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CSF-ApoER2 fragments as a read-out of reelin signaling: Distinct patterns in sporadic and autosomal-dominant Alzheimer disease

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ABSTRACT

Reelin is a glycoprotein associated with synaptic plasticity and neurotransmission. The malfunctioning of reelin signaling in the brain is likely to contribute to the pathogenesis of Alzheimer's disease (AD). Reelin binding to Apolipoprotein E receptor 2 (ApoER2) activates downstream signaling and induces the proteolytic cleavage of ApoER2, resulting in the generation of soluble fragments. To evaluate the efficiency of reelin signaling in AD, we have quantified the levels of reelin and soluble ectodomain fragments of ApoER2 (ectoApoER2) in the cerebrospinal fluid (CSF). CSF from sporadic AD patients (sAD; $n = 14$, age 54–83 years) had lower levels of ecto-ApoER2 (~31% reduction; $p = .005$) compared to those in the age-matched controls ($n = 10$, age 61–80), and a higher reelin/ecto-ApoER2 ratio. In contrast, autosomal dominant AD patients, carriers of *PSEN1* mutations (ADAD; $n = 7$, age 31–49 years) had higher ecto-ApoER2 levels (~109% increment; $p = .001$) and a lower reelin/ecto-ApoER2 ratio than the non-mutation carriers from the same families ($n = 7$, age 25–47 years). Our data suggest that the levels of ecto-ApoER2 in CSF could be a suitable read-out of an impaired reelin signaling in AD, but also indicate differences between sAD and ADAD.

1. Introduction

Reelin and its downstream pathway components are associated with synaptic plasticity and neurotransmission [1–4]. The pathway begins with the binding of reelin to the transmembrane lipoprotein-receptor, apolipoprotein E receptor 2 (ApoER2) [5,6], and the binding of the intracellular adaptor Dab1 (Disabled-1) to the cytoplasmic domain of the receptor [7–10]. Reelin signaling transduction in the adult brain includes interaction with NMDA receptors (NMDARs). For recent reviews about the role of ApoER2 in the reelin-mediated signaling pathway see Lee and D'Arcangelo [11] and Lane-Donovan and Herz [12].

The ligand binding or reelin to ApoER2 elicits proteolytic processing of the receptor by α-secretases and γ-secretases [13,14], generating a soluble ectodomain together with intracellular fragments [15,16]. Therefore, the determination of the levels of the soluble ApoER2 ectodomain fragments could be a suitable *in-vivo* readout of reelin signaling.

In this context, it is important to note that reelin levels are higher in the brain of Alzheimer's disease (AD) individuals than in those of non-demented individuals, but there is a lower generation of intracellular ApoER2 fragments, indicating that reelin signaling pathway is likely to be disrupted in AD [17,18]. Moreover, the amyloid-β peptide 1–42 (Aβ42) alters reelin glycosylation and compromises its capacity to bind to ApoER2 [19,20]. Therefore, to evaluate reelin activity in AD, it is important to examine, not only reelin, but also the level of the downstream signaling components.

Reelin is a 3460 amino-acid residue extracellular glycoprotein. The full-length 420 kDa protein is cleaved *in vivo* at two sites, resulting in the production of several fragments whose relative abundance differs among tissues [21]. Reelin proteolytic cleavage by metalloproteinases occurs after secretion, and the fragments generated can regulate its function [22–25]. Classically, two main cleavage sites have been described for reelin (see Fig. 1A and reference [26]), and recently an

Abbreviations: Aβ, β-amyloid protein; AD, Alzheimer's disease; ApoER2, apolipoprotein E receptor 2; CSF, cerebrospinal fluid; Dab1, Disabled-1; ICD, intracellular domain fragment; PS1, presenilin 1 protein; *PSEN1*, presenilin 1 gene.

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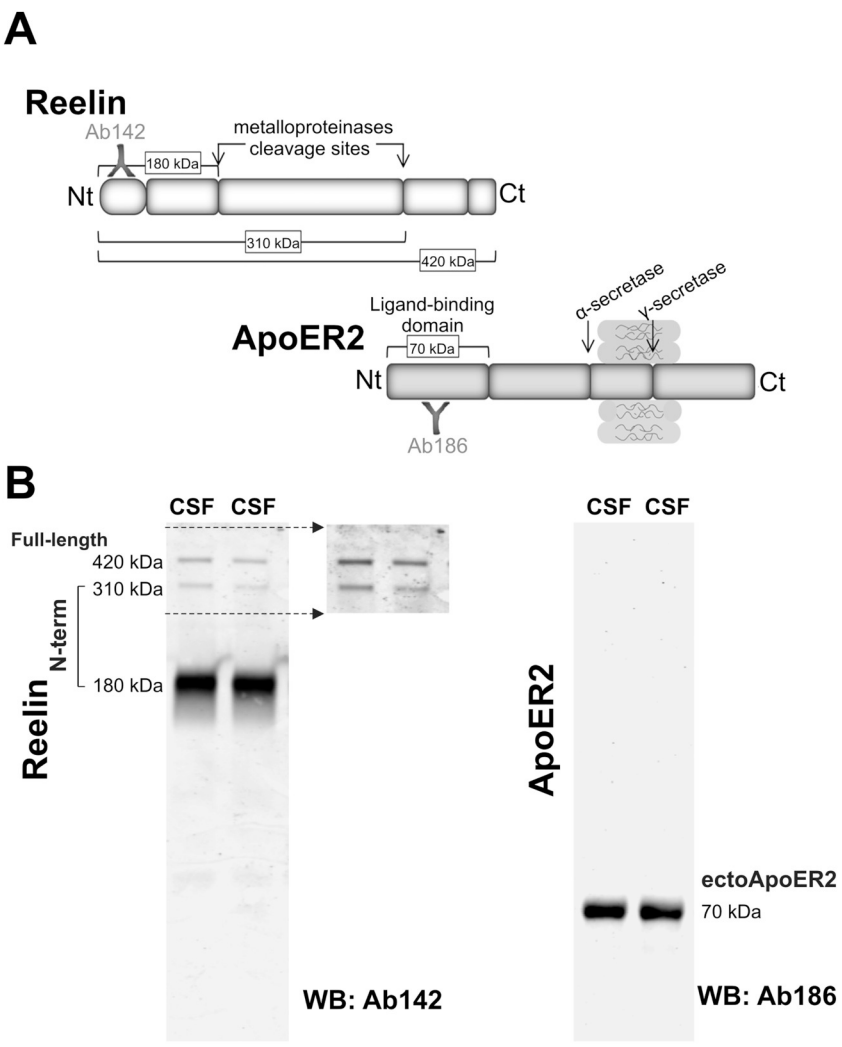

Fig. 1. Reelin and ecto-ApoER2 are present in human CSF. (A) Schematic repesentation of full-length reelin and ApoER2. Binding of reelin to ApoER2 regulates their processing by proteases at the indicated regions (under physiological conditions dimers of reelin bind dimers of ApoER2). Reelin can be also processed by matrix metalloproteinases, independent of ApoER2 binding. The epitopes for the antibodies used in this study are also indicated. (B) Western blotting of two human CSF samples from non-demented controls subjects, resolved with the indicated antibody for reelin and ApoER2 (immunoreactivities were resolved simultaneously by multiplex fluorescence imaging). Since the predominant 180 kDa reelin band displayed greater immunoreactivity than the 410 and 320 kDa fragment, a higher contrast image is also showed as an insert. For further quantitative analysis (Figs. 2 and 3) immunoreactivities for all fragments were quantified with the same contrast.

additional proteolytic cleavage site within the C-terminal region has been described [27]. Reelin proteolytic cleavage also occurs after binding to its receptor, and this generates soluble reelin fragments [28], which do not differ from those generated by proteolysis in cell-free conditions [24].

ApoER2 is a type I glycoprotein containing 963 amino-acid residues (see scheme in Fig. 1A). The full length protein has not been described yet in human CSF, however, ecto-ApoER2 fragments have been reported in human and bovine CSF [20].

In this study, we determined simultaneously by multiplex fluorescence the levels of reelin and soluble proteolytic fragments of ApoER2 ectodomain (ecto-ApoER2) in CSF of sporadic AD patients (sAD) and in subjects with autosomal dominant AD (ADAD) linked to *PSEN1* mutations.

2. Materials and methods

2.1. Patients

Lumbar CSF samples were obtained from autosomal dominant AD (ADAD) subjects who were all carriers of *PSEN1* mutations and who were part of the Genetic Counseling Program for familiar dementia (PICOGEN) at the Hospital Clínic (Barcelona, Spain). This group included 7 subjects carrying *PSEN1* mutations. In addition, 7 age-matched non-mutation carriers from the same families were used as non-disease controls. We also included 14 subjects with sporadic AD (sAD) and 10 age-matched controls defined as patients with non-specific symptoms without neurochemical evidence of AD. All AD patients fulfilled the NIA-AA criteria for dementia [28]. CSF data from these patients have been reported in previous studies [29,30]. This study was approved by the ethic committee at the Miguel Hernandez University and it was carried out in accordance with the Declaration of Helsinki. All patients (or their nearest relatives) and controls gave informed consent to participate in the study.

2.2. Western blotting

CSF reelin and ApoER2 fragments levels were determined as previously described [17,20] with minor modifications. Previous studies have demonstrated that methodological factors and storage conditions influence the measurement and fragmentation of reelin [17,31]. Thus, all analyses were performed on samples stored frozen at −80 °C, avoiding thawing-freezing cycles. Samples in sample buffer were heated before electrophoresis for 3 min at 98 °C and electrophoresis was performed at low voltage to minimize excessive heat generation. CSF samples (13 µL) were resolved on 4–15% gradient SDS-PAGE (Mini-PROTEAN® TGX™ Precast Gels; Bio-Rad) and transferred to 0.45 µm nitrocellulose membranes (Bio-Rad). Then, the membrane was blocked with Odyssey Blocking Buffer (PBS) and incubated with a mouse monoclonal anti-N-terminal reelin 142 antibody (1:1000 dilution; MAB5366 Merck Millipore) and a rabbit monoclonal anti-N-terminal ApoER2 antibody clone 186 (1:4000) [32]. Finally, blots were washed and incubated with the appropriate conjugated secondary antibodies (IRDye 680RD goat anti-mouse and IRDye 800CW goat anti-rabbit, LI-

COR Biosciences) and imaged on an Odyssey Clx Infrared Imaging System (LI-COR Biosciences). Multiplex fluorescence with the two independent antibodies served to assess simultaneously reelin and ApoER2. Band intensities were analyzed using LI-COR software (Image Studio Lite). A control CSF sample was used to normalize the immunoreactive signal between blots. All samples were analyzed at least in duplicate. To estimate the quotient between reelin and ApoER2 for each sample, the unprocessed immunoreactivity was considered for each of the band (see Results section).

2.3. Statistical analysis

All the data were analyzed using SigmaStat (Version 3.5; Systac Software Inc.) using a Student's *t*-test (two-tailed) or a Mann-Whitney *U* test for single pairwise comparisons, and determining the exact *p* values. The results are presented as means ± SEM and the correlation between variables was assessed by linear regression analyses.

3. Results

3.1. Reelin and ecto-ApoER2 in the human CSF

Initially, we examined whether full-length reelin and its N-terminal fragments were present in the human CSF by western blotting, using the anti-reelin 142 antibody. As expected, we distinguished three reelin bands: the full-length 420 kDa and two N-terminal fragments of 310 and 180 kDa. The 180 kDa fragment was particularly abundant (Fig. 1B). The results are in accordance with previous observations by our group and others [17,32,33].

We also used the 186 antibody, raised against the entire ligand binding domain of ApoER2 [32] and we confirmed the presence of a ~70 kDa ecto-ApoER2 fragment in the human CSF (Fig. 1B).

3.2. Ecto-ApoER2 levels are decreased in CSF from sAD patients

To assess whether reelin and ecto-ApoER2 levels are altered in AD, we first analyzed CSF samples from sporadic AD subjects (sAD; $n = 14$) and compared the results to those obtained from CSF of age-matched non-demented controls ($n = 10$) (Table 1). Neither the 420 kDa full-length reelin nor the most abundant 180 kDa reelin N-terminal fragment, resolved with the anti-reelin 142 antibody, displayed significant differences between sAD and controls (Fig. 2A,B). The anti-ApoER2 antibody 186 detected in all CSF samples a ~70 kDa band attributable to the ecto-ApoER2 fragment. The immunoreactivity of this ecto-ApoER2 fragment in the CSF from sAD subjects was low (~31% decrease; $p = .005$) compared to controls (Fig. 2A,B). Since both reelin and ecto-ApoER2 immunoreactivities were detected simultaneously by multiplex fluorescence, we could also calculate the ratio of reelin to ecto-ApoER2 for each sample. The full-length reelin/ecto-ApoER2 ratio was statistically significant higher in sAD than in controls (Fig. 2C).

3.3. Ecto-ApoER2 levels are increased in CSF from ADAD patients

Finally, we assessed the potential differences in the levels of reelin and ecto-ApoER2 in CSF samples from autosomal dominant AD (ADAD) subjects. The ADAD patients were all carriers of *PSEN1* mutations, and the age-matched controls were individuals from the same families but non-carriers of the mutation. The information about their clinical parameters, demographic data and classic CSF biomarker levels are included in Table 1. Similar to sAD, ADAD subjects displayed a subtle, but not statistically significant increase in full-length reelin and the 180 kDa fragment (Fig. 3A, B). However, unexpectedly, ADAD subjects displayed higher ecto-ApoER2 levels (~109% increment; $p = .001$) than those in age-matched controls (Fig. 3A, B), which affected the reelin/ecto-ApoER2 quotient (Fig. 3C).

4. Discussion

In this study, we have evaluated the levels of full-length reelin and its N-terminal fragments, together with the soluble ecto-ApoER2 proteolytic fragment in CSF samples from sAD and ADAD subjects. In previous studies, we demonstrated high reelin protein and mRNA levels in brain extracts from sAD individuals [17,18,20,34]. However, our studies in CSF do not show such consistency. We found high reelin levels in a cohort of CSF samples from AD subjects compared to control subjects, while in a different cohort of samples we found only an incremental trend, that did not reach statistical significance [17,34]. The discrepancy in the reelin levels between the post-mortem brain and CSF samples in sAD could be explained by the neurodegeneration stage of the individuals. The elevated reelin expression occurs at advanced Braak stages, from Braak stage III to VI [18,20]. Therefore, the lack of steady changes in earliest pathological stages may be responsible for the inconsistent variations in CSF-reelin levels. It is also important to note that in the AD brain considerable amounts of reelin are “trapped” into insoluble aggregates containing Aβ [20]. Consequently, it is likely that reelin levels variations in brain do not match reelin levels in CSF in AD subjects, and therefore, the potential of reelin in CSF as a diagnostic biomarker for AD is questionable. However, in a previous study in which the CSF samples were selected based on similar reelin levels among them, the ecto-ApoER2 levels were lower in sAD as compared with those in age-matched controls [20], similar to what we found in this cohort.

Reelin binding to ApoER2 initiates a signaling cascade that induces the proteolytic processing of both reelin and ApoER2. This proteolysis releases soluble extracellular (ecto-ApoER2) and intracellular domain (ApoER2-ICD) fragments of the receptor [15,16], as well generating reelin fragments [27]. Extracellular reelin and ApoER2 soluble fragments may have an auto-regulatory function, due to their capacity to directly interfere in the binding of functional reelin to its receptor [15,22,35–37]. While ApoER2 proteolytic processing is dependent on ligand binding, similarly to other type-I membrane receptors [38], processing of reelin can also be exerted extracellularly by matrix metalloproteinases, independently of any receptor interaction [26,39–41]. Moreover, many studies indicate an upregulated expression of some matrix metalloproteinases during AD progression [42–45]. Accordingly, in the search for a specific read-out of reelin signaling, the determination of the ligand-binding dependent ecto-ApoER2 fragments in CSF, may be more reliable than the estimation of reelin fragments.

Table 1
Clinical, demographic data and classic CSF biomarker levels.

Group	Age (years)	Gender	CSF Aβ42 (pg/mL)	CSF T-tau (pg/mL)	CSF P-tau (pg/mL)
sAD	67 ± 2 [55–83]	10F/4M	348 ± 16**	844 ± 82**	136 ± 16**
Ctrl	66 ± 2 [60–80]	6F/4M	711 ± 30	205 ± 18	46 ± 3
ADAD	43 ± 2 [31–49]	5F/2M	266 ± 49**	883 ± 204**	168 ± 69*
Ctrl	39 ± 3 [25–47]	5F/2M	809 ± 94	245 ± 29	46 ± 4

Each ADAD subject carried one of the following *PSEN1* mutations (autosomal dominant), S169P, L173F, L235R, L282R and 3 individuals carried the L286P mutation. The data represent the means ± SEM; the range of ages is also indicated. All the pathological groups were compared with age-matched controls (Ctrl). $^{**}p < .001$; $^{*}p < .05$ from the control group.

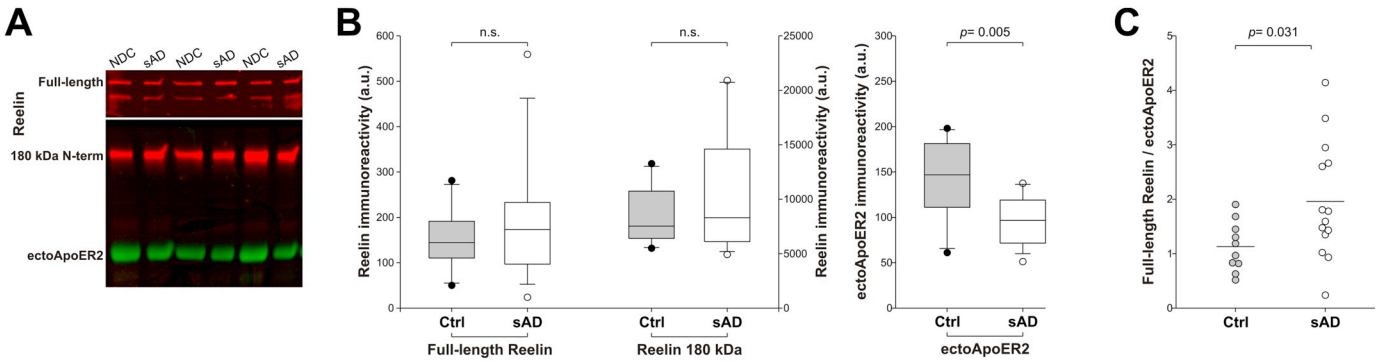

Fig. 2. The ectoApoER2 is decreased in sporadic AD (sAD). (A) Reelin and the ectodomain fragment of ApoER2 (ectoApoER2) were assayed by multiplex fluorescence western blotting in patients with dementia due to sporadic AD (sAD; $n = 14$, age 54–83 years) and age-matched controls (Ctrl; $n = 10$, age 61–80), Representative multiplex blot of the reelin (in red) and ecto-ApoER2 (in green) are shown. The upper area of the gel is shown with higher contrast to show the 420 and 310 kDa reelin bands more clearly, but quantification was performed under the same exposure conditions. (B) Densitometric quantification of the individual immunoreactivities for the 420 kDa full-length and major 180 kDa reelin fragment and the ecto-ApoER2 (box plots). (C) The full-length reelin/ecto-ApoER2 quotient was determined for each sample. Each determination was made in duplicate. The data represent the means ± SEM. *Significantly different ($p < .01$) from the Ctrl group (n.s.: non significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

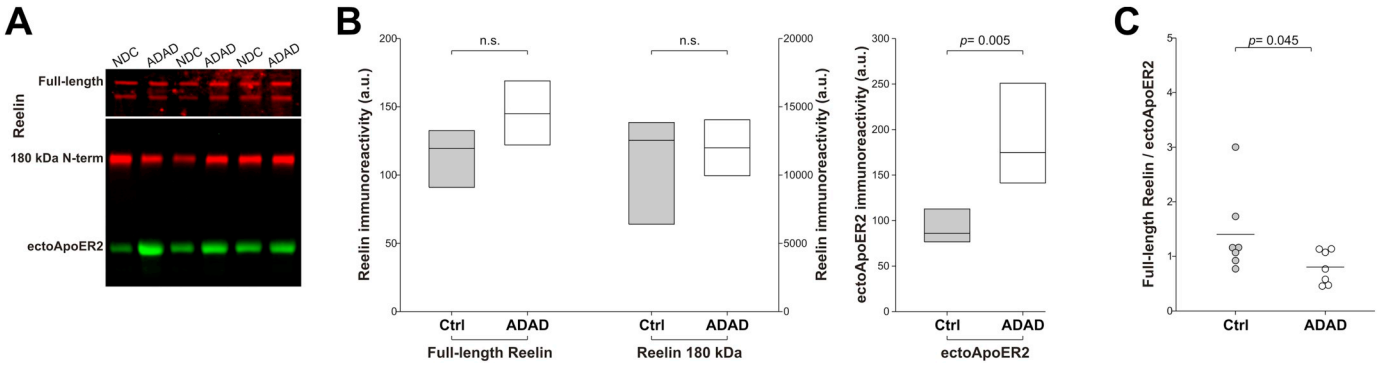

Fig. 3. The ectoApoER2 is increased in autosomal dominant AD (ADAD). (A). Representative multiplex blot of the reelin (in red) and ecto-ApoER2 (in green) in the CSF samples from 7 symptomatic ADAD (age 31–49 years) and 7 control siblings (Ctrl; age 25–47 years), not carriers of the mutations. The upper area of the gel is shown with higher contrast for reasons described in Fig. 2. (B) Densitometric quantification of the individual immunoreactivities for the 420 kDa full-length and major 180 kDa reelin fragment and the ecto-ApoER2 (box plots). (C) The full-length reelin/ecto-ApoER2 quotient was determined from each sample. The results were confirmed in two independent determinations. The data represent the means ± SEM. *Significantly different ($p < .01$) from the Ctrl group (n.s.: not significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We have also included in our analysis a small cohort of ADAD subjects, carriers of *PSEN1* mutations. While ADAD subjects showed similar CSF-reelin levels to age-matched controls, interestingly ADAD patients displayed a robust increase in ecto-ApoER2 levels. This difference between sAD and ADAD was unexpected. Cleavage of ApoER2 induced by reelin binding occurs through the sequential processing by α-secretase and γ-secretase [46,47]. Presenilin-1 (PS1) is the catalytic subunit of the γ-secretase complex (for review of γ-secretase structure and function see references [48, 49]). Experiments in cellular models have shown that some pathological *PSEN1* mutations (R278I, M146V) induce an impaired γ-secretase cleavage of ApoER2, while others (L282V) affect the trafficking and cell-surface levels of ApoER2 [50]. Unfortunately, there is a lack of reports about ApoER2 and reelin levels in human ADAD brains.

Mutations in *PSEN1* that affect the γ-secretase activity, although do not alter the downstream phosphorylation of Dab1 [51], could generate less ApoER2-ICD, which downregulates the transcription of synaptic plasticity genes [4], including reelin [16]; therefore, these mutations would hamper the downregulation of these gene expression. When the γ-secretase activity is inhibited in a sustained manner, this leads to a rebound increase in PS1 levels that accordingly, increases ApoER2 processing [52] and therefore it could induce an increment of the ecto-ApoER2 levels. We cannot discard the possibility that, in those subjects carrying *PSEN1* mutations, the increment in ApoER2 processing could results from the combination of a failure in the downregulation of reelin transcription and an increase in PS1 activity on ApoER2.

5. Conclusions

Here, we have explored and confirmed that estimation of ecto-ApoER2 levels in the CSF constitutes a suitable read-out of impaired signaling function in sAD (for a complete discussion see Cuchillo-Ibañez [53]). Ecto-ApoER2 soluble fragments are cleavage products of the reelin receptor whose generation depends on ligand binding, but also on activity of secretases. In subjects with ADAD carrying *PSEN1* mutations, we observed a noticeable increase in ecto-ApoER2 levels in the CSF, indicating particularities on the regulation of the reelin pathway that require further analysis.

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Disclosure

None of the authors have any actual or potential financial conflicts or conflict of interest related with this study.

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