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Alpha- and beta-secretase activity as a function of age and beta-amyloid in Down syndrome and normal brain

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Abstract

Aged individuals with Down syndrome (DS) develop Alzheimer's disease (AD) neuropathology by the age of 40 years. The purpose of the current study was to measure age-associated changes in APP processing in 36 individuals with DS (5 months–69 years) and in 26 controls (5 months–100 years). Alpha-secretase significantly decreased with age in DS, particularly in cases over the age of 40 years and was stable in controls. The levels of C-terminal fragments of APP reflecting alpha-secretase processing (CTF-alpha) decreased with age in both groups. In both groups, there was significant increase in beta-secretase activity with age. CTF-beta remained constant with age in controls suggesting compensatory increases in turnover/clearance mechanisms. In DS, young individuals had the lowest CTF-beta levels that may reflect rapid conversion of beta-amyloid (A β) to soluble pools or efficient CTF-beta clearance mechanisms. Treatments to slow or prevent AD in the general population targeting secretase activity may be more efficacious in adults with DS if combined with approaches that enhance A β degradation and clearance.

Keywords

Alzheimer's disease; Beta-amyloid; Oldest old; Trisomy 21

1. Introduction

Individuals with Down syndrome (DS) develop Alzheimer disease (AD) pathology in a progressive age-dependent manner [26,34,35,68] and as such, are at high risk for the development of dementia [29,33]. Clinical signs of dementia are more commonly observed when individuals are over 50 years of age [4,29,49,63]. By age 40 years, however, all individuals with DS have neuropathological changes including senile plaques and neurofibrillary tangles consistent with AD [35,67,68]. Senile plaques contain the β -amyloid peptide that is derived from a longer precursor protein, β -amyloid precursor protein (APP), the gene for which is on chromosome 21. In the most common form of DS, trisomy 21, chromosome 21 (21q21.2) is present in triplicate and leads to overexpression of APP [52]. However, despite life-long overexpression of APP in brain and in peripheral lymphocytes [47,52], A β accumulation in plaques does not typically begin until after the age of 30 years

[35]. However, A β within diffuse plaques and compact plaques has also been observed occasionally in the temporal cortex of individuals under 40 years (i.e. in childhood, teens and in early adulthood) [30,31]. These reports suggest that APP processing may lead to the production of nonamyloidogenic fragments at younger ages and shift to favor production of A β in later years.

APP is cleaved by alpha-secretase leading to a nonamyloidogenic pathway [56]. The amyloidogenic pathway leading to A β production occurs by sequential cleavage of APP by β -secretase and subsequently by γ -secretase [43,56]. β -secretase has now been identified as beta-site APP cleaving enzyme or BACE [65]. Once A β is cleaved from APP it may first appear in soluble form either within neurons or in the extracellular space. Higher levels of soluble A β are observed in DS fetal brain and in brains from adults up to 61 years of age than in tissue from controls [61]. Thus, individuals with DS exhibit higher levels of soluble A β , which may interact with both developmental and aging processes but conversion into more insoluble forms with age may be a critical event related to plaque accumulation. Additionally, A β degradation and clearance mechanisms may also be critically involved with the accumulation of plaques in DS with increasing age [16]. To our knowledge, no studies have shown age related increases in insoluble A β in the brains of DS subjects nor linked functional changes in secretase activity to A β . Further evidence that A β and APP processing play a critical role in AD pathogenesis and relevant for DS is a recent report of several families with early onset AD associated with duplication of the APP locus [51]. These individuals develop dementia around the age of 50 years and develop both parenchymal and vascular A β deposition.

We hypothesized that age-dependent increases in beta-secretase and/or decreases in alpha-secretase activity may be associated with increased production of amyloidogenic fragments of APP and with A β deposition. We predicted prior to the age of 30 years, when A β typically begins to accumulate in plaques, that secretase activity may be relatively stable. After this age, we hypothesized that secretase activity to show either decreases (alpha-secretase) or increases (beta-secretase). Thus, we assayed secretase activity in the midfrontal cortex of DS cases ranging in age from 5 months to 69 years in comparison to a series of control cases. Last, we measured A β 1–40 and A β 1–42 in formic acid soluble fractions of frozen frontal cortex to link A β to secretase activity. The midfrontal cortex was selected because it is vulnerable to age-associated AD pathology in DS and may be an early site of A β pathogenesis in DS [22].

2. Methods

2.1. Subjects

Frozen tissue blocks from the midfrontal cortex were obtained from a total of 35 individuals (22M, 13F) with DS ranging in age from 5 months to 69 years of age. A series of 18 control cases (11M, 7F) ranging in age from 5 months to 69 years were used as an age-matched group for comparison. An additional series of “oldest old” autopsy cases were also included to provide a broad range of ages for normal aging and included eight subjects ranging in age from 93 to 100 years (4M, 4F). DS and young or nondemented aged control cases were obtained from three sources: (1) the UCI-ADRC Brain tissue repository; (2) Brain and Tissue Banks for Developmental Disorders, University of Miami and; (3) The University of Washington Alzheimer's Disease Research Center. Detailed individual data are presented in Table 1. Either 10% buffered formalin or 4% paraformaldehyde fixed sections from the contralateral midfrontal cortex (Brodmann Area 9) was available for a total of 19/36 DS cases and for 20/26 control cases for immunohistochemistry. Frozen samples from the contralateral hemisphere and additional cases were taken from the same region but dissected into three blocks to assay A β concentration and secretase activity levels.

2.2. Immunolabeling experiments

Fixed samples from the midfrontal cortex were placed on blocks for sectioning. Free-floating 50 μm -thick serial sections were subsequently collected using a vibratome. Senile plaque formation was visualized using a polyclonal antibody against A β 1–42 (Biosource International, CA) and neurofibrillary tangles were visualized using AT8 (Endogen, Woburn, MA) as published previously [23]. All sections to be incubated in anti-A β 42 were pretreated with 90% formic acid for 4 min [27]. They were then treated with 3% hydrogen peroxide in 10% methanol in Tris solution for 30 min to block endogenous peroxidase activity prior to incubation in the primary antibody overnight at room temperature. Sections were subsequently incubated in either biotinylated anti-mouse or anti-rabbit IgG secondary antibodies, followed by incubation in avidin-biotin complex (Vector Laboratories, Burlingame, CA). Positive immunoreactivity was visualized using a brown 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories). Sections were mounted on glass slides, allowed to dry overnight, dehydrated in a series of increasing concentrations of alcohol and coverslipped with Depex mounting media. Controls were performed to rule out nonspecific immunostaining by eliminating the primary or secondary antibody. All controls were negative. In three control cases (#14–16), serial sections were also mounted on glass slides and stained with thioflavin-S using previously published methods [21] to establish if A β positive (cases #14 and 15) immunostaining represented diffuse (thio-S negative) or neuritic plaques (thio-S-positive) relative to a case without A β immunostaining (case #16).

2.3. A β ELISA

A β was sequentially extracted from approximately 200 mg of frozen midfrontal cortex in 0.1 M Tris pH 6.8 with 1% SDS and a Protease inhibitor Cocktail Kit (MP Biochemicals Inc.) containing 0.4 mg/ml AEBSF, 1mg/ml EDTA-Na₂, 1 $\mu\text{g}/\text{ml}$ Leupeptin and Pepstatin A at 1ml buffer/150 mg wet weight tissue using Potter Elevehjem 10 ml Wheaton glass tube and centrifuged at 4°C at 100,000 $\times g$ for 1h. The pellet was resuspended in 70% formic acid and sonicated on ice. After centrifugation at 4 °C at 100,000 $\times g$ for 1 h, the supernatant was collected and stored at –80 °C until assayed. Brain samples were run in triplicate on ELISA plates coated with a monoclonal anti-A β 1–16 antibody (kindly provided by Dr. William Van Nostrand, Stony Brook University, Stony Brook, NY) and detection was by monoclonal HRP conjugated anti-A β 1–40 (MM32-13.1.1) and anti-A β 1–42 (MM40-21.3.1) antibodies (kindly provided by Dr. Christopher Eckman, Mayo Clinic Jacksonville, Jacksonville, CA) [9,28,37]. For standards, A β 1–40 and A β 1–42 (Bachem California, Inc., Torrance, CA) were used after a pretreatment with HFIP to prevent fibril formation.

2.4. Measurement of secretase activity

Two samples of frozen midfrontal cortex immediately adjacent to the sample taken for A β ELISAs were processed according to the protocols provided by the commercial supplier of the Secretase Activity Kits (R&D Systems, Inc., Minneapolis, MN) and reported in another publication [50]. The assay tests for secretase activity by providing secretase-specific peptide conjugated to the reporter molecules EDANS and DABCYL. In the uncleaved form, the fluorescent emissions from EDANS are quenched by the physical proximity of the DABCYL moiety. Cleavage of the peptide by secretase leads to a separation of the two reporter molecules and releases a fluorescent signal that is proportional to the enzymatic activity. All assays were completed in triplicate. α - and β -secretase activity was assayed after first conducting preliminary studies to establish the amount of protein to use and the duration of the reaction for individual assays. The data presented reflect the enzymatic activity of each secretase at a protein concentration and reading time that falls on the unsaturated and linear portion of the enzyme reaction curve. For the α -secretase activity assay, 150 μg frontal cortex total protein was used (3.0 $\mu\text{g}/\mu\text{l}$ in 50 μl total, OD read at 25 min) and for β -secretase, 2.5 μg (0.05 $\mu\text{g}/\mu\text{l}$ in 50 μl total, OD read at 25 min) was used.

2.5. A β and NFT load quantification

The procedure for obtaining A β loads has been reported previously [24]. Briefly, A β or NFT immunostaining was captured using a 2.5 photo eyepiece, a Sony high-resolution CCD video camera (XC-77), the built-in video capture capabilities of a Macintosh 8100/80AV, and NIH Image 1.59b5. For every case, 10 images (525 $\mu\text{m} \times 410 \mu\text{m}$ each) were captured with a 20 \times objective. Sampling consisted of five adjacent images from the superficial cortical layers and five from the deep cortical layers (for a total of 10 images per case \times 24 cases = 240 samples). The cross-sectional area occupied by A β or AT8 in each individual image was quantified using gray scale thresholding, which separated positive staining from background and calculated the percentage of area occupied by A β immunoreactivity. These measures were intended to provide an estimate of the extent of senile plaque and neurofibrillary tangle accumulation for case characterization. Note that loads of less than 1% typically represents background levels of noise.

2.6. Total APP and C-terminal fragments Western blots and quantification

Total APP and C-terminal fragments were quantified from the same brain homogenate that A β was extracted, using the SDS soluble fraction. For total APP detection, samples were diluted in a loading buffer containing 2-mercaptoethanol (β ME) as a reducing agent and SDS as a denaturing agent, were boiled 5 min, and loaded onto a 4–20% Tris-HCl criterion gel. The running buffer used is generic for the SDS-PAGE buffer system: Glycine-Tris-SDS. A 10 μg total protein was loaded in each well. Proteins were transferred onto polyvinylidene difluoride (Sequi-blot PVDF from BioRad) for immunoblotting. For detection of the C-terminal fragments (CTFs) of APP, samples were diluted in a loading buffer containing 20 \times XT reducing agent and 4 \times XT sample buffer, then were boiled 5 min, and loaded onto a 10% Bis-Tris Criterion gel that has a near neutral pH for improved protein stability. A 30 μg of total protein was loaded into each well. The running buffer used is XT-MES to improve the resolution of the low molecular proteins. Proteins were transferred onto nitrocellulose (Trans-blot 0.2 μm from BioRad) for immunoblotting.

For both Westerns, the membranes were blocked 1 h at room temperature in 5% non-fat milk in TTBS (10 mM Tris pH 7.6, 100 mM NaCl, and 0.1% Tween 20) then incubated overnight at 4 $^{\circ}\text{C}$ in mouse monoclonal anti-APP A4 Clone 22C11 1:2000 (Chemicon) for APP, or in CT20 at 1:500 (kindly provided by Dr. Pritam Das [40]) for the CTFs. CT20 (also called O443) detects CTF- α , CTF- β and CTF- γ and full length APP [48]. After a series of washes in TTBS, membranes were incubated for an hour at room temperature in horseradish peroxidase conjugated goat anti-mouse or anti-rabbit respectively (Biorad). Proteins were visualized using Supersignal West Pico chemoluminescent substrate (Pierce). Membranes were exposed to CL-Xposure film (Pierce) and for quantification, exposure times used were established as being below the saturation range in preliminary studies. Proteins of interest were quantified by measuring the intensity using Scion Image software and CTFs were expressed as a proportion of full length APP measured on the same Western blot experiments.

2.7. Data analysis

Initially a one-way analysis of variance (ANOVA) was used to test differences in average formic acid extracted A β , secretase activity and CTF among cases in four groups—young controls, aged controls, young DS, and aged DS. Age groups were separated into under 40 years and over 40 years with the rationale that virtually all individuals with DS have the neuropathology of AD after 40 years. Thus, this classification was used to represent pre-AD and post-AD pathology in the DS cases. Post hoc comparisons were made using a Bonferonni correction. Linear regression models were used to test whether age and/or post mortem interval (PMI), if included as covariates would change our initial conclusions. That

is, the significance of the slope of these potential covariates with each outcome was tested as well as the need for an age-by-group (DS and CTL) or a PMI-by-group interaction term (to check the assumption of parallelism). Outcome measures of interest were A β or secretase activity and all initial models included age, group, and PMI as independent variables. To detect a significant relationship between A β with secretase activity both Pearson product moment correlations or partial correlations were calculated that corrected for age. When necessary, raw data were log transformed to satisfy the equality of variance assumption.

3. Results

As shown in Table 1, the average age of the control cases (mean = 36.9, S.E. = 5.6) was not significantly different from the DS cases (mean = 37.5, S.E. = 3.5), ($t(51) < 1$, $p = 0.93$). Control cases over the age of 90 years were of an average age of 95.8 years (S.E. = 0.75). The post mortem interval in the DS cases (mean = 12.4, S.E. = 1.4) was higher than in the controls (mean = 8.65, S.E. = 1.2) and the difference approached significance ($t(51) = 1.70$, $p = 0.095$). This is due to the long post mortem intervals in the youngest DS cases. Thus, in subsequent analyses, the effect of post mortem interval was evaluated for each outcome measure. If there was a significant contribution of PMI to an outcome measure of interest it was included as a covariate in subsequent analyses. Table 1 also shows plaque loads (A β 42) and neurofibrillary tangle loads (AT8) for individual cases. In the control group, one 67-year-old subject (case #17) showed a moderate tangle load in the midfrontal cortex and two subjects, a 64 (case #14) and 67-year-old (case #15), showed mild plaque accumulation (data not shown). Thioflavine-S staining was negative in these two older control cases suggesting that plaques were of the diffuse subtype (data not shown). In the DS group, we observed little plaque and tangle formation by immunohistochemistry in three subjects 24 years and younger that had available fixed tissues. In a 31-year-old subject (case #12), we observed virtually no tangle accumulation but diffuse plaque accumulation as reported in a previous study [21] although fixed tissue from this case was not available for the current study (data not shown). In a 37-year-old case (case #13), significant plaque but not tangle accumulation was observed. In cases 40 years and older ($n = 13$ with available fixed tissues), plaques were consistently observed but neurofibrillary tangles were only observed in 10/13 cases (data not shown).

3.1. A β 1–40/1–42 as a function of age

To detect age and group differences in formic acid extracted A β , subjects were first compared using a one-way ANOVA with four groups formed by diagnosis (DS versus CTL) and age (<40 years and >40 years) (Fig. 1). Post hoc comparisons with Bonferroni adjustment indicate that average A β 1–40/1–42 concentration for young control cases, young DS cases and aged control cases did not differ but aged DS individuals had significantly higher average A β values ($p < 0.0005$) than those of the other groups. Thus, age was a significant factor in the accumulation of A β in DS but not in control cases.

We next checked whether or not the inclusion of PMI as a covariate would change our conclusions by fitting regression models with A β 1–40 and A β 1–42 as outcome measures and age, diagnosis group, and PMI as independent variables. Age-by-group (DS and CTL) and PMI-by-group interaction terms were also included to check for parallelism. For models of each outcomes measure, the age-by group interaction term was highly significant ($p < 0.0005$) indicating the groups were differentially affected by the aging process. Because of this, subsequent analyses to test diagnosis group differences (and also the influence of PMI) were stratified into younger and older age groups.

In agreement with our initial analyses, no differences between young DS and young control individuals were identified in average A β 1–40 and A β 1–42 concentrations and also, the

PMI slope was not significant for either measure. However, when focusing on A β 1–40 for aged control and aged DS individuals, PMI has a significant negative slope ($F(1,17) = 9.9, p = 0.006$) while the interaction between PMI and group was not significant. This suggests that in older subjects, A β 1–40 declines as the PMI interval increases possibly suggesting instability in A β 1–40 in these samples. In an ANCOVA after adjusting for PMI, a highly significant difference between DS and CTL groups in average A β remains ($F(1,18) = 44.5, p < 0.0005$). When modeling A β 1–42, PMI was not a significant predictor, so our initial conclusion regarding significant DS /CTL differences is not altered.

Fig. 2 further shows the strong association between age and A β 1–40 ($r = 0.855, p < 0.0005$) and A β 1–42 ($r = 0.890, p < .0005$) in the DS subjects but not in the control subjects. If the A β 1–40 calculations are adjusted to remove the effect of PMI, the partial correlation drops to 0.718 which still remains highly significant ($p < 0.0005$). Adjusting the correlation of age with A β 1–42 for the effect of PMI, the partial correlation is 0.767 ($p < 0.0005$). The relatively stable amounts of A β 1–40 and A β 1–42 in controls and progressively increasing levels as a function of age in DS is also reflected in the ratio of A β 1–42:A β 1–40. In control cases, the ratio between A β 1–40 and A β 1–42 remains stable with age with approximately 80% of A β consisting of A β 1–42. In contrast, in DS, the ratio of A β 1–42 to A β 1–40 is stable until after age 30 years and then shows a rapid decrease (Fig. 3). In three DS cases over the age of 40 years, A β 40 level was higher than A β 42 but overall, we observed higher levels of A β 42 in the insoluble fraction than A β 40.

3.2. Secretase activity

To detect age and group effects on alpha-secretase activity in the frontal cortex, a one-way ANOVA was used. Fig. 4A shows that there is considerable overlap in alpha-secretase activity across patient groups and age groups. Neither these age and group differences nor PMI contributed significantly to the outcome. To further confirm the lack of age effect in control cases, additional cases from individuals over the age of 90 years were included. Average values of alpha-secretase activity for control cases over 90 years of age did not differ significantly from those of young control cases or old control cases ($F(2,20) = 0.64, p = 0.54$). However, Fig. 4B shows a trend towards lower alpha-secretase activity with age when the individual ages of subjects were considered and classifying control subjects into two groups based on a cut off of 40 years may not be optimal for testing age effects. Thus, an ANOVA was repeated with age at death included as a covariate. With this approach, group differences (control versus DS) did not affect alpha-secretase activity but age was a significant covariate ($F(1,42) = 8.75, p = 0.005$). As shown in Fig. 4B, the contribution of age appears to be primarily within the DS group particularly in individuals over the age of 40 years. When alpha-secretase activity and age is analyzed in this subset of individuals, there is a significant negative correlation ($r = -0.58, p = 0.009$) with activity decreasing markedly with age.

A similar analysis was used to detect age and group effects on beta-secretase activity (Fig. 5A). Beta-secretase activity varied as a function of both group (control versus DS) and with age ($F(3,41) = 25.97, p < 0.0005$). Post hoc comparisons with Bonferroni adjustment show that aged controls had significantly higher average beta-secretase activity than that of young controls ($p < 0.0005$) and that aged DS cases had higher activity than young DS cases ($p < 0.0005$). We extended these findings out to include cases over the age of 90 years. A one-way ANOVA comparing young, old and 90+ cases showed a significant main effect of age ($F(2,20) = 13.0, p < 0.0005$) and as Fig. 5B shows, beta-secretase activity appeared to be stable in normal aged controls even into advanced age but higher than young controls. To confirm that the PMI would not alter these conclusions, regression models as described above for A β 1–40 and A β 1–42 were undertaken to identify possible covariates and to check for parallelism. The positive slope for beta-secretase against age was highly significant

($F(1,41) = 36.4, p < 0.0005$) while the slope for PMI was negative ($F(1,41) = 1.99, p = 0.165$). The interaction terms were not significant. When age was used as a covariate in an ANCOVA, similar to the approach above, the DS average beta-secretase was significantly higher than that of the control group ($F(1,42) = 9.5, p = 0.004$). Thus, beta-secretase activity increased with age in both groups and reached higher levels overall, particularly in the aged DS cases relative to controls.

3.3. Secretase-associated C-terminal fragments of APP and steady state levels of APP

To confirm and extend the results of the secretase activity assays, an antibody directed against the C-terminus of APP (CT-20) was used to identify alpha and beta-C-terminal fragments resulting from the cleavage of APP by individual enzymes (Fig. 6A). For these analyses, CTF-alpha or beta was corrected for total APP measured by CT20 and is expressed as a percentage. A one-way ANOVA reveals a significant main effect of age group ($F(1,39) = 4.89, p = 0.034$) on the accumulation of CTF-alpha in relation to total APP. There was general trend towards lower percentage of CTF-alpha to total APP in both normal and DS aged groups (Fig. 6B). When CTF-alpha levels are plotted as a function of age in the control (Fig. 6E) or the DS group (Fig. 6F), a significant decline with age can be observed in the DS cases ($r = -0.44, p = 0.031$) but no significant effect in controls ($r = -0.30, p = 0.28$). The statistical significance of the age effect in controls may be reduced due to increased individual variability observed in older subjects.

The accumulation of CTF-beta as a percentage of total APP varied as a function of age group ($F(1,39) = 14.25, p = 0.001$) and of diagnostic group ($F(1,39) = 19.21, p < 0.0005$). But, there is no significant interaction between age group and diagnostic group ($F(1,39) = 1.82, p = 0.186$). Overall, young individuals with DS had the lowest percentage of CTF-beta to total APP (Fig. 6C). When CTF-beta is plotted as a function of age for each diagnostic group, there is significant increase in CTF-beta with age in DS ($r = 0.69, p < 0.0005$) and not in controls (Fig. 6E and F).

We next compared the percentage of CTF-beta to CTF-alpha as a function of age and diagnostic group. An ANOVA reveals a significant main effect of age group ($F(1,29) = 18.23, p < .0005$) and of diagnostic group ($F(1,39) = 5.76, p = 0.022$) but no interaction between these two variables. Fig. 6D shows that there was a large amount of individual variability in the old controls cases and that overall, the young DS cases had a lower CTF-beta to CTF-alpha percentage. When the controls are considered separately, there was no correlation between age and the percentage of CTF-beta to alpha ($r = 0.38, p = 0.16$), primarily due to the large spread in individual ratios in the oldest subjects (Fig. 6G). However, in DS cases the correlation was significant ($r = 0.75, p < 0.0005$) (Fig. 6H).

We further hypothesized that the level of CTFs would reflect secretase activity. Within the DS group, although there appeared to be little association between alpha-secretase activity measures and the levels of CTF-alpha, there was a significant correlation between beta-secretase activity and CTF-beta level ($r = 0.57, p = 0.003$).

To determine if one mechanism underlying increased formic acid extracted A β was associated with age-dependent increases in the steady state levels of total APP, Western blotting experiments were conducted. In general, DS cases exhibited higher steady state levels of APP relative to controls (Fig. 7A), but these levels were not statistically significantly different after correcting for total protein loading. Further, as reported previously, there appears to be no consistent changes in APP protein levels with age in either normal controls ($r = -0.36, p = 0.19$) or in DS ($r = 0.25, p = 0.23$) (Fig. 7B and C, respectively).

3.4. Association between plaque loads, A β and secretase activity

In control subjects, there was a significant correlation between plaque load and insoluble A β 1–40 ($r = 0.96$, $p < .0001$) and insoluble A β 1–42 ($r = 0.96$, $p < .001$) however, this was primarily driven by a single case #15 that was high in both plaque load and A β by ELISA. When corrected for age, these correlations remained. No other correlations were found between plaque load and secretase activity (alpha or beta) or level of CTFs. In DS subjects, plaque load was correlated with insoluble A β 1–40 ($r = 0.84$, $p = 0.001$), insoluble A β 1–42 ($r = 0.96$, $p < 0.0001$) but not with secretase activity or with CTF levels. Further, as in the control group, when corrected for age these correlations remained significant and higher plaque loads were associated with increasing levels of formic acid soluble A β 1–40 and A β 1–42.

Formic acid extracted A β 1–42 and A β 1–40 levels were significantly correlated with beta-secretase but not alpha-secretase activity in DS subjects. No correlations were observed in controls. In DS, there was no association between formic acid extracted A β 1–40 ($r = -0.13$, $p = 0.55$) and A β 1–42 ($r = -0.26$, $p = 0.22$) and alpha-secretase activity. Higher beta-secretase activity was associated with more extensive A β 1–40 ($r = 0.67$, $p < 0.0005$) and A β 1–42 ($r = 0.74$, $p < 0.0005$). However, when correcting for age, partial correlation coefficients were not significant suggesting that a significant part of the association between beta-secretase activity and the accumulation of A β was that both increase with age.

4. Discussion

In the most common form of DS, trisomy 21, three copies of chromosome 21 (21q21.2) leads to the overexpression of APP in brain and in peripheral lymphocytes [47,52]. As a consequence, plasma A β is significantly higher in individuals with DS (17–58 years) than in age-matched controls [8,38,55]. However, reports of further age-dependent increases in plasma A β in DS are variably reported as increased [38] or unchanged [8,55]. In CSF, there is an age dependent decrease in A β 42 that may reflect increasing A β deposition in the brain [26].

Previous reports in DS also show age-dependent increases in soluble A β and intracellular A β in brain [20,25] that precedes the deposition of A β in plaques [7,61]. Soluble A β is higher in DS early in development and continues to rise with age [61]. The data presented in the current study extends these previous findings and shows that insoluble A β increases with age in DS but at older ages (>40 years) than that reported for soluble A β [61] and reaches significantly higher levels than in normal aging but closer to levels reported in AD [14,32]. We did not observe significant differences in the levels of insoluble A β in younger DS cases as compared to controls suggesting that insoluble A β reflects either an aging or a disease process. However, our ELISA capture antibody will not detect N-terminally truncated species of A β that have been previously reported to also rise with age in DS [30] and thus our measures may be conservative.

We next determined that total APP levels did not increase with age leading to the observed increases in insoluble A β in DS, which is consistent with previous reports [13,69] despite individuals with DS having higher APP levels than controls [52]. This led us to the hypothesis that the activity of enzymes involved with the cleavage of APP may shift with age to favor the production of amyloidogenic fragments [10,56,57]. In normal aging, beta-secretase activity increases [15] and correlates with increased amounts of insoluble A β 1–40 and A β 1–42 [15]. In AD autopsy samples, BACE protein and beta-secretase activity levels are increased relative to age-matched controls [14,32,62,69] and alpha-secretase activity is reduced [62]. However, in AD brain, beta-secretase activity does not correlate with insoluble A β peptide [14]. The current study confirms increased beta-secretase activity with normal

aging but also in DS. Further, we did not observe a correlation between beta-secretase activity and insoluble A β when covarying for age. This is not entirely surprising given that insoluble A β may reflect accumulation over an extended period of times and may be more tightly coupled to degradation and clearance pathways [16]. Thus, A β accumulation in the DS brain may reflect both increased production and decreased degradation and clearance although the relative contributions of each of these pathways is unknown.

Increased beta-secretase activity in DS with age may reflect BACE1 activity [64], but also possibly BACE2, which has approximately a 64% amino acid similarity to BACE1 [1] and is present on chromosome 21 in the DS obligate region [1]. Developmentally, BACE2 protein levels are significantly higher in DS fetal tissue relative to controls [2]. In addition, in cultured fibroblasts from adults with DS, BACE2 mRNA (i.e. protein expression) was 2.6 fold higher than normal controls [2]. In the brain, BACE2 immunoreactivity was observed within frontal cortex neurons of adults with DS and AD but not in younger DS individuals nor in control cases [39]. However, overall, BACE2 is present in low levels in the brain [3,36] and within select subcortical brain nuclei and not as ubiquitously expressed as BACE1. Further, a recent paper suggests that the cleavage site on APP of BACE2 may be more consistent with alpha-secretase activity [12]. We also would have predicted that had BACE2 been a significant contributor to A β production in DS, then beta-secretase activity may have been significantly higher than controls; our observations did not support this hypothesis. Thus it is most likely that beta-secretase changes with age in DS reflect BACE1 rather than BACE2 activity.

To extend and confirm the results of the secretase activity assays, we measured protein levels of the resulting C-terminal fragments of APP representing alpha- or beta-secretase cleavage, CTF-alpha and CTF-beta, respectively. We observed unexpected dissociations between secretase activity levels and the levels of CTFs in relation to total APP that were different in controls and in DS. In normal aging, despite little or no change in alpha-secretase activity with age, we observed a decrease in CTF-alpha. Reduced CTF-alpha may be due to a shift of APP processing towards cleavage by beta-secretase. Although beta-secretase activity does increase with age in controls, which is consistent with previous reports [15], CTF-beta levels remained stable. A rapid cleavage of CTFs to A β in soluble pools, which were not measured in this study, or intact CTF clearance mechanisms in normal aging may account for this result. In DS, we observed age-dependent increases in beta-secretase activity that were consistent with an increase in CTF-beta, similar to previous reports [41,53]. These results suggest that in DS, increased CTF-beta levels more closely parallel reports in AD cases [69] rather than that observed in normal aging.

Increased APP CTF-beta with age in DS may be due to the combined effects of APP overexpression and increased beta-secretase activity that is not present in normal brain. APP overproduction in cell culture leads to abnormally large endosomes, increased A β 40, A β 42, and CTF-beta [17]. In DS brain, the number of neurons containing abnormally large endosomes rises from 21 to 40% between 5 months and 12 years of age [7]. However there is also evidence that APP overexpression alone may be insufficient to cause endosomal dysfunction suggesting that other genes on chromosome 21 may be a contributor [6].

An unexpected outcome in the current study was the lower amounts of CTF-beta in young DS cases relative to young controls. This is not due to lower beta-secretase or higher alpha-secretase activity relative to controls. There are two possible explanations. The first is that CTF-beta may be rapidly cleaved to form soluble A β , which we did not measure in the current study but in a previous report is increased in young DS brain [61]. Alternatively, lower CTF-beta may be associated with enhanced degradation and clearance mechanisms in DS that may subsequently be linked to the lack of A β plaque accumulation despite

overexpression of full length APP. The pathways that may be involved with enhanced CTF-beta turnover in DS have yet to be determined but may involve the proteasome [42,44] and it is interesting to note that proteins involved with the 20S proteasome are increased in DS fetal brain tissue [11].

We had the opportunity to include several nondemented autopsy cases between the ages of 90 and 100 years to fully extend the range of age to study alpha- and beta-secretase activity in normal aging. We had hypothesized that the oldest old may be protected from A β accumulation by increased alpha-secretase activity or decreased beta-secretase activity. Individuals over the age of 90 years exhibited consistent alpha- and beta-secretase activities as individuals between 40 and 69 years of age but higher than young controls (<40 years). Despite these changes in enzyme activity, there was very little A β plaque accumulation in these successful agers as none of the subjects included in the study had sufficient pathology for a diagnosis of AD. However, it is possible that A β may be increased in insoluble or soluble pools that were not analyzed in the current study. Thus, against a background of increasing beta-secretase activity, there may be compensatory responses such as increased A β degradation and clearance that protects these individuals from developing AD pathology.

By combining previous studies and the results of the current study the temporal events in AD pathogenesis in DS can be further clarified. Early in development there is an overproduction of APP and soluble A β [61]. Soluble A β may reflect A β accumulating within neurons [20] in association with endosomes [5]. In the brains of DS individuals 30 years and older, intracellular A β begins to decline as extracellular A β accumulates in the form of diffuse plaques [5,20]. The data collected in the current study suggests that insoluble A β levels are similar in DS and controls under the age of 30 years but increases as individuals approach their 40s and as A β plaques begin to accumulate. Increased A β in the insoluble pools occurs at approximately the same age as a shift in APP processing to favor the production of amyloidogenic fragments. Overall, A β 42 in the insoluble pool was higher than A β 40, which is consistent with immunohistochemical studies of A β in the plaques of DS brain [30].

However, increased incidence of clinical signs of dementia in DS after the age of 50 years [29,54,63,66] appear to be at a later age than the first signs of significant insoluble A β accumulation or plaque accumulation and also after the first signs of neurofibrillary tangle pathology [35]. Thus, downstream events that may be directly or indirectly related to A β or tangle formation may lead to neuronal dysfunction and cognitive decline. For example, synaptic dysfunction may be a consequence of APP overexpression or increased A β [58,60], or other pathological molecular cascades may be engaged in DS that are associated with AD pathology such as neuroinflammation [18,19,21,59], endosomal dysfunction [6,7] and oxidative damage [45,46].

When these temporal events are considered together, DS involves the co-occurrence of features of aging and of AD pathogenesis. Treatments to slow or prevent AD in the general population targeting secretase activity may also be successful when translated to the DS population at high risk for developing the disease. However, beta-secretase activity is not significantly higher in aged DS cases than that observed in aged control cases, which do not accumulate significant insoluble A β . Thus, targeting secretase activity may be more efficacious for adults with DS if combined with interventions to promote degradation and clearance of insoluble A β .

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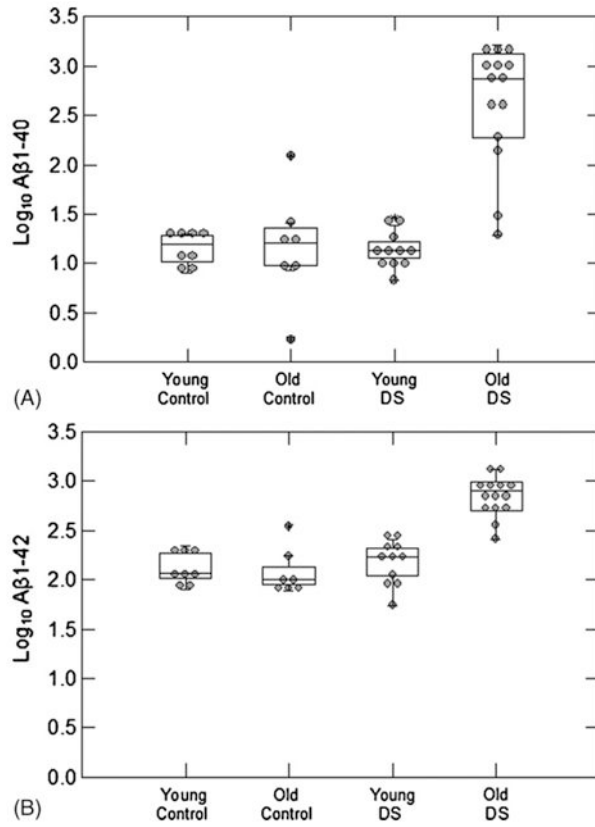


Fig. 1. Insoluble Aβ is higher in DS brain than in control brain after the age of 40 years of age. Both Aβ1-40 (A) and Aβ1-42 (B) are consistent in young controls, aged controls and in young individuals with DS. However, DS cases over the age of 40 years (old DS) show significantly higher formic acid extracted Aβ relative to the other three groups. Individual data points are shown, horizontal lines represent means and the top of the box illustrates the 75th percentile and the bottom the 25th percentile. Young controls <40 years, old controls >40 years, young DS <40 years, old DS >40 years.

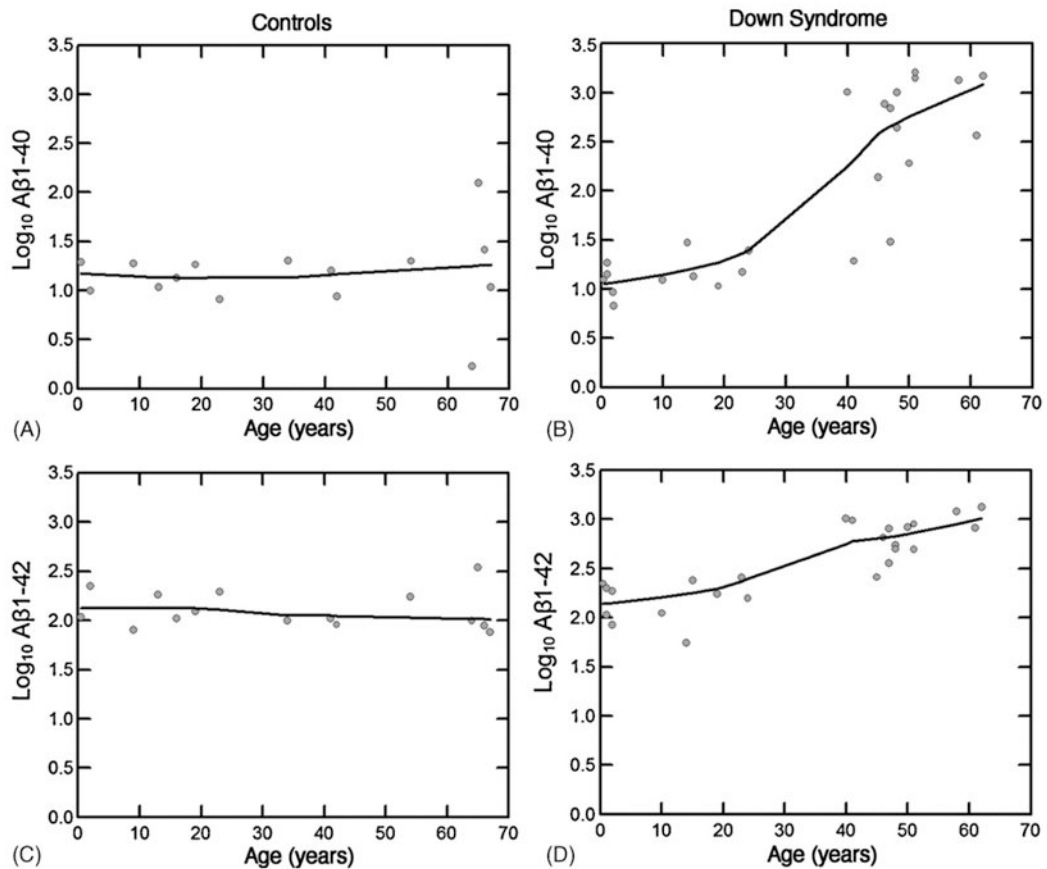


Fig. 2. Progressive rise in insoluble Aβ42 with age and a rapid rise in Aβ40 after 40 years of age in DS. Aβ40 remains constant with age in controls (A) but shows a rapid increase after the age of 40 years in DS (B). Aβ1-42 remains constant with age in controls (C) and rises progressively with age in DS (D).

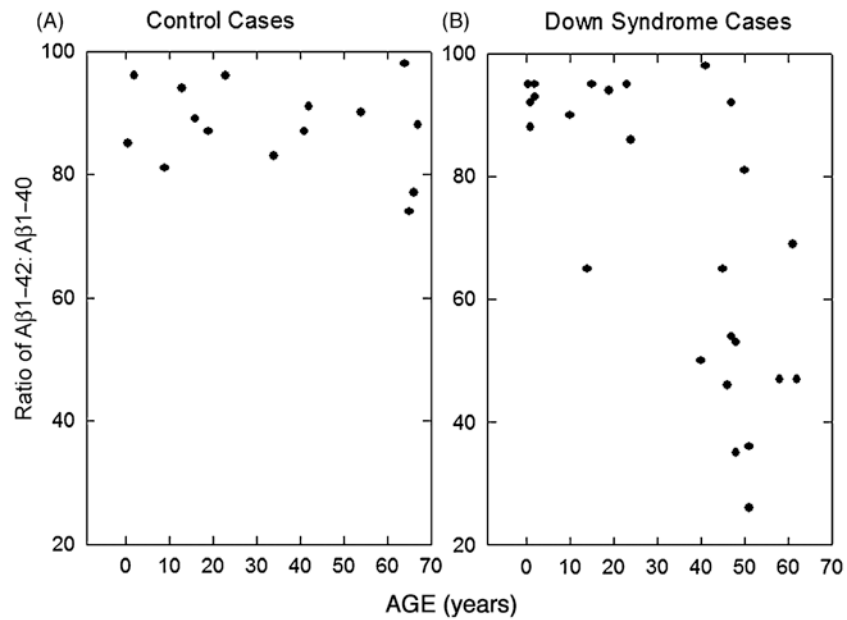


Fig. 3.

A decrease in the ratio of Aβ1-42:Aβ1-40 occurs in individuals with DS after the age of 40 years. (A) The ratio of Aβ1-42:Aβ1-40 remains constant at greater than 80% in control subjects. (B) In contrast, the ratio of Aβ1-42 to Aβ1-40 is stable until approximately 40 years of age in DS and then shows a steep drop with increasing age.

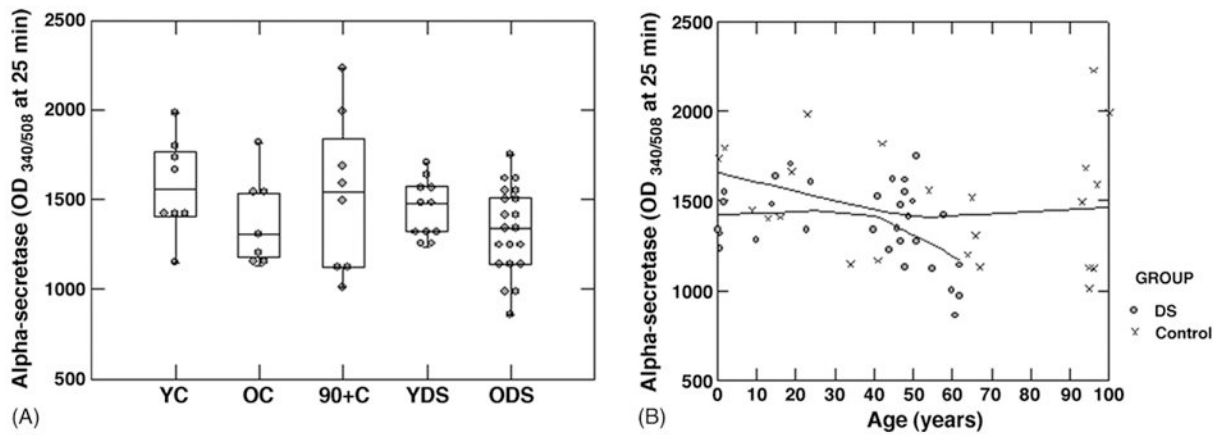


Fig. 4.

Average alpha-secretase activity remains relatively stable in controls and in DS. (A) Alpha-secretase activity was similar in control and DS cases under the age of 40 years and over the age of 40 years. Further, control cases over the age of 90 years were not different from younger controls. Individual data points are shown, horizontal lines represent means and the top of the box illustrates the 75th percentile and the bottom the 25th percentile. Young controls <40 years, old controls >40 years, young DS <40 years, old DS >40 years. (B) Alpha-secretase is plotted as a function of age and shows relatively consistent activity levels in both controls and DS. Note that a subset of DS cases over 40 years of age has lower alpha-secretase activity.

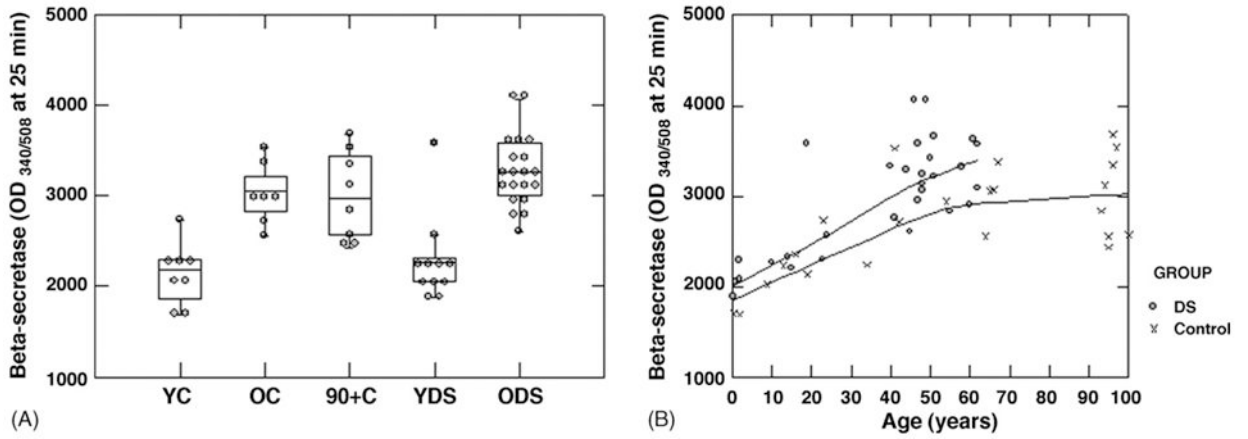


Fig. 5.

Beta-secretase activity increases with age in both controls and in DS cases. (A) Beta secretase activity increases with age in both controls and in DS, however, no further increases are observed in control cases when the age range is extended to include subjects over 90 years of age. Note that a subset of old DS cases had higher levels of beta-secretase than other cases. Individual data points are shown, horizontal lines represent means and the top of the box illustrates the 75th percentile and the bottom the 25th percentile. Young controls <40 years, old controls <40 years, young DS <40 years, old DS >40 years. (B) Beta-secretase activity is plotted as a function of age and illustrates the relatively parallel rise in activity with age in both controls and DS but maintenance of activity levels in individuals over 90 years of age.

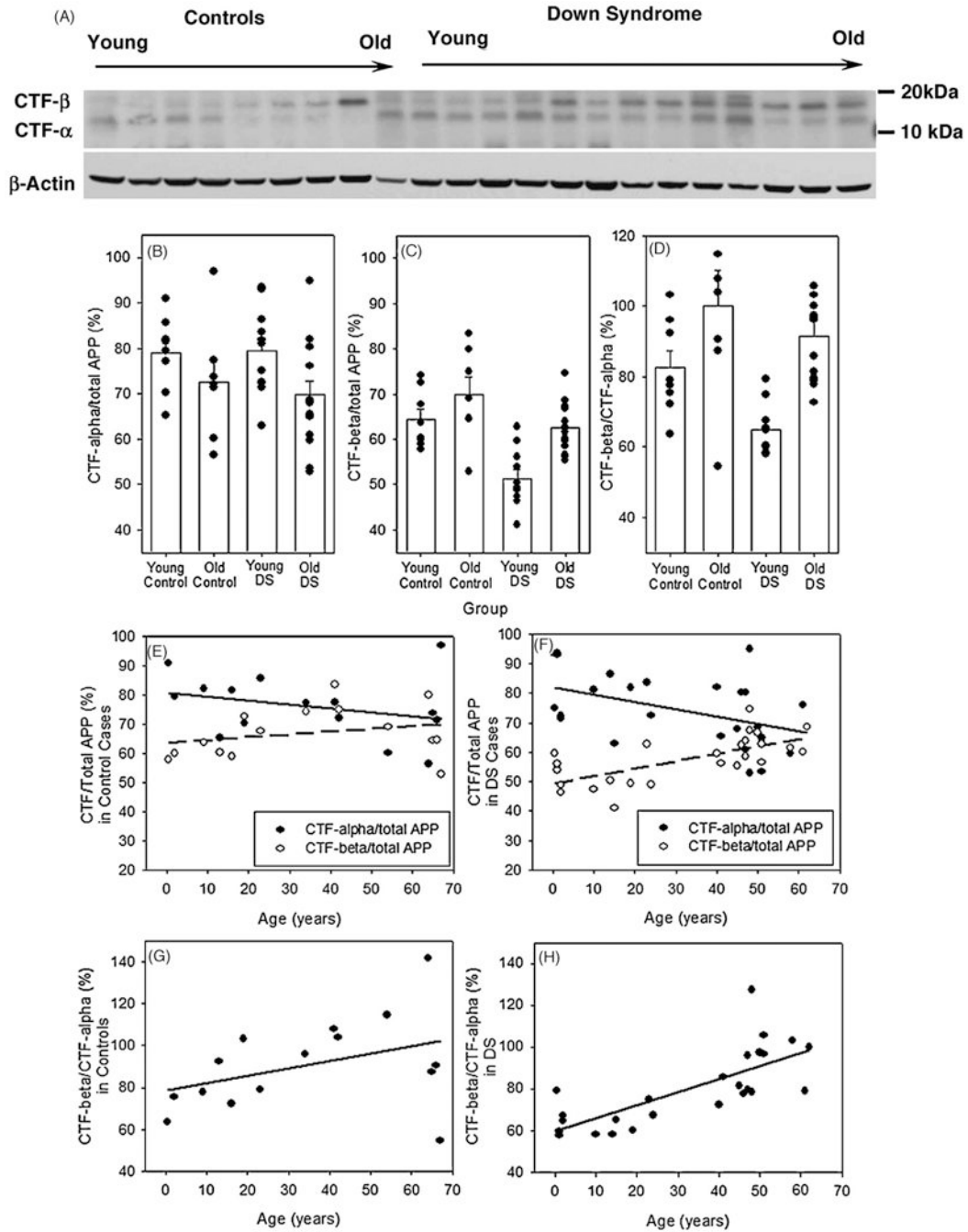


Fig. 6. APP CTF-alpha and CTF-beta with age in controls and in DS. (A) A western blot illustrates CTF-alpha and CTF-beta decreasing and increasing, respectively in both controls and DS. β -actin illustrates relatively equal loading across all cases. (B) Quantification of Western blots after correcting for protein loading and expressed as a proportion of total full length APP from the larger set of samples reveals an age dependent decrease in CTF-alpha to total APP in old controls and a similar decline in old DS cases. (C) In contrast, CTF-beta to total APP did not appear to increase appreciably in control cases and appeared lower in young DS cases when quantified. (D) The ratio of CTF-beta to alpha rose with age in controls and in DS but was also lower overall in young DS cases. (E) In controls, CTFs of APP as a result

of alpha- or beta-secretase shows stable CTF beta levels relative to total APP and a progressive decline in CTF-alpha. (F) In DS, a similar effect was observed except that the differences were more pronounced particularly in cases under 40 years of age. (G) The percentage of CTF-beta to CTF-alpha rose with age in controls but was variable in cases over 60 years of age. (H) In DS, the percentage of CTF-beta to alpha increased progressively with age. To detect CTF, the antibody CT20 was used and the specificity has been described previously [48].

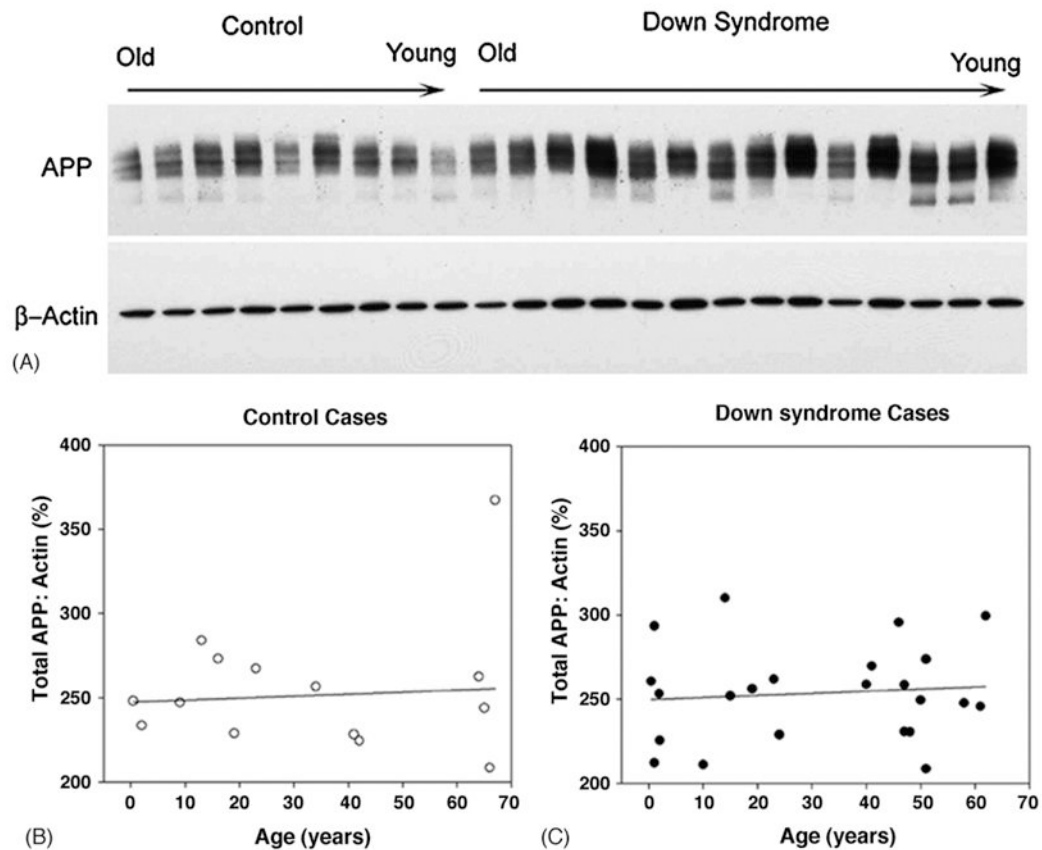


Fig. 7. Steady state levels of APP show no direct relationship with age but are higher overall in the DS cases. (A) Full length APP was detected using 22C11 in this illustration showing that overall, individuals with DS had higher levels of APP but there were no apparent trends towards an increase or decrease in APP with age in either group. Total APP corrected for actin protein loading is plotted as a function of age for controls (B) and for individuals with DS (C) showing a lack of change with increasing age.

Table 1

Case demographics

| Case | Age | Sex | PMI | Diagnosis | Formic acid (pM/mg) | | | IHC | | |
|---------------|------|-----|------|-----------|---------------------|--------|-------|-------|-------|-------|
| | | | | | AP1-42 | AP1-40 | AP42 | AT8 | AT8 | AP42 |
| Controls | | | | | | | | | | |
| 1 | 0.45 | M | 2 | Control | 107.90 | 19.50 | N/A | N/A | N/A | N/A |
| 2 | 2 | M | 10 | Control | 222.90 | 9.80 | 0.03 | 0.03 | N/A | N/A |
| 3 | 9 | F | 20 | Control | 79.90 | 18.80 | 0.26 | 0.26 | 0.00 | 0.00 |
| 4 | 13 | F | 17 | Control | 181.10 | 10.80 | 0.29 | 0.29 | 0.00 | 0.00 |
| 5 | 16 | F | 10 | Control | 105.30 | 13.50 | 0.28 | 0.28 | 0.02 | 0.02 |
| 6 | 19 | F | 7 | Control | 124.30 | 18.40 | 0.05 | 0.05 | 0.00 | 0.00 |
| 7 | 23 | M | 7 | Control | 194.50 | 8.10 | 0.04 | 0.04 | 0.02 | 0.02 |
| 8 | 34 | M | 6 | Control | 98.90 | 20.10 | 0.02 | 0.02 | 0.00 | 0.00 |
| 9 | 39 | M | 10.5 | Control | N/A | N/A | 0.02 | 0.02 | N/A | N/A |
| 10 | 41 | F | 5 | Control | 104.70 | 16.00 | 0.01 | 0.01 | N/A | N/A |
| 11 | 41 | M | 6 | Control | N/A | N/A | 0.48 | 0.48 | N/A | N/A |
| 12 | 42 | M | 18 | Control | 90.40 | 8.60 | 0.15 | 0.15 | 0.00 | 0.00 |
| 13 | 54 | F | 5 | Control | 174.00 | 20.00 | 0.02 | 0.02 | 0.00 | 0.00 |
| 14 | 64 | F | 8 | Control | 99.70 | 1.70 | 0.01 | 0.01 | 1.19 | 1.19 |
| 15 | 65 | M | 4.5 | Control | 349.00 | 123.60 | 0.02 | 0.02 | 11.89 | 11.89 |
| 16 | 66 | M | 10.4 | Control | 89.00 | 26.10 | 0.01 | 0.01 | 0.00 | 0.00 |
| 17 | 67 | M | 2.7 | Control | 76.40 | 10.80 | 1.52 | 1.52 | 0.00 | 0.00 |
| 18 | 69 | M | 6.6 | Control | N/A | N/A | 0.01 | 0.01 | 0.00 | 0.00 |
| Mean | 36.9 | | 8.65 | | 139.87 | 21.72 | 0.191 | 0.191 | 1.009 | 1.009 |
| Down syndrome | | | | | | | | | | |
| 1 | 0.42 | F | 23 | DS | 222.50 | 12.50 | N/A | N/A | N/A | N/A |
| 2 | 1 | F | 28 | DS | 106.20 | 14.20 | 0.04 | 0.04 | 0 | 0 |
| 3 | 1 | F | 24 | DS | 199.50 | 18.50 | N/A | N/A | N/A | N/A |
| 4 | 1.9 | M | 18 | DS | 185.60 | 9.20 | N/A | N/A | N/A | N/A |
| 5 | 2 | M | 17 | DS | 84.00 | 6.70 | 0.10 | 0.10 | N/A | N/A |
| 6 | 10 | M | 17 | DS | 111.20 | 12.40 | N/A | N/A | N/A | N/A |

| Case | Age | Sex | PMI | Diagnosis | Formic acid (pM/mg) | | | IHC | |
|------|-----|-----|------|-----------|---------------------|----------------|--------------|-------|--------------|
| | | | | | A β 1-42 | A β 1-40 | A β 42 | AT8 | A β 42 |
| 7 | 14 | M | 25 | DS | 55.10 | 29.70 | N/A | N/A | N/A |
| 8 | 15 | M | 14 | DS | 240.70 | 13.50 | N/A | N/A | N/A |
| 9 | 19 | M | 14 | DS | 172.20 | 10.70 | N/A | N/A | N/A |
| 10 | 23 | M | 15 | DS | 256.40 | 14.90 | N/A | N/A | N/A |
| 11 | 24 | M | 24 | DS | 157.40 | 24.60 | 0.01 | 0.01 | 0 |
| 12 | 31 | M | 4 | DS | N/A | N/A | N/A | 0.03 | <i>a</i> |
| 13 | 37 | M | 11 | DS+AD | N/A | N/A | N/A | 0.21 | 16.82 |
| 14 | 40 | M | 10 | DS+AD | 1021.70 | 1017.70 | 21.84 | 21.84 | 15.75 |
| 15 | 41 | M | 24 | DS | 974.70 | 19.40 | N/A | N/A | N/A |
| 16 | 42 | M | 16 | DS+AD | N/A | N/A | 0.02 | 0.02 | 14.2 |
| 17 | 44 | F | 16 | DS+AD | 2659.78 | 225.83 | N/A | N/A | N/A |
| 18 | 45 | F | 13 | DS | 259.20 | 138.30 | 0.24 | 0.24 | 12.61 |
| 19 | 46 | M | 6.3 | DS+AD | 652.40 | 771.20 | 29.60 | 29.60 | 17.01 |
| 20 | 47 | F | 5 | DS+AD | 805.80 | 697.40 | 30.47 | 30.47 | N/A |
| 21 | 47 | F | 24 | DS+AD | 357.80 | 30.30 | N/A | N/A | N/A |
| 22 | 48 | F | 1.5 | DS+AD | 545.70 | 1008.90 | 0.01 | 0.01 | 9.64 |
| 23 | 48 | M | 3 | DS+AD | 499.10 | 440.20 | 6.86 | 6.86 | 11.14 |
| 24 | 48 | F | 3.3 | DS+AD | 1621.44 | 181.20 | N/A | N/A | N/A |
| 25 | 49 | M | 5 | DS+AD | 790.27 | 328.07 | N/A | N/A | N/A |
| 26 | 50 | F | 5 | DS+AD | 831.30 | 190.60 | 49.08 | 49.08 | N/A |
| 27 | 51 | M | 20 | DS+AD | 896.60 | 1607.60 | N/A | N/A | N/A |
| 28 | 51 | M | 4.8 | DS+AD | 492.60 | 1405.40 | 43.22 | 43.22 | 23.7 |
| 29 | 54 | F | 5.25 | DS+AD | N/A | N/A | 1.91 | 1.91 | 7.54 |
| 30 | 55 | M | 3.1 | DS+AD | 1216.87 | 1304.42 | N/A | N/A | N/A |
| 31 | 58 | M | 3.3 | DS+AD | 1205.40 | 1337.60 | 29.25 | 29.25 | 22.56 |
| 32 | 60 | M | 3.3 | DS+AD | 1905.59 | 2241.33 | N/A | N/A | N/A |
| 33 | 61 | M | 10.5 | DS+AD | 818.00 | 366.70 | 56.19 | 56.19 | 14.79 |
| 34 | 62 | F | 3 | DS+AD | 633.68 | 3425.79 | 28.17 | 28.17 | 19.58 |
| 35 | 62 | F | 3 | DS+AD | 1330.00 | 1486.20 | N/A | N/A | N/A |
| 36 | 69 | F | 24 | DS+AD | N/A | N/A | N/A | N/A | 20.9 |

| Case | Age | Sex | PMI | Diagnosis | Formic acid (pM/mg) | | | IHC | |
|------------|------|-----|------|-----------|---------------------|----------------|-------|--------------|--|
| | | | | | A β 1-42 | A β 1-40 | AT8 | A β 42 | |
| Mean | 37.7 | | 12.4 | | 687.38 | 593.26 | 16.51 | 13.75 | |
| Oldest old | | | | | | | | | |
| 1 | 93 | F | 2.2 | Control | N/A | N/A | N/A | N/A | |
| 2 | 94 | M | 5.3 | Control | N/A | N/A | N/A | N/A | |
| 3 | 95 | F | 3.2 | Control | N/A | N/A | N/A | N/A | |
| 4 | 95 | F | 4.2 | Control | N/A | N/A | N/A | N/A | |
| 5 | 96 | M | 3.8 | Control | N/A | N/A | N/A | N/A | |
| 6 | 96 | F | 5 | Control | N/A | N/A | N/A | N/A | |
| 7 | 97 | M | 7 | Control | N/A | N/A | N/A | N/A | |
| 8 | 100 | M | 3.8 | Control | N/A | N/A | N/A | N/A | |
| Mean | 95.8 | | 4.31 | | | | | | |

PMI, post mortem interval in hours; IHC, immunohistochemistry; ELISA, cases used for A β and secretase experiments with individual values being reported for amounts of insoluble (formic acid extracted) A β 1-40 and A β 1-42; AT8/A β 42, "loads" for individual subjects for AT8 (neurofibrillary tangles) and for A β 1-42 (senile plaques); N/A, not available.

^aDiffuse plaques were observed and reported in a previous publication [21].