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Profiles of β-Amyloid Peptides and Key Secretases in Brain Autopsy Samples Differ with Sex and *APOE* ε4 Status: Impact for Risk and Progression of Alzheimer Disease

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Abstract—The *APOE* ε4 allele was originally reported to contribute to risk of Alzheimer's disease (AD) in women, yet male and female AD patient-derived data are routinely pooled. Histopathological hallmarks of AD include neurofibrillary tangles centered on hyperphosphorylated Tau and plaques composed of the β-amyloid (Aβ) peptide that is derived by sequential secretase-mediated cleavage of the Amyloid Protein Precursor (APP). We chose to examine profiles of Aβ(1–40), Aβ(1–42), and N-truncated (*i.e.,* p3-related) fragments in the plaque-associated fraction of autopsied cortical and corresponding hippocampal samples from donors with a diagnosis of early-onset (EOAD) and late-onset (LOAD) AD. Levels of Aβ(1–40), Aβ(1–42), and the p3 fragment-enriched pool were increased in EOAD and LOAD samples, and correlated well within –but not between– regions. Counterintuitively, these increases were similar regardless of the AD donor's *APOE* ε4 status. Focusing on the donor's sex and *APOE* ε4 status as nominal variables (*i.e.,* omitting diagnosis from the stratification) revealed that increases in Aβ peptides were specific to female carriers of the ε4 allele and correlated with the proportional expression of BACE1/β-secretase and ADAM10/α-secretase in the cortex and with nicastrin (γ-secretase) expression in the hippocampus. These data preliminarily support the possibility that AD follows distinct amyloidogenic processes in males and females, and that the *APOE* ε4 allele exerts a major influence on the disease process, particularly in women. This knowledge could significantly impact the (re)interpretation of unsuccessful outcomes of clinical interventions targeting either Aβ peptides directly or the secretases implicated in APP processing.

Key words: APOE e4 allele, gender-risk, amyloid plaque, secretase, Alzheimer disease.

INTRODUCTION

Alzheimer disease (AD) is identified by overt behavioral and cognitive phenotypes that are thought to reflect pathology centered on the hippocampal formation and associated structures (West, 1993). Unfortunately, symptoms invariably present at an advanced stage of disease progression that precludes effective intervention (Prins et al., 2010). Risk factors for AD-related dementia, including sex, advancing age, and/or genetics (involving either autosomal mutations or allelic variations) are helping to identify modifiable events within the earlier stages of AD progression.

The 'β-amyloid cascade hypothesis', which champions β-amyloid (Aβ) as a causative factor in AD, grew out of observations such as the age-dependent cognitive deficits and AD-like pathology in Down's Syndrome patients (triplication of the gene for APP; Amyloid Protein Precursor) (Glenner and Wong, 1984)

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Abbreviations: AD, Alzheimer disease; Aβ, β-amyloid; ADAM10, A disintegrin and metalloproteinase domain-containing protein 10/α-secretase (brain); APP, Amyloid Protein Precursor; *APOE* ε4, Apolipoprotein, allele epsilon4; BACE1, beta-site amyloid precursor protein cleaving enzyme 1/β-secretase; bp, base pair; BSA, bovine serum albumin; CHSA, Canadian Study of Health and Aging; CSF, cerebrospinal fluid; CTL, control; DS, Down's Syndrome; ELISA, enzyme-linked immunosorbent assay; EOAD, early-onset AD; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; IB, immunoblotted; ID, immunodepleted; IP, immunoprecipitated; LOAD, late-onset AD: p3: the 3 kDa C-terminal fragment of the Aβ peptide, usually resulting from γ-secretase-mediated cleavage of the C83/α-secretase fragment of APP; PS-1, presenilin-1 (catalytic core of the γ-secretase complex); sAPPα, soluble APP-α; sAPPβ, soluble APP-β; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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as well as subsequent reports on the link between autosomal dominant mutations in the genes coding for either APP (Citron et al., 1992) or presenilins (the catalytic core of the γ-secretase complex) (Rogaev et al., 1995; Sherrington et al., 1995), and aggressive, familial forms of AD-related amyloidosis. Histopathological confirmation of a diagnosis of AD continues to rely on evidence of amyloid plaque burden (Glenner and Wong, 1984) and is supported by evidence of insoluble neurofibrillary tangles that consist primarily of hyperphosphorylated Tau (Iqbal et al., 2010). Although there is substantial interindividual variability in terms of rate of disease progression, the amyloid and tauopathy appear to develop in a predictable manner. Indeed, amyloid burden tends to arise first in cortex and spread to the hippocampus, while neurofibrillary tangle pathology originates in the hippocampus and spreads through the entorhinal cortex into the neocortex (Oddo et al., 2003; Braak and Del Tredici, 2015).

The Aβ peptide is derived from sequential cleavage of APP by β- and γ-secretases, and Aβ species of varying lengths are generated *via* imprecise γ-secretase cleavage. It is the longer, more hydrophobic Aβ(1–42) (Placanica et al., 2009) (*vs*. the physiological Aβ(1–40)) that is more likely to form toxic, soluble oligomeric intermediates before aggregating as the insoluble plaque in AD brains (Lemere et al., 1996). The diminishing cerebrospinal fluid (CSF) ratio of Aβ(1–42)/Aβ(1–40) –purported to reflect the retention of Aβ(1–42) in the brain in the form of the plaque– is thought to have a greater predictive value for AD progression than that afforded by levels of individual peptides (discussed in (Hoglund et al., 2008)). Alternatively, APP can be initially cleaved between residues 16 and 17 within the Aβ sequence by α-secretase/ADAM10, which precludes the generation of an intact Aβ fragment from that specific APP molecule. Physiological roles for APP –such as synaptic maintenance (Yang et al., 2005) and memory retention (Senechal et al., 2008)– would rely on a delicate balance between these secretases. ADAM10 activity has been reported to both increase (Bernstein et al., 2003) and decrease (Colciaghi et al., 2002) with age/AD, but it is an increase in BACE1 (Vassar et al., 1999; Fukumoto et al., 2002), the rate-limiting enzyme in APP processing, as well as increased activity of γ-secretase (Placanica et al., 2009) that is thought to drive amyloidogenic APP cleavage in the aging and AD brains.

While age does remain the predominant risk factor for AD and associated cognitive decline (Richard et al., 2012; Zahodne et al., 2016), one's biological sex is also a putative risk factor for AD, although the risk of AD in women is not consistent with a post-menopausal loss of the neuroprotective hormone, estradiol (Hogervorst et al., 2000). A 1994 Canadian Study of Health and Aging (CHSA) report estimates a female/male ratio of 2.7 in AD (McDowell et al., 1994), whereas a more recent CHSA report (Lindsay et al., 2002) and the Framingham study (Bachman et al., 1993) find no sex difference in AD. It is important to note that there is a similar prevalence in males and females in the early stages of AD, but a strong female prevalence reported in severe cases; thus, gender-risk might be similar, but disease progression might be exacerbated in males, who might die sooner after their AD becomes severe (Aguero-Torres et al., 1998; Hy and Keller, 2000).

Several novel genetic factors associated with brain amyloid deposition and the sporadic, late-onset form of AD (LOAD) have been identified recently (Hollingworth et al., 2011; Naj et al., 2011). However, *APOE*, specifically the ε4 allele, remains the most robust genetic risk factor to influence brain amyloidosis in LOAD patients (Poirier et al., 1993; Tanzi, 2012). Although the original studies revealed that a single ε4 allele could increase the risk of AD fourfold in women, but had little risk in men (Poirier et al., 1993; Payami et al., 1996; Farrer et al., 1997; Bretsky et al., 1999), reviews on the topic –discussed in (Altmann et al., 2014)– as well as clinical AD research, in general, continue to view male and female *APOE* ε4 carriers as having equal risk. It is not clear whether the ApoE4 gene product alters risk *via* an influence on Aβ peptide clearance/turnover (Patterson et al., 2015), but it is clear that the influence of ApoE4 is not necessarily generalizable across neurodegenerative processes. For example, a large population study did not find any differences between *APOE* genotypes in Parkinson's disease patients and neurologically normal controls (Federoff et al., 2012).

Studies of conditioned cell culture medium, CSF samples, and insoluble isolates from AD brain have revealed numerous Aβ species with extensive amino (N)- and carboxy (C)-terminal heterogeneity. Aβ(1–40) and Aβ(1–42) represent the major species, but isolates also include diverse pools of C-truncated peptides (*e.g.,* the 28- to 39-mers) (Seubert et al., 1992; Suzuki et al., 1994; Wang et al., 1996) as well as N-truncated Aβ peptides, including the p3 fragment, *i.e.*, Aβ(17–40/42) (Kummer and Heneka, 2014). Reports based on ELISAs in this context often refer to Aβ peptide fragments of unspecified lengths –for example Aβ(*x*–42), Aβ(11–*x*), Aβ(1–4*x*)– because of an inability of the ELISA(s) to effectively discriminate the myriad N- and C-terminally modified variants.

Based on these collective notions, we designed a sequential immunoprecipitation protocol supported by the Urea/SDS–PAGE system (Wiltfang et al., 1997) so as to visualize the expression pattern of Aβ and p3-related peptides in autopsied early-onset AD (EOAD) and late-onset AD (LOAD) cortical samples and the corresponding hippocampal samples. Our analyses reveal significantly higher levels of Aβ peptides and the p3-enriched pool of fragments in female carriers of the *APOE* ε4 allele, regardless of their diagnosis. Changes in expression of key secretases implicated in APP cleavage also display distinct patterns of expression between the sexes and the regions, with an inferred influence by the ε4 allele. This strongly suggests different amyloidogenic processes in the male and female brain, and can be extrapolated to suggest that secretase-mediated interventions might benefit one sex, while exerting little or no effect on the other sex. This must be considered when developing biomarker profiles and intervention strategies for the earliest phases of the disease process.

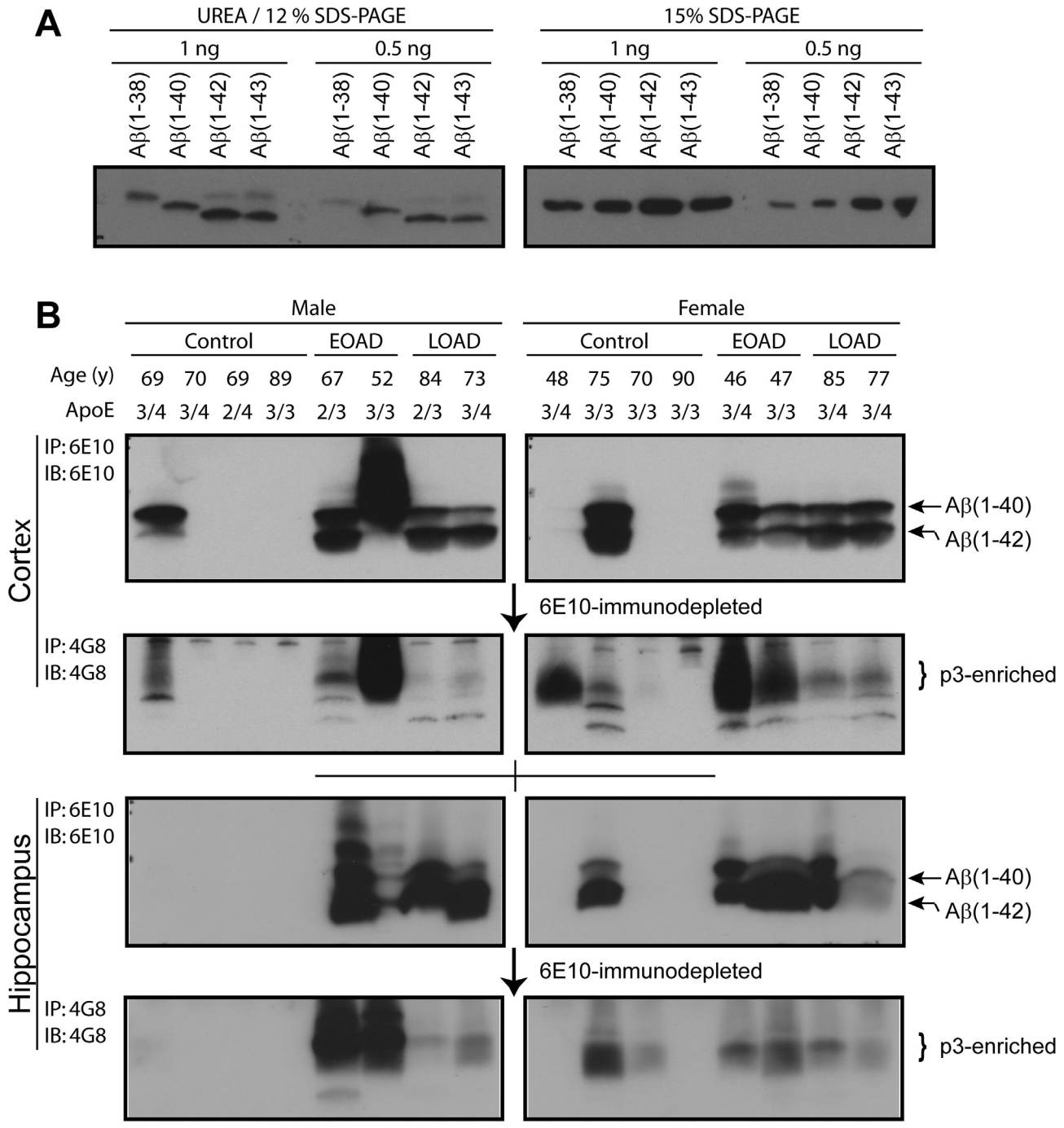

Fig. 1. Immunoblots for Aβ peptides and p3-related fragments isolated from human cortical and hippocampal extracts. (A) Comparison of synthetic Aβ(1–38), Aβ(1–40), Aβ(1–42), and Aβ(1–43) peptides resolved on (*left*) an 8-M Urea/SDS–PAGE gel and (*right*) a standard SDS–PAGE gel. (B) Representative gels from resolved 6E10-immunoprecipitates immunoblotted for 6E10, and 4G8-immunoprecipitates immunoblotted for 4G8 in cortical (*upper panels*) and corresponding hippocampal (*lower panels*) samples. The experiment included control samples as well as samples from male and female donors diagnosed with Early-Onset AD (EOAD) or Late-Onset AD (LOAD). Respective ages and *APOE* genotypes are indicated, with carriers (*i.e.,* 2/4, 3/4) and non-carriers (*i.e.,* 2/3, 3/3) of the ε4 allele being represented. Bands corresponding to Aβ(1–40), Aβ(1–42), and to the N-truncated/p3 fragment-enriched pool are identified.

EXPERIMENTAL PROCEDURES

Human brain samples

The use of autopsied tissues in this study is covered by the University of Saskatchewan's Research Ethics Office *Certificate of Approval* 'Bio 06-124' (PI: Mousseau).

Sixty male and female (M/F) samples matched as close as possible for age and sex were obtained from the Douglas-Bell Canada Brain Bank (McGill University, Canada). These included 26 controls (12 M/14F), 16 early-onset/EOAD (*i.e.,* age of onset < 65 years: 7 M/9F), and 18 LOAD (*i.e.,* age of onset 65 + years: 8

M/10F) (donor summaries are available under 'Publications' at: http://researchers.usask.ca/darrell-mousseau/). Cortical samples corresponded to a mix of superior and middle frontal cortices (Brodmann Areas 9/46, respectively). These areas are associated with executive function and cognition, and show clear evidence of relative hypoperfusion in AD patients (Levy-Cooperman et al., 2008). Histopathological diagnoses were based on staining with H&E, modified Bielschowsky, and alkaline Congo red by on-site pathologists. All AD donors had both a clinical and a neuropathological diagnosis according to the CERAD criteria. Regional variation was explored by comparing these cortical samples with, if available, the corresponding hippocampal samples from each donor. Our hippocampal set contained 18 controls (5M/13F), 15 early-onset/EOAD (7M/8F), and 18 LOAD (8M/10F).

Antibodies and reagents

The 6E10 antibody [targets Aβ(1–16): cat# SIG-39320], the 4G8 antibody [targets Aβ(17–24): cat# SIG 39220] were obtained from Cedarlane Laboratories Ltd. The anti-mouse/anti-rabbit IgG-HRP conjugates were obtained from Bio-Rad Laboratories (Canada) Ltd. The 22C11 antibody [recognizes amino acid residues 66–81 of APP: cat# MAB348], the anti-ADAM10 antibody (*i.e.,* α-secretase: cat# AB19026], and the antibodies raised against two components of the γ-secretase complex, *i.e.,* presenilin-1 [loop region: cat# MAB5232] and Nicastrin [cat# MAB5556], were obtained from Millipore. The anti-BACE1 (*i.e.,* β-secretase: cat# D10E5) antibody was obtained from Cell Signaling Technology and the antibody raised against the C-terminal region of human APP695 (amino acids 676–695: cat# A8717) was obtained from Sigma–Aldrich. Protein-A/G Sepharose and the enhanced chemiluminescence kit were obtained from GE Healthcare Bio-Sciences Inc. All other reagents were obtained from standard commercial sources.

APOE genotyping

APOE restriction isotyping for two non-synonymous single-nucleotide polymorphisms –*i.e.,* rs429358 (APOE-C112R) and rs7412 (APOE-R158C)– was based on a combination of PCR amplification and electrophoretic analysis of restriction fragments on a non-denaturing polyacrylamide gel system (Hixson and Vernier, 1990). Genotyping identified ε3/ε3 and ε4/ε4 homozygotes as well as ε2/ε3, ε2/ε4, and ε3/ε4 heterozygotes, with the frequency of ε4 carriers across cases corroborating previous reports (Poirier et al., 1993). The rare ε2/ε2 homozygote was not represented in our sample set.

PCR amplification was performed on 500 ng of genomic DNA using Platinum *Taq* (Qiagen) and the following primer pair: (F) 5′-CAC GGC TGT CCA AGG AGC TGC-3′ and (R) 5′-GCC CCG GCC TGG TAC ACT GCC A-3′. Each reaction mixture (50 μL, containing 10% DMSO) was first denatured at 94 °C for two minutes and then subjected to 35 cycles of amplification through 94 °C (30 s), 60 °C (30 s) and 72 °C (30 s). A final elongation step (72 °C; 5 min) was included. This protocol yielded a 226-bp amplicon.

Restriction isotyping involved mixing 10 μL of the PCR reaction mix with 10 U of *Afl*III and 10 U of *Hae*II in the appropriate NEB buffer containing 1.5% BSA and incubating overnight at 37 °C. The restriction fragments were resolved on a 10% non-denaturing, polyacrylamide gel and electrophoresed (50 V; 180 min). DNA

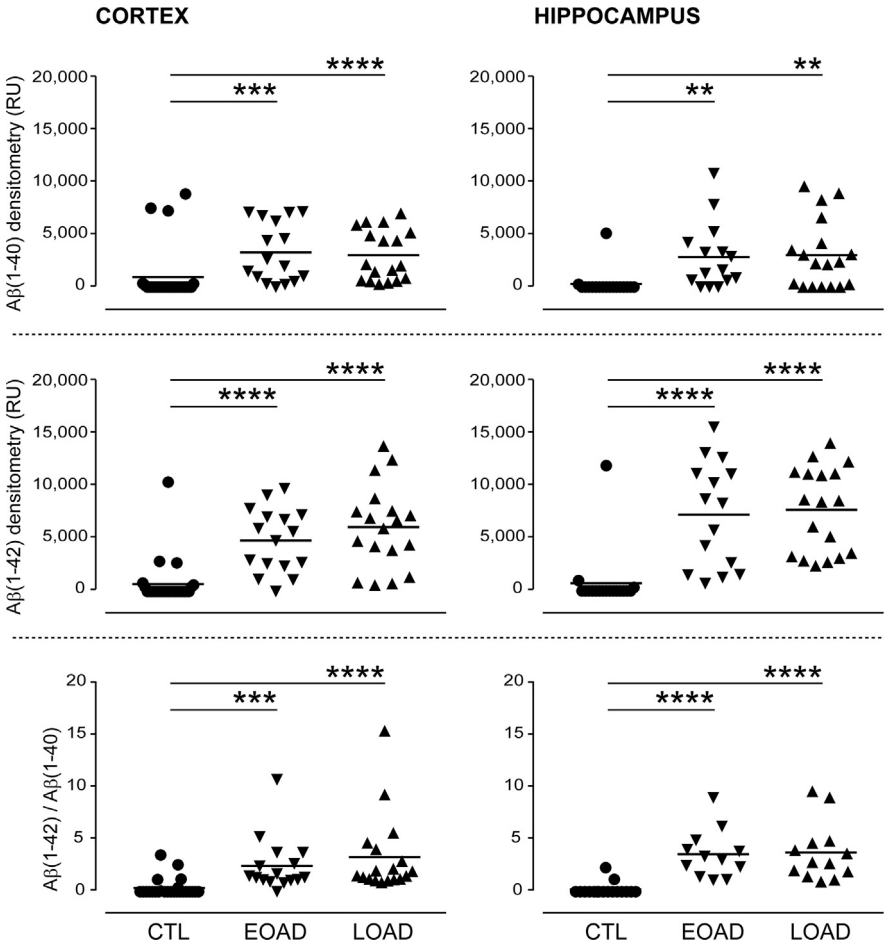

Fig. 2. Densitometric analyses of Aβ peptides fragments. The levels of Aβ(1–40) and Aβ(1–42) were determined in control (CTL: $n = 26$), Early-Onset AD (EOAD: $n = 16$), and Late-Onset AD (LOAD: $n = 18$) samples by densitometric analysis of probed western blots. Levels from (*left*) pooled (male + female) cortical samples and (*right*) corresponding 18 CTL, 15 EOAD, and 18 LOAD hippocampal samples are shown. The ratio of Aβ(1–42)/Aβ(1–40) in each sample is included. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ between indicated groups.

fragments were visualized by staining with GelRed (Biotium) (30 min; room temperature with agitation).

Immunodetection and immunoprecipitation

Samples (20–30 mg wet weight) were homogenized in 20 volumes of ice-cold RIPA buffer and then centrifuged at 12,000×*g* (10 min; 4 °C). An aliquot of the RIPA supernatant was used for protein determination based on the Lowry (Folin–Ciocalteu reagent) assay (Lowry et al., 1951). This fraction, the *soluble* fraction, was used for standard SDS–PAGE immunodetection of full-length-APP (fl-APP) and for the C-terminally truncated soluble APP alpha (sAPPα) and sAPPβ fragments as well as the major secretases involved in APP processing, *i.e.,* BACE1/β-secretase, ADAM10/α-secretase, and PS-1/γ-secretase. The proteins (15–20 μg protein/lane) were resolved on standard 10% or 12% SDS–PAGE systems (Wei et al., 2012).

After centrifugation of the homogenized tissue lysate, the corresponding pellet was dissolved in 5 M guanidine·HCl (1:20, wt:vol) by rocking at room temperature for 2 h. This was then diluted with TBS (1:1, vol:vol) and Aβ peptides were separated by sequential immunodepletion and immunoprecipitation strategies based on 300 μg of input protein. This *insoluble* fraction was used first to immunoprecipitate Aβ (1–40/42) [using the 6E10 antibody, which targets an intact Aβ(1–17) sequence] and the resulting 6E10-immunodepleted supernatant was then used to isolate any N-truncated/p3 fragment-enriched pool [immunoprecipitated using the 4G8 antibody, which targets Aβ(17–24)]. The Aβ-enriched and the p3-enriched pools of peptides were resolved on a discontinuous 8 M urea gel system (Wiltfang et al., 1997).

Protein bands were detected using enhanced chemiluminescence and densitometric analysis of scanned blots was performed by multiple individuals using ImageJ 1.32j. Immunoblotting conditions were optimized and initial examinations of our protein expression levels were done on selected subsets of our samples, cortical as well as hippocampal. For final densitometric analyses, all samples from cortex and, whenever possible, all hippocampal samples were run concurrently. This allowed for semi-quantitative comparison between the two regions. In the event that the two sets, *i.e.,* cortex and hippocampus, were scheduled to be run on separate days, several random cortical samples were included with the hippocampal set and allowed for factoring of day-to-day differences in exposure times, immunoblotting variability, and any densitometric differences.

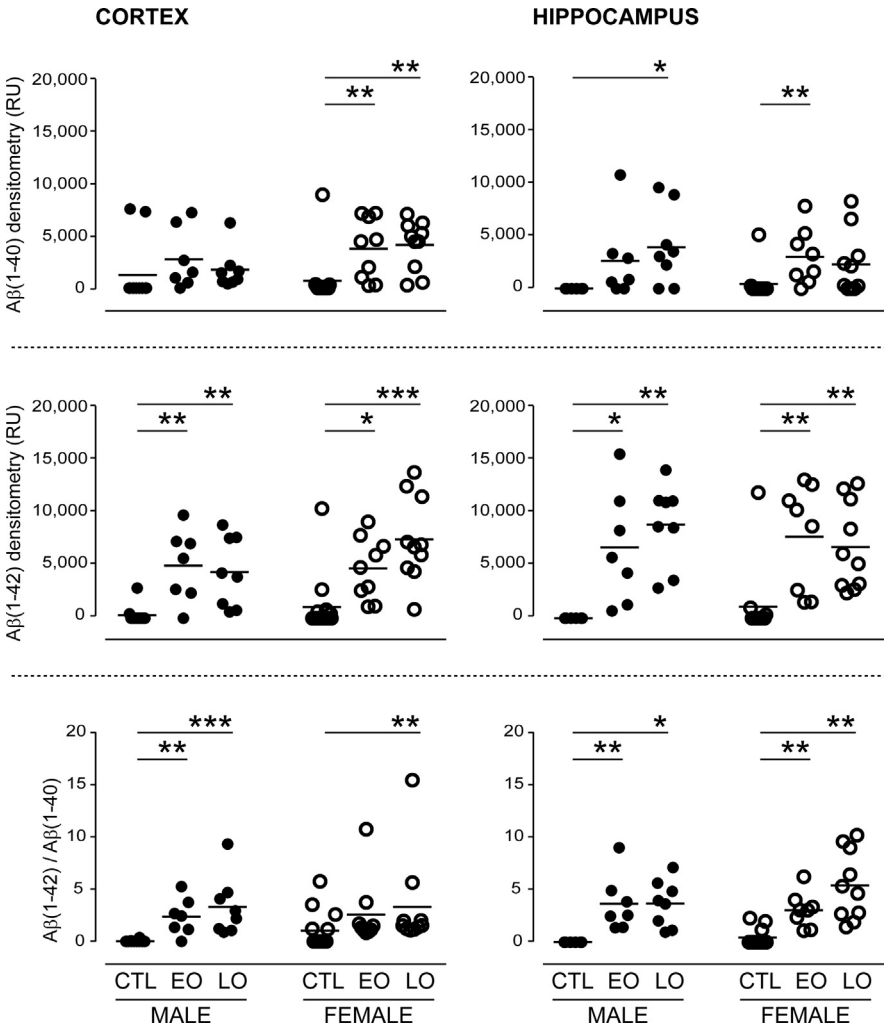

Fig. 3. Densitometric analyses of Aβ peptides fragments separated by sex of the donor. The data from Fig. 2 were separated by the sex of the donor. Cortical male samples represent control (CTL: $n = 12$), and Early-Onset (EO: $n = 7$) and Late-Onset (LO: $n = 8$) AD, while cortical female samples represent CTL ($n = 14$), and EO ($n = 9$) and LO ($n = 10$) AD. Hippocampal male samples represent control (CTL: $n = 6$), and Early-Onset (EO: $n = 7$) and Late-Onset (LO: $n = 8$) AD, while hippocampal female samples represent CTL ($n = 12$), and EO ($n = 8$) and LO ($n = 10$) AD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ between indicated groups.

Statistical analyses

These autopsy-derived data were analyzed using non-parametric models and relied on either the Mann–Whitney *U* test or ANOVA (Kruskal–Wallis) with adjustment for multiple comparisons using Dunn's test. Outliers were identified using Grubbs' test (extreme Studentized deviate method: GraphPad). Significance was set at $P < 0.05$. However, analyses in which *P* values fell between 0.05 and 0.1 were discussed as *tendencies*. Data are represented as scatter plots with the line representing the sampling mean.

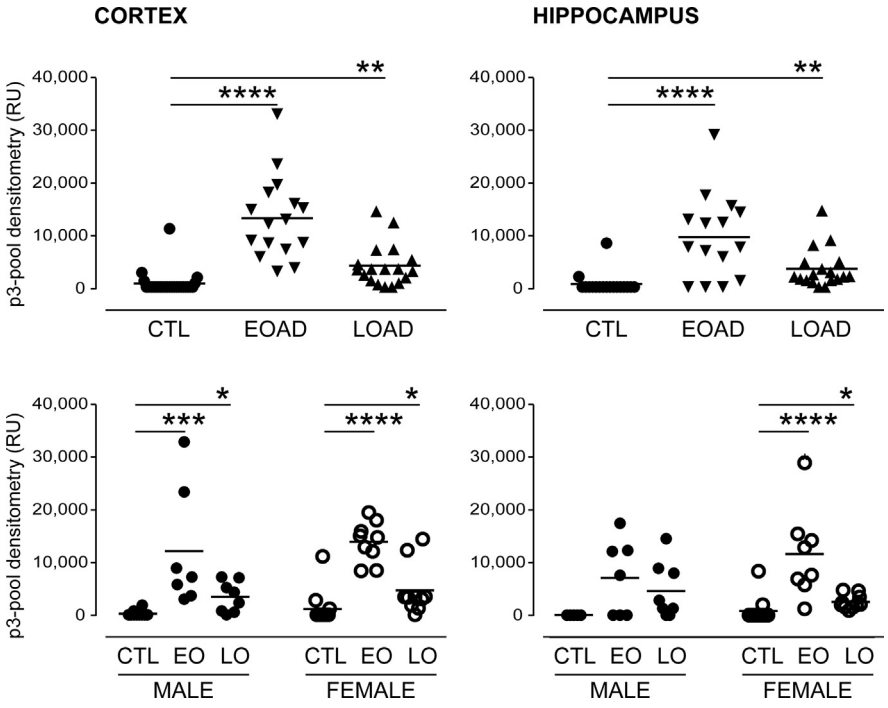

Fig. 4. Densitometric analyses of p3-related fragments. The levels of N-truncated/p3 fragment-enriched bands isolated by 4G8 immunoprecipitation of 6E10-immunodepleted lysates were determined in control (CTL), Early-Onset AD (EOAD), and Late-Onset AD (LOAD) samples (*top panels*). The same data were separated by sex of the donor (*bottom panels*). Sample sizes are as indicated in Fig. 2 (pooled) and Fig. 3 (separated by sex). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ between indicated groups.

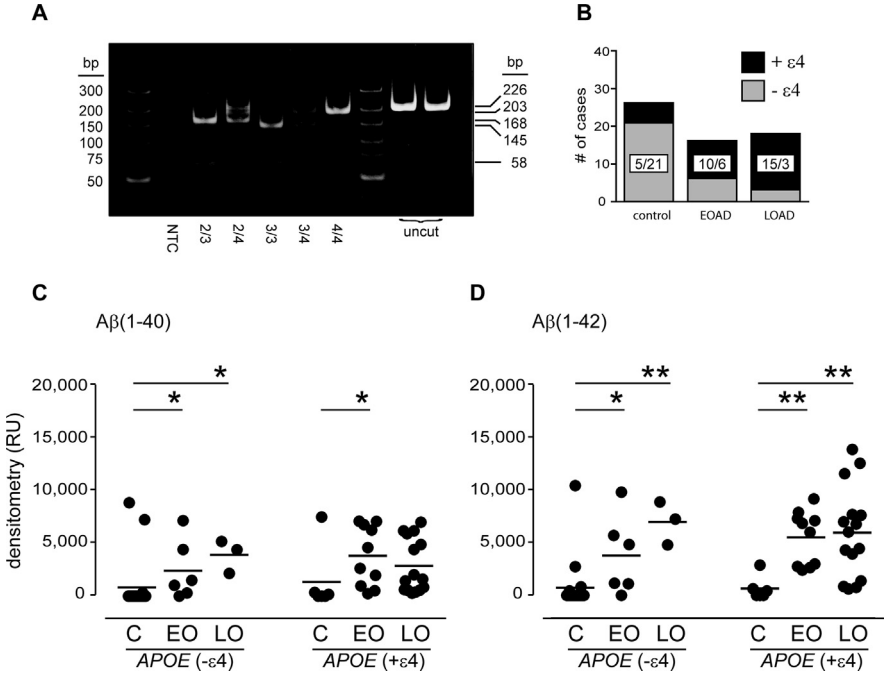

Fig. 5. Samples stratified by the diagnosis and *APOE* ε4 status of the donor. (A) Genomic DNA was PCR amplified for *APOE* variants. These were then restricted and resolved by non-denaturing PAGE. A representative gel is shown. [NTC: no template control; bp: base pair/ladder]. (B) The frequency of carriers of the *APOE* ε4 allele separated by diagnosis, with the relative frequency identified as, for example, '5/21' (controls were represented by 5 carriers of the ε4 allele and 21 non-carriers). The levels of (C) Aβ(1–40) and (D) Aβ(1–42) in cortical samples depicted in Fig. 2 were stratified by diagnosis and *APOE* ε4 status. These included non-carriers (-ε4: 20 CTL, 6 EOAD, 3 LOAD) and carriers (+ε4: 6 CTL, 10 EOAD, 15 LOAD). $^{*}P < 0.05$; $^{**}P < 0.01$ between groups.

Given the number of figures already included in this report, we sometimes opted to simply refer to non-significant differences between sample means as '*data not shown*'. *P* values are given in the corresponding figure legend. Correlation statistics were based on Pearson's correlation coefficient.

Note that our sample set was not sufficiently large to allow for stratification based on individual *APOE* allele heterozygosity and homozygosity. Hence, we used *APOE* ε4 status as a dichotomous nominal variable, *i.e.,* a carrier (has at least one ε4 allele) *versus* non-carrier.

RESULTS

The post-mortem interval did not differ between control, EOAD and LOAD samples [$P = 0.5697$]. As expected, the age of the donor at autopsy [$P < 0.0001$] was lower in EOAD donors compared to LOAD donors. The age of onset was significantly different between EOAD and LOAD donors [$P < 0.0001$], with males [$P = 0.0012$] and females [$P < 0.0001$] both contributing to this difference. The duration of the disease was not different between EOAD and LOAD donors [$P = 0.7915$], regardless of sex [males: $P = 0.9225$; females: $P = 0.9128$]. Brain weight was impacted by a diagnosis of EOAD/LOAD [$P < 0.0001$], with contribution from male LOAD, but not EOAD, donors [$P = 0.0268$] and female EOAD as well as LOAD donors [$P = 0.0001$].

The proportion of *APOE* ε4 carriers in control donors (21 non-carriers (n-c) *vs.* 5 carriers (c)), in EOAD donors (6n-c *vs.* 10c), and LOAD donors (3n-c *vs.*15c) is in keeping with the literature (Poirier et al., 1993; Tanzi, 2012). The age of onset was not influenced by *APOE* ε4 status [$P = 0.4046$], regardless of the donor's sex [males: $P = 0.8392$; females: $P = 0.5797$]. Similarly, the duration of the disease was not influenced by *APOE* ε4 status [$P = 0.4554$], regardless of the donor's sex [males: $P = 0.7448$; females: $P = 0.2143$]. Brain weight was only modestly affected by *APOE* ε4 status if using pooled male/female data

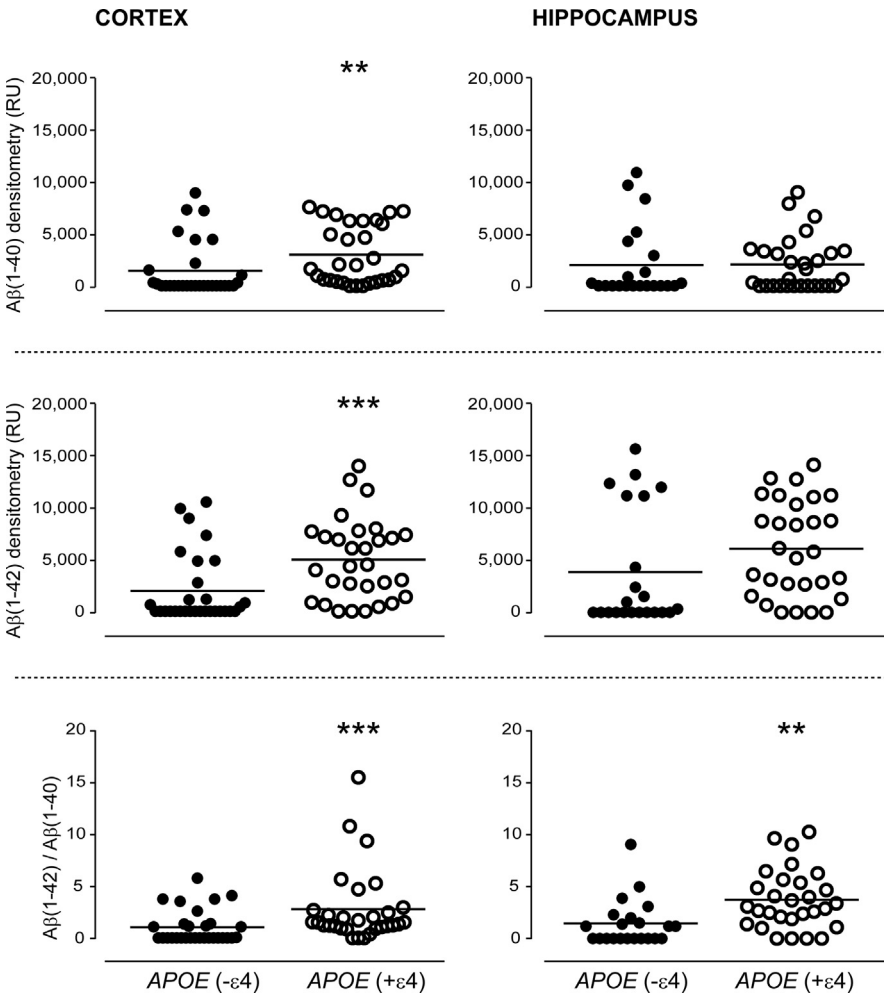

Fig. 6. Aβ peptide levels stratified by *APOE* ε4 status of the donor. The levels of Aβ(1–40) and Aβ (1–42) from (*left*) cortical and (*right*) hippocampal samples were stratified solely according to whether the donor was a non-carrier (-ε4: cortex: $n = 29$; hippocampus: $n = 21$) or a carrier (+ε4: cortex: $n = 31$; hippocampus: $n = 30$) of the *APOE* ε4 allele. The ratios of Aβ(1–42)/Aβ(1–40) are included for both regions. $^{**}P < 0.01$; $^{***}P < 0.001$ between groups.

[$P = 0.1041$], but was significantly less in female carriers of the ε4 allele [$P = 0.0213$], but not in male carriers of the ε4 allele [$P = 0.4341$].

We sequenced the APP and PS-1 cDNA in our EOAD sample set and were unable to identify any genetic variant, which suggests that our EOAD donors were likely not representing familial AD, but rather had a sporadic form of AD (which would explain the higher incidence of *APOE* ε4-positive donors in this cohort) with a younger age of onset. The donors' AD progression might have been precipitated by an environmental factor such as traumatic brain injury (Mendez et al., 2015).

Wiltfang's Urea/SDS–PAGE system (Wiltfang et al., 1997) easily discriminated between synthetic Aβ peptides of varying lengths, *i.e.,* Aβ(1–38), Aβ(1–40), Aβ(1–42) and Aβ(1–43), in contrast with standard 15% SDS–PAGE, which did not (Fig. 1). The Urea/SDS–PAGE system allowed us to clearly differentiate Aβ(1–40) and Aβ(1–42) in anti-6E10 immunocomplexes from the insoluble (guanidium) fractions of human samples. The 6E10-immunodepleted supernatants were then used to isolate the p3-enriched pool of fragments using the 4G8 antibody (Fig. 1). Data were first examined as a *pool* of male and female data, and then were separated based on the donor's sex. Densitometry revealed that Aβ(1–40), Aβ (1–42), and the Aβ(1–42)/Aβ(1–40) ratio were all significantly increased in *pooled* (*i.e.,* M + F) cortical as well as in *pooled* hippocampal EOAD and LOAD samples (Fig. 2), but these increases were not uniform between the sexes (Fig. 3). Interestingly, the Aβ(1–42)/Aβ(1–40) ratio in female EOAD and LOAD cortical samples clustered toward '1'; in contrast, the ratio was significantly higher in the corresponding female hippocampal samples –and in all male AD samples– indicating that the Aβ(1–42) peptide was being preferentially generated in these donors/samples (Fig. 3). The p3-enriched pool was also increased in cortical and hippocampal EOAD and LOAD samples, and differences between the sexes were also evident (Fig. 4).

In general, the production of the Aβ(1–40) and the Aβ(1–42) peptides in LOAD samples correlated strongly within a given region (Cortex: Aβ(1–42) *vs* Aβ(1–40): $P = 0.0014$; Hippocampus: Aβ(1–42) *vs* Aβ(1–40): $P < 0.0008$), whereas there was very little correlation in the production of either Aβ(1–40) or Aβ (1–42) between the cortical LOAD samples and the corresponding hippocampal LOAD samples (Cortex Aβ(1–40) *vs* Hippocampus Aβ(1–40): $P = 0.9758$; Cortex Aβ(1–42) *vs* Hippocampus Aβ(1–42): $P = 0.5680$).

Unexpectedly, when the data were stratified for diagnosis-by-*APOE* ε4 status, the increases in levels of Aβ(1–40) and Aβ(1–42) in cortical EOAD and LOAD samples were very similar and could not be used to differentiate between carriers and non-carriers of the ε4 allele (Fig. 5). The same trend held for hippocampal Aβ (1–40) and Aβ(1–42) expression (*data not shown*). We were intrigued by this lack of a clear association of the Aβ peptides with the combination of a diagnosis of AD/ *APOE* ε4 allele. As our sample set was not large enough to allow for adequate sample sizes within each level of a triple stratification, *i.e.,* by diagnosis (CTL, EOAD, LOAD) by sex (male *vs.* female) by *APOE* ε4 status (carrier *vs.* non-carrier), we chose to re-examine the data by focusing on the donor's sex and *APOE* ε4 status, and exclude diagnosis from the analysis (*i.e.,* we

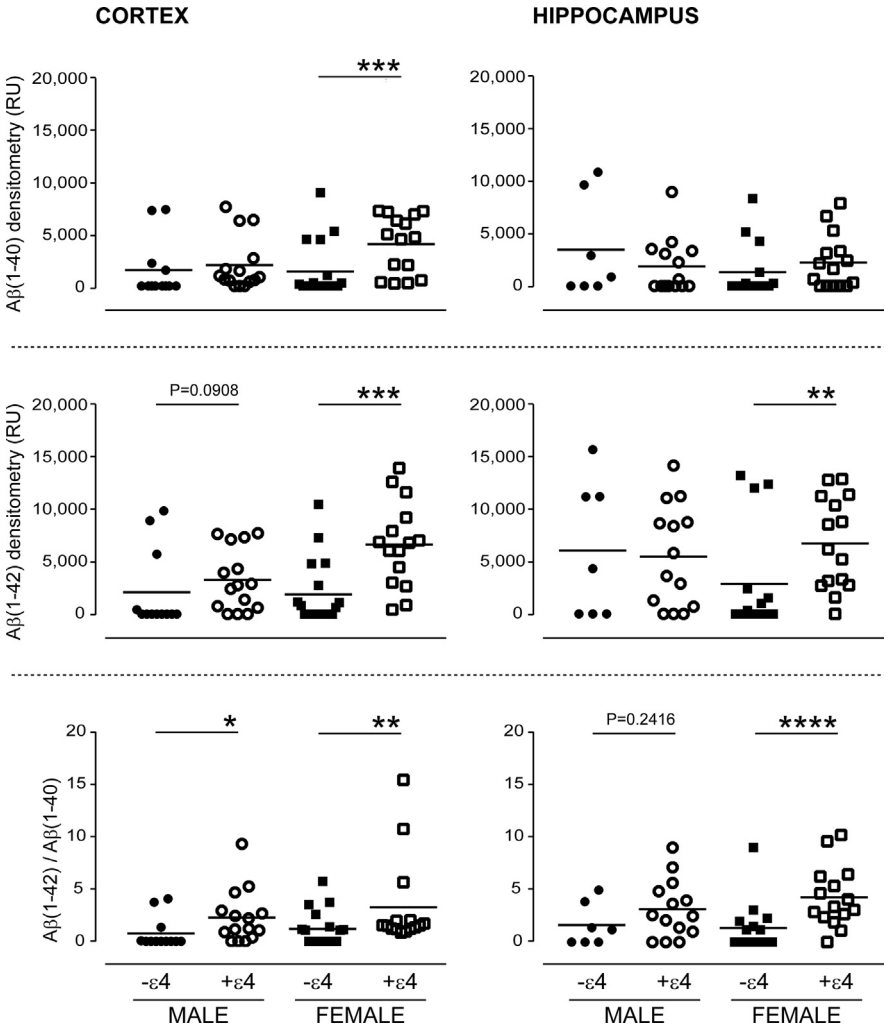

Fig. 7. Aβ peptide levels stratified by *APOE* ε4 status and sex of the donor. (*left*) The cortical data from Fig. 6 were separated according to the donor's and sex. Male samples represent non-carriers (−ε4: $n = 12$) or carriers (+ε4: $n = 15$) of the allele. Female samples represent non-carriers (−ε4: $n = 17$) or carriers (+ε4: $n = 16$) of the allele. (*right*) The corresponding hippocampal data are represented by male non-carriers (−ε4: $n = 7$) or carriers (+ε4: $n = 14$) of the allele, while female samples represent non-carriers (−ε4: $n = 14$) or carriers (+ε4: $n = 16$) of the allele. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ between indicated groups.

did not consider the diagnosis as a nominal variable in the stratification).

This stratification revealed significant increases in immunodetection of Aβ(1–40) and Aβ(1–42) (Fig. 6) in cortical samples from ε4-positive donors; these increases invariably were aligned with female carriers of the ε4 allele (Fig. 7). Interestingly, in the hippocampus, there was no overall change in the levels of Aβ(1–40) in ε4-positive donor samples (Fig. 6) and, in keeping with this, the level of Aβ(1–40) in the hippocampus of female carriers of the ε4 allele was not different from that in females who did not carry the allele (Fig. 7). The majority of cortical Aβ(1–42)/Aβ(1–40) ratios in female carriers of the ε4 allele again tended to average around '1', whereas the ratio was significantly higher in the other cortical and hippocampal samples. The detection of the p3 fragment-enriched pool was higher in ε4-positive cortical and hippocampal samples, and in both regions the contribution to these changes was primarily from female carriers of the ε4 allele (Fig. 8).

The levels of Aβ(1–40) and Aβ(1–42) correlated strongly within a given region, whether the donor (male or female) was a carrier of the ε4 allele (Cortex: Aβ(1–42) *vs* Aβ(1–40): $P < 0.0001$; Hippocampus: Aβ(1–42) *vs* Aβ(1–40): $P < 0.0001$) or not (Cortex: Aβ(1–42) *vs* Aβ(1–40): $P < 0.0001$; Hippocampus: Aβ(1–42) *vs* Aβ(1–40): $P < 0.0001$) (*data not shown*). In contrast, while there was a strong correlation between the level of Aβ(1–40) in cortex and hippocampus in individuals who did not carry the ε4 allele ($P < 0.0001$), this did not hold true for carriers of the ε4 allele ($P = 0.2834$). The regional levels of the Aβ(1–42) peptide were also significantly correlated in non-carriers of the ε4 allele ($P < 0.0001$), but not in carriers of the ε4 allele ($P = 0.1796$). The loss of any interregional correlation (for either peptide) in carriers of the ε4 allele was evident regardless of the sex of the donor (*data not shown*).

Next, we examined the levels of the key secretases implicated in APP processing, *i.e.* β-secretase (BACE1), α-secretase (ADAM10), and components of the γ-secretase complex (*e.g.* presenilin-1, nicastrin). Representative immunoblots are provided in Fig. 9.

Cortical levels of ADAM10 were significantly decreased with a diagnosis of AD (EOAD as well as LOAD) [$P = 0.0002$] and while a decrease was observed in both sexes, the effect was only significant in females (Fig. 10). A lower ADAM10 expression was also observed in hippocampal AD samples, but this was not significant [$P = 0.2706$] (Fig. 10). Although there was an indication of lower levels of BACE1 in male AD donors and higher levels in female AD donors, this was not significant either in cortex [$P = 0.6451$] or in hippocampus [$P = 0.8943$] (*data not shown*).

Stratifying the data by sex and *APOE* ε4 status did not alter BACE1 expression levels in either cortex [$P = 0.8505$] or hippocampus [$P = 0.6345$] (Fig. 11). In contrast, this stratification revealed that both male and female carriers of the ε4 allele contributed to a decrease in cortical ADAM10 expression [$P = 0.0047$], while the ε4 allele did not exert any effect on hippocampal ADAM10 expression, regardless of the sex of the carrier [$P = 0.7457$] (Fig. 11).

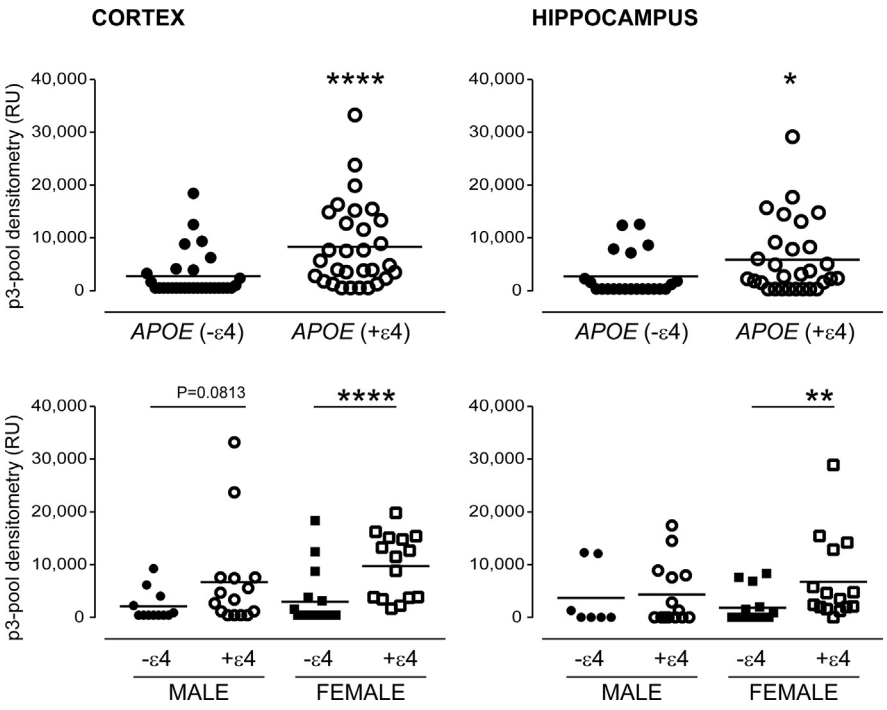

Fig. 8. Levels of the p3-related fragment pool stratified by *APOE* ε4 status and sex of the donor. (*top panels*) The levels of (*left*) cortical and (*right*) hippocampal N-truncated, p3 fragment enriched pool were re-examined according to the donor's *APOE* ε4 status (*i.e.,* as non-carriers (-ε4) or carriers (+ε4) of the allele). (*bottom panels*) The same data were stratified according to the donor's sex. Sample sizes as in Figs. 6 & 7. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$ between groups.

Given that the balance between amyloidogenic and non-amyloidogenic processing relies, in great part, on the contributions of BACE1 and ADAM10, we examined how the relative amount of BACE1 to ADAM10 aligned with sex and *APOE* ε4 status. The BACE1/ADAM10 ratio was significantly increased only in cortical samples from female carriers of the ε4 allele (Fig. 11) and while it did not correlate with cortical levels of Aβ(1–40) or Aβ (1–42), it was positively correlated with the Aβ(1–42)/Aβ (1–40) ratio, again specifically in female carriers of the allele (Fig. 12). This pattern was not observed in the corresponding hippocampal samples (Figs. 11 and 12).

We next examined selected components of the γ-secretase complex. There was a modest loss of PS-1 expression in cortical LOAD samples [$P = 0.0492$], but any significance was lost when samples were separated by sex (*data not shown*). In hippocampal samples, there was a significant increase in PS-1 expression in EOAD samples [$P = 0.0120$], which was due to a change in female EOAD samples (*data not shown*). Nicastrin expression did not change with diagnosis in either cortical samples [$P = 0.1317$] or hippocampal samples [$P = 0.3790$], although any variation in nicastrin expression in the latter sample set was clearly aligned with female AD donor samples (*data not shown*). The levels of PS-1 were unaffected by the ε4 allele in cortical [$P = 0.4382$] as well as in hippocampal [$P = 0.3466$] samples (Fig. 13). Similarly, the ε4 genotype exerted no effect on either cortical [$P = 0.6301$] or hippocampal [$P = 0.8247$] nicastrin expression, although any variation in the latter sample set once again aligned clearly with the female carriers of the ε4 allele (Fig. 13).

We investigated whether the variability in nicastrin expression was meaningful to the regional Aβ profiles. Nicastrin levels were not correlated with either Aβ(1–40) or Aβ (1–42) levels in cortex, although there was a correlation with the Aβ(1–42)/Aβ(1–40) ratio in male carriers ($P = 0.0232$) and female carriers ($P = 0.0453$) of the ε4 allele. This correlation was modest in the corresponding male hippocampal samples ($P = 0.0479$) and lost in the female hippocampal samples ($P = 0.8802$) (*data not shown*). What was more telling was the lack of any correlation between nicastrin levels and Aβ(1–42) levels in cortex, but dramatically different (negative and positive) correlations in the corresponding male and female hippocampal samples (Fig. 14).

Note that we did not explore the correlation between secretases and the N-truncated/p3 fragment-related pool of fragments as an outcome, if it were to exist, would be difficult to interpret in light of the heterogeneity of the fragment pool.

Finally, we examined the levels of fl-APP and the C-terminally truncated fragments, *i.e.,* sAPPα and sAPPβ, which are soluble N-terminal fragments that correspond respectively to α -secretase- and β-secretase-mediated processing of APP.

The fl-APP was isolated by immunoprecipitation of the RIPA/soluble fraction with a C-terminus-directed antibody. The resolved immunocomplex was then probed with the N-terminus-directed 22C11 antibody (see representative immunoblot, Fig. 9). There was a significantly lower detection of fl-APP in cortical AD samples [$P = 0.0272$]. This pattern aligned specifically with female AD samples [$P = 0.0462$] and was not observed in corresponding male samples [$P = 0.5321$]. The detection of fl-APP was similar between control and EOAD/LOAD hippocampal samples [$P = 0.5923$], regardless of sex [males: $P = 0.8653$; females: $P = 0.4907$]. Stratifying the data by sex and *APOE* ε4 status did not reveal any significant patterns [cortex: $P = 0.1516$; hippocampus: $P = 0.1018$] (*data not shown*).

The C-terminally immunodepleted supernatant was then immunoprecipitated for 6E10 so as to isolate the sAPPα fragment (which contains the 6E10 epitope, *i.e.,* residues 1–16 of the Aβ sequence). The resulting 6E10-immunodepleted supernatant contained the sAPPβ fragment, which lacks the 6E10 epitope. Both sAPPα and sAPPβ were identified using the 22C11 antibody (see representative immunoblots, Fig. 9). A diagnosis of

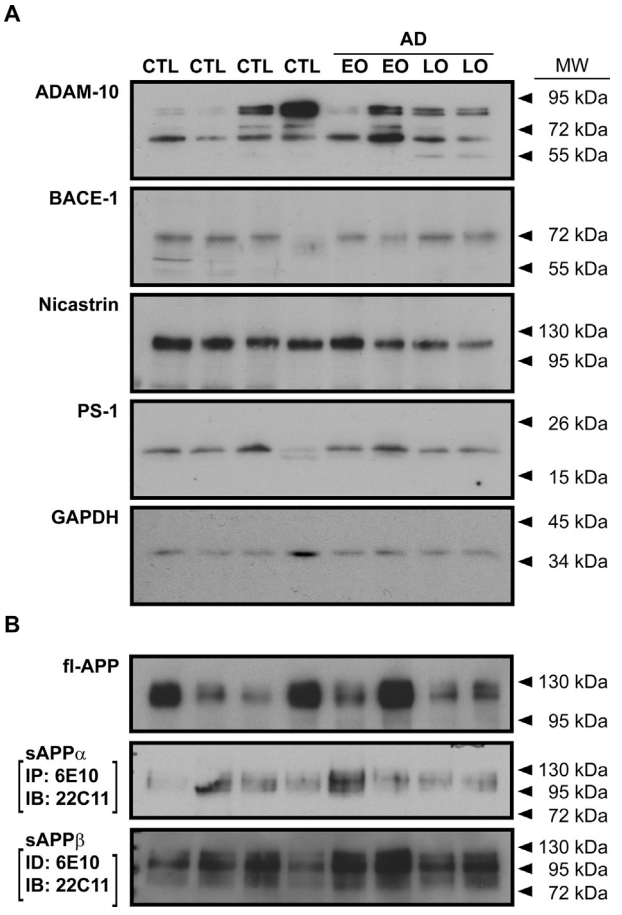

Fig. 9. Representative immunoblots of key secretases in APP processing. (A) The expression of key secretases in APP processing and the housekeeping protein, GAPDH, are shown; these samples are the male samples from Fig. 1. (B) Levels of full-length APP (fl-APP) and of the two major soluble N-terminal fragments, *i.e.,* sAPPα and sAPPβ, are shown. IP: immunoprecipitated; ID: immunodepleted; IB: immunoblotted.

AD did not alter levels of sAPPα in either cortical [$P = 0.2255$] or hippocampal [$P = 0.3753$] extracts. Similarly, levels of sAPPβ in cortical [$P = 0.2351$] and hippocampal [$P = 0.0521$] extracts were unchanged. Stratifying the data by sex and *APOE* ε4 status revealed similar trends. Although there was a tendency for more variability/higher levels of sAPPα and sAPPβ in female, ε4-positive hippocampal samples, overall levels of sAPPα were unaltered in cortical [$P = 0.4737$] or hippocampal [$P = 0.1800$] extracts, and levels of sAPPβ in cortical [$P = 0.8967$] or hippocampal [$P = 0.2501$] extracts were also statistically unchanged (*data not shown*).

DISCUSSION

Our analysis confirms the expected increases in Aβ(1–42), Aβ(1–40), and the N-truncated/p3 fragment-enriched pool in the guanidine-soluble fraction of autopsied AD cortical and hippocampal extracts. These changes are more evident in EOAD samples than in LOAD samples, thus confirming the aggressive amyloidosis in this early-onset cohort. The hydrophobic Aβ(1–42) is increased in EOAD as well as LOAD, regardless of sex of the donor. In contrast, any increase in cortical Aβ(1–40) and hippocampal p3 fragment-enriched fragments is driven primarily by female EOAD and LOAD samples. A strength of the current study was our ability to compare regional profiles from the same set of donors. Correlational analyses indicate that the levels of the Aβ(1–42) and Aβ(1–40) peptides are tightly co-regulated within a given region, but not between regions, thus suggesting region-specific influences on the production and/or accumulation/clearance of these peptides.

Interestingly, the peptide profiles are not influenced by the donor's *APOE* ε4 status, if the diagnosis of AD is considered as a nominal factor in the stratification. This unexpected, and certainly counterintuitive, lack of an interaction between *APOE* ε4 status and a diagnosis of AD led us to re-evaluate our dataset. Evaluating the influence of *APOE* ε4 itself, independent of any consideration of diagnosis of AD, was not an uninformed strategy; indeed, there has been suggestion that the ApoE protein might simply influence the generation of the Aβ peptide (Prince et al., 2004), although the report did not consider sex as a variable. We now demonstrate that levels of Aβ(1–42), Aβ(1–40), and the p3-related pool are all increased in carriers of the ε4 allele and, specifically, in female carriers of the ε4 allele (and independent of their diagnosis). This observation reconciles the notions of a role for the ε4 allele in generation of the Aβ peptide (independent of a diagnosis of AD, *i.e.,* (Prince et al., 2004)) and the acknowledged gender-risk of the ε4 allele in such amyloidosis (Poirier et al., 1993; Payami et al., 1996; Farrer et al., 1997; Bretsky et al., 1999). Furthermore, the effect of the ε4 allele is more evident in the cortical samples, which also supports the temporal pattern of amyloid burden that has been associated with AD progression, *i.e.,* cortex first, followed by hippocampus (Oddo et al., 2003). We also demonstrate that there is a very strong correlation between the levels of Aβ(1–42) and Aβ(1–40) within a given region. However, the strong correlation between cortical and hippocampal Aβ(1–40) levels –or cortical and hippocampal Aβ(1–42) levels– that is evident in non-carriers of the ε4 allele, is absent in carriers of the allele. This suggests a disruption of inter-regional regulation of these peptides by the ε4 allele.

Our analyses revealed additional intriguing region-dependent observations, including one relating to a putative biomarker of AD progression, *i.e.,* the Aβ42/Aβ40 ratio. A lower plasma Aβ(1–42)/Aβ(1–40) ratio (*i.e.,* a lower Aβ(1–42) signal) is thought to reflect the retention of Aβ(1–42) –*via* aggregation as plaques– in the brain. In support of this, two large population studies, *i.e.,* the Rotterdam and the Mayo Clinic cohorts, have shown that a low CSF (or plasma) Aβ(1–42)/Aβ(1–40) ratio corresponds with higher risk of AD (van Oijen et al., 2006; Graff-Radford et al., 2007) or advanced cognitive dysfunction (Piccinni et al., 2013). Therefore, it is interesting that the Aβ(1–42)/Aβ(1–40) ratios in cortical samples from female, ε4-positive donors clustered mostly

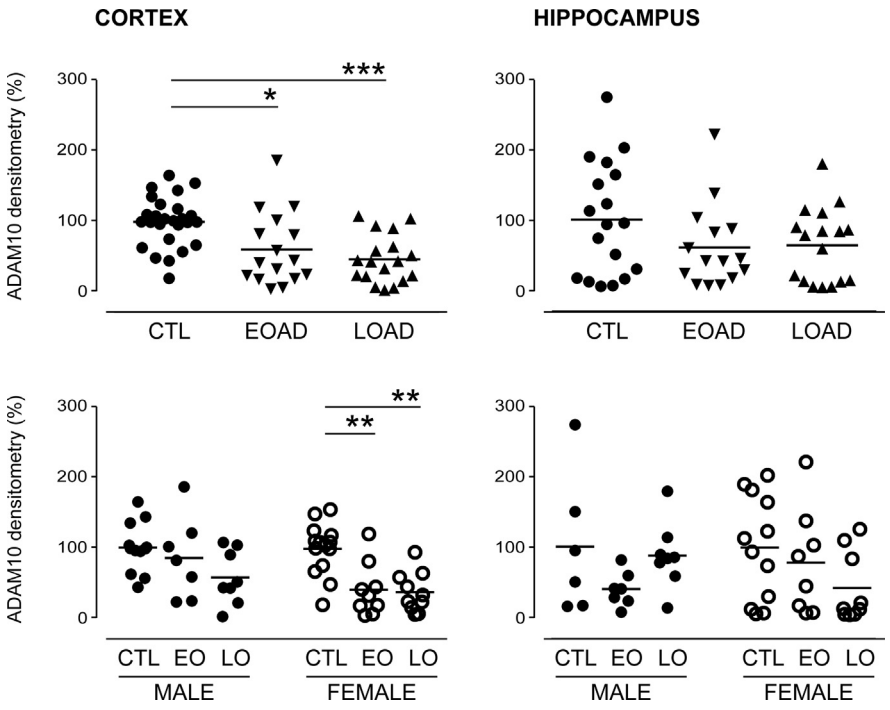

Fig. 10. ADAM10 levels stratified by diagnosis and sex of the donor. The levels of (*left*) cortical and (*right*) hippocampal ADAM10 (α-secretase) expression were analyzed according to (*top panels*) diagnosis and then separated by (*bottom panels*) sex and diagnosis. Samples sizes are as indicated in Figs. 2 and 3. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ between groups.

around '1', thus suggesting that comparable amounts of Aβ(1–40) and Aβ(1–42) were being deposited –or generated– in this region of the female brain; in contrast, the ratio was significantly higher in the corresponding female hippocampal samples (and in all male AD samples), indicating that the Aβ(1–42) was being preferentially accumulated in these donors/samples. This confirms a previous report of regional differences in relative amounts of Aβ (1–42) and Aβ(1–40) in frontal cortex *versus* hippocampus of AD patients, although gender was not factored into that particular analysis (Beffert et al., 1999). Clearly, any change in a plasma or CSF Aβ(1–42)/Aβ(1–40) ratio would only identify cumulative changes of the ratio across brain regions and certainly could not identify any specific regional changes or disease processes. Furthermore, neuroimaging with amyloid plaque-targeting tracers would certainly identify regions of high amyloid burden (Johnson et al., 2012), but would be unable to differentiate regions – or more specifically plaques within a given region– with differing Aβ(1–42), Aβ(1–40), and N-truncated/p3 fragment composition.

We did observe a modest tendency for increases in the N-terminal soluble APPα in female patients with AD, which does align with an observation made in a similar cohort by Wiltfang's group, who left it as '*data not shown*' (Lewczuk et al., 2010).

These observations raise the very likely possibility of distinct disease processes in the female and male brain, and that any difference would be influenced strongly by a sex-by-*APOE* ε4 status interaction. Perhaps the male brain might suffer from a shift toward a primarily BACE1-mediated, amyloidogenic phenotype with age/AD and an exacerbation of disease progression, as seen elsewhere (Aguero-Torres et al., 1998; Hy and Keller, 2000). In contrast, perhaps the female brain –particularly that influenced by the ε4 allele– could see an increased, indiscriminate processing of APP through both amyloidogenic (*e.g.,* BACE1) and non-amyloidogenic (*e.g.,* ADAM10) pathways, which might explain the significant reduction in fl-APP levels in female cortical EOAD and LOAD extracts (current study). Physiological roles for APP –such as synaptic maintenance (Yang et al., 2005) and memory retention (Senechal et al., 2008)– would be disrupted in both sexes and AD could certainly be a common clinical outcome, but the disease would follow distinct, sex-dependent trajectories.

We expected our observed regional Aβ peptide profiles to reflect concurrent changes in BACE1 expression as reported previously, *e.g.,* (Vassar et al., 1999; Fukumoto et al., 2002). Yet, BACE1 expression was not changed with either a diagnosis of AD or the *APOE* ε4 status. In retrospect, this is not surprising as the one study, *i.e.,* (Fukumoto et al., 2002), did not actually find any correlation between BACE1 activity and Aβ peptide burden in the *insoluble* (plaque-associated) fraction (similar to what we measured in the current study), while other studies have found a decrease in BACE1 expression in the AD brain, *e.g.,* (Decourt et al., 2013). *In vitro*, BACE1 can cleave APP at either Asp1 of the Aβ sequence (thus leading to full-length Aβ peptides) or at Glu11 of the Aβ sequence (Benjannet et al., 2001; Bodendorf et al., 2002), which would contribute to the pool of N-terminally truncated Aβ species detected *ex vivo* (Kummer and Heneka, 2014). ADAM10 cleavage of APP targets residues 16 and 17 of the Aβ sequence, which would also promote N-truncated Aβ variants.

Interestingly, activated BACE1 tends to generate Aβ (1–40), whereas changes in PS-1/γ-secretase function favor the generation of the Aβ(1–42) species (Herzig et al., 2007). Therefore, our observed loss of cortical ADAM10 expression, and the resulting increase in the BACE1-to-ADAM10 ratio (*i.e.,* a relative increase in BACE1) could help explain the increased Aβ(1–40) in female cortical AD samples or in female ε4-positive cortical samples. Alternatively, the strong positive correlation between nicastrin (a component of the γ-secretase complex) and Aβ(1–42) specifically in hippocampal samples of female AD patients or female carriers of the ε4 allele could explain some of the associated region-dependent pathology. In contrast, there was a significant negative correlation between nicastrin and Aβ(1–42) in the male, ε4-positive hippocampal samples. Nicastrin stabilizes

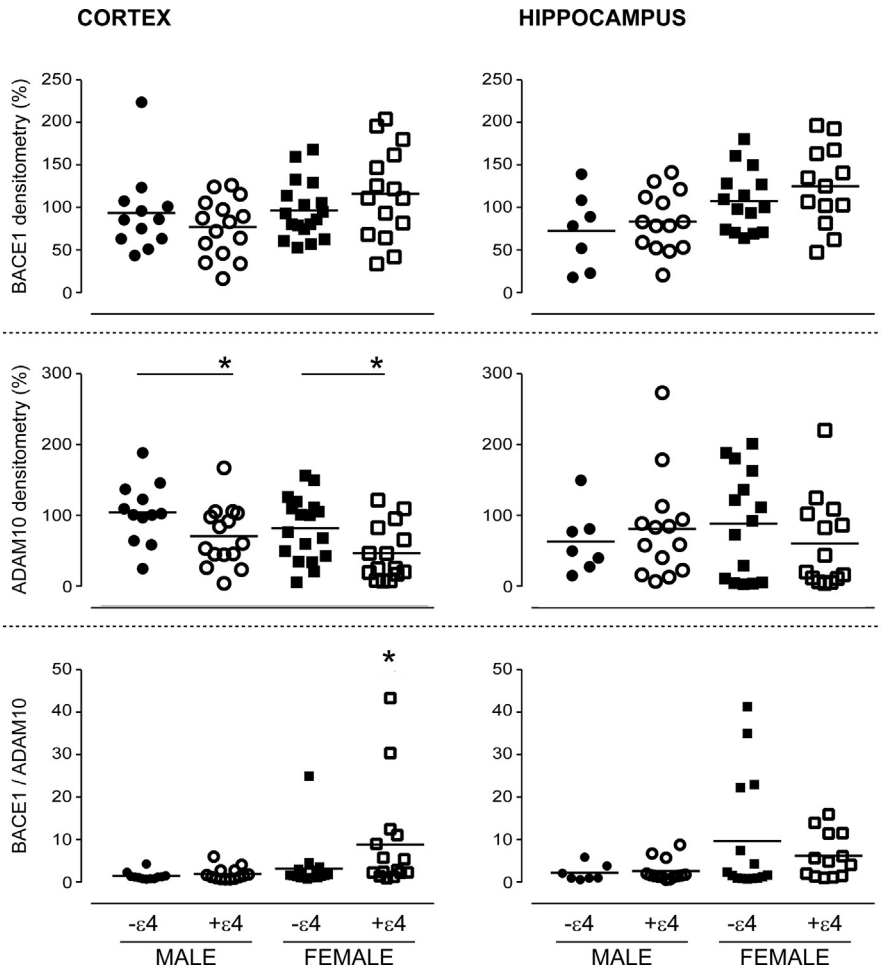

Fig. 11. BACE1 and ADAM10 expression levels stratified by *APOE* ε4 status and sex of the donor. The levels of (*top panels*) BACE1 (β-secretase) and (*middle panels*) ADAM10 (α-secretase) were stratified by sex and *APOE* ε4 status. (*bottom panels*) These data were used to determine the ratio of BACE1 to ADAM10 in corresponding cortical (*left*) and (*right*) hippocampal samples. Samples sizes are as indicated in Fig. 7, except for hippocampal female + ε4, where a data point was excluded as an outlier ($n = 15$). $^{*}P < 0.05$ between groups.

the conformation of the γ-secretase complex and acts as a substrate gatekeeper for the complex's catalytic core (Bolduc et al., 2016). Perhaps what our data are reflecting is a nicastrin-sensitive change in γ-secretase affinity for its substrates, *i.e.,* APP, Notch, etc., in an *APOE* ε4 background, and progression through to sex- and substrate-specific phenotypes, as has already been suggested for the aging mouse brain (Placanica et al., 2009). Similarly, processing of APP through BACE1, possibly through cleavage at Glu11, would also support our observed increases in N-truncated Aβ species in cortical female lysates (AD or ε4-positive) as well as a modest change in male carriers of the allele. Of course, it should be borne in mind that insoluble Aβ peptides in the human AD brain likely represent a composite of accumulation, degradation, and clearance, and our data cannot discern the relative contribution of these distinct events. We are currently extending our study to Aβ peptide profiles in the RIPA/soluble fraction so as to determine how this pool of peptides (presumably being the ones available for clearance into CSF and/or blood) align with diagnosis and/or *APOE* ε4 status. In light of a recent report suggesting that *APOE* ε4 can also significantly impact cell viability and hippocampal volume in a knock-in mouse model of tauopathy (and supported by recombinant treatments in AAV2/P301S-tau-mixed cultures) (Shi et al., 2017), it will be interesting to determine how neurofibrillary tangle/Tau-related indices are altered in our sample set.

Given that BACE1 function might not actually change (or only change relative to a loss of ADAM10 function), this might mitigate any benefit to targeting BACE1 in the clinical context, particularly if one's sex and/or *APOE* ε4 status is not duly considered. Perhaps this is what led to the recent termination of Merck's EPOCH Phase III drug trial after it was concluded that the BACE inhibitor verubecestat was ineffective in treating mild-to-moderate AD [http://www.sciencemag.org/news/2017/02/another-alzheimers-drug-flops-pivotal-clinical-trial].

The initial reports on the risk of AD being associated with the *APOE* ε4 allele clearly stated a female-specific risk (Poirier et al., 1993; Payami et al., 1996; Farrer et al., 1997; Bretsky et al., 1999) Yet, in spite of this, the literature has persisted in pooling male and female data, and inexplicably continues to do so, even when *APOE* ε4 status is available. This supports the fallacy that male and female carriers of the ε4 allele carry similar risk for AD and, furthermore, pooling male and female *APOE* ε4-related data –seemingly to increase statistical power– would certainly dilute effect size, introduce heterogeneity in any test cohort, and could, ultimately, lead to misinterpretation and contention in the relevant literature. Indeed, a recent review has categorized some of these discrepancies. For example, the *APOE* ε4 allele has been linked to both acceleration as well as slowing the clinical course of AD and any associated cognitive decline and behavioral outcomes, and to discrepancies in hippocampal volume loss over time, or perhaps hippocampal baseline volume, all of which might rely on whether the carrier is heterozygous or homozygous (Ungar et al., 2014). Our current data do not support an association between *APOE* ε4 status and age of onset or duration of disease progression. Fortunately, the interaction between *APOE* ε4 genotype and gender has not always been ignored, and imaging, pathological, and psychological testing independently support more pronounced AD-related phenotypes primarily in female carriers of the allele (compared to male carriers or non-carriers in general) (Ungar et al., 2014).

A

	Goodness of fit	Male (+ε4)	Female (+ε4)
Cortex: BACE1/ADAM10 *vs.* Aβ42/40	Slope	0.4698 ± 0.2822	0.3140 ± 0.03981
	R^2	0.1877	0.8272
	P value	0.1218	**<0.0001**
Hippocampus: BACE1/ADAM10 *vs.* Aβ42/40	Slope	0.2776 ± 0.3016	-0.1056 ± 0.1445
	R^2	0.06594	0.04630
	P value	0.3755	0.4802

B

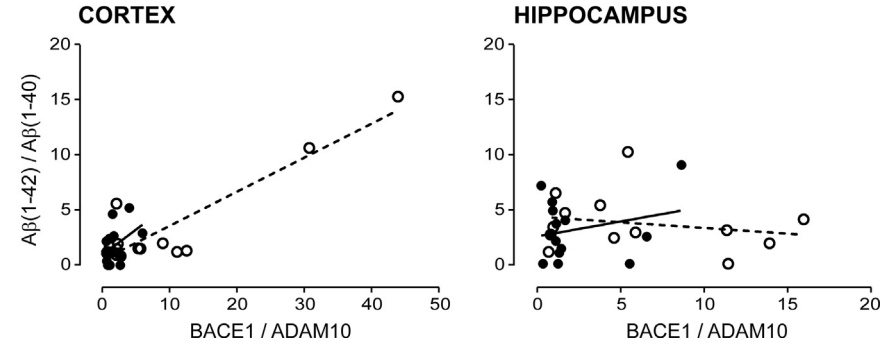

Fig. 12. The BACE1/ADAM10 ratio affects APP processing in female/ε4 hippocampus. The influence of the *APOE* ε4 allele on the relation between the BACE1/ADAM10 ratios and the corresponding Aβ(1–42)/Aβ(1–40) ratios were analyzed (A) in the cortex and hippocampus of carriers of the allele. Note that analyses of non-carriers are not included as the majority of samples did not contain any Aβ peptide, thereby rendering any interpretation of statistics meaningless. (B) The corresponding graphic representation of the data. (●): Male carriers of the ε4 allele; (○): Female carriers of the ε4 allele.

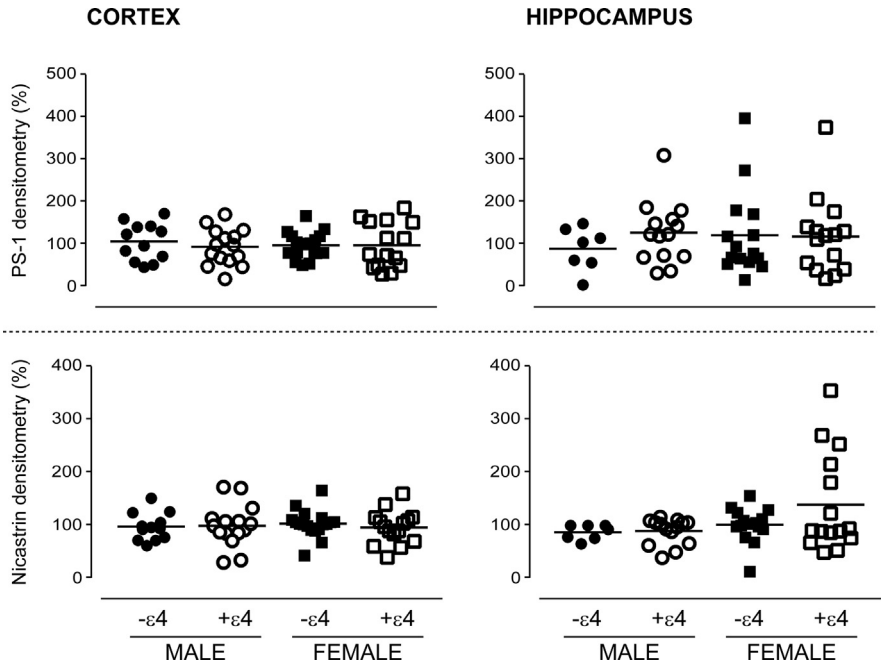

Fig. 13. Components of the γ-secretase complex stratified by *APOE* ε4 status and sex of the donor. (*top panels*) Levels of 23 kDa C-terminal fragment of Presenilin-1 and (*bottom panels*) levels of nicastrin were stratified by sex and *APOE* ε4 status. Levels from cortical samples are on the left and levels in corresponding hippocampal samples are on the right. Samples sizes are as indicated in Fig. 7.

In support of gender-risk associated with the ε4 genotype, female carriers (but not males) are more likely to be depressed (Muller-Thomsen et al., 2002) and to have been depressed prior to developing AD (Delano-Wood et al., 2008), while the combination of ε4 genotype and depression increases the risk of incident dementia (Geda et al., 2006). It is interesting to note that older age, depression, and *APOE* ε4 status each have been associated with distinct trajectories in cognitive and memory decline (Zahodne et al., 2016). The effects of *APOE* ε4 status on cognitive function might be far more subtle and, if triggered, might emerge far sooner than previously thought, if one considers the influence of environmental pollutants in Mexico City on cognitive decline in local teenage female carriers of the ε4 allele (Calderon-Garciduenas et al., 2016). Furthermore, telomere integrity is compromised and accelerated aging of cells is observed, specifically in female carriers of the allele (Jacobs et al., 2013), which corroborates the highly significant association between the ε4 genotype and non-pathological, aging-associated cognitive changes in women revealed during a multicohort GWAS meta-analysis study (Davies et al., 2014). More recently, a retrospective analysis of data obtained from cognitively normal individuals recruited through the Australian Imaging, Biomarker and Lifestyle Study of Ageing revealed that the *APOE* ε4 allele was not necessarily a risk factor for AD *per se*, but rather was a risk factor for accumulation of Aβ and that this subsequently exacerbated the rate of disease progression (Hollands et al., 2017). The difference, albeit subtle, supports the notion that ApoE4 might simply promote the generation of Aβ (Prince et al., 2004) and explains, in part, why cognitively normal carriers of the ε4 allele are more likely to present with a clinical phenotype if they also had a significant Aβ burden (discussed in (Hollands et al., 2017)). The role of ApoE4 in cholesterol transport/clearance is widely acknowledged and it is therefore interesting that a polymorphism in the *HMGCR* gene (encodes for the reductase involved in cholesterol synthesis) can delay the age of onset of AD, particularly in women, and that this same polymorphism can attenuate risk of conversion from MCI to AD in carriers of the *APOE* ε4 allele (Leduc et al., 2015). Parenthetically, 'super seniors' –those over 85 years of age with no history of any

A

		Male (+ε4)	Female (+ε4)
Cortex: Nicastrin *vs.* Aβ(1-42)	Slope	0.007292 ± 20.34	-15.80 ± 35.06
	R^2	9.886e-009	0.01539
	P value	0.9997	0.6596
Hippocampus: Nicastrin *vs.* Aβ(1-42)	Slope	-121.9 ± 44.17	25.01 ± 10.90
	R^2	0.3884	0.2881
	P value	**0.0173**	**0.0391**

B

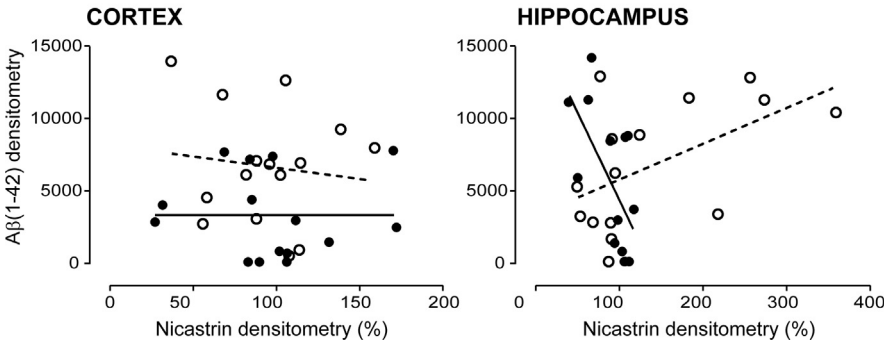

Fig. 14. The *APOE* ε4 allele influences the relation between the levels of nicastrin and the corresponding levels of Aβ(1–42). (A) Correlation data from the cortex and hippocampus of carriers of the allele. Note that analyses of non-carriers are not included as the majority of samples did not contain any Aβ peptide, thereby rendering any interpretation of statistics meaningless. (B) The corresponding graphic representation of the data. (●): Male carriers of the ε4 allele; (○): Female carriers of the ε4 allele.

significant medical complications– are far less likely to be carriers of the ε4 allele (Tindale et al., 2017).

The high Aβ(1–42)/Aβ(1–40) ratios in male EOAD and LOAD samples indicate an evident shift to amyloidogenic processing of the APP molecule. In contrast, the clustering of the ratio around '1' and the higher levels of the N-truncated/p3-pool of fragments in the female cortex is intriguing as this suggests that in this region – in the female ε4-positive AD brain– APP processing involves less of a shift in secretase-mediated processing, but rather more of an increase in APP processing through both amyloidogenic and non-amyloidogenic pathways. This is perhaps not surprising given that the *APOE* ε4 allele is a far stronger activator of APP transcription than are the 'neuroprotective' ε3 and ε2 alleles (Huang et al., 2017).

Interestingly, triplication of the wild-type APP gene (carried on Ch21) –*i.e.,* Trisomy 21/Down Syndrome (DS)– exerts age-dependent cognitive deficits and AD-like Aβ pathology [74] and promotes neuropsychiatric sequelae, such as depression, particularly in female patients [79]. Of further relevance to our findings, the fragment composition of plaques in DS brains indicates an age-dependent shift, likely due to a loss of ADAM10 (Nistor et al., 2007), from primarily N-truncated/p3-related species to Aβ-related products of APP (Lalowski et al., 1996). Given that glia tend to provide more of an influence in the DS brain (Chen et al., 2014) as well as in the female brain in aging and pathology (Schwarz and Bilbo, 2012), that ApoE proteins are synthesized by glia (Boyles et al., 1985), and that N-terminally modified Aβ peptides, including p3, are glial in origin (Lalowski et al., 1996; Oberstein et al., 2015), a preliminary conclusion could be that a DS-related Aβ profile might stem from a similar mechanism as that leading to the Aβ peptide profiles we see in cortical samples obtained from female carriers of the *APOE* ε4 allele. Given our knowledge of the disease process in clinical DS, this could be very important for extrapolating markers and possible interventions into the female AD patient cohort.

Finally, we must acknowledge that our study does present with certain limitations. First, we do not know how well the donors represent the general population and we do not have any information on the donors' medication usage and/or co-morbid phenotypes, any of which might be significant confounds. Although we are not aware of any reason why our findings –based on 60 samples that cover early-onset and late-onset AD donors as well as age- and sex-matched controls– would not be representative of a broader trend in the general population, it will be important to have our findings replicated independently, with larger control and AD sampling sizes, and preferably supported by CSF/plasma biomarker data and/or premortem neuroimaging data. Second, it is well known that age is a strong risk factor for AD. Given the substantial sex difference in longevity, this can complicate studies of sex differences in the disease. Stratified sampling reduces error variance and increases power. Our 60 samples strongly suggest, based on insoluble (*i.e.,* plaque-associated) Aβ peptide levels, that AD follows distinct neurobiological processes in women and men, and that *APOE* ε4 status influences Aβ behavior in a region-dependent manner. Both processes, however, ultimately lead to a clinical AD phenotype. This is perhaps not unexpected, given the well-known sex-dependent differences in amyloid burden and behaviors in mouse models of AD (discussed in (Turner, 2001)), but it certainly needed to be demonstrated in the human context, and underscores the seemingly complex nature of the disease process and the inherent difficulty in developing a one-size-fits-all means of intervention or a generalizable biomarker (whether it be based on CSF/plasma or neuroimaging correlates).

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