ApoER2 processing by Presenilin-1 modulates

Reelin expression

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Abbreviations: A β : β -amyloid protein; AD: Alzheimer's disease; AcH2B: acetylated histone H2B; APP: β -amyloid precursor protein; ApoER2: apolipoprotein E receptor 2; ChIP: chromatin immunoprecipitation; CTF: C-terminal fragment; Dab1: Disabled-1; DAPT: N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; DMSO: Dimethyl Sulfoxide; ICD: intracellular domain; MTS: tetrazolium assay; NGS: normal goat serum; PB: phosphate buffer; PBS: Phosphate-saline buffer; PF: paraformaldehyde; PS1: presenilin 1.

Abstract

The Reelin signaling protein and its downstream components have been associated with the etiopathogenesis of Alzheimer's disease (AD). The Reelin signaling pathway begins with the binding of Reelin to the transmembrane lipoprotein-receptor, apolipoprotein E receptor 2 (ApoER2), which in turns 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 demonstrate 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 demonstrate that ApoER2 processing occurs in the presence of PS1, resulting in an intracellular C-terminal ApoER2 fragment. In addition, the pharmacological inhibition of γ -secretase in SH-SY5Y cells leads to increased Reelin levels. Over-expression of ApoER2 decreases Reelin mRNA levels in these cells. A luciferase reporter gene assay and nuclear fractionation confirm that increased amounts of intracellular fragment of ApoER2 suppress Reelin expression at a transcriptional level. Chromatin immunoprecipitation experiments corroborate that the intracellular fragment of ApoER2 binds to the *RELN* promoter region. Our studies suggest that PS1/ γ -secretase-dependent processing of the Reelin receptor ApoER2 inhibits Reelin expression and may regulate Reelin signaling.

Key words: Alzheimer's disease; Reelin; ApoER2; Presenilin-1; processing; transcription.

Introduction

Alzheimer's disease (AD), the most common cause of dementia among the elderly, is characterized by the presence in the brain of senile plaques and neurofibrillary tangles. The major component of the extracellular senile plaques is the β -amyloid protein ($A\beta$). This small polypeptide is generated by processing of the larger transmembrane β -amyloid precursor protein (APP; 1,2), by the successive action of proteolytic enzymes known as secretases (3). The intracellular neurofibrillary tangles are composed of paired helical filaments of the microtubule-associated protein tau, which is abnormally hyperphosphorylated (4).

Reelin is a large signaling protein which plays an important role in the adult brain, influencing neurotransmission and synaptic plasticity. Normal Reelin levels are essential for some forms of long-term memory (5-7). Increasing evidence suggests that Reelin signaling is altered in the AD brain (8,9), but the pathophysiological significance and subsequent implications of these changes in the diseased brain remains unknown.

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) (10,11). Reelin receptors are also receptors for apolipoprotein E; which ApoE4 variant is the largest known genetic risk factor for late-onset sporadic AD (12). Reelin binding induces the cleavage of Reelin receptors by the sequential processing of α - and γ -secretases which also process APP (13-15). Binding of Reelin to its receptor relays the signal into the cell via the adapter Dab1 (Disabled-1;16-18). Reelin-dependent induction of Dab1 tyrosine phosphorylation triggers an intracellular kinase cascade, which ultimately inhibits glycogen synthase kinase-3 β (GSK-3 β), preventing tau hyperphosphorylation (19). Reelin and Dab1 have both been shown to interact with APP (20-22) and influence its trafficking and processing (23-24).

We have also recently demonstrated that A β alters Reelin expression in the AD brain (8-9).

In this study, we investigated if 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 (25), in this modulation, as ApoER2 cleavage after its binding to Reelin is mediated by secretases. We used a PS1 conditional knockout mouse model (*PS1* cKO; 26) to study the effect of PS1 on ApoER2, and on protein and mRNA levels of Reelin *in vivo*. Using SH-SY5Y cells over-expressing ApoER2, we establish that the C-terminal ApoER2 fragment (CTF) is processed by γ -secretase, generating an intracellular domain fragment (ICD) that has transcriptional activity.

Methods

PS1 conditional knockout mice

Generation and characterization of *PS1* cDKO mice have been previously described (26). *PS1* cDKO mice lack expression of *PS1* gene in glutamatergic neurons of the forebrain at postnatal stage starting around P18. *PS1* cDKO mice (fPS1/fPS1;CaMKII α -Cre) were obtained by crossing floxed *PS1* (fPS1/fPS1) males to *PS1* cKO females (fPS1/fPS1;CaMKII α -Cre). Mice used in this study were age-matched littermate control (fPS1/fPS1) and *PS1* cDKO (fPS1/fPS1; CaMKII α -Cre) mice (background C57BL/6). Experimental procedures were conducted according to the Animal and Human Ethical Committee of the Universitat Autònoma de Barcelona (protocol CEEAH 475) following the European Union guidelines.

Brain samples were collected from 3-4 month old *PS1* cKO (n=10) and age-matched control littermates (n=10). No PS1 was detectable in the forebrain glutamatergic neurons of 2–3 month old mice, and no significant alterations in general behaviour, motor coordination, and exploratory anxiety were observed. Brain cytoarchitecture, neuronal numbers and morphology was also normal (26,27).

Pharmacological treatment of SH-SY5Y neuroblastoma cells

SH-SY5Y cells (220,000 cells/ well) were grown on 12 well-plates for 24 h in D-MEM+GlutaMAX™ (Dulbecco's Modified Eagle medium; Gibco®, life technologies Paisley, UK) supplemented with 10% fetal bovine serum (Gibco) and 100 μ g/mL penicillin/streptomycin (Gibco). Cells were treated with 5 μ M of γ -secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT; Calbiochem®, Merck KGaA, Darmstadt, Germany) or vehicle Dimethyl Sulfoxide (DMSO). Following 22 h of treatment, the cells were washed twice with cold

Phosphate-saline buffer (PBS) and resuspended in 100 μ L of ice-cold extraction buffer: 50 mM Tris-HCl, pH 7.4 / 150 mM NaCl / 5 mM EDTA / 1% (w/v) Nonidet P-40 / 0.5% (w/v) Triton X-100 supplemented with a cocktail of protease inhibitors. Cell lysates were sonicated and centrifuged at 20,000 $\times$ g at 4°C for 12 min, and extracts frozen at -80°C for future analysis.

Cell viability was measured using the tetrazolium assay (MTS; CellTiter 96® Aqueous Assay, Promega, Southampton, UK) according to the manufacturer's instructions. SH-SY5Y cells were cultured in 96-well plates (19,000 cells/well) and treated with DAPT as previously described. MTS was added after DAPT treatment and cells were incubated for 4 h, before viability was determined by measuring the absorbance at 490nm in a microplate reader (Infinite M200, Tecan; Männedorf, Switzerland).

ApoER2 over-expression, RNA interference and luciferase reporter assay

SH-SY5Y cells were transfected with a construct encoding full-length ApoER2 (pEGFPN1-*Mus musculus* ApoER2). Alternative ApoER2 constructs were also used: ApoER2-myc expressing the transmembrane and cytoplasmic domains (amino acids 825-963); and ApoER2-HA expressing only the cytoplasmic domain (amino acids 728-842) (both generously provided by Dr W. Rebeck; see ref. 28, 29). Empty vectors (Promega) were used as control. Cells (650,000 cells/well) were grown in 6 well plates and transfected using Lipofectamine 2000 (Invitrogen™, life technologies Paisley, UK) according to the manufacturer's instructions. After two days in culture, cells and culture supernatants were separately harvested. Cell culture supernatants were cleared by centrifugation for 10 min, 5000 $\times$ g at 4°C. Cells were extracted as previously described

and ApoER2 levels were assayed by Western blotting to determine transfection efficiency.

For the luciferase reporter assay, cells were transfected with a pGL3 Basic Vector-*RELN* promoter (-2600 region which contains 2.6 kb of 5' flanking sequence of human *RELN* promoter (a kind gift of Dr. D. Grayson; see ref. 30) in the presence of ApoER2-pEGFP or a pEGFP empty vector. 2-4 µg of each cDNA was co-transfected with 10-30 ng of the pRL-CMV vector (Renilla luciferase, Promega) in each well of a 12-well plate (200,000 cells/well). 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 for determination of luciferase activity in a SIRUS Luminometer V3.1 (Berthold Detection Systems GmbH, Germany). Firefly luciferase data were normalized to Renilla luciferase activity.

Nuclear protein fractionation

SH-SY5Y cells were cultured in 10 cm² culture plates (4,000,000 cells/ plate). 36 h after transfection with ApoER2-pEGFP or the pEGFP empty vector, cells were washed twice with cold PBS, and gently scraped 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 using the QProteome Nuclear Protein kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Cytosolic and nuclear protein fractions were assayed by Western Blotting.

Western blotting

Reelin levels were determined as previously described (8). 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 bicinchoninic assay (Pierce, Thermo Scientific; IL USA). Electrophoresis was allowed to proceed at low voltage to minimize excessive heat generation (8,31).

Proteins were blotted onto nitrocellulose membranes, blocked with 5% non-fat milk and incubated with 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 recognise epitopes located in the region of amino acids 164-189 (clone 142) and 164-496 (clone G10) (32).

To detect ApoER2, brain and cell extracts were boiled for 7 min at 98°C, and full length and C-terminal fragments 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 amino acids 928-945 located near the C-terminus of ApoER2, was used to detect ApoER2 and C-terminal fragments. 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). 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. Loading control was estimated using mouse α -tubulin (1:5000 dilution; Sigma-Aldrich). A polyclonal antibody against acetylated histone H2B (AcH2B; 1:5000 dilution) (generous gift of Dr. A. Barco) 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) using SuperSignal (Pierce) in a Luminescent Image Analyzer LAS-1000 Plus (Fujifilm, Tokyo, Japan). The intensity of the Reelin bands was measured by densitometry using the Science Lab Image Gauge v 4.0 software provided by Fujifilm.

Immunohistochemistry and immunocytochemistry

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SH-SY5Y cells used for confocal microscopy were transfected with either ApoER2-pEGFP or a vector containing pEGFP alone using Lipofectamine 2000 (Invitrogen) according to the manufacturers's instructions. After 4 hours, media was replaced with fresh media containing 5 μ M DAPT, or the equivalent volume of DMSO as control. After 24 hours, cells were washed with cold Hank's buffered salt solution and fixed with methanol for 10 min at -20°C. Non-specific sites were blocked with 2% (w/v) BSA and 40 μ g/ml digitonin in PBS for 30 min. Cells were then incubated with primary antibodies (rabbit anti-GFP 1:100 Invitrogen; mouse anti-Emerin 1:200 Santa Cruz Biotechnology, Santa Cruz, CA, USA; mouse anti-N Cadherin 1:200; BD Biosciences, Erembodegem, Belgium) for 1 hour followed by secondary antibodies (Alexa Fluor® 546 anti-mouse 1:200; Alexa Fluor® 488 anti-rabbit 1:200, Invitrogen) for 1 h. To study the location of ApoER2-EGFP in the vicinity of the inner nuclear membrane, cells were incubated with cycloheximide (50 μ M) (Sigma-Aldrich) for 2 hours after transfection and before immunocytochemistry, to block synthesis of new

proteins. Pictures were acquired in a Leica upright TCL-SL confocal microscope (Leica Microsystems, Wetzlar, Germany) using an HCX Plan Apochromat 63×/1.32-0.6 NA oil objective (Leica). To measure the intensity of fluorescence of ApoER2-EGFP co-localizing with emerin, a circle was hand drawn over the green fluorescence which overlaps the red fluorescent nuclear membrane in the merge channel, and fluorescence intensity analyzed by the 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™ Micro-to-Midi Total RNA Purification System (Invitrogen) following the manufacturer's instructions. cDNAs were synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions using 5 µg of total RNA and oligo (dT)12-18. Quantitative PCR amplification was performed using a StepOne™ Real-Time PCR System (Applied Biosystems; life technologies Paisley, UK) with TaqMan probes specific for mouse *RELN* (assay ID: Mm00465200_m1 Applied Biosystems) and human *RELN* (assay ID: HS01022646_m1 Applied Biosystems), and mouse and human GAPDH as endogenous controls (Applied Biosystems). Reelin transcript levels were calculated using the relative standard curve method normalized to GAPDH.

Chromatine Immunoprecipitation (ChIP) assay

SH-SY5Y cells plated in 100-mm tissue culture dishes (4,300,000 cells/plate) were transfected with 20 µg of ApoER2-HA according protocol described above. After 36 h, cells were fixed in freshly prepared 1% paraformaldehyde for 10 min at room temperature. Cross-linking was halted by addition of 2.5 M glycine, 5 min. Cells were

washed twice with ice-cold PBS plus protease inhibitors. The chromatin was extracted using EZ Magna ChIP A/G (Millipore, Merck KGaA Darmstadt, Germany) according to manufacturer's protocol.

Extracted chromatin was sheared to 100-500 bp using the BiorruptorTM (Diagenode Inc., USA), 30 s cycles for 10 min. After centrifugation at 10,000×g at 4°C for 10 min, the supernatant was collected. For immunoprecipitation, protein A/G magnetic beads (Millipore) were coupled to anti HA-antibody (Abcam) or to rabbit IgG antibody as a control (Millipore). Supernatants were incubated overnight with the beads. Immunoprecipitates were incubated with Proteinase K (Millipore) at 62°C for 2 h (Thermomixer system; Eppendorf AG, Hamburg, Germany) to reverse the formaldehyde cross-linking, and purified DNA was analyzed by PCR, using primers for human GAPDH as control (Millipore). PCR amplification was performed using 30 ng of immunoprecipitated DNA in a 20 µl reaction volume containing 0,3 µM of sense and antisense primers, 10% v/v DMSO, and Kod Hot Start DNA Polymerase kit (Novagen, Merck KGaA, Darmstadt) (0.3 mM of each dNTP, 1X KOD Polymerase buffer, 1,5 mM MgSO₄ and 1 U of KOD Hot Start DNA Polymerase). Primers were designed to amplify 5'UTR human *RELN* at different positions of the promoter: [-515 to -395 bp] *sense* 5'- CTC GCT CAT TCA GTT TTG GAG-3', *antisense* 5'- GGG GCT TTA AGA AGG TGT GG-3'; [-393 to -181 bp] *sense*: 5'- CTC AGC GGT CCT CGA CAG-3', *antisense*: 5'-CCG CTC CAC ACC TTC TTA AA-3'; [-191 to +110 bp] *sense* 5'-CTC AGC GGT CCT CGA CAG-3', *antisense* 5'- CCG CTC CAC ACC TTC TTA AA-3'. The assembly PCRs were carried out by an initial denaturation of DNA at 95°C for 5 min. Cycling conditions were 32-35 cycles 30 s 94°C, annealing at 61°C for 30s, 72°C for 30 s. A final extension period of 2 min at 72°C completed the thermocycling before

cooling. PCR products were analyzed by gel electrophoresis on a 2% Tris acetate-EDTA (TAE) agarose gel.

Statistical Analysis

All data were analyzed by a Student's t-test for single pair-wise comparisons using SigmaStat (Version 2.0; SPSS Inc.). Results are presented as means $\pm$ SEM. *p* values < 0.05 were considered significant.

Results

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

To determine if PS1 is involved in the control of Reelin receptor levels *in vivo*, brain homogenates from *PS1* cKO mice (26) and littermate controls were first analyzed by SDS-PAGE followed by Western blotting. Immunoblotting for the main brain Reelin receptor, ApoER2, revealed a complex banding pattern probably reflecting the several ApoER2 isoforms (33), and mature and precursor forms (15,34). The levels of ApoER2 appear to be significantly increased in cortical extracts from *PS1* cKO mice (~80% increase) compared with controls (Fig. 1A). It is known that Reelin bound to ApoER2 induces subsequent ApoER2 cleavage by the sequential action of α - and γ -secretases (13,15). α -Secretase cleaves the ApoER2 extracellular domain generating a membrane bound C-terminal fragment (CTF) which subsequently acts as a substrate for γ -secretase. Consistent with a role of PS1 in the proteolysis of ApoER2, we found a significant accumulation of ApoER2-CTF in cortical extracts from *PS1* cKO mice (~300% increase) compared with controls littermate animals (Fig. 1A). Levels of ApoER2 in the cerebellum, a region in which PS1 is not silenced, did not differ between *PS1* cKO mice and littermate controls (Fig. 1A).

We next measured the levels of the ApoER2 ligand, Reelin, and found that full-length Reelin (420 kDa) display a large increase in cortex of *PS1* cKO mice (~200%), while N-terminal 310 kDa (~70%) and 180 kDa (~50%) Reelin fragments show moderate increase (Fig. 1B). Reelin levels in the cerebellum were similar in *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 labelled tissue sections with a specific antibody against Reelin and counted the number of stained cells (Fig. 2A). Density of Reelin-

positive cells was similar in the cortex of control and *PS1* cKO mice (Fig. 2B), indicating that differences in levels detected by Western blotting may be attributed 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 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 if altered ApoER2 processing influences Reelin levels, and if this effect is mediated by γ -secretase. SH-SY5Y cells express significant levels of Reelin and PS1 (9,35), and have been used as cellular models of ApoER2 over-expression (36). As previously mentioned, Reelin bound 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 intracellular domain fragment (ICD) (for an illustrative scheme see Fig. 3A). An ~25 kDa ApoER2-CTF fragment was observed in cell extracts from untransfected cells using a C-terminal ApoER2 antibody, in agreement with previous studies (Fig 3B) (13,15,29). Endogenous ApoER2-ICD was not detected, probably due to its low levels and inherent instability (13). 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 over-expressed in SH-SY5Y cells. Another chimeric ApoER2 protein containing only the cytoplasmic domain tagged to HA (ApoER2 ICD-HA) was also expressed (28,29). Cell extracts were then analyzed by Western blotting. An anti-myc antibody detected the ApoER2-CTF-myc at 25 kDa. A faint band of ~18 kDa was also

detected. This 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 over-expressing 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 (37). The γ -secretase inhibitor DAPT has been demonstrated to reduce A β levels *in vivo* (38). The efficiency of DAPT to inhibit γ -secretase activity was monitored by measuring the accumulation of APP-CTF in SH-SY5Y cells (Fig. 4A). There was no cell death in cultures treated with 5 μ M DAPT, as evaluated by the MTS assay ($3 \pm 4\%$ reduction, $p = 0.7$). Under identical conditions, treatment of the cells with DAPT inhibited γ -secretase-mediated processing of the ApoER2-CTF, resulting in increased levels of the endogenous 25 kDa ApoER2-CTF fragment (Fig. 4A). Interestingly, the γ -secretase inhibitor induced an increase of cellular full-length Reelin levels ($\sim 40\%$ increase, $p = 0.003$; Fig. 4B); by contrast, the 180 kDa Reelin fragment was not changed ($\sim 20\%$ decrease; $p = 0.3$; Fig. 4B). 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 (39). 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 over-expression on endogenous Reelin levels, SH-SY5Y cells were transfected for 48 hours 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. 5A). Over-expression of ApoER2 leads to a significant decrease in secreted full-length Reelin measured in the culture medium ($84 \pm 3\%$, decrease; Fig. 5B). The 180 kDa Reelin N-terminal fragment which is generated by extra and intracellular proteolysis (39-43) was not decreased (Fig. 5B), suggesting the increased levels of full-length Reelin is not dependent on the Reelin processing. Anyhow, the influence of ApoER2 in Reelin expression is difficult to address by measuring Reelin protein levels alone, since increases in the ApoER2 protein will 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. 4A). We then investigated the effect of γ -secretase inhibition in the production of ApoER2-ICD in SH-SY5Y cells over-expressing full-length ApoER2-EGFP. ApoER2-EGFP over-expression resulted in immunoreactive bands with a shift in size due to the expected size of the EGFP tag (~ 25 -30 kDa; Fig. 6A). 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. 6B).

Immunofluorescence labelling of emerin, a protein associated to the intranuclear lamina and located at the inner nuclear membrane (44), allowed us to examine the presence of ApoER2-EGFP at the internal side of the nuclear membrane and in the nuclear lamina (Fig. 6C). Co-localization of ApoER2-EGFP and emerin is decreased in transfected cells treated with DAPT compared to controls ($35 \pm 2\%$ decrease, $p = 0.01$; Fig. 6C). Western blotting and staining of cellular nuclear extracts with anti-GFP and anti-ApoER2 C-terminal antibodies confirm the presence of an ICD-

EGFP in this fraction (Fig. 6D). These results suggest that the ApoER2-ICD is translocated to the nucleus.

Quantitative PCR was used to determine whether increased ApoER2 levels, including the ApoER2-ICD, influences Reelin mRNA expression. Levels of the Reelin transcripts were significantly decreased ($12 \pm 3\%$, $p = 0.02$) in ApoER2-EGFP over-expressing cells compared to controls (Fig. 7A). 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 expression at the transcriptional level. We observed that over-expression of ApoER2 alone greatly reduced expression of Reelin-luc in transfected cells ($47 \pm 8\%$ decrease in luciferase activity, $p < 0.001$; Fig. 6B). Similar results were obtained when an ICD-HA was over-expressed instead of the ApoER2 full-length protein ($37 \pm 5\%$ decrease in luciferase activity, $p < 0.001$; Fig. 7B).

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 Figure 7C, 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 and subsequent generation of an ICD.

Discussion

A growing number of studies now demonstrate the participation of Reelin and elements of its signaling pathway in the pathophysiology of AD. Therefore, there is increasing interest in the interaction of members of the Reelin cascade with pathways affected in AD. We have investigated the influence of PS1, a key protein involved in AD pathogenesis, in Reelin processing and expression.

Reelin binds to ApoER2, a transmembrane protein which belongs to the LDL-receptor family, and this induces its cleavage in a similar manner to APP by secretases. In this study, we first confirmed that the γ -secretase catalytic subunit, PS1, participates in ApoER2 cleavage. The total level of full-length ApoER2 is increased in *PS1* cKO mice, suggesting a role for PS1 in affecting expression or processing of ApoER2. The ApoER2-CTF accumulates in *PS1* cKO mice and in cultured SH-SY5Y cells (untransfected or transfected with full-length ApoER2) treated with the γ -secretase inhibitor DAPT. This CTF is likely to represent the penultimate proteolytic fragment before the final cleavage by γ -secretase, releasing the soluble intracellular ICD (13). Our data also indicate that the modulation of γ -secretase activity not only affects PS1-processing of ApoER2, but also Reelin levels. We also demonstrate an effect on Reelin transcription.

In *PS1* cKO mice with decreased γ -secretase activity, levels of full-length Reelin are increased. Furthermore, inhibition of γ -secretase with DAPT increases the levels of full-length Reelin in cell extracts; although the amounts of the 180 kDa N-terminal fragment of Reelin do not appear to be affected. In the *PS1* cKO mouse brain, twice the amount of full-length Reelin is present compared to its N-terminal fragments. Any change in the level of full-length Reelin or its N-terminal fragments will reflect on Reelin processing which occurs in parallel with ApoER2 processing. Discrepancies between *in vivo* and *in*

vitro findings may also be related to the complex regulation of Reelin expression and signaling, as the capacity of the functional full-length Reelin to bind to its receptors is regulated by competition with the truncated Reelin fragments (45). Therefore, the *in vivo* control of Reelin expression and signaling might be intricate.

Interestingly, the deficiency of γ -secretase in *PS1* cKO mice also leads to an increase in Reelin mRNA. These results suggest that ApoER2 processing, mediated by Reelin binding and catalysed 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 acts as a transcriptional regulator (46). In contrast, the cleavage of other substrates seems to merely be a degradative function (for a review see 47). The Notch ICD represents the best γ -secretase substrate studied to date (48), and an ICD transcriptional activity has been also associated with APP (49). The generation of ICD fragments has been shown for other LRP family members (50-52), and its nuclear translocation inferred. Whether the ICD generated from ApoER2 processing has a transcriptional activity has been unknown, to date.

Our current studies demonstrate that the inhibition of PS1-mediated ApoER2 processing, and hence, the increase of CTF levels, also results in an increase in Reelin levels. Furthermore, when cells overexpress full length ApoER2, ApoER2-CTF levels are increased in comparison with untransfected cells, with a parallel rise in the ApoER2 ICD to detectable levels in the cell extract. We observe a parallel reduction in levels of the extracellular Reelin protein, indicating that over-expression of full-length ApoER2 promotes an increase in Reelin processing. Both, over-expression of full-length ApoER2 (and the subsequent increase in the ApoER2-ICD), or over-expression of the ApoER2-ICD, down-regulate *RELN* promoter activity through binding in the *RELN*

promoter region. Accordingly, an ApoER2-ICD is found in the soluble nuclear fraction after transfection of SH-SY5Y cells with full length-ApoER2. Interestingly, when cells overexpressing full length-ApoER2 are incubated with DAPT, the amount of ApoER2-ICD is decreased, and is accompanied by a decrease in ApoER2-GFP fluorescence at the nucleus. These results suggest that the ApoER2-ICD can act as an inhibitor of Reelin synthesis.

The potential modulation of the Reelin signaling pathway by γ -secretase has been previously explored in conventional *PS1* KO mice (53). In this work, no difference in Reelin levels was observed between embryonic PS1 null mutant and wild type mice. The cellular pattern of Reelin expression has been demonstrated to differ between embryonic and adult brain. At the end of the neuronal migration period in embryos, Reelin is predominantly expressed by Cajal-Retzius neurons. These cells become eventually replaced principally by a subset of GABAergic interneurons in the adult brain (54). Indeed, it is thought that Reelin has distinct functions in the developing and in the adult brain (5). Changes in embryonic and adult expression pattern for the Reelin receptor ApoER2 are also expected. Therefore, differences between embryonic and adult brain in the cellular pattern of expression and in the regulation of Reelin and its receptors, may contribute to the divergent results observed in our studies.

To our knowledge, this study is the first report demonstrating an autocrine loop modulating Reelin levels where Reelin binding activates the processing of its receptor, which in turns, 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 (55). Our studies also demonstrate 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 (56), has attracted much attention in neuropathology. Similar ligands for lipoprotein receptors such as ApoE have also been shown to act as an autocrine/paracrine factor (57). Evidence also suggests a role for Reelin in autocrine signaling. A dual role for Reelin in both autocrine and paracrine signaling has been recently suggested in neuroblastomas (58). Glutamatergic cerebellar granule neurons which synthesize Reelin, have been shown also to co-express ApoER2 (59). In addition, Reelin and Dab1, the intracellular component of the Reelin signaling cascade, are co-expressed in Cajal-Retzius neurons during cortical development and in cortical pyramidal neurons after neuronal migration is complete (60). Reelin and ApoER2 also co-localize in some cells in the adult mouse brain (not shown), and in ApoER2 enriched areas of the retina (33). In situ hybridization studies demonstrate as well co-expression of Reelin and ApoER2 which are expressed in the same brain areas in the adult canary brain (61). These data support the idea that in the adult brain, the action of Reelin may take place within close vicinity of its synthesis.

Interestingly, the processing of ApoER2 by γ -secretase is regulated by the glycosylation state of the receptor, which in turn is dependent on tissue-specific alternative splicing (13). Modulation of synaptic plasticity and memory by Reelin involves the differential splicing of ApoER2 (5). Although the association of ApoER2 polymorphisms with AD is not clear (62,63), altered glycosylation of Reelin in the Alzheimer’s brain may influence Reelin signaling and appears to be associated with progression of the disease (8,9). Binding affinities of Reelin for its receptors can also be modulated by competition with other ligands. The ApoE4 variant, the major known genetic risk factor for AD, is one of its strongest competitors (10). It has been recently

demonstrated that ApoE4 selectively impairs synaptic plasticity and NMDA receptor phosphorylation by Reelin (64). The isoform-specific role of ApoE in the localization and intracellular trafficking of ApoER2 and glutamate receptors reveals an alternative mechanism by which ApoE4 may accelerate onset of dementia and neuronal degeneration by differentially impairing the Reelin-dependent maintenance of synaptic stability (64).

In conclusion, our studies suggest that PS1, a key enzyme in AD pathophysiology, influences Reelin expression. This is mediated by γ -secretase processing of the Reelin receptor ApoER2. Our data and other recent findings suggest a scenario where Reelin signaling, in conjunction with APP trafficking and PS1 processing of ApoER2, are cornerstones of an integrated signaling network that may be disrupted in the AD brain, compromising synaptic responsiveness, plasticity and survival.

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Figure Legends

Figure 1. Increased levels of ApoER2 and Reelin in the cortex of *PSI* cKO mice.

(A) Representative blot for immunodetection of ApoER2 full-length isoforms and CTF in the cortex (left panel) and the cerebellum (right panel) of control wild-type (WT; n= 6) and *PSI* cKO mice (n= 6). The densitometric quantification from the sum of all the bands close to 160 and 140 kD, and of the 25-kDa CTF band are shown. (B) Western blots and densitometric quantification of Reelin fragments from the same WT and *PSI* cKO samples presented in (A). The N-terminal Reelin antibody G10 detects full-length 420 kDa Reelin and the N-terminal 310 and 180-kDa fragments. Reelin immunoreactivity from each fragment is shown, and expressed as a percentage relative to immunoreactivity of the 420-kDa band from WT mice. Experiments were performed in triplicate, and α -tubulin was used as a loading control. The data represent the means $\pm$ SEM. *Significantly different ($p < 0.01$) from the WT group.

Figure 2. Unaltered number of cells expressing-Reelin in *PSI* cKO mice and increased levels of Reelin mRNA. (A) Immunostaining of cortical tissue sections from control wild-type (WT) and *PSI* cKO mice with the Reelin specific antibody G10. Scale bar: 100 μ m. (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 per group), including mean $\pm$ SEM. (C) Relative mRNA levels were analyzed by QRT-PCR in brain cortex from WT and *PSI* cKO mice (n= 8 each). Values were calculated using relative standard curves and normalized to GAPDH from the same cDNA preparations. Specificity of the PCR products was confirmed by dissociation curve analysis. The results were confirmed in two independent determinations. The data represent the means $\pm$ SEM. *Significantly different ($p < 0.05$) from the WT group.

Figure 3. Diagram of ApoER2 and identification of intracellular fragments. (A)

Diagram of the wild-type ApoER2 and chimeric proteins containing the ligand-binding domain (LBD), transmembrane domain (TMD) and cytoplasmic domain (CD). The two main processing sites by α - and γ -secretase are indicated, sequential secretase cleavage generates a membrane bound C-terminal fragment (CTF) and an intracellular domain fragment (ICD). (*)The epitope for the C-terminal antibody is approximately located as indicated. C-terminal tags for ApoER2 constructs are myc, HA and EGFP. **(B)** The endogenous ApoER2 fragments from untransfected SH-SY5Y cells (-) were identified by blotting with a C-terminal ApoER2 antibody; while chimeric proteins expressed in transfected cells (+) were identified by blotting with the anti C-terminal ApoER2 antibody and with antibodies against the specific tag, as indicated.

Figure 4. Inhibition of γ -secretase enhances Reelin levels. SH-SY5Y cells were treated for 24 hours in the presence of 5 μ M of DAPT (+) or the vehicle (control; -), and ApoER2 and Reelin were assayed by Western blotting of cell extracts (equivalent amounts of protein were loaded in each lane). **(A)** Assessment of APP CTF levels serves as a control of the efficiency of DAPT to inhibit γ -secretase activity. The accumulation of ApoER2 CTF was monitored with a C-terminal anti-ApoER2 antibody. Data represent percentage relative to control cells. **(B)** Reelin from cell extracts was detected with an N-terminal antibody. Immunoreactivity of full-length Reelin at 420 kDa and the predominant 180 kDa N-terminal fragment is shown, and expressed as a percentage relative to immunoreactivity of the 420-kDa band from control cells. The data represent the means $\pm$ SEM (determinations by duplicate). *Significantly different ($p < 0.005$) from the control group.

Figure 5. ApoER2 over-expression decreases full-length Reelin levels. (A) Confocal images of a SH-SY5Y cells transfected with full-length ApoER2-EGFP. Cells were incubated with an antibody against GFP to label ApoER2-EGFP and with an antibody against N-Cadherin to stain the plasma membrane. The merged image shows ApoER2 localized in the plasma membrane (yellow) as well as intracellularly. The insets show magnification of the selected area. (B) ApoER2 over-expression influences Reelin secretion, assessed by an antibody against the N-terminal. Immunoreactivity of full-length Reelin at 420 kDa and the predominant 180 kDa N-terminal fragment is shown, and expressed as percentage relative to immunoreactivity of the 420-kDa band from control cells. The data represent the means $\pm$ SEM (determinations by duplicate). *Significantly different ($p < 0.001$) from the control group transfected with an empty vector (-).

Figure 6. An ApoER2-ICD is identified in the nucleus of full-length ApoER2-EGFP over-expressing cells. (A) SH-SY5Y cells were transfected with ApoER2-EGFP. Endogenous and exogenous proteins were identified by blotting with anti C-terminal ApoER2 and anti GFP antibodies (note the shift due to the EGFP tag). (B) The 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. Treatment with DAPT results in an increase of ApoER2-CTF (closes arrow-head) in cell extracts, whereas the ApoER2-ICD band (open arrow-head) is more abundant when γ -secretase activity is not inhibited. (C) Confocal images of SH-SY5Y cells transfected with full-length ApoER2-EGFP in the presence of DAPT. Cells were incubated for 1 hour 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 to lamina. The insets show magnification of the selected area. Quantification of the ApoER2-EGFP fluorescence that co-localizes with Emerin is shown (for control n=166 cells, 2 independent experiments; for DAPT, n=131 cells, 2 independent experiments). $*p < 0.001$. **(D)** Nuclear fractions from SH-SY5Y cells transfected with full-length ApoER2-pEGFP or a pEGFP vector were analyzed by Western blotting with an anti-ApoER2 C-terminal antibody or anti-GFP. ApoER2-ICD was detected in the nuclear fraction of over-expressing cells. Acetylated histone H2B (AcH2B) and tubulin were used as markers for nuclear and cytoplasmic fractions respectively.

Figure 7. ApoER2 over-expression 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 using relative standard curves and normalized to GAPDH from the same cDNA preparations. Specificity of the PCR products was confirmed by dissociation curve analysis. The results were confirmed in two independent determinations. $*p < 0.01$. **(B)** SH-SY5Y cells were co-transfected with *RELN* reporter plasmid and either full-length ApoER2-EGFP (n= 12, 3 independent experiments) or ICD-HA (n= 8, 2 independent experiments). Empty vectors for each ApoER2 plasmid were used as controls. The activity was related to Renilla luciferase reporter. Over-expression of full-length ApoER2 and ApoER2-ICD diminishes the activity of the *RELN* promoter. $**p < 0.001$. **(C)** Scheme of the proximal region of the *RELN* promoter. The approximate positions of the known transcription factor binding sites are also indicated (CREB: ▲; SP1: Δ; N-Myc: ↑; Mzf1: ▲; Tbr1: ^), 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 control, followed by PCR amplification of various regions of the *RELN* promoter indicated in the schematic above. Unprecipitated chromatin preparations were similarly analyzed and used as “input” control.

Fig. 1

A **B** **C**

Fig. 2

Fig. 3

Fig. 4

A

B

Fig. 5

Fig. 6

A **B** **C** **Fig. 7**