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Decreased generation of C-terminal fragments of ApoER2 and increased reelin expression in Alzheimer's disease

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ABSTRACT: Increasing evidence indicates that altered reelin signaling could contribute to synaptic dysfunction in Alzheimer's disease (AD). We found that reelin protein and mRNA levels were increased in the AD brain (particularly at advanced Braak stages in apolipoprotein E4 noncarriers), compared with that of control subjects. The β-amyloid (Aβ) protein impairs reelin activity and increases reelin expression through a mechanism that is not yet understood. To explore that mechanism, we examined the effect of Aβ aa 1–42 ($A\beta_{42}$) on DNA methylation of the *RELN* promoter and the processing of reelin receptor apolipoprotein E receptor 2 (ApoER2) in differentiated SH-SY5Y cells because ApoER2 C-terminal fragments (CTFs), generated after reelin binding, regulate reelin expression. We found that $A\beta_{42}$ decreased nuclear levels of DNA-methyltransferase 1. However, *RELN* promoter methylation did not change in $A\beta_{42}$-treated cells or in AD brain extracts. Instead, the levels of ApoER2-CTF appeared significantly lower in $A\beta_{42}$-treated cells and in AD extracts from advanced Braak stages of apolipoprotein E4 noncarriers. Our data show that ApoER2-CTF levels are decreased, whereas reelin expression is increased in AD brain at advanced Braak stages and after Aβ treatment, supporting the view that ApoER2-CTF exerts a modulatory role on reelin expression.—Mata-Balaguer, T., Cuchillo-Ibañez, I., Calero, M., Ferrer, I., Sáez-Valero, J. Decreased generation of C-terminal fragments of ApoER2 and increased reelin expression in Alzheimer's disease. FASEB J. 32, 3536–3546 (2018). www.fasebj.org

KEY WORDS: brain · ApoE · DNMT1 · methylation

Alzheimer's disease (AD) is characterized by the presence of extracellular amyloid plaques, comprising the β-amyloid protein (Aβ) and intracellular neurofibrillary tangles, which contain the abnormally hyperphosphorylated, microtubule-associated protein τ (1). It is generally accepted that Aβ and hyperphosphorylated τ are pathologic effectors of AD. Therefore, pathways that facilitate the interaction between these proteins are of particular interest, both for deciphering the pathologic mechanisms involved in AD causation and for the design of successful therapeutic interventions.

Reelin is a large, signaling glycoprotein, which has been implicated in the regulation of synaptic neurotransmission, plasticity, and memory in the adult brain (2). Secreted reelin transduces intracellular signals through the disabled-1 (Dab1) adapter by binding to the apolipoprotein E receptor 2 (ApoER2) or the very low-density lipoprotein receptor (LDLR) (3). Dab1 phosphorylation initiates an intracellular kinase cascade that ultimately inhibits glycogen synthase kinase-3β (GSK3β) and prevents τ hyperphosphorylation (4). Reelin, ApoER2, and Dab1 all interact with the β-amyloid precursor protein (APP) (5–10). In addition, genetic risk factors for late-onset AD, such as apolipoprotein E (ApoE) and apolipoprotein J/clusterin, are competitors for binding to

ABBREVIATIONS: Aβ, β-amyloid; $A\beta_{42}$, β-amyloid aa 1–42; Aβsc, β-amyloid scrambled control; AD, Alzheimer's disease; ApoE, apolipoprotein E; ApoER2, apolipoprotein E receptor 2; ApoER2-CTF, ApoER2 C-terminal fragment; ApoER2-ICD, ApoER2 intracytoplasmic domain; APP, amyloid β precursor protein; CpG, cytosine-phosphate-guanine; CSF, cerebrospinal fluid; CTF, C-terminal fragment; Dab1, disabled-1; DNMT, DNA-methyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GSK3β, glycogen synthase kinase-3β; HEK, human embryonic kidney; ICD, intracytoplasmic domain; LDLR, low-density lipoprotein receptor; ND, nondemented; qRT-PCR, quantitative RT-PCR; Reln, reelin

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reelin receptors (3, 11, 12). ApoE is a major constituent of very LDL, and it binds to all members of the LDLR family (13). Three major isoforms, ApoE2, ApoE3, and ApoE4, are encoded by different alleles of human *APOE* gene, with ApoE3 being the most common isoform. Interestingly, the *APOE*-ε4 allele (encoding ApoE4), the major susceptibility genetic factor for AD (14), is one of the strongest competitors for reelin binding to ApoER2 (3).

Recently, we demonstrated that Aβ increases reelin levels (15) and also disrupts reelin capacity to form homodimers, which are necessary to bind to reelin receptors, thereby compromising reelin signal transduction and, possibly, contributing to synaptic dysfunction in AD (16). Thus, altered reelin glycoforms induced by Aβ are less capable of down-regulating τ phosphorylation *via* Dab1 and GSK3β signaling (16). Furthermore, the direct interaction between reelin and Aβ precludes the proteolytic processing of ApoER2 induced after reelin binding in nonpathologic conditions, possibly because of interference in the initiation of the intracellular signaling (17). However, the mechanism by which Aβ triggers the increase in reelin levels is not yet understood.

In the present study, we have examined the effect of Aβ aa 1–42 ($A\beta_{42}$) on cytosine methylation of the reelin promoter and on the levels of DNA-methyltransferases (DNMT1, 3a and 3b), enzymes that modulate the methylation of the *RELN* promoter, influencing reelin expression (18). We also examined the effect of $A\beta_{42}$ on ApoER2 proteolytic processing because the intracellular ApoER2 C-terminal fragments (ApoER2-CTF) generated after reelin binding have a key role in regulating reelin transcription (19). We extended our analysis on brains from patients with AD to considering the patient Braak stage and the *APOE* genotype.

MATERIALS AND METHODS

Collection of human brains

This study was approved by the ethics committee of Universidad Miguel Hernández de Elche (Alicante, Spain) and was performed in accordance with the World Medical Association (WMA) Declaration of Helsinki. Brain samples (frontal cortex) were obtained from the Brain Bank at the Institute of Neuropathology, Bellvitge University Hospital (Barcelona, Spain). Late-onset AD cases (12 women, 18 men; mean ± SD age, 75 ± 10 yr) were categorized according to the Braak stage of neurofibrillary tangle pathology (20): Braak stage I–II, $n = 10$; Braak stage III–IV, $n = 10$; and Braak stage V–VI, $n = 10$. Special care was taken not to include cases with combined pathologies to avoid bias in the pathologic series. Samples from nondemented (ND) controls (2 women, 6 men; mean ± SD age, 54 ± 4 yr) corresponded to individuals with no clinical dementia and no evidence of brain pathology. The mean postmortem interval of the tissue was ~8 h in all cases, with no significant difference between the groups.

Protein extraction from human brain

Frontal cortex (0.1 g) was homogenized (10% w/v) in Tris-HCl (50 mM, pH 7.4), NaCl (150 mM), Triton X-100 (0.5% w/v), Nonidet P-40 (0.5% w/v), 0.5 mM fresh PMSF, and a cocktail of protease inhibitors (1:25 v/v; MilliporeSigma, Billerica, MA, USA), for solubilization of reelin, or in Tris-HCl (50 mM, pH 7.4), NaCl (150 mM), EDTA (5 mM), Triton X-100 (0.5% w/v), Nonidet P-40 (1% w/v), fresh 0.5 mM PMSF, and the cocktail of protease inhibitors for solubilization of the transmembrane reelin receptor, ApoER2, and other membrane-associated proteins. The homogenates were then sonicated (15 pulses × 2, on ice, at intensity 4, Ultrasonic Cell Disruptor; Misonix, Farmingdale, NY, USA), centrifuged for 20 min at 20,000 *g* and 4°C, and the supernatant fractions were recovered for further analysis by Western blotting.

Cell culture

SH-SY5Y cells were seeded at a density of 1×10^5 cells/well in 6-well plates and cultured in DMEM supplemented with Glutamax (GIBCO; Thermo Fisher Scientific, Waltham, MA, USA), 1% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 incubator. Cells were differentiated with all-*trans*-retinoic acid (MilliporeSigma), which was added the day after plating, at a final concentration of 10 μM in DMEM with 1% fetal bovine serum. The medium was changed every 2 d. After 6 d in the presence of all-*trans*-retinoic acid, cells were treated with amyloid proteins. Suspensions of $A\beta_{42}$ or β-amyloid scrambled control (Aβsc) peptide (AIAEGDSHVLKEGAYMEIFDVQGHVFGGKIFRVVDLGSHNVA) (both from American Peptide Co., Sunnyvale, CA, USA) were dissolved in sterilized, distilled water at a concentration of 1 mg/ml and added to the cells to yield a final concentration of 5 μM; moreover, that treatment was performed 2 d consecutively, without changing the medium. The culture medium was collected, and the cells were suspended in 120 μl/well of solubilization buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% (w/v) Triton X-100, 1% (w/v) Nonidet P-40, 0.5 mM fresh PMSF, and cocktail of protease inhibitors] and were processed as described for the cortical extracts. For measurement of cell viability, SH-SY5Y cells were cultured in 96-well plates, then differentiated and assayed using the tetrazolium assay (MTS assay; CellTiter 96 Aqueous Assay; Promega, Madison, WI, USA), according to the manufacturer's instructions.

For studies involving the characterization of ApoER2-CTF, differentiated SH-SY5Y cells were treated with reelin (~10 nM) obtained from conditioned medium of human embryonic kidney (HEK)-293T cells stably-transfected with reelin cDNA or green fluorescent protein (GFP) cDNA (mock), as described (17).

For studies involving the transcriptional activity of ApoER2, SH-SY5Y cells were transfected with Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's protocol, for 48 h using cDNA of human full-length ApoER2 (provided by Dr. J. Nimpf, Max F. Perutz Laboratories, Department of Medical Biochemistry, Medical University of Vienna, Vienna, Austria; see ref. 19), or a chimeric ApoER2 protein expressing only the intracytoplasmic domain (ICD: aa residues 728–842) (ApoER2-ICD; provided by Dr. W. Rebeck, Georgetown University Medical Center, Washington, DC, USA), or an empty vector (Promega) as control.

The effect of $A\beta_{42}$ on cellular reelin levels and ApoER2 processing was also tested on primary cortical neuron cultures, performed as previously described (16). Briefly, neurons from cortical lobes of E17.5 mice embryos were plated in 6-well plates (2×10^6 cells/dish) and maintained in Neurobasal medium (Thermo Fisher Scientific) containing B27 supplements (GIBCO; Thermo Fisher Scientific), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. After 7 d, cortical neurons were treated with 5 μM $A\beta_{42}$ or 5 μM Aβsc for 2 d consecutively, following the same schedule as that for SH-SY5Y cells. The cells were suspended in 120 μl/well of solubilization buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% (w/v) Triton X-100, 1% (w/v) Nonidet P-40, 0.5 mM fresh PMSF, and a cocktail of protease inhibitors] and processed as previously described.

Extraction of nuclear proteins

Cultured SH-SY5Y cells were washed twice with cold PBS and gently scraped off the plates with cold PBS. Cell debris were discarded by centrifugation for 5 min at 500 *g* and 4°C. Cells were then lysed, and nuclei were isolated with the QProteome Nuclear Protein Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Western blotting

For western blotting, brain or SH-SY5Y cell extracts (each sample containing 40 μg protein) were heated at 98°C for 7 min or for 3 min exclusively for reelin denaturation (21), in 6× Laemmli sample buffer. After SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose membranes and detected with antibodies against reelin (clone142, 1:400; MilliporeSigma), ApoER2 C-terminal (1:2000; Abcam, Cambridge, United Kingdom), Dab1 (1:1000; Abcam), 4G10 platinum anti-phosphotyrosine antibody (1:1000; MilliporeSigma), ErbB4 C-terminal (1:400; Santa Cruz Biotechnology, Dallas, TX, USA), DNMT1 (1:1000; Abcam), DNMT3a (1:2000; Abcam), DNMT3b (1:500; Abcam. α-Tubulin (1:4000; MilliporeSigma), or Lamin B2 (1:200; Thermo Fisher Scientific) were used as a loading control. To improve detection of ApoER2-CTF by the ApoER2 C-terminal antibody, the membranes were boiled in PBS in a microwave at maximum power for 5 min before the blocking step. Primary antibody binding was visualized with fluorescently (IRDye) labeled secondary antibodies (1:10,000), and images were acquired with an Odyssey CL× Infrared Imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

Quantitative RT-PCR analysis and *APOE* genotyping

RNA was extracted from human brains and differentiated SH-SY5Y cells with Trizol reagent in the PureLink Microto-Midi Total RNA purification system (Thermo Fisher Scientific), following the manufacturer's instructions. SuperScript III reverse transcriptase (Thermo Fisher Scientific) was used to synthesize cDNAs from that total RNA (2 μg) with random primers, according to the manufacturer's instructions. Quantitative PCR amplification was performed on a StepOne Real-Time PCR System (Thermo Fisher Scientific) with TaqMan probes specific for human *RELN* (HS01022646_m1; Thermo Fisher Scientific), human *ApoER2* (HS00182998_m1; Thermo Fisher Scientific), and human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; HS02758991_g1; Thermo Fisher Scientific) as an endogenous control. The transcripts were quantified using the relative standard-curve method normalized to GAPDH from the same cDNA preparation to confirm the specificity of the PCR products from the dissociation curves.

Genomic DNA was isolated from brain tissue by with the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions. *APOE* polymorphisms (rs429358 and rs7412), which determine the ε2/ε3/ε4 haplotypes, were analyzed by real-time PCR using specific oligonucleotides, according to a previously described method (22). Details of *APOE* genotype and the age and gender for individual patients with AD and ND subjects are shown in Supplemental Table 1.

Methylation-specific PCR and sequencing

Genomic DNA was extracted from human brain and differentiated SH-SY5Y cells with DNeasy Blood and Tissue kit. Bisulfite conversion of unmethylated cytosine was performed with the EZ DNA methylation Gold kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions.

The sequences of the PCR primers used to distinguish between the methylated and unmethylated genomic DNA in the *RELN* promoter were the following: methylated forward primer: 5′-CGGGGTTTTGACGTTTTTCG-3′ (−602 to −582), methylated reverse primer: 5′-CGCCCTCTCGAACTAACTCGACG-3′ (−418 to −441), unmethylated forward primer: 5′-GTGGGGTTTTGATGTTTTTTG-3′ (−603 to −582), and methylated reverse primer: 5′-CACCCTCTCAAACTAACTCAACA-3′ (−418 to −441).

The primers used for the amplification of the *RELN* promoter region from the bisulfite-modified genomic DNA were as follows: forward primer: 5′-GTATTTTTTTAGGAAAAATAGGGTATATTGA-3′ (−687 to −656), and reverse primer: 5′-ACTCCCAAAATTACTTTAAACC-3′ (−180 to −202).

The PCR reaction was performed with 50 ng bisulfite-modified genomic DNA and 2.5 U of platinum Taq DNA polymerase (Thermo Fisher Scientific). Water was used as a negative control and a fully cytosine-phosphate-guanine (CpG) methylated human genomic DNA (EpiGentek, Farmingdale, NY, USA) was used as a positive control. Each sample was run in triplicate.

Statistical analysis

The distribution of data was tested for normality using a D'Agostino-Pearson test. Data were analyzed by unpaired Student's *t* test, applying a Welsh's correction when variances were not equal, or ANOVA, with a Dunnett's multiple comparison test. When normality was rejected, a Mann-Whitney *U* test was used. The results are presented as the means ± SEM, and all the analyses were performed using GraphPad Prism (v.5; GraphPad Software, La Jolla, CA, USA). A value of $P < 0.05$ was considered significant.

RESULTS

Increased reelin expression in the brain of patients with AD and in $A\beta_{42}$-treated cells

Reelin expression in the frontal cortex of patients with AD and ND subjects was evaluated by quantitative RT-PCR (qRT-PCR). We found that reelin mRNA levels were statistically increased in patients with AD, compared with ND subjects ($P = 0.0003$) (**Fig. 1*A***). When patients with AD were classified by their Braak stage, we found that, at Braak stage III–IV ($P = 0.01$) and Braak stage V–VI ($P = 0.003$), reelin expression was significantly greater than it was for ND subjects, but was not so at Braak stages I–II. This result corroborates previous analyses obtained from various cohorts (15, 17, 21). Interestingly, when the *APOE* genotype was considered within the group with AD and Braak stages III–VI, only ApoE4 noncarriers (ε2/ε3 and ε3/ε3; $n = 14$; $P = 0.0003$) displayed increased reelin mRNA levels with respect to controls (Fig. 1*A*), although ApoE4 carriers (ε3/ε4 and ε4/ε4, $n = 6$; $P = 0.4$) did not exhibit significant increases in reelin mRNA levels compared with controls. Indeed, patients with AD who were ApoE4 carriers exhibited significantly lower reelin mRNA levels compared with ApoE4 noncarriers (30% decrease; $P = 0.026$). The differences in age between patients with AD and ND subjects did not account for the differences observed in the reelin mRNA levels because the correlation between age and reelin mRNA levels was not significantly different in the ND group ($R^2 = 0.075$; $P = 0.5$), for all patients with AD ($R^2 = 0.002$; $P = 0.8$), for those with Braak

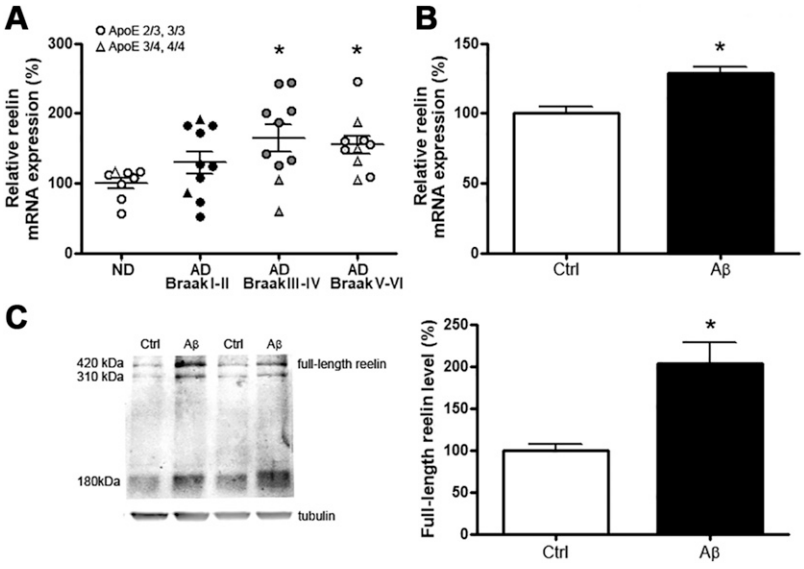

Figure 1. Increased reelin expression in AD brain at various Braak stages and in SH-SY5Y cells after $A\beta_{42}$ treatment. *A*) Relative reelin mRNA expression analyzed by qRT-PCR in frontal cortex samples from ND subjects (n = 8) and patients with AD (Braak stage I–II, n = 10; Braak stage III–IV, n = 10; and Braak stage V–VI, n = 10). ○, ApoE4 noncarrier (ApoE2/3 and 3/3) subjects; Δ, ApoE4 carrier (ApoE 3/4 and 4/4) subjects. The values were calculated from relative standard curves and normalized to *GAPDH* from the same cDNA preparation and are expressed as means ± SEM. $^*P < 0.05$ with respect to ND; 1-way ANOVA plus Dunnett's test. *B*) Relative reelin mRNA expression analyzed by qRT-PCR in differentiated SH-SY5Y cells treated with Aβsc control (ctrl; n = 6 from 2 independent experiments) or $A\beta_{42}$ (n = 6 from 2 independent experiments). The values were calculated from relative standard curves and normalized to *GAPDH* from the same cDNA preparation and are expressed as means ± SEM. $^*P < 0.01$, Mann-Whitney *U* test. *C*) Western blots of differentiated SH-SY5Y cell extracts, after treatment with 5 μM Aβsc (ctrl; n = 6 from 2 independent experiments) or 5 μM $A\beta_{42}$ (n = 8 from 2 independent experiments) and probed for reelin (with a N-terminal antibody that detects 420-kDa full-length reelin and 310 and 180 kDa N-terminal fragments) and tubulin. Data from quantification of full-length reelin were normalized to tubulin and are expressed as means ± SEM. $^*P < 0.01$, Mann-Whitney *U* test.

III–VI ($R^2 = 0.0006$; $P = 0.7$), or for ApoE4–only noncarriers ($R^2 = 0.02$; $P = 0.6$).

In previous studies, performed in differentiated SH-SY5Y cells (cells expressing all reelin signaling components), treatment with 2 doses of 10 μM $A\beta_{42}$ triggered increases in reelin protein and mRNA levels (15, 17). In the present study, we used 2 × 5 μM $A\beta_{42}$ doses, and the results were similar to the previous studies, resulting in an increase in reelin mRNA (Fig. 1*B*; $P = 0.008$) and protein levels, relative to cells treated with Aβsc peptide (Fig. 1*C*; $P = 0.008$). The cell death in cultures treated with 2 × 5 μM $A\beta_{42}$ doses, evaluated with an MTS assay, was not significant (6.8 ± 5.4% reduction in cell viability compared with cells treated with Aβsc peptide, $P = 0.2$).

Analysis of *RELN* promoter methylation and DNMT1 in SH-SY5Y cells and AD brain

DNA methylation has a critical role in the regulation of gene expression. In the mammalian brain, promoter methylation on CpG islands is a postreplicative process mediated by a group of DNMTs, such as DNMT1, DNMT3a, and DNMT3b (23). The *RELN* promoter is potentially modifiable by the 3 DNMTs (24), but in particular, DNMT1 has been related to the down-regulation of reelin expression and hypermethylation of *RELN* promoter in several neuropsychiatric disorders (25, 26). Therefore, we examined whether 5 μM $A\beta_{42}$ would be able to modulate DNMT1, DNMT3a, and DNMT3b levels and to alter the methylation pattern of *RELN* promoter in differentiated SH-SY5Y cells. We found that levels of DNMT1 were lower in the nuclear fraction in $A\beta_{42}$-treated cells compared with control Aβsc-treated cells (P = 0.002), whereas the levels of DNMT isoforms 3A ($P = 0.2$) and 3B ($P = 0.4$) remained unchanged (**Fig. 2*A***).

We further employed methylation-specific PCR to distinguish methylated from unmethylated DNA after bisulfite modification, taking advantage of the differences in the nucleotide sequence that resulted after that treatment. We analyzed the bisulfite-modified *RELN* promoter (region −602 to −418) in SH-SY5Y cells treated with $A\beta_{42}$. However, only the specific primer for unmethylated DNA amplified the *RELN* promoter, indicating that there was no change in the methylation in this region (Fig. 2*B*). The data suggest that the decrease in DNMT1 levels induced by $A\beta_{42}$ does not influence *RELN* promoter methylation. Sequencing of the *RELN* promoter region between bases −687 and −202 confirmed that there was no change in the methylation pattern of the bisulfite-modified DNA extracted from cells treated with $A\beta_{42}$ compared with those cells treated with Aβsc (Fig. 2*C*).

Samples of the frontal cortex of patients with AD and ND subjects were also analyzed by methylation-specific PCR and sequencing (same promoter region as that for SH-SY5Y cell sequencing). Again, no differences in the methylation pattern were found between patients with AD and ND subjects in the *RELN* promoter after bisulfite modification (Fig. 2*B*, *C*). Overall, the data support the view that the up-regulation of reelin expression in AD cortex and in $A\beta_{42}$-treated cells is not a consequence of hypomethylation of the *RELN* promoter.

Levels of CTFs of ApoER2 in $A\beta_{42}$-treated cells and in the brain of patients with AD

The possibility that $A\beta_{42}$ may influence reelin expression by affecting the proteolysis of ApoER2 was also investigated. The binding of reelin to ApoER2 is known to induce the clustering and subsequent cleavage of ApoER2 by α- and γ-secretases (27, 28). The processing by α-secretase generates membrane-bound CTFs, which can be further cleaved by γ-secretase to generate soluble ICD

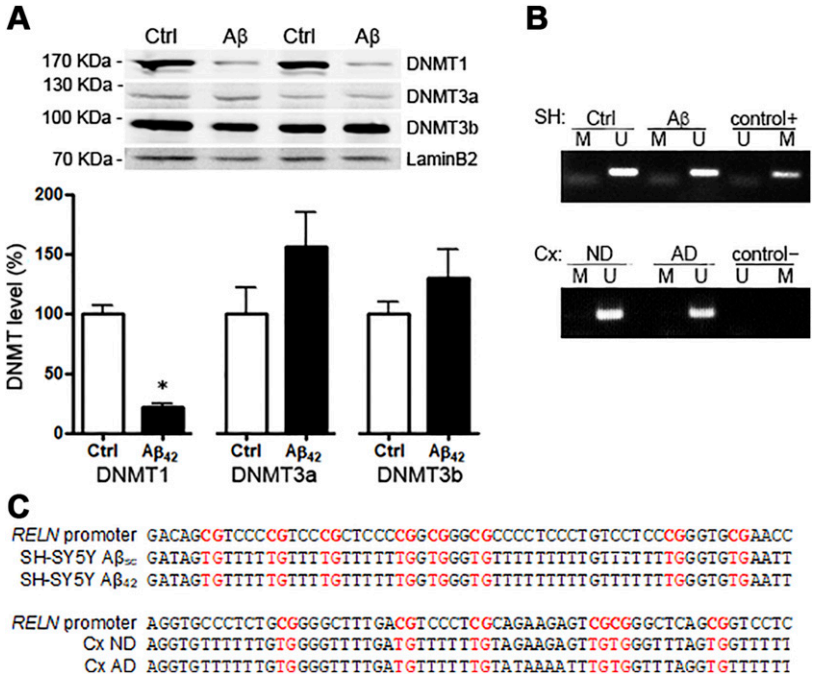

Figure 2. Aβ increases nuclear levels of DNMT1, but does not alter methylation of the reelin promoter. *A*) Western blots of nuclear extracts of differentiated SH-SY5Y cells, after treatment with control (ctrl) 5 μM Aβsc (n = 6) or 5 μM $A\beta_{42}$ (n = 6) stained for DNMT1, DNMT3a, and DNMT3b. DNMT levels were quantified by image analysis, and the results were normalized to Lamin B2 levels and are expressed as means ± SEM. *$P < 0.05$, Student's t test. *B*) Amplification of bisulfite-modified reelin promoter (−602 to −418) from cells treated with 5 μM Aβsc (ctrl) or 5 μM $A\beta_{42}$, and from frontal cortex extracts from ND subjects and patients with AD, using a specific primer for unmethylated DNA (U) or for methylated DNA (M). *In vitro* methylated DNA was the positive control (control +), and a saline buffer was the negative control (control −). *C*) Reelin promoter regions before and after bisulfite modification, from cells treated with 5 μM Aβsc or 5 μM $A\beta_{42}$; and frontal cortex extracts of ND subjects and patients with AD. The same promoter region was sequenced in SH-SY5Y cells and brain extracts [−687 to −202; see Abdolmaleky *et al.* (56)]; however, 2 different enriched-CpG regions are shown to demonstrate the absence of changes in the methylation patterns after bisulfite modification along the promoter. The first line (*RELN* promoter) shows the sequence before bisulfite modification.

fragments. The ApoER2-ICD is able to regulate reelin expression through a down-regulation of reelin mRNA transcription (17). Given that endogenous ApoER2-ICD is hardly detectable, probably because of its low levels and inherent instability (24), we decided to determine the levels of ApoER2-CTF as an indicator of the interaction of reelin and ApoER2. To confirm that, we first examined whether reelin induces the generation of ApoER2-CTF. Reelin treatment of differentiated SH-SY5Y cells increased the levels of that fragment with respect to mock-treated cells, probably because of changes in the processing of ApoER2 after reelin binding to the receptor (**Fig. 3*A***; P = 0.039). Then, we found that treatment with 5 μM $A\beta_{42}$ reduced the levels of ApoER2-CTF compared with cells treated with the Aβsc peptide (Fig. 3*B*; P = 0.0001). However, the level of the full-length ApoER2 protein (Fig. 3*C*; P = 0.7) and its transcript quantified by qRT-PCR (Fig. 3*D*; P = 0.7) did not change following Aβ treatment, indicating that the reduction in ApoER2-CTF was probably due to decreased processing of full-length ApoER2 and not to a decrease in the levels of the receptor.

Because $A\beta_{42}$ treatment can induce an increase in the cellular content of the γ-secretase catalytic subunit presenilin 1 (29), we also examined whether the reduction in ApoER2-CTF resulted from an increase in γ-secretase activity. Therefore, we examined the levels of the full-length ErbB4 and its CTFs. ErbB4 is a single-pass, type I transmembrane receptor, a member of the epidermal growth factor receptor subfamily that binds to neuregulins, and it is also a γ-secretase substrate (30). Our data indicate that the levels of ErbB4-CTF remain unaltered, suggesting that $A\beta_{42}$ treatment does not alter the γ-secretase processing of ErbB4 (Supplemental Fig. 1).

After binding to its receptor, intracellular transduction of reelin signaling begins with the phosphorylation of intracellular adaptor protein Dab1 (3). Thus, we first validated the reelin-dependent induction of Dab1 phosphorylation in differentiated SH-SY5Y cells (Fig. 3*E*; P = 0.028). To corroborate the effect of $A\beta_{42}$ on reelin signaling, we also analyzed the phosphorylation of Dab1. In cells treated with $A\beta_{42}$, we found that the tyrosine phosphorylation of Dab1 decreased (Fig. 3*F*; P = 0.035) after treatment, compared with control cells, which is in accordance with a lower activation of reelin signaling through ApoER2 in the presence of $A\beta_{42}$.

We confirmed the influence of $A\beta_{42}$ on the generation of ApoER2-CTF in primary neuronal cultures. Neurons treated with 2 × 5 μM $A\beta_{42}$ doses displayed an increase in reelin protein levels relative to treated cells with Aβsc peptide (**Fig. 4*A***; P = 0.019); however, despite that increment, the presence of the $A\beta_{42}$ peptide lowered the levels of ApoER2-CTF (Fig. 4*B*; P = 0.035), whereas full-length ApoER2 levels remained unchanged (Fig. 4*C*; P = 0.39).

We next analyzed ApoER2-CTF levels in brain extracts from patients with AD and ND subjects. The amount of ApoER2-CTF was significantly lower in the AD cortex compared with ND cortex (**Fig. 5*A***, P = 0.021). When patients with AD were classified by their Braak stage, we found that, at Braak stages III–IV (P = 0.046) and V–VI (P = 0.020), the levels of ApoER2-CTF were significantly reduced compared with ND samples, but not at Braak stage I–II (P = 0.055). Similar to reelin expression, the differences were only observed when comparing ND with AD extracts from ApoE4 noncarriers (P = 0.019) but not from ApoE4 carriers (P = 0.28). No significant differences were found between patients with AD and ND subjects in the amount of full-length ApoER2 protein (Fig. 5*B*; P = 0.87) or in the level of ApoER2 mRNA (either when all the AD samples were compared with the ND

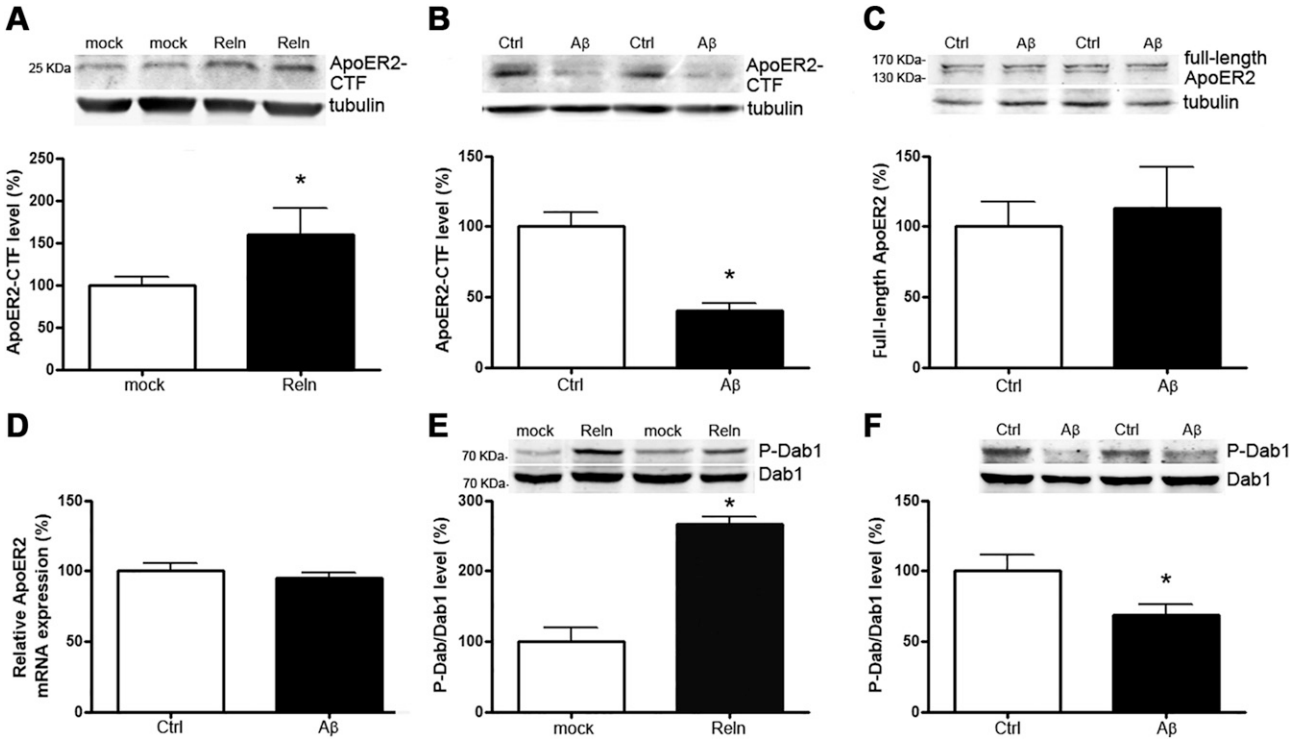

Figure 3. Aβ treatment decreases ApoER2-CTF generation and alters Dab1 phosphorylation. *A*) Western blots of SH-SY5Y cell extracts after treatment for 45 min with ~10 nM reelin (Reln) from conditioned medium from reelin stably transfected HEK-293T cells (n = 9 from 2 independent experiments) or conditioned medium from GFP stably transfected HEK-293T cells (mock; n = 6 from 2 independent experiments) and probed for ApoER2 C-terminal and tubulin. Data from quantification of the 25 kDa ApoER2-CTF band were normalized to tubulin and are expressed as means ± SEM. $^*P < 0.05$, Student's t test. *B*) Western blots of SH-SY5Y cell extracts after treatment with control 5 μM Aβsc (ctrl; n = 12 from 3 independent experiments) or 5 μM $A\beta_{42}$ (n = 12 from 3 independent experiments) and probed for ApoER2 C-terminal and tubulin immunoreactivity. Data obtained after quantification of the 25 kDa ApoER2-CTF band intensity were normalized to tubulin staining intensity, and the resulting normalized values expressed as means ± SEM. $^*P < 0.0001$, Student's t test. *C*) Western blots of SH-SY5Y cell extracts, after treatment with control (ctrl) 5 μM Aβsc (n = 12 from 3 independent experiments) or 5 μM $A\beta_{42}$ (n = 12 from 3 independent experiments) and probed for ApoER2 C-terminal and tubulin immunoreactivity. Data obtained after quantification of the intensity of 2 ApoER2 bands between 170 and 130 kDa were normalized to tubulin staining intensity and the resulting normalized values are expressed as means ± SEM. $P > 0.05$, Student's t test. *D*) Relative ApoER2 mRNA expression analyzed by qRT-PCR from differentiated SH-SY5Y cells treated with scrambled Aβsc (ctrl, n = 12 from 3 independent experiments) or $A\beta_{42}$ (n = 12 from 3 independent experiments). The values were calculated from relative standard curves, normalized to GAPDH from the same cDNA preparation, and expressed as means ± SEM. $P > 0.05$, Student's t test. *E*) Western blots of SH-SY5Y cell extracts after treatment with ~10 nM reelin (Reln; n = 6) or conditioned medium as in *A* (mock; n = 6), probed for Dab1 and with an anti-phosphotyrosine antibody (P-Dab1). Data obtained after quantification of the intensity were expressed as means ± SEM. $^*P < 0.05$, Mann-Whitney U test. *F*) Western blots of SH-SY5Y cell extracts, after treatment with control 5 μM Aβsc (n = 12 from 2 independent experiments) or 5 μM $A\beta_{42}$ (n = 12 from 3 independent experiments), were probed for Dab1 and with an anti-phosphotyrosine antibody (P-Dab1). Data obtained after quantification of the intensity are expressed as means ± SEM. $^*P < 0.05$, Mann-Whitney test.

samples or when specific Braak stages were compared separately) (Fig. 5C).

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

Overexpression of full-length ApoER2 in SH-SY5Y cells leads to an increase in ApoER2-CTF (15) and a decrease in reelin mRNA, and it is also evident when cells overexpressing ApoER2 are treated with recombinant reelin (**Fig. 6*A***; P = 0.006). When the level of transcripts were analyzed in SH-SY5Y cells overexpressing a chimeric ApoER2-ICD (aa residues 728–842), we confirmed that reelin expression was reduced by a small (10%) but significant extent compared with controls (Fig. 6*B*; P = 0.038).

DISCUSSION

Our results suggest that an abnormal intracellular transduction of the reelin signal, induced by Aβ, is related with increased reelin expression and that this impaired signaling may underlie some of the changes that occur in AD. A previous study demonstrated that the generation of CTFs of ApoER2 (ApoER2-ICD), associated with binding of reelin to its receptor and processing by secretases, can regulate reelin transcription (19). In the frontal cortex of

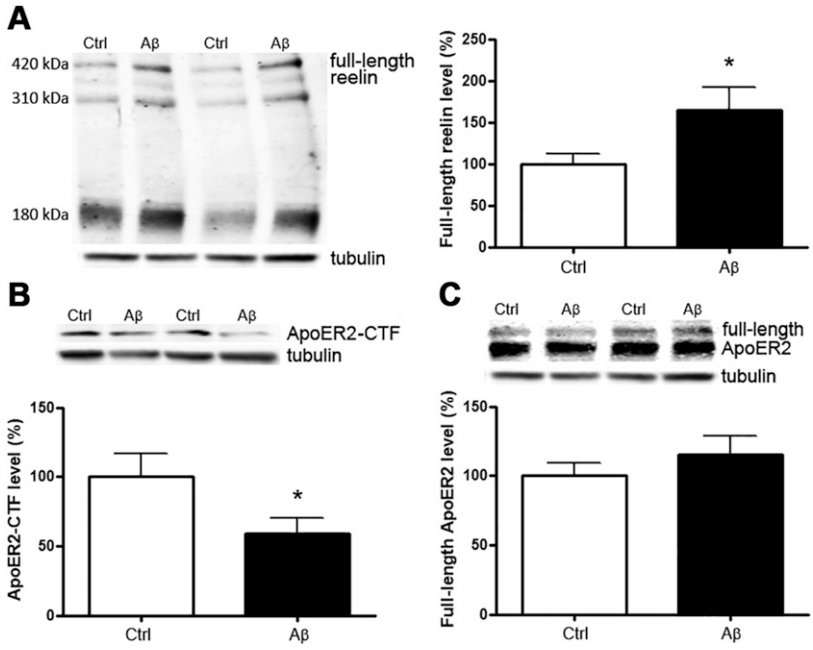

Figure 4. Decreased generation of ApoER2-CTF in Aβ-treated neurons. Western blots of primary neuronal cultures after treatment with control (ctrl) 5 μM Aβsc (n = 8 from 2 independent experiments) or 5 μM $A\beta_{42}$ (n = 8 from 2 independent experiments), probed for reelin (A), ApoER2 C-terminal (B, C), and tubulin immunoreactivity. Data obtained after quantification of the immunoreactivity intensity were normalized to tubulin staining intensity, and the resulting normalized values are expressed as means ± SEM. *$P < 0.05$, Mann-Whitney U test.

control subjects increased levels of CTFs of ApoER2 correlated with less expression of reelin, whereas, in patients with AD, particularly those at late Braak stages, and in differentiated SH-SY5Y cells treated with $A\beta_{42}$, we found that there was decreased generation of ApoER2-CTF and a correspondingly higher level of reelin expression. Therefore, we postulate that the mechanism by which Aβ increases reelin expression is by inhibiting generation of ApoER2-ICD and that this inhibition consequently results in up-regulation of reelin expression. However, in the presence of Aβ, reelin, despite its high levels, is inefficient in activating signaling, even when the lower generation of ApoER2-CTF in the presence of Aβ maintains high reelin expression. Therefore, the reelin signaling and autoregulatory mechanisms may be ineffective in AD (see a graphical summary of our model in **Fig. 7**).

In the context of impaired reelin function, ApoE4 could contribute to exacerbate neuropathology. Both reelin and ApoE compete for binding to ApoER2, and previous results in cultured neurons indicated that ApoE4 was more effective in dampening reelin signaling than ApoE3 was (3). Thus, the possibility that ApoE4 exacerbates AD progression, as indicated in several studies (31, 32), by contributing to the impairment of reelin signaling, should be considered. Interestingly, reelin expression is less in AD brain samples from ApoE4 carriers than it is in samples

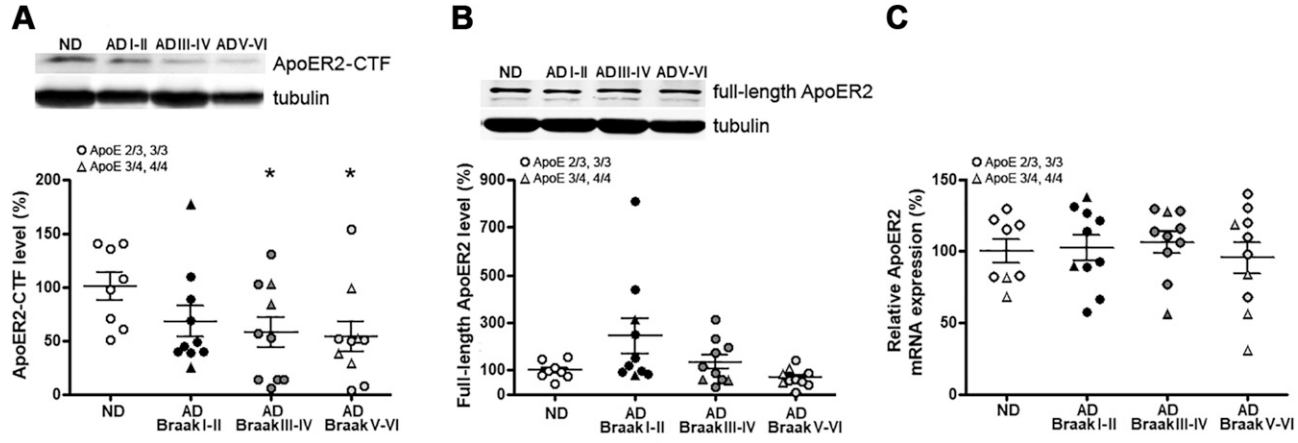

Figure 5. ApoER2-CTF expression is decreased in AD brain. A, B) Western blots of frontal cortex extracts from ND subjects (n = 8) and patients with AD (Braak stage I–II, n = 10; Braak stage III–IV, n = 10; Braak stage V–VI, n = 10) and probed for ApoER2 C-terminal and tubulin immunoreactivity. ○, ApoE4 noncarrier (ApoE2/3 and 3/3) subjects; Δ, ApoE4 carrier (ApoE 3/4 and 4/4) subjects. Data obtained after quantification of ApoER2-CTF (A) or full-length ApoER2 (B) bands were normalized to tubulin staining intensity, and the resulting normalized values are expressed as means ± SEM. *$P < 0.05$ with respect to ND; 1-way ANOVA plus Dunnett's test. C) Relative ApoER2 mRNA expression analyzed by qRT-PCR from frontal cortex samples from ND subjects (n = 8) and patients with AD (Braak stage I–II, n = 10; Braak stage III–IV, n = 10; and Braak stage V–VI, n = 10). The values were calculated from relative standard curves and normalized to GAPDH from the same cDNA preparation and are expressed as means ± SEM. *$P > 0.05$ respect to ND; 1-way ANOVA plus Dunnett's test.

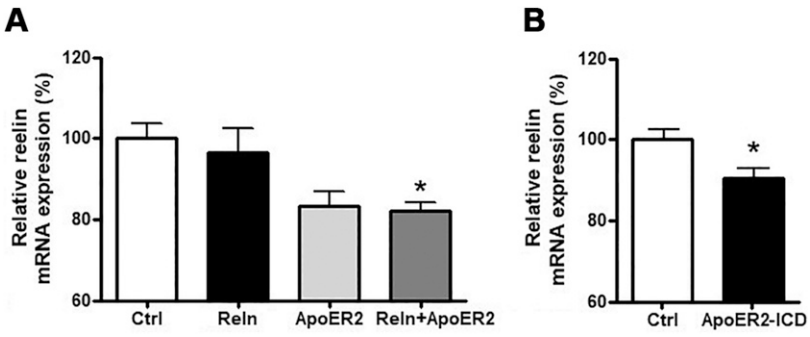

Figure 6. Overexpression of ApoER2-ICD increases reelin expression. *A*) Relative reelin mRNA expression, analyzed by qRT-PCR from SH-SY5Y cells after overexpressing for 48 h of either an empty vector or treatment for 45 min with mock-conditioned medium from GFP-stably transfected HEK-293T cells [control (ctrl)], either an empty vector and treatment for 45 min with ~10 nM reelin from conditioned medium from reelin stably transfected HEK-293T cells (Reln), either full-length ApoER2 or treatment with mock conditioned medium (ApoER2), either full-length ApoER2 or treatment for 45 min with ~10 nM recombinant reelin (ApoER2 + Reln). The values ($n = 7$ for each condition) were calculated from relative standard curves and normalized to GAPDH from the same cDNA preparation and are expressed as means ± SEM. $^*P < 0.05$ with respect to ctrl, 1-way ANOVA plus Dunnett's test. *B*) Relative expression of *RELN* by qRT-PCR from SH-SY5Y cells overexpressing an empty vector (ctrl, $n = 10$) or ApoER2-ICD ($n = 10$). The values were calculated from relative standard curves and normalized to *GAPDH* from the same cDNA preparation and are expressed as means ± SEM. $P < 0.05$ with respect to ctrl, Student's t test.

from ApoE4 noncarriers. In this study, AD-ApoE4 carriers did not display significant differences in reelin mRNA levels compared with ND controls, and they did not display differences in ApoER2-CTF levels. Our hypothesis is that ApoE4 carriers display differences in reelin/ApoE balance and that, influenced by Aβ, the regulatory mechanisms for reelin expression are affected during AD progression. The few ND-ApoE4 carriers in our cohort did not allow us to evaluate differences with ApoE4 noncarriers in nonpathologic conditions. Further characterization of the proteolytic processing of ApoER2 in the presence of various ApoE isoforms is yet to be conducted, but it has been shown that ApoER2 clustering and processing are less upon ApoE binding than they are upon reelin binding (33). Indeed, previous studies indicated that the ApoE isoforms promoted proteolysis of ApoER2 to different degrees, with ApoE2 resulting in a greater accumulation of the CTFs of ApoER2 compared with ApoE4 (28). Although other study indicated that ApoE can inhibit γ-secretase cleavage of ApoER2 preventing the release of CTFs of ApoER2 (34), in this context, expression of ApoER2 splicing variants is altered in the brains from patients with AD and in a transgenic mouse model of AD (35). Specific ApoER2 ligands may have a role in AD pathology. Along with ApoE4, apolipoprotein J/clusterin is a ligand of reelin receptors, which is a genetic risk factor for AD (12; reviewed in Yu and Tan 36).

The possibility that human ApoE isoforms have differential effects on brain function has been largely suspected (37), and some of these isoform-specific effects could be explained by differential interactions of isoforms with lipoprotein receptors (38). An isoform-specific role of ApoE4 in the localization and intracellular trafficking of ApoER2 (and, therefore, impairing in reelin activity) has been suggested (39). The observed effects of ApoE4 on the levels of ApoER2 probably act at a posttranscriptional level (40). An ApoE isoform–dependent effect on lipoprotein receptor (LRP1 and LDLR) shedding in the brain has been also demonstrated (41). In that regard, it has been proposed that an impairment in reelin/ApoE receptor–dependent neuromodulation may contribute to AD progression (42). That hypothetical model, in which the

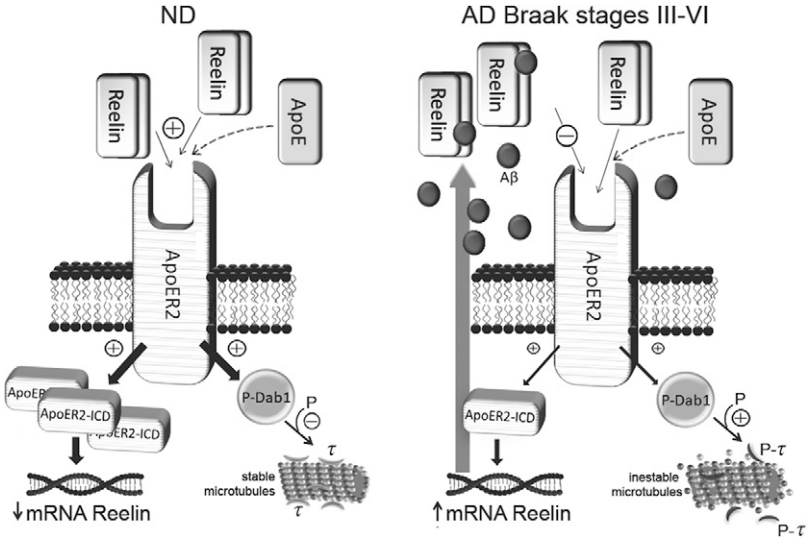

Figure 7. Schematic representation of reelin signaling in healthy brains and in brains in advances stages of AD. In the healthy condition, reelin binds to ApoER2 as a covalent homodimer. ApoE isoforms are competitors of that binding. Effective reelin binding to ApoER2 induces tyrosine phosphorylation of the intracellular adapter Dab1. Phosphorylated Dab1 (P-Dab1) activates a kinase cascade that ultimately inhibits GSK3β and prevents τ hyperphosphorylation. Reelin binding also induces clustering and further ApoER2 processing by secretases, generating intracellular ApoER2-ICD fragments, which can modulate reelin expression by inhibiting reelin transcription. In late Braak stages of AD, Aβ impairs reelin's ability to form active covalent homodimers. In that situation, reelin complexes display a weaker affinity for the ApoER2 receptor, and they fail to induce tyrosine phosphorylation of Dab1 and to transduce the signaling that modulates τ phosphorylation. The processing of ApoER2 after reelin binding is also decreased in AD, resulting in lower levels of ApoER2-ICD fragments. The failure of the ApoER2- ICD fragments to control reelin transcription results in an increase in reelin. A hypothesis that would explain the different contributions of ApoE isoforms to AD progression could be the diverse affinities of those isoforms for ApoER2 and, consequently, their different capacities to generate ApoER2 proteolysis.

promotion of memory dysfunction by ApoE4 is related with an impairment of ApoE receptor-dependent signaling pathways, is consistent with our present data.

Another consequence of the lesser reelin levels in AD-ApoE4 carriers is the difficulty of interpreting altered reelin expression in various AD cohorts if the *APOE* genotype is not investigated. Although we previously found increased reelin associated to AD in various cohorts, we also noted large intersubject variability in reelin protein and mRNA levels, and reports from other laboratories have been controversial regarding reelin changes in AD; now, in the light of our results, these differences could be explained by the presence or lack of the *APOE*-ε4 allele (discussed in Cuchillo-Ibañez *et al.* 43).

The level of full-length ApoER2 in Aβ-treated cells and in AD cortex was not significantly different from that of controls, indicating that the decrease in ApoER2-CTF was not a consequence of decreased expression of ApoER2. Instead, the decrease in ApoER2-CTF was likely due to altered proteolytic processing of ApoER2. Consistent with previous reports (17, 19), treatment of cells with recombinant reelin induced proteolytic cleavage of ApoER2 leading to an increase in ApoER2-CTF and decreased in reelin expression. In support of that view, overexpression of ApoER2-ICD was found to induce a discrete but significant decrease in reelin in SH-SY5Y cells. The specificity of the effect of Aβ on ApoER2-CTF generation is supported by the observation that the levels of another γ-secretase substrate, ErbB4, were not affected by Aβ.

In a previous study, we found that, in primary cortical neuron cultures, altered glycoforms of reelin affected its signaling pathway, including Dab1 phosphorylation (16), similar to our present finding in SH-SY5Y cells. In this study, we corroborated, in primary neuronal cultures, decreased generation of ApoER2-CTF in the presence of Aβ, despite reelin protein levels being increased, again, indicating impaired reelin signaling in an amyloid condition. We cannot discard the possibility that higher reelin levels in later-stage AD brains may be due to relative sparing of particular subtypes of interneurons (reviewed in 44, 45), such as the cells expressing reelin in the adult brain (46–48).

The capacity of Aβ to disturb the self-regulatory pathway that controls reelin levels is explained by the ability of Aβ to alter reelin glycosylation and formation of biologic-active reelin dimers, which, in turn, affect the capacity of reelin to bind to ApoER2 and its capacity to generate ApoER2 intracellular fragments that down-regulate reelin expression (16, 17). This interference by Aβ in reelin biologic function and in the autoregulatory control of reelin expression is likely to occur in the AD brain, in which reelin levels are greater than that found in ND subjects, particularly for those AD cases in stage III–VI. Soluble N-terminal fragments of ApoER2 resulting from proteolysis, which are also generated after binding of reelin, have been characterized in human cerebrospinal fluid (CSF) (17). These N-terminal ApoER2 fragments were found to be decreased in the CSF from patients with AD. We postulate that the quantification of soluble N-terminal ApoER2 fragments in CSF and intracellular C-terminal ApoER2 fragments in frontal cortex extracts could give a credible readout of reelin signaling impairment in patients with AD. Alterations in reelin levels can contribute to the progression of AD pathology (49) and probably affect the capacity of reelin to protect against Aβ toxicity (50, 51). The crosstalk between APP/Aβ and reelin has been shown by various groups, but the effect of Aβ on reelin function should be considered when interpreting changes in expression in AD (discussed in Cuchillo-Ibañez *et al.* 43).

In this study, we also explored the possibility that Aβ could affect reelin expression by affecting the methylation of the *RELN* promoter. DNA methylation is probably the most intensively studied mechanism of epigenetic regulation. Aberrant methylation of cytosine nucleotides can alter gene transcription and, therefore, potentially give rise to neurodegenerative and neuropsychiatric diseases (52–54), including AD (55). The possibility that hypermethylation of the *RELN* promoter contributes to the down-regulation of reelin has been suggested for neuropsychiatric disorders, such as schizophrenia, bipolar disorders, and other psychoses (25, 26, 56), as well for other pathologies, such as epilepsy (57) and hypothyroidism (58). However, hypermethylation of the *RELN* promoter in patients with neuropsychiatric disorders has not been not confirmed in all reports (59). Nevertheless, a relation between high expression of reelin and hypomethylation of the *RELN* promoter region in myeloma cell lines as well as in patients with cancer has been reported (60). Similarly, up-regulation of reelin expression in mouse colon in response to acute colitis has been associated with a decrease in DNMT1 and in *RELN* promoter methylation (61).

In our cell culture studies, $A\beta_{42}$ promoted a decrease in the nuclear levels of DNMT1, although DNMT3a and DNMT3b were unaffected. Cerebral levels of DNMT1 have been shown to be reduced in AD mouse models (62, 63). However, we did not observe a decrease in the methylation of the *RELN* promoter, either in frontal cortex from patients with AD or in our cell culture studies. Indeed, very little methylation was found in the promoter region assessed (region −687 to −202 bp). In another study, in which DNMT1 levels were modulated, the resulting epigenetic changes did not include hypermethylation of the *RELN* promoter (64). Nevertheless, the possibility of altered methylation in other regions of the *RELN* promoter cannot be dismissed (65). Furthermore, it has also been suggested that down-regulation of reelin in neuropsychiatric disorders may be mediated, at least in part, by a change in DNMT1 activity, which is independent of the enzyme's DNA methylation activity (66). Accordingly, cytosine methylation may not be the only mechanism by which DNMT1 regulates gene expression, and we cannot yet discount the possibility that the Aβ-mediated decrease in DNMT1 participates in the up-regulation of reelin.

In summary, our studies confirm that reelin expression is up-regulated in the brain of patients with AD. Aβ is able to trigger a decrease in nuclear DNMT1 levels. However, the methylation of the *RELN* promoter is not altered by Aβ. The reduced level of proteolytic fragments of ApoER2 in the AD brain compared with the ND brain suggests an inefficient interaction with ApoER2 and, therefore, a deficit in further processing. Because ApoER2-ICD exerts

transcriptional control on reelin expression, decreased production of ApoER2-ICD may be responsible for increased reelin expression. The possibility that reelin dysfunction and the alteration of ApoER2 processing ultimately influences synaptic function (67, 68) points to a role for these proteins in AD progression. FJ

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AUTHOR CONTRIBUTIONS

T. Mata-Balaguer performed research and analyzed data; I. Cuchillo-Ibañez performed research, analyzed data, and wrote the paper; M. Calero and I. Ferrer contributed new reagents or analytic tools; and J. Sáez-Valero designed the research and wrote the paper.

REFERENCES

1. Scheltens, P., Blennow, K., Breteler, M. M., de Strooper, B., Frisoni, G. B., Salloway, S., and Van der Flier, W. M. (2016) Alzheimer's disease. *Lancet* **388**, 505–517
2. Förster, E., Bock, H. H., Herz, J., Chai, X., Frotscher, M., and Zhao, S. (2010) Emerging topics in reelin function. *Eur. J. Neurosci.* **31**, 1511–1518
3. D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D. S., Sheldon, M., and Curran, T. (1999) Reelin is a ligand for lipoprotein receptors. *Neuron* **24**, 471–479
4. Beffert, U., Morfini, G., Bock, H. H., Reyna, H., Brady, S. T., and Herz, J. (2002) Reelin-mediated signaling locally regulates protein kinase B/Akt and glycogen synthase kinase 3β. *J. Biol. Chem.* **277**, 49958–49964
5. Trommsdorff, M., Borg, J. P., Margolis, B., and Herz, J. (1998) Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J. Biol. Chem.* **273**, 33556–33560
6. Howell, B. W., Lanier, L. M., Frank, R., Gertler, F. B., and Cooper, J. A. (1999) The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids. *Mol. Cell. Biol.* **19**, 5179–5188
7. Hoe, H. S., Tran, T. S., Matsuoka, Y., Howell, B. W., and Rebeck, G. W. (2006) DAB1 and reelin effects on amyloid precursor protein and ApoE receptor 2 trafficking and processing. *J. Biol. Chem.* **281**, 35176–35185
8. Fuentealba, R. A., Barría, M. I., Lee, J., Cam, J., Araya, C., Escudero, C. A., Inestrosa, N. C., Bronfman, F. C., Bu, G., and Marzolo, M. P. (2007) ApoER2 expression increases Aβ production while decreasing amyloid precursor protein (APP) endocytosis: possible role in the partitioning of APP into lipid rafts and in the regulation of gamma-secretase activity. *Mol. Neurodegener.* **2**, 14
9. Hoe, H. S., Lee, K. J., Carney, R. S., Lee, J., Markova, A., Lee, J. Y., Howell, B. W., Hyman, B. T., Pak, D. T., Bu, G., and Rebeck, G. W. (2009) Interaction of reelin with amyloid precursor protein promotes neurite outgrowth. *J. Neurosci.* **29**, 7459–7473
10. Rice, H. C., Young-Pearse, T. L., and Selkoe, D. J. (2013) Systematic evaluation of candidate ligands regulating ectodomain shedding of amyloid precursor protein. *Biochemistry* **52**, 3264–3277
11. Hiesberger, T., Trommsdorff, M., Howell, B. W., Goffinet, A., Mumby, M. C., Cooper, J. A., and Herz, J. (1999) Direct binding of reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. *Neuron* **24**, 481–489
12. Leeb, C., Eresheim, C., and Nimpf, J. (2014) Clusterin is a ligand for apolipoprotein E receptor 2 (ApoER2) and very low density lipoprotein receptor (VLDLR) and signals via the reelin-signaling pathway. *J. Biol. Chem.* **289**, 4161–4172
13. Mahley, R. W. (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* **240**, 622–630
14. Strittmatter, W. J., and Roses, A. D. (1995) Apolipoprotein E and Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **92**, 4725–4727
15. Botella-López, A., Cuchillo-Ibáñez, I., Cotrufo, T., Mok, S. S., Li, Q. X., Barquero, M. S., Dierssen, M., Soriano, E., and Sáez-Valero, J. (2010) β-Amyloid controls altered reelin expression and processing in Alzheimer's disease. *Neurobiol. Dis.* **37**, 682–691
16. Cuchillo-Ibáñez, I., Balmaceda, V., Botella-López, A., Rabano, A., Avila, J., and Sáez-Valero, J. (2013) β-Amyloid impairs reelin signaling. *PLoS One* **8**, e72297
17. Cuchillo-Ibañez, I., Mata-Balaguer, T., Balmaceda, V., Arranz, J. J., Nimpf, J., and Sáez-Valero, J. (2016) The β-amyloid peptide compromises reelin signaling in Alzheimer's disease. *Sci. Rep.* **6**, 31646
18. Noh, J. S., Sharma, R. P., Veldic, M., Salvacion, A. A., Jia, X., Chen, Y., Costa, E., Guidotti, A., and Grayson, D. R. (2005) DNA methyltransferase 1 regulates reelin mRNA expression in mouse primary cortical cultures. *Proc. Natl. Acad. Sci. USA* **102**, 1749–1754
19. Balmaceda, V., Cuchillo-Ibáñez, I., Pujadas, L., García-Ayllón, M. S., Saura, C. A., Nimpf, J., Soriano, E., and Sáez-Valero, J. (2014) ApoER2 processing by presenilin-1 modulates reelin expression. *FASEB J.* **28**, 1543–1554
20. Braak, H., and Braak, E. (1991) Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol.* **82**, 239–259
21. Botella-López, A., Burgaya, F., Gavín, R., García-Ayllón, M. S., Gómez-Tortosa, E., Peña-Casanova, J., Ureña, J. M., Del Río, J. A., Blesa, R., Soriano, E., and Sáez-Valero, J. (2006) Reelin expression and glycosylation patterns are altered in Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* **103**, 5573–5578
22. Calero, O., Hortigüela, R., Bullido, M. J., and Calero, M. (2009) Apolipoprotein E genotyping method by real time PCR, a fast and cost-effective alternative to the TaqMan and FRET assays. *J. Neurosci. Methods* **183**, 238–240
23. Smith, Z. D., and Meissner, A. (2013) DNA methylation: roles in mammalian development. *Nat. Rev. Genet.* **14**, 204–220
24. Kundakovic, M., Chen, Y., Guidotti, A., and Grayson, D. R. (2009) The reelin and GAD67 promoters are activated by epigenetic drugs that facilitate the disruption of local repressor complexes. *Mol. Pharmacol.* **75**, 342–354
25. Grayson, D. R., Jia, X., Chen, Y., Sharma, R. P., Mitchell, C. P., Guidotti, A., and Costa, E. (2005) Reelin promoter hypermethylation in schizophrenia. *Proc. Natl. Acad. Sci. USA* **102**, 9341–9346
26. Guidotti, A., Ruzicka, W., Grayson, D. R., Veldic, M., Pinna, G., Davis, J. M., and Costa, E. (2007) *S*-adenosyl methionine and DNA methyltransferase-1 mRNA overexpression in psychosis. *Neuroreport* **18**, 57–60
27. May, P., Bock, H. H., Nimpf, J., and Herz, J. (2003) Differential glycosylation regulates processing of lipoprotein receptors by γ-secretase. *J. Biol. Chem.* **278**, 37386–37392
28. Hoe, H. S., and Rebeck, G. W. (2005) Regulation of ApoE receptor proteolysis by ligand binding. *Brain Res. Mol. Brain Res.* **137**, 31–39
29. Silveyra, M. X., García-Ayllón, M. S., Serra-Basante, C., Mazzoni, V., García-Gutierrez, M. S., Manzanares, J., Culvenor, J. G., and Sáez-Valero, J. (2012) Changes in acetylcholinesterase expression are associated with altered presenilin-1 levels. *Neurobiol. Aging* **33**, 627.e27–627.e37
30. Ni, C. Y., Murphy, M. P., Golde, T. E., and Carpenter, G. (2001) γ-secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* **294**, 2179–2181

31. Liu, D. S., Pan, X. D., Zhang, J., Shen, H., Collins, N. C., Cole, A. M., Koster, K. P., Ben Aissa, M., Dai, X. M., Zhou, M., Tai, L. M., Zhu, Y. G., LaDu, M., and Chen, X. C. (2015) APOE4 enhances age-dependent decline in cognitive function by down-regulating an NMDA receptor pathway in EFAD-Tg mice. *Mol. Neurodegener.* **10**, 7
32. Shi, Y., Yamada, K., Liddelow, S. A., Smith, S. T., Zhao, L., Luo, W., Tsai, R. M., Spina, S., Grinberg, L. T., Rojas, J. C., Gallardo, G., Wang, K., Roh, J., Robinson, G., Finn, M. B., Jiang, H., Sullivan, P. M., Baufeld, C., Wood, M. W., Sutphen, C., McCue, L., Xiong, C., Del-Aguila, J. L., Morris, J. C., Cruchaga, C., Fagan, A. M., Miller, B. L., Boxer, A. L., Seeley, W. W., Butovsky, O., Barres, B. A., Paul, S. M., and Holtzman, D. M.; Alzheimer's Disease Neuroimaging Initiative. (2017) ApoE4 markedly exacerbates tau-mediated neurodegeneration in a mouse model of tauopathy. *Nature* **549**, 523–527
33. Divekar, S. D., Burrell, T. C., Lee, J. E., Weeber, E. J., and Rebeck, G. W. (2014) Ligand-induced homotypic and heterotypic clustering of apolipoprotein E receptor 2. *J. Biol. Chem.* **289**, 15894–15903
34. Hoe, H. S., Pocivavsek, A., Dai, H., Chakraborty, G., Harris, D. C., and Rebeck, G. W. (2006) Effects of apoE on neuronal signaling and APP processing in rodent brain. *Brain Res.* **1112**, 70–79
35. Hinrich, A. J., Jodelka, F. M., Chang, J. L., Brutman, D., Bruno, A. M., Briggs, C. A., James, B. D., Stutzmann, G. E., Bennett, D. A., Miller, S. A., Rigo, F., Marr, R. A., and Hastings, M. L. (2016) Therapeutic correction of ApoER2 splicing in Alzheimer's disease mice using antisense oligonucleotides. *EMBO Mol. Med.* **8**, 328–345
36. Yu, J. T., and Tan, L. (2012) The role of clusterin in Alzheimer's disease: pathways, pathogenesis, and therapy. *Mol. Neurobiol.* **45**, 314–326
37. Raber, J., Wong, D., Buttini, M., Orth, M., Bellosta, S., Pitas, R. E., Mahley, R. W., and Mucke, L. (1998) Isoform-specific effects of human apolipoprotein E on brain function revealed in ApoE knockout mice: increased susceptibility of females. *Proc. Natl. Acad. Sci. USA* **95**, 10914–10919
38. Hussain, A., Luong, M., Pooley, A., and Nathan, B. P. (2013) Isoform-specific effects of apoE on neurite outgrowth in olfactory epithelium culture. *J. Biomed. Sci.* **20**, 49
39. Chen, Y., Durakoglugil, M. S., Xian, X., and Herz, J. (2010) ApoE4 reduces glutamate receptor function and synaptic plasticity by selectively impairing ApoE receptor recycling. *Proc. Natl. Acad. Sci. USA* **107**, 12011–12016
40. Marzolo, M. P., and Bu, G. (2009) Lipoprotein receptors and cholesterol in APP trafficking and proteolytic processing, implications for Alzheimer's disease. *Semin. Cell Dev. Biol.* **20**, 191–200
41. Bachmeier, C., Shackleton, B., Ojo, J., Paris, D., Mullan, M., and Crawford, F. (2014) Apolipoprotein E isoform-specific effects on lipoprotein receptor processing. *Neuromolecular Med.* **16**, 686–696
42. Weeber, E. J., Beffert, U., Jones, C., Christian, J. M., Forster, E., Sweatt, J. D., and Herz, J. (2002) Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning. *J. Biol. Chem.* **277**, 39944–39952
43. Cuchillo-Ibañez, I., Balmaceda, V., Mata-Balaguer, T., Lopez-Font, I., and Sáez-Valero, J. (2016) Reelin in Alzheimer's disease, increased levels but impaired signaling: when more is less. *J. Alzheimers Dis.* **52**, 403–416
44. Palop, J. J., and Mucke, L. (2016) Network abnormalities and interneuron dysfunction in Alzheimer disease. *Nat. Rev. Neurosci.* **17**, 777–792
45. Saiz-Sanchez, D., Flores-Cuadrado, A., Ubeda-Bañon, I., de la Rosa-Prieto, C., and Martinez-Marcos, A. (2016) Interneurons in the human olfactory system in Alzheimer's disease. *Exp. Neurol.* **276**, 13–21
46. Alcántara, S., Ruiz, M., D'Arcangelo, G., Ezan, F., de Lecea, L., Curran, T., Sotelo, C., and Soriano, E. (1998) Regional and cellular patterns of reelin mRNA expression in the forebrain of the developing and adult mouse. *J. Neurosci.* **18**, 7779–7799
47. Martínez-Cerdeño, V., Galazo, M. J., Cavada, C., and Clascá, F. (2002) Reelin immunoreactivity in the adult primate brain: intracellular localization in projecting and local circuit neurons of the cerebral cortex, hippocampus and subcortical regions. *Cereb. Cortex* **12**, 1298–1311
48. Abraham, H., and Meyer, G. (2003) Reelin-expressing neurons in the postnatal and adult human hippocampal formation. *Hippocampus* **13**, 715–727
49. Kocherhans, S., Madhusudan, A., Doehner, J., Breu, K. S., Nitsch, R. M., Fritschy, J. M., and Knuesel, I. (2010) Reduced Reelin expression accelerates amyloid-β plaque formation and tau pathology in transgenic Alzheimer's disease mice. *J. Neurosci.* **30**, 9228–9240
50. Pujadas, L., Rossi, D., Andrés, R., Teixeira, C. M., Serra-Vidal, B., Parcerisas, A., Maldonado, R., Giralt, E., Carulla, N., and Soriano, E. (2014) Reelin delays amyloid-β fibril formation and rescues cognitive deficits in a model of Alzheimer's disease. *Nat. Commun.* **5**, 3443
51. Lane-Donovan, C., Philips, G. T., Wasser, C. R., Durakoglugil, M. S., Masiulis, I., Upadhaya, A., Pohlkamp, T., Coskun, C., Kotti, T., Steller, L., Hammer, R. E., Frotscher, M., Bock, H. H., and Herz, J. (2015) Reelin protects against amyloid β toxicity in vivo. *Sci. Signal.* **8**, ra67
52. Lu, H., Liu, X., Deng, Y., and Qing, H. (2013) DNA methylation, a hand behind neurodegenerative diseases. *Front. Aging Neurosci.* **5**, 85
53. Day, J. J., Kennedy, A. J., and Sweatt, J. D. (2015) DNA methylation and its implications and accessibility for neuropsychiatric therapeutics. *Annu. Rev. Pharmacol. Toxicol.* **55**, 591–611
54. Sanchez-Mut, J. V., Heyn, H., Vidal, E., Moran, S., Sayols, S., Delgado-Morales, R., Schultz, M. D., Ansoleaga, B., Garcia-Esparcia, P., Pons-Espinal, M., de Lagran, M. M., Dopazo, J., Rabano, A., Avila, J., Dierssen, M., Lott, I., Ferrer, I., Ecker, J. R., and Esteller, M. (2016) Human DNA methylomes of neurodegenerative diseases show common epigenomic patterns. *Transl. Psychiatry* **6**, e718
55. Klein, H. U., Bennett, D. A., and De Jager, P. L. (2016) The epigenome in Alzheimer's disease: current state and approaches for a new path to gene discovery and understanding disease mechanism. *Acta Neuropathol.* **132**, 503–514
56. Abdolmaleky, H. M., Cheng, K. H., Russo, A., Smith, C. L., Faraone, S. V., Wilcox, M., Shafa, R., Glatt, S. J., Nguyen, G., Ponte, J. F., Thiagalingam, S., and Tsuang, M. T. (2005) Hypermethylation of the reelin (*RELN*) promoter in the brain of schizophrenic patients: a preliminary report. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* **134B**, 60–66
57. Kobow, K., Jeske, I., Hildebrandt, M., Hauke, J., Hahnen, E., Buslei, R., Buchfelder, M., Weigel, D., Stefan, H., Kasper, B., Pauli, E., and Blümcke, I. (2009) Increased reelin promoter methylation is associated with granule cell dispersion in human temporal lobe epilepsy. *J. Neuropathol. Exp. Neurol.* **68**, 356–364
58. Sui, L., and Li, B. M. (2010) Effects of perinatal hypothyroidism on regulation of reelin and brain-derived neurotrophic factor gene expression in rat hippocampus: role of DNA methylation and histone acetylation. *Steroids* **75**, 988–997
59. Tochigi, M., Iwamoto, K., Bundo, M., Komori, A., Sasaki, T., Kato, N., and Kato, T. (2008) Methylation status of the reelin promoter region in the brain of schizophrenic patients. *Biol. Psychiatry* **63**, 530–533
60. Lin, L., Wang, P., Liu, X., Zhao, D., Zhang, Y., Hao, J., Liang, X., Huang, X., Lu, J., and Ge, Q. (2017) Epigenetic regulation of reelin expression in multiple myeloma. *Hematol. Oncol.* **35**, 685–692
61. Carvajal, A. E., Vázquez-Carretero, M. D., García-Miranda, P., Peral, M. J., Calonge, M. L., and Ilundain, A. A. (2017) Reelin expression is up-regulated in mice colon in response to acute colitis and provides resistance against colitis. *Biochim. Biophys. Acta* **1863**, 462–473
62. Eid, A., Bihaqi, S. W., Renehan, W. E., and Zawia, N. H. (2016) Developmental lead exposure and lifespan alterations in epigenetic regulators and their correspondence to biomarkers of Alzheimer's disease. *Alzheimers Dement. (Amst.)* **2**, 123–131
63. Griñán-Ferré, C., Sarroca, S., Ivanova, A., Puigoriol-Illamola, D., Aguado, F., Camins, A., Sanfeliu, C., and Pallàs, M. (2016) Epigenetic mechanisms underlying cognitive impairment and Alzheimer disease hallmarks in 5XFAD mice. *Aging (Albany N.Y.)* **8**, 664–684
64. D'Aiuto, L., Di Maio, R., Mohan, K. N., Minervini, C., Saporiti, F., Soreca, I., Greenamyre, J. T., and Chaillet, J. R. (2011) Mouse ES cells overexpressing DNMT1 produce abnormal neurons with upregulated NMDA/NR1 subunit. *Differentiation* **82**, 9–17
65. Lintas, C., Sacco, R., and Persico, A. M. (2016) Differential methylation at the RELN gene promoter in temporal cortex from autistic and typically developing post-puberal subjects. *J. Neurodev. Disord.* **8**, 18
66. Dong, E., Ruzicka, W. B., Grayson, D. R., and Guidotti, A. (2015) DNA-methyltransferase1 (DNMT1) binding to CpG rich GABAergic and BDNF promoters is increased in the brain of schizophrenia and bipolar disorder patients. *Schizophr. Res.* **167**, 35–41
67. Lee, G. H., and D'Arcangelo, G. (2016) New insights into Reelin-mediated signaling pathways. *Front Cell Neurosci.* **10**, 122
68. Lane-Donovan, C., and Herz, J. (2017) ApoE, ApoE receptors, and the synapse in Alzheimer's disease. *Trends Endocrinol. Metab.* **28**, 273–284

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