Proteins involved in endocytosis are upregulated by ageing in the normal human brain: implications for the development of Alzheimer's disease.

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Abstract

The greatest risk factor for Alzheimer's disease (AD) is advanced age, but the reason for this association remains unclear. Amyloid-beta (Aβ) is produced from amyloid precursor protein (APP) primarily after APP is internalised by clathrin-mediated or clathrin-independent endocytosis. Changes in endocytosis in AD have been identified. We hypothesised that endocytic protein expression is altered during ageing, thus influencing the likelihood of developing AD by increasing Aβ production. We explored how levels of endocytic proteins, APP, its metabolites, secretase enzymes and tau varied with age in cortical brain samples from men of three age ranges (young (20-30), middle-aged (45-55) and old (70-90)) with no symptoms of dementia. Aβ40 and Aβ42 were significantly increased in old brains, while APP and secretase expression was unaffected by age. Phosphorylated GSK3β increased significantly with age, a possible precursor for neurofibrillary tangle production, although phosphorylated tau was undetectable. Significant increases in clathrin, dynamin-1, AP180, Rab-5, caveolin-2 and flotillin-2 were seen in old brains. Rab-5 also increased in middle-aged brains prior to changes in Aβ levels. This age-related increase in endocytic protein expression, not described previously, suggests an age-related up-regulation of endocytosis which could predispose older individuals to develop AD by increasing APP internalisation and Aβ generation.

Keywords

Age, amyloid precursor protein, amyloid-beta, human brain.
Introduction

Alzheimer's disease (AD) is the most common form of dementia accounting for an estimated 60-80% of all cases (1). The brain pathology of AD is characterised by two hallmarks; extracellular senile plaques made of amyloid-beta (Aβ) and intracellular neurofibrillary tangles (NFTs) composed of hyper-phosphorylated tau (2). Aβ is produced in the amyloidogenic pathway where amyloid precursor protein (APP) is cleaved by β-secretase APP-cleaving enzyme (BACE1) to liberate soluble APPβ (sAPPβ) and the intracellular β-carboxy-terminal fragment (βCTF) (2). γ-Secretase then cleaves βCTF to produce Aβ and a C-terminal fragment (2-3), primarily after APP has undergone endocytosis (4). In the non-amyloidogenic pathway α-secretase cleaves APP within Aβ to release soluble APPα (sAPPα) therefore precluding the production of Aβ (5).

A variety of Aβ peptides exist with Aβ40 and Aβ42 the most abundant isoforms (6). Aβ40 is more soluble and found in high levels in both healthy and AD brains, whereas Aβ42 is more hydrophobic, considered more toxic and found at significantly higher levels in AD brains (6). Tau, a microtubule-associated protein essential for protein transport, becomes hyper-phosphorylated in AD due to the activity of a number of kinases including glycogen synthase kinase3β (GSK3β) (7).

One of the earliest reported abnormalities in AD is a change in endocytic processes (8). Cells use a variety of highly complex endocytic pathways to internalise material but it is generally considered that most cargos enter cells via clathrin-mediated endocytosis (CME) (9). Thus we have concentrated on this pathway and two others considered important in the CNS that may be linked to AD; clathrin-independent endocytosis (CIE) via caveolae or flotillins (8, 10). During CME, plasma membrane proteins are packaged into clathrin-coated vesicles which are internalised into the cell and fuse with early/sorting endosomes. Material may then be delivered to a variety of compartments including the trans-Golgi network (TGN), the lysosomes or recycled back to the plasma membrane (11). Besides clathrin, CME involves a number of proteins including the scission protein dynamin, adaptor or assembly proteins (phosphatidylinositol binding clathrin assembly protein (PICALM), adaptor protein complex 2 (AP-2), bridging integrator-1 (BIN-1) and clathrin coat assembly protein (AP180))
Caveolae are invaginations of the plasma membrane, located in ordered lipid raft domains and enriched with caveolin-1, -2 or -3 (11, 14). Once internalised, their contents may interact with early and late endosomes (15). Another route for CIE occurs through the lipid raft-associated proteins, flotillin-1 and -2 which form non-caveolar microdomains highly expressed in neurones (16-17).

Both CME and CIE have been implicated in the process of Aβ generation (18-19). A number of genes of small risk for AD were identified in genome-wide association studies (GWAS), including genes coding for proteins involved in endocytosis, PICALM, BIN-1 and sorLA (sorting protein-related receptor) (20). In addition, early endocytic changes such as enlarged Rab5-positive endosomes and increased volume of total endosomes were reported in AD brains (21-22). Furthermore, we have shown that the levels of several CME proteins including clathrin, dynamin-2 and PICALM were significantly higher in aged 22-month transgenic Tg2576 mice expressing the Swedish mutation of human APP compared to age-matched wild-type mice (23). Functionally, inhibition of CME by a dynamin dominant-negative inhibitory peptide resulted in lowered brain interstitial Aβ levels (24).

Changes in the CIE pathway are also implicated in AD since levels of caveolin-1 in the hippocampus and cortex were elevated two-fold in AD brains compared to controls (10). In contrast, decreased expression of sorLA, associated with APP recycling and normally linked with controlling Aβ levels, was found in neurons in AD (25).

It is well established that ageing is the major risk factor for AD. Approximately 11% of people over 65 have the disease and this rises to 32% over the age of 85 (1), but the reason for this association remains to be elucidated (26). Since changes in Aβ levels in AD patients are known to start 10-15 years before symptoms associated with memory problems appear (27), it is highly likely that the ageing process affects the expression of proteins associated with endocytosis as this is so closely linked to the production of Aβ. We therefore hypothesised that the expression of endocytic proteins is altered during ageing predisposing older individuals to develop AD. We have investigated this hypothesis using brain samples from young, middle-aged and old men with no known history of
dementia to examine the expression of a range of endocytic proteins involved in CME and CIE, APP metabolites and tau. Our data provide evidence for the first time of wide-ranging changes in several endocytic proteins with increasing age, suggesting that our hypothesis is indeed correct and that alterations in endocytic processes with age could underlie the development of AD.

Materials and methods

All chemicals and reagents were purchased from Sigma–Aldrich (Poole, UK) or Fisher Scientific (Leicester, UK) unless specified. Antibodies used in Western blotting were: anti N-APP, 22C11 and anti-GSK-3α/β (Millipore, Watford, UK); anti-clathrin heavy chain (CHC), Clone 23, anti-caveolin-2, Clone 65, anti-flotillin-1, Clone 18 and anti-dynamin-2 (BD Biosciences, Oxford, UK); anti-BACE1 (Merck Chemicals Ltd. Nottingham, UK); anti-AP180 (LP2D11), anti-PICALM, anti-flotillin-2 and anti-AP-2 (Novus Biologicals, Littleton, CO, USA); anti-caveolin-1 (Cell Signalling Technology, Beverly, MA, USA); anti-caveolin-3, anti-presenilin-1 (PS-1), anti-Disintegrin and Metalloproteinase domain-containing protein 10, (ADAM10), anti-sorLA (EPR14670), anti-dynamin-1 and anti-BIN-1 (Abcam, Cambridge, MA); anti-Rab5, S-19 (Santa Cruz Biotechnology, SantaCruz, CA, USA) and total tau (Dako, Hamburg, Germany). Monoclonal anti-phospho-tau Ser396/Ser404 (PHF-1) was a generous gift from Prof. Peter Davies, Albert Einstein College of Medicine, Bronx, NY, USA.

Human brain samples

Fresh frozen human frontal cortex brain samples from subjects with no recorded history of dementia were obtained from the Sudden Death Edinburgh Brain and Tissue Bank and the Newcastle Brain Tissue Resource (Table 1). None of the subjects presented with any neuropathological findings after death indicative of AD and the individuals died from the following causes: suicide, fatal overdoses of drugs or alcohol, road traffic accidents, various types of cardiovascular disease, cancer and 1 of an unknown cause. All subjects were male of three different age ranges; young 20-30, middle-aged 45-55 and old 70-90 and the mean age ± SD for each group was 26.4 ± 2 years, 49.7 ± 2.2 years and 75.1
± 4.9 years. The mean post-mortem interval was 57.2 ± 3.5 hours for all samples (Table 1). All samples were stored at -80°C prior to use. All procedures were performed in accordance with the U.K. Human Tissue Act (2004).

Tissue processing

Soluble and insoluble proteins were extracted from cortex samples to allow quantification of Aβ40 and Aβ42 from each fraction. All other proteins were investigated in the soluble fraction. Proteins were extracted using 2% sodium dodecyl sulphate with protease inhibitor cocktail III (Roche) adapted from Rees et al. (23,28). Total protein concentration was determined with the BCA Protein Assay Kit (Thermo Scientific, Waltham, USA).

Western Blotting

Western blotting was performed using standard methods. Briefly, samples were resolved on 7.5-10% polyacrylamide gels, transferred on to 0.45µm nitrocellulose membranes (Amersham Biosciences, Little Chalfont, U.K.), incubated with the relevant primary antibody and detected as previously described (23, 29).

Quantification of APP, Aβ, βCTF, sAPPα and sAPPβ

ELISA quantification was performed to detect APP (APP DuoSet ELISA, R&D Systems, Abingdon, Oxon., UK), Aβ40, Aβ42, βCTF, sAPPα and sAPPβ (IBL International GmbH, Hamburg, Germany) according to the suppliers’ guidelines and as described previously (23, 29-30). Data were presented for Aβ as pg/mg total protein concentration, APP, sAPPα and sAPPβ as ng/mg total protein concentration and βCTF as pmol/mg total protein concentration.

APOE genotyping

Brain tissue DNA extractions were performed using DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA). DNA samples were quantified using a Nanodrop spectrophotometer and normalised to a concentration of 5ng/µl prior to genotyping. APOE genotypes were determined by TaqMan
genotyping of single nucleotide polymorphism (SNP) rs7412 and KASP genotyping of SNP rs429358 as previously described (31).

**Statistical Analysis**

ELISA data were quantified using standard curves with Graphpad Prism5 and normalised to total protein concentrations. Western blots were quantified using ImageJ (www.imagej.nih.gov). The expression of proteins within each age group were analysed across more than one Western blot. All protein bands were expressed as the relative density of the same first human brain sample and normalised for GAPDH levels. ELISA results and Western blot data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or Kruskal-Wallis followed by Dunn’s post-hoc test to determine whether protein levels differed significantly between young, middle-aged or old groups. Data are presented as mean ± SEM. *p* values < 0.05 were considered significant. Where necessary, data were transformed to fit the assumptions of normality and equal variances.

**Results**

**APP metabolites and their inter-relations are altered by ageing**

APP was measured by ELISA (Figure 1A) and Western blot (Supplementary Figure 1A,B) and its levels were not affected by age.

Levels of both soluble and insoluble Aβ40 were very low in young and middle-aged brain samples, while a significant increase was detected in old brains compared to the younger two ages (*p* < 0.05, Figure 1B). Soluble Aβ42 was below the detection limits of the ELISA in young and middle-aged brains, but was significantly increased in old brains compared to the other ages (*p* < 0.05, Figure 1C). The level of insoluble Aβ42 was very low in young and middle-aged brains, but a large, significant
increase was seen in old subjects compared to younger men (p< 0.05, Figure 1C). The soluble Aβ42/Aβ40 ratio appeared to increase with ageing but was not statistically significant due to the large variation in the old subjects (Figure 1D). The insoluble Aβ42/Aβ40 ratio was significantly higher in old subjects compared to the younger cases (p< 0.05, Figure 1D).

βCTF levels were not significantly increased by ageing (Figure 1E). However, there was a weak positive correlation between the levels of βCTF and those of soluble/insoluble Aβ40 and soluble Aβ42 at certain ages (Supplementary Figure 2A-I), but this correlation was more pronounced with insoluble Aβ42 at all ages (r² = 0.71, p< 0.05 for young samples, Figure 2A, r² = 0.70, p< 0.05 for middle-aged samples, Figure 2B and r² = 0.98, p< 0.001 for old samples, Figure 2C). While the absolute levels of sAPPα and sAPPβ did not change with age (Supplementary Figure 2J,K), their inter-relation did. In the young brains, levels of sAPPα were strongly, significantly correlated with those of sAPPβ (r² = 0.82, p< 0.001, Figure 2D) but there was no correlation between sAPPα and sAPPβ in middle-aged or old subjects (r² = 0.011, p> 0.05 for middle-aged, Figure 2E and r² = 0.006, p> 0.05 for old, Figure 2F).

Since the secretases are essential enzymes for APP processing and Aβ production, we investigated whether their expression was altered with ageing. The expression of all three secretases, ADAM10 a main α-secretase candidate (5), BACE1 (mature or immature) (32) and presenilin-1 (PS-1, representative of γ-secretase) (3) was not affected by age (Supplementary Figure 1A,B).

Levels of phosphorylated-GSK-3β are increased but total tau is not altered with ageing

The other main pathology of AD, tau phosphorylation, was also studied and the expression of total tau was detected at approximately 45-60 kDa due to multiple tau isoforms (Figure 3A). Tau expression was not quantified due to the multiple number of bands, however, the level of total tau was clearly not altered with age (Figure 3A). The expression of phosphorylated tau was examined using the widely recognised PHF-1 antibody (pSer196/pSer404) (33) but no phosphorylated tau was detected in our brain samples even in old individuals (data not shown). We also examined how the
expression of GSK-3α and GSK-3β may change with ageing as it is one of the most important enzymes involved in the phosphorylation of tau. Total GSK-3α and GSK-3β were detected at 49.1 ± 0.4 kDa and 45.1 ± 0.4 kDa, respectively (7) (Figure 3A). Phosphorylation of GSK-3 was examined using a phospho-GSK-3α and -β-specific antibody. There was no change with ageing in the levels of total GSK-3α and -β nor did the ratio of phospho-GSK-3α:GSK-3α differ with age. However, the phospho-GSK-3β:GSK-3β ratio increased significantly in old brains compared to young and middle-aged subjects (p< 0.05, Figure 3A,B).

Levels of CME-related proteins are upregulated with ageing

Since Aβ levels have been linked to endocytosis, the expression of proteins involved in CME was examined to identify whether there were alterations with ageing. The expression of CHC was significantly increased with ageing; increasing by 1.7 and 2.8 times in middle-aged and old groups, respectively, in relation to the young group (p< 0.05, Figure 4A). Dynamin-1 was also affected by ageing as its level was approximately 3.6 times higher in old subjects compared to young subjects (p< 0.05, Figure 4B), while the level in middle-aged samples was not significantly different to the other ages. In contrast, dynamin-2 expression did not differ with ageing (Supplementary Figure 1A,B).

Three bands were detected for PICALM with molecular masses of 71.1 ± 0.1 kDa, 66.8 ± 0.4 kDa and 61 ± 0.4 kDa (Figure 4C). Four main isoforms of PICALM have been identified in man (NCBI Reference Sequence Database, RefSeq) NP_009097.2, NP_001008660.1, NP_001193875.1, and NP_001193876.1). It is likely that the 71.1 kDa band equates to isoform 1 with a predicted mass of 70.6 kDa, the 66.8 kDa band to isoform 2 with a predicted mass of 66.3 kDa and the 61 kDa band to isoform 4 with a predicted mass of 59.8 kDa (Figure 4C). Isoform 3 with a predicted mass of 69.9 kDa, was not well resolved from isoform 1 due to their similar masses, so they were analysed together. While PICALM isoforms 1 and 2 were not altered with ageing, the level of PICALM isoform 4 was
about 2.3 times higher in middle-aged subjects in comparison to young cases (p< 0.05, Figure 4C,D) with no significant change seen in old subjects.

AP180, a PICALM homologue (12), exists in multiple isoforms (NCBI Reference Sequence Database, RefSeq) with molecular masses ranging from 78.6 to 92.4 kDa. Here AP180 was detected as multiple bands ranging from 83.7 to 145.5 kDa (Figure 4E). Levels of AP180 increased significantly with ageing being 1.5 times higher in old subjects compared to middle-aged cases (p< 0.05, Figure 4E) while levels in young subjects were not significantly different to the other groups. Since AP-2 appears essential and specific for the early stages of endocytosis (12), its expression was also examined but no change was seen with age (Supplementary Figure 1A,B).

There are at least 15 different isoforms of BIN1, most of which are expressed in the brain including the largest, isoform 1, believed to be expressed exclusively in neurons (34). Here BIN-1 was detected as three separate bands; the largest band at 77.4 ± 56.1 kDa is likely to equate to isoform 1, while the other bands at 64.2 ± 0.5 kDa and 56.1 ± 0.4 kDa equate to two of the smaller isoforms (53). None of the BIN-1 isoforms were changed with age (Supplementary Figure 1A,B).

The expression of Rab5, an early endosome marker (21) was significantly increased in old and middle-aged groups by approximately 1.9 and 1.8 times, respectively, in comparison to the young subjects (p< 0.05, Figure 4F). SorLA levels, however, did not vary with ageing (Supplementary Figure 1A,B).

Levels of some CIE-related proteins are altered with ageing

We then examined how the level of proteins involved in CIE change with age. While caveolin-1 remained constant with age (Figure 5A), caveolin-2 was significantly elevated in old subjects by approximately 1.9 and 1.8 times in comparison to middle-aged and young cases, respectively (p<0.05, Figure 5B). Conversely, caveolin-3 was significantly decreased with ageing; 2.6 times lower in old subjects in comparison to young cases (p< 0.05, Figure 5C). The expression of flotillin-1 was not
significantly altered with age (Figure 5D). In contrast, the level of flotillin-2 was 2.3 times higher in older subjects compared to young cases (p< 0.05, Figure 5E).

**APOE genotypes do not correlate with any parameter**

**APOE** genotypes were investigated to determine whether there was any correlation between genotype and the levels of APP metabolites or endocytic protein expression. We found that 46% of the cases carried **APOE** e3/3, 34.5% carried e3/4, 8% carried e2/4 and 11.5% carried e2/3 alleles. In total 11 (42%) participants carried the **APOE** e4 allele, but there was no significant relationship between carriers of the **APOE** e4 allele collapsed across all ages and any parameter examined in the current study.

**Discussion**

This study demonstrates for the first time an age-related increase in the levels of a number of endocytic proteins involved in CME in non-diseased male human brains. Proteins involved in CIE were also increased with age. Combined with these findings, there was an age-linked increase in the absolute levels of Aβ40 and Aβ42 as well as an increase in the ratio of Aβ42 to Aβ40. In contrast, levels of APP, its metabolites and the relevant secretases were unchanged with age. The expression of total tau and p-GSK-3α was not affected by ageing, but levels of p-GSK-3β were elevated in old brains.

We obtained our brain samples from the Sudden Death Edinburgh Brain and Tissue Bank and the Newcastle Brain Tissue Resource which collect brain samples from individuals dying for a variety of reasons. We know that these individuals did not have a known history of dementia before death, did not present with any neuropathological symptoms of AD after death and died from a number of causes unrelated to AD. Thus we have considered these samples to come from non-diseased subjects representative of normal ageing. We compared human brain samples representing periods in life
before the start of any signs of future AD pathology such as the accumulation of Aβ (20-30 years), potentially during the early pre-clinical stages of disease initiation (45-55 years) and at the time when clinical symptoms of AD pathology often manifest (70-90 years). Thus our study allows us to draw conclusions about factors affected by ageing which could predispose a person to develop AD. In addition to a broad age range, we also used brains only from men to control for the effects of gender on any age-related changes we saw. There were no significant differences in post-mortem delay or brain pH between our age groups (data not shown) thus making it highly unlikely that our data can be explained by either of these factors.

The expression of different APOE alleles is associated with differential risk of developing AD. APOE e4 is associated with increased prevalence of AD and lower age of onset (35). In contrast, e2 alleles are protective against the development of AD (55). The distribution of the APOE alleles here agrees with previous studies (36). We did not see any correlation between expression of the e4 allele and any of the proteins we examined suggesting that APOE genotype does not affect the expression of endocytic proteins. However, as only 11 of our cases across all ages expressed the e4 allele, it is possible that we lacked the power to detect any correlations.

Previously, ageing has been associated with increased Aβ load in human brain samples (37). These observations were replicated here where the levels of soluble and insoluble Aβ40 and Aβ42 were significantly higher in brains of old individuals in relation to all younger subjects. We saw a wide range of Aβ expression which we believe is representative of the natural variation in Aβ40 and Aβ42 levels in individuals as there was no correlation between the levels of Aβ40 or Aβ42 with age. This was consistent with another study showing that the levels of Aβ increased in parallel with increasing age in both humans and APP transgenic mice (38). The level of insoluble Aβ42 was approximately double that of Aβ40, consistent with earlier studies indicating that Aβ42 is more prone to form aggregates than Aβ40, and hence Aβ42 is considered as an initial and major component of senile plaque deposits (6). Interestingly, we did not find any correlations between the levels of soluble and
insoluble Aβ40 and Aβ42 and the endocytic proteins we examined suggesting that the changes in endocytic proteins we see are the cause of amyloid accumulation rather than a consequence. An increase in the Aβ42/Aβ40 ratio is a useful diagnostic marker for early-onset familial AD in the brain, since the age of onset of familial AD was correlated inversely with the Aβ42/Aβ40 ratio and the level of Aβ42 but directly with Aβ40 levels (39). In AD patients the Aβ42/Aβ40 ratio is often shifted to a higher percentage of Aβ42 and associated with high synapto-toxicity in brains (40). Since similar changes were identified in normal ageing here, our findings indicate a higher likelihood, as expected, for older subjects to develop AD.

Although levels of Aβ increased with age, we saw no corresponding changes in the levels of APP in agreement with other studies (37). Interestingly, the other major APP metabolites, βCTF, sAPPα and sAPPβ, were also unchanged with age. This was surprising given the increases seen in Aβ40 and Aβ42. However, importantly, the level of βCTF was positively correlated with Aβ, particularly with insoluble Aβ42 in old brains, indicating that the level of βCTF is indeed associated with those of Aβ. Accumulation of βCTF can occur early in the pathogenesis of AD and may induce toxicity resulting in synaptic loss and cell death, hence contributing to AD pathology (41). βCTF is also very likely to be involved in the development of the enlarged endosomes seen in both AD and Down Syndrome (41) via activation of Rab5 leading eventually to endosomal enlargement and accelerated endocytosis (41). Since the levels of Aβ40 and Aβ42 increased in old subjects, the proportion of APP undergoing beta cleavage may be altered with age but absolute changes in βCTF were too small to detect. The levels of sAPPα and sAPPβ were not correlated with either Aβ or βCTF. However, there was a strong positive correlation between sAPPα and sAPPβ in young subjects which disappeared in middle-aged and old subjects. These data suggest that the control of APP metabolism is tightly regulated earlier in life with coordinated activity of α- and β–secretases but this co-ordination is lost as we age, one possible cause of the increase in Aβ seen with ageing.
Our data suggest that the changes in APP metabolites with age were not due to alterations in expression of the secretases responsible for APP metabolism. Levels of ADAM10, one of the most important α-secretases and reported to be involved in the molecular pathogenesis of AD (5), were unaltered with age implying that the increase in Aβ in old brains was not due to a decrease in α-secretase activity. This agrees with the unchanged levels of sAPPα. BACE1 mediates β-secretase cleavage of APP but again its expression did not differ significantly with age, possibly suggesting that its activity may not be altered with ageing. In previous reports, although the level of BACE1 was constant with age in human brain cortices, its activity was significantly increased with ageing (37), indicating a potential role of BACE1 in the production of Aβ in older brains. The positive correlation of βCTF and Aβ levels suggests that BACE1 activity is also increased here. PS-1, one of the catalytic subunits of γ-secretase (3), was investigated as a marker of γ-secretase. Its expression did not differ with age suggesting that the high level of Aβ in older subjects is not due to an increase in γ-secretase activity. Our data agree with an earlier study showing that the level of PS-1 and PS-2 mRNA was highest in embryonic brains and then declined to remain constant with increasing age (37).

As abnormal hyper-phosphorylation of tau is important in AD (2), we looked for phosphorylated tau in our brain samples but it was undetectable despite using a well-characterised anti-phosphorylated tau antibody (31) at a relatively high dilution (1:100). These findings suggest a very low level of this protein in non-diseased human brains. In contrast, the levels of Aβ were dramatically higher in the old group in relation to the other group consistent with the amyloid cascade hypothesis where Aβ deposition develops prior to tau tangle pathology (27). Thus, since none of our subjects were known to have AD ante-mortem, it is unlikely that they would have developed NFTs.

GSK-3 plays a central role in the pathogenesis of AD (42) since it can phosphorylate tau at multiple sites (7). It exists as two isoforms, GSK-3α and GSK-3β, which have functional differences (42) and the majority of AD-related studies have focussed on the role of GSK-3β (7). Interestingly, we observed a significant increase in the level of active phosphorylated GSK-3β at Tyr279/216 (7) in old
brains in relation to the other ages, whereas GSK-3α levels were not altered with ageing, supporting the functional differences between these two isoforms. This finding indicates that, although there was no phosphorylated tau in the old brains, the increase in active GSK-3β could be a precursor for the development of NFT along with other events.

Since the elevation in the level of Aβ in old subjects was not associated with any changes in APP or the secretase enzymes, we hypothesised that this increase could be attributed to alterations in endocytosis with age, as APP is internalised from the cell surface into endosomes prior to Aβ generation (4). Increased APP internalisation would thus result in a rise in Aβ production as the substrate would come into contact more with the enzymes.

Our data clearly show that it is indeed highly likely that CME is increased with ageing. Firstly, and most crucially, the level of clathrin was significantly increased with age. Further support for our hypothesis was provided by our data on dynamin. Clathrin-coated vesicle budding depends on dynamin (12) and neurones express three closely related dynamin proteins (43). Dynamin-2 is ubiquitously expressed across tissues, while dynamin-1 and dynamin-3 are selectively enriched in the brain, with dynamin-1 more predominant than dynamin-3 (43). We found that, while dynamin-2 was unaffected by age, the level of dynamin-1 was significantly higher in old brains. Clathrin relies on adaptor proteins and complexes (AP-2) and accessory proteins (AP180) for it to be recruited to the plasma membrane (12). We observed a significant increase in the level of AP180 in old brains adding extra support to the possibility of increased CME with ageing. Interestingly, the level of AP180 was reported to be decreased in several regions of AD brains (44), thus AP180 expression may be differentially affected by disease status. Our findings for PICALM, a highly homologous protein, are also supportive of an increase in endocytosis with ageing. PICALM aids in the formation of clathrin-coated pits (45) and is involved in the fusion of clathrin-coated vesicles with endosomes and subsequent trafficking (45). It has been linked to AD pathogenesis as discussed above (29) and supported by evidence showing that PICALM immunoreactivity was significantly higher in brains from AD cases compared to controls (19). We found that the levels of PICALM isoforms 1 and 2
were not altered with age, whereas PICALM isoform 4 was significantly increased in middle-aged brains suggesting it may contribute to the initial rise seen in Aβ in pre-clinical AD (27). The level of PICALM may increase in middle-aged brains prior to that of clathrin as PICALM has a crucial role in CME (45). PICALM was found to be abnormally cleaved with a band at 50 kDa in AD samples (19) but we only detected full-length PICALM in our brain samples. Our final evidence for an increase in CME with ageing comes from Rab5. Rab5 is an essential effector molecule which facilitates early endosome fusion and targets endosomes to the lysosomes (21). The association of increased Rab5 expression with AD was described 20 years ago (21-22). We saw significantly increased Rab5 levels in both middle-aged and older brains suggesting that endocytic uptake is elevated. Interestingly, the increase in Rab5 in the middle-aged brains occurred prior to the elevations observed for Aβ and the other CME proteins except PICALM agreeing with previous findings showing that Rab5-positive endosome enlargement is a pathological feature preceding Aβ deposition and subsequent pathological changes (22). Thus, we believe that an increase in Rab5 levels may be involved in the initiation of changes in endocytosis which could contribute to increased Aβ levels and the subsequent development of AD.

Interestingly, not all proteins associated with CME were affected by ageing. BIN-1 is a major binding partner of dynamin (12) and is linked to AD as discussed above. Further, the level of BIN-1 was found to be altered in AD but was not correlated with those of Aβ or phosphorylated tau (46). We detected BIN-1 as multiple isoforms with no changes in any of the isoforms seen with age, suggesting that the activity of BIN-1 was unaffected by age. Likewise, no correlation was observed between the level of BIN-1 and age in human brain samples of various ages (46). However, the lack of effect of ageing on BIN-1 could be due to the complexity of the many isoforms of this protein with subtle changes in isoforms being very difficult to detect. Furthermore, the expression of AP-2 was not altered with age although a recent study showed that AP-2 in addition to clathrin is involved in APP internalisation and trafficking in mouse hippocampal tissue (47). There are multiple adaptor proteins involved in CME (12) which could explain the lack of effect seen for AP2.
CIE has been also implicated in AD, particularly via caveolins and flotillins (10, 49). Our data indicate that CIE may also be increased by ageing although to a lesser extent than CME. Several studies demonstrated that caveolins are important for APP processing in the brain. The three isoforms of caveolin are all found in the nervous system of mammals (49). APP was enriched in caveolae, and caveolin-1 was shown to be physically associated with APP (50). α-Secretase cleavage was also described in caveolae and over-expression of caveolin-1 was associated with α-secretase-mediated proteolysis of APP (49-50). Here the expression of caveolin-1 was not altered with ageing agreeing with a previous report showing no difference in levels in human frontal cortex between AD brains and controls (51). Caveolin-2 forms a stable hetero-oligomeric complex with caveolin-1 (51). Surprisingly, given the lack of change in caveolin-1, we found that the level of caveolin-2 significantly increased with ageing suggesting a possible increase in the process of endocytosis via caveolae and thus an increase in APP endocytosis. Caveolin-3 is also a principal component of caveolae (49). Caveolin-3 interacts and colocalises with APP and its immuno-reactivity increased in astroglial cells surrounding senile plaques in AD brains (52). In contrast to caveolin-1 and -2, we observed a significant fall in caveolin-3 in older brains. As caveolin-3 is predominantly expressed in brain astroglial cells (49), it is possible there may be decreased caveolin-mediated endocytosis in these cells or fewer of these cells, and thus less APP may be internalised.

Flotillin is also associated with cholesterol-rich membranes involved in the generation of Aβ (53). Accumulation of flotillin-1 was observed in the endosomes of neurons from AD patients (53). We found that the level of flotillin-2 was significantly higher in old brains while flotillin-1 was unaffected by ageing. The effect of ageing on flotillin-2 is relevant as it is involved in the endocytosis of APP while flotillin-1 is not (54) and flotillin-2-dependent clustering of APP has been found to initiate its internalisation into the CME pathway (54). Thus, the increase in flotillin-2 observed in old brains may trigger greater internalisation of APP and its subsequent metabolism to Aβ which could partially account for the higher level of Aβ detected in old brains.
In conclusion, we have demonstrated for the first time clear evidence that the levels of several proteins involved in CME and CIE are increased with age in the human brains. Increased levels of these proteins in old brains suggest an age-related up-regulation in the process of endocytosis which would affect many proteins including APP. This could explain the elevation seen in the level of Aβ in the old brains. Critically, the increase in Rab5 occurred earlier in life compared to the increase in Aβ. Rab5 has been implicated in protein transport from the plasma membrane to early endosomes during CME in a variety of cells. We therefore propose that there is an age-related increase in endocytosis activity starting with Rab5 and followed later in life by increases in various CME and CIE proteins. This increase in endocytosis, when combined with other factors, could predispose older individuals to develop AD by increasing the internalisation of APP and thus its subsequent metabolism to generate Aβ. Therefore these novel findings could explain in part why ageing is the most important risk factor for AD.

**Conflicts of interest**

None of the authors have any conflicts of interest.

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References


Table 1 Summary of the sample identifiers, ages, gender, post-mortem intervals and pH of the human frontal cortex brain samples

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<sup>a</sup>MRC – Medical Research Council

<sup>b</sup>ND – not determined
Figure 1 Comparison of the levels of (A) APP, (B) soluble and insoluble Aβ40, (C) soluble and insoluble Aβ42, (D) the soluble and insoluble Aβ42/Aβ40 ratio and (E) βCTF in male human brain frontal cortex samples of old (70-90) non-diseased subjects compared to middle-aged (45-55) and young (20-30) non-diseased subjects. (A) Levels of APP were not changed with ageing. (B-D) Levels of soluble/insoluble Aβ40, soluble/insoluble Aβ42 and the insoluble Aβ42/Aβ40 ratio were significantly increased with age in human brains, while the soluble Aβ42/Aβ40 ratio was not significantly affected by age. (E) Levels of βCTF did not differ significantly with age. Data are represented as mean ± S.E.M. *p< 0.05 non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test. n=6-10.
Figure 2 Linear regression analysis of insoluble Aβ42 and βCTF levels in the (A) young (20-30) group, (B) middle-aged (45-55) group and (C) old (70-90) group; sAPPα and sAPPβ levels in the (D) young group, (E) middle-age group and (F) old group. (A-C) Levels of Aβ42 were positively
correlated with those of βCTF in all age groups. (D) The level of sAPPα was positively correlated with that of sAPPβ in the young group, while there was no correlation between sAPPα and sAPPβ levels in middle-aged and old groups (E, F). Pearson correlation analysis. n=6-7.
Figure 3 Comparison of total tau, phospho-GSK-3α:GSK-3α and phospho-GSK-3β:GSK-3β in male human brain frontal cortex samples of old (70-90) non-diseased subjects compared to middle-aged (45-55) and young (20-30) non-diseased subjects. (A) Representative immunoblots of the proteins in brain samples of different ages and (B) densitometric analysis of the immunoblots. Levels of phospho-GSK-3β were significantly increased with ageing while levels of phospho-GSK-3α and total tau were unchanged with age. Data are represented as mean ± S.E.M. *p< 0.05, one-way ANOVA followed by Tukey’s post-hoc tests. n=6-7.
Figure 4 Comparison of CME-related proteins in male human brain frontal cortex samples of old (70-90) non-diseased subjects compared to middle-aged (45-55) and young (20-30) non-diseased subjects. Each section shows a representative immunoblot of a protein in brain samples of different
ages and densitometric analysis of the immunoblots: (A) clathrin heavy chain, (B) dynamin-1, (C) PICALM isoforms 1 and 2, (D) PICALM isoform 4, (E) AP180 and (F) Rab5. Levels of clathrin, dynamin-1, PICALM (isoform 4), AP-180 and Rab-5 were all significantly increased with ageing in human brains. Levels of PICALM (isoforms 1 & 2) were not altered with age. Data are represented as mean ± S.E.M. *p< 0.05, ***p< 0.001, one-way ANOVA followed by Tukey's post-hoc tests. n=6-7.
Figure 5 Comparison of CIE-related proteins in male human brain frontal cortex samples of old (70-90) non-diseased subjects compared to middle-aged (45-55) and young (20-30) non-diseased subjects. Each section shows a representative immunoblot of a protein in brain samples of different
ages and densitometric analysis of the immunoblots: (A) caveolin-1, (B) caveolin-2, (C) caveolin-3, (D) flotillin-1 and (E) flotillin-2. Levels of caveolin-1 and flotillin-1 were unchanged with age, while the level of caveolin-3 was significantly decreased with age and caveolin-2 and flotillin-2 levels were significantly increased with age. Data are represented as mean ± S.E.M. *p< 0.05, **p< 0.01, one-way ANOVA followed by Tukey's post-hoc tests. n=6-7.
Supplementary Figure 1 Comparison of APP, BIN1, AP-2, dynamin-2, sorLA and α-, β- and γ-secretase enzymes in male human brain frontal cortex samples of old (70-90) non-diseased subjects
compared to middle-aged (45-55) and young (20-30) non-diseased subjects. (A) Representative immunoblots of the proteins in human brain samples of different ages and (B) densitometric analysis of the immunoblots. Levels of APP, BIN1, AP-2, dynamin-2, sorLA, mature/immature ADAM10 and BACE1 and PS-1 (full length and cleaved derivatives) were not significantly altered with age. Data are represented as mean ± S.E.M. n=6-7.
Supplementary Figure 2 Linear regression analysis of soluble Aβ42 and βCTF levels in the (A) young (20-30) group, (B) middle-aged (45-55) group and (C) old (70-90) group; soluble Aβ40 and
βCTF levels in the (D) young group, (E) middle-aged group and (F) old group; insoluble Aβ40 and
βCTF levels in the (G) young group, (H) middle-aged group and (I) old group. Comparison of the
levels of (J) sAPPα and (K) sAPPβ in male human brain frontal cortex samples of old non-diseased
subjects compared to middle-aged and young non-diseased subjects. No significant differences were
found between any of these comparisons. n=6-10.