ApoER2 processing by presenilin-1 modulates reelin expression

Valeria Balmaceda,*† Inmaculada Cuchillo-Ibáñez,*† Lluís Pujadas,†,‡,§ María-Salud García-Ayllón,*† Carlos A. Saura,†,‡ Johannes Nimpf,# Eduardo Soriano,†,‡,§ and Javier Sáez-Valero*†,1

*Instituto de Neurociencias de Alicante, Universidad Miguel Hernández-Consejo Superior de Investigaciones Científicas (CSIC), Sant Joan d’Alacant, Spain; †Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain; ‡Institut de Recerca Biomèdica (IRB)-Barcelona, Parc Científic de Barcelona, Barcelona, Spain; §Department of Cell Biology, University of Barcelona, Barcelona, Spain; †Unidad de Investigación, Hospital General Universitario de Elche, Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana (FISABIO), Elche, Spain; #Institut de Neurociències, Departament Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Bellaterra, Spain; and #Max F. Perutz Laboratories, Department of Medical Biochemistry, Medical University of Vienna, Vienna, Austria

ABSTRACT The reelin signaling protein and its downstream components have been associated with synaptic plasticity and neurotransmission. The reelin signaling pathway begins with the binding of reelin to the transmembrane lipoprotein receptor apolipoprotein E receptor 2 (ApoER2), which in turn induces the sequential cleavage of ApoER2 by the sequential action of α- and γ-secretases. Using conditional-knockout mice of the catalytic component of the γ-secretase complex, presenilin 1 (PS1), we demonstrated increased brain ApoER2 and reelin protein and transcript levels, with no changes in the number of reelin-positive cells. Using the human SH-SY5Y neuroblastoma cell line, we showed that ApoER2 processing occurs in the presence of PS1, producing an intracellular ApoER2 C-terminal fragment. In addition, the pharmacologic inhibition of γ-secretase in SH-SY5Y cells led to increased reelin levels. Overexpression of ApoER2 decreased reelin mRNA levels in these cells. A luciferase reporter gene assay and nuclear fractionation confirmed that increased amounts of intracellular fragment of ApoER2 suppressed reelin expression at a transcriptional level. Chromatin immunoprecipitation experiments corroborated that the intracellular fragment of ApoER2 bound to the RELN promoter region. Our study suggests that PS1/γ-secretase-dependent processing of the reelin receptor ApoER2 inhibits reelin expression and may regulate its signaling.—Balmaceda, V., Cuchillo-Ibáñez, I., Pujadas, L., García-Ayllón, M.-S., Saura, C. A., Nimpf, J., Soriano, E., Sáez-Valero, J. ApoER2 processing by presenilin-1 modulates reelin expression. FASEB J. 28, 1543–1554 (2014). www.fasebj.org

Key Words: transcription • Alzheimer’s disease • Reelin

Reelin is a large signaling protein that plays an important role in the adult brain, influencing neurotransmission and synaptic plasticity, thereby favoring memory formation. Indeed, normal reelin levels are essential for some forms of long-term memory (1–3). The reelin signaling pathway is initiated by binding of reelin to transmembrane lipoprotein receptors: the apolipoprotein E receptor 2 (ApoER2) and/or the very low density liporeceptor (VLDLR; refs. 4, 5). Binding of reelin to its receptor relays the signal into the cell via the adapter Disabled 1 (Dab1; refs. 6–8). Reelin-dependent induction of Dab1 tyrosine phosphorylation triggers an intracellular kinase cascade that includes the activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway and that ultimately inhibits glycoprotein synthase kinase-3β (GSK-3β), preventing hyperphosphorylation of the microtubule-associated protein tau (9).

Disruption of reelin, Dab1, or both ApoER2 and VLDLR leads to similar anatomic alterations during brain development, resulting in a reeler phenotype (8, 10, 11). Downstream consequences of an impaired reelin signaling pathway are well established during brain development and become clearer in the adult

Abbreviations: Aβ, β-amyloid; AcH2B, acetylated histone H2B; AD, Alzheimer’s disease; ApoER2, apolipoprotein E receptor 2; APP, β-amyloid precursor protein; cDKO, conditional double knockout; ChIP, chromatin immunoprecipitation; cKO, conditional knockout; CTF, C-terminal fragment; Dab1, Disabled-1; DAPT, N-[(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; DMSO, dimethyl sulfoxide; ICD, intracellular domain; MTS, tetrazolium; NGS, normal goat serum; PB, phosphate buffer; PBS, phosphate-buffered saline; PF, paraformaldehyde; PS1, presenilin 1; VLDLR, very low density liporeceptor

1 Correspondence: Instituto de Neurociencias de Alicante, Universidad Miguel Hernández-CSIC, Av. Ramón y Cajal s/n, E-03550 Sant Joan d’Alacant, Spain. E-mail: j.saez@umh.es doi: 10.1096/fj.13-239350
(12). Afterward, it is important to determine the mechanisms of modulation and regulation of reelin signaling, including putative autoregulatory mechanisms, which control the expression and function of the reelin protein level in normal and pathological conditions. In this context, both reelin and ApoER2-soluble fragments, resulting from proteolysis during receptor activation, may represent a putative autoregulatory mechanism by directly interfering in the binding of reelin to its receptor (13–17).

In this study, we investigated whether modulation of ApoER2, the major reelin receptor in the brain, influences reelin expression. We also studied the role of presenilin 1 (PS1), the active proteolytic component of the γ-secretase complex (18), in this modulation, in that ApoER2 cleavage after its binding to reelin is mediated by the sequential processing of the proteolytic enzymes α- and γ-secretases (19–21). Finally, we studied the transcriptional activity of the intracellular domain (ICD) fragment, generated by the process of the ApoER2 C-terminal fragment (CTF) by γ-secretase.

MATERIALS AND METHODS

**PS1 conditional-knockout (PS1 cKO) mice**

Generation and characterization of the PS1 cKO mice have been previously described (22). PS1 conditional double-knockout (cDKO) mice lack expression of PS1 gene in glutamatergic neurons of the forebrain in the postnatal stage starting around P18. PS1 cDKO mice (iPS1/iPS1;CaMKII-Cre) were obtained by crossing floxed PS1 (iPS1/iPS1) males to PS1 cKO females (iPS1/iPS1;CaMKII-Cre). Mice used in this study were age-matched littermate control (iPS1/iPS1) and PS1 cDKO (iPS1/iPS1;CaMKII-Cre) mice (C57BL/6 background). Experimental procedures were conducted according to the Human Ethics Committee of the Universitat Autònoma de Barcelona (protocol CEEAH 475), according to European Union guidelines.

Brain samples were collected from 3- to 4-mo-old PS1 cKO (n=10) and age-matched control littermates (n=10). No PS1 was detectable in the forebrain glutamatergic neurons of 2- to 3-mo-old mice, and no significant alterations in general behavior, motor coordination, and exploratory anxiety were observed. Brain cytoarchitecture, the number of neurons, and morphology were also normal (22,23).

**Pharmacologic treatment of cells with the γ-secretase inhibitor DAPT**

SH-SY5Y cells (2.2×10^5 cells/well) were grown on 12-well plates for 24 h in Dulbecco’s modified Eagle’s medium (DMEM) plus GlutaMAX (Life Technologies, Inc., Paisley, UK) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin (Life Technologies, Inc.). The cells were treated with 5 μM γ-secretase inhibitor N-[(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT; Calbiochem, Merck KGaA, Darmstadt, Germany) or the dimethyl sulfoxide (DMSO) vehicle. After 22 h of treatment, the cells were washed twice with cold phosphate-buffered saline (PBS) and resuspended in 100 μl of ice-cold extraction buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% (wv) Nonidet P-40, and 0.5% (wv) Triton X-100, supplemented with a cocktail of protease inhibitors. The cell lysates were sonicated and centrifuged at 20,000 g at 4°C for 12 min, and extracts were frozen at −80°C for future analysis. Alternatively, for analysis of the effect of DAPT on reelin transcriptional activity, HEK293 cells were used.

Cell viability was measured using the tetrazolium (MTS) assay (CellTiter 96 AQueous Assay; Promega, Southampton, UK) according to the manufacturer’s instructions. SH-SY5Y cells were cultured in 96-well plates (1.9×10^4 cells/well) and treated with DAPT, as described previously. MTS was added after DAPT treatment and the cells were incubated for 4 h before viability was determined by measuring the absorbance at 490 nm in a microplate reader (Infiniti M200; Tecan, Männedorf, Switzerland).

**ApoER2 and PS1 overexpression and luciferase reporter assay**

SH-SY5Y cells were transfected with a construct encoding full-length ApoER2 (pEGFP-N1-Mus musculus ApoER2). Alternative ApoER2 constructs were also used: ApoER2-myc, expressing the transmembrane and cytoplasmic domains (aa 825–963), and ApoER2-HA, expressing only the cytoplasmic domain (aa 728–842) (both generously provided by Dr. W. Rebeck, Georgetown University Medical Center, Washington, D.C., USA; see refs. 24, 25). Alternatively, SH-SY5Y cells were transfected with a construct encoding human full-length PS1 (26). Empty vectors (Promega) were used as the control. Cells (6.5×10^5 cells/well) were grown in 6-well plates and transfected with Lipofectamine 2000 (Invitrogen; Life Technologies), according to the manufacturer’s instructions. After 2 days in culture, the cells and culture supernatants were separately harvested. The cell culture supernatants were cleared by centrifugation for 10 min, 5000 g at 4°C. The cells were extracted as described previously, and ApoER2 levels were assayed by Western blot to determine transfection efficiency.

For the luciferase reporter assay, the SH-SY5Y cells were transfected with a pGL3 Basic Vector-RELN promoter (−2600 region, which contains 2.6 kb of the 5’ flanking sequence of the human RELN promoter; a kind gift from Dr. D. Grayson, University of Illinois, Chicago, IL, USA; see ref. 27) in the presence of ApoER2-pEGFP or a pEGFP empty vector. cDNA (2–4 μg) was cotransfected with 10–30 ng of the pRL-CMV vector (Renilla luciferase; Promega) in each well of a 12-well plate (2×10^5 cells/well). Alternatively, some luciferase reporter assays were performed in HEK293 cells. Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Cell lysates were prepared 36 h after transfection using Passive Lysis Buffer (Promega), and aliquots (~20 μl) were used to determine luciferase activity in a SIRUS Luminometer (Titer-Tek-Berthold; Berthold Detection Systems GmbH, Pforzheim, Germany). Firefly luciferase data were normalized to Renilla luciferase activity.

**Nuclear protein fractionation**

SH-SY5Y cells were cultured in 10 cm² culture plates (4×10^6 cells/plate). At 36 h after transfection with ApoER2-pEGFP or the pEGFP empty vector, the cells were washed twice with cold PBS and gently scapped off the plates with 10 ml of cold PBS. Cell debris was discarded by centrifugation for 5 min at 500 g, 4°C. Cell lysis and isolation of cellular nuclei were performed with the QProteome Nuclear Protein kit (Qiagen, Crawley, UK), according to the manufacturer’s instructions. Cytosolic and nuclear protein fractions were assayed by Western blot.
Western blot analysis

Reelin levels were determined as described elsewhere (28, 29). Brain extracts (30 μg), SH-SY5Y cell extracts (30 μg), or cell culture media (~30 μl) were boiled for 3 min, then resolved on 6% polyacrylamide slab gels. Total protein concentration was determined by the bichinchoninic assay (BCA; Pierce-ThermoScientific, Rockford, IL, USA). Electrophoresis was allowed to proceed at low voltage, to minimize excessive heat generation (28, 30). Proteins were blotted onto nitrocellulose membranes, blocked with 5% nonfat milk, and incubated with the monoclonal mouse anti-reelin antibodies 142 (1:1000 dilution; Chemicon International, Merck KGaA, Darmstadt, Germany) for SH-SY5Y samples and G10 (1:500 dilution; Chemicon) for mouse samples. These antibodies recognize epitopes located in the region of aa 164–189 (clone 142) and 164–496 (clone G10) (31).

To detect ApoER2, brain and cell extracts were boiled for 7 min at 98°C, and full-length and CTFs of ApoER2 were resolved on 6 and 10% polyacrylamide gels, respectively. A rabbit polyclonal antibody to ApoER2 (1:1000 dilution; Abcam, Cambridge, UK), raised to a synthetic peptide corresponding to aa 928–945 located near the C terminus of ApoER2, was used to detect the ApoER2 and CTFs. The β-amyloid precursor protein (APP)-CTF was analyzed by 10% Tris-tricine SDS-PAGE and detected with a polyclonal rabbit anti-C-terminal antibody (1:5000 dilution; Sigma-Aldrich, Poole, UK). Rabbit polyclonal antibodies anti-HA (1:3000 dilution), anti-myc (1:3000 dilution; Sigma-Aldrich), and mouse anti-GFP (1:3000 dilution; Abcam) were also used. The loading control was estimated with mouse α-tubulin (1:5000 dilution; Sigma-Aldrich). A polyclonal antibody against acetylated histone H2B [AcH2B; 1:5000 dilution; generous gift from Dr. A. Barco, Instituto de Neurociencias de Alicante, Universidad Miguel Hernández-Consejo Superior de Investigaciones Científicas (CSIC), Sant Joan d’Alacant, Spain] was used to characterize the nuclear fraction. Individual Western blots were used for each antibody, to avoid stripping of immunoblots and hence loss of signal. Immunoblots were developed with enhanced chemiluminescence (ECL) by SuperSignal (Pierce-ThermoScientific) in a Luminescent Image Analyzer LAS-1000 Plus (Fujifilm, Tokyo, Japan). The intensity of the reelin bands was measured by densitometry with the Science Lab Image Gauge 4.0 software provided by Fujifilm.

Immunohistochemistry and immunocytochemistry

Animals were anesthetized and perfused for 20 min with 0.1 M phosphate buffer (PB) containing 4% parformaldehyde (PF). The brains were removed, postfixed overnight with PB-4% PF, cryoprotected with PB containing 30% sucrose, and frozen. They were then sectioned coronally at a thickness of 30 μm, and tissue sections were collected in series of 10 sections and maintained at ~20°C in PB containing 30% glycerol and 30% ethylene glycol. Nuclei were detected by using Nissl staining. For immunohistochemistry, the sections were blocked for 2 h at room temperature with PBS containing 10% normal goat serum (NGS), 0.2% gelatin, and 0.1% F(ab’)2 fragment of anti-mouse IgG (1:300; Jackson ImmunoResearch, West Grove, PA, USA). Reelin was detected by the antibody G10 (Chemicon). Sequential incubation with biotinylated secondary antibody (1:200; Dako, Carpinteria, CA, USA) and streptavidin-HRP (1:400; GE Healthcare, Piscataway, NJ, USA) were performed in PBS-5% NGS. Bound antibodies were visualized by using dianaminobenzidine (DAB) reagent and H2O2 as peroxidase substrates (Sigma-Aldrich), and the sections were dehydrated and mounted (Eukitt; EMS, Hatfield, PA, USA). To quantify the number of reelin-positive cells in the cingulate and motor cortex (bregma +1.1/−0.5 mm; lateral ±1 mm), we counted every 10th section for each animal. Data were normalized to the area counted in 30-μm sections (n=6 sections/animal, 4 animals/group). Positively stained areas were quantified with ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA).

SH-SY5Y cells used for confocal microscopy were transfected with either ApoER2-pEGFP or a vector containing pEGFP alone with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. After 4 h, medium was replaced with fresh medium containing 5 μM DAPT, or the equivalent volume of DMSO as control. After 24 h, the cells were washed with phosphate-buffered saline solution and fixed with methanol for 10 min at −20°C. Nonspecific sites were blocked with 2% (w/v) BSA and 40 μg/ml digoxin in PBS for 30 min. The cells were then incubated with primary antibodies (rabbit anti-GFP 1:100, Invitrogen; mouse anti-merlin 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA; mouse anti-N cadherin 1:200, BD Biosciences, Erembodegem, Belgium) for 1 h followed by secondary antibodies (Alexa Fluor 546 anti-mouse 1:200; Alexa Fluor 488 anti-rabbit 1:200; Invitrogen-Molecular Probes, Paisley, UK) for 1 h. To study the location of ApoER2-pEGFP in the vicinity of the inner nuclear membrane, we incubated cells with cycloheximide (50 μM; Sigma-Aldrich) for 2 h after transfection and before immunocytochemistry, to block synthesis of new proteins. Micrographs were acquired with a Leica upright TCI-SL confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with an HCX Plan Apochromat ×63/1.32/0.6 NA oil objective (Leica). To measure the intensity of fluorescence of ApoER2-pEGFP colocalizing with emerin, we drew a circle by hand over the green fluorescence that overlapped the red fluorescent nuclear membrane in the merged channel, and the fluorescence intensity was analyzed with LAS AF Lite software (Leica).

RNA isolation and real-time RT-PCR analysis

RNA was extracted from mouse brains and SH-SY5Y cells by TRIzol Reagent in the PureLink Microto-Midi Total RNA Purification System (Invitrogen), according to the manufacturer’s instructions. cDNAs were synthesized with SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions, with 5 μg of total RNA and oligo (dT)12–18. Quantitative PCR amplification was performed with a StepOne Real-Time PCR System (Applied Biosystems; Life Technologies) with TaqMan probes specific for mouse RELN (assay ID: Mm00465200_m1 and human RELN (assay ID: HS01022646_m1) and mouse and human GAPDH as endogenous controls (all from Applied Biosystems). Reelin transcript levels were normalized to GAPDH.

Chromatin immunoprecipitation (ChIP) assay

SH-SY5Y cells plated in 100 mm tissue culture dishes (4.3×10⁶ cells/plate) were transfected with 20 μg of ApoER2-HA, according to the protocol described above. After 36 h, the cells were fixed in freshly prepared 1% parformaldehyde for 10 min at room temperature. Cross-linking was halted by the addition of 2.5 M glycine for 5 min. The cells were washed twice with ice-cold PBS plus protease inhibitors. The chromatin was extracted by using EZ Magna ChIP A/G (Millipore; Merck KGaA, Darmstadt, Germany) according to the manufacturer’s protocol.

The extracted chromatin was sheared to 100–500 bp with the Bioruptor (Diagenode Inc., Denville, NJ, USA) in 30 s cycles for 10 min. After centrifugation at 10,000 g at 4°C for 10 min, the supernatant was collected. For immunoprecipitati-
Primers were designed to amplify 5′ different positions of the promoter: (5′-H11002 to 3′), and PS1 of ApoER2 full-length isoforms and CTF in the cortex (hemicortex; left) and the cerebellum (right) of control wild-type (WT) mice. Increased levels of ApoER2 and reelin in the cortex of cKO mice are shown. Experiments were performed in triplicate; α-tubulin was used as a loading control. Data represent means ± se. *Values of $P < 0.05$ were considered to be statistically significant.

**RESULTS**

### Reelin and ApoE receptor levels are altered in brains of PS1 cKO mice

To determine whether PS1 is involved in the control of reelin receptor levels in vivo, brain homogenates from PS1 cKO mice (22) and littermate controls were analyzed by SDS-PAGE followed by Western blot. Immunoblot analysis for the main brain reelin receptor, ApoER2, revealed a complex banding pattern, probably reflecting the several ApoER2 isoforms (32) and the mature and precursor forms (21, 33). The levels of ApoER2 appeared to be significantly increased in cortical extracts (hemicortex) from the PS1 cKO mice (~80% increase; $P=0.03$) compared with the control animals (Fig. 1A). It is known that reelin bound to ApoER2 induces subsequent ApoER2 cleavage by the sequential action of α- and γ-secretases (19, 20). α-Secretase cleaves the ApoER2 extracellular domain, generating a membrane-bound CTF that subsequently acts as a substrate for γ-secretase. Consistent with the role of PS1 in the proteolysis of ApoER2, we found a significant accumulation of ApoER2-CTF in cortical extracts from the PS1 cKO mice (~300% increase; $P<0.001$) compared with the control littermates (Fig. 1A). Levels of ApoER2 in the cerebellum, a region in which PS1 is not silenced, did not differ between the PS1 cKO mice and the littermate controls.

We next measured the levels of the ApoER2 ligand reelin and found that full-length reelin (420 kDa) displayed a large increase in the cortices of the PS1 cKO mice.
mice (~20%; \( P=0.02 \)), whereas N-terminal 310-kDa (~50%; \( P=0.02 \)) and 180-kDa (~74%; \( P=0.005 \)) reelin fragments showed a moderate increase (Fig. 1B). Reelin levels in the cerebellum were similar in the PS1 cKO mice and controls (Fig. 1B).

To assess whether increased reelin levels are due to a higher number of cells expressing reelin in PS1 cKO mice, we labeled tissue sections with a specific antibody against reelin and counted the number of stained cells (Fig. 2A). The density of reelin-positive cells was similar in the cortices (cingulate and motor cortex) of the control and PS1 cKO mice (Fig. 2B). Data obtained from immunohistochemistry images did not allow us to quantify the amount of reelin expressed, but the similar number of reelin-expressing cells in the PS1 cKO and littermate controls indicates that the differences in levels detected by Western blot analysis may be attributable to changes in the level of expression. A quantitative PCR assay was designed to determine whether changes in the reelin protein corresponded to alterations in mRNA expression. Reelin mRNA levels were significantly increased (~20%) in the cortices from the PS1 cKO mice compared to those from the controls (\( P=0.01 \); Fig. 2C).

**Inhibition of PS1/γ-secretase enhances reelin levels in neuronal cells**

The SH-SY5Y neuroblastoma cell line was used to determine whether altered ApoER2 processing influences reelin levels and whether the effect is mediated by γ-secretase. SH-SY5Y cells express significant levels of reelin (29) and PS1 (34) and have been used as cellular models of ApoER2 overexpression (35). As previously mentioned, the binding of reelin to ApoER2 induces subsequent ApoER2 cleavage by α- and γ-secretases. The CTF generated after α-secretase cleavage acts as a substrate for γ-secretase, which in turn yields an ICD. (For an illustrative scheme, see Fig. 3A.) Use of a C-terminal ApoER2 antibody identified an ~25-kDa ApoER2-CTF fragment in cell extracts from nontransfected cells, in agreement with previous studies (Fig. 3B and refs. 19, 20, 25). Endogenous ApoER2-ICD was not detected, probably because of its low levels and inherent instability (19). To further characterize the banding pattern for the ApoER2 CTF and the expected ICD fragment, a chimeric ApoER2 protein expressing the transmembrane and cytoplasmic domains tagged to myc (ApoER2 CTF-myc) was overexpressed in SH-SY5Y cells. Another chimeric ApoER2 protein containing only the cytoplasmic domain tagged to HA (ApoER2 ICD-HA) was also expressed (24, 25). Cell extracts were then analyzed by Western blot. An anti-myc antibody detected the ApoER2-CTF-myc at ~25 kDa. A faint band of ~18 kDa was also detected that may correspond to an ICD-myc fragment resulting from processing of the longer ApoER2-CTF-myc. The specificity of the bands was confirmed by a C-terminal ApoER2 antibody (Fig. 3B). An 18-kDa band was also detected by the anti-HA antibody and the anti-C-terminal ApoER2 antibody in extracts of cells overexpressing ApoER2 ICD-HA, corresponding with the expected size of an ApoER2-ICD fragment (Fig. 3B).

Previous studies show a similar processing mechanism for both APP and ApoER2 (36). Thus, in cells overexpressing PS1, γ-secretase activity was monitored by measuring the content in their well-known substrate APP-CTF by Western blot (Fig. 4A). Accordingly, γ-secretase activity increased when the level of the endogenous 25-kDa ApoER2-CTF fragment decreased.

**Figure 2.** Unaltered number of cells expressing reelin in PS1 cKO mice and increased levels of reelin mRNA. A) Immunostaining of cortical tissue sections from cingulate and motor cortices of control wild-type (WT) and PS1 cKO mice with the reelin-specific antibody G10. Scale bar = 100 μm. B) Scatter plots representing the number of reelin-positive cells quantified by counting the number of stained cells in each section (n=36–40 sections, 4 animals/group), including means ± SE. C) Relative mRNA levels were analyzed by qRT-PCR in brain cortex (hemicortex) from WT and PS1 cKO mice (n=8 each). Values were calculated with relative standard curves and normalized to GAPDH from the same cDNA preparations. Specificity of the PCR products was confirmed by dissociation curve analysis. Results were confirmed in 2 independent determinations. Data represent means ± SE. *\( P < 0.05 \) vs. WT group.
The efficiency of the γ-secretase inhibitor DAPT, a well-known γ-secretase inhibitor that targets PS1 (37), was also monitored by measuring the accumulation of APP-CTF in SH-SY5Y cells (Fig. 4B). Under identical conditions, treatment of the cells with DAPT inhibited γ-secretase-mediated processing of the ApoER2-CTF, resulting in increased levels of the 25-kDa fragment (~47% increase, P=0.01; Fig. 4B). There was no cell death in cultures treated with 5 μM DAPT, as evaluated by the MTS assay (3±4% reduction, P=0.7). Of note, the γ-secretase inhibitor induced an increase in cellular full-length reelin levels (~40% increase, P=0.003; Fig. 5), by contrast, the 180-kDa reelin fragment did not change (~20% decrease; P=0.3; Fig. 5). Recent evidence indicates that this 180-kDa reelin fragment is generated, at least in part, after interaction of full-length reelin with ApoER2, initiating subsequent endocytosis and proteolysis (38). Our results suggest that reelin processing may depend on ApoER2 binding.

Modulation of ApoER2 expression in SH-SY5Y cells influences cellular reelin expression

To study the effect of ApoER2 overexpression on endogenous reelin levels, SH-SY5Y cells were transfected for 48 h with full-length ApoER2-EGFP. ApoER2-EGFP was detected both intracellularly and at the plasma membrane (identified by staining with N-cadherin, a plasma membrane protein; Fig. 6A). Overexpression of ApoER2 led to a significant decrease in secreted full-length reelin measured in the culture medium (~84%, decrease; P<0.001; Fig. 6B). The 180-kDa reelin N-terminal fragment which is generated by extra and intracellular proteolysis (16, 17, 38–40) did not decrease (P=0.9; Fig. 6B), suggesting that the decreased levels of full-length reelin is not dependent only on reelin processing. In any event, the influence of ApoER2 in reelin expression is difficult to address by measuring reelin protein levels alone, since increases in the ApoER2 protein would also result in an increased binding to reelin and subsequent processing.

We have shown that treatment of ApoER2-transfected SH-SY5Y cells with the γ-secretase inhibitor DAPT inhibits ApoER2-CTF processing (Fig. 4B). We then investigated the effect of γ-secretase inhibition in the production of ApoER2-ICD in SH-SY5Y cells overexpressing full-length ApoER2-EGFP. ApoER2-EGFP overexpression resulted in immunoreactive bands with a shift in electrophoretic mobility due to the expected size of the EGFP tag (~25–30 kDa; Fig. 7A). Treatment of transfected cells with DAPT induced accumulation
of CTF-EGFP (~50 kDa) and parallel decreased generation of the ICD-EGFP (~42-kDa band) (Fig. 7B).

Finally, we studied whether these ApoER2 fragments could translocate to the nucleus to regulate transcriptional activity. SH-SY5Y cells overexpressing ApoER2-EGFP were coimmunostained with emerin, a protein associated with the intranuclear lamina and located at the inner nuclear membrane (41). The analysis of the confocal images revealed the presence of EGFP fluorescence at the internal side of the nuclear membrane and in the nuclear lamina (Fig. 7C). Colocalization of EGFP fluorescence and emerin was decreased after treatment with DAPT, compared to that in the controls (35±2% decrease, P=0.01; Fig. 7C). Assays of nuclear extraction in cells overexpressing ApoER2-EGFP were also performed. Western blots of the nuclear extracts revealed with anti-GFP and anti-ApoER2 C-terminal antibodies confirmed the presence of an ICD-EGFP in this fraction (Fig. 7D). These results suggest that the ApoER2-ICD translocates to the nucleus.

Quantitative PCR was used to determine whether increased ApoER2 levels, including the ApoER2-ICD, influence reelin mRNA expression. Levels of the reelin transcripts were significantly decreased (12±3%, P=0.02) in ApoER2-EGFP-overexpressing cells compared to the controls (Fig. 8A). A luciferase reporter gene assay under the control of the RELN 5' upstream region (reelin-luc) was designed to further confirm whether ApoER2 modulates reelin transcription. We observed that overexpression of an ICD-HA greatly reduced expression of reelin-luc in transfected cells (~37% decrease in luciferase activity, P<0.001; Fig. 8B). Similar results were obtained when the ApoER2 full-length protein was overexpressed (~47% decrease in luciferase activity, P<0.001; Fig. 8B). Conversely, in cells treated with DAPT, reelin-luc was increased, compared to the level in the controls (~38% increase, P=0.02; Fig. 7B). The specificity of the role of ApoER2 on RELN transcriptional activity was corroborated by the lack of effect of DAPT in HEK293 cells (P=0.9), a cell line that expresses very low levels of endogenous ApoER2 (14).

To confirm that an ApoER2-ICD binds to the endogenous RELN promoter, ChIP assays were performed. Lysates of SH-SY5Y cells transfected with ApoER2-ICD were immunoprecipitated with an anti-HA antibody, and the DNA that bound to HA was amplified by PCR with different primers designed for distinct regions of the RELN promoter. As shown in Fig. 8C, ApoER2-ICD binds the RELN promoter between the positions −191 to +110 bp, with no binding elsewhere.

Altogether, these results demonstrate that ApoER2 influences reelin at the transcriptional level, and this process may be mediated by the processing of ApoER2 and subsequent generation of an ICD.

DISCUSSION

Reelin binds to ApoER2, a transmembrane protein that belongs to the LDL-receptor family, and this binding induces its cleavage by secretases. In the PS1 cKO mice, the total level of full-length ApoER2 increased with respect to the littermate controls; and, interestingly, the greater increase was observed for ApoER2-CTF, suggesting a role for PS1 in affecting expression or processing of ApoER2. This CTF is likely to represent the penultimate proteolytic fragment before the final cleav-
ApoER2 and anti-GFP antibodies (note the shift due to the EGFP tag). Transfected with ApoER2-EGFP. Endogenous and exogenous proteins were identified by blot analysis with anti-C-terminal antibody. Treatment with DAPT results in an increase of ApoER2-CTF (solid arrowhead) in cell extracts, whereas the ApoER2-ICD band (open arrowhead) is more abundant when γ-secretase activity is not inhibited. Confocal images of SH-SY5Y cells transfected with full-length ApoER2-EGFP in the presence of DAPT. Cells were incubated for 1 h with or without DAPT (control) before fixation with methanol, followed by incubation with an antibody against GFP to label ApoER2-EGFP fragments and with an antibody against emerin, a nuclear protein associated with lamina. Insets show magnification of the selected area. Quantification of the ApoER2-EGFP fluorescence that colocalized with emerin is shown (for control, n=166 cells, 2 independent experiments; for DAPT, n=131 cells, 2 independent experiments). *P < 0.001.

Figure 7. An ApoER2-ICD is identified in the nucleus of full-length ApoER2-EGFP-overexpressing cells. A) SH-SY5Y cells were transfected with ApoER2-EGFP. Endogenous and exogenous proteins were identified by blot analysis with anti-C-terminal antibody. B) Generation of CTF-EGFP and ICD-EGFP fragments was monitored in transfected cells treated with the γ-secretase inhibitor DAPT. ApoER2 fragments were detected with an anti-ApoER2 C-terminal antibody. C) Confocal images of SH-SY5Y cells transfected with full-length ApoER2-EGFP in the presence of DAPT. D) Nuclear fractions from SH-SY5Y cells transfected with full-length ApoER2-EGFP or a pEGFP vector were analyzed by Western blot with an anti-ApoER2 C-terminal antibody or anti-GFP. ApoER2-ICD was detected in the nuclear fraction of overexpressing cells. AcH2B and tubulin were used as markers for nuclear and cytoplasmic fractions, respectively.

Figure 8. Changes in embryonic and adult expression pattern for reelin and its receptors are also expected. Therein, the deficiency of PS1 cKO mice also affected. Discrepancies between in vivo and in vitro studies. Human SH-SY5Y neuroblastoma cells have enabled us to study whether variation in the levels or processing of ApoER2 could affect reelin expression in a “single” neuron-like cell type. We first confirmed in this cell line that the γ-secretase catalytic subunit PS1 participated in ApoER2 cleavage. ApoER2-CTF accumulated in cultured SH-SY5Y cells treated with the γ-secretase inhibitor DAPT (both, nontransfected and transfected with full-length ApoER2), but decreased in cells that overexpressed PS1. Furthermore, inhibition of γ-secretase with DAPT increased the levels of full-length reelin in cell extracts; although the amounts of the 180-kDa N-terminal fragment of reelin did not appear to be affected. Discrepancies between in vivo and in vitro studies.
Transmembrane proteins undergo influence reelin expression at the transcriptional level. 

Figure 8. ApoER2 overexpression down-regulates reelin expression. A) Reelin transcript levels were analyzed by qRT-PCR in SH-SY5Y cells transfected with full-length ApoER2-pEGFP or a pEGFP vector (n=10 each). Values were calculated by using relative standard curves and were normalized to GAPDH from the same cDNA preparations. Results were confirmed in 2 independent determinations. *P < 0.01. B) SH-SY5Y cells were cotransfected with RELN reporter plasmid and ApoER2 (left), either ICD-HA (n=8, 2 independent experiments) or full-length ApoER2-EGFP (n=12, 3 independent experiments). Empty vectors for each ApoER2 plasmid were used as controls. SH-SY5Y and HEK293 cells transfected with RELN reporter plasmid were treated for 24 h in the presence of 5 μM of DAPT or the vehicle control (right). In all cases, firefly luciferase activity was related to the Renilla luciferase reporter. Data (n=8 for each condition, obtained from ≥2 experiments) were analyzed by 1-way analysis of variance with the Bonferroni post hoc test to compare all groups, and exact P values were determined by Student’s t test. *P < 0.001. C) Scheme of the proximal region of the RELN promoter. Approximate positions of the known transcription factor binding sites are also indicated (CREB: solid triangle; SP1:open triangle; N-Myc: vertical arrow; Mzf1: shaded triangle; Tbr1: caret), together with the position of the primers used for the ChIP analysis (horizontal arrows). SH-SY5Y cells transfected with ApoER2 ICD-HA were also processed for ChIP with an antibody specific for HA or an unrelated rabbit IgG antibody as the control, followed by real-time PCR amplification of various regions of the RELN promoter indicated in the schematic. Unprecipitated chromatin preparations were similarly analyzed and used as the input control.

findings regarding the influence of decreasing γ-secretase activity on the generation of reelin fragments may also be related to the complex in vivo regulation of reelin expression and signaling. Moreover, we should keep in mind that different cell populations in the brain may respond differently to reelin signaling and to subsequent regulation by a mechanism mediated by reelin and ApoER2 processing.

We also addressed in SH-SY5Y cells whether ApoER2 processing, mediated by PS1/γ-secretase, can in turn influence reelin expression at the transcriptional level. Transmembrane proteins undergo γ-secretase cleavage, culminating in the release of their ICDs, which translocate to the nucleus and act as transcriptional regulators (45). In contrast, the cleavage of other substrates seems to be merely a degradative function (for a review, see ref. 46). The Notch ICD represents the best γ-secretase substrate studied to date (47), and an ICD transcriptional activity has also been associated with APP (48). The generation of ICD fragments has been shown for other LRP family members (49–51) and its nuclear translocation is inferred. To date, whether the ICD generated from ApoER2 processing has transcriptional activity is unknown.

When cells overexpressed full-length ApoER2, ApoER2-CTF levels were increased in comparison with those in the nontransfected cells, with a parallel increase in the ApoER2-ICD to detectable levels in the cell extract. We observed a parallel reduction in levels of the extracellular reelin protein, indicating that overexpression of full-length ApoER2 promoted an increase in reelin processing. Both overexpression of full-length ApoER2 (and the subsequent increase in the ApoER2-ICD) and overexpression of the ApoER2-ICD downregulated RELN promoter activity through binding in the RELN promoter region. Accordingly, an ApoER2-ICD was found in the soluble nuclear fraction after transfection of SH-SY5Y cells with full length-ApoER2. Of note, when cells overexpressing full-length ApoER2 were incubated with DAPT and the amount of ApoER2-ICD decreased, the ApoER2-EGFP fluorescence at the nucleus also decreased. DAPT treatment, which inhibited the generation of ApoER2-ICD, triggered an increase in RELN transcriptional activity. These results suggest that the ApoER2-ICD can act as an inhibitor of reelin synthesis.

To our knowledge, this study is the first to report an autocrine loop that modulates reelin levels where reelin binding activates the processing of its receptor, which in turn regulates the levels of reelin. Previous studies have shown that binding of reelin to its receptors results in processing of the complex, releasing a secreted soluble ApoER2 fragment containing the entire ligand-binding domain. This domain acts in a dominant-negative fashion in the regulation of reelin signaling (14). Our study also demonstrated that ApoER2 modulation of reelin levels could occur within the cells themselves. The secretory nature of reelin and the evidence of a “paracrine” biological activity in distal cells expressing ApoE receptors (52) has attracted much attention in neuropathology. Similar ligands for lipoprotein receptors such as ApoE have also been shown to act as autocrine/paracrine factors (53). Evidence also suggests a role for reelin in autocrine signaling. A dual role for reelin in both autocrine and paracrine signaling has recently been suggested in...
neuroblastomas (54). Glutamatergic cerebellar granule neurons, which synthesize reelin, have been shown also to coexpress ApoER2 (55). In addition, reelin and Dab1, the intracellular component of the reelin signaling cascade, are coexpressed in Cajal-Retzius neurons during cortical development and in cortical pyramidal neurons after neuronal migration is complete (56). Reelin and ApoER2 also colocalize in some cells in the adult mouse brain (results not shown), and in ApoER2-enriched areas of the retina (32). In situ hybridization studies also demonstrate coexpression of reelin and ApoER2, which are expressed in the same areas in the adult canine brain (57). These data support the idea that, in the adult brain, the action of reelin takes place in proximity to its synthesis. We have observed that transfection of ApoER2 in HEK293 cells, which express very low endogenous levels of reelin and its receptors, triggers a substantial increase in reelin expression (unpublished observation).

A growing number of studies have demonstrated the participation of reelin and elements of its signaling pathway in the pathophysiology of Alzheimer’s disease (AD). Therefore, there is increasing interest in the interaction of members of the reelin cascade with pathways affected in AD. Reelin receptors are also receptors for ApoE, and the ApoE4 variant is the largest known genetic risk factor for late-onset, sporadic AD (58). Secretases also process the transmembrane APP (59), generating the small β-amyloid (Aβ) peptide, a major AD effector and component of the senile plaques found in AD (60, 61). Reelin and Dab1 have both been shown to interact with APP (62–64) and to influence its trafficking and processing (65, 66). We have demonstrated that Aβ alters reelin expression (29) and have more recently suggested that reelin signaling is altered in the brain in AD (67). We have shown that reelin expressed in the presence of Aβ is functionally impaired, since its ability to bind efficiently to ApoER2 is decreased. Impaired reelin binding to its receptors may result in altered generation of ApoER2-ICD and subsequent influence on reelin expression. This effect may be related to the increased reelin expression observed in the AD brain (28, 29). The pathophysiological significance and implications of the altered reelin levels in the diseased brain remain unknown.

In summary, our study suggests that the level of the reelin receptor ApoER2 and its processing influence reelin expression. This effect may be mediated by γ-secretase processing of ApoER2. Our data and other recent findings suggest a scenario where reelin signaling, in conjunction with parallel processing of ApoER2, serves as an autoregulatory mechanism that may ultimately influence synaptic plasticity and responsiveness.

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Valeria Balmaceda, Inmaculada Cuchillo-Ibáñez, Lluis Pujadas, et al.

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