Research Article

Characterization of cerebrospinal fluid BACE1 as a biomarker for Alzheimer's disease

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Abstract

Background: The primary neuronal β-secretase in the brain is the β-site amyloid precursor protein cleaving enzyme 1 (BACE1). BACE1 is the main protease responsible for the amyloidogenic processing of the amyloid precursor protein (APP) leading to Aβ generation. Previous studies have suggested the potential of cerebrospinal fluid (CSF) β-secretase activity to be a diagnostic biomarker for Alzheimer's disease (AD), but these studies have not demonstrated consistent changes in AD CSF. Therefore, further biochemical characterization of BACE1 protein and β-secretase activity in AD CSF is needed.

Methods: β-Secretase activity was determined in CSF from AD patients (n= 19) and age-matched non-AD controls (n= 19) using a fluorogenic substrate in the absence and presence of a BACE1 inhibitor. BACE1 protein was characterized by lectin-binding, immunoprecipitation, blue native-PAGE and western blotting using specific antibodies.

Results: We show that the majority of the β-secretase activity in the CSF is not inhibited by a specific BACE1 inhibitor, and that β-secretase activity attributable to BACE1 is higher in AD subjects. BACE1 is present in human CSF, both as immature forms of ~50 kDa that retain the prodomain and mature forms of ~70 kDa that probably comprise truncated and full-length species. CSF-BACE1 forms large hetero-complexes containing distinct species. Immunoblotting with an antibody against the C-terminus of BACE1 revealed significantly higher levels of the 70-kDa full-length BACE1, while the 50 kDa immature form remained unaltered. When the 70-kDa band was probed with an antibody against the N-terminus of BACE1, which does not discriminate between truncated and full-length forms, no increase was observed, suggesting that truncated forms of BACE1 do not increase in AD.

Conclusions: The activity of β-secretase in the CSF cannot be attributed only to BACE1. Both mature and immature species of BACE1 occur as heteromers. Only mature full-length BACE1 shows increased levels in AD CSF. The complexity of BACE1 species in CSF has to be taken into consideration for the determination of BACE1 activity and protein levels in CSF as biomarkers of AD.

1. Introduction

Alzheimer disease (AD) is an age-related neurodegenerative disorder that is the most common cause of dementia among the elderly. The pathologic characteristics of AD are proteinaceous deposits, including extracellular plaques, composed mostly of β-amyloid protein (Aβ), and intracellular tangles containing an abnormally hyperphosphorylated form of the microtubule-associated protein tau (P-tau) (Braak and Braak, 1991). The proteins, Aβ, P-tau and total tau, can be monitored in cerebrospinal fluid (CSF) and constitute the so-called "core" biomarkers (Blennow et al., 2006). Numerous laboratories have reported an increase in levels of P-tau and total tau (T-tau) in CSF, but these biomarkers lack specificity, since they are also increased in other neurological diseases (Blennow et al., 2010). In contrast, abnormal metabolism of Aβ is considered a more specific phenomenon related to AD.

The primary neuronal β-secretase responsible for Aβ generation in the brain is an aspartyl protease type-I transmembrane protein, the β-site amyloid precursor protein cleaving enzyme 1 or BACE1 (Bennett et al., 2000;Hussain et al., 1999;Vassar et al., 2009). Previous studies demonstrated the existence of β-secretase activity in AD CSF (Alexopoulos et al., 2018; Ewers et al., 2011; Holsinger et al., 2004; Mulder et al., 2010; Pera et al., 2013; Perneczky and Alexopoulos, 2014; Rosen et al., 2012; Savage et al., 2015; Wu et al., 2008; Zetterberg et al., 2008; Zhong et al., 2007). Most published results suggest that β-secretase activity is increased in AD, and particularly so in amnestic mild cognitive impairment (MCI) cases, which can be considered as prodromal AD, but these studies have failed to demonstrate consistent changes in AD [reviewed in (Perneczky et al., 2014)].

It has been assumed that the β-secretase activity present in CSF is attributable to a truncated BACE1 species produced by cleavage of the full-length membrane-bound

form, thus that it is missing the transmembrane and intracellular domains (Verheijen et al., 2006). In fact, some studies, using anti-BACE1 C-terminus antibodies, have demonstrated the presence of full-length BACE1 in human CSF (Garcia-Ayllon et al., 2013; Holsinger et al., 2004), indicating that an unprocessed BACE1 form exists in CSF. The presence of full-length BACE1, together with its truncated form, has also been demonstrated in conditioned media from cultured neurons (Murayama et al., 2005). The presence in CSF of an immature form of BACE1 protein, poorly active or inactive, has also been suggested (Zhong et al., 2007).

In order to define the potential of BACE1 as a biomarker for AD it is necessary to perform a more precise characterization of the soluble BACE1 species present in the CSF, as well to determine if the activity levels of the β-secretase present in the CSF reflect levels of particular forms of BACE1. Determining the oligomerization state of the soluble forms of BACE1 in the CSF is also relevant for development of ELISA kits.

2. Materials and methods

2.1. CSF samples

CSF samples were obtained from the Clinical Neurochemistry Laboratory (Mölndal, Sweden) from patients who sought medical advice because of cognitive impairment. In total, 19 patients with AD (5 man and 14 women, 72 ±2 years) and 19 age-matched non-AD controls (NADC; 11 men and 8 women, 71 ±2 years) were included. Patients were designated as AD or non-AD controls (NADC) according to CSF biomarker levels (Hansson et al., 2006). AD patients have a CSF biomarker profile indicative of AD including increased T-tau (>730 pg/mL) and P-tau (>90 pg/mL) together with low Aβ42 (< 405 pg/mL) concentrations in CSF; while NADC subjects had normal levels of all three CSF biomarkers (T-tau < 235 pg/mL, P-tau < 37 pg/mL, and Aβ42 > 758 pg/mL), thereby excluding AD pathology. All AD patients fulfilled the 2011 NIA-AA criteria for dementia (McKhann et al., 2011). The CSF samples used for the present study were de-identified leftover aliquots from clinical routine analyses, following a procedure approved by the Ethics Committee at University of Gothenburg. The CSF samples were aliquoted and frozen at −80ºC until use, avoiding freezing and thawing cycles. This study was also approved by the Ethics Committee at the Miguel Hernandez University.

2.2. Preparation of human brain samples

Brain frontal cortex samples (post-mortem interval 4-8 hours) were obtained from the UIPA neurological tissue bank (Unidad de Investigación Proyecto Alzheimer; Madrid, Spain). Samples were homogenized (10% w/v) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% Triton X-100, supplemented with a cocktail of protease inhibitors (cat# P834; Sigma Aldrich). The homogenates were sonicated and centrifuged at 70,000×*g* at 4 °C for 1 h; the supernatant was collected,

aliquoted, and frozen at −80°C until use. Total protein concentrations were determined using the bicinchoninic acid method (Pierce).

2.3. Cell culture

For obtaining conditioned cell-culture medium, CHO cells (450,000 cells/well) were grown in six-well plates for 48 h in Dulbecco's modified Eagle's medium (DMEM) plus GlutaMAX™ (Gibco® Life Technologies, Paisley, UK) supplemented with 5% fetal bovine serum (FBS; Gibco) and 100 µg/mL penicillin/streptomycin (Gibco). After 48h, the cell medium was recollected, centrifuged for 15 min at 1500×*g* at 4ºC, and frozen at −80°C for future analysis.

2.4. β-Secretase-specific enzymatic activity assay

β-Secretase activity in human CSF was measured by incubating 10 µL of human CSF with 90 µL of fluorogenic substrate (40 µM; Millipore, cat# 565758) at 37ºC in reaction buffer (50 mM acetic acid pH 4.1, 100 mM NaCl) (Pera et al., 2013;Zhong et al., 2007). Fluorescence was measured at different time points with an Infinite M200 (Tecan) microplate reader with an excitation wavelength of 355 nm and emission wavelength of 486 nm. The enzymatic activity was calculated as ΔUF/min from the linear phase of the reaction. We also used β-secretase inhibitor III (Millipore, cat# 565780) to block β-secretase activity in solubilized membrane fractions of BACE-transfected cells.

2.5. Western blotting

For the analysis of BACE1 in denaturing conditions, proteins in CSF (30 µL), brain extracts (30 µg) and cell medium (30 µL) were separated by 10 % SDS-PAGE and transferred electrophoretically to 0.45 µm nitrocellulose membranes (Bio-Rad). For

some experiments, CSF samples were depleted of albumin and immunoglobulins prior to electrophoresis, as described below. BACE1 was detected on blots using the following antibodies: C-terminus B0806 (rabbit polyclonal; 1:1000; Sigma Aldrich; epitope 485-501), C-terminus ab2077 (rabbit polyclonal; 1:1000; Abcam; epitope 485-501); N-terminus (rabbit polyclonal; 1:1000; Sigma Aldrich; epitope 46-62), ectodomain (mouse monoclonal; 1:1000; R&D Systems; epitope 22-460) and propeptide (rabbit polyclonal; 1:1000; GenScript; epitope 26-45). Albumin was detected using an anti-albumin N-terminus antibody (mouse monoclonal, epitope 39-164). Previous determination of serum and CSF albumin concentrations, measured by immunonephelometry on a Beckman Immage Immunochemistry system (Beckman Instruments, Beckman Coulter), indicated non differences (p= 0.40) in the CSF albumin (mg/L)/serum albumin (g/L) ratio between NADC (6.1 ± 0.3) and AD (6.6 ± 0.6) groups. Ponceau staining served to monitor potential loading inaccuracies in individual blots. The immunoreactivity of BACE1 signal for each band was normalized to the immunoreactivity of the corresponding band from the same control CSF sample that was resolved in all the blots. All samples were analyzed at least in duplicate. Band intensities were analyzed using LI-COR software (Image Studio Lite).

2.6. Albumin and immunoglobulins immunodepletion procedure

The Seppro® IgY14 spin Columns technology (Sigma, SEP010) were used for immunodepletion of albumin and immunoglobulins. These columns employ a mixture of antibodies designed to remove highly abundant proteins from human fluids (albumin, IgG, α1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α2-macroglobulin, fibrinogen, complement C3, α1-acid glycoprotein, aplopoproteins A-1, A-II and B). Thirty µL of CSF samples were mixed in dilution buffer to a final volume of 500 µL and processed

according to the manufacturer instructions. The depleted samples were then concentrated with an Amicon® filter to a final volume of 30 µL.

2.7. Immunuprecipitation

Immunoprecipitations were performed at 4ºC by incubating 150 µL of CSF or cell medium overnight with 120 µL of hydrated Sepharose A beads coupled to specific BACE antibody. Precipitated proteins were eluted with 0.1M glycine buffer at pH 2.5. After pH neutralization with 1M Tris-HCl pH 9.5, supernatant fractions were denatured and analyzed by western blotting.

2.8. Lectin binding

CSF samples were incubated overnight at 4ºC in Sepharose or agarose coupled to individual immobilized lectins with specificity for terminal sugars: the mannose-binding lectins Con A (lectin from *Canavalia ensiformis*), LCA (lectin from *Lens culinaris*) and GNA (lectin from *Galanthus nivalis*); and lectins that recognize more complex type N-glycans, the biantennary galactosylated N-glycan with bisecting N-acetylglucosamine binding lectin PHA (lectin from *Phaseolus vulgaris*), and the sialic acid / N-acetylglucosamine binding lectin WGA (lectin from *Triticum vulgaris*). The fraction of proteins not linked to lectins was separated by centrifugation and analyzed by western blotting using anti-BACE1 ectodomain and propeptide antibodies. The percentage of non-lectin-bound BACE1 was calculated as the ratio of non-lectin-bound BACE immunoreactivity to total immunoreactivity (aliquot maintained under the same conditions without interacting with any lectin). The same tests were repeated in duplicate.

2.9. Blue Native gel analysis

For analysis of protein molecular size under native conditions, the Life Technologies NativePAGE Novex 4-16% Bis-Tris Gel System was used in accordance with the manufacturer's protocol. Samples were prepared in a 30 µL total volume containing 7.5 µL of NuPage LDS sample buffer (4×), 3 µl of 5% G-250 sample additive and 1.5 µl of DDM 10% (n-dodecyl-β-D-maltoside). The NativeMark™ unstained protein standard (Life Technologies, Carlsbad, CA, USA) was used as a protein ladder.

2.10. Measurement of T-tau, P-tau and Aβ42 by ELISA

Total tau (T-tau), phosphorylated tau (P-tau) and Aβ1-42 (Aβ42) concentrations in CSF were measured using INNOTEST ELISA methods (Fujirebio Europe). All samples were analyzed as part of a clinical routine, by board-certified laboratory technicians following strict procedures for batch-bridging, analyses and quality control of individual ELISA plates, as described preciously (Palmqvist et al., 2014) .

2.11. Statistical analysis

All data were analyzed using SigmaStat (Version 2.0; SPSS Inc.). A Student's *t* test (two-tailed) or Mann-Whitney U test were used for single pairwise comparisons, and determining the *p* values. Normal distribution was checked using the Kolmogorov–Smirnov test. Correlations were investigated using the Pearson correlation coefficient. The results are presented as means ±SEM and the correlations between variables were assessed by linear regression analyses.

3. Results

3.1. β-Secretase activity in human CSF

Biochemical analysis of β-secretase activity in CSF and other fluids often relies on the hydrolysis of fluorogenic substrates with modified APP β-cleavage sites, but these substrates do not discriminate between BACE1 and other β-secretase-like enzymes (Decourt and Sabbagh, 2011). In our preliminary analysis, estimating β-secretase activity in human CSF samples similarly that in previous reports, by the cleavage of a fluorogenic peptide substrate containing the BACE1 cleavage site, we found intra-assay coefficients of variation of ~4%, but inter-assay assays coefficients of 38%. To avoid great variation between assays, we performed the analysis of all the samples simultaneously. Also, we attempted to estimate the contribution of other proteases to the standard determination of β-secretase activity in human CSF by measuring activity in the presence 16 µM concentration of the β-secretase inhibitor III (Calbiochem), a concentration that is 3 times higher than the concentration needed to block entirely the proteolytic activity of solubilized membrane fractions from BACE1-transfected cells (Capell et al., 2002). Unexpectedly, we found that ~67% of the apparent β-secretase activity that was detectable in CSF samples was not inhibited in presence of the specific inhibitor (Fig 1A).

Next, we evaluated whether the activity of β-secretase is altered in our cohort of 19 AD samples and 19 age-matched non-AD controls (NADC) previously characterized for core AD biomarkers (Supplemental Table 1). Our data indicated that there was a mild (~13%) increase in β-secretase activity in the CSF of AD subjects, compared with NADC (p= 0.02; Fig. 1B), a level of increase that was small to the increase (~30 %) found for β-secretase activity attributable to BACE1, determined indirectly after subtracting activity in presence of the β-secretase inhibitor III (p=0.02; Fig 1C).

3.2. Characterization of BACE1 forms in human CSF

The previous studies suggested the presence of soluble full-length and truncated BACE1 species in the human CSF (Garcia-Ayllon et al., 2013; Holsinger et al., 2004). Therefore, in order to determine the possible co-existence of several species of BACE1 in human CSF, we used western blotting using antibodies that recognize different domains of BACE1 to examine CSF control samples to compare the profile of BACE1 species with those found in brain extracts. Fig. 2A shows a schematic representation of full-length BACE1 and the epitopes recognized by the antibodies.

An antibody raised against a synthetic peptide corresponding to the N-terminus of human BACE1 (residues 46-62), revealed bands of ~50 and 70 kDa in both brain extracts and CSF aliquots. In the CSF samples, the 70-kDa band was poorly resolved probably because it overlapped with albumin, but in some CSF samples the presence of a double band was observed (Fig. 2B). We obtained an improved separation of the 70-kDa doublet in albumin-immunodepleted CSF samples (Supplemental Fig. 1A). A second antibody (ectodomain) raised against a recombinant human N-terminal BACE1 ectodomain (residues 22-460), revealed the 70-kDa band, but not the 50-kDa band (Fig. 2B). A third antibody (propeptide) raised against a synthetic N-terminal peptide covering part of the prodomain (residues 26-45) of BACE1 detected only the 50 band (Fig. 2B). Finally, immunoblotting with two anti-C-terminus antibodies, B0806 and ab2077, both raised against a synthetic peptide corresponding to the C-terminus of human BACE1 (residues 485-501) detected both the 70-kDa and 50-kDa bands (Fig. 2B).

Based on the pattern of immunoreactivity with the different antibodies, and the apparent molecular mass of mature and immature BACE1 forms reported previously

(Capell et al., 2000;Gonzales et al., 2011;Holsinger et al., 2004;Wu et al., 2008), we attributed the 70-kDa band to the mature form of BACE1 lacking the prodomain, and the 50-kDa band to the immature form that retains the prodomain, but displaying incomplete glycosylation. Differences in the conformation of mature and immature forms may explain the lack of immunoreactivity of the 50-kDa form with the ectodomain antibody (Ermolieff et al., 2000). Based only on the immunoreactivity with different antibodies, we could not discriminate between truncated and full-length forms of BACE1. Although the N-terminus antibody indicated the existence of a doublet around 70-kDa and the C-terminus antibodies revealed that at least one of the 70-kDa bands should correspond to a mature full-length BACE1 species.

To ensure that the supposedly full-length BACE1 in CSF retained the intracellular C-terminal domain, we performed immunoprecipitation/western blotting assays. Human control CSF samples were immunoprecipitated using the C-terminus B0806 antibody and BACE1 was then detected with a second C-terminus antibody ab2077. The results demonstrated that both the 70-kDa and 50kDa bands in the pull-down fraction contained an intact C-terminus (Fig. 2C). Similar results were obtained when CSF samples were immunoprecipitated using the N-terminus antibody and detected with the C-terminus B0806 antibody (Fig. 2D). These results confirmed the presence of soluble mature (and immature) forms of BACE1 full-length in human CSF. Immunoprecipitation/western blotting assays of CHO cell-conditioned medium using the same antibodies, revealed a similar banding patter, confirming the existance of soluble full-length BACE1 (Supplemental Fig. 2).

Next, we performed a glycosylation analysis to determine whether the immature BACE1 band (~50 kDa) is glycosylated differently from the mature BACE1 band (~70 kDa). We compared the glycosylation pattern of five human control CSF sampes by

lecting-binding analysis with several inmobilized lectins. The carbohydrate-binding proteins known as lectins have very high binding specificity and are powerful tools for distinguishing differences in the carbohydrate composition of glycoproteins (Varki et al., 1999). For this purpose, we used several immobilized lectins and the lectin-sample interactions were then analyzed in the unbound fraction by western blotting with the two antibodies exclusive for mature (anti-ectodomain) and immature (anti-propeptide) forms. The 70-kDa BACE1 species bound more strongly to the mannose specific lectins, Con A, LCA and GNA than the 50-kDa BACE1 form (see the complete percentages of lectin binding in Table 1). These differences in the lectin binding properties confirmed a different pattern of glycosylation for the 70-kDa and 50-kDa BACE1 forms.

3.3. Characterization of BACE1 oligomers in CSF

We addressed the possibility that BACE1 species may form oligomers in human CSF samples by blue native-PAGE (Fig. 3A). Because the determination of the molecular mass of amphiphilic proteins by blue native-PAGE is complicated, we included a CSF sample denatured under reducing conditions to locate the electrophoretic migration of monomeric BACE1. We identified a major immunoreactive band of ~240 kDa with all the antibodies tested, which indicated that the CSF-BACE1 was present as higher molecular mass complexes. Weaker bands with still higher molecular mass were also identified at ~480 and ~720 kDa with the N-terminus and C-terminus antibodies, but were not immunoreactive with the propeptide antibody, indicating that mature BACE1 may be more predisposed to form large complexes in CSF than the soluble immature species.

Finally, to address the possibility that CSF-BACE1 complexes are heteromeric aggregates, control CSF samples were immunoprecipitated using an anti-propeptide

antibody, which recognizes exclusively the 50-kDa immature forms of BACE1. Forms were detected with an anti-N-terminus antibody. The presence of 70-kDa immunoreactive bands, corresponding to mature forms of BACE1 in the pull-down fraction, together with immature forms, demonstrated that oligomers of BACE1 are heteromeric complexes (Fig. 3B). Moreover, co-immunoprecipitation using a C-terminus antibody (B08006) and subsequent detection with the N-terminus antibody showed the presence of dimers of ~70 kDa (Fig. 3C), probably both full-length and also truncated species since blots resolved with the alternative C-terminal antibody ab2077 detected a unique immunoreactive band (Fig. 2C).

3.4. Levels of BACE1 forms in AD CSF

To assess whether the levels of different BACE1 forms are altered in AD CSF, we analyzed by western blotting the same 19 AD and 19 NADC samples previously assayed to determine the β-secretase activity and core AD biomarkers (Fig. 1B and Supplemental Table 1). Blots were resolved with the N-terminus, ectodomain, propeptide and C-terminus ab2077 antibodies that were used to characterize different BACE1 forms in CSF (Fig. 2B). No differences were detected between AD and NADC samples for the immunoreactivity levels of the immature 50-kDa band resolved with the propeptide antibody (Supplemental Fig. 3A), nor were differences observed in the blots resolved with the N- or C-terminus antibodies (Fig. 4A and 4B). The amount of the 50-kDa band correlated when levels were determined for all three antibodies, in both NADC and AD groups (multiple lineal regression NADC: R= 0.82; $p< 0.001$ / AD: R = 0.95; $p< 0.001$). Therefore, we assumed that the three antibodies probably detected a unique 50-kDa immature form of BACE1.

With respect to the immunoreactivity of mature BACE1 forms, we found that the levels of the 70-kDa form were higher (~73%; p= 0.04) in AD CSF compared to the CSF of NADC subjects, but only when the blots were resolved with the C-terminus antibody, thus revealing an increase in full-length forms in AD (Fig. 4B). In blots resolved with the N-terminus antibody, the doublet of ~70 kDa was poorly resolved in most of the samples, so the quantification was estimated as a single band. Considering a unique N-terminal immunoreactive band, a non-significant decrease (~30%; p= 0.19) in immunoreactivity was observed in the level of the 70 kDa band in the AD samples compared with the NADC samples (Fig. 4A). No significant changes were detected in the 70 kDa doublet-band when resolved with the ectodomain antibody (~10% decrease in AD compared with NADC, p= 0.39; Supplemental Fig. 3B). Indeed, no correlation was observed for the 70 kDa band resolved with C-terminus and N-terminus antibodies, neither in the NADC or AD groups (multiple lineal regression NADC: R= 0.40; p= 0.24 / AD: R = 0.32; p= 0.43).

Taking these results into account, we attribute this difference to the level of immunoreactivity of the ~70 kDa BACE1 form(s), resolved with C- or N-terminus antibodies, because the C-terminus antibody only detects the full-length form while the N-terminus detects both, full-length and truncated forms. However, even being unable to distinguish and quantify separately the doublet attributable to full-length and truncated forms, we could deduce that levels of truncated forms do not parallel levels of full-length BACE1 forms, at least in AD. In fact, when we defined a "70 kDa (C-terminus / N-terminus) quotient" for each sample, we obtained the best discrimination between NADC and AD groups (Fig. 4C).

We were also interested in knowing whether the activity of the β-secretase attributed to BACE1 correlated with BACE1 protein levels determined by western

blotting. As expected, no correlation was found between β-secretase activity that attributed to BACE1 and the level of immunoreactivity of the immature form resolved with the tree antibodies. This was the case for the NADC group (e.g. for the propeptide: R= 0.30; *p*= 0.21), and for the AD group (e.g. for the propeptide: R= 0.05; *p*= 0.84). Regarding the correlation between the β-secretase activity levels attributed to BACE1 and the immunoreactivity levels of the 70-kDa band of mature full-length BACE1 resolved with the C-terminus antibody, no correlation was found in the NADC group (R= 0.42; *p*= 0.07) or in the AD group (R= 0.35; *p*= 0.15). In addition, no correlation was found between the β-secretase activity levels attributed to BACE1, and the immunoreactivity levels of the 70-kDa band when resolved with the N-terminus antibody, which cannot discriminate full-length and truncated species, either for the NADC group (R= 0.07; *p*= 0.78), or for the AD group (R= 0.21; *p*= 0.40).

The amount of the 70-kDa band resolved with the C-terminus antibody correlated with age of the NADC subjects (R = 0.54; *p*= 0.017), but not of the AD subjects (R = 0.16; *p*= 0.51). No changes were associated with 70-kDa band resolved with the C-terminus with gender, and no correlations were observed with the classical AD biomarkers Aβ42, T-tau or P-tau (not shown).

4. Discussion

In this study, we characterized the forms of the BACE1 protein present in human CSF and the relative levels of these forms in relation to the β-secretase activity. This biochemical characterization may serve for a better evaluation of BACE1/β-secretase activity as a potential biomarker for AD. BACE1 has been considered as a promising candidate biomarker due to its role in the generation of Aβ and the demonstration that the β-secretase activity can be assayed in CSF and plasma (Gonzales et al., 2011;Holsinger et al., 2004;Manzine et al., 2016;Shen et al., 2018;Wu et al., 2008;Wu et al., 2012;Zetterberg et al., 2008). However, to date, little is known about the cell biology and biochemistry of BACE1 in CSF.

As in previous reports, we found a slight increase in β-secretase activity in CSF samples from subjects with AD, both when the total activity of β-secretase was measured and when only activity attributable to BACE1, as determined by measuring the activity in the presence of a specific BACE1 inhibitor, was measured. In fact, since most of the previous studies aimed at determining changes in the activity of the β-secretase in plasma or CSF have measured total activity, *i.e.*, activity in the absence of BACE inhibitors, this activity should be refer as β-secretase activity and not as BACE1 activity. Nonetheless, a surprising finding has been that a large proportion of the activity of the β-secretase present in the CSF was not attributable to BACE1. In this regard, a recent study revealed that the maximum inhibition of β-secretase activity in human plasma only reached ~80% (instead of 100% inhibition achieved with a recombinant protein), indicating the presence of other proteins in human plasma that interfere with the assay of β-secretase activity (Shen et al., 2018).

BACE1 and BACE2 are two highly homologous proteins that can process APP at the β-secretase site. Although both enzymes exhibit many of the characteristics

expected for β-secretase, it has been demonstrated that BACE1 is the major protease involved in cleavage of APP into the brain (Bennett et al., 2000;Hussain et al., 1999;Lin et al., 2000;Sinha et al., 1999;Vassar et al., 1999;Yan et al., 1999). However, BACE2 (Lin et al., 2000) and other sheddases such as cathepsin B (Tagawa et al., 1991) and meprin β (Bien et al., 2012), can also act as alternative β-secretases. All these enzymes can cleave fluorogenic APP-derived peptides employed as substrates to determine BACE1 activity in CSF. In fact, while proteases of the "a disintegrin and metalloprotease" (ADAM) and BACE families are widely considered as sheddases, it has been shown that a much wider range of proteases, including intramembrane and soluble proteases, catalyze similar cleavage reactions (Andrew et al., 2016;Lichtenthaler et al., 2018).

Other studies have avoided the issue of interference by enzymes other than BACE1 in the determination of CSF β-secretase activity by using enzyme-linked immunosorbent assay (ELISA) methods to first capture the BACE1 protein and then to measure activity (Ewers et al., 2008;Zhong et al., 2007). Despite this being a more appropriate approach, we and others (Zhong et al., 2007) have found that that β-secretase activity and BACE1 protein levels do not correlate well in CSF.
The lack of correlation between protein levels and activity may be related to the presence of mature and immature forms of BACE1, as well as to the fact that all the soluble species are capable of forming heteromeric aggregates, which may influence immunodetection. Early studies have indicated that in human brain tissue BACE1 occurs as a dimer (Jin et al., 2010;Schmechel et al., 2004;Westmeyer et al., 2004), or as an oligomer (Liebsch et al., 2017). Therefore, the presence of heteromeric aggregates of BACE1 in CSF is not an unpredictable finding due to the existence of protein domains that favour homophilic interactions. Methodologically, the nature of the antibodies, with

different epitope specificities, and the potential variability in the composition and stoichiometry of the BACE1 aggregates, that may hinder the access of the antibodies, could compromise their determination by ELISA. In this regard, we previously demonstrated that quantification of CSF-sAPPα and sAPPβ by ELISA is affected by assembling of APP into heteromers (Cuchillo-Ibanez et al., 2015).

How BACE1 reaches the CSF or the functional significance of the soluble β-secretase activity are not currently known. As previously suggested, active secretion is unlikely, and it is still unclear whether passive release from brain cells or neuronal death could be important contributing factors (Barao et al., 2013); in this sense, the presence of immature forms of BACE1 in CSF and in culture cell media is intriguing. Moreover, BACE1 has also been identified along with other secretases in human plasma brain-derived exosomes (Goetzl et al., 2016), so an exosomic contribution of CSF-BACE1 cannot be ruled out. Whether different forms of BACE1 reach the CSF by different mechanisms is relevant to the interpretation of changes in levels and the potential of BACE1 as a biomarker.

Another important question is whether the β-secretase activity attributable to BACE1 in CSF arises from a full length and/or a truncated form of the protein, and if both forms express similar enzymatic properties. Earlier studies suggested that BACE1 may be shed from the plasma membrane resulting in the release of its large catalytic ectodomain into the extracellular milieu (Murayama et al., 2005;Verheijen et al., 2006;Wu et al., 2008). Consequently, subsequent studies assumed that truncated BACE1 forms were responsible for the activity of β-secretase in CSF. BACE1 has been identified as a ~54 kDa transmembrane glycoprotein with heterogeneous oligosaccharides that contribute to the apparent molecular weight of ~70 kDa of the mature form of BACE1 (Hampel and Shen, 2009). The increased maturation of BACE1

contributes to the increase in the enzymatic activity of BACE1, so we can expect that only the mature form expresses activity.

There are no data available on the enzymatic activity of the truncated species of BACE1, nor are there data on the capacity of the soluble mature full-length forms in human CSF to act as enzymatically active species, outside their natural membrane environment. In this regard, due to the existence of heteromeric complexes compromising the several species present in the CSF, we have not been able to analyze separately the activity of the β-secretase of the full-length and truncated forms. Nevertheless, the effect of the membrane lipid microenvironment on secretase cleavage of substrates appears critical. For example, increasing evidence indicates that the physicochemical properties of the lipid bilayer govern the sheddase function of ADAM-proteases (Reiss and Bhakdi, 2017), as well of the γ-secretase (Holmes et al., 2012). Thus, it is reasonable to hypothesize that truncated BACE1 and/or soluble full-length BACE1 forming heteromeric complexes may display a distorted conformational state which affects β-secretase activity. The characterization of the catalytic activities of the individual CSF-BACE1 species is needed.

Regarding the determination of protein levels, when using the electrophoresis/western blot analysis, we were not able to obtain an adequate separation that allowed us to determine and quantify mature full-length and truncated forms separately with N-terminus antibodies, a common epitope on both species. This difficulty could be due to proximity, after electrophoretic separation, of albumin. Preliminary attempts to use albumin-immunodepleted CSF samples for BACE1 analysis showed low reliability and reproducibility for using this strategy as a preparative approach for further western blot quantification. Moreover, to our knowledge, there are no pan BACE1-specific antibodies that recognize the C-terminus of the truncated

protein. Hence, we only estimated individual full-length species with antibodies directed to the unprocessed C-terminus, and assumed that the quantification with N-terminal antibodies was indicative of the measured the sum of both the full-length and truncated forms. Interestingly, the 70-kDa full-length mature species, resolved with a C-terminus antibody, is the only form for which there was an increase in the AD samples, whereas the co-determination of full-length and truncated mature species displayed no significant changes. We can surmise, but not prove, that the truncated species do not increase in parallel to the mature full-length BACE1 forms. Interestingly, the 70 kDa (C-terminus/N-terminus) quotient displayed the largest differences between AD and NADC groups.

Also in the context of the existence of different BACE1 species in regard of the β-secretase activity, we can hypothesize that most of the β-secretase activity in CSF attributed to BACE1 is due to mature full-length BACE1, but we failed to demonstrate a correlation. Both, increases in BACE1 protein and in β-secretase activity have been demonstrated in AD brain, but lacking correlations (Fukumoto et al., 2002). We cannot discard differences in the kinetics of CSF-BACE1 in AD subjects respect to non-disease subjects, as previously suggested (Stockley et al., 2006). Whether BACE1 protein levels or BACE1/β-secretase activity levels in CSF is the more accurate indicator of brain changes during AD progression is still unknown.

In conclusion, the precise determination of BACE1/β-secretase in CSF remains as a great challenge. In view of the evidence of different forms of the enzyme and their assembly into heteromers, much work remains to be achieve for reinforcing the interest of using CSF-BACE1 protein levels and/or β-secretase activity levels as biomarkers of AD, as well to design appropriate methodologies for their analysis.

Disclosure statement

The authors have no competing interests to disclose in relation to this article.

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Figure legends

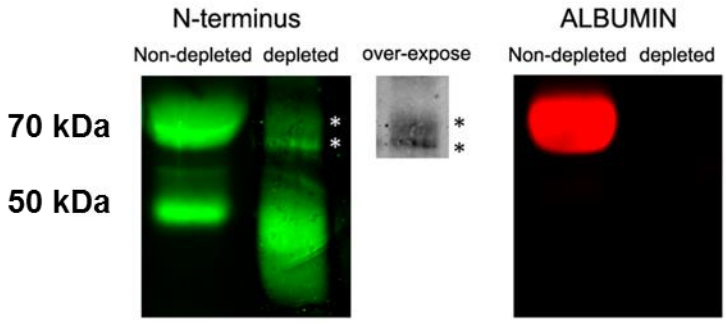
(the final version of the figures is not ready, so labels, * and figure will change)

Figure 1. **Detection of β-secretase activity in human CSF.** The activity of β-secretase was measured using a fluorogenic peptide substrate containing the BACE1 cleavage site. **(A)** β-Secretase activity was detectable in human CSF [the mean values for 19 age-matched non-AD controls (NADC) and 19 AD are represented] in the absence (- BSI) and presence (+ BSI) of the β-secretase inhibitor III at 16 µM. **(B)** β-Secretase activity levels in the CSF of 19 AD subjects (open symbol) compared with 19 NADC (closed symbol). **(C)** β-secretase activity levels attributable to BACE1 estimated in the 19 AD and 19 NADC cases after subtracting the activity in the presence of the β-secretase inhibitor III. The results are presented as means ± SEM. *p* values are indicated.

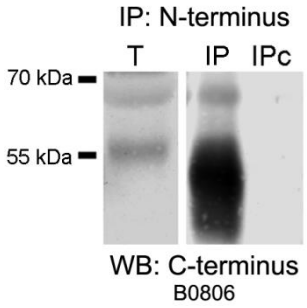
Figure 2. **Different BACE1 species are present in human CSF. (A)** Schematic representation of transmembrane type I BACE1 protein and of the epitope region in the different protein domains recognized by the antibodies used in this study [the domains represented are the signal peptide (SP), the pro-domain (PD), the transmembrane domain and the cytoplasmatic domain (CD)]. **(B)** Western blotting of the cortical extract (Cx) obtained from human brain and CSF samples (non-disease control cases are represented), resolved with the indicated anti-BACE1 antibodies. The existence of a ~70-kDa dimeric band detected with N-terminus antibodies is indicated (*). **(C) (D)** Control CSF aliquots (Total, T) were immunoprecipitated using the anti-BACE1 antibody indicated, and precipitated proteins (IP) were immunoblotted with an alternative ani-BACE1 antibody, as indicated. No bands were observed in the absence of capture antibody (IPc).

Figure 3. **Characterization of BACE1 complexes**. **(A)** BACE1 complexes from human control CSF samples were analyzed by blue native-PAGE. Incubation of blots with antibodies specific for the different BACE1 epitopes confirmed that BACE1 associates into complexes. An aliquot of each sample was denatured by boiling at 95°C for 5 min (dn) and also analyzed to determine the migration of the monomeric BACE1 band. **(B)** Human control CSF aliquots (Total, T) were immunoprecipitated using the propeptide antibody (exclusive for immature species of ~50 kDa; see Fig. 2B) and precipitated proteins (IP) were immunoblotted with the N-terminus antibody. The presence of 70-kDa immunoreactive bands (*), corresponding to mature forms of BACE1, demonstrated that different BACE1 species can form complexes. In the absence of a capture antibody, no bands were observed (IPc). **(C)** Human control CSF aliquots (Total, T) were immunoprecipitated using the C-terminus antibody B0806 and precipitated proteins (IP) were immunoblotted with the N-terminus antibody. The presence of a two bands of ~70 kDa (*) indicated that other N-terminal BACE1 immunoreactive band that the full-length specie is co-precipitated and present in the IP complexes. In absence of capture antibody no bands were observed (IPc).

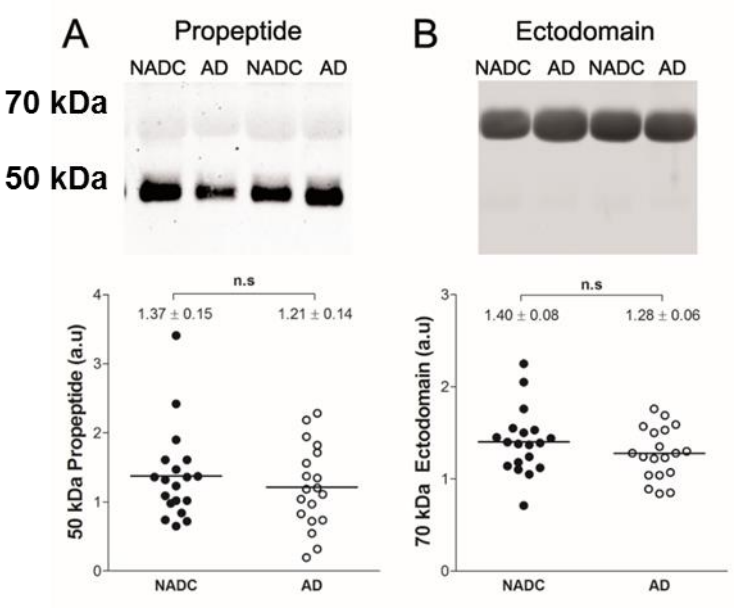
Figure 4**. Levels of BACE1 in AD CSF.** Representative western blots of human CSF samples from NADC (closed symbol; n=19) and AD (open symbol; n=19) using N-terminus **(A)** and C-terminus ab2077 **(B)** antibodies. The densitometric quantification of the 50 and 70-kDa bands are shown. **(C)** Graph of the quotient obtained by dividing the level of immunoreactivity of the 70-kDa band resolved with the C-terminus antibody by the level of immunoreactivity the 70-kDa band resolved with the N-terminal antibody [70 kDa (C-terminus / N-terminus)], for each sample. The data represent the means ± SEM (calculations performed in duplicate). *p* values are indicated; n.s.: non-significant *p* value.



***Supplemental Figure 1*. Albumin interferes in the electrophoretic migration of the 70-kDa BACE1 species of CSF-BACE1.** Representative western blot of human CSF sample before (non-depleted) and after albumin immunodepletion (depleted) using the antibodies anti-BACE1 N-terminus (green) and anti-albumin (red). When albumin is not present in the CSF (depleted), a double BACE1-immunoreactive band can be observed at ~70 kDa (*). The 70-kDa doublet-bands are also shown in an over-exposed image.



***Supplemental Figure 2*. Mature and immature full-length form of BACE1, retaining the C-terminal domain are present in the conditioned medium of CHO cells.** CHO cell-conditioned medium (Total, T) were immunoprecipitated using the N-terminus antibody and the precipitated proteins (IP) were immunoblotted with the C-terminus antibody B0806. No bands were observed in the absence of capture antibody (IPc).



***Supplemental Figure 3*. Levels of BACE1 in AD CSF.** Representative western blots of human CSF samples from NADC (closed symbol; n=19) and AD (open symbol; n=19) using the propeptide **(A)** and the ectodomine **(B)** antibodies (same samples analyzed in Fig. 4). The densitometric quantificationa of the 50-kDa band (propeptide) and 70-kDa band (ectodomain) are shown. The data represents the means ±SEM. Calculations were performed in duplicate. n.s.: non-significant *p* value.

Tables

	ConA	LCA	GNA	PHA	WGA
70 kDa BACE1 (ectodomain)	75±9 (51-100)	81±6 (70-100)	80±5 (67-95)	59±8 (59-100)	71±6 (54-93)
50 kDa BACE1 (propeptide)	44±4 (31-56)	57±4 (45-64)	63±6 (43-74)	67±8 (48-93)	65±4 (50-79)
***p* value**	0.015	0.024	n.s.	n.s.	n.s.

***Table 1*. Glycosylation of BACE1 species in CSF.** Five non-pathological CSF and samples were incubated with immobilized lectins Con A (*Canavalia ensiformis*), LCA (*Lens culinaris*), GNA (*Galanthus nivalis*), PHA (*Phaseolus vulgaris*) and WGA (*Triticum vulgaris*). The unbound BACE1 was assayed in the supernatant fraction by western blotting (blots not shown) to compare differences in lectin binding between 70-kDa mature (detected with the ectodomain antibody exclusive for the 70-kDa band; see Fig. 1B) and the 50-kDa immature (detected with the prodomain antibody). The data represent the percentages of each non-lectin-bound BACE1 specie, calculated as the ratio of non-lectin-bound BACE1 immunoreactivity to total BACE1 immunoreactivity (non-incubated with lectins). The data represents the means ±SEM; the ranges of values for each variable are also indicated. *p* value was calculated comparing percentages of the mature 70-kDa BACE1 unbound to a concrete lectin with percentages of the immature 50-kDa BACE1 unbound to the same lectin, as assessed by the Student's *t* test (n.s.: non-significant *p* value).

	Age (years)	Gender	CSF Aβ42 (ng/L)	CSF T-tau (ng/L)	CSF P-tau (ng/L)
NADC	72±2	11M/8F	758±35	232±15	36±2
AD	72±21	5M/14F	404±18*	731±69*	94±9*

Supplemental Table 1: **Demographic data and core AD CSF biomarkers.** Patients were designated as NADC or AD according to CSF biomarker levels using cutoffs as described in the Material and Method section. F= female; M= male. The data represent the means ±SEM. *Significantly different ($p< 0.001$) from the NADC group.