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Effects of hypertension and anti-hypertensive treatment on Aβ plaque load, Aβ–synthesising and –degrading enzymes in frontal cortex

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Running title: Effects of hypertension and its treatment on Aβ

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Abstract

Epidemiological data associate hypertension with a predisposition to Alzheimer's disease (AD) and a number of post-mortem and *in vivo* studies also demonstrate that hypertension increases amyloid-beta (Aβ) pathology. In contrast anti-hypertensive medications reportedly improve cognition and decrease the risk of AD, while certain classes of anti-hypertensive drugs are associated with decreased AD-related pathology. We investigated the effects of hypertension and anti-hypertensive treatment on Aβ plaque load in post-mortem frontal cortex in AD. Aβ load was significantly increased in hypertensive (N = 20) relative to normotensive cases (N = 62) and was also significantly higher in treated (N = 9) than untreated hypertensives (N = 11). We then looked into mechanisms by which hypertension and treatment might increase Aβ load, focusing on Aβ-synthesising enzymes, β- and γ-secretase, and Aβ-degrading enzymes, angiotensin-converting enzyme (ACE), insulin-

degrading enzyme (IDE) and neprilysin. ACE and IDE protein levels were significantly lower in hypertensive (N = 21) than normotensive cases (N = 64), perhaps translating to decreased Aβ catabolism in hypertensives. ACE level was significantly higher in treated (N = 9) than untreated hypertensives (N = 12), possibly reflecting feedback upregulation of the renin-angiotensin system. Prospective studies in larger cohorts stratified according to anti-hypertensive drug class are needed to confirm these initial findings and to elucidate the interactions between hypertension, anti-hypertensive treatments and Aβ metabolism.

Keywords: Alzheimer's disease, amyloid β protein, anti-hypertensive, hypertension, β-secretase, BACE, γ-secretase, angiotensin-converting enzyme, insulin-degrading enzyme

Abbreviations: Aβ = amyloid-beta; AβPP = amyloid-beta precursor protein; ACE = angiotensin-converting enzyme; ACEI = angiotensin-converting enzyme inhibitor; AD = Alzheimer's disease; AngII = angiotensin II; ARB = angiotensin receptor blocker; BBB = blood brain barrier; CCB = calcium channel blocker; IDE = insulin-degrading enzyme; NEP = neprilysin; NFT = neurofibrillary tangle

Introduction

Epidemiological studies suggest that midlife hypertension is a risk factor for later development of Alzheimer's disease (AD), the most common form of dementia in the elderly [1, 2]. Although the mechanistic links between hypertension and AD remain elusive, there is evidence of association between hypertension and the classical neuropathological hallmarks

of AD; particularly the accumulation of Aβ in plaques. In the Honolulu Heart Program/Honolulu-Asia aging Study cohort, elevated midlife systolic blood pressure (≥ 160 mm Hg) was associated with a significant increase in the number of neocortical and hippocampal Aβ plaques at post-mortem examination, and elevated midlife diastolic blood pressure (≥ 95 mm Hg) was associated with a higher number of neurofibrillary tangles (NFTs) in the hippocampus [3]. Similar associations were reported in other post-mortem studies [4, 5]. In living participants with or without dementia, the amount of cerebral Aβ detectable by amyloid positron emission tomography (amyloid-PET) increased with diastolic blood pressure [6] and was significantly greater in cognitively normal hypertensive participants who had at least 1 *APOE* ε4 allele [7], an established risk factor for AD [8, 9]. Plasma Aβ42:Aβ40 ratio (mainly driven by Aβ40 level) correlated with systolic blood pressure [10] and with severity of white matter damage [11], which predicts the incidence [12] and progression of decline in AD [13]. Experimental studies in mice [14-16] and rats [17] provided further evidence that hypertension promotes Aβ accumulation.

At present the mechanism by which hypertension enhances Aβ accumulation is unknown. Hypertension induced by infusion of angiotensin II (AngII) did not alter the expression of mRNAs encoding multiple proteins involved in Aβ production: the α-secretases *Adam9*, *Adam10*, and *Adam17*, the β-secretases *Bace1* and *Bace2* and the γ-secretase components *Psen1*, *Psen2*, *Aph1a*, *Aph1b*, *Psenen* and *Ncstn* [18] in C57/BL6 mice. However, intravenous infusion of AngII enhanced β-secretase cleavage of amyloid-β precursor protein (AβPP) in Tg2576 mice, which overexpress a mutant form of APP bearing the Swedish mutation (KM670/671NL) [15]. Intracerebroventricular infusion of AngII in adult wild-type Sprague-Dawley rats increased activity of Aβ synthesising enzymes and Aβ42 production and also increased tau phosphorylation, and all of which was inhibited by an

AngII receptor antagonist, losartan [19, 20]. Similarly AngII exposure increased β-secretase activity in Chinese hamster ovary cells producing Aβ [15].

In recent years, the potential protective effect of anti-hypertensive therapies on cognitive decline and AD has become a topic of increasing interest and a number of reviews have attempted to evaluate the somewhat inconsistent evidence from longitudinal cohort studies and clinical trials [21-23]. The data are encouraging and discrepancies between the studies mainly concern differential effects of the anti-hypertensive drug classes. Fournier *et al* [23] concluded that the angiotensin receptor blockers (ARBs) and dihydropyridine calcium channel blockers (CCBs) have greater protective effects than do angiotensin-converting enzyme inhibitors (ACEIs) and diuretics, and that β-blockers may be neutral or even worsen cognitive decline. The amount of cerebral Aβ detected by amyloid-PET in cognitively normal adults was higher in those with untreated hypertension than in the treated or normotensive participants [7].

There is also evidence from post-mortem studies that antihypertensive treatment reduces Aβ plaque pathology [3, 5, 24]. In one of these studies, Hajjar *et al* [24] found less Aβ deposition after treatment with ARBs than other classes of anti-hypertensives or in brain tissue from people with a history of untreated hypertension. The ARBs valsartan [25] and candesartan [26] reduced oligomerisation of Aβ *in vitro*, as did the diuretic furosemide and the CCB nitrendipine [26], while the ARBs valsartan [25] and losartan [27] reduced Aβ plaque formation in transgenic mouse models of Aβ accumulation. The mechanism(s) by which anti-hypertensive drugs might decrease Aβ accumulation are largely unstudied. Paris *et al* [28] showed that dihydropyridine CCBs improved Aβ clearance across the blood-brain

barrier (BBB) in a mouse model of Aβ accumulation and Wang *et al* [25] showed that ARB treatment increased activity of an Aβ-degrading enzyme, insulin-degrading enzyme (IDE).

In this study of post-mortem tissue we initially performed a retrospective analysis to investigate whether hypertension and anti-hypertensive treatment was associated with Aβ plaque load in the frontal cortex of a series of AD brains in which the plaque load had been routinely measured. Since there is some evidence for a relationship between *APOE* genotype and hypertension [29], we included an analysis of *APOE* ε4 allele presence or absence. Our finding in this study of significantly increased Aβ load in the AD cases with a history of hypertension compared to normotensive cases, and in treated than untreated hypertensive cases guided a series of subsequent investigations of possible mechanisms by which hypertension and anti-hypertensive treatment might have increased Aβ production or proteolytic degradation. In particular, we investigated the following with respect to hypertensive status and treatment, in AD and neuropathologically normal controls: activity of β- and γ-secretases, responsible for sequential cleavage of AβPP to Aβ, and concentration and activity of three Aβ-degrading enzymes, neprilysin (NEP) [30-32], IDE [33, 34] and angiotensin-converting enzyme (ACE) [35, 36]. Levels of ACE and IDE were decreased in cortex from people with a history of hypertension, suggesting that hypertension may affect Aβ catabolism, and the level of ACE was increased in cases where hypertension was treated, suggesting feedback upregulation of the renin-angiotensin system. Prospective studies in larger cohorts stratified according to anti-hypertensive drug class are needed to confirm these initial findings.

Materials and Methods

Brain tissue

Brain tissue was obtained from the South West Dementia Brain Bank (SWDBB), University of Bristol, with research ethics committee approval from NRES Committee South West – Central Bristol. The brains had been divided mid-sagittally at autopsy, the left cerebral hemisphere sliced and frozen at -80°C, and the right hemisphere fixed in 10% buffered formalin for 3 weeks before its embedding for paraffin histology. All cases had been subjected to detailed neuropathological assessment and diagnosis had been made following standard protocols [37, 38]. According to National Institute on Aging-Alzheimer's Association guidelines, AD neuropathological changes were a sufficient explanation for the dementia in all of the AD cases [37] and the controls had no clinical history of cognitive decline and showed no or minimal AD changes (up to Braak tangle stage II) or other neuropathological abnormalities.

For immunohistochemical analysis we used 7-µm paraffin sections from the right frontal lobe. For biochemical analysis, frozen tissue (200 mg) from left midfrontal cortex (Brodmann area 46) was homogenised in either 0.5% Triton X-100 lysis buffer or 1% sodium dodecyl sulphate (SDS) lysis buffer (see individual assays) in a Precellys 24 homogeniser (Stretton Scientific, Derbyshire, UK) for 2 x 15 s at 6000 x *g*, centrifuged at 13,000 x *g* for 15 min at 4°C and stored at -80°C. Total protein was measured using the Total Protein kit (Sigma Aldrich).

Study cohorts

Diagnostic criteria for retrospective diagnosis of hypertension were: sustained SBP ≥ 140 mm Hg and/or DBP ≥ 90 mm Hg recorded on three or more occasions. Cases in which all blood pressure readings recorded in the medical notes were within the normal range were classified as normotensive; furthermore these cases had no history of any prescriptions for anti-hypertensive drugs. The hypertensive cases were stratified into those with a history of anti-hypertensive treatment (prescription of one or more anti-hypertensive drugs listed in the medical records) and an untreated group (no history of any anti-hypertensive drug prescriptions). MRC database identifiers, pathological and demographic data and details of measurements available for individual cases are listed in Supplementary Table 1.

Measurement of Aβ plaque load

For initial analysis, we used measurements of frontal Aβ plaque load that had previously been determined in 82 AD brains: 62 cases from normotensive patients and 20 from patients with a history of hypertension. Of the hypertensive cases, only 9 had been treated. Age and post-mortem delay did not differ significantly between the groups used for pairwise comparisons. Previous studies by our group found no effect of simulated post-mortem delay of up to 72 h on Aβ measurements [39]. Braak tangle stage was not significantly different between groups used for pairwise comparisons; however the normotensive group tended to include more cases with lower Braak tangle pathology (including 3 cases with Braak tangle stage III) (Supplementary Figure 1). Demographic data for this cohort are summarised in Table 1.

The field fraction (percentage area) immunopositive for Aβ (labelled with antibody to Aβ residues 17-24, clone 4G8 from BioLegend, London, UK; 1:16000 overnight incubation

after immersion of sections in formic acid, visualised with avidin-biotin horseradish peroxidase complex kit from Vector Laboratories, Burlingame, CA) was measured in 10 areas of cortex covering 4 mm^2. Histometrix software (Kinetic Imaging, Wirral, UK) driving a Leica DM microscope with a motorised stage was used to make an unbiased selection of the 10 areas, as previously described [40]. Aβ-laden blood vessels were excluded from analysis.

Measurement of β-and γ-secretase activity

Retrospective analysis of β-secretase and prospective analysis of γ-secretase activity was performed in 108 brains from AD and neuropathologically normal controls: 77 cases from normotensive patients (50 AD and 27 controls) and 31 from patients with a history of hypertension (18 AD and 13 controls). Frontal Aβ plaque load was available for all AD cases. Of the hypertensive cases, 13 had been treated. Post-mortem delay did not differ significantly between groups used for pairwise comparisons but age was significantly higher in the hypertensive cohort. Demographic and neuropathological data for this cohort are summarised in Table 2.

β-secretase activity had been measured previously [31, 41, 42]. As described, the fluorogenic substrate Mca-SEVNLDAEFRK(Dnp)RR-NH_2 (R&D Systems), containing part of human AβPP sequence modified by the Swedish double mutation, was used according to the manufacturer's guidelines to measure β-secretase activity in brain homogenates prepared in 0.5% Triton X-100 lysis buffer diluted 1:200 in 0.1 M sodium acetate buffer (pH 4, 37°C). Each homogenate was assayed in duplicate in the absence, and once in the presence, of the β-secretase inhibitor III (Millipore, Durham, UK, 5 mM). Seven two-fold serial dilutions of recombinant human β-secretase (R&D Systems, 20000 – 156 ng/ml) were

also assayed, in the absence and in the presence of β-secretase inhibitor III, on each plate. After a 3 h incubation at 37°C, fluorescence was measured by excitation at 320 and emission at 405 nm in a FLUOstar Optima plate reader (BMG LABTECH Ltd, Aylesbury, UK). β-secretase-specific activity was determined by subtracting the fluorescent signal after inhibition from that in the paired uninhibited wells. β-secretase activity in relative fluorescence units (rfu) was interpolated from the standard curve and adjusted for total protein content.

γ-secretase activity was measured in the same homogenates. The fluorogenic substrate Nma-GGVVIATVK(Dnp)*D*R*D*R*D*R-NH_2 (Merk Millipore, Darmstadt, Germany), containing the C-terminal AβPP amino acid sequence that is cleaved by γ-secretase, was used according to the manufacturer's guidelines to measure γ-secretase activity in brain homogenates prepared in 0.5% Triton X-100 lysis buffer. Brain homogenates were diluted 1:50 in 0.1 M sodium acetate buffer (pH 4, 37°C) and seven two-fold serial dilutions of a standard reference brain homogenate were included on each plate. Each sample was assayed in duplicate in the absence, and once in the presence, of two γ-secretase-specific inhibitors, L-685, 458 (Tocris Bioscience, Bristol, UK, 100 µM) and DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester (Enzo Life Sciences, Exeter, UK, 200 µM) in black F16 Nunc Maxisorp plates (Fisher Scientific). Following a 2 h incubation at 37°C (with agitation, protected from light), fluorescence was measured by excitation at 360 and emission at 440 nm in a FLUOstar Optima plate reader (BMG LABTECH Ltd). γ-secretase-specific activity was determined by subtracting the fluorescent signal after inhibition from that in the paired uninhibited wells. γ-secretase activity (in rfu) was interpolated from the standard curve and adjusted for total protein content. The mean intra-assay CV was 4.41% (s.d. 3.33) and the inter-assay CV was 7.64% (s.d. 3.66).

Measurement of ACE, IDE and NEP concentration and activity

Retrospective analysis of ACE, IDE and NEP concentration and activity was performed in 85 brains from AD and neuropathologically normal controls: 64 cases from normotensive patients (43 AD and 21 controls) and 21 from patients with a history of hypertension (12 AD and 9 controls). Frontal Aβ plaque load was available for all AD cases. Of the hypertensive cases, 9 had been treated. Post-mortem delay did not differ significantly between groups. In previous studies by our group ACE, IDE and NEP activities were found to be stable for at least 72 h under conditions of simulated post-mortem delay [34, 43]. Age was significantly higher in the hypertensive cohort. Demographic and neuropathological data for this cohort are summarised in Table 3.

ACE, IDE and NEP protein and activity in these cases had been previously measured and published [30, 31, 33-36, 41]. ACE protein had been measured using the ACE Duoset ELISA kit (R&D systems) and ACE activity had been measured using the ACE1-specific fluorogenic substrate Abz-FRK(Dnp)-*P* (Biomol International, Exeter, UK) in the presence of captopril (1 mM) in brain homogenates prepared in 1% SDS lysis buffer, as detailed in Miners *et al* [35, 36]. IDE protein had been measured by sandwich ELISA with rabbit anti-human IDE capture antibody (Abcam, Cambridge,UK) and mouse anti-IDE detection antibody (R&D Systems, Abingdon, UK) in brain homogenates prepared in 1% SDS lysis buffer, and IDE activity had been measured using an immunocapture-based assay with an IDE-specific capture antibody (rabbit polyclonal anti-IDE, Abcam) and the fluorogenic peptide substrate Mca-RPPGFSAFK-OH (R&D Systems) in brain homogenates prepared in 0.5% Triton X-100 lysis buffer, as detailed in Miners *et al* [33, 34]. NEP protein had been measured using the Neprilysin

Duoset ELISA kit (R&D systems) in brain homogenates prepared in 1% SDS lysis buffer, and NEP activity had been measured using an immunocapture-based assay with a NEP-specific capture antibody (goat anti-human NEP, R&D Systems) and the fluorogenic peptide substrate Mca-RPPGFSAFK-OH (R&D Systems) in brain homogenates prepared in 0.5% Triton X-100 lysis buffer, as detailed in Miners *et al* [30-32].

Statistical analysis

Pairwise comparisons between groups were made by Mann-Whitney U test. Spearman's test was used to assess correlations between post-mortem delay and all measured proteins (for results see Supplementary Table 2). Post-hoc statistical power calculations were made using G*Power 3.1.9.2 software [44]. P values < 0.05 were considered statistically significant.

Results

Increased frontal Aβ load in AD with history of hypertension

Frontal Aβ load was significantly higher in the hypertensive than normotensive AD cases (p = 0.020; Figure 1 A). Frontal Aβ load tended, although not significantly so, to be higher in treated than untreated hypertensive cases (p = 0.07; Figure 1 B). In this cohort possession of *APOE* ε4 was associated with a non-significantly greater frontal Aβ load. Frontal Aβ load was significantly higher in the ε4+ hypertensive than ε4+ normotensive cases (p = 0.009) (Supplementary Figure 2).

Aβ-synthesising enzyme activities are not altered in AD or controls with history of hypertension, or affected by anti-hypertensive treatment

β- and γ-secretase activities in midfrontal cortex were higher in AD than control cases (β-secretase p = 0.031; γ-secretase p = 0.058) (Supplementary Figure 3), therefore analysis of the influence of history of hypertension was performed both with the diagnosis groups combined and also separately in AD and controls. β- and γ-secretase activities were similar in hypertensive and normotensive cases, and in treated and untreated hypertensive cases in all diagnosis groups (Figure 3).

Hypertension, anti-hypertensive treatment and Aβ-degrading enzymes

Analysis was performed both with diagnosis groups combined and also separately in AD and controls, as there is evidence that ACE, IDE and NEP are altered in AD [31]. Levels and activities of ACE, IDE and NEP were compared between hypertensive and normotensive groups (Supplementary Table 3). ACE protein was significantly lower in the combined hypertensive group (p = 0.010) and in hypertensive AD cases (p = 0.009) but not hypertensive controls (Figure 3A – C). Similar but non-significant trends were observed for ACE activity (Figure 3D – F). IDE protein was significantly lower in the combined hypertensive group (p = 0.036) but not when cases were stratified according to diagnosis. IDE activity was significantly higher in hypertensive controls (p = 0.027) but not in the combined or AD group (Figure 3 G - L). NEP protein and activity did not differ significantly between groups.

Levels and activities of ACE, IDE and NEP were also compared between treated and untreated hypertensive cases (Supplementary Table 4). ACE protein was significantly higher in treated cases with diagnosis groups combined (p = 0.008) and in treated AD cases (p = 0.016), and a similar trend

was observed in treated controls (Figure 4 A - C). However, the increases in ACE protein were not reflected in ACE activity (Figure 4 D – F). IDE and NEP protein and activity did not differ significantly between groups. Post-hoc analysis estimating the power of the test to reject a type II error (reject the hypothesis falsely) at the $p = 0.05$ level for ACE protein between hypertensive and normotensive groups was 70% in the combined cohort and 79% in the AD cohort. For ACE protein between treated and untreated groups the figures were 81% in the combined cohort and 83% in the AD cohort.

Discussion

This retrospective study has shown significantly higher frontal Aβ load in AD cases with a history of hypertension, in keeping with prior evidence from other human post-mortem [3, 4] and neuroimaging studies [6, 7], and studies in mice [14-16] and rats [17]. Although Braak tangle stage did not differ significantly between the hypertensive and normotensive group, the hypertensive group did tend to have slightly more advanced Braak tangle stages, raising the possibility that hypertension may also exacerbate this aspect of AD pathology as well. We have expanded on previous studies by investigating several mechanisms by which hypertension might increase Aβ load, focusing on the synthesis and enzymatic degradation of Aβ.

Our findings indicate that β- and γ-secretase activities are unchanged by hypertensive status, arguing against increased Aβ synthesis as an explanation for increased Aβ load in hypertensives. This is in keeping with a study in mice in which hypertension induced by infusion of AngII had no effect on the expression of AβPP or the secretase enzymes [18], but in contrast to other AngII infusion studies in mice [15] and rats [20]. Of the three Aβ-degrading enzymes we investigated, the concentration of two – ACE and IDE –was significantly decreased in the frontal cortex in hypertensive cases, particularly those with

AD. Although we did not find that the activity of these enzymes was significantly reduced in hypertension, this finding raises the possibility that one contributor to the increased Aβ load in hypertension may be a decrease in Aβ catabolism. Studies that have shown that ACE converts Aβ43 (recently found to be the earliest form of Aβ deposited in a mouse model of AD [45]) to Aβ41 [45], and Aβ42 to the less amyloidogenic form Aβ40 [46, 47].

In this study we examined only a few of the potential contributors to increased accumulation of Aβ in AD. Several other effects of hypertension may also play a role. These could include impaired perivascular drainage as a consequence of collagenous thickening and reduced pulsatility of arteries and arterioles [48-50]; reduced receptor-mediated transport of Aβ across the endothelium into the bloodstream; degeneration of pericytes [51], leading to reduced clearance of soluble Aβ40 and Aβ42 from the interstitial fluid [52]; altered expression of AβPP [53, 54]; or reduction in the activity of any of several Aβ-degrading enzymes other than those measured in the present study [55-57].

In previous post-mortem [3, 5, 24] and clinical studies [7], anti-hypertensive treatment was reported to reduce Aβ pathology. Unexpectedly, we found frontal Aβ load to be higher in the treated than untreated hypertensives. One possible explanation is that treatment was more likely to be prescribed to people with more marked or more persistent hypertension. However, there were several limitations to the present study that should be considered in interpreting the findings. The cohort size was small, particularly that of the treated hypertensive group, and although post-hoc power analyses indicated reasonable statistical power, this type of power calculation cannot be used to support findings retrospectively. Furthermore, the retrospective nature of the study imposed multiple constraints on the data acquisition and quality assurance: in many cases we lacked information on the duration and effectiveness of anti-hypertensive treatment, the criteria for diagnosis and treatment of

hypertension have changed over time, and there was inconsistency in the way in which blood pressure was measured – in some cases automated, in others manual. A final consideration is that the cohort was too small for meaningful stratification of cases according to anti-hypertensive drug class, whilst polypharmacy (i.e. being on more than one type of blood pressure drug concurrently) is also often common. We noted that 89% of treated cases had received either diuretics or β-blockers, which have little protective effect on cognition and may even exacerbate cognitive decline [23], and 33% were prescribed ACE inhibitors, which were found to have ambiguous effects on cognition [23] and may even increase mortality rate in AD [22]). Only 22% were prescribed a CCB and none were prescribed an ARB, the drug classes shown to have the greatest protective potential against AD [23]. For all of these reasons, and the reported differential effects of various anti-hypertensive drugs on AD, the findings on subgroup analysis in the current study cannot be taken to indicate that Aβ accumulation is increased by treatment of hypertension per se.

As far as we are aware, this is the first study to investigate the possible influence of anti-hypertensive treatment on the level or activity of Aβ-synthesising and -degrading enzymes in human brain tissue. ACE protein level in the frontal cortex was significantly increased in treated hypertensives, particularly in the AD group. Whilst for the reasons discussed above, caution is warranted in interpreting this finding, it raises the possibility that antihypertensive therapy may cause feedback upregulation of ACE production within the brain. Although this is unlikely to be have an impact on the control of blood pressure, it is noteworthy that ACE has a wide range of substrates and probable physiological functions in the CNS [58] that have the potential to be affected if the production of this enzyme is increased.

In conclusion we have shown that hypertension increases plaque-associated Aβ in the frontal cortex. This may be partly attributable to decreased Aβ catabolism but other mechanisms are likely to contribute and warrant further investigation. Prospective studies of larger cohorts are needed to assess the influence of different classes of anti-hypertensive drug on Aβ pathology, and the impact this has on their efficacy in reducing cognitive decline and protecting against AD.

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Author contributions

EA and JM performed and analysed experiments and EA wrote the manuscript. SL devised the study and PK and SL supervised the work. All authors contributed to the critical review of the writing. None of authors has any conflict of interest to declare.

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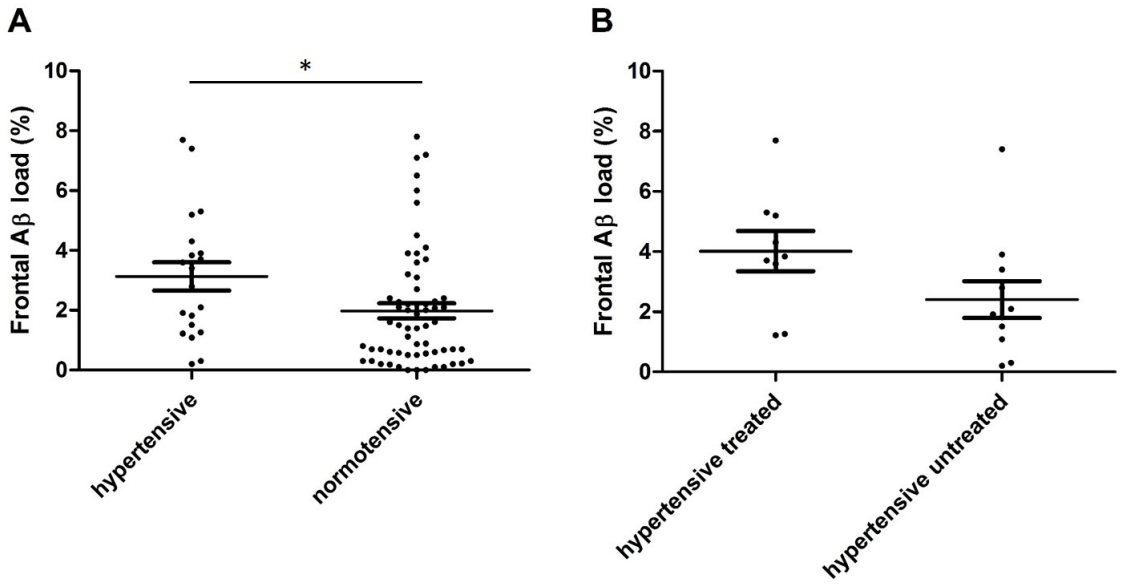


Figure 1. Frontal Aβ load (%) in AD cases with and without hypertension (**A**) and in the hypertensive group stratified into treated and untreated cases (**B**). Frontal Aβ load was significantly higher in the hypertensive group ($p = 0.020$) and was also higher in cases that had been treated with anti-hypertensives. The horizontal bars indicate the mean and SEM. Each point represents a separate brain.

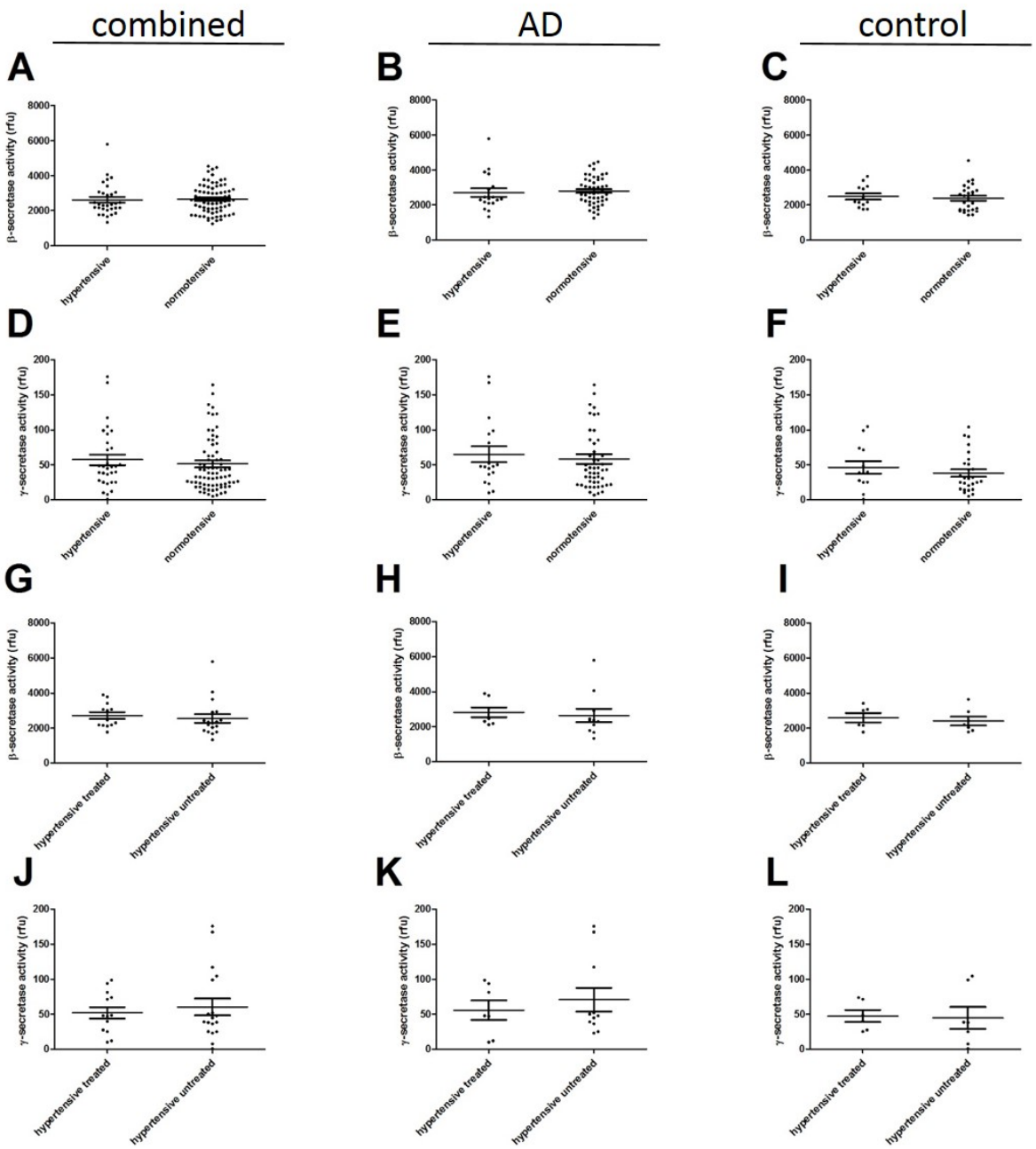


Figure 2. A – C and **D – F** show β- and γ-secretase activities in hypertensive and normotensive cases, and **G – I** and **J – L** show β- and γ-secretase activities, in treated and untreated hypertensives, with diagnosis groups combined (column 1), in AD (column 2) and in controls (column 3). β- and γ-secretase activities were similar in the hypertensive and normotensive groups and were not affected by anti-hypertensive treatment. The horizontal bars indicate the mean and SEM. Each point represents a separate brain.

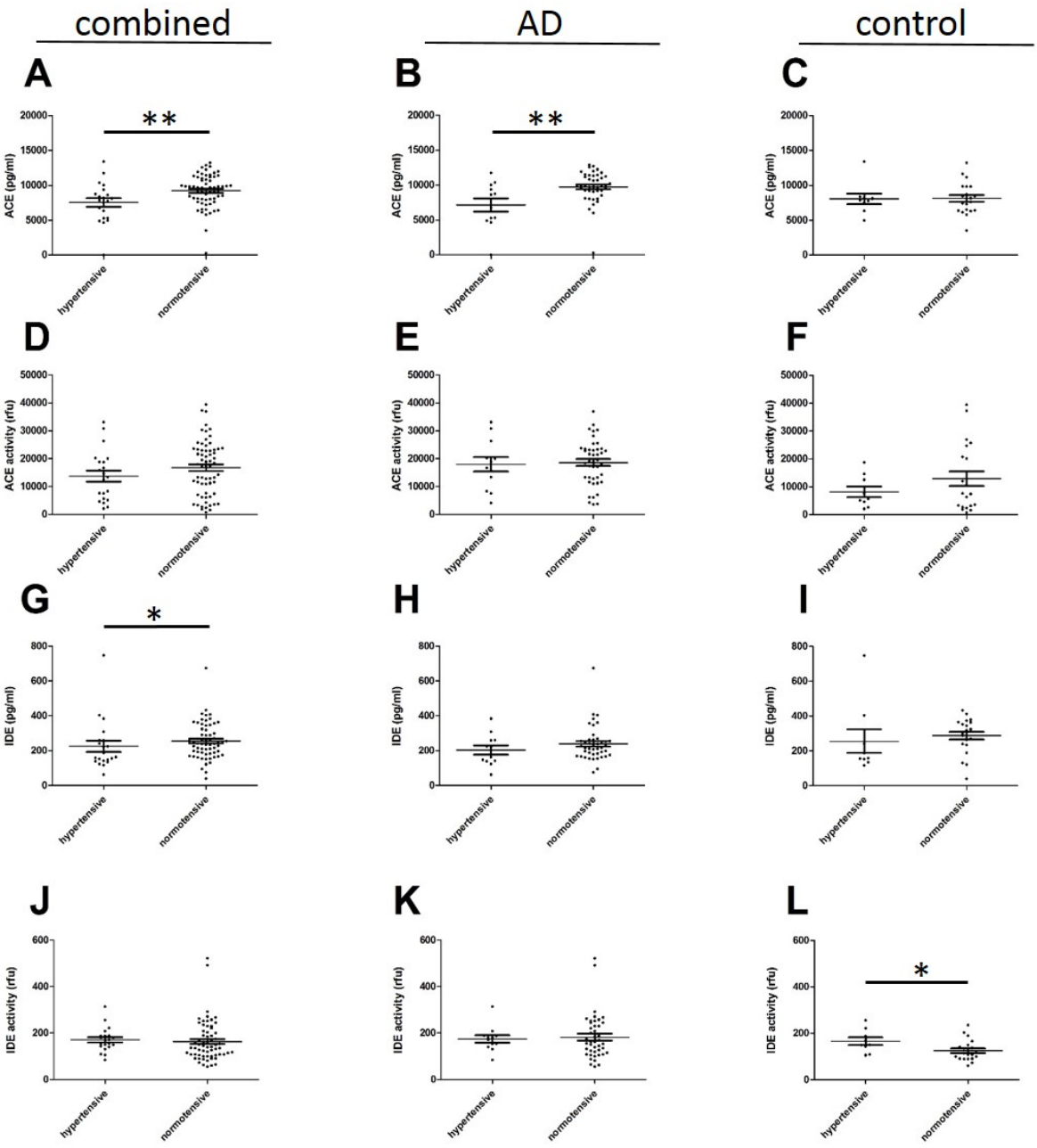


Figure 3. A – C and **D – F** show ACE protein and activity, and **G – I** and **J – L** show IDE protein and activity with respect to hypertensive status, with diagnosis groups combined (column 1), in AD (column 2) and controls (column 3). ACE protein was significantly lower in hypertensive cases in the combined diagnosis ($p = 0.010$) and AD groups ($p = 0.009$). IDE protein was significantly lower in hypertensive cases in the combined diagnosis group (0.036) and IDE activity was significantly increased in hypertensive control cases ($p = 0.027$). The horizontal bars indicate the mean and SEM. Each point represents a separate brain.

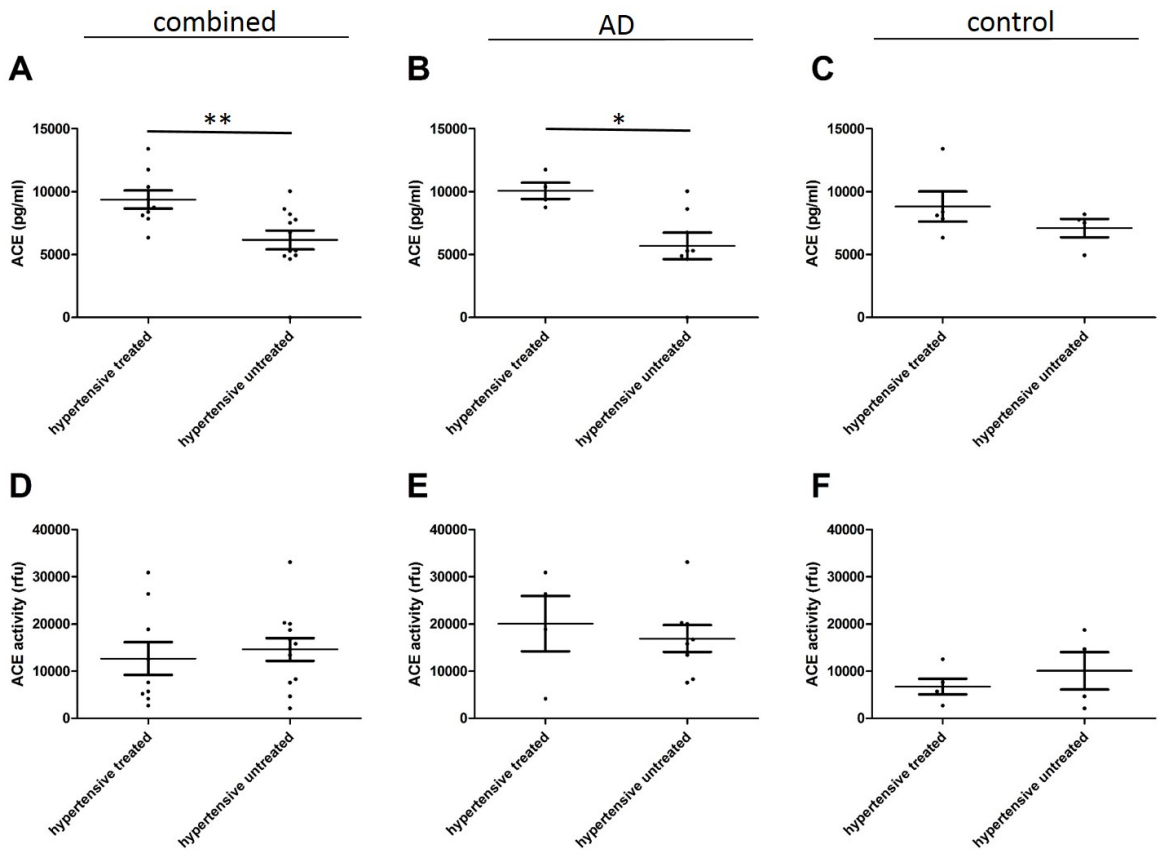


Figure 4. A - C and **D - F** show ACE protein and activity with respect to hypertensive treatment status, with diagnosis groups combined (column 1), in AD (column 2) and controls (column 3). ACE protein was significantly increased in treated hypertensives in the combined diagnosis ($p = 0.008$) and AD groups ($p = 0.016$). The horizontal bars indicate the mean and SEM. Each point represents a separate brain.

Table 1 Summary demographic data of the hypertensive and normotensive AD brains and hypertensive treated and untreated AD brains used in the retrospective analysis of frontal Aβ load.

	hypertensive group (N = 20)		normotensive group (N = 62)
age, y ± s.d.	82.30 ± 5.01		79.42 ± 8.95
gender, F/M	13/7		38/24
post-mortem delay, h ± s.d.	37.15 ± 19.75		48.69 ± 25.33
	treated (N = 9)	**untreated** (N = 11)	
age, y ± s.d.	83.89 ± 5.21	81.00 ± 4.67	
gender, F:M	4/5	2/9	
post-mortem delay, h ± s.d.	43.56 ± 22.27	31.91 ± 16.65	
Braak tangle stage			

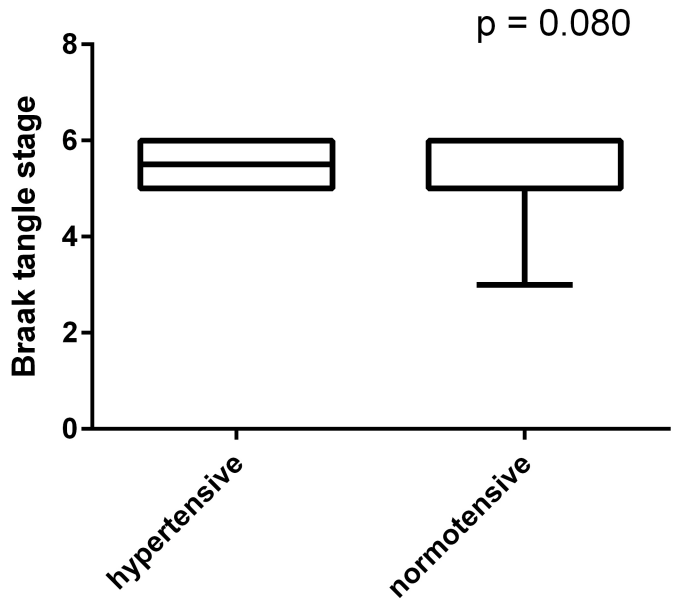
Table 2 Summary demographic and neuropathological data of the hypertensive and normotensive AD brains and hypertensive treated and untreated AD brains used in the retrospective and prospective analysis of β- and γ-secretase activity, respectively.

	hypertensive group (N = 31)		**normotensive group** (N = 77)	
age, y ± s.d.	82.97 ± 6.96		78.40 ± 10.11	
gender, F/M	15/16		44/33	
post-mortem delay, h ± s.d.	41.45 ± 23.95		44.86 ± 26.99	
	AD (N = 18)	**control** (N = 13)	**AD** (N = 50)	**control** (N = 27)
age, y ± s.d.	82.50 ± 5.25	83.62 ± 9.01	79.68 ± 9.27	76.04 ± 11.32
gender, F:M	12/6	3/10	31/19	13/14
post-mortem delay, h ± s.d.	35.28 ± 19.89	50.00 ± 27.15	49.96 ± 25.25	35.41 ± 28.02
	treated (N = 13)	**untreated** (N = 17)		
age, y ± s.d.	85.85 ± 5.96	80.89 ± 7.04		
gender, F:M	5/8	10/8		
post-mortem delay, h ± s.d.	40.85 ± 22.21	41.89 ± 25.76		

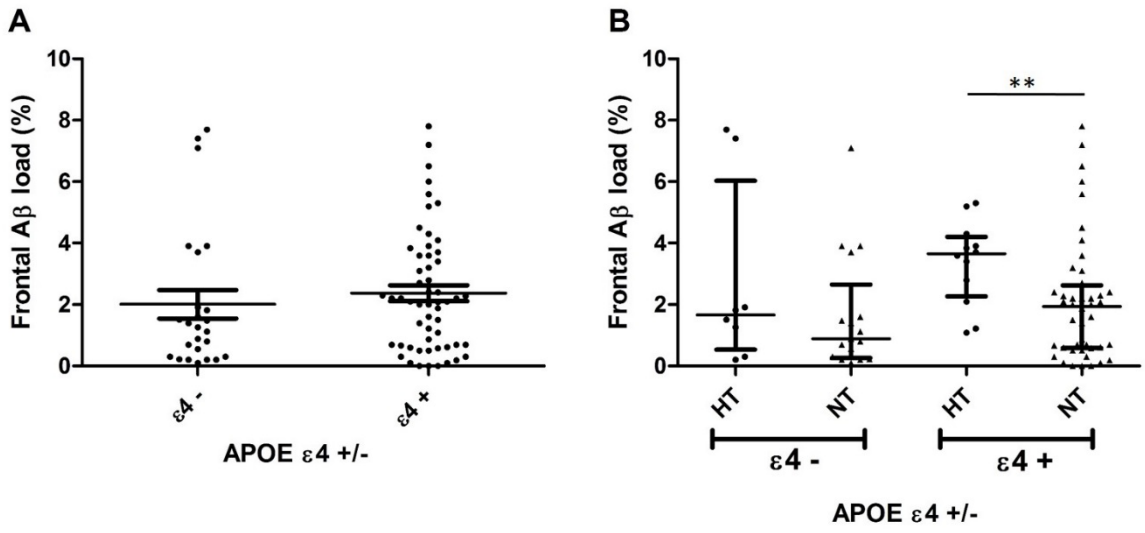
Table 3 Summary demographic and neuropathological data of the hypertensive and normotensive AD brains and hypertensive treated and untreated AD brains used in the retrospective analysis of ACE, IDE and NEP protein and activity.

	hypertensive group (N = 21)		**normotensive group** (N = 64)	
age, y ± s.d.	83.57 ± 7.57		78.00 ± 10.10	
gender, F/M	11/10		36/28	
post-mortem delay, h ± s.d.	35.71 ± 22.57		43.47 ± 27.71	
	AD (N = 12)	**control** (N = 9)	**AD** (N = 43)	**control** (N = 21)
age, y ± s.d.	83.08 ± 4.58	84.22 ± 10.65	79.72 ± 9.44	74.48 ± 10.69
gender, F:M	9/3	2/7	27/16	9/12
post-mortem delay, h ± s.d.	31.08 ± 17.58	41.89 ± 27.80	47.21 ± 25.63	35.81 ± 30.77
	treated (N = 9)	**untreated** (N = 12)		
age, y ± s.d.	87.22 ± 5.12	80.83 ± 8.12		
gender, F:M	3/6	8/4		
post-mortem delay, h ± s.d.	39.11 ± 20.12	33.17 ± 24.80		

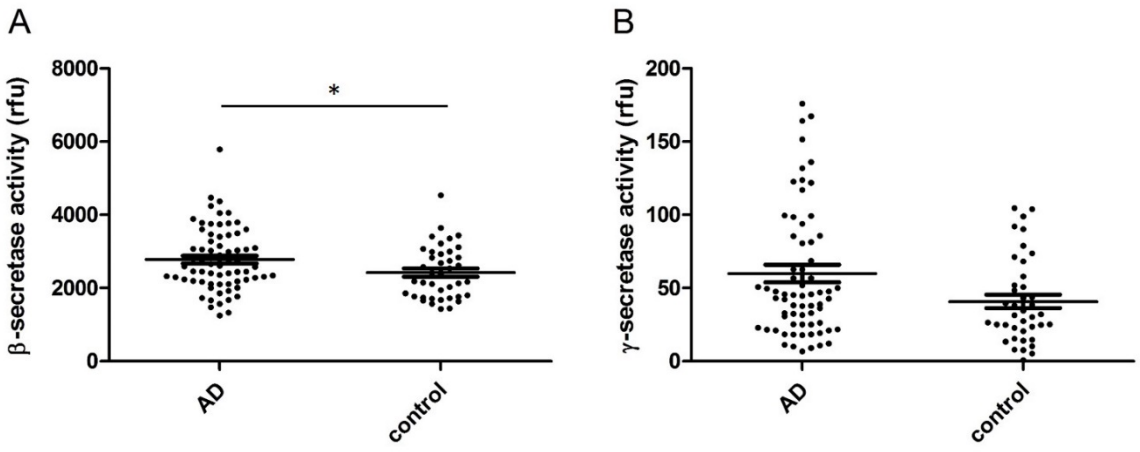
Supplementary Material



Supplementary Figure 1. Box and whisker plot of Braak tangle stages in the hypertensive and normotensive group. The difference between groups was not significant (Mann-Whitney U test, p = 0.080) but it was noteworthy that the normotensive group included a few cases with less extensive Braak tangle pathology.



Supplementary Figure 2. Frontal Aβ load analysed with respect to the absence or presence of *APOE* ε4 in AD cases before (**A**) and after stratification into hypertensive (HT) and normotensive (NT) cases (**B**). Frontal Aβ load was not significantly greater in cases with one or two *APOE* ε4 alleles. There was no difference in the distribution of *APOE* genotype between hypertensive and normotensive cases (data not shown). Frontal Aβ load was significantly higher in the ε4+ hypertensive than ε4+ normotensive cases ($p = 0.009$). The horizontal bars indicate the mean and SEM. Each point represents a separate brain.



Supplementary Figure 3. β- (**A**) and γ-secretase activities (**B**) in midfrontal cortex in AD and controls. β-secretase activity was significantly higher in the AD group ($p = 0.031$). A similar but non-significant trend was found for γ-secretase activity ($p = 0.058$). The horizontal bars indicate the mean and SEM. Each point represents a separate brain.

Supplementary Table 1 MRC database identifiers, pathological and demographic data and summary of measurements available for individual cases included in all retrospective analyses and prospective analysis of γ-secretase activity in relation to hypertensive status.

				delay (h)	*stage*			treatment		activity	activity					
HT1	BBN_8644	84	M	72	III	3.3	C	diuretic		+	+	+	+	+	+	+
HT2	BBN_8651	95	F	46	II	2.3	C	diuretic		+	+	+	+	+	+	+
HT3	BBN_8964	82	F	37	II	4.4	C	β-blocker, CCB, diuretic		+	+	+	+	+	+	+
HT4	BBN_8965	80	F	21	V	3.4	AD	diuretic	+	+	+	+	+	+	+	+
HT5	BBN_8983	78	M	48	I	3.3	C	CCB, diuretic		+	+					
HT6	BBN_9106	93	M	20	VI	3.3	AD	ACEI, diuretic	+	+	+					
HT7	BBN_9113	81	F	59	V	3.4	AD	ACEI, diuretic	+							
HT8	BBN_9119	80	M	49	V	3.4	AD	ACEI, diuretic, nitrates	+							
HT9	BBN_9136	77	F	26	VI	3.4	AD	β-blocker, CCB	+	+	+					
HT10	BBN_9200	84	M	64	V	3.4	AD	CCB, diuretic	+	+	+	+	+	+	+	+
HT11	BBN_9222	90	M	32	VI	3.3	AD	nitrates	+	+	+	+	+	+	+	+
HT12	BBN_9248	83	F	85	VI	4.4	AD	diuretic	+	+	+					
HT13	BBN_9275	87	M	36	VI	4.4	AD	β-blocker	+	+	+	+	+	+	+	+
HT14	BBN_9299	90	M	6	II	2.3	C	ACEI, diuretic, nitrates		+	+	+	+	+	+	+
HT15	BBN_9311	93	M	38	III	2.3	C	ACEI, CCB		+	+	+	+	+	+	+
HT16	BBN_8639	62	M	4	0	3.4	C	-		+	+	+	+	+	+	+
HT17	BBN_8709	83	M	86	II	3.3	C	-		+	+					
HT18	BBN_8723	80	M	67	III	3.4	C	-		+	+					
HT19	BBN_8735	88	F	72	0	3.3	C	-		+	+					
HT20	BBN_8757	90	M	48	II	3.3	C	-		+	+	+	+	+	+	+
HT21	BBN_8887	89	M	91	I	3.4	C	-		+	+	+	+	+	+	+
HT22	BBN_8906	78	F	35	VI	3.3	AD	-	+	+	+					
HT23	BBN_8947	78	F	4	V	3.3	AD	-	+	+	+	+	+	+	+	+
HT24	BBN_8997	74	F	12	VI	3.4	AD	-	+	+	+	+	+	+	+	+
HT25	BBN_9076	84	F	20	V	3.4	AD	-	+	+	+	+	+	+	+	+
HT26	BBN_9150	87	F	55	V	3.4	AD	-	+	+	+	+	+	+	+	+
HT27	BBN_9167	79	F	48	V	3.4	AD	-	+	+	+	+	+	+	+	+
HT28	BBN_9261	83	M	48	V	3.3	AD	-	+	+	+					
HT29	BBN_9263	74	M	48	V	2.3	AD	-	+	+	+					
HT30	BBN_9292	73	M	35	III	3.3	C	-		+	+	+	+	+	+	+

HT31	BBN_9298	83	F	32	VI	3.4	AD	-	+	+	+	+	+	+	+	+
HT32	BBN_9320	87	F	28	VI	3.3	AD	-	+	+	+	+	+	+	+	+
HT33	BBN_9323	84	F	21	VI	2.3	AD	-	+	+	+	+	+	+	+	+
NT1	BBN_8671	78	F	24	II	3.3	C	-		+	+					
NT2	BBN_8684	71	M	25	I	3.3	C	-		+	+	+	+	+	+	+
NT3	BBN_8691	82	F	35	III	2.3	C	-		+	+					
NT4	BBN_8702	58	M	20	0	2.3	C	-		+	+					
NT5	BBN_8703	64	M	16	0	3.3	C	-		+	+	+	+	+	+	+
NT6	BBN_8706	72	M	42	I	3.3	C	-		+	+	+	+	+	+	+
NT7	BBN_8708	90	M	45	II	2.3	C	-		+	+					
NT8	BBN_8712	81	F	103	II	3.3	C	-		+	+	+	+	+	+	+
NT9	BBN_8717	77	M	55	I	3.3	C	-		+	+	+	+	+	+	+
NT10	BBN_8722	78	M	12	II	3.3	C	-		+	+	+	+	+	+	+
NT11	BBN_8725	73	M	36	II	3.3	C	-		+	+	+	+	+	+	+
NT12	BBN_8728	88	F	62	II	3.3	C	-		+	+					
NT13	BBN_8732	76	F	106	II	3.3	C	-		+	+	+	+	+	+	+
NT14	BBN_8739	93	F	18	II	3.3	C	-		+	+					
NT15	BBN_8741	80	F	92	0	3.3	C	-		+	+	+	+	+	+	+
NT16	BBN_8749	88	F	28	II	3.3	C	-		+	+	+	+	+	+	+
NT17	BBN_8751	82	M	30	II	3.3	C	-		+	+	+	+	+	+	+
NT18	BBN_8770	89	F	15	II	3.3	C	-		+	+	+	+	+	+	+
NT19	BBN_8776	73	M	33	I	2.3	C	-		+	+	+	+	+	+	+
NT20	BBN_8819	89	F	71	V	3.3	AD	-	+	+	+	+	+	+	+	+
NT21	BBN_8825	78	F	77	VI	4.4	AD	-	+	+	+	+	+	+	+	+
NT22	BBN_8834	78	F	9	V	3.4	AD	-	+	+	+	+	+	+	+	+
NT23	BBN_8835	73	F	59	I	3.3	C	-		+	+	+	+	+	+	+
NT24	BBN_8839	82	M	69	V	4.4	AD	-	+							
NT25	BBN_8841	81	F	80	III	3.4	AD	-	+	+	+	+	+	+	+	+
NT26	BBN_8842	81	F	42	VI	3.4	AD	-	+	+	+	+	+	+	+	+
NT27	BBN_8845	91	F	37	IV	3.4	AD	-	+	+	+	+	+	+	+	+

NT28	BBN_8848	77	F	43	IV	3.4	AD	-	+	+	+	+	+	+	+	+
NT29	BBN_8852	71	F	67	V	4.4	AD	-	+	+	+					
NT30	BBN_8853	96	F	53	IV	2.3	AD	-	+	+	+	+	+	+	+	+
NT31	BBN_8857	87	F	72	V	3.4	AD	-	+	+	+	+	+	+	+	+
NT32	BBN_8870	87	F	67	V	2.4	AD	-	+	+	+	+	+	+	+	+
NT33	BBN_8871	79	F	70	III	3.3	AD	-	+	+	+	+	+	+	+	+
NT34	BBN_8872	88	F	79	VI	3.3	AD	-	+	+	+	+	+	+	+	+
NT35	BBN_8885	68	M	28	V	3.3	AD	-	+	+	+	+	+	+	+	+
NT36	BBN_8886	81	M	29	IV	3.4	AD	-	+	+	+	+	+	+	+	+
NT37	BBN_8892	91	F	70	V	2.4	AD	-	+	+	+	+	+	+	+	+
NT38	BBN_8898	83	F	24	II	3.3	C	-		+	+	+	+	+	+	+
NT39	BBN_8905	72	M	61	IV	3.4	AD	-	+	+	+					
NT40	BBN_8912	82	F	24	VI	3.4	AD	-	+	+	+	+	+	+	+	+
NT41	BBN_8915	85	M	58	IV	3.4	AD	-	+	+	+	+	+	+	+	+
NT42	BBN_8916	83	F	43	V	3.4	AD	-	+	+	+					
NT43	BBN_8917	91	M	43	III	3.3	AD	-	+	+	+	+	+	+	+	+
NT44	BBN_8918	89	F	82	V	3.3	AD	-	+	+	+					
NT45	BBN_8923	82	M	3	II	3.3	C	-		+	+	+	+	+	+	+
NT46	BBN_8930	70	F	25	VI	3.4	AD	-	+							
NT47	BBN_8949	79	M	24	0	3.3	C	-		+	+	+	+	+	+	+
NT48	BBN_8954	69	M	48	V	4.4	AD	-	+	+	+	+	+	+	+	+
NT49	BBN_8956	43	F	12	0	3.3	C	-		+	+	+	+	+	+	+
NT50	BBN_8958	74	M	50	V	3.4	AD	-	+	+	+	+	+	+	+	+
NT51	BBN_8966	53	M	7	III	3.3	C	-		+	+	+	+	+	+	+
NT52	BBN_8980	72	F	24	0	3.3	C	-		+	+	+	+	+	+	+
NT53	BBN_9005	89	F	4	VI	3.4	AD	-	+							
NT54	BBN_9026	79	M	28	VI	3.4	AD	-	+	+	+	+	+	+	+	+
NT55	BBN_9031	85	M	66	VI	3.4	AD	-	+	+	+					
NT56	BBN_9037	81	F	66	IV	3.3	AD	-	+	+	+	+	+	+	+	+
NT57	BBN_9043	80	M	31	VI	3.4	AD	-	+							

NT58	BBN_9050	90	F	21	IV	4.4	AD	-	+	+	+	+	+	+	+	+
NT59	BBN_9092	75	M	6	III	2.3	C	-		+	+	+	+	+	+	+
NT60	BBN_9112	74	F	53	V	4.4	AD	-	+							
NT61	BBN_9114	82	M	64	IV	3.3	AD	-	+							
NT62	BBN_9125	84	F	23	VI	4.4	AD	-	+							
NT63	BBN_9132	80	M	5	V	3.4	AD	-	+							
NT64	BBN_9156	79	M	39	V	4.4	AD	-	+	+	+	+	+	+	+	+
NT65	BBN_9165	60	F	68	VI	3.3	AD	-	+							
NT66	BBN_9182	74	M	24	V	3.4	AD	-	+	+	+	+	+	+	+	+
NT67	BBN_9188	68	F	87	VI	3.4	AD	-	+	+	+	+	+	+	+	+
NT68	BBN_9189	78	F	21	VI	4.4	AD	-	+	+	+	+	+	+	+	+
NT69	BBN_9193	79	M	84	V	3.4	AD	-	+	+	+	+	+	+	+	+
NT70	BBN_9194	89	F	39	V	4.4	AD	-	+	+	+	+	+	+	+	+
NT71	BBN_9201	65	M	90	VI	3.3	AD	-	+	+	+	+	+	+	+	+
NT72	BBN_9205	85	F	85	VI	3.4	AD	-	+							
NT73	BBN_9207	80	F	71	VI	3.3	AD	-	+	+	+	+	+	+	+	+
NT74	BBN_9209	73	F	38	V	4.4	AD	-	+	+	+	+	+	+	+	+
NT75	BBN_9212	65	F	22	VI	3.3	AD	-	+	+	+	+	+	+	+	+
NT76	BBN_9221	68	M	61	VI	3.3	AD	-	+	+	+					
NT77	BBN_9242	85	F	14	IV	3.3	AD	-	+	+	+	+	+	+	+	+
NT78	BBN_9243	88	F	75	V	3.4	AD	-	+	+	+	+	+	+	+	+
NT79	BBN_9265	60	F	5	VI	-	AD	-	+	+	+	+	+	+	+	+
NT80	BBN_9269	83	M	99	IV	3.4	AD	-	+	+	+	+	+	+	+	+
NT81	BBN_9284	76	F	43.5	VI	3.4	AD	-	+							
NT82	BBN_9291	62	M	25	VI	3.4	AD	-	+	+	+	+	+	+	+	+
NT83	BBN_9293	78	M	50	VI	3.4	AD	-	+							
NT84	BBN_9295	85	M	50	VI	3.3	AD	-	+	+	+	+	+	+	+	+
NT85	BBN_9296	98	F	21	V	3.4	AD	-	+	+	+	+	+	+	+	+
NT86	BBN_9301	84	F	11	V	3.4	AD	-	+	+	+	+	+	+	+	+
NT87	BBN_9303	69	M	12	V	3.4	AD	-	+	+	+	+	+	+	+	+

Supplementary Table 2 Non-parametric analysis of frontal Aβ load and of Aβ–synthesising and –degrading enzymes in relation to post-mortem delay

Dataset vs post-mortem delay	*Spearman*	*Mann-Whitney test p value*
Frontal Aβ load	0.755	0.035
β-secretase activity	0.963	-0.005
γ-secretase activity	0.677	-0.041
ACE protein	0.309	0.112
ACE activity	0.499	0.156
IDE protein	0.211	0.137
IDE activity	0.114	-0.173
NEP protein	0.571	0.062
NEP activity	0.789	0.029

Supplementary Table 3 Non-parametric analysis of levels and activity of Aβ-degrading enzymes in hypertensive and normotensive groups, with diagnosis groups combined, in AD and in controls.

Dataset compared between hypertensive and normotensive group	*cohort*	*Mann-Whitney test p value*
ACE protein	combined	0.001**
	AD	0.009**
	control	0.856
ACE activity	combined	0.239
	AD	0.783
	control	0.587
IDE protein	combined	0.036
	AD	0.233
	control	0.148
IDE activity	combined	0.208
	AD	0.799
	control	0.027*
NEP protein	combined	0.412
	AD	0.927
	control	0.258
NEP activity	combined	0.676
	AD	0.508
	control	0.189

*significant at the 95% confidence interval, ** significant at the 99% confidence interval

Supplementary Table 4 Non-parametric analysis of levels and activity of Aβ-degrading enzymes in treated and untreated hypertensive groups, with diagnosis groups combined, in AD and in controls**.**

Dataset compared between hypertensive treated and hypertensive untreated group	*cohort*	*Mann-Whitney test p value*
ACE protein	combined	0.008**
	AD	0.016*
	control	0.286
ACE activity	combined	0.500
	AD	0.683
	control	0.905
IDE protein	combined	0.594
	AD	0.683
	control	0.191
IDE activity	combined	0.749
	AD	0.808
	control	0.905
NEP protein	combined	0.859
	AD	0.461
	control	1.000
NEP activity	combined	0.749
	AD	0.570
	control	0.730

*significant at the 95% confidence interval, ** significant at the 99% confidence interval

NT88	BBN_9304	61	M	38	V	3.4	AD	-	+	+	+	+	+	+	+	+
NT89	BBN_9309	88	F	88	V	3.3	AD	-	+	+	+					

ACE = angiotensin-converting enzyme; ACEI = angiotensin-converting enzyme inhibitor; AD = Alzheimer's disease; C = control; CCB = calcium channel blocker; HT = hypertensive; IDE = insulin-degrading enzyme; NEP = neprilysin; NT = normotensive; - = untreated; + = measured