritic spine density under the conditions in G (*p<0.05). (I) Primary hippocampal neurons (DIV18) were transfected with GFP + PLL or GFP + VLDLR shRNA for 72 hours. Cells were then immunostained for CaMKIIβ. Quantification of CaMKIIβ levels (n= 12–17 neurons/group, *p<0.05). (J) Primary hippocampal neurons (DIV18) were transfected with GFP + Vector + PLL (n=12 neurons), GFP + VLDLR + Vector (n=11 neurons), GFP + CaMKIIβ shRNA +Vector (n=10 neurons), or GFP + VLDLR + CaMKIIβ shRNA (n=10 neurons) for 72 hours. Cells were then immunostained for GFP to visualize dendritic spines and quantification of dendritic spine density (*p<0.05).

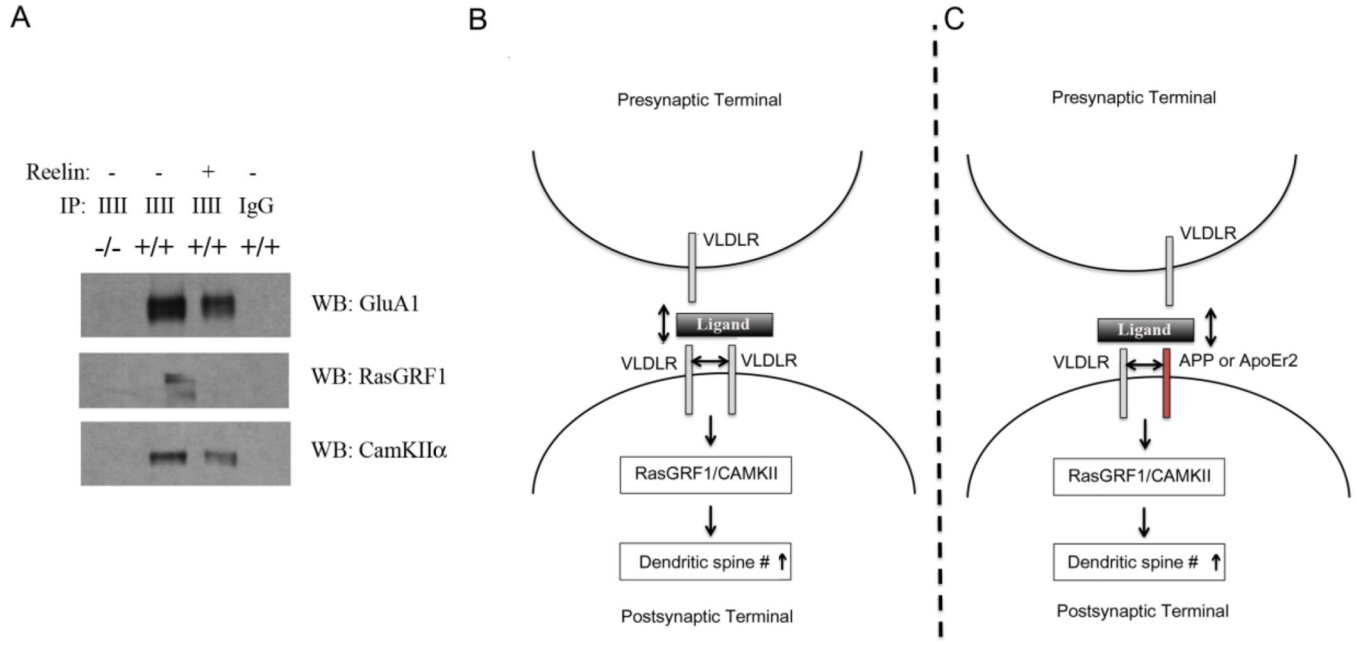

Fig. 8.

Reelin alters the interaction between VLDLR and its new interaction partners. (A) Primary hippocampal neurons (DIV21) were treated with control or Reelin (50 ng/ml). Following 1 hour treatment, cell lysates were immunoprecipitated with IIII or IgG and probed for GluA1 (upper panel), RasGRF1 (middle panel), CamKIIα (lower panel). As an additional negative control, brain lysate from VLDLR knock out (−/−) were also immunoprecipitated with IIII, and loaded in the first lane of the western. (B–C) A working model for how VLDLR can promote dendritic spine formation. VLDLR (light gray rectangle) can be expressed pre- or post-synaptically. The ligand binding domain of VLDLR is necessary for the VLDLR-mediated increase in dendritic spines. A recent study demonstrated that the ligands for VLDLR can promote homo- and hetero-clustering with itself or another receptor, respectively. Therefore, it is possible that these ligands can help promote the (B) homo- or (C) hetero- clustering of VLDLR cis- or trans-synaptically. This clustering would serve as an amplification of receptor signaling, which downstream impacts the interaction of CaMKII and RasGRF1 to VLDLR, which subsequently leads to the increase in dendritic spine number.